The Effect of Maternal Asthma During Pregnancy on Placental Function and Fetal Development

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Declaration

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution

Signed .....

Vanessa E Murphy

January 2004

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Philippians 4:13

I can do all things through Christ who strengthens me

Publication List

The work presented in this thesis has directly resulted in the following publications:

1. **Murphy VE**, Zakar T, Smith R, Giles WB, Gibson PG, Clifton VL. Reduced 11β-hydroxysteroid dehydrogenase type 2 activity is associated with decreased birth weight centile in pregnancies complicated by asthma. Journal of Clinical Endocrinology and Metabolism 2002; 87(4), 1660-1668.
2. **Murphy VE** and Clifton VL. Alterations in human placental 11β-hydroxysteroid dehydrogenase type 1 and 2 with gestational age and labour. Placenta 2003; 24(7), 739-744.
3. **Murphy VE**, Gibson PG, Giles WB, Zakar T, Smith R, Bisits AM, Kessell CG and Clifton VL. Maternal asthma is associated with reduced female fetal growth. American Journal of Respiratory and Critical Care Medicine 2003; 168(11), 1317-1323.
4. Clifton VL and **Murphy VE**. Maternal asthma as a model for examining fetal sex-specific effects on maternal physiology and placental mechanisms that regulate human fetal growth. Placenta (Supplement: Trophoblast Research), invited review to be published in April 2004.

The work presented in this thesis has directly resulted in the following abstracts/conference presentations:

1. **Murphy VE**, Zakar T, Smith R and Clifton VL. Placental 11β-HSD2 activities in asthmatic women. Human Parturition on the Beach, International Congress of Endocrinology Satellite Meeting, Newcastle, Australia, 27-28 October 2000 (Poster).
2. **Murphy VE**, Zakar T, Gibson PG, Giles W, Smith R and Clifton VL. Placental 11β-HSD2 activity in asthmatic women. 48th Annual Meeting of the Society for Gynecologic Investigation, Toronto, Canada, 14-17 March 2001 (Poster).
3. **Murphy VE**, Zakar T, Smith R, Giles WB, Gibson PG and Clifton VL. The effects of asthma during pregnancy on placental 11β-hydroxysteroid dehydrogenase type 2 and neonatal birth weight centile. 10th NSW Scientific

Meeting of the Australian Society for Medical Research, Sydney, Australia, 4 June 2001 (Oral).

4. **Murphy VE**, Zakar T, Smith R, Giles WB, Gibson PG and Clifton VL. A potential mechanism for growth retardation in pregnancies complicated by asthma. 44th Annual Scientific Meeting of the Endocrine Society of Australia, Gold Coast, Australia, 9-12 September 2001 (Oral - Finalist for the Novartis Junior Scientist Award).

5. Clifton VL, **Murphy VE**, Giles WB, Zakar T, Gibson PG and Smith R. 11β-hydroxysteroid dehydrogenase type 2 activity in pregnancies complicated by asthma. 8th Meeting of the International Federation of Placenta Associations, Sorrento, Italy, 19-23 September 2001 (Presented by Dr Vicki Clifton).

6. **Murphy VE**, Zakar T, Smith R, Giles WB, Gibson PG and Clifton VL. Female fetal growth is adversely affected by maternal asthma in the absence of glucocorticoid therapy. 40th National Scientific Conference of the Australian Society for Medical Research, Gold Coast, Australia, 24-27 November 2001 (Poster - won the Medibank Private Student Researcher Award).

7. **Murphy VE**, Gibson PG, Smith R, Giles WB, Kessell CG and Clifton VL. Maternal asthma affects fetal growth in a gender specific manner and is associated with reduced placental 11β-HSD2 activity and altered sensitivity to cortisol. 49th Annual Meeting of the Society for Gynecologic Investigation, Los Angeles, CA, USA, 20-23 March 2002 (Poster).

8. Clifton VL, **Murphy VE**, Zakar T, Smith R, Giles WB and Gibson PG. Female fetal growth is adversely affected by maternal asthma in the absence of inhaled glucocorticoid (ICS) therapy. 98th Annual Scientific Meeting of the American Thoracic Society, Atlanta, GA, USA, 17-22 May 2002 (Presented by Prof Peter Gibson).

9. **Murphy VE**, Gibson PG, Giles WB, Zakar T, Smith R, Kessell CG and Clifton VL. Maternal asthma affects female fetal growth and is associated with reduced placental 11β-HSD2 activity and altered sensitivity to cortisol. 11th NSW Scientific Meeting of the Australian Society for Medical Research, Sydney, Australia, 3 June 2002 (won Best Poster Presentation by a Student).

10. **Murphy VE**, Gibson PG, Giles WB, Zakar T, Smith R, Kessell CG and Clifton VL. Inflammatory factors have sex-specific effects on fetal growth in humans. 29th Annual Meeting of the Fetal and Neonatal Physiological Society, Prague, Czech Republic, 8-11 September 2002 (won Best Oral Presentation by a Student).

11. **Murphy VE**, Gibson PG, Giles WB, Zakar T, Smith R, Kessell CG and Clifton VL. Sex-specific changes in placental function and fetal growth and development in pregnancies complicated by asthma. 45th Annual Scientific Meeting of the Endocrine Society of Australia, Adelaide, Australia, 22-25 September 2002 (Oral).

12. **Murphy VE**, Gibson PG, Giles WB, Zakar T, Smith R, Kessell CG and Clifton VL. Inflammatory factors have sex-specific effects on fetal growth in humans. Perinatal Society of Australia and New Zealand, Fetal and Neonatal Physiology Workshop at the Australian Health and Medical Research Congress, Melbourne, Australia, 22-25 November 2002 (Poster).

13. **Murphy VE**, Gibson PG, Giles WB, Smith R and Clifton VL. A novel model for examining fetal growth restriction in human pregnancy. 50th Annual Meeting of the Society for Gynecologic Investigation, Washington DC, USA, 25-30 March 2003 (Poster).

14. **Murphy VE**, Gibson PG, Giles WB, Smith R and Clifton VL. The effect of fetal gender on maternal inflammation and lung function in asthmatic pregnancies. 46th Annual Scientific Meeting of the Endocrine Society of Australia, Melbourne, Australia, 14-17 September 2003 (Oral).

15. **Murphy VE**, Gibson PG, Giles WB, Smith R and Clifton VL. Placental and maternal inflammatory pathways in pregnancies complicated by asthma. 9th Meeting of the International Federation of Placenta Associations, Mainz, Germany, 24-27 September 2003 (Oral - won the New Investigator Award).

16. **Murphy VE**, Johnson RF, Wang Y-C, Akinsanya K, Gibson PG, Smith R and Clifton VL. A proteomic analysis of maternal plasma, umbilical cord plasma and placenta in asthmatic and non-asthmatic pregnancies. 42nd National Scientific Conference of the Australian Society for Medical Research, Glenelg, Australia, 22-24 November 2003 (Oral).

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Abbreviations

11β-HSD	11 beta-hydroxysteroid dehydrogenase
2D-PAGE	Two dimensional-polyacrylamide gel electrophoresis
^3H	Tritium (Tritiated)
ACTH	Adrenocorticotropic hormone
AME	Apparent mineralocorticoid excess
AMS	Asthma Management Service
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
β_2-agonist	Beta two adrenergic receptor agonist
BDP	Beclomethasone dipropionate
BMI	Body Mass Index
BPD	Biparietal diameter
BSA	Bovine serum albumin
BWC	Birth weight centile
C section	Caesarean section
cAMP	Cyclic 3',5'-adenosine monophosphate
cGMP	Cyclic 3',5'-guanosine monophosphate
CHAPS	3-[(3-chloamidopropyl)-dimethylammonio]-1-propanesulfonate
CHCA	α-cyano-4-hydroxycinnamic acid
cpm	Counts per minute
CRH	Corticotropin releasing hormone
C_T	Threshold cycle
Da	Daltons
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DHEA-S	Dehydroepiandrosterone sulfate
dNTPs	Deoxynucleotide triphosphates
dpm	Disintegrations per minute
DTT	Dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
EAM	Energy absorbing molecule
EDTA	Ethylene-diamine-tetra-acetic acid
EGTA	Ethylene-glycol-bis(β-aminoethyl ether)-tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FEV_1	Forced expiratory volume at one second
FRI	Ferring Research Institute
FSH	Follicle stimulating hormone
FVC	Forced vital capacity
fwd	Forward (primer)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GM-CSF	Granulocyte macrophage-colony stimulating factor
GR	Glucocorticoid receptor
HC:AC	Head circumference to abdominal circumference ratio

hCG	Human chorionic gonadotropin
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IMAC	Immobilised metal affinity capture
IQ	Intelligence quotient
IU	International units
IUGR	Intrauterine growth restriction
KCl	Potassium chloride
K_m	Michaelis constant
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MALDI	Matrix assisted laser desorption/ionisation
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
MS	Mass spectrometry
MW	Molecular weight
m/z	Mass to charge ratio
n	Number of subjects
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NBT/BCIP	Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate
Ni-NTA	Nickel nitrilotriacetic acid
No.	Number
NO	Nitric oxide
NTC	No template control
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PCO_2	Partial pressure of carbon dioxide
PEF	Peak expiratory flow
PEFR	Peak expiratory flow rate
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
pI	Isoelectric point
PIH	Pregnancy induced hypertension
PO_2	Partial pressure of oxygen
PPROM	Preterm premature rupture of membranes
PVDF	Polyvinylidene fluoride
RDS	Respiratory distress syndrome
rev	Reverse (primer)
RIA	Radioimmunoassay
rRNA	Ribosomal RNA

RT-PCR	Reverse transcriptase-polymerase chain reaction
SAX	Strong anion exchange
SD	Systolic/diastolic ratio
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SELDI-TOF	Surface enhanced laser desorption/ionisation-time of flight
SEM	Standard error of the mean
SGA	Small for gestational age
SOD	Superoxide dismutase
SPA	Sinapinic acid
TBE	Tris borate EDTA
TBS	Tris buffered saline
TFA	Trifluoroacetic acid
TGF-β	Transforming growth factor-beta
Th1/2	T helper type 1/2
TLC	Thin layer chromatography
T_m	Melting temperature
TNF-α	Tumour necrosis factor-alpha
TOF	Time of flight
TTTS	Twin to twin transfusion syndrome
TX-100	Triton X-100
UV	Ultraviolet
VC	Vital capacity
WCX	Weak cation exchange

Abstract

Maternal asthma is associated with low birth weight, a risk factor for disease in adult life. To determine the mechanisms involved, the relationships between mother, placenta and fetus were examined in asthmatic and non-asthmatic pregnancies.

Maternal asthma and its treatment (no glucocorticoid or glucocorticoid) was monitored throughout pregnancy. Fetal growth was examined during gestation, and at birth, neonatal size and sex were determined. Placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme activity and umbilical vein plasma cortisol and estriol concentrations were measured. Placental cytokine, growth factor and glucocorticoid receptor (GR) mRNA were determined using quantitative RT-PCR.

Birth weight of female neonates in the no glucocorticoid asthmatic group only, was significantly reduced compared to females of the non-asthmatic group. Male neonates were unaffected by asthma or its treatment. Asthmatic women pregnant with a female fetus showed a significant increase in circulating monocytes and glucocorticoid treatment as pregnancy progressed, while those pregnant with a male fetus did not, suggesting that maternal asthma worsens in the presence of a female fetus. 11β-HSD2 activity was significantly reduced in placentae from female neonates of the no glucocorticoid group compared to other female neonates and was associated with a trend towards higher plasma cortisol, reduced fetal adrenal activity demonstrated by lower cord blood estriol, reduced placental GR expression, no alteration in placental or fetal insulin-like growth factors or their binding proteins and a significantly increased Th2:Th1 cytokine mRNA ratio, which was inversely correlated with 11β-HSD2 activity in all females. Reduced placental 11β-HSD2 activity may be an important component leading to decreased female fetal growth in pregnancies complicated by asthma.

This study provides strong evidence for a fetal sex-specific effect on the maternal immune system which can have adverse effects on the female fetus. The female fetus alters maternal inflammatory pathways, which when not controlled by the use of inhaled glucocorticoids results in reduced placental 11β-HSD2 activity, contributing to suppressed fetal adrenal function and a late gestation decrease in female fetal growth.

Chapter 1 **Literature Review**

Literature Review Part 1: Asthma and Pregnancy

1.1 The pathophysiology of asthma

Asthma is a complex respiratory disease characterised by acute exacerbations and chronic alterations in airway structure and function. Symptoms of asthma include wheeze, cough and an inability to breath caused by narrowing of the airways due to smooth muscle contraction (1). Asthma is often characterised by bronchial responsiveness to stimuli (2), in the form of hypersensitivity to foreign substances resulting in the inappropriate production of IgE antibodies. Exposure to triggers, specific or non-specific, including allergens such as pollen, cold air, exercise and pollution results in the release of histamine and other factors from mast cells (3). Edema of the bronchioles and production of mucous follows, along with spasm of the bronchial smooth muscle, which makes breathing difficult (1, 3).

Diagnosis of asthma relies on an objective demonstration of variable airway obstruction which may be spontaneously reversible or reversible with treatment (4). Spirometry is used for the diagnosis of asthma and for monitoring its progress over time, by comparing the degree of airway obstruction to predicted normal values (4, 5). By measuring inspired and expired volumes over time, spirometry allows an evaluation of how effectively and quickly the lungs fill and empty with air (6). The forced expiratory volume at one second (FEV_1) is the volume of air maximally expired in the first second following a maximal inspiration, while the forced vital capacity (FVC) is the maximum volume of air exhaled or inspired (6). The FEV_1:FVC ratio is used to gauge the degree of airflow limitation (6). Peak expiratory flow (PEF) refers to the maximum expiratory flow rate achieved (6). An example spirogram is shown in Figure 1.1. Asthmatics have a reduction in FEV_1 relative to the FVC (6) and a reduced peak expiratory flow rate (PEFR) (4).

Chronic alterations in the airway such as epithelial damage and collagen deposition, are known to occur in all asthmatics, including those with mild asthma (7-9). Inflammation of the airways is also a prominent feature of mild asthma (10) and mediates many of the changes in airway structure and function observed in asthmatic patients.

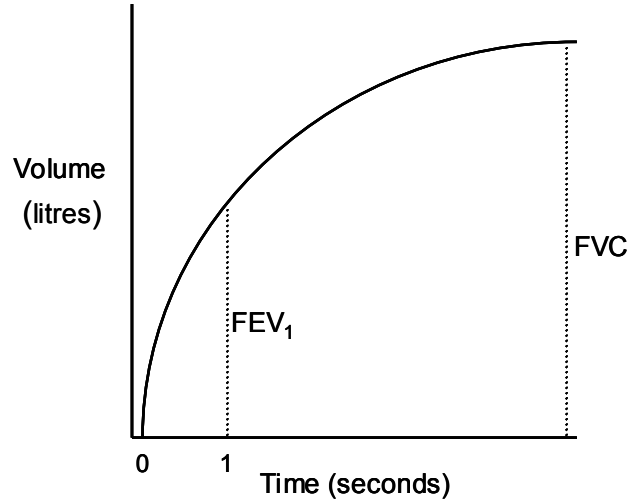

Figure 1.1 Example spirogram showing the FEV_1 and FVC

In spirometry, the patient performs a maximum inspiration followed by a maximum forced expiration until no more air can be exhaled (4). The FEV_1 is the volume of air exhaled in the first second, while the FVC is the total volume of air expired during the procedure. Diagram adapted from Pierce and Johns, 1995 (6).

1.2 The role of inflammation in asthma

Asthma is a chronic inflammatory disease, where inflammatory cells are either recruited to the airway or activated at the site (11). The major cells which infiltrate the airway and are involved in cell to cell signalling include mast cells, macrophages, eosinophils, T lymphocytes, basophils, neutrophils, epithelial cells and dendritic cells (2, 4). Inflammation causes the symptoms of wheezing, coughing and chest tightness in susceptible individuals (4).

Airway epithelial cells and smooth muscle cells are themselves able to synthesise and release inflammatory mediators (11). However, T cells have an important role in the induction and maintenance of airway inflammation, particularly through their production of cytokines and chemokines (2). No inflammatory cytokine alone is responsible for all the characteristic effects of asthma (12). T helper cells can be subdivided broadly into T helper type 1 (Th1) and T helper type 2 (Th2) subsets, based upon their production of different kinds of cytokines (13-15). The Th1 cells produce interferon (IFN)-γ, tumour necrosis factor (TNF)-α, TNF-β, interleukin (IL)-2 and IL-12, while the Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (2, 13). It is thought that the Th2 cytokines (16), particularly IL-4, IL-5, IL-9 and IL-13 are the major inflammatory cytokines associated with asthma (12).

Increased circulating concentrations of IgE in asthmatics are a result of IL-4-stimulated synthesis by B cells (12, 17, 18). IL-4 also has an important role in the early stages of Th2 cell development (19) and contributes to the accumulation of macrophages, lymphocytes, eosinophils and neutrophils within the airway in mice, but does not appear to have a role in airway hyperreactivity (20, 21). In transgenic mice deficient in both IL-4 and IL-13, eosinophil infiltration, IgE and IL-5 production are abolished, while the absence of only one of these cytokines leads to a reduction in these processes (22).

Eosinophils are important inflammatory cells which may be responsible for many chronic effects in asthmatic patients (23). IL-5 has a role in promoting the activation and survival of eosinophils (24). Over-expression of IL-5 in transgenic mice results in eosinophilia in the blood and tissues including spleen, bone marrow and lymph nodes (25). Conversely, IL-5 knockout mice do not develop blood and tissue eosinophilia following infection or allergen challenge (26, 27) and do not show signs of lung damage or airway hyperreactivity normally observed with allergen challenge (27).

Anti-inflammatory cytokines may also have a role in the pathogenesis of asthma (28). IL-10, IL-12 and IFN-γ may play a role by suppressing Th1 cytokines (29), Th2 clone expansion (30) and Th2 cell differentiation, respectively (28). Asthmatic patients have reduced T cell production of IFN-γ which correlates with disease severity (31) and can be increased by glucocorticoid therapy (32). Production of IL-12 is also reduced in asthmatics (33). IL-12 may have a role in reducing IgE synthesis following allergen exposure (28). Reduced mRNA and protein expression of the anti-inflammatory cytokine, IL-10 is observed in alveolar macrophages of asthmatics and can be restored by glucocorticoid therapy (34). IL-10 inhibits eosinophil survival and the production of IL-4 and IL-5 (28, 35).

Asthma is a complex disease involving a large number of inflammatory mediators, which operate directly and through interactions with other cells.

1.3 The prevalence of asthma

The prevalence of asthma worldwide is on the rise, particularly among children (36) and it is also increasing in the pregnant population (37-39). Recent estimates in the United States, based on national health surveys, suggest that between 3.7 and 8.4% of pregnant women had asthma between 1997 and 2001 (39). This was increased from 3.2%

between 1988 and 1994 (39). Similarly, earlier reports in the literature suggested that between 1% and 4% of pregnant women in North America had asthma (40-42), while more recent studies report a prevalence of around 7% (37, 43). The large variation in prevalence statistics is partly due to differences in the methodology used to assess current asthma status, which may range from physician diagnosed asthma to whether the patient has experienced an episode of asthma or asthma attack in the previous 12 months. Kwon *et al.* found that of the women of child-bearing age who responded as having current asthma, only 61.3% also responded positively to having an episode of asthma in the previous year (39). Thus, the definition of current asthma is important in determining prevalence data. In 2001, the Centers for Disease Control and Prevention found that the self-reported asthma prevalence among women across the United States was 9.1% (44).

In Australia, the rate of asthma is one of the highest in the world (36, 45). A 1995 study from Western Australia found that 12.4% of pregnant women currently had asthma, although only 8.8% had an attack or used asthma medication during pregnancy (46). At the John Hunter Hospital (Newcastle, New South Wales), asthma was estimated to affect approximately 12% of women (1995-1998 obstetrics database, unpublished data). Asthma is the most common respiratory disorder to complicate pregnancy and therefore represents a significant public health issue. The high prevalence of asthma in Australia makes this an ideal place for research into the effects of asthma during pregnancy on fetal development.

1.4 The effect of asthma on pregnancy outcome

In 1961, Schaefer and Silverman stated that "The pregnant woman can be reassured that her asthma will have no bearing on her pregnancy or on the outcome of her delivery" (47). However, between 1950 and 1962 there were 19 maternal deaths associated with asthma and pregnancy in England and Wales alone (48). Indeed, in the decades which followed, numerous epidemiological studies have demonstrated that asthmatic women are more at risk of many poor pregnancy outcomes including preterm labour or delivery, low birth weight, delivery by caesarean section, gestational diabetes, chorioamnionitis and pre-eclampsia.

The following sections contain a detailed review of over 20 studies which have previously examined pregnancy outcomes in pregnancies complicated by asthma. The methodology used in each of these studies differs widely. Many are retrospective, while others are prospective. These studies have produced conflicting results and many have not used standardised treatment, clinical management or classification systems. My thesis will address these deficiencies in the existing literature on asthma and pregnancy, by using standard asthma management protocols to prospectively study pregnant women with asthma who have been well-characterised and classified both by disease severity and treatment, independently. My study will concurrently collect data from the mother, placenta and fetus, which is a unique approach in this field of study.

1.4.1 Population based studies

Most studies of asthma during pregnancy are population based, large epidemiological studies; some with little information available about asthma severity, disease progression and medication use during pregnancy (49). Studies based upon medical record review have several disadvantages including the possibility that mild asthma may not have been documented (42, 49, 50), with such cases possibly included in the control population (51). Nonetheless, these studies may more clearly reflect asthma as it is managed in the general population (42, 50). Possible confounders such as maternal smoking or socioeconomic status are not always present in administrative records (50) and coding or data entry errors are possible (42, 50, 52). Furthermore, since these studies rely on retrospective analysis of data, no possibility exists to study the mechanisms involved (43). Despite these drawbacks, the large number of subjects used in these studies give them more power to detect associations between maternal asthma and adverse pregnancy outcomes, which may then be followed up with smaller prospective studies. Most of these prospective studies take many years to conduct (53), especially when the incidence of asthma is small in the study population. A summary of the adverse outcomes detected in population based or prospective studies of maternal asthma during pregnancy is shown in Table 1.1. Pregnancy outcomes which were investigated, but were not found to be significantly associated with maternal asthma are also given in Table 1.1. Studies in which asthmatic women were divided primarily based upon medication use will be discussed in Section 1.6.

Table 1.1 Results of studies on adverse outcomes in pregnancies complicated by asthma

Author, year (reference)	Population	Asthmatic (n)	Control (n)	Definition of asthma	Poor outcomes associated with asthma	Poor outcomes not associated with asthma
Gordon *et al.*, 1970 (40)	New York, USA Prospective Study	277	30861	Actively treated asthma	Maternal mortality Perinatal mortality	Preterm labour Low birth weight Low Apgar score
Bahna & Bjerkedal, 1972 (54)	Norway Medical Birth Registry 1967-1968	381	112530	Self report of asthma before or during the pregnancy	Hyperemesis gravidarum Hemorrhage Toxemia Complicated labour Neonatal mortality Preterm delivery Low birth weight Hypoxia at birth	Still-birth Perinatal mortality Infant mortality
Stenius-Aarniala *et al.*, 1988 (55)	Finland Prospective study 1978-82	198	198	American Thoracic Society criteria	Pre-eclampsia Caesarean section	Gestational diabetes Premature rupture of membranes Hemorrhage Preterm labour Low birth weight Low Apgar score Congenital anomalies Perinatal mortality
Lao & Huengsburg, 1990 (56)	Hong Kong Retrospective study 1984-1987	87	87	History of asthma	Low birth weight Caesarean section	Postpartum hemorrhage Perinatal mortality Preterm delivery Post-term delivery Complicated labour
Mabie *et al.*, 1992 (57)	Tennessee, USA 1986-1989	200	22651	Documented in hospital records		Preterm delivery Low birth weight Pre-eclampsia
Perlow *et al.*, 1992 (41)	California, USA Case controlled study 1985-1990	183	130	Doctor diagnosis	Caesarean section for fetal distress Preterm labour/ delivery Premature rupture of membranes Low birth weight (steroid users) Gestational diabetes (steroid users)	Congenital anomalies Low Apgar score
Doucette & Bracken, 1993 (58)	USA Prospective study 1980-1982	32	3859	Self-report in 1st trimester	Preterm delivery	Low birth weight

Schatz *et al.*, 1995 (53)	**USA Prospective study 1978-1990**	**486**	**486**	**Spirometry & doctor diagnosis during study**		**Preterm labour/delivery Low birth weight Pre-eclampsia Perinatal mortality**
Jana *et al.*, 1995 (59)	**India Prospective study 1983-1992**	**182**	**364**	**Doctor diagnosis**	**Low birth weight (severe asthmatics requiring hospitalisation)**	**Gestational diabetes Antepartum hemorrhage Premature rupture of membranes Preterm labour Caesarean section Fetal distress Perinatal mortality**
Minerbi-Codish *et al.*, 1998 (60)	**Israel 1993-1994**	**101**	**77**	**Interview at 1 day post-partum**	**Respiratory & urinary tract infections Caesarean section (when presentation not cephalic)**	**Low birth weight Preterm delivery Pregnancy induced hypertension Gestational diabetes Low Apgar score**
Demissie *et al.*, 1998 (38, 42)	**New Jersey, USA Historical cohort study 1989-1992**	**2289**	**9156**	**Hospital records**	**Low birth weight Preterm labour/delivery Congenital anomalies Prolonged infant hospital stay Placenta previa Pre-eclampsia Caesarean section Transient tachypnea of the newborn**	
Alexander *et al.*, 1998 (37)	**Nova Scotia, Canada Retrospective cohort study 1991-1993**	**817**	**13709**	**Self-report at hospital admission**	**Antepartum hemorrhage Postpartum hemorrhage Infant hyperbilirubinemia**	**Low birth weight Neonatal respiratory distress syndrome (RDS) Congenital anomalies Pregnancy induced hypertension Caesarean section**
Kallen *et al.*, 2000 (61)	**Sweden Medical Birth Registry 1984-1995**	**15512**	**36985**	**Midwife interview & hospital discharge records**	**Preterm delivery Low birth weight Prolonged pregnancy (>41 weeks) Pre-eclampsia Gestational diabetes Infant hypoglycemia**	**Congenital anomalies**

Liu *et al.*, 2001 (49)	**Quebec, Canada Retrospective cohort study 1991-1996**	**2193**	**8772**	**Hospital records**	**Preterm birth Small for gestational age neonates Large for gestational age neonates Hypertension Chorioamnionitis Caesarean section**	**Prolonged hospital stays Congenital anomalies**
Wen *et al.*, 2001 (50)	**Canada Historical cohort Study 1989-1996**	**8672**	**34688**	**Hospital records**	**Preterm labour Pre-eclampsia Gestational diabetes Premature rupture of membranes Amniotic infection Caesarean section Hemorrhage**	**Fetal death**
Sobande *et al.*, 2002 (62)	**Saudi Arabia (high altitude) Prospective case control study 1997-2000**	**88**	**106**	**Emergency room admission for asthma**	**Low birth weight Low placental weight Pre-eclampsia Spontaneous abortion Induced labour Caesarean section Perinatal mortality Congenital anomalies Low Apgar score**	**Preterm labour**
Sorensen *et al.*, 2003 (63)	**USA Case control study 1994-1995**	**20 (preterm) 14 (term)**	**292 (preterm) 410 (term)**	**Lifetime history of asthma diagnosis**	**Preterm delivery**	
Mihrshahi *et al.*, 2003 (51)	**Sydney, Australia Prospective Study (>36 weeks gestation only)**	**340**	**271**	**Doctor or hospital diagnosis**	**Hypertension**	**Low birth weight Gestational diabetes Complicated labour/delivery Low Apgar score**
Bracken *et al.*, 2003 (64)	**Connecticut & Massachusetts, USA Prospective study 1997-2001**	**832**	**1266**	**Lifetime history of doctor diagnosed asthma**	**Preterm delivery (related to treatment) Intrauterine growth restriction (IUGR) (related to symptoms)**	
Dombrowski *et al.*, 2004 (65)	**USA Prospective study**	**1739**	**881**	**Doctor diagnosis**	**Neonatal sepsis (mild asthma group) Caesarean section (moderate and severe asthma group)**	**Preterm delivery**

The first large study of pregnant asthmatic women was published by Gordon *et al.* in 1970 (40). Only patients with actively treated asthma were included in their analysis (n=277) and 16 of these patients had severe asthma characterised by regular attacks during pregnancy. When corrected for ethnic background, there was no increase in the incidence of preterm delivery or low birth weight in asthmatic mothers. The most striking finding of this study was the relatively large number of maternal (n=5) or perinatal deaths (n=16) from asthmatic mothers, which were more likely to occur in the severe asthmatics. Infants from this study were examined after birth and at 8 months of age, but no differences in Apgar scores, or psychological parameters were observed at these times. However, at one year of age, neurological examination revealed a tendency for more abnormalities to be found in infants of asthmatic mothers. Interestingly, seven times as many infants from asthmatic mothers compared to control mothers, had developed asthma within the first year of life (40).

Following soon after Gordon *et al.*'s study was that of Bahna and Bjerkedal which used the Norwegian medical birth registry (1967-1968) to examine the pregnancies of 381 asthmatics and more than 112000 controls who did not suffer from any diseases before or during pregnancy (54). Pregnancy complications including hyperemesis gravidarum, hemorrhage and toxemia were more frequent in asthmatic patients, as were interventions during labour, induced labour and complicated labour. There was a higher rate of neonatal mortality, low birth weight, premature birth and hypoxia at birth in infants from asthmatic mothers. A disadvantage was that information about asthma treatment was not available in this study (54). Numerous other groups have since carried out retrospective studies to examine adverse pregnancy outcomes in asthmatic women, with conflicting results.

Lao and Huengsburg studied 87 asthmatic patients who delivered between 1984 and 1987 in Hong Kong (56). Many of these patients (n=33) did not require medication for asthma and were considered to be in remission during the study period. All other patients were treated with bronchodilators and some with oral or inhaled steroids. Mothers with asthma were significantly more likely to have a low birth weight baby, epidural analgesia or a caesarean section. Taking into account asthma treatment, those women who did not use any medication had a higher incidence of low birth weight, and those taking medication had a higher incidence of caesarean section (56).

A self-report questionnaire administered in Italy in 1987 found that maternal asthma was a risk factor for low birth weight (66). However, when other variables were considered, this relationship was only found to hold in male infants, mothers who smoked and those who lived in an industrial town, suggesting that other risk factors contribute to the effect of maternal asthma on pregnancy outcome (66).

The effects of asthma and asthma medication on pregnancy outcome were examined in a Californian perinatal database study between 1985 and 1990 (41). There were 31 steroid dependent asthmatic women and 50 non-steroid dependent asthmatic women and 130 controls were selected from the reference population. Asthmatics who used steroids were more likely to have or develop diabetes during pregnancy. Asthmatics of both groups were more at risk of caesarean section, preterm labour or delivery and preterm premature rupture of the membranes (PPROM), compared to the control group. A statistically significant increase in low birth weight was only observed in the steroid dependent asthmatics, although there was a trend for this in the non-steroid dependent asthmatics also. Patients who only used over the counter medications were excluded and thus, this study represented a group of more severe asthmatics (41).

Two studies from New Jersey, based upon analysis of hospital records between 1989 and 1992 have examined neonatal outcomes (38) and maternal and pregnancy outcomes (42) in asthmatic women. Data from 2289 asthmatic women were collected and compared to 9156 control subjects. Maternal asthma was significantly associated with low birth weight, preterm delivery, small for gestational age (SGA) neonates, congenital anomalies and prolonged infant hospital stay. After adjustment for potential confounders, asthmatic mothers also had an increased risk of preterm labour, placenta previa, caesarean section, prolonged hospital stay (greater than the median of 3 days) and hypertensive disorders of pregnancy, including pre-eclampsia (42). An analysis of almost 25000 pregnant women in Canada also found a significant association between pregnancy induced hypertension (PIH) and asthma which was treated with inhaled steroids during pregnancy (67).

Transient tachypnea of the newborn is a condition causing respiratory distress in the neonate and has been linked to the development of asthma in childhood (38, 68). Demissie *et al.* examined this neonatal outcome in the same group of asthmatic patients used to examine other pregnancy outcomes (42). There was no increased risk of

respiratory distress syndrome (RDS) or neonatal or infant death in the asthmatic population (38). Neonates from asthmatic mothers were more likely to have transient tachypnea after accounting for confounding risk factors such as caesarean delivery and premature birth. This association was stronger for male infants than female infants of asthmatic mothers, possibly because male sex is a known risk factor for this condition, as well as others such as RDS (69), due to differences in fetal lung maturation between the sexes (70).

Schatz *et al.* (1991) earlier described an increased risk of transient tachypnea of the newborn, but not RDS, in a prospective study of 294 asthmatic women and 294 controls (71). This study did not find a link with either asthma severity or medication use. The association is of interest, since transient tachypnea of the newborn has previously been shown to be related to a higher incidence of asthma and atopic symptoms at 5 years of age (68). It is possible that there are links between the *in utero* environment in asthmatic pregnancies and the risk of developing childhood asthma in the offspring, independent of genetic factors. This is demonstrated by the fact that the development of atopy in children is more closely associated with maternal asthma or IgE levels rather than paternal asthma or IgE (72-74). Therefore, as well as the immediate implications of poor pregnancy outcomes on the offspring of asthmatic mothers, there may be long-term implications for these children. Some studies have investigated the long-term effects of asthmatic pregnancies on offspring and found an increase in the incidence of left-handedness (75), wheezing at 15 months of age (71) and childhood respiratory diseases in general (76). Others have found no long-term developmental effects (77). Investigating the longer-term outcomes of pregnancies complicated by asthma is beyond the scope of this thesis, but is being followed up in our Australian cohort by Dr Vicki Clifton and colleagues.

Mabie *et al.* examined aspects of asthma in 200 pregnancies in Tennessee from 1986 to 1989 by medical record review (57). There was no increased rate of preterm delivery or low birth weight among asthmatic women compared to the general population rates, which were very high (17.7% and 6.3% respectively). However, intrauterine growth restriction (IUGR) was significantly more likely in women with moderate or severe asthma, who required hospitalisation during pregnancy, compared to women with mild asthma, who were not hospitalised for asthma during pregnancy. The caesarean section rate and incidence of postpartum exacerbations were also significantly increased in

moderate and severe asthmatics compared to mild asthmatics. Interestingly, this group found that asthmatic women who had a caesarean delivery were 18 times more likely to have exacerbations of asthma postpartum compared to asthmatics who had a vaginal delivery. The mechanism for this effect is unknown (57).

A retrospective cohort study from Nova Scotia, Canada, studied 817 asthmatic women and 13709 non-asthmatic women between 1991 and 1993 (37). Medical records were examined to assess medication use, which may be subject to errors, due to underestimation or overestimation by pregnant patients. Overall, the prevalence of asthma in this population increased from 4.8% in 1991 to 6.9% in 1993. Over 45% of asthmatic women did not use any medication to treat asthma, while 37.1% used β_2-agonists and 17% used steroids. Regardless of medication use, asthmatic women were found to be at increased risk of antepartum or postpartum hemorrhage, possibly due to alterations in platelet function in asthmatics (78, 79). In addition, neonates from asthmatic mothers who used steroids were found to be at increased risk of hyperbilirubinemia (37). This outcome was also examined by Stenius-Aarniala *et al.*, who did not find an alteration in neonatal risk for hyperbilirubinemia in either mild or severe asthmatics (55).

The effect of maternal asthma on pregnancy outcomes was examined in a population based study, using administrative data available from the Canadian Institute for Health Information from 1989 to 1996 (50). Maternal asthma was associated with all adverse outcomes examined; namely, preterm labour, PIH, pre-eclampsia, gestational diabetes, antepartum hemorrhage, membrane disorders (including PPROM), postpartum hemorrhage and caesarean delivery. The association of maternal asthma with preterm labour, abruptio placenta, PPROM, intra-amniotic infection and fetal death was found to be stronger in teenage mothers than adult mothers (50). Apter *et al.* also examined adolescents with severe asthma and found a high rate of exacerbations, hospitalisations and emergency room visits in these patients, which were associated with respiratory tract infections and lack of medication compliance (80). However, no evidence of an increased rate of PIH, preterm delivery or IUGR was found in asthmatic adolescents compared to general estimates for adolescent pregnancies (80).

A study of 2193 asthmatic and 8772 non-asthmatic singleton pregnancies in Quebec was carried out from 1991 to 1996 (49). After adjusting for confounders such as

maternal age and pre-existing diabetes or hypertension, this study found that maternal asthma was associated with an increased incidence of preterm labour and delivery, SGA and large for gestational age neonates, PIH, chorioamnionitis, abruptio placentae and caesarean delivery. Interestingly, this group also analysed data separately based upon fetal sex and found that the risks of preterm birth and pre-eclampsia were higher in asthmatic women pregnant with a female fetus, compared to asthmatic women pregnant with a male fetus. The prevalence of asthma in this population was very low and similar among women pregnant with a female fetus (0.52%) and women pregnant with a male fetus (0.51%) (49). The cause of the increased risk to the female fetus is unknown and has not been examined in any other studies.

Kallen *et al.* (2000) examined the effect of asthma on pregnancy outcomes using the medical birth registry and the hospital discharge register in Sweden (61). The medical birth registry identified women with asthma from midwife interviews during the first trimester, while a combination of this and the hospital discharge register was used to identify women who had been hospitalised for asthma and also had a pregnancy between 1984 and 1995. Asthmatic women identified by either or both of these methods were found to have an increased risk of preterm delivery, low birth weight, or prolonged pregnancy (>41 weeks gestation) (61). The approach used in this study would have identified women with very mild asthma as well as those with severe asthma requiring hospitalisation. However, despite this, the study did not separate subjects based on disease severity which may have been a contributing factor to the outcomes examined.

A retrospective analysis of medical records by Beckmann for the period 1992 to 1997 in the USA assessed outcomes in 782 asthmatic women (43). Over 90% of these women were mild asthmatics (according to hospital records) and almost half did not use any asthma medication during pregnancy. Only 5.6% of the asthmatics used a β_2-agonist and inhaled steroid for treatment. Lung function was assessed by peak flow measurements in each trimester in a small number of women and did not change during pregnancy, as has previously been described in pregnant women without asthma (81). There was an increased incidence of meconium staining, preterm labour and oligohydramnios among asthmatic women. This study lacked the numbers to be able to convincingly demonstrate a relationship between steroid use and outcomes associated with altered placental function, such as IUGR, PIH and oligohydramnios (43).

In 1993, over 1000 parents of children aged 5-11 years were surveyed in the United Kingdom with regard to the child's history of respiratory disease and pregnancy related factors including birth weight and preterm delivery (76). Preterm birth was significantly more likely to be reported when the mother was asthmatic, but not when the father was asthmatic, compared to children of non-asthmatic parents. An obvious drawback of this study was the potential for recall errors on the part of the parent, given the amount of time that had passed since the pregnancy. In addition, preterm birth was assessed by asking the question "Was your baby born prematurely?" which could lead to errors as a result of the participant's misunderstanding of the clinical definition of preterm birth. Maternal smoking was an additional risk factor for preterm delivery and no association was found between parental asthma and low birth weight (76).

The risk of preterm delivery in asthmatic women was recently assessed in the USA by Sorensen *et al.* (63). Women participating in a prenatal screening program between 1994 and 1995 were included in this case control study. The prevalence of asthma, defined as a lifetime history of asthma diagnosis, was compared in 312 women who delivered preterm and 424 control subjects who delivered at term. Significantly more preterm cases had a positive asthma history (6.4%) compared to control cases (3.3%), after adjustment for multiple confounders. A similar study was previously reported by Kramer *et al.*, with histories of asthma symptoms or asthma diagnosis more commonly associated with idiopathic preterm labour and idiopathic recurrent preterm labour (82). However, the risk of preterm labour was not associated with increased serum IgE or altered response to inhaled methacholine challenge in the mother (82). A two-fold increase in the risk of preterm labour or delivery with no increased risk of low birth weight was also reported by Doucette and Bracken (58).

The results of these population based studies indicate that maternal asthma is a risk factor for some poor pregnancy outcomes, particularly low birth weight, preterm labour or delivery, PIH or pre-eclampsia.

1.4.2 Prospective studies

There are few studies which have prospectively examined pregnant women with asthma in order to study the effects of asthma on pregnancy outcome and the changes in asthma which occur during pregnancy. These studies have the distinct advantage of being able

to assess lung function, treatment and asthma symptoms during pregnancy, while the intervention and/or close follow-up of asthmatic women ensures that asthma is well managed throughout pregnancy and is often well-characterised (53). However, due to the smaller sample sizes, significant associations between maternal asthma and adverse outcomes are frequently not observed. While the intervention itself may alter the risk of an adverse outcome, this remains the most ethical approach to asthma management. One problem when comparing prospective studies is that each population of asthmatics examined varies with regard to steroid use, general treatment and asthma severity, with some studies focussed on mild asthmatics and others on women with severe asthma. This makes comparison between studies difficult. In addition, standard classification systems are often not employed and the criteria used to assess disease severity differ between studies.

A prospective study of asthmatic mothers was conducted by Stenius-Aarniala *et al.* in Finland between 1978 and 1982 (55). The study followed 181 asthmatic women during pregnancy, with 17 of these women having two pregnancies in the study. Data on the control population was obtained retrospectively from labour records and subjects were matched for age, parity and date of delivery. One of the disadvantages of this study was the fact that only 20% of study subjects were recruited during the first trimester, with as many as 26% of subjects recruited in the third trimester, making it difficult to follow changes in asthma during pregnancy. However, an advantage was that subjects were classified according to asthma severity as very mild, mild, moderately severe or severe. In addition, skin-prick tests and serum IgE was used to assess atopy in these women, with 62% being classified as atopic. Of these women, 59% had very mild or mild asthma. During pregnancy, 42% of subjects required more maintenance therapy than before pregnancy, while 18% required less therapy than before pregnancy. Postpartum, 26% of subjects reported a worsening of asthma, while 34.5% reported an improvement. With regard to pregnancy outcomes, this study found a significantly higher incidence of pre-eclampsia in asthmatics (14.6%) compared to control subjects (4.5%). Mild pre-eclampsia occurred more often in women with severe asthma (29%) compared to women with very mild asthma (8.7%). The use of systemic steroids may also have contributed to the high frequency of pre-eclampsia, which was 25% in asthmatic women using systemic steroids and 10% in asthmatic women who did not use systemic steroids.

Asthmatic subjects also had a higher rate of caesarean section, but no differences in perinatal outcome, including birth weight, were found (55).

Over many years, Schatz and colleagues have performed the most comprehensive studies of the effects of asthma on pregnancy outcome as well as the effects of pregnancy on asthma progression. This group prospectively managed asthmatic women during their pregnancies, measured lung function by spirometry at several times and related these measurements to pregnancy outcome (83). In 352 asthmatic women who had at least three lung function measurements made during pregnancy, there was a correlation between mean percent predicted FEV_1 and birth weight. Subjects with an FEV_1 in the lowest quartile (<83% predicted) were significantly more likely to have an infant with a birth weight in the lowest quartile (<3150 g) or a ponderal index <2.2, indicative of asymmetric IUGR (84, 85). This finding was not related to steroid use. There was no relationship between low FEV_1 and preterm delivery, PIH or pre-eclampsia. A later publication by this group on 486 women with actively managed asthma and 486 controls, found no significant differences in the incidences of pre-eclampsia, perinatal mortality, low birth weight, IUGR, preterm delivery or congenital malformations (53). This study was conducted over a period of 12 years and the asthmatic subjects were well-characterised and their asthma was actively managed. A further advantage was that control subjects also underwent pulmonary function testing, and were matched for maternal age, parity, smoking and delivery date (53). Despite the negative findings regarding adverse perinatal outcomes, many prospective studies from this group have provided valuable information about the physiological mechanisms which may be involved in altering outcome and course of asthma during pregnancy.

Jana *et al.* examined 182 pregnancies complicated by asthma in India between 1983 and 1992 and compared outcomes to 364 non-asthmatic pregnancies (59). Most women had well controlled asthma (90.5%) and were using medications including oral or inhaled β_2-agonists, theophylline or steroids. There was no significant increase in the rate of preterm labour, low birth weight, caesarean section, perinatal mortality, hemorrhage or fetal distress in the asthmatic group compared to the control group. However, 15 of the asthmatics had a severe asthma attack during pregnancy which required hospitalisation and in these women there was a significant reduction in birth weight (59).

A prospective study in Israel comparing asthmatic mothers (n=101) and control mothers (n=77), collected data by interview at 1 day postpartum and from medical records (60). Asthmatic women were classified as mild (no inhaled steroid use), moderate (inhaled steroid use, no hospitalisations for asthma) or severe (inhaled steroid use with periodic or permanent systemic steroid use and possibly hospitalisations for asthma). Many women believed their asthma worsened during pregnancy (38%), while 20% believed it had improved. Significantly more asthmatic women suffered from urinary tract or respiratory infections (30.6% of women with mild or moderate asthma and 68.8% of women with severe asthma) compared to non-asthmatic women (approximately 5%). The marked effect of severity on infections may be related to suppression of the immune system following prolonged glucocorticoid use. There was no significant effect of asthma on other outcomes including preterm delivery, gestational age, birth weight and PIH (60).

Pregnancy outcome of asthmatic patients residing at high altitude in Saudi Arabia has been studied (62). Sobande *et al.* hypothesised that the low oxygen environment at high altitude may further contribute to complications associated with asthma and pregnancy through a worsening of asthma. The pregnancies of 88 asthmatic women and 106 non-asthmatic women were studied between 1997 and 2000. Asthmatic patients were managed by a medical specialist and were treated with the β_2-agonist, salbutamol alone (n=57), in combination with oral theophylline (n=20) or with oral prednisolone (n=11). Asthmatic pregnancies were more likely to be complicated by pre-eclampsia, congenital malformations, low Apgar score or perinatal mortality and mean birth weights and placental weights were significantly reduced in asthmatics compared to non-asthmatics. Gestational age at delivery was not different between the groups. Asthma control was considered poor in 17% of asthmatic subjects. It is possible that the hypoxic environment contributed to an amplification of poor outcomes in these asthmatic women. However, women were selected for the study because they had visited the emergency room with asthma while they were pregnant, and the outcomes may simply have been observed due to the severity of the asthma. No comparison with a similar group of asthmatic women at low altitude was made (62) and thus the effect of high altitude on maternal asthma could not be properly examined.

As part of the childhood asthma prevention study in Sydney, Australia, pregnant women with physician diagnosed asthma, and non-asthmatic pregnant women whose partners or

other children had asthma, were prospectively studied (51). In this study, women were recruited at 36 weeks; hence there was no evaluation of the effect of asthma on preterm labour or delivery. Of the 340 asthmatic women, 31% did not use any medication to control asthma during pregnancy, while 35% of women used short-acting β_2-agonists alone and 31% used inhaled steroids. This study was complicated by the fact that several (21 out of 271) non-asthmatics were using short acting β_2-agonists for wheezing during pregnancy, despite no previous doctor diagnosis of asthma. These women may have had mild asthma, but were not assessed during the study or excluded from analysis. Hypertension was significantly increased in the asthmatic group compared to the non-asthmatic group, after adjustment for confounders. There was no significant effect of asthma on other outcomes including pre-eclampsia, gestational diabetes, induced labour, caesarean delivery or any neonatal outcomes, including birth weight, which the authors conclude suggests that when the asthmatic pregnancy progresses to term there may be no major problems for the neonate (51).

A recent prospective study from the USA examined pregnancy outcomes in 832 asthmatic women and 1266 non-asthmatic controls (64). Asthma was defined as a lifetime history of doctor diagnosed asthma, and symptoms and medication use during pregnancy were recorded and each rated as intermittent, mild persistent, moderate persistent or severe persistent, according to the 2002 global initiative for asthma guidelines. Asthmatic women using at least two controller medications including an inhaled glucocorticoid (plus long acting β_2-agonist or theophylline or leukotriene inhibitor and/or oral glucocorticoid) had a three-fold increase in risk of preterm delivery. The use of oral steroids daily resulted in a 2.2 week decrease in gestational age at delivery, while the use of theophylline daily resulted in a 1.1 week reduction in gestational age at delivery. There was no relationship between symptom scores and preterm delivery risk. However, there was an increased risk of IUGR in asthmatic women with high symptom and severity scores (symptoms daily) but no association with treatment. Interestingly, women who had not been diagnosed as asthmatic by a doctor, but who were experiencing symptoms and/or using medication for asthma were also found to be at significantly increased risk for IUGR compared to non-asthmatic women (64).

The results of these prospective studies have not always supported epidemiological findings. However, associations between asthma and pre-eclampsia, and asthma and

low birth weight have been demonstrated. The association between maternal asthma and reduced fetal growth seems to be associated mostly with hospitalisations (59, 62), or reduced lung function in the mother (83). Fetal sex may be a confounder, and studies reporting no adverse perinatal outcomes may have done so due to a lack of separate data analysis for women pregnant with male and female fetuses. There are deficiencies in classification approaches in some prospective studies. Classification at enrolment (65), rather than constant monitoring of women, and classification which considers asthma throughout pregnancy is one difference between studies. In some studies, control patients were not assessed for the absence of asthma (51, 64). The use of standard classification systems and close monitoring of all patients, will improve the quality of data obtained from prospective studies of asthma and pregnancy.

1.4.3 Possible mechanisms for the effect of maternal asthma on pregnancy outcomes

Despite the conflicting results, it is apparent that asthmatic women may be at increased risk of poor pregnancy outcomes. However, the majority of the studies discussed did not directly examine the possible mechanisms involved, with the exception of the study of Schatz *et al.*, where maternal lung function was measured and related to birth size (83). Nonetheless, the following mechanisms have been presented by several authors to explain the adverse pregnancy outcomes observed with maternal asthma (42, 49, 50, 53, 61, 63). A common pathway leading to hyperactivity of the smooth muscle in both the bronchioles and the myometrium has been proposed to explain the increased incidence of preterm labour in asthmatics (58, 82, 86). Bertrand *et al*. initially suggested this mechanism after finding evidence of airway hyperreactivity in the mothers of premature infants (86), but this was not found by another group examining airway responsiveness in mothers of premature or low birth weight children (87). At least one study has suggested an additional risk of prolonged pregnancy in asthmatic women (61), which could not be explained by this same mechanism.

Hypoxia has been proposed to be a contributor to low birth weight, pre-eclampsia, congenital malformations, spontaneous abortions and placenta previa in asthmatic women (62). Hypoxemia or reduced PO_2 is a feature of acute severe asthma or status asthmaticus (88-90) and of maternal smoking (91). A small decrease in maternal PO_2

can have serious effects on the fetus (92), since the slope of the fetal oxygen dissociation curve is steep in the 50% oxygen saturation range (93). McClure and James demonstrated that administration of oxygen to mothers in labour resulted in increased umbilical cord O_2 values at delivery, suggesting that there is a relationship between maternal and fetal oxygen (93). However, maternal hypoxia during asthmatic pregnancies has not previously been directly investigated in relation to fetal outcome.

The release of bioactive mediators, such as inflammatory products could also be involved in these mechanisms. Poor pregnancy outcomes including low birth weight and preterm delivery are also features of many other inflammatory diseases including rheumatoid arthritis (94-96), malaria (95, 97-99), systemic lupus erythematosus (100), inflammatory bowel disease (101-103) and periodontal disease (104, 105). Moreover, elevated maternal serum levels or placental gene expression of inflammatory cytokines has also been associated with IUGR (98, 106-109). Placental membrane inflammation (histological chorioamnionitis and funisitis) was increased in HIV positive women and correlated with the high risk of preterm delivery (110). Bowden *et al.* found that women with active inflammatory arthritis during pregnancy had smaller babies at birth and up to 8 months of age compared to healthy control women or women whose disease was in remission (95). These data indicate that active inflammation during pregnancy may contribute to low birth weight. Despite this evidence, an inflammatory mechanism for reduced fetal growth has not previously been examined in asthmatic pregnancies, but will be investigated in this thesis.

Asthma treatment, particularly with inhaled or systemic steroids has been widely investigated as a possible mediator of the adverse effects and will be discussed further in Section 1.6. Smoking, a contributor to low birth weight, has consistently been reported to be more common among asthmatics than non-asthmatics (37, 38, 43, 46, 51, 60, 61). However, most studies have found that maternal smoking does not fully explain the association between asthma and adverse pregnancy outcomes.

In this thesis, maternal asthma severity, inflammation, lung function and treatment with inhaled steroids will be examined in relation to fetal growth in asthmatic pregnancies. This an important area to research given the discrepancies in results for low birth weight risk with maternal asthma and the lack of mechanistic data related to this outcome. Uniquely, this study of asthma and pregnancy at the Mothers and Babies Research

Centre (Newcastle, Australia) is the first to examine possible placental mechanisms contributing to adverse outcomes in pregnancies complicated by asthma. Initial studies involved an examination of placental blood flow in asthmatic pregnancies using a perfusion method (111). The results showed that corticotropin releasing hormone (CRH)-induced vasodilation and potassium chloride (KCl) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced vasoconstriction were significantly reduced in placentae collected from women with moderate and severe asthma, but unaffected in placentae from women with mild asthma compared to a non-asthmatic control group (111). Further investigation into alterations of placental function in asthmatic pregnancies is the subject of this thesis.

The large number of adverse outcomes associated with asthma suggests there is a complex interaction of factors associated with the disease and possibly its treatment which may alter normal maternal physiology during pregnancy.

1.5 The effect of pregnancy on asthma severity

Physiological changes in the respiratory system occur during pregnancy. Specifically, alterations in pulmonary function and maternal-fetal gas exchange occur as a result of the presence and growth of the fetus as well as alterations in circulating hormones during the pregnancy. Although lung function as measured by FEV_1, PEFR, vital capacity (VC) or FEV_1:FVC does not appear to change significantly during pregnancy in asthmatics or non-asthmatics (43, 81, 112-116), there may be a decrease in the total lung capacity and in residual volume and functional residual capacity (112, 117, 118). Beginning in the first trimester and continuing throughout pregnancy, there is a significant rise in the minute ventilation or amount of gas expelled from the lungs each minute (112, 117-120). This is due to an increased tidal volume and unchanged respiratory rate (121-123), which contributes to dyspnea (124, 125) in up to 75% of women (112, 126). These changes are thought to be influenced by increases in circulating progesterone (117, 127, 128), since progesterone administration to non-pregnant adults resulted in an increased sensitivity of the respiratory centre, in a similar manner to that observed in pregnant women (127). As a consequence of altered minute ventilation, respiratory alkalosis occurs with an increase in blood pH to 7.40-7.47 (129, 130). In asthmatic women, any changes in FEV_1 or FEV_1:FVC during pregnancy are likely to be due to asthma itself, rather than normal changes associated with pregnancy

(122, 123). In addition, changes in PO_2 or PCO_2 may be more severe in a pregnant patient than a non-pregnant patient (120, 123, 131). The normal alkalosis of pregnancy may be further aggravated by maternal asthma, potentially resulting in alterations in placental blood flow and oxygen supply to the fetus (122). There has been one report of maternal alkalosis following hospitalisation with severe asthma which resulted in fetal demise and still-birth (132). It is important to understand how maternal asthma changes during pregnancy since this could impair fetal gas exchange and seriously affect fetal development.

The consensus has remained for many years that one third of women experience a worsening of asthma during pregnancy, one third improve and one third remain unchanged (133, 134). A variety of methods have been used to obtain this data, including subjective patient questionnaires, daily symptom recording and objective measures of lung function. Few studies have employed more than one type of analysis of maternal asthma alterations during pregnancy. In 1964, Fein and Kamin assessed asthma severity according to requirements for treatment and reported that 12% of subjects improved, 21% worsened and 67% remained the same (135). Gibbs *et al.* reported that 43% of asthmatics improved, 18% deteriorated and 39% remained the same during pregnancy (136). Despite the variation in results reported by individual studies, it is clear that pregnancy itself can have a major impact on asthma in some women. Cases of severe life threatening asthma requiring first trimester termination have been reported and an improvement in maternal asthma within 24 hours of termination has been observed (137, 138). However, the course of an individual woman's asthma during pregnancy remains unpredictable. Therefore, an understanding of the mechanisms which contribute to worsening or improved asthma during pregnancy is important for ensuring the best outcome for both mother and baby and this issue will be addressed in this thesis.

In 1976, Sims and De Swiet performed lung function tests on asthmatic women during pregnancy and post-partum (115). With small study numbers, they found that there were no pregnancy-related changes in FEV_1:VC ratio (an indicator of bronchospasm), or VC in either non-asthmatic or asthmatic women. Asthmatic women with abnormal spirometry had a tendency to improve post-partum, but this was not statistically significant (115).

Improvement in asthma during pregnancy has been reported by Juniper *et al.* (116). This study was conducted with 16 subjects, who were recruited prior to conception. They demonstrated an overall improvement in airway responsiveness to methacholine challenge in the second trimester compared to pre-conception, but no significant changes in FEV_1 or FEV_1:VC were observed. There was individual variation between the subjects, with the majority showing an improvement in objective measures and clinical symptoms of asthma during pregnancy, with a reversion to pre-pregnancy status post-partum. However, no relationship between airway responsiveness and serum progesterone or estriol concentrations was found (116, 139).

Schatz *et al.* examined the progression of asthma in 330 women who recorded symptoms, wheezing and sleep/activity interference daily during pregnancy and up to 12 weeks post-partum (134). Women subjectively rated their asthma as having improved, remained the same or worsened during the pregnancy. In women whose asthma worsened, there was a significant increase in the number of days of wheezing and interference with sleep and activity between 25 and 32 weeks gestation. In asthmatic women who felt their asthma improved during pregnancy, there was a decrease in wheezing and little change in interference with sleep or activity between 25 and 32 weeks gestation. In all women, there was a fall in wheezing and interference with sleep and activity between 37 and 40 weeks. Most women who felt their asthma worsened had a subjective improvement post-partum, with significantly fewer days of wheezing at 5-12 weeks postpartum compared to 29-32 weeks. Conversely, most women who felt their asthma improved during pregnancy had a subjective worsening of asthma after pregnancy, with significantly more days of interference of activity in this period compared to 29-36 weeks gestation (134).

Some women were assessed in two successive pregnancies and 60% followed the same course of asthma (worse, improved or the same) in the second pregnancy as the first (134). Williams reported a similar finding in 1967, with 63% of women followed for more than one pregnancy having a similar change in asthma each time (48). Early reports suggested that women whose asthma was not affected by pregnancy have "pollen-sensitive" asthma, those who improve during pregnancy have "ovarian asthma" (improved by the high hormone environment of pregnancy) and those who worsen during pregnancy must be sensitive to a product of the conceptus (140, 141). In the study from Schatz *et al.*, a substantial minority of patients did not follow the same

course of asthma in subsequent pregnancies, suggesting that there must be a determinant of asthma which differed in the different pregnancies (134). A further study from this group has examined maternal and pregnancy characteristics such as smoking, maternal body weight, fetal sex, season of delivery and changes in nasal symptoms in pregnancy, to determine whether any of these factors may be causing the pregnancy-associated changes in asthma (142). Season of pregnancy or delivery has not been found to effect asthma progression (48, 142). Kircher *et al.* found that only the course of rhinitis during pregnancy correlated with the course of asthma during pregnancy (142). Rhinitis worsened or improved in more than 50% of patients whose asthma had also worsened or improved, respectively (142). This was not surprising, since there may be common systemic inflammatory mechanisms in asthma and rhinitis (143). The authors suggest that factors such as IgE which affect both the upper and lower airways may be important in changes that occur in asthma during pregnancy (142). Another group found that cockroach-specific IgE levels in serum were linked to clinical asthma severity during pregnancy, and may be useful as a predictive measure (144). Early studies from Gluck and Gluck (1976) also found a correlation between an increase in serum IgE and worsening asthma during pregnancy (133).

Similar trends on asthma progression during pregnancy have been obtained from other studies (48, 56, 145). Lao and Huengsburg reported that amongst treated asthmatics, 38.9% had no change, 29.6% had an increase and 31.5% had a decrease in the frequency and severity of symptoms or attacks during pregnancy (56). When compared to the group whose asthma did not change, asthmatic women who reported a worsening of asthma during pregnancy had a lower (not significant) percent predicted PEFR, while those women who reported an improvement in asthma during pregnancy had significantly higher percent predicted PEFRs (56).

Data obtained in Western Australia indicated that during pregnancy, 16.4% of women experienced improved asthma, 35.4% experienced worsening asthma, 35.4% experienced no change in asthma and the remainder were unable to comment (46). Wheezing or asthma attacks were experienced by 62% of women during pregnancy. A large number of asthmatics were also smokers in this study, although smoking was not related to the changes in asthma during pregnancy (46).

Hospitalisation of asthmatic patients during pregnancy has been reported to occur in 1.6% of patients and emergency room visits in 12.6% of patients (134). Wendel *et al.* reported that, with the use of objective pulmonary function tests, 62% of exacerbations during pregnancy required hospitalisation of the asthmatic patient (146). Stenius-Aarniala *et al.* found that 9.3% of subjects had an acute asthma attack during pregnancy and this was more common in women who did not use inhaled steroids (147). They concluded that a mild attack of asthma, if promptly treated does not affect pregnancy or perinatal outcome (147).

Several studies indicate that women with severe asthma are more likely to show signs of worsening asthma during pregnancy than women with milder asthma (48, 133, 148). In a recent study from Schatz and colleagues, the relationship between asthma severity classification and subsequent changes in asthma during pregnancy was assessed in over 1700 pregnant asthmatics (148). Exacerbations of asthma occurred in over half of all severe asthmatics, while only 12% of patients with mild asthma had exacerbations during pregnancy. Re-classification of asthma from mild to either moderate or severe occurred in 30% of patients, while only 23% of patients who were initially moderate or severe were later re-classified as mild. Asthma morbidity, encompassing hospitalisations, symptoms, steroid requirements and unscheduled doctor visits, was found to be closely related to the pregnancy classification of asthma (148).

There is little evidence that labour and delivery themselves have any major effect on maternal asthma. If an acute attack occurs at this time, normal medication use is recommended (149). The prospective study of 198 asthmatic women by Stenius-Aarniala *et al.* found that 14% of patients with atopic asthma and 22% of patients with non-atopic asthma experienced asthma symptoms during labour (55). They reported that in all women, symptoms during labour were mild and well controlled by inhaled β_2-agonists (55). Similar data has been reported by other groups (57, 59) including Schatz *et al.* (1988) where 10% of women experienced mild symptoms during labour and delivery (134). The larger multi-centre study by Schatz *et al.* (2003) found that asthma symptoms were present during labour in 17.9% of all patients, with 46% of women with severe asthma experiencing symptoms during this time (148).

1.5.1 Possible mechanisms for the effect of pregnancy on maternal asthma

The mechanisms which contribute to changes in asthma during pregnancy are not well understood, although increases in maternal circulating hormones may be involved. The pregnancy-associated rise in serum free cortisol may contribute to improvements in asthma during pregnancy (48, 126), since cortisol has anti-inflammatory properties. In addition, estradiol and progesterone concentrations increase significantly during pregnancy (92). Progesterone is known to contribute to increased minute ventilation during normal pregnancy (127) and is also a potent smooth muscle relaxant (150) and may therefore be expected to contribute to improved asthma during pregnancy. Alternatively, changes in β_2-adrenoreceptor responsiveness and airway inflammation as a result of circulating progesterone may contribute to worsening asthma during pregnancy (151). Tan *et al.* found that in female asthmatics, there was a desensitisation and down-regulation of lymphocyte β_2-adrenoceptors following administration of medroxyprogesterone (152). Alterations in asthma associated with changes in sex steroid production during the menstrual cycle have previously been observed (136, 153), with up to 40% of women experiencing an exacerbation around the time of menstruation when progesterone and estradiol levels are low (154). However, the role of progesterone and estradiol in premenstrual exacerbations of asthma remains controversial (153). No correlation has been found between the occurrence of premenstrual asthma and the progression of asthma during pregnancy (48, 136).

During pregnancy, exposure to fetal antigens, or alterations in immune function may predispose some women to worsening asthma. Successful pregnancy has previously been described as a Th2 phenomenon (155-158), and asthma itself is primarily a Th2 mediated disease (16). Although in both asthma and pregnancy, the distinction between Th2 and Th1 is not definitive (159, 160), in this sense, asthma may be expected to become worse during pregnancy. Another inflammatory disease, rheumatoid arthritis, which is Th1 mediated, is known to go into remission during pregnancy in 75% of patients (161, 162).

The fact that some women experience an improvement in asthma during pregnancy, while others experience a deterioration of asthma and that different patterns are observed in different pregnancies in the same mother (134, 145) casts doubt on the

contribution of these major common hormonal or immune changes of pregnancy. However, studies in non-pregnant women have shown that a high proportion of asthmatics have an abnormal concentration of either progesterone or estradiol compared to non-asthmatics, and these changes are not consistent across the entire group (163). Such individual abnormalities may explain why the progression of asthma during pregnancy differs between women.

A recent study suggested that maternal asthma symptoms during pregnancy may be influenced by the fetus. In a blind prospective study, Beecroft *et al.* (164) studied 34 pregnant women with moderate or severe asthma who were using regular treatments. A questionnaire was administered in the second trimester which assessed symptoms, cough, nocturnal waking, frequency and amount of drug treatment and history of doctor visits. There were significantly more mothers of girls who reported shortness of breath, nocturnal waking and a worsening of cough and asthma in general, while mothers of boys were more likely to report an improvement in asthma (164). In a letter to the British Medical Journal, Dodds *et al.* (165) reported that re-analysis of their Canadian population based study (37) indicated that fewer asthmatic women pregnant with boys required steroids for treatment (14%) compared to asthmatic women pregnant with girls (20%). Although equal proportions of women pregnant with males or females used no drug treatment for asthma, there was a trend towards more women pregnant with a male to use β_2-agonists alone (40% of subjects) compared to women pregnant with a female (35%), suggesting better managed asthma in the women pregnant with a male fetus (165). These data require further clarification in larger sample groups.

Although there is little information in the recent literature, the possible influence of fetal sex on maternal asthma during pregnancy may not be a novel concept. In 1961, the following comment was made by Dr Schaefer in a discussion of his publication on seven cases of asthma in pregnancy: “There have been reports that asthma becomes worse only when the patient is pregnant with a female child and shows no change or gets better when she is pregnant with a male child” (47). These authors and others (48) did not find any data to support this statement in their own patients, nor did they give any references to identify this older literature. However, reference to this older literature was also made by Green in 1934 (140) and Derbes and Sodeman in 1946 (166). They reviewed much of the non-English language literature dating back to the 1920s and found that in several studies sex of the fetus had an effect on maternal asthma during

pregnancy. In particular, a study by Lami (1937) found that in a very small number of asthmatic patients, those pregnant with a female fetus had mild cases of asthma early in pregnancy, while those pregnant with a male fetus were more likely to have more severe asthma (167). On the other hand, Derbes and Sodeman referred to work by Weinstein where one woman was unaffected by her asthma during two pregnancies with a male fetus, and had problems with her asthma during two pregnancies with a female fetus (166). No consistent pattern was observed in their own series of observations of 22 asthmatic women during a total of 60 pregnancies (166). Green suggested that where asthma attacks during pregnancy were associated with a particular fetal sex, the factor responsible came from the sexual organs of the fetus (140), since in 1932, Dorn and Sugarman had found that fetal sex could be predicted by the change that occurred when urine from pregnant women was injected into immature rabbit testicles (168). There were few other reports of asthma and pregnancy in the English language literature from 1904-1961 (Index Medicus, 1904-1926, Cumulated Index Medicus 1928-1956, Medline 1953-1961) and the others did not examine the effect of fetal sex on maternal asthma (141, 145, 169). However, in 1930, Williamson reported case histories of 13 women with asthma and 14 women with hay fever (170). He was surprised to find that some women had differing histories of urticaria (an allergic skin condition) during their pregnancies which were related to the sex of the child. He commented "It is curious to note, that the same mother during gestation with a male child would be free from the discomfort of urticaria or 'food poisonings' and yet be most sensitive if pregnant with a female child or *vice versa*" (170). Further investigations into the effects of fetal sex on maternal asthma are warranted and will be carried out as part of this thesis.

1.6 The treatment of asthma during pregnancy

Many studies confirm that asthma which is well controlled is less likely to result in adverse outcomes than poorly controlled asthma (57, 59, 171, 172). For example, one study found that birth weight was decreased in asthmatics who had at least one asthma attack during pregnancy compared to asthmatics who did not have an attack or require emergency therapy (172). Thus, in addition to avoiding possible asthma triggers (131), treatments may have an important role to play in controlling maternal asthma exacerbations during pregnancy. On the other hand, they may have undesirable effects on the fetus, which should be examined.

Several types of medication are used to treat asthma. In this review, theophylline, β_2-agonists and glucocorticoids will be discussed. Most emphasis will be placed on the use of inhaled glucocorticoids during pregnancy, since these were the preventative medications used by asthmatic women in my study and are of most interest in possibly having an effect on fetal growth and development.

1.6.1 Theophylline

Theophylline is a bronchodilator, which directly relaxes the smooth muscle of the bronchi and pulmonary vessels (4). It acts by inhibiting phosphodiesterase, an enzyme which degrades cAMP and through this mechanism leads to smooth muscle relaxation (173). Theophylline is no longer widely used, except in a sustained release formula for treating nocturnal asthma. It has generally been replaced by the more effective and safer long acting β_2-agonists (4).

Dombrowski *et al.* found that among a small number of asthmatics, the use of theophylline was associated with a decrease in the development of pre-eclampsia (174). They suggested that the ability of theophylline to reduce vascular reactivity and platelet aggregation via increasing cAMP may be responsible for this trend (174). However, another study examined theophylline use in pregnant asthmatics and found that patients using this drug were more likely to have an asthma exacerbation than patients not using theophylline, and more likely to develop pre-eclampsia (175). Although asthma severity was not specifically described in these women, the authors explain these findings as being possibly due to the higher prevalence of severe asthmatics among the theophylline users and therefore the effect on pre-eclampsia may have been independent of theophylline use (175).

The pharmacokinetics of theophylline changes with pregnancy, with a significant increase in half life during the third trimester as a result of a 25% reduction in clearance (176). Consequently, the doses of theophylline used during pregnancy require alteration to maintain appropriate plasma levels (177). Schatz *et al.* found no effect of first trimester use or any exposure to theophylline during pregnancy on congenital malformations (178). However, there was a relationship between theophylline use and preterm birth in this study (178). Theophylline crosses the placenta as shown by similar serum levels in maternal and cord blood at delivery (179, 180) and has been shown to

have a prolonged half life in premature infants (181). In a study of asthmatic women, theophylline was present in neonates at similar levels as their mother, but there was no evidence of its toxic effects, as demonstrated by normal Apgar scores and neonatal heart rates (180).

1.6.2 β_2-agonists

β_2-adrenergic receptor agonists (β_2-agonists) are bronchodilators used for the immediate relief of asthma symptoms (4, 173). The most commonly used short-acting inhaled β_2-agonist is salbutamol (albuterol), while long-acting β_2-agonists such as salmeterol (serevent) are also becoming more commonly used (4). They induce relaxation and bronchodilation in airway smooth muscle by binding to β_2-adrenergic receptors (173), which causes the release of intracellular cAMP (182).

Schatz *et al.* confirmed the safety of inhaled β_2-agonists in 259 pregnant women with asthma (183). Most of these women used metaproterenol (83%), while other β_2-agonists used included isoetharine (27%), epinephrine (13%), albuterol (8%) and isoproterenol (4% of subjects). More than one bronchodilator was used by 32% of subjects. The incidence of adverse outcomes was compared in women who used regular (one or more puffs per day) or intermittent (less than one puff per day) β_2-agonists during pregnancy, with asthmatic women who did not use β_2-agonists and control non-asthmatic women. There was no effect of use or dose of β_2-agonists on congenital malformations, perinatal mortality, preterm delivery, low birth weight or complications during labour. A later study confirmed that first trimester use or any exposure to β_2-agonists during pregnancy was not associated with congenital malformations (178).

The short-term effect of inhaled albuterol on the maternal and fetal circulation was examined in 12 asthmatic women by Rayburn *et al.* (184). They found no alterations in maternal blood pressure, maternal heart rate, fetal umbilical artery systolic/diastolic (SD) ratios or fetal heart rate within 2 hours of albuterol inhalation, the time at which serum concentrations were expected to peak (184).

No studies have examined the use of the long acting β_2-agonists (such as salmeterol), either alone or in combination with inhaled steroids (for example, the fluticasone propionate and salmeterol combination drug, seretide) in asthmatics during pregnancy. An epidemiological study of salmeterol use in over 15000 patients, reported that among

this population, there were 65 women who used salmeterol while pregnant (185). No adverse outcomes were reported; however, no information was given about the analysis of outcomes as this was not the primary aim of the study (185).

1.6.3 Glucocorticoids

Inhaled glucocorticoids (corticosteroids, steroids) are the major anti-inflammatory preventative medication used to treat asthma (4), while oral glucocorticoids are used as emergency therapy, when asthma is non-responsive to β_2-agonists and inhaled glucocorticoids (4). Inhaled glucocorticoids are effective for the long-term management of asthma as they have effects on many cells including the bronchial epithelium, mucosal inflammatory cells and the submucosa (186). The inhaled route of administration is preferred over the oral route, as direct inhalation to the lung results in fewer systemic side effects (187).

Pregnant asthmatic women may use several inhaled synthetic glucocorticoid derivatives to control asthma, including budesonide, beclomethasone dipropionate and fluticasone propionate. In some pregnant women, prednisolone or prednisone may be used orally (usually periodically) to treat severe cases of asthma. The glucocorticoid drugs have similar chemical structures to cortisol as shown in Figure 1.2 (188-190).

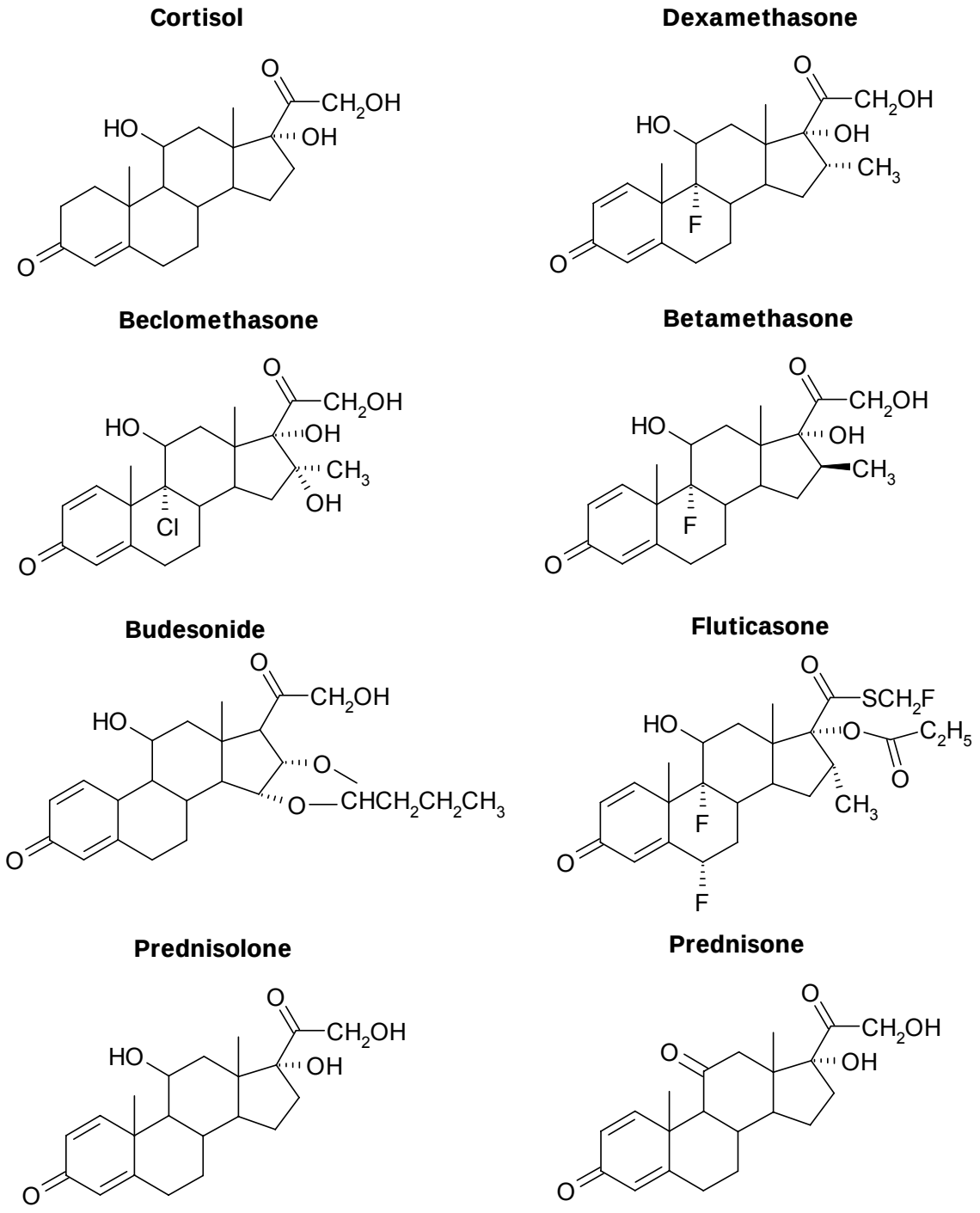

Figure 1.2 Chemical structures of cortisol and synthetic glucocorticoid drugs

Early studies in the literature focussed on the effects of glucocorticoid medication used by pregnant women on fetal development. Warrell and Taylor reported an increase in risk to the fetus attributable to oral prednisolone use (2.5-30 mg per day) during human pregnancy (191). Women using prednisolone had a higher incidence of still-birth, fetal

distress or placental insufficiency than women with the same disease who did not use prednisolone (191). A study of women using prednisone treatment for infertility and pregnancy maintenance demonstrated an increase in low birth weight infants (192). However, in this study, prednisone was administrated at a relatively high dose of 10 mg daily throughout pregnancy. In other studies where prednisone was ceased after conception, no alteration in birth weight was found (193, 194). Similarly, neonates of pregnant women treated with dexamethasone for congenital adrenal hyperplasia were not found to have reduced birth weights compared to their untreated counterparts (195). Asthmatic women using oral steroids during pregnancy (an average of 10 mg per day, n=70) were found to have a slightly higher incidence of preterm delivery, but no significant increases in congenital malformations were observed compared to general population estimates (196). Another study found no adverse outcome in 37 infants up to 2 years of age, of asthmatic mothers who used prednisone (5-60 mg per day) during pregnancy (197). In 1997, oral steroids were found to be independently associated with pre-eclampsia in asthmatic women (178). Recent epidemiological data demonstrated a significant association between first trimester systemic glucocorticoid use and an increased risk of cleft lip (with or without cleft palate) in neonates (198).

Inhaled glucocorticoid use has also been examined in pregnant asthmatic women. Inhaled beclomethasone to treat severe asthma was found to be safe in 45 pregnancies (199). In this study, the average dose used was 400 μg per day. However, withdrawal from oral prednisolone was not possible in most patients due to the severity of asthma. As a result, in this study and others (200), examination of the safety of the inhaled glucocorticoid medication is complicated by the concomitant use of additional drugs during pregnancy.

Schatz *et al.* have analysed the effect of commonly used asthma and allergy medications in prospectively studied pregnant asthmatic women (178). An advantage of this study was that the analysis took into consideration the effects of multiple medication use, asthma severity and other risk factors. The use of inhaled, oral or intranasal steroids, inhaled or oral β_2-agonists, theophylline, cromolyn, antihistamines and decongestants was not associated with congenital malformations or any adverse perinatal outcomes, including reduced birth weight (178).

A randomised controlled trial has been conducted comparing β_2-agonist use alone (albuterol, n=27) with the combination of β_2-agonist and inhaled steroid (albuterol plus beclomethasone, n=33) in asthmatic women who experienced an acute exacerbation requiring hospitalisation during pregnancy (146). Although all women in both groups required re-admission, the addition of inhaled beclomethasone therapy reduced asthma exacerbations and the re-admission rate by 55% (146).

A study conducted by Stenius-Aarniala *et al.* examined the relationship between inhaled steroid use, acute asthma exacerbations during pregnancy and pregnancy outcome (147). Patients who were already using anti-inflammatory medication (inhaled beclomethasone or budesonide) were less likely to have an acute mild asthma attack during pregnancy, than patients who were not using inhaled steroids. Approximately one third of patients who had an acute exacerbation were using inhaled steroids before the exacerbation, while overall, 61.5% of patients who did not have an exacerbation during pregnancy used inhaled steroids. The use of steroids was therefore observed to reduce the risk of asthma attacks during pregnancy. In this study, no details of asthma classification were given, making it difficult to assess the influence of asthma severity on these exacerbations (147). Studies in pregnant women indicate the effectiveness of inhaled steroid medications in controlling asthma exacerbations (146, 147).

A Danish population based study collected data from a birth registry and a prescriptions database (1991-1996) to study the use of asthma medications by pregnant women and relate this to perinatal outcome (201). Data was restricted to primiparae to ensure that prescribed medications were intended for the mother and not for any of her other children. Less than 2% of all Danish women were prescribed asthma medications during pregnancy. Asthma treatment was defined as one of five levels: 1) inhaled β_2-agonist, 2) inhaled steroid, 3) systemic β_2-agonist, 4) systemic steroid, 5) theophylline. Data was analysed based on whether asthmatic women increased medication during pregnancy (prescribed treatment of a higher level than prior to pregnancy), or decreased medication during pregnancy (prescribed treatment of a lower level than prior to pregnancy). The results showed that asthmatic women who decreased medication during pregnancy (78 out of 342 asthmatics) had smaller babies in terms of birth weight and length, with a lower mean gestational age compared to non-asthmatic women, or asthmatic women who increased medication during pregnancy. This was particularly evident among the 22 women who decreased their medication use from inhaled steroid

to inhaled β_2-agonist. This study was limited by a lack of information regarding compliance and the reasons for alterations in asthma management (201). These may have included a clinical improvement in lung function and asthma symptoms, or may have been due to a pregnancy-related reduction in prescribing as a result of fears of drug use during pregnancy.

A recent study from Sweden confirms that the use of budesonide in pregnant women does not affect gestational age, birth weight, birth length or the rate of still-births or multiple births (202). This data came from 2968 women who used inhaled budesonide during pregnancy and was compared to 7719 women who used asthma medications other than steroids, and a control population of over 293000 women. The authors acknowledged that comparison with asthmatic mothers of similar severity who did or did not use budesonide would be of benefit since inadequate asthma control may be a confounder. Another Swedish medical birth registry study previously reported no increase in congenital malformations in women who used inhaled budesonide during early pregnancy (203). Similarly, Silverman *et al.* reported no adverse outcomes in pregnant women using budesonide (400 μg daily) in a randomised trial of treatment in newly diagnosed mild asthmatics (204).

No studies have examined the use of fluticasone propionate for asthma during pregnancy. However, Ellegard *et al.* examined the use of a fluticasone nasal spray in women with pregnancy rhinitis and found no influence on circulating maternal cortisol, or on fetal growth or pregnancy outcome (205).

Despite reports indicating the safety of glucocorticoid use for asthma treatment during human pregnancy, there still remains apprehension about using these medications during pregnancy, both in pregnant women themselves (206-208) and in doctors (122, 126, 147, 206, 207, 209). Patterson *et al.* presented a case report of a pregnant asthmatic woman who continued with theophylline treatment rather than taking inhaled beclomethasone, due to her obstetrician's advice against using additional medication (210). In this case, the advice led to fear and non-compliance in the patient and she was subsequently hospitalised with an acute episode of asthma (210). A recent survey of 501 asthmatic women of child-bearing age reported that 82% of women who used inhaled glucocorticoids were concerned about their effects on the fetus, including congenital malformations, fetal death, low birth weight and preterm delivery (211). However,

women also felt concern about the consequences of discontinuing medication on their own health, but despite this, many were likely to discontinue medication while pregnant, without first seeking advice from their physician (211). The problem of unfounded fears of the effects of asthma drugs on the fetus was acknowledged by the working group on asthma and pregnancy from the National Institutes of Health (149). Publicity surrounding teratogenic effects of drug use in early pregnancy and concern about litigation contributes to these fears (209, 212). A comparison of emergency department visits by pregnant and non-pregnant asthmatic women found that although there were similar symptom durations and PEFRs in both groups, those who were pregnant were significantly less likely to be treated with systemic steroids either in the emergency department or following discharge from hospital (122). In addition, the pregnant asthmatics were more likely to experience an on-going exacerbation in the following two week period compared to non-pregnant asthmatics (122). These studies suggest that despite continuing advice that pregnant women with asthma should be treated in the same way as non-pregnant asthmatic women, this has not completely translated into clinical practice. Further data demonstrating the safety of inhaled glucocorticoid use for both the fetus and mother in asthmatic pregnancies may facilitate improved asthma management in pregnant women.

In spite of the lack of randomised trials of asthma treatments in large groups of pregnant asthmatic women, inhaled glucocorticoids are considered safe to use during pregnancy, as the risk of a poor pregnancy outcome appears greater when asthma is not well controlled. Some early reports (135) and most recent reviews and recommendations on asthma management suggest treating asthma in pregnant women in a similar manner to non-pregnant women (4, 149). Recent literature has also highlighted the importance of educating pregnant women about their asthma (123, 212, 213). Education has numerous benefits including improvement of patient compliance with medications (131). Incorporating patient education into the clinical management of pregnant asthmatic women was conducted in conjunction with my study. These strategies are designed to result in the best possible outcome for both mother and fetus.

Despite conflicting results in previous epidemiological and prospective studies, it is clear that maternal asthma is a risk factor for poor pregnancy outcomes and that asthma itself may be altered by pregnancy. However, the mechanisms, both maternal and placental, which contribute to changes in asthma during pregnancy and changes in

pregnancy outcomes with asthma, remain unknown. Although some authors have proposed maternal mechanisms which may contribute to poor outcomes and to changes in asthma with pregnancy, no group has previously collected data simultaneously from the mother, placenta and fetus in asthmatic pregnancies. This approach will provide more detailed information of the changes and interactions which occur in these pregnancies, leading to more effective treatment and management of asthmatic women and improved outcomes for their babies.

The major focus of this thesis will be to address whether maternal asthma is associated with reduced fetal growth and to examine the maternal and placental mechanisms contributing to this outcome.

Literature Review Part 2: Fetal Growth

1.7 Low birth weight

Low birth weight is defined by the World Health Organization as birth weight less than 2500 g (214). A small for gestational age (SGA) neonate is less than the 10th centile (percentile) for its gestational age in relation to the reference population (85). Low birth weight may also be referred to as intrauterine growth restriction (IUGR) (85). The incidence of low birth weight at term is 11% in developing countries with an estimated 13.7 million low birth weight infants born each year, a rate six times higher than in developed countries (215). In Taiwan, the prevalence of low birth weight was approximately 5% in 1996-1997 (216), while in the USA it has recently been estimated to affect approximately 5-6% of live births in Caucasians and 10-12.5% of live births in African Americans (217, 218), with 3% and 7% representing SGA infants in each population, respectively (218).

Cell number increases maximally during embryonic and fetal development up to 16 weeks gestation, with very little change after 32 weeks (85). From 16 to 32 weeks there is also an increase in cell size, which becomes more dominant after 32 weeks (85). Fetal growth restriction may be symmetrical (Type I) or asymmetrical (Type II). Symmetrical growth restriction, where the entire body is proportionally small (219), accounts for 25% of IUGR cases and often results from an alteration in growth in early gestation, during the period of cellular hyperplasia and may be the result of genetic anomalies, severe malnutrition, or maternal smoking (85). Sub-optimal first trimester growth, represented by a small crown-rump length measurement, is a good predictor of birth weight less than 2500 g at term, or birth weight below the 5th centile (220). Asymmetrical fetal growth was first recognised by Gruenwald in 1963 who found that growth restricted infants had higher brain weight and lower thymus weight than premature infants of the same size (221). Such asymmetric growth restriction may occur during the periods of cellular hypertrophy later in gestation and is often the result of uteroplacental insufficiency secondary to other maternal complications (85). In asymmetric growth restriction, there is sparing of the brain and other vital organs such as the heart, with other parts of the body such as the liver and muscle reduced in size (85, 219).

Fetal growth can be assessed in several ways with common measurements including crown-heel length, head circumference, weight-height ratio, skin fold thickness and ponderal index (85). The ponderal index (birth weight (g) / [birth length (cm)]^3 × 100), which is unaffected by race or infant sex, is used to assess the thinness or obesity of the neonate, with symmetrically small neonates having a normal ponderal index and those with asymmetric growth restriction having a reduced ponderal index due to a normal length and low weight (85).

Low birth weight independent of prematurity, is a significant contributor to neonatal morbidity and mortality (85, 221-224) and is responsible for a large health care cost, both in economic and social terms (225). A population increase of just 100 g in mean birth weight via maternal nutritional supplementation has been shown to reduce neonatal mortality by 30-50% (226). The risk of postnatal death in term infants weighing 2000-2499 g has been estimated to be increased two-fold compared to infants weighing 2500-2999 g and increased four-fold compared to infants weighing 3000-3499 g (227). In Gambia, a randomised controlled trial of maternal dietary supplements significantly increased mean birth weight by 136 g and mean head circumference by 3.1 mm, which was associated with a significant overall reduction in still-births and perinatal mortality (228). Low birth weight has been shown to be associated with an increased risk of mortality up to 15 years of age, which is mostly accounted for by higher infant mortality rates (229). Low birth weight also contributes to increased morbidity including birth asphyxia, meconium aspiration, persistent fetal circulation, hypoglycemia, hypothermia and hypocalcemia (85, 222, 224, 230, 231).

1.8 Developmental origins of adult disease

Recent interest in the developmental origins or fetal origins of adult disease, also known as the Barker hypothesis, has revealed that low birth weight or small size at birth is a predictor for the development of and/or death from diseases in adult life, including diabetes (232), cardiovascular disease (233), atherosclerosis (234), hypertension (235), stroke (236) and coronary heart disease (237, 238) and may also be related to markers of ageing (239). This concept, which proposes that events *in utero* can determine long-term outcomes into adulthood, is known as fetal programming. It is thought that glucocorticoids may play a major role in programming. Adaptation of the fetus to its

environment *in utero* is believed to lead to changes in body structure, physiology and metabolism which persist into extra-uterine life. Thus, low birth weight infants of asthmatic mothers may be at increased risk of mortality in both neonatal and adult life.

Barker's initial studies examined the geographical relationship in England and Wales between current death rates from heart disease or stroke and prior infant or maternal mortality rates (240, 241). The rate of ischemic heart disease in 1968-78 was closely correlated with neonatal and post-neonatal mortality in 1921-25 (240). In addition, the geographical distribution of death rates from stroke was more closely correlated with past maternal mortality than with any other cause of death, suggesting that the health of mothers may be linked to the risk of disease in their offspring (241). Similar data has been reported recently and prenatal factors have been proposed to contribute to the geographical distribution of stroke mortality in both the United States and England and Wales which cannot be fully explained by adult lifestyle (236).

In 1989, Barker *et al.* published results of follow-up studies of almost 10000 children at age 10, born in 1970 and over 3000 adults at 36 years of age, born in 1946 (237). They found an inverse relationship between birth weight and systolic blood pressure, which was stronger in the 36 year old adults and independent of current weight. In the children, increased systolic blood pressure was not related to gestational age and therefore was associated only with a reduction in fetal growth (237). A study from another group found a similar relationship between systolic blood pressure in children aged 5-8 years and birth weight but only when standardised for current weight (242).

Further studies from Barker *et al.* examined over 5000 men born in Hertfordshire between 1911 and 1930 and found that mortality from ischemic heart disease was more common in men with low weights at birth and one year of age (238). A similar trend was noted for death from chronic obstructive lung disease, but not death from lung cancer (238). The relationship between higher systolic blood pressure and low birth weight has been found to be consistent in children aged 0-10 years, and adults at 36 years, 46-54 years and 59-71 years but to become more pronounced with age (243).

Blood pressure in adult life is also linked to placental size (244) and to the ratio of fetal to placental size (245). In 449 men and women born in Lancashire, England between 1935 and 1943, systolic and diastolic blood pressures were strongly related to both placental weight and birth weight independent of gestational age, current alcohol

consumption and current body mass index (BMI), with the highest blood pressures in those with a low birth weight but high placental weight (244). Within each social class, the relationships between blood pressure and placental and birth weights were similar (244). Placental weight was inversely correlated with length to head circumference ratio, suggesting the possibility that changes in the fetal circulation such as diversion of blood flow to the brain at the expense of other parts of the body may permanently alter arterial structure and blood vessel development (232, 244, 246, 247).

Barker *et al.* also studied the Hertfordshire and Lancashire cohorts in relation to the development of Syndrome X, or the combination of non-insulin dependent diabetes mellitus, hypertension and hyperlipidemia (232). In Hertfordshire, men aged 64 years with higher birth weights had lower 2 hour plasma glucose and insulin concentrations and lower blood pressures, while 56 of the men had Syndrome X and this was related to lower weights at birth and one year of age (248). In Lancashire, men and women at age 50 with Syndrome X had lower birth weights as well as a small head circumference and low ponderal index at birth. The proportion of subjects with Syndrome X fell with increasing birth weight in the Hertfordshire and Lancashire cohorts (232). The association between fetal growth and diabetes may be related to alterations in fetal pancreas development and a reduction in insulin secreting capacity (232).

Other studies have found higher plasma glucose in children who were thin at birth with a low ponderal index (249) and in adults of reduced birth weight (250, 251). Alterations in β-cell development and function during undernutrition in fetal life may result in permanent changes such as a reduced capacity for insulin production which becomes a disadvantage when nutrition is abundant (248). Alternatively, a genetic predisposition to low insulin production may result in both reduced fetal growth and glucose intolerance later in life (248).

It is becoming increasingly clear that the effects of small size at birth on adult diseases, is compounded by rapid rates of childhood growth (252-254). Barker *et al.* found that adults from Helsinki who had been born small and had the largest BMIs in childhood were at greatest risk for type 2 diabetes, hypertension and death or hospitalisation due to coronary heart disease (252). They suggested that developmental plasticity allows an appropriate phenotype for the current environment (*in utero*); however, when nutrition improves after birth, compensatory growth occurs. The combination of these events

results in physical and physiological changes which contribute to the increased risk of developing metabolic and cardiovascular diseases later in life (252). Evidence for a mechanism connecting small size at birth to obesity later in life comes from a study of "thin-fat" Indian babies, in which low birth weight was associated with low ponderal index (thinness) and reduced abdominal and mid-arm circumference, but marked sparing of subscapular skin fold thickness, a representative depot of central fat (255). Babies in India and the United Kingdom with birth weights less than the 10th centile exhibited both brain-sparing and fat-sparing characteristics, possibly putting them at risk of insulin resistance and cardiovascular disease in adulthood (255).

Maternal diet during pregnancy may have an adverse effect on fetal growth and consequences for adult blood pressure. Several studies have demonstrated that a particularly high or low protein diet during pregnancy has adverse effects on blood pressure in offspring (256, 257). Maternal undernutrition has been examined in women who were pregnant at the time of the Dutch famine in 1944 and 1945, where babies exposed during mid or late gestation were found to have reduced birth weights compared to babies born before the famine or conceived after the famine (258, 259). Follow up studies of the offspring indicate that at approximately 50 years of age, the highest rates of impaired glucose tolerance and type 2 diabetes were in those exposed to maternal famine in late gestation (258). In addition, offspring exposed to maternal famine during early gestation had an increased prevalence of coronary heart disease, respiratory disease, hypertension, diabetes and cancer and a poorer perception of their own health at 50 years of age, which is a predictor of mortality (259).

Numerous other adult consequences of small size at birth have been described in humans, including an increased risk of renal failure (260), depression in men but not women (261), atherosclerosis (262) and the development of pre-eclampsia while pregnant (263). Women of low birth weight were found to be 2.3 times more likely to develop pre-eclampsia than those who weighed 2500-2999 g at birth, with the risk further decreasing with increasing birth size (263). However, there was also an important effect when adult weight was considered, with lean women of low birth weight having no increased risk and overweight women of low birth weight having a 16-fold increase in risk for pre-eclampsia (263). Reduced birth weight in combination with high adult weight may produce the greatest risk for disease in adult life.

Low birth weight has also been linked to behavioural problems at school such as lack of motivation, aggression and concentration difficulty at age 10 (264) and low IQ at 6 years of age in children with no neurologic impairment (265). The study by Breslau *et al.* examined outcomes for children across a range of birth weights, in an inner city area and suburban area of Michigan (264). In both populations, low birth weight was associated with an average IQ score five points lower than normal birth weight children, resulting in 10% of low birth weight children having an IQ more than one standard deviation below the mean. In addition, a gradient effect was observed, with the largest reduction in the very low birth weight group (<1500 g) (264).

Reduced fetal growth may have an effect on the development of respiratory diseases in childhood and adult lung function. However, the available data is contradictory, with some studies showing an increased risk of developing asthma or having reduced lung function in smaller neonates (266-271) and others showing an increased risk of asthma or atopy in larger neonates (272, 273). A study from Barker *et al.* demonstrated that lower birth weight was associated with reduced adult FEV_1 at 59-70 years of age and death from chronic obstructive airways disease was also related to lower birth weight (274). In an Indian study, adult lung function (FEV_1), was reduced with decreasing birth weight in men and women, while a small head circumference at birth was associated with reduced FEV_1:FVC ratio in men but not women (275). These changes in adult lung function may be related to permanent effects of maternal undernutrition on lung development and structure, and differences between men and women may relate to sex-specific differences in lung growth *in utero* (274, 275). Lopuhaa *et al.* found that men and women who had been exposed to famine in mid-gestation had a higher rate of obstructive airways disease, suggesting that fetal nutrition affects lung development, although they found no evidence of changes in serum IgE or lung function in adulthood (276). Potential mechanisms linking low birth weight to poor lung function later in life have been studied in the sheep model of IUGR induced by chronic placental insufficiency or maternal anemia (277). In these studies, fetal growth restriction resulted in alterations of lung structure and function, including a thickened air-blood barrier, enhanced surfactant gene expression (277) and a reduction in total lung capacity (278).

Alterations in susceptibility to disease in childhood and adulthood in relation to fetal growth may be related to impaired development *in utero* or in infancy, or increased susceptibility to postnatal disease (265). Children of asthmatic mothers may be at

particular risk of disease in adult life due to an increased likelihood of low birth weight. Therefore, an understanding of the mechanisms which cause low birth weight is important for the development of future interventions which may give these and other small infants a better chance of a healthy life, both in their immediate future and in the long-term. The mother, placenta and fetus are all integral components in the regulation of fetal growth during human pregnancy.

1.9 The role of the mother in fetal growth regulation

The mother has a very important role to play in supplying oxygen and essential nutrients to the fetus via the placental blood supply. Maternal genes have an important specific influence over fetal growth (219), and maternal size, (particularly height which represents genetic potential for growth and uterine capacity), is a major determinant of fetal size (279, 280). In a study of pregnancies involving ovum donation, Brooks *et al.* found that the only factors contributing to birth weight were gestational age and recipient mother's weight, while the weight of the donor mother was not related to birth weight (281). This study suggested that the uterine environment was very important to the control of fetal growth (281). Many maternal factors influence fetal growth, including height and weight, race and parity (85, 282, 283), age (216, 283), energy intake and gestational weight gain (217).

The mother is the supplier of nutrients to the fetus and maternal diet and caloric intake have an essential role to play (214, 284). The placenta also has an essential role in transferring these nutrients to the fetus, which will be discussed in section 1.10. Adequate caloric intake is essential during all of pregnancy and an increase is necessary during the second and third trimesters when most fetal and placental growth occurs (284, 285). Additional protein intake is specifically required for growth of maternal, placental and fetal tissues (285). Some studies have suggested that protein supplementation does not in itself increase fetal growth in undernourished communities (286), while others have shown a relationship between low protein intake in late pregnancy and reduced birth weight (287).

Nutrient availability in maternal blood is clearly an important regulator of fetal growth and supplementation of calories or vitamins to undernourished women increases birth weight (228, 286). In Nepal, pregnant women supplemented with folic acid and iron had

an increased mean birth weight of 37 g and a 16% reduction in the rate of low birth weight compared to control subjects given vitamin A alone (288). The effect of multiple micronutrient supplementation (folic acid, zinc, iron, vitamin A and 10 other micronutrients) was not found to be of additional benefit compared to folic acid and iron (288). This study suggested that iron deficiency may be an important cause of reduced fetal growth (288).

Glucose is an important nutrient in the control of fetal growth. Studies of diabetic women have shown that low blood glucose levels during pregnancy as a result of excessively tight glycemic control leads to a greater incidence of SGA neonates, while having high blood glucose levels contributes to a high incidence of macrosomia (289, 290).

During the Dutch famine in 1944 and 1945, pregnant women were undernourished due to compulsory food rationing, which was as low as 400-800 calories per day at the height of the famine (258). Consequently, maternal weight gain and fetal growth were significantly reduced, when the exposure to famine occurred during the second or third trimester (258). In the 1960s, Scottish women in Motherwell were advised to increase their red meat intake and decrease consumption of carbohydrates during pregnancy in an attempt to avoid pre-eclampsia (257). As pregnancy progressed women doubled their meat consumption, while carbohydrate consumption fell by one third. As a consequence these women had reduced weight gain during pregnancy compared to women in other parts of Scotland and their babies were of reduced birth weight (257).

Many other maternal factors unrelated to nutrition can also alter fetal growth. For example, living at altitude (291, 292) or having low arterial oxygenation (293), having pre-eclampsia (294), PIH, anemia (295, 296) or infections such as malaria (95, 97-99) or rubella (85), cigarette smoking (297-301), excess alcohol consumption (302) or using drugs such as cocaine (303-305) also increase the risk of having a low birth weight baby (217). Many of these conditions may contribute to altered fetal growth via changes in placental function.

It has been understood for many years that cigarette smoking is associated with reduced birth weight, with early reports suggesting a doubling of low birth weight rate in smokers compared to non-smokers and an increase in low birth weight with increasing number of cigarettes smoked (297, 298, 306). The entire birth weight distribution curve

is shifted to the left, such that maternal smoking affects the entire range of birth weights (307). MacMahon *et al.* (1965) established that women who smoked before pregnancy but not during pregnancy had babies of similar size to non-smoking mothers and that paternal smoking also had no influence on birth weight (307). Infants born to smoking mothers are approximately 150-200 g lighter than infants of non-smokers (297, 300, 308-310), representing one of the largest preventable effects on birth weight (280). Neonates born to smoking mothers are usually symmetrically growth restricted, having reduced weight, head circumference and abdominal circumference (85). Higher levels of carbon monoxide in maternal blood which cross the placenta to the fetus (311), leading to fetal tissue hypoxemia along with the vasoconstrictive effects of nicotine (299) are thought to contribute to these changes in fetal growth (297, 300). Astrup *et al.* found a negative relationship between levels of carbon monoxide bound to hemoglobin and birth weight in 176 smoking mothers (309). Smoking may reduce fetal growth by altering uteroplacental blood flow, as Lehtovirta and Forss demonstrated reduced placental blood flow at the time of smoking a cigarette which returned to normal within 15 minutes (312). Zaren *et al.* found reductions in ultrasound measured biparietal diameter (BPD) and mean abdominal diameter were evident from 33 weeks and 25 weeks gestation respectively (313). Mean birth weight was reduced by 167 g in light smokers (1-9 cigarettes per day) and by 241 g in heavy smokers (≥ 10 cigarettes per day) (313).

Sexton *et al.* conducted a randomised clinical trial to investigate whether a reduction in maternal smoking would improve fetal growth (314). In this trial, women in the intervention group received health information and counselling and 43% stopped smoking by late pregnancy, compared to only 20% in the control (no intervention) group. The significant reduction in the number of cigarettes smoked per day corresponded to a decrease in salivary thiocyanate levels, a biochemical marker of smoking and associated with this was a significant increase in birth weight (by 92 g) and birth length (by 0.6 cm) compared to the control group, confirming that smoking cessation overcomes some of the reduction in fetal growth in smokers (314). Smoking is known to be more prevalent among asthmatics than non-asthmatics (37, 38, 43, 46, 51, 60, 61) and this may be a confounder, further contributing to reduced fetal growth in some women.

Maternal arterial oxygenation and high altitude residence also have an effect on fetal growth. Babies born at high altitude are of lower birth weight than their low altitude counterparts (291, 315) regardless of socioeconomic status (316) and the effect of altitude on birth weight is independent of existing risk factors such as maternal smoking, PIH and nulliparity (317). The mean difference in birth weight between high altitude (2744-3350 m) and lower altitude (915-1524 m) in Colorado was 241 g, a difference which could not be explained only by a reduction in gestational age (317). A study from Yip found a three-fold increase in the low birth weight rate at the highest altitudes in the US (2500-3100 m) compared to the lowest altitudes (<500 m) with a far greater increase in the proportion of low birth weights due to IUGR than prematurity (291). This study also demonstrated that the entire birth weight distribution was shifted to the left, so that a greater proportion of births fell below 2500 g, indicating that altitude affected all births and not just an "at risk" sub-group (291). Altitude is a strong predictor of IUGR (291, 318), through changes in third trimester fetal growth (292, 319). Krampl *et al.* performed serial ultrasound measurements of fetal size from 14 to 42 weeks gestation in several hundred women at sea level and at 4300 m in Peru and found that the reduction in fetal growth occurs from approximately 25 weeks gestation (320). The effect of altitude was greater on abdominal circumference than on head circumference and mean birth weight was reduced by approximately 400 g (320).

Hypoxia is an important factor involved in altering fetal growth at altitude and may also be involved in asthmatic pregnancies. The combination of hypoxia and pregnancy appears to be important in alterations in maternal physiology, including changes in immune pathways (321). Coussons-Read *et al.* found that maternal serum levels of the pro-inflammatory cytokines, TNF-α and IL-6 were increased at high altitude (3100 m) compared to moderate altitude (1600 m), and the anti-inflammatory cytokine, IL-10 was decreased by the third trimester, while none of these parameters differed between moderate and high altitude residents at 3 months postpartum (321). Moore *et al.* found that maternal hypoventilation and a decreased maternal arterial O_2 content in the third trimester was directly related to infant birth weight at 3100 m (293). In women living at high altitude in Peru, an increased ventilatory response to hypoxia during pregnancy was associated with a rise in birth weight through increases in maternal oxygenation (322).

Decreased arterial oxygen content as a result of high altitude exposure may also decrease uterine blood flow which could contribute to reduced nutrient transport to the fetus (323). Uterine blood flow is altered at high altitude with less common iliac flow reaching the uterine artery (324). Although uterine artery flow velocity increased, the uterine artery diameter was smaller, resulting in lower volumetric flow in late pregnancy (324). Uterine blood flow velocity was found to be correlated with birth weight at 1600 m (324). In 2001, Moore *et al.* reported that despite lower arterial O_2 content in Tibetan compared to Han Chinese residents at high altitude (3658 m), the Tibetan women had a higher uterine artery blood flow velocity and a greater distribution of blood flow to the uterine artery, which contributed to babies of higher birth weight compared to the Han Chinese (325). Women who develop pre-eclampsia at high altitude have less blood flow distributed to the uterine artery compared with normotensive women (324). Studies of the placenta from high altitude pregnancies have demonstrated that there is less remodelling of the uteroplacental arteries compared to those at moderate altitude (326). These studies suggest that physiological adaptations to high altitude residence which increase blood flow to the feto-placental unit are beneficial for fetal growth.

1.10 The role of the placenta in fetal growth regulation

The placenta is the site where nutrients and waste products are exchanged between mother and fetus. Morphometric studies have shown that the placental villous surface area for exchange is approximately 11 m^2 at term (327). This surface area is decreased in cases of fetal growth restriction (327) as is the mid-pregnancy or term placental volume (327, 328). Placental weight is an important predictor of fetal weight, with SGA neonates having significantly reduced placental weights and placental weight to birth weight ratios than appropriately grown neonates of the same birth weight (329). This suggests that adequate placental growth is required for adequate fetal growth. Several aspects of placental function are critical for human fetal growth. They include adequate trophoblast invasion, an increase in uteroplacental blood flow during gestation, transport of nutrients such as glucose and amino acids from mother to fetus and the production and transfer of growth regulating hormones. In addition, the placenta plays a

very important role in limiting the transfer of the maternal hormone cortisol which may have a negative effect on the growth of the fetus.

1.10.1 Trophoblast invasion and uteroplacental blood flow

One week after fertilisation, the blastocyst enters the uterus (330). The blastocyst contains the inner cell mass, which will develop into the fetus, and an outer layer of trophoblast, which will become the placenta (330). The trophoblast layer implants into the uterus, by releasing proteolytic enzymes which digest cells of the endometrium allowing subsequent penetration by the trophoblast (1, 330). Adequate trophoblast invasion is required to sustain fetal growth. When the blastocyst adheres to the uterus, the fetal trophoblast cells differentiate into villous or extravillous cells (331). The extravillous cytotrophoblasts migrate and invade into the maternal uterine epithelium, a process which is essential for increased uteroplacental blood flow as pregnancy progresses (331). In this process, maternal uterine spiral arteries are transformed into larger, low resistance vessels (332), capable of transporting the increased maternal blood to the placenta (333). Part of the modification and remodelling of spiral arteries involves a replacement of the muscular and elastic walls of the arteries with a fibrinoid layer embedded with trophoblast cells (334-336), allowing low pressure intervillous flow (333, 336). The absence of trophoblast induced changes in decidual or myometrial segments of spiral arteries is a feature of some pregnancies complicated by fetal growth restriction (337).

Growth of the uterus, placenta and fetus requires an increase in uterine blood flow during pregnancy in order to meet metabolic demand (333, 338). During pregnancy, total blood volume (339) and cardiac output increase by approximately 40% (331) and the total uteroplacental blood flow represents 25% of cardiac output (333). Thaler *et al.* found that uterine artery volume flow rate increased by over three-fold during pregnancy, partly influenced by an increased artery diameter and reduced resistance to flow (340). Palmer *et al.* found that the diameter of the uterine artery had increased two-fold by 21 weeks gestation, and further increased between 30 and 36 weeks gestation (341). In addition, flow velocity of the uterine artery increased throughout gestation and was eight times greater by 36 weeks compared to non-pregnant values (341). Uteroplacental blood flow was shown to be reduced by up to 50% in women with pre-eclampsia (342), a group susceptible to IUGR, and uterine artery volumetric flow was

also reduced by one third in late gestation in high altitude pregnancies (324). In addition to increased uterine blood flow during pregnancy, the development of new blood vessels also occurs in the uterus, possibly promoted by human chorionic gonadotropin (hCG) (343) and IGF-II (344). In IUGR, there is a decrease in number and surface area of terminal villi, representing a malfunction of vascularisation in these pregnancies (344-346).

Umbilical vein blood flow can be measured by Doppler ultrasound techniques and has been shown to be decreased in IUGR fetuses in relation to fetal size (347), representing reduced perfusion of the fetal tissues (348). In a study of 70 human fetuses, Barbera *et al.* found a strong correlation between absolute umbilical vein flow and fetal head and abdominal circumferences, with an increase in umbilical vein diameter and mean velocity throughout pregnancy (349). They also found an exponential increase in flow from 97.3 ml/min at mid-gestation to 529.1 ml/min in late gestation, but no corresponding increase in flow per kg of fetal weight, suggesting that increasing flow is driving the increase in fetal size in late gestation (349). Di Naro *et al.* also demonstrated reduced umbilical vein flow in IUGR fetuses, both in absolute terms and when adjusted for abdominal circumference (350). In addition, they found that the cross-sectional area of the umbilical cord and of the umbilical vein itself was lower in IUGR fetuses than normally grown fetuses (350). These studies suggest the importance of uteroplacental blood flow in maintaining appropriate fetal growth through the supply of oxygen and nutrients. A previous study from our group demonstrated alterations in placental blood flow in some women with asthma and this potentially contributes to changes in fetal growth (111).

1.10.2 Nutrient transport

Glucose, amino acids and lipids are some of the most important nutrients which are transported from mother to fetus via the placenta. These nutrients may be delivered by passive diffusion; however, concentrations of many amino acids are higher in fetal than maternal plasma, suggesting the existence of active transport mechanisms across the placenta (351, 352). Amino acid transporters within the fetal (basal) and maternal (microvillous) facing syncytiotrophoblast plasma membranes actively transport numerous amino acids across the placenta (353). System A, found mostly on the microvillous membrane, is sodium dependent and transports neutral amino acids such as

alanine, proline, glycine and serine (353, 354). Neutral amino acids may also be transported by system ASC, found mostly on the basal membrane (355). System L is sodium independent, transporting phenylalanine and branched chain amino acids (353). Systems y^+ and y^+L transport cationic amino acids such as arginine across the microvillous and basal membranes respectively (353, 355, 356). Amino acids may also be metabolised and processed by the placenta. For example, leucine is deaminated in the placenta, and the deaminated product and leucine itself are both transferred to the fetus (357).

In SGA fetuses there are alterations in amino acid transport by the placenta and uptake by the fetus. Jansson *et al.* found that *in vitro* uptake of lysine in the basal membrane and leucine in both the basal and microvillous membranes was decreased in placentae from IUGR pregnancies, suggesting reduced activity of amino acid transporters (358). Fetal plasma collected at mid-gestation from SGA fetuses showed a reduction in essential amino acids with lower levels of alpha-aminonitrogen and decreases in branched chain amino acids such as valine, leucine and isoleucine along with lycine, serine (359, 360) and phenylalanine (361). Economides *et al.* found that fetal concentrations of many amino acids including branched chain, basic and essential amino acids that cannot be produced by the fetus, were reduced (352). Moreover, the ratio of non-essential to essential amino acids was increased with increasing fetal hypoxemia, assessed by umbilical vein PO_2 (352). In IUGR, the activity of system A in the microvillous membrane is reduced (362, 363), while the expression and activity of glucose transporters in the syncytiotrophoblast is not changed (355, 364).

Glucose transport from mother to fetus is related to the concentration gradient and is carried out by transporters found on the maternal and fetal sides of the trophoblast (365). Nicolini *et al.* found that while maternal glucose concentrations were similar between normal and growth restricted pregnancies, the fetal glucose concentration was significantly reduced in the growth restricted group (366). Hypoglycemia in SGA fetuses may be related to reduced supply and transfer of glucose across the placenta (367). The glucose transporter, GLUT1, is found in abundance in the microvillous membrane of the syncytiotrophoblast at levels three times higher than the basal membrane (364, 368). In a perfusion study of preterm IUGR placentae, it was found that baseline glucose consumption was two-fold higher in IUGR, suggesting that placental consumption of glucose may contribute to alterations in maternal-fetal

concentration differences in glucose (369). However this study also demonstrated no change in glucose transfer to the fetal side of the placenta (369), confirming previous studies showing no alteration in glucose transporter expression or activity in IUGR placentae (364, 370, 371). Another study found that in IUGR, the maternal-fetal glucose concentration gradient was increased in relation to clinical severity, possibly representing an adaptation to maintain glucose uptake across the placenta (365).

Fatty acids, which are essential components of plasma membranes and used for energy, are also transported to the fetus across the placenta (372, 373). In the third trimester, fatty acids are required for changes in fetal tissue composition, particularly that of the brain and adipose tissue (374). The n-3 and n-6 fatty acid structures can only be acquired from the maternal diet and placental transfer (373). Free fatty acids may be transferred across the placenta via passive diffusion, due to the concentration gradient between mother and fetus (375) and there are fatty acid binding proteins and fatty acid transfer proteins in the microvillous and basal membranes (373, 376). The essential fatty acid, linoleic acid was found to be significantly higher in IUGR placentae compared to those from appropriately grown fetuses (377) which may have implications for fetal brain development (378). However, there is no clear evidence for reduced fatty acid concentrations or placental transport in fetal growth restriction (373).

While the transport of amino acids and other nutrients is clearly critical for fetal growth, alterations in placental transport in asthmatic pregnancies will not be examined in this thesis. However, the placental production and metabolism of key growth regulating hormones such as the insulin-like growth factors and cortisol will be examined in this study.

1.10.3 Placental production of growth factors and growth regulating hormones

Insulin-like growth factors (IGFs)

The IGF axis is of major importance in both fetal and placental growth. Insulin-like growth factors I and II (IGF-I and IGF-II) are polypeptides with a sequence similar to that of insulin (379), which have mitogenic properties, inducing somatic cell growth and proliferation (380, 381). They may also have the ability to influence the transport of

glucose and amino acids across the placenta (382). Alterations in the IGF axis are associated with fetal growth restriction in animal models and human studies.

Knockout and transgenic mice studies have demonstrated that IGF-I and IGF-II are required for optimal fetal and placental growth (383-386). Null mutations in the gene encoding IGF-I result in mice that are 60% smaller than their wild-type littermates without altering placental size (384, 385). Inactivation of the IGF-II gene also results in a 60% reduction in fetal weight (383) with reduced placental growth also evident from embryonic day 13.5 (383, 384). When both IGF-I and IGF-II were knocked out, the birth weight was further reduced to 30% of normal size (385). Knocking out the IGF-I receptor either alone, or in combination with IGF-I or IGF-II, resulted in postnatal death due to respiratory failure and a 50% reduction in fetal size (385). Recent work has demonstrated that selective mutation of the placental promoter of the IGF-II gene (P0 in mice) results in a proportionate reduction in size of all parts of the placenta by embryonic day 12 and in fetal size by day 16, despite the fact that this transcript comprises only 10% of all placental IGF-II mRNA (386, 387). The reduced placental growth was as great as when all IGF-II was absent, suggesting that the P0 transcript is essential for determining the action of IGF-II on the placenta (386). This study also showed that mice carrying the mutation had reduced placental passive transport but increased active transport of amino acids, possibly reflecting a compensatory mechanism to increase fetal growth (386). Overexpression of the IGF binding protein, IGFBP-1 in transgenic mice results in a transient decrease in mid-gestation fetal growth (388).

The type 1 IGF receptor is similar in structure to the insulin receptor (389), being a transmembrane heterotetrameric ($\alpha_2\beta_2$) glycoprotein (390-393) with disulfide links and an intracellular tyrosine kinase domain (393, 394). It is able to bind both IGF-I and IGF-II through an extracellular α subunit; however its affinity for IGF-I is 15-20 times greater than for IGF-II (395). The type 2 IGF receptor is a single-chain polypeptide which has a high affinity for IGF-II, but does not bind IGF-I or insulin (385, 396). This receptor is identical to the mammalian mannose-6-phosphate receptor (397). Recent studies in humans have indicated that a mutation in the IGF type 1 receptor gene which results in reduced functioning of the receptor is associated with poor prenatal and postnatal growth (398, 399).

IGF-I and IGF-II circulate in pregnant women at higher levels than in non-pregnant women (400) and concentrations increase even further by the third trimester (401-404), suggesting that these hormones may have a role in fetal growth regulation in addition to their well characterised effects on postnatal growth (401). Levels of IGF-I and IGF-II in the maternal circulation are mainly derived from the liver (405). Fetal serum concentrations of IGF-I, IGF-II and IGFBP-3 increase significantly with advancing gestation, with the greatest rise in IGF-I (406).

The actions of the IGF-I and IGF-II are regulated by one of six insulin-like growth factor binding proteins (IGFBP-1-6) (407). IGFBP-2, 4, 5 and 6 are present in low concentrations in plasma (407). IGFBP-3 complexes with IGF-I or II and an acid-labile subunit acting as a reservoir for IGFs in the circulation (407, 408) and increases in maternal plasma during pregnancy (409). IGFBP-1 is dynamically regulated in human plasma and its levels can vary more than 10-fold in response to changes in insulin (407). IGFBP-1 binds IGF-I and II with greater affinity than either of the IGF receptors and thus prevents the IGFs from exerting their mitogenic actions (407).

During pregnancy, IGFBP-1 is the major regulator of IGF-I action, since it is the main product of the decidua (410, 411), the main IGFBP in the amniotic fluid (412-415) and a major binder of IGFs in fetal plasma (412, 416, 417). IGFBP-1 can exist in one of several phosphorylated forms. Jones *et al.* first described the existence of up to five phosphorylated forms in addition to a non-phosphorylated form of IGFBP-1 finding that amniotic fluid and fetal serum contained large amounts of the non-phosphorylated form, while decidual cells contained only the phosphorylated forms (418). This group also showed that the mix of phosphorylated forms of IGFBP-1 had six-fold higher affinity for IGF-I than the non-phosphorylated form (418). Subsequently, Westwood *et al.* demonstrated the importance of post-translational phosphorylation of IGFBP-1 in pregnancy by showing that plasma from non-pregnant adults only contained the highly phosphorylated species, while pregnant plasma also contained a non-phosphorylated and three less phosphorylated variants, with concentrations at least double that of non-pregnant individuals and higher in multi-fetal pregnancies (417, 419, 420). The highly phosphorylated isoform has the highest affinity for IGF-I which is greater than that of the IGF type 1 receptor, resulting in an inhibition of IGF activity, while the non-phosphorylated form has a similar affinity for IGF-I as its receptor (417, 421). Dephosphorylation of IGFBP-1 may represent a mechanism by which IGF-I is released

and its bioactivity increased during pregnancy. Maternal serum concentrations of IGFBP-1 increase in the first trimester, peak at mid-gestation and remain constant until delivery, falling after birth (422).

The human placenta produces IGF-I and IGF-II which may act as local growth regulators (423). The mRNA abundance of IGF-II is greater than that of IGF-I in the placenta at all gestational ages (411). IGF-II is found throughout the chorionic villi, chorionic plate, basal plate and fetal membranes, while all IGFBPs are found in the decidua, with IGFBP-1 in greatest abundance (410, 411). IGFBP-1 produced by the maternal decidua may be involved in cell to cell communication with IGF-II produced by fetal trophoblast cells, due to the close spatial positioning of the two mRNAs (411). The autocrine or paracrine actions of IGF-II and IGFBP-1 may be especially important during implantation and trophoblast invasion (424, 425). In the syncytiotrophoblast, type 1 IGF receptors are found mainly on the microvillous membrane, facing the maternal side (426). IGFBP-3 has been localised to both the microvillous and basal membranes and IGFBP-1 is predominantly found on the basal surface, facing the fetal side (427).

Immunohistochemistry and *in situ* hybridisation studies have shown that placental expression of IGF-I is increased in some cases of IUGR, possibly as a compensatory mechanism for reduced fetal growth (428, 429). However, another study showed that secretion of IGF-I from decidual explants is reduced in cases of IUGR and a correlation with birth weight was observed (430). However, across the birth weight spectrum, no correlation between decidual secretion of either IGF-I or IGFBP-1 and birth weight was noted by the same group, suggesting that reduced IGF-I in IUGR represents a discrete hormonal profile (431). Abnormal production of IGF-I from the placenta has been proposed to play a role in some cases of IUGR (432). Across a group of normal and diabetic pregnancies, placental IGF-II mRNA was positively correlated with placental weight (433).

The role of the IGF axis in fetal growth has been studied in monozygotic twin pregnancies where the twins are genetically identical and share a common uterine environment. Twin to twin transfusion syndrome (TTTS) accounts for a high rate of perinatal mortality in monochorionic twins and causes the growth of one twin to be compromised as it donates blood to the other (434, 435). Fetal serum IGF-I

concentrations are thought to be primarily determined by genetic influences, while IGF-II and IGFBP-1 concentrations are determined both by maternal environment and genetic factors (436). Bajoria *et al.* found that donor twins with TTTS had significantly lower levels of IGF-II and significantly higher levels of IGFBP-1, particularly the inhibitory phosphorylated isoform, than their recipient twin (435). In addition, there was a positive correlation between birth weight and IGF-II and a negative correlation with IGFBP-1 (435). Similarly, another study of monozygotic twins with discordant growth found lower IGF-II, similar IGF-I and increased total IGFBP-1 in the growth restricted twin compared to the normally growth co-twin (437). Given that the IGF-I levels in cord blood were similar and are thought to be genetically determined, altered placental production or placental regulation was proposed to contribute to changes in IGF-II and IGFBP-1 in growth restricted twins (435). Inadequate placental dephosphorylation of IGFBP-1 may lead to alterations in the mitogenic activity of IGF-I and of placenta nutrient transfer stimulated by IGF-I (435, 438).

Alterations in the IGF axis are also observed in dichorionic twins and in singletons of low birth weight. Two studies in dichorionic twins with discordant birth weight have found that the smaller twin had lower cord blood levels of amino acids and IGF-I and higher levels of IGFBP-1 (437, 439) and that IGFBP-1 concentration was negatively associated with total essential amino acids (439). Numerous studies have found a positive relationship between cord blood IGF-I and birth weight in normal term singleton infants (440-450) and some have also found a relationship between cord blood IGF-I and other parameters of size such as birth length (444, 449), crown-rump length (451), ponderal index (442) or placental weight (442, 443, 451), but not head circumference (452). In pregnancies complicated by IUGR, umbilical cord blood IGF-I is reduced compared to pregnancies with normal fetal growth (403, 453-456). These differences may be apparent earlier in gestation as measurements of fetal IGF-I and IGF-II by cordocentesis showed that fetal IGF-I and third trimester IGF-II were reduced in cases of growth restriction (403). Some studies have not been able to demonstrate any relationship between cord blood IGF-I and birth weight in normal term infants (457, 458). In addition, the relationship between IGF-II and birth weight is not clear with some groups finding a positive correlation in term singletons (441, 448) and others finding no significant correlation (440, 442) or no difference between normally grown

and growth restricted groups (453). A correlation has been demonstrated between cord blood IGF-II and placental weight (445).

The relationship between cord blood IGFBPs and fetal growth has also been examined. IGFBP-3 correlates positively with birth weight (442, 446, 448, 459), while IGFBP-1 is inversely correlated with birth weight in term (443, 450, 460) and preterm infants (461, 462). Increased cord blood IGFBP-1 (456) and reduced IGFBP-3 have been observed in IUGR neonates (403, 454, 456). Iwashita *et al.* also observed an increase in phosphorylated isoforms of IGFBP-1 and a reduced proportion of non-phosphorylated to total IGFBP-1 in SGA fetuses, suggesting that the bioactivity of IGFBP-1 is increased in cases of poor fetal growth (463).

Many studies have described some relationship between cord blood IGFs or IGFBPs and birth weight, but whether there is any relationship between fetal growth and maternal concentrations of these factors is more controversial. Boyne *et al.* found a positive correlation between maternal IGF-I concentration and birth weight and a negative correlation between maternal IGFBP-1 and birth weight, at 35 weeks gestation but not earlier in gestation (450). Reduced maternal IGF-I (404, 464-466), IGF-II (466) and elevated IGFBP-1 (465) in cases of fetal growth restriction have been described. However, other studies could not demonstrate any association between maternal IGF-I or IGFBP-1 measured at any stage of pregnancy, with neonatal birth weight or the development of IUGR (454, 467, 468). Similarly, several studies report no correlation between maternal IGFBP-3 and fetal growth (446). Despite this, it is clear that the IGF axis has a crucial role to play in modulating normal fetal growth during human pregnancy, with IGF-I and IGFBP-1 implicated as having central roles in fetal growth and IGF-II possibly having an important role in placental growth. These factors potentially play a role in altered fetal growth in asthmatic pregnancies and will be investigated in this thesis.

1.10.4 Placental metabolism of glucocorticoids

Glucocorticoids and the fetus

Glucocorticoids are essential for the development and maturation of fetal organs before birth. Late pregnancy in humans and in many animal species is characterised by a rise in cortisol levels, which parallels the increased maturity of fetal organs (469). Studies in

the sheep demonstrated that infusion of adrenocorticotropic hormone (ACTH), cortisol or dexamethasone into the preterm fetus resulted in delivery of lambs within 4-7 days (470, 471). These animals had accelerated adrenal growth and maturation of the lungs comparable to term lambs, suggesting an effect of glucocorticoids on fetal lung development (470, 471). Glucocorticoids also contribute to maturation of other organs including the thymus, gastrointestinal tract (472, 473), liver (474) and kidney (475). Incubation of human fetal lung explants with dexamethasone stimulates fatty acid synthesis and fatty acid synthetase activity, which are involved in surfactant production (476). Many of the studies on fetal organ maturation by glucocorticoids have been carried out in animals such as the sheep, which may differ significantly from the human.

In humans, betamethasone administration to women at risk of preterm delivery has confirmed the effectiveness of glucocorticoids in maturing the fetal lungs since it lowers the incidence of neonatal RDS and its associated mortality (477, 478). Glucocorticoid treatment has been shown to result in an increase in the ratio of lecithin to sphingomyelin in amniotic fluid, an indicator of fetal lung development and surfactant synthesis (479, 480). Today antenatal glucocorticoids are commonly given to women in preterm labour to mature the fetal lungs and successfully reduce the risk of neonatal morbidity and mortality (481). Recent research interest, however, has focussed on the potentially harmful effects of these treatments on the fetus and particularly on fetal growth.

Glucocorticoids may have adverse effects on the fetus. Women using prednisone during pregnancy have been reported to have an increase in still-birth, fetal distress, placental insufficiency (191) and low birth weight neonates (192). Antenatal dexamethasone treatment has been associated with a reduction in birth weight, by as much as 161 g in infants delivered between 30 and 32 weeks (482). In addition, multiple doses of antenatal glucocorticoids have been linked to reduced fetal growth compared to single doses (483). However, recent evidence from randomised controlled trials suggests that there is no additional decrease in fetal growth when repeated courses of antenatal steroids are used compared to single doses (484, 485). French *et al.* found that repeated courses of betamethasone were associated with a 9% reduction in birth weight and a 4% reduction in head circumference in preterm infants born prior to 33 weeks gestation (486). In addition to reduced growth, neonates who receive antenatal glucocorticoids have also been shown to have an increased incidence of gastroesophageal reflux (487)

and modifications in fetal heart rate (488, 489). *In vitro* studies suggest that effects on the fetal vascular system may be due to the vasodilatory properties of glucocorticoids (490, 491). The use of oral or inhaled glucocorticoids by pregnant women with asthma may contribute to reduced fetal growth or altered fetal development.

Numerous animal studies have demonstrated that synthetic glucocorticoids can inhibit fetal growth. Synthetic glucocorticoid treatment to pregnant ewes in mid-gestation results in reduced fetal weight (492), with the greatest effect in animals receiving repeated doses (493). Four doses of betamethasone reduced birth weight by 27% in sheep (494). Reduced body weight or organ weight at birth following glucocorticoid treatment during pregnancy has also been demonstrated in mice (192), rats (495), rabbits (496), rhesus monkeys (497) and guinea pigs (498). Other effects of glucocorticoid administration in animals included decreased brain weight, neurological damage (499-501) and placental lesions (502). Fowden *et al.* examined the mitogenic effect of endogenous cortisol on sheep fetal growth (503). In late gestation, the crown-rump length decreased in parallel with the fetal cortisol surge and this decrease in growth was prevented by fetal adrenalectomy (503). This study linked the rise in cortisol in late gestation with a reduction in fetal growth in sheep. The effects of glucocorticoids on fetal growth may be mediated by changes in IGF-I. In pregnant rats, treatment with betamethasone or dexamethasone decreased maternal plasma IGF-I, which was related to reduced liver to body weight ratio (504, 505). Indirectly or directly, glucocorticoids have a beneficial effect on fetal organ maturation before birth but also have the potential to reduce fetal growth.

Placental 11β-hydroxysteroid dehydrogenase (11β-HSD)

Maternal cortisol concentrations are 5-10 times higher than fetal cortisol concentrations (506-508). This difference is maintained by the presence of the placental enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which controls the passage of cortisol from mother to fetus. Two isoforms of 11β-HSD have been cloned and characterised in humans (509, 510) which interconvert glucocorticoids with their 11-keto metabolites (Figure 1.3). The type 1 enzyme (11β-HSD1) is NADP(H) dependent, catalyses the bi-directional interconversion of cortisol and cortisone, but acts primarily as an oxoreductase, converting cortisone to cortisol (511). This is due to its higher affinity for cortisone (K_m in the nanomolar range) compared to cortisol (K_m in the

micromolar range) (512). The type 2 enzyme (11β-HSD2) is a high affinity, NAD dependent, uni-directional enzyme, catalysing only the dehydrogenase reaction, converting cortisol to cortisone (513). 11β-HSD1 and 2 are members of the short-chain alcohol dehydrogenase super family (514), are quite different in amino acid sequence, sharing about 21% homology (515) and the genes encoding them are found on chromosome 1 (509) and 16 (516) respectively.

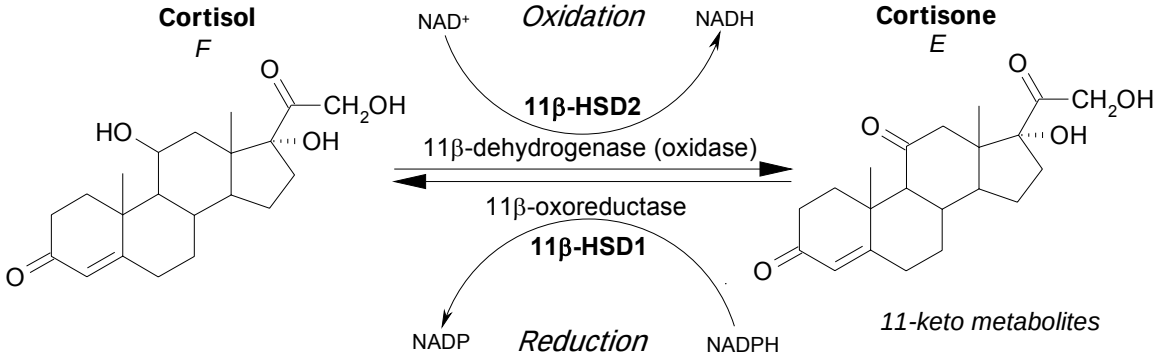

Figure 1.3 Interconversion of cortisol and cortisone by 11β-HSD

11β-HSD2 catalyses the oxidation of cortisol to its inactive 11-keto derivative, cortisone, with the use of NAD as a cofactor. 11β-HSD1 catalyses the reduction of cortisone to cortisol with NADPH as a cofactor.

The type 1 11β-HSD isozyme is mostly found in tissues such as the liver (517), adipose tissue (517), lung (518) and testis (519) with its main function being to increase the availability of glucocorticoids for the glucocorticoid receptor (GR), allowing pre-receptor control of local glucocorticoid action (515). 11β-HSD1 is also found in gestational tissues; predominantly the decidua (517, 520) and chorion (521), as well as the endothelium of placental villous tissue (521), where it modulates the effect of cortisol on other placental pathways including prostaglandin biosynthesis and metabolism (522, 523).

The type 2 11β-HSD isozyme is found in specific tissues such as the kidney (524-526), colon (527), adrenal (517) and the placenta (526, 528). Its presence in mineralocorticoid target tissues, especially the kidney, is necessary to protect the mineralocorticoid receptor (MR) from occupation by cortisol (529, 530). Cortisol has a much higher plasma concentration than aldosterone, the "natural" mineralocorticoid, but the two compounds have equal affinity for the MR (531). The 11-keto metabolites formed by 11β-HSD2 are unable to bind to the MR, while aldosterone is not metabolised by 11β-HSD2 and therefore remains active. Over activation of the MR by cortisol leads to

sodium retention and potassium excretion in the renal tubules, resulting in hypertension and suppression of the renin-angiotensin system (532). This can occur following excess ingestion of licorice which contains the 11β-HSD inhibitor, glycyrrhetinic acid (533) and in a congenital disease known as apparent mineralocorticoid excess (AME) which results from mutations of the 11β-HSD2 gene (534, 535).

In the placenta the main function of 11β-HSD2 is to protect the fetus from the potentially harmful effects of endogenous maternal glucocorticoids (536). Synthetic glucocorticoids such as dexamethasone and betamethasone are not thought to be extensively metabolised by placental 11β-HSD2, possibly due to protection from their 9-halogen group (537, 538) (see Figure 1.2). No studies have been published regarding the placental metabolism of synthetic glucocorticoids used for asthma treatment during pregnancy such as beclomethasone, budesonide and fluticasone. Preliminary studies from our group suggest that beclomethasone is partially metabolised by the placenta, but that budesonide and fluticasone are not (539). If these glucocorticoids reach the placenta but are not metabolised into inactive forms, they may have adverse affects on the fetus and may contribute to reduced fetal growth in asthmatic women using inhaled or oral glucocorticoid medication.

In addition to its barrier role, 11β-HSD2 in the placenta may also protect the MR as in tissues such as the kidney (540, 541). Hirasawa *et al.* co-localised 11β-HSD2 and MR immunoreactivity and mRNA in the placenta (540). This group suggests a role for 11β-HSD2 in regulation of maternal-fetal electrolyte and water transport in the placenta in addition to its barrier role (540). Driver *et al.* have also found mineralocorticoid-responsive genes and a functional MR in human cytotrophoblast cells, suggesting that 11β-HSD2 is involved in placental sodium transport (541, 542).

The ability of the placenta to metabolise cortisol and other glucocorticoids to 11-keto products was first described by Osinski in 1960 (528). An immunohistochemical study by Krozowski *et al.* found that 11β-HSD2 was localised to syncytiotrophoblast cells lining the chorionic villi (526). Similarly, Hirasawa *et al.* detected 11β-HSD2 immunoreactivity in syncytiotrophoblast from 5 weeks to term (540). In placental bed biopsies, 11β-HSD2 immunoreactivity was found in fused syncytiotrophoblast, invasive extravillous trophoblast and trophoblast lining the maternal spiral arteries (542). Sun *et al.* found 11β-HSD2 mRNA (but not 11β-HSD1) in the placenta and no expression of

11β-HSD2 in the amnion, chorion or decidua (521). Activities of both type 1 and 2 enzyme were demonstrated in the human perfused placenta by Sun *et al.* (543), while Benediktsson *et al.* found that most of the maternally administered cortisol was converted to cortisone with no cortisone to cortisol conversion detected (536). Dodds *et al.* also demonstrated cortisol to cortisone conversion in the perfused placenta, which could be eliminated by co-perfusion with the inhibitor glycyrrhetinic acid (544).

Immunohistochemical studies have localised 11β-HSD1 to the chorion trophoblast, amnion epithelial cells, the endothelium of placental and umbilical blood vessels and the decidua (521). Others have confirmed the presence of 11β-HSD1 in decidual stromal cells (517, 542). 11β-HSD1 mRNA was detected in the greatest amount in chorion and also in amnion and placenta (521).

Human studies on the expression and activity of the 11β-HSD isozymes in the placenta and fetal membranes throughout gestation have produced conflicting results. In 1973, Beitins *et al.* demonstrated that at term, 75% of the cortisol found in the fetus was of fetal origin, while all the cortisone in the fetus was of maternal origin (545). This suggested that placental 11β-HSD2 was acting as an effective glucocorticoid barrier at term and that fetal cortisol was mainly derived from the fetal adrenal and not from a maternal source (545). Similar work from Murphy *et al.* (1974) indicated that high levels of 11β-HSD activity were present in early gestation (13-18 weeks), with 85% of infused maternal cortisol converted to cortisone by the placenta (546). Giannopoulos *et al.* (1982) examined placental 11β-HSD activity and found that type 2 activity predominated and that this activity decreased from early (8-12 weeks) to late (38-40 weeks) gestation (547). Similarly, Blasco *et al.* (1986) described a decrease in placental 11β-HSD2 activity from early to late gestation (548). Studies have shown an increase in 11β-HSD1 conversion of cortisone to cortisol in the fetal membranes with advancing gestational age (549, 550). No labour-associated changes in 11β-HSD2 mRNA abundance or enzyme activity have been described (521, 551, 552).

More recent studies have described an increase in 11β-HSD2 activity (553, 554) and mRNA abundance (555) in the placenta from mid to late gestation. Shams *et al.* compared samples collected in the first and second trimester from terminations with preterm samples (27-36 weeks gestation) and term placenta (39-40 weeks gestation) (554). They did not examine any trends within the term group, but found an overall

increase in placental 11β-HSD2 activity across the whole of pregnancy (554). Similarly, Schoof *et al.* compared a preterm group with a term group, with a wide range of gestational ages from 18 to 41 weeks, finding an overall increase in 11β-HSD2 mRNA (555). In 2003, Kajantie *et al.* published a report on 107 small preterm placentae (22-32 weeks) and demonstrated a fall in placental 11β-HSD2 activity rate as gestation progressed (556). In the guinea pig, a species with a similar hemomonochorial placental structure to the human, 11β-HSD2 activity falls significantly in late gestation (557). Our group has proposed that there is a decrease in 11β-HSD2 activity in the last few weeks of human gestation and an increase in placental 11β-HSD1 mRNA abundance with spontaneous labour (552). This may be a mechanism by which cortisol concentrations rise at term to regulate fetal maturation and activate pathways associated with labour (552).

Placental 11β-HSD2 and fetal growth

Reductions in 11β-HSD2 activity have been associated with reduced human fetal growth. Shams *et al.* demonstrated that there was a significant reduction in enzyme activity in placentae from pregnancies complicated by IUGR compared to normally grown term deliveries and appropriately grown preterm deliveries (554). Further work demonstrated that there were also reductions in 11β-HSD2 mRNA levels but no mutations in the gene (558). Studies in rats have demonstrated a relationship between 11β-HSD2 activity and birth weight (495). One human study found a positive correlation between placental 11β-HSD2 activity and birth weight (559). However, a larger study from this group was unable to confirm this result (560). In the latter report, all but one neonate weighed more than 2500 g, suggesting that the correlation of 11β-HSD2 activity and birth weight may not be apparent within the normal weight range, but may become more obvious when studying low birth weight infants (560). Hofmann *et al.* found no correlation between placental 11β-HSD2 activity and birth weight in healthy term pregnancies, or in pregnancies complicated by PIH or IUGR (561). However, others have reported reduced 11β-HSD2 activity or mRNA in placentae from patients with pre-eclampsia, where there was decreased fetal growth, compared to normotensive pregnancies (562, 563). In small preterm infants (22-32 weeks gestation), a positive correlation between relative birth weight (expressed in standard deviation units compared to population standards) and placental 11β-HSD2 total activity and activity rate was observed (556). In addition, lower birth weight was

associated with reduced umbilical cord vein cortisone, also suggesting a reduction in transplacental cortisol to cortisone conversion in association with reduced fetal growth (556).

One of the clinical features of patients suffering from AME, which results from mutations of the 11β-HSD2 gene, is moderate IUGR (564, 565). Kitanaka *et al.* found that 17 out of 18 AME patients had a birth weight less than 2700 g (564). Stewart *et al.* studied 11β-HSD2 activity in placenta obtained from a 28 week twin still-birth in a family with two other children with AME (565). Placental 11β-HSD2 activity was approximately 15% of that in five gestational-age matched controls and immunohistochemical staining for 11β-HSD2 was virtually absent in the AME placenta (565). Both siblings with AME and the placenta were shown to have a point mutation in exon V of the 11β-HSD2 gene (565). There have been other reports of still-birth in families with 11β-HSD2 mutations and AME (566). These studies suggest that reduced 11β-HSD2 activity may be related to reduced fetal growth and possibly an increased risk of fetal death. The role of placental 11β-HSD2 in fetal growth regulation in asthmatic pregnancies will be a focus of this thesis, as decreased activity or mRNA expression may be associated with reduced fetal growth.

Glucocorticoids and fetal programming

Glucocorticoids are thought to be involved in the fetal programming of adult disease. Although no human studies have investigated placental 11β-HSD2 in relation to outcomes in later life, animal studies have implicated decreased 11β-HSD2 activity as having a role in fetal programming.

In rats, Benediktsson *et al.* showed a positive correlation between placental 11β-HSD2 activity and term fetal weight and a negative correlation with placental weight (495). Treatment of pregnant rats with dexamethasone, a steroid not extensively metabolised by placental 11β-HSD2, resulted in a decrease in maternal weight gain, reduced birth weight and significantly raised blood pressure 140-150 days after birth compared to untreated rats (495). This study proposed that the relationship between low birth weight, high placental weight and increased adult blood pressure may be mediated by glucocorticoid exposure *in utero* (495).

Levitt *et al.* found that administration of dexamethasone to rats in late pregnancy resulted in an 11% reduction in birth weight and elevated blood pressure in offspring at

16 weeks of age (567). The same group later demonstrated that inhibition of placental 11β-HSD2 by carbenoxolone treatment throughout pregnancy gave similar results (568). They observed a 20% decrease in birth weight and elevated blood pressure in adult offspring. When mothers were adrenalectomised, this effect did not occur, highlighting the importance of exposure to maternally derived glucocorticoids (568). Male offspring were also found to be hyperglycemic later in life (569). Similar studies by another group found that maternal carbenoxolone treatment in pregnant rats resulted in smaller offspring with glucose intolerance in later life and reduced hepatic 11β-HSD1 and reduced renal 11β-HSD2 gene expression (570). In sheep, mid-gestation dexamethasone treatment inhibited placental 11β-HSD2 gene expression and reduced birth weight (493).

Maternal protein restriction has been shown to decrease birth weight and placental 11β-HSD2 activity in rats (571). In early adulthood, offspring also had raised systolic blood pressure (571). This study proposed that maternal undernutrition results in fetal glucocorticoid exposure and this leads to the programming of hypertension in later life (571). Further work demonstrated that a low protein maternal diet reduced 11β-HSD2 gene expression in the rat placenta and in the fetal and neonatal kidney and adrenal (572). The authors suggested that altered exposure of the fetus and in particular, the fetal kidney, to glucocorticoids may lead to the observed increase in GR protein and mRNA expression in the kidney, which was a possible mechanism for raised blood pressure in later life (572).

Prenatal exposure to maternally derived glucocorticoids may also alter other aspects of postnatal development. Smith and Waddell found that treatment of pregnant rats with either dexamethasone or carbenoxolone led to reduced birth weight and a delay in the onset of puberty in females (573). Particular research attention has focussed on glucocorticoid-mediated alterations to the fetal hypothalamic-pituitary-adrenal (HPA) axis. Carbenoxolone treatment during pregnancy in rats leads to increased basal corticosterone levels, reduced GR mRNA in the hypothalamus and alterations in stress induced behaviour in the offspring (574). Similarly, maternal undernutrition in rats leads to reduced placental 11β-HSD2 mRNA, reduced neonatal weight, adrenal atrophy, reduced GR and MR mRNA in the hippocampus and increased neonatal plasma corticosterone at birth (575).

Treatment of pregnant guinea pigs with dexamethasone at the time of rapid fetal brain development (days 50 and 51 of a 70 day gestation) results in an immediate increase in plasma cortisol in females and a decrease in plasma cortisol in male fetuses (576). In addition, increased hippocampal MR and GR gene expression was observed, but only in female fetuses (576). When pregnancies progressed to term, female offspring exhibited a reduced brain to body weight ratio, while male and female offspring both had reduced heart to body weight ratios (498). Fetal exposure to dexamethasone resulted in reduced plasma ACTH in females, increased plasma cortisol in males and decreased plasma cortisol during isolation stress in female offspring at 18 days of age (498). There are sex-specific differences in the regional and temporal expression of brain GR and MR (577) and sex-specific changes in hippocampal GR expression were observed in offspring exposed to dexamethasone (498). Repeated doses of dexamethasone or betamethasone during guinea pig pregnancy also resulted in inhibition of the fetal HPA axis (578). Similar alterations in HPA function of offspring can be induced by prenatal exposure to stress, such as noise and light stress (579) and restraint stress (580) in rats. Evidence from all this work demonstrates that fetal exposure to glucocorticoids during the period of rapid brain development permanently alters HPA function (498, 581).

Studies in humans have also related low birth weight and prenatal stress to altered HPA activity in later life. Low birth weight has been associated with elevated cortisol levels at birth (582) and elevated plasma cortisol concentrations or HPA activity in adult life in several populations (583, 584). Another group has found that the effect of birth weight on adult plasma cortisol is dependent on gestational age at birth (585, 586). In those born before 39 weeks gestation, lower birth weight was associated with higher total and free plasma cortisol, while in those born after 40 weeks gestation, lower birth weight was associated with lower plasma cortisol (585). In children, increased urinary excretion of glucocorticoids was found in those who had the lowest or highest birth weights (587). Maternal first trimester exposure to the stress of war has been associated with an increased risk of the offspring developing schizophrenia in adult life (588). Lou *et al.* found that maternal stress during mid-gestation affected birth weight and was associated with small head circumference, suggesting a specific effect on the brain (589), thus linking prenatal stress, reduced growth and altered brain development. Approximately 10% of maternal cortisol does cross to the fetus and increases in maternal cortisol levels may therefore contribute to increased fetal cortisol levels during

pregnancy (508). Therefore, despite the presence of the placental 11β-HSD2 enzyme barrier, an increase in maternal glucocorticoids as a result of stress could contribute to a significant change in fetal glucocorticoid exposure (508), which would be compounded by reduced placental 11β-HSD2 activity.

Regulation of placental 11β-HSD2

Placental 11β-HSD2 is clearly an important modulator of fetal glucocorticoid exposure and it is regulated by many hormones and factors associated with pregnancy, including estradiol, progesterone and prostaglandins. Studies in other tissues and cell lines have demonstrated regulation of this enzyme by inflammatory cytokines. Some of these regulators are also associated with asthma and therefore it is possible that women with asthma are particularly susceptible to altered placental 11β-HSD2 during pregnancy.

In syncytiotrophoblast cell cultures, progesterone dose-dependently reduced 11β-HSD2 activity through a non-receptor mediated mechanism and also reduced 11β-HSD2 mRNA abundance, an effect which was reversed by treatment with progesterone receptor antagonists (590). In addition, Pepe and Albrecht reported that 11β-HSD2 activity in human and baboon placental homogenates was inhibited by progesterone (591). Estradiol was found to significantly decrease activity but not mRNA of 11β-HSD2 in placental cells (590). Nitric oxide (NO) donors (sodium nitroprusside and S-nitroso-N-acetyl penicillamine) have been shown to inhibit 11β-HSD2 mRNA and activity in syncytiotrophoblast cells cultured for 72 hours, through a cGMP mediated pathway (592). Activators of the cAMP pathway such as forskolin were demonstrated to increase 11β-HSD2 activity and mRNA expression in JEG-3 choriocarcinoma cells (593) and syncytiotrophoblast cells (590), while activation of the protein kinase C pathway by phorbol 12-myristate 13-acetate (PMA) had no effect on placental 11β-HSD2 (590, 593).

Hardy *et al.* examined the effect of the prostaglandins, PGE_2 and $PGF_{2\alpha}$ and the leukotriene, LTB_4 on 11β-HSD2 activity and gene expression in JEG-3 cells (594). They found that PGE_2 and $PGF_{2\alpha}$ reduced 11β-HSD2 activity to 75% of the untreated level. Blocking prostaglandin synthesis with the cyclo-oxygenase inhibitor indomethacin, however, did not reverse the effect, but also resulted in inhibition. LTB_4 treatment resulted in a dose dependent inhibition of 11β-HSD2 activity. Importantly, this study showed that there were no corresponding changes in the mRNA abundance of

11β-HSD2 by treatment with PGE_2, $PGF_{2\alpha}$ or LTB_4, indicating that their effect was post-translational (594).

Recent work from Alfaidy *et al.* showed that oxygen may be an important regulator of placental 11β-HSD2 (595). In this study, incubation of first trimester placental villous explants or trophoblast cell cultures from term placentae under 20% O_2 led to a significant increase in 11β-HSD2 protein expression and activity compared to incubation under 3% O_2 (595). Similarly, Hardy and Yang found that 11β-HSD2 protein and activity more than doubled when cytotrophoblast cells differentiated into syncytiotrophoblasts under 20% O_2 (596). However, when cells were cultured under 1% O_2, they did not differentiate and 11β-HSD2 was not increased (596).

11β-HSD2 activity is inhibited by calcium in placental microsomes and in JEG-3 cells via a post-translational mechanism (597). Calcium is a common second messenger for leukotrienes and prostaglandins, previously shown to inhibit placental 11β-HSD2 activity (594). Inhibition by calcium was reversed by the addition of a calcium chelator and inhibition did not alter the binding capacity for cortisol and could not be overcome by the addition of extra cofactor, indicating that the effect was mediated through a change in the enzyme's catalytic efficiency (597). The catecholamines epinephrine and norepinephrine also inhibit placental 11β-HSD2 through a decrease in mRNA in trophoblast cells (598). Since catecholamines are released during stress, this may be a mechanism linking prenatal stress and altered fetal development.

ATP has been shown to increase placental 11β-HSD2 activity in microsomes via a mechanism independent of phosphorylation (599). Tremblay *et al.* found that retinoic acids, the major metabolites of vitamin A, dose-dependently stimulated 11β-HSD2 activity in JEG-3 cells via an increase in mRNA expression (600).

The regulation of 11β-HSD2 activity has been studied in other cell types. In the kidney, progesterone and its metabolites such as 5α-dihydro-progesterone have been shown to inhibit microsomal 11β-HSD2 (601). Hypoxia also inhibited 11β-HSD2 activity in a renal epithelial cell line and this study also demonstrated reduced renal 11β-HSD2 in healthy men as a result of ascending to high altitude (602). In bronchial epithelial cells, dexamethasone was found to increase 11β-HSD2 mRNA and protein and dose dependently increase activity over 72 hours (603). Previous work in osteosarcoma cells indicated that the pro-inflammatory cytokines TNF-α and IL-1β dose-dependently

inhibit both activity and mRNA expression of 11β-HSD2 (604). However, the effect of these and other inflammatory cytokines on 11β-HSD2 have not previously been examined in the placenta.

Glucocorticoids have an important role to play during fetal development, promoting maturation of organs required for extra-uterine survival. An important pre-receptor mechanism exists to control the actions of glucocorticoids during pregnancy in the form of placental and fetal 11β-HSD2. Alterations in the activity of the placental 11β-HSD2 barrier which result in an increase in maternal glucocorticoids crossing to the fetus, can have a deleterious effect on fetal growth and postnatal development. This pathway will be examined in asthmatic placentae.

1.11 The role of the fetus in growth regulation

The fetus itself may have a role in growth regulation. The fetal tissues also express IGFs and 11β-HSD2, allowing the fetus to adjust local levels of growth factors and glucocorticoids, thereby modulating cellular growth and differentiation in an autocrine or paracrine manner.

The mid-gestation human fetus (16-19 weeks) contains 11β-HSD2 mRNA and activity in the kidney, lung (605), gonad, liver, adrenal (606) and colon (607-609), while 11β-HSD1 mRNA has not been found in any fetal tissues at mid-gestation (608). In many fetal tissues, 11β-HSD2 is co-localised with the GR (610) or with the MR (611). The presence of placental 11β-HSD2, high levels of 11β-HSD2 activity in fetal tissues and the absence of 11β-HSD1 in the fetus all contribute to a predominance of cortisone over cortisol in the fetal circulation (612). The presence of 11β-HSD2 enzyme in the fetal tissues may serve to locally regulate the positive and negative effects of glucocorticoids on the fetus.

Receptors for IGFs have also been identified in the human fetus from as early as the first trimester (613, 614), which allow IGF-I and IGF-II to exert growth promoting effects on fetal cells (615). IGF-I and IGF-II have mitogenic actions in cultures of fetal fibroblasts, fetal myoblasts (616, 617) and fetal adrenal cortical cells (618). IGF-I itself has been localised to many human fetal tissues, with high expression in the lung and intestine (617, 619). In addition, IGF-II has been found in the fetal kidney, liver, adrenal and muscle (617, 620) and may be present in larger quantities than IGF-I (621). IGF-II

is thought to be the dominant regulator of fetal adrenal growth, due to high expression in mid-gestation and regulation by ACTH (618). IGF-I and IGF-II mRNA expression was also found in all fetal tissues examined by Han *et al.* except cerebral cortex (622). IGFBP-1 has been localised to most fetal tissues including liver, lung, muscle, kidney, pancreas, adrenal and intestine (623) and *de novo* synthesis of IGFBP-1 to 4 has been observed in fetal liver and kidney explants (624). IGFBP mRNA expression studies suggest that IGFBP-1 is predominantly found in the fetal liver, while the other IGFBPs are located in most tissues of the fetus (625). IGFs may be complexed to IGFBP-1 on the surface of fetal cells as the pattern of immunostaining for fetal IGFs and IGFBP-1 was found to be similar in most sites (623). The presence of IGF-I and IGF-II mRNA and protein in most fetal tissues suggests a local role for them in modulating growth.

Fetal sex is known to affect fetal growth, with male fetuses on average being larger than female fetuses (280, 626, 627). This difference may not be evident until after 30 weeks (279, 626), but increases as gestation progresses (627) with some studies reporting a 150-200 g weight difference by 38 weeks (279, 627). Cogswell and Yip reported that among white neonates, males were 135 g heavier than females, while among black neonates there was a 125 g difference between males and females (280). In addition, males had a greater variation in birth weight distribution, with an increased tendency for higher birth weights (280). Despite the difference in birth weight between male and female infants, the survival rate is greater for females than males (280, 628).

There are fetal sex differences in the IGF axis. IGF-II concentrations in umbilical cord serum from male neonates were significantly higher than female neonates (440) and cord plasma IGF-I was higher in female neonates than males (449). A recent study of 987 healthy singletons found that IGF-I and IGFBP-3 concentrations in cord blood were higher in females than males (629). In this study, there was no difference in IGF-II between male and female neonates, while growth hormone (GH) concentrations were higher in males than females (629). Given that males are larger at birth and both IGF-I and IGFBP-3 correlate with birth size, these findings are counterintuitive, but do suggest that there are sexually dimorphic patterns of fetal growth regulation.

One possible mechanism by which the male fetus becomes larger than the female fetus was recently proposed by Tamimi *et al.*, who studied maternal dietary intake during the second trimester of pregnancy (630). They suggested that the fetus may be able to

modulate its mother's nutritional input, since women pregnant with a male fetus had a higher energy intake compared to women pregnant with a female fetus (630). After adjustment for confounding factors, this related to an extra 796 kJ per day contributed by 8% higher protein, 9.2% higher carbohydrates and over 10% higher lipid intakes in women pregnant with a male fetus compared to women pregnant with a female fetus (630).

Other studies have demonstrated that maternal smoking (631, 632) or caffeine consumption (633) have different effects on growth of the male and female fetus. Vik *et al.* studied second and third trimester caffeine consumption of mothers with SGA infants and mothers with normally grown infants (633). They found that the risk of having an SGA infant was increased in women who consumed high levels of caffeine at 33 weeks gestation. However, when data was analysed based on fetal sex, only the male fetus was at risk of being born small in association with high maternal caffeine intake (633). Spinillo *et al.* examined a variety of risk factors for fetal growth restriction and found that overall, IUGR was more frequent in female fetuses, and that females were more sensitive to hypertension-induced growth restriction (632). On the other hand, males were more affected by low maternal pre-pregnancy weight or BMI and maternal smoking (632). Other groups have also found that maternal smoking was more likely to affect males than females (313, 631). Zaren *et al.* made serial ultrasound measurements throughout pregnancy and showed a significant decrease in biparietal diameter (BPD) from 18 weeks gestation with heavy maternal smoking in males fetuses, but no significant difference in BPD in female fetuses (313). However, mean abdominal diameter in female fetuses of heavy smokers was significantly decreased from 25 weeks, while no decrease was observed in males until 33 weeks gestation (313). All aspects of male neonatal size were reduced in the presence of maternal smoking and while females had reduced birth weight and length, there was no significant difference in their head circumference, skin fold thickness or femur length compared to female neonates from non-smoking mothers (313). These studies suggest that the regulation of fetal growth may be different for male and female fetuses.

Placental 11β-HSD2 may also differ depending on the sex of the fetus, thus possibly contributing to different fetal growth regulation in males and females. In mice, there is greater placental 11β-HSD2 activity when the fetus is female than when the fetus is male (634). It is possible that factors responsible for fetal growth regulation in asthmatic

pregnancies are also altered in a fetal sex-specific manner. This could contribute to increased susceptibility to low birth weight in the male fetus, as observed with maternal smoking (632) and caffeine intake (633), or to an increased susceptibility to low birth weight in the female fetus, as observed with hypertension-associated IUGR (632).

Together the mother, placenta and fetus interact during pregnancy to modulate fetal growth. Maternal nutrients are essential for growth and development of the fetus, and transport of these nutrients occurs via the placental blood supply. The placenta is also important in the production and transport of growth promoting hormones. A barrier function for the placenta, through the activity of 11β-HSD2 is of major importance in preventing the high concentrations of anti-mitogenic glucocorticoids found in the mother from reaching the fetus in an active form. Disturbances in fetal growth regulation can result in adverse outcomes for the neonate and these adverse outcomes may persist into adult life. It is therefore important to understand the regulation of fetal growth, and particularly the role of mother, placenta and fetus in complicated pregnancies, such as those in asthmatic women. As a result, a better outcome for the fetus may be achieved, which may have long-term health benefits into adulthood.

Chapter 2 **Background, Hypotheses and Aims**

2.1 Background

Asthma is a disease which affects a large number of pregnant women around the world. Epidemiological studies have highlighted the fact that asthmatic women are more at risk of a poor pregnancy outcome than non-asthmatic women. These poor outcomes include low birth weight and preterm delivery. Low birth weight is known to be a risk factor for the development of diseases in adulthood, including diabetes and heart disease. The offspring of asthmatic mothers are therefore more at risk of morbidity and disease both in the short-term and the long-term. Currently the mechanisms which contribute to low birth weight in asthmatic pregnancies are unknown. Fetal sex is known to influence fetal growth, with males on average born larger than females. Fetal growth regulation may be fetal sex-specific. However, no other group has previously examined alterations in placental function in relation to maternal asthma and fetal sex.

Much of the literature concerning the effects of asthma on pregnancy outcomes is population based and does not address the underlying mechanisms which contribute to these outcomes in asthmatic women. The literature has focussed on a discussion of the epidemiological facts, the necessity of asthma control and the safety of glucocorticoid therapy during pregnancy. By taking the approach of studying large cohorts of women, subtle changes in birth weight in sub-groups of asthmatic women are not observed, and therefore the mechanisms contributing to such an outcome cannot be properly examined. Thus, the purpose of this study is to address an area which is lacking in the literature by closely examining the roles which the mother, placenta and fetus have to play in the regulation of fetal growth in pregnancies complicated by asthma.

My study of pregnant women with asthma will follow Australian asthma management guidelines, which are comparable to those of the National Heart Lungs and Blood Institute. The study will prospectively follow asthmatic women during pregnancy, collecting data on the mother's asthma, fetal and neonatal growth and placental function, from the same group of women. Allowing pregnant women to be managed according to individual needs, will produce a broad spectrum of subjects with range of asthma severities and medication requirements. Consequently, this approach will allow data to be analysed based on asthma severity and asthma treatment (inhaled glucocorticoid use) separately, which will improve our understanding of the role these factors play in altering fetal growth in pregnant women with asthma.

2.2 Hypotheses

This study will address the following hypotheses:

1. That fetal growth is reduced in pregnant women with asthma and that reduced birth weight is specifically related to either asthma severity or inhaled glucocorticoid treatment.
2. That susceptibility to altered fetal growth in asthmatic pregnancies differs between male and female fetuses.
3. That changes in maternal inflammation and lung function associated with asthma contribute to reduced fetal growth.
4. That alterations in placental function including reduced placental 11β-HSD2 activity contribute to changes in fetal growth and altered HPA axis function in asthmatic pregnancies, via increased maternal cortisol reaching the fetus.

2.3 Aims

This study will examine fetal growth in asthmatic pregnancies and address the following aims:

1. To determine whether alterations in fetal growth in pregnant women with asthma relate to a sub-group of women taking a particular treatment or to a sub-group of women based on asthma severity.
2. To examine the susceptibility of male and female fetuses to altered fetal growth associated with maternal asthma.
3. To determine if changes in fetal growth can be detected at 18 and 30 weeks gestation by ultrasound, or by measurements made at birth.

In order to investigate the mechanisms regulating fetal growth in pregnancies complicated by asthma, the endocrine and immune relationships between mother, placenta and fetus will be addressed further in the following specific aims:

The Mother

1. To determine whether there are changes in the requirement for inhaled glucocorticoids for asthma treatment as pregnancy progresses.
2. To determine whether there are changes in maternal lung function as gestation progresses.
3. To determine whether there are changes in circulating inflammatory cells in the mother as gestation progresses.
4. To profile maternal plasma proteins at 18 and 30 weeks gestation using a proteomic technique to determine the effect of fetal sex and maternal asthma on circulating proteins.

The Placenta

1. To determine the activity, mRNA abundance and protein expression of placental 11β-HSD2 and establish whether alterations in 11β-HSD2 are involved in fetal growth regulation in asthmatic pregnancies.
2. To determine the mRNA abundance of placental IGF-I, IGF-II and IGFBP-1 and establish whether alterations in the IGF axis are involved in decreased fetal growth in asthmatic pregnancies.
3. To determine the mRNA abundance of placental cytokines, including the Th1 cytokine, TNF-α, Th2 cytokines involved in asthma including IL-4, IL-5 and IL-10 and other cytokines such as IL-1β, IL-6 and IL-8 and establish whether these change in the presence of maternal asthma or are related to alterations in 11β-HSD2 activity.
4. To determine the mRNA abundance of the glucocorticoid receptor sub-types, GR-α and GR-β and the mineralocorticoid receptor (MR) in the placenta in the presence of maternal asthma.
5. To profile proteins present in the placenta using a proteomic technique to determine the effect of fetal sex and maternal asthma on placental proteins.

The Fetus

1. To examine fetal and neonatal outcome in asthmatic pregnancies.
2. To determine whether there are alterations in the levels of cortisol crossing the placenta in asthmatic pregnancies and to determine whether the amount of maternal cortisol crossing to the fetus is related to placental 11β-HSD2 activity.
3. To determine whether there are alterations in fetal HPA axis function in asthmatic pregnancies by measuring cord blood estriol.
4. To determine whether there are alterations in cord blood concentrations of the IGF binding proteins, IGFBP-1 and IGFBP-3 and establish whether these relate to fetal growth in asthmatic pregnancies.
5. To profile proteins present in the cord blood using a proteomic technique to determine the effect of fetal sex and maternal asthma on circulating fetal proteins.

The major premise of this study is that the mother, placenta and fetus all contribute to the regulation of human fetal growth and that the relationships between mother, placenta and fetus may be altered in pregnant women with asthma. Figure 2.1 outlines the major aspects of mother, placenta and fetus which will be examined in this study.

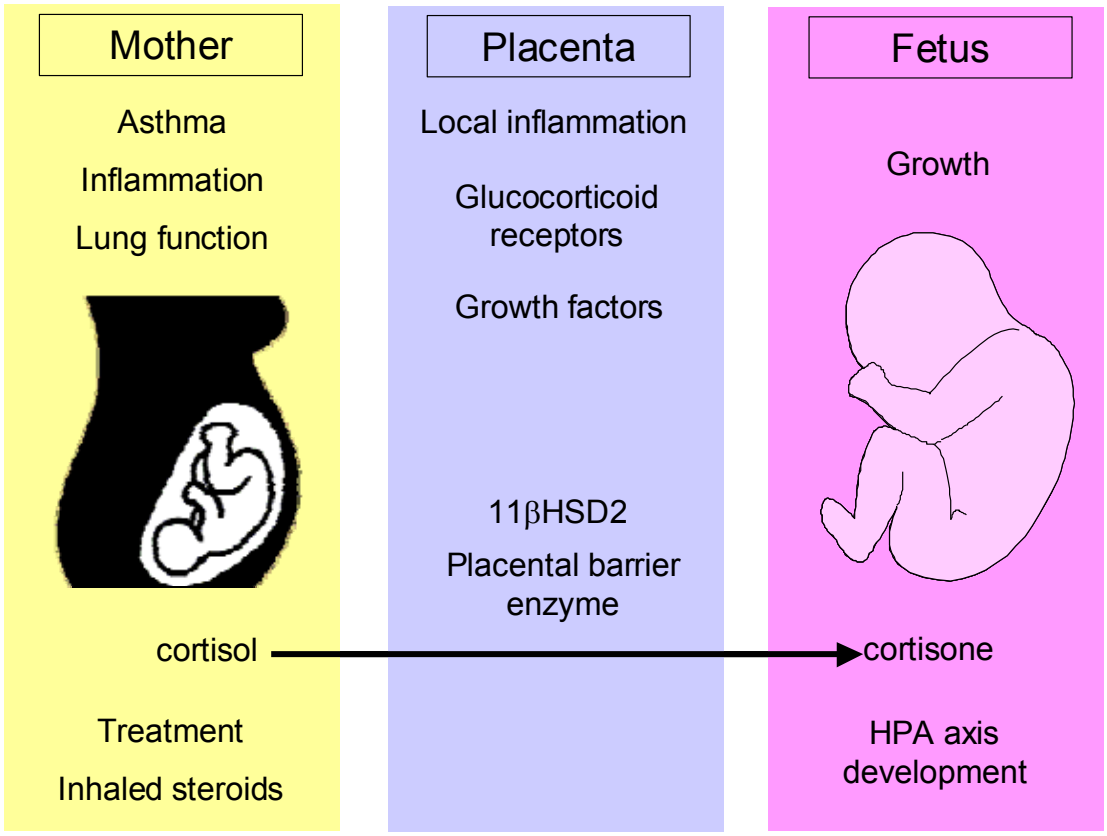

Figure 2.1 Aspects of the relationship between mother, placenta and fetus to be examined in this study

This study will examine the endocrine and immune relationships between mother, placenta and fetus in asthmatic pregnancies. In the mother, various aspects of asthma will be investigated including inflammation, lung function and treatment with inhaled steroids. In the placenta, local inflammatory pathways, glucocorticoid receptor and growth factor expression will be examined along with the mRNA and protein expression and activity of the placental barrier enzyme 11β-HSD2, which prevents the high levels of cortisol found in the mother from reaching the fetus by inactivating cortisol to cortisone. In the fetus, growth and HPA axis development will be examined.

Chapter 3 **Methods**

3.1 Subject recruitment

Pregnant asthmatic and non-asthmatic women were recruited from the John Hunter Hospital Antenatal Clinic in the first trimester by Sr Carolyn Kessell (Research Nurse, Department of Respiratory and Sleep Medicine, John Hunter Hospital, Newcastle). All women gave written, informed consent for participation (Appendix 1, Appendix 2) and ethical approval was provided by the Human Research Ethics Committees of the University of Newcastle (ethics approval number H3901097, Appendix 3) and Hunter Area Health Service (ethics approval number 9709173.07). Information on maternal age, weight, height, parity, gravidity, blood pressure and pregnancy outcomes was obtained from the patient's medical records. Women donated plasma at approximately 18 and 30 weeks gestation. Plasma was stored at –20°C until use.

3.2 Assessment of maternal asthma

Subjects were managed according to a standard treatment procedure in a combined antenatal/asthma management clinic (635). This standard procedure has been demonstrated to be beneficial for the implementation of asthma management guidelines within our hospital setting, leading to improved delivery of health care to asthmatic patients (635). The major steps of this program include assessing asthma severity during a stable period, achieving best lung function by altering bronchodilator and anti-inflammatory treatment, maintaining best lung function by avoiding triggers and using the minimum required medication, developing an action plan to manage acute asthma exacerbations, educating patients and reviewing individual asthma management regularly (635, 636). The use of this standard management procedure was ethically sound, and designed to provide maximum care for pregnant asthmatic women. It has previously been found to result in significantly improved asthma control and management skills in asthmatic adults at the John Hunter Hospital (635).

Most control, non-asthmatic subjects attended the Asthma Management Service (AMS) once at approximately 18 weeks to have lung function assessed, while asthmatic women visited the AMS two or more times depending on asthma severity and individual requirements. At these visits, FEV_1 and FVC were measured by Sr Kessell using a Vitalograph Spirometer (Vitalograph Ltd, Buckingham, UK). The percent predicted FEV_1 was calculated based upon reference values and corrected for sex, age and height

(5). In addition, a history of asthma was taken, medication use and compliance assessed (Appendix 4) and asthmatics received education about asthma control and management skills and an asthma action plan (Appendix 5). The degree of asthma control was assessed using women's self-report of the frequency of night symptoms, morning symptoms and limitation of physical activity in the previous week. Women also reported the number of days per week and times per day that reliever medication (β_2-agonist) was used. Sr Kessell assessed the women's inhaler technique and categorised this as optimal, adequate or inadequate. Asthmatic women with poor asthma control or requiring extra attention attended the AMS up to eight times as required. Classification of asthma severity was assigned by Prof Peter Gibson (Department of Respiratory and Sleep Medicine, John Hunter Hospital, Newcastle) according to the Australian asthma management guidelines (637), which are comparable to the guidelines of the National Heart Lungs and Blood Institute (638). Women were assigned an asthma severity rating of mild, moderate or severe according to symptoms, asthma history and other features including FEV_1 and PEF, as outlined in Table 3.1. Women were assigned to the most severe category which applied for any one of these criteria.

Table 3.1 Criteria used to assign asthma severity classifications

Criteria	Mild Asthma	Moderate Asthma	Severe Asthma
FEV_1	>80% predicted	60-80% predicted	<60% predicted
PEF variability		<25%	>25%
Night-time symptoms	None	Up to once per week	Frequent
Symptoms on awakening	None		Every day
β_2-agonist use	Infrequent	Most days	3-4 times per day
Daytime symptoms	<4 times per week	Most days	Every day
Other	No severe attacks in the previous year		Limited physical activity

3.3 Assessment of maternal asthma treatment

Asthmatic women used a variety of inhaled glucocorticoid medication to treat their asthma. The drugs used were budesonide, beclomethasone dipropionate or fluticasone propionate. Oral steroid medication (prednisone) was used periodically by a small number of subjects. All asthmatic women used the short acting inhaled β_2-agonist salbutamol for symptom relief when required. Women who used a long acting β_2-agonist or the combination inhaled glucocorticoid and long acting β_2-agonist medication

(fluticasone propionate plus salmeterol) were excluded from all analyses presented in this thesis.

Inhaled glucocorticoid use and oral prednisone intake before pregnancy were recorded retrospectively and intake during pregnancy was monitored during the AMS visits with Sr Kessell. Inhaled glucocorticoid dose was calculated for each trimester and expressed as the mean daily dose of beclomethasone dipropionate (BDP) or equivalent, where 1 µg BDP was considered equivalent to 1 µg budesonide or 0.5 µg fluticasone propionate. This calculation takes into account the increased potency of fluticasone propionate (639). Subjects were classified according to average cumulative dose throughout pregnancy as no glucocorticoid (no inhaled glucocorticoids used during pregnancy), low (<400 µg inhaled glucocorticoids per day), moderate (400-1500 µg inhaled glucocorticoids per day) or high (>1500 µg inhaled glucocorticoids per day). The calculation used to derive average cumulative dose is given in Appendix 6. For some data analysis, the low, moderate and high dose groups were combined into one group (glucocorticoid).

3.4 Assessment of maternal inflammation

Information from full blood counts taken during pregnancy was obtained from the medical records. Data was analysed from samples taken in early pregnancy (<20 weeks gestation) and late pregnancy (>30 weeks gestation). The number of circulating white blood cells, lymphocytes, neutrophils, eosinophils, monocytes and basophils was noted and the percentage of each was calculated.

3.5 Assessment of fetal growth

Fetal growth was assessed by ultrasound at approximately 18 and 30 weeks gestation by Prof Warwick Giles (Department of Obstetrics and Gynaecology, John Hunter Hospital, Newcastle). Measurements of femur length, abdominal circumference, head circumference and biparietal diameter (BPD = diameter of the head between the two parietal lobes) were obtained. The ratio of head circumference to abdominal circumference (HC:AC) was calculated as an indicator of proportionate growth (640). Umbilical artery flow velocity waveforms, expressed as the systolic to diastolic (SD)

ratio were also assessed by Prof Giles using Doppler ultrasound (Acuson XP4, Acuson Corporation, Mountain View, CA, USA).

Details of weight, length and head circumference at birth were obtained from the medical records. Ponderal index was calculated as birth weight (g) / [birth length (cm)]^3 × 100. Birth weight, head circumference and length centiles were calculated using John Hunter Hospital intrauterine growth charts (641), based on gestational age determined by date of the last menstrual period and 18 week ultrasound (Appendix 7). These charts were derived from a study of all live births at the Royal Women's Hospital in Melbourne in 1979, along with preterm births during 1977 and 1978 (641). Although this study reported that male infants were always slightly heavier than female infants, the authors compared the curves from 37 weeks, where the sample number was sufficient for comparison between males and females, and considered it to be impractical to present separate curves based on fetal sex (641). Consequently, the growth charts used by the John Hunter Hospital and in this study were composite male/female charts.

3.6 Assessment of pregnancy outcomes

Fetal and maternal parameters related to labour and delivery were collected from the medical records. The presence of fetal heart rate decelerations during delivery and neonatal Apgar scores at 1 minute and 5 minutes were noted. Apgar scores are used to evaluate the physical condition of the newborn immediately after delivery. They were first described by Dr Virginia Apgar in 1953 (642) and involve scoring for each of the following signs: heart rate, respiratory effort, muscle tone, reflex irritability (response to catheter in nostril) and colour (Table 3.2). Total Apgar scores of 0-3 signify a poor prognosis, while a total score of 10 indicates that the neonate is in the best possible condition (642) and the 5 minute Apgar score is considered useful for the prediction of neonatal mortality (643).

Table 3.2 Apgar scoring

Signs	Score = 0	Score = 1	Score = 2
Heart Rate (beats per minute)	Absent	Below 100	Above 100
Respiratory Effort	Absent	Slow, irregular	Good, crying
Muscle Tone	Limp	Some flexion of extremities	Active motion
Reflex irritability	No response	Grimace	Cough, sneeze
Colour	Blue, pale	Pink body, blue extremities	Completely pink

Information was collected about the type of labour (spontaneous, induced, augmented or no labour), the total duration of labour, the duration of ruptured membranes, blood loss during delivery, the presence of meconium staining and type of delivery of baby and placenta. Maternal complications such as pregnancy induced hypertension (PIH) and pre-eclampsia were noted. Calculations of group means for the total duration of labour or the duration of ruptured membranes and calculation of the percentage of subjects with a spontaneous or induced labour onset excluded women who had an elective caesarean section before labour onset.

3.7 Placenta and cord blood collection

The placenta was collected from the John Hunter Hospital Delivery Suite within 45 minutes of delivery, from a subset of subjects. Samples were obtained at any time of the day or night. The whole placenta was weighed prior to the collection of samples. Cord blood was collected from the umbilical vein into heparinised tubes (Figure 3.1). Blood was centrifuged at 1000 g for 15 minutes (J-6B centrifuge, Beckman Coulter, Palo Alto, CA, USA) for the collection of plasma. Plasma was stored at –20°C until further use. Small pieces of placental tissue were collected, snap frozen in liquid nitrogen and stored at -80°C until required.

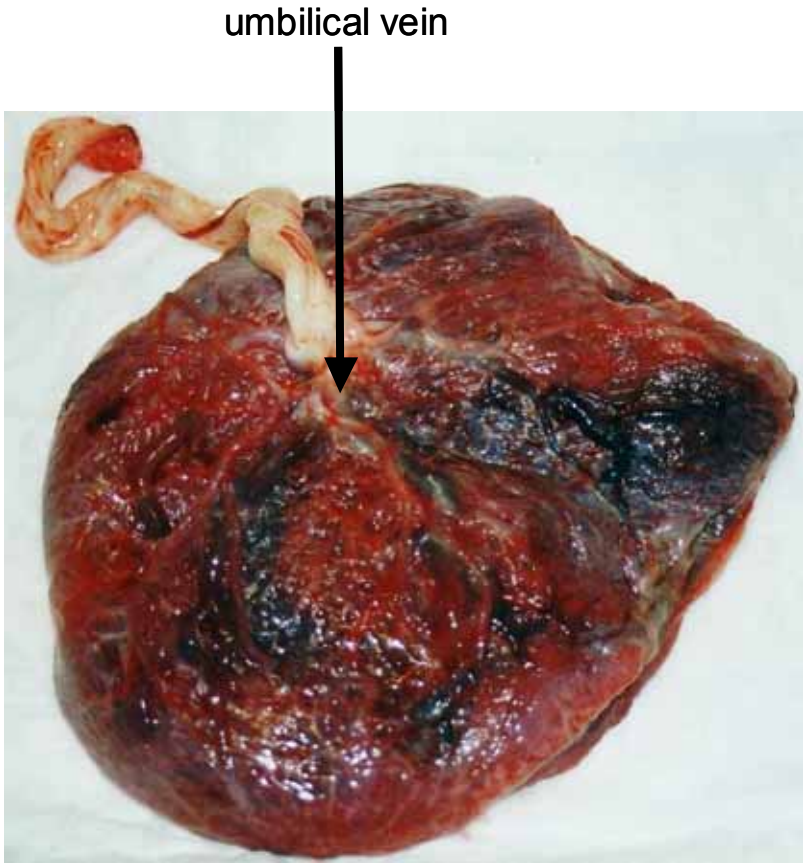

Figure 3.1 Placenta from an asthmatic woman showing location of the umbilical vein

Blood was collected from the umbilical vein, as close to the umbilical cord as possible.

3.8 Measurement of placental 11β-HSD2 activity

3.8.1 Principles of 11β-HSD2 activity assay

Radiometric conversion assay

Placental 11β-HSD2 activity was measured by radiometric conversion assay, using ^{3}H-cortsiol as a tracer, following its conversion by 11β-HSD2 into ^{3}H-cortisone. The method was based on that described by Sun *et al.* (592), which was similar to methods previously used by other authors to measure 11β-HSD2 activity in human placental homogenates or cell lines (547, 553, 559, 560, 593, 644, 645).

Radiometric enzyme assays have many advantages over non-radiometric methods, as they are more sensitive, specific, quantitative, rapid and can be performed on both crude and purified enzyme samples (646). Conversion of the radiolabelled substrate to

labelled product should occur during the initial linear rate of reaction, before the rate of product formation declines, due to substrate depletion or product inhibition (646, 647). Upon completion of the reaction, substrate and product are separated and their radioactivity measured. Quantification is possible by converting the disintegrations per minute (dpm) of product formed into a molar amount, using the known specific activity of the radiolabelled substrate (646).

Thin layer chromatography (TLC)

Various methods have previously been used to separate the cortisol and cortisone components when measuring 11β-HSD2 activity, including high performance liquid chromatography (HPLC) (513, 548), sephadex column chromatography (648) and TLC. In my study, TLC was used to separate the ^{3}H-cortisol substrate and ^{3}H-cortisone product.

TLC is a planar chromatography method, involving the use of a stationary and mobile phase to separate compounds found in a single sample (649). Glass backed plates coated in an adherent layer of silica were used as the stationary phase. The mobile phase (chloroform:ethanol mixture) moves through the stationary phase by capillary action (649). The samples are spotted onto the TLC plate and one end (below the position of samples) is immersed in mobile phase inside a developing tank which is saturated with vapours (649). After the mobile phase has travelled up most of the plate, it is removed from the tank and allowed to dry. The position of the compounds can be visualised under short wave (254 nm) ultraviolet (UV) light, due to the presence of a fluorescent material in the stationary phase (649). This material causes the TLC plate to fluoresce, except where sample components are found, since they quench the fluorescence of the material and appear as dark spots (649). Due to their differing polarities, cortisol and cortisone run to different positions on the TLC plate. Cortisone runs higher than cortisol due to its greater affinity for the organic mobile phase.

Scintillation Counting

The amounts of ^{3}H-cortisol substrate and ^{3}H-cortisone product remaining in the placental 11β-HSD2 activity assay were quantified using liquid scintillation counting, as tritium is a beta emitter. In this method, the sample (silica scraping from the TLC plate) is dissolved in a scintillation cocktail, or solution containing fluors, which produce

detectable flashes of light (scintillations) when they interact with the radiation (650, 651). The scintillations are measured by photomultiplier tubes which convert them into electronic pulses which can be counted (651).

3.8.2 Materials

The following chemicals were obtained from Sigma (St Louis, MO, USA): acetic acid, cortisol (hydrocortisone), cortisone, carbenoxolone, β-nicotinamide adenine dinucleotide (NAD), β-nicotinamide adenine dinucleotide reduced form (NADH), β-nicotinamide adenine dinucleotide phosphate (NADP), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), bovine serum albumin fraction V (BSA), pepstatin A, benzamidine (hydrochloride hydrate), bacitracin, ethylene-diamine-tetra-acetic acid (EDTA), dithiothreitol (DTT) and sucrose. Complete protease inhibitor cocktail tablets were from Roche Diagnostics (Mannheim, Germany), trasylol was from Bayer Corp (Leverkusen, Germany) and Bio-Rad protein assay reagent (for Bradford assay) was from Bio-Rad Laboratories (Hercules, CA, USA). [1,2,6,7-^{3}H]-cortisol (1 mCi/ml) and biodegradable liquid scintillation cocktail were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Ethyl acetate, chloroform, ethanol and chromium trioxide were obtained from BDH Laboratory Supplies (Dorset, UK). Glass backed TLC plates (Adsorbosil Plus-IP containing 254 nm UV fluorescent indicator) and TLC tanks were from Alltech (Deerfield, IL, USA).

3.8.3 Protein Extraction

Approximately 1-2 g of snap frozen placenta was crushed with a mortar and pestle under liquid nitrogen. Crushed placental tissue was homogenised in 10 volumes of 0.1 M sodium phosphate buffer (pH 7.4) containing 2 mM EDTA, protease inhibitor cocktail tablets (20/L), 50000 KIU/l trasylol, 100 μM dithiothreitol, 1 μM pepstatin A, 1 mM benzamidine, 65000 U/L bacitracin and 0.25 M sucrose (Appendix 8), using a polytron homogeniser (Kinematica AG, Switzerland). The homogenate was centrifuged at 1000 g for 10 minutes (J-6B Centrifuge, Beckman Coulter, Palo Alto, CA, USA) to remove cellular debris. The supernatant was centrifuged at 105000 g for 1 hour (Optima XL-A Analytical Centrifuge, Beckman Coulter, Palo Alto, CA, USA) and the pellet which contained the microsomal fraction was re-homogenised in 1 ml of sodium

phosphate buffer containing protease inhibitors, but no sucrose. Protein was aliquoted into small volumes (100-500 µl) which were stored at –80°C until further use.

The protein concentration was determined by Bradford Assay (652) against a standard curve of bovine serum albumin (BSA). A 1 mg/ml stock solution of BSA was prepared and 500 µl aliquots stored at –20°C until use. The standard curve was prepared in duplicate with the following volumes of 1 mg/ml stock solution, which were made up to 50 µl with distilled water: 1, 2.5, 5, 7.5, 10, 15 and 20 µl. The placental homogenates were diluted 1:20 and 1:5 and assayed in duplicate using 10 µl and 30 µl volumes made up to 50 µl with distilled water. Bio-Rad protein assay reagent was diluted 1:5 with distilled water and 1 ml added to each sample. Optical density was measured in a spectrophotometer at 595 nm (Cary50 UV-Visible Spectrophotometer, Varian, Palo Alto, CA, USA).

3.8.4 11β-HSD2 enzyme activity assay

11β-HSD2 activity was determined using a radiometric conversion assay, adapted from the work of Sun *et al.* (592). The microsomal protein fraction was incubated in triplicate at three protein concentrations (usually 100 µg/ml, 150 µg/ml and 200 µg/ml) to ensure that the experiment was carried out in the linear range of enzyme activity. Incubated with the protein was a saturating concentration of cofactor (NAD^+, 1 mM), a saturating concentration of cold substrate (cortisol, 5 µM) and approximately 200000 counts per minute (cpm) ^{3}H-cortisol made up to 1 ml with sodium phosphate buffer containing protease inhibitors. The incubation was carried out at 37°C for 15 minutes in a shaking water bath. The reaction was stopped by removing the solution from the water bath into a tube containing 2 ml of ice-cold ethyl acetate. The solution was thoroughly mixed and the organic phase containing the steroids removed and dried overnight under high speed vacuum (Speedvac SC200, Savant Instruments, Holbrook, NY, USA). The dried steroids were reconstituted in 100 µl of ethyl acetate and 10 µl each of 10 mM cold cortisol and 10 mM cold cortisone were added as markers. This solution was spotted onto a glass-backed TLC plate and chromatographed with 95:5 chloroform:ethanol (100 ml total volume) as the mobile phase. The bands (cortisone upper band and cortisol lower band) were visualised under UV light (portable short wave 254 nm UV light, Alltech, Deerfield, IL, USA) and the silica bands scraped into a vial to which 10 ml of scintillation fluid was added. Steroids were quantified using a liquid scintillation

counter (1217 Rackbeta, LKB-WALLAC, Turku, Finland). Disintegrations per minute (dpm) were adjusted for experimental losses and enzyme activity was expressed as nmol cortisone formed per mg protein per hour (nmol/mg/h). Some data is also presented as the percentage (%) conversion from cortisol to cortisone.

3.8.5 Optimisation of 11β-HSD2 enzyme activity assay

Initial studies using the method outlined above were performed using at least two placental samples each in triplicate, to optimise the assay procedure. These included a time optimisation where the incubation was carried out for 2, 10, 15, 30, 60 and 120 minutes. A zero time control in which the reaction components were added directly to ice-cold ethyl acetate was included. In this experiment, 100 nM cortisol was incubated with 1 mM NAD and 150 µg protein. As shown in Figure 3.2, the reaction was under conditions of initial velocity up to 15 minutes. Consequently, 15 minutes was chosen as the optimal incubation time for further experiments.

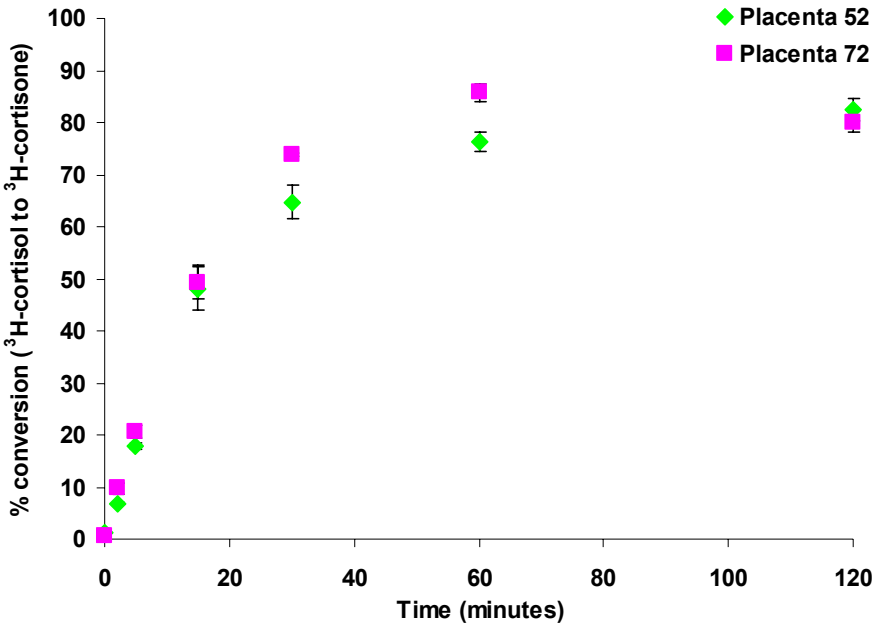

Figure 3.2 Optimisation of 11β-HSD2 activity over time

This figure shows a representative time course experiment for two placental samples (tested in triplicate) measuring % conversion of ^{3}H-cortisol to 3H-cortisone after 0, 2, 5, 15, 30, 60 and 120 minutes.

The concentration of NAD cofactor used was optimised by incubating 100 nM cortisol and 50 µg protein for 15 minutes with 0, 1 µM, 10 µM, 100 µM, 1 mM or 10 mM NAD. The enzyme was considered saturated with cofactor at 1 mM (Figure 3.3).

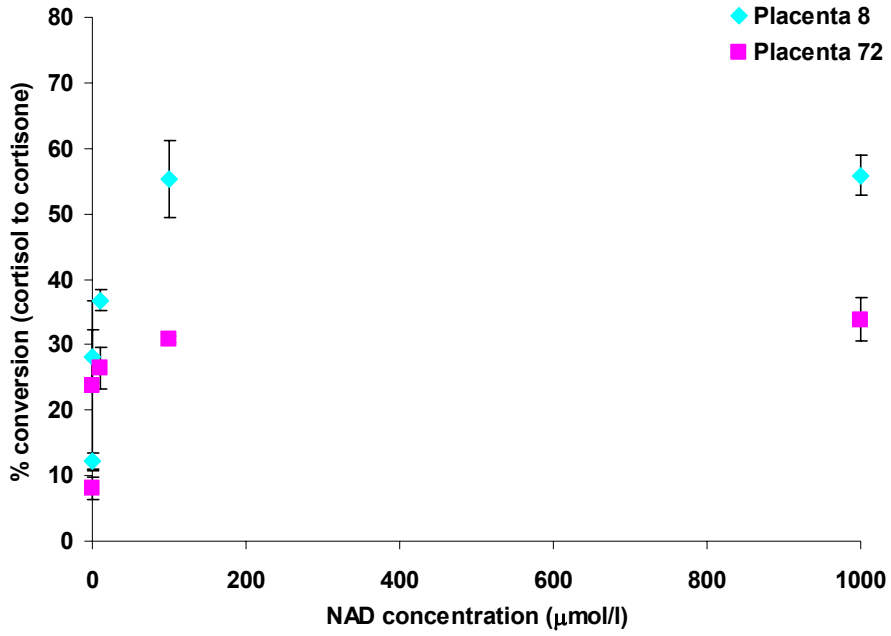

Figure 3.3 Cofactor dependence of 11β-HSD2 activity

This figure shows a representative plot of NAD concentration dependence of 11β-HSD2, for two placental samples (tested in triplicate). The % conversion of cortisol to cortisone is shown over increasing concentrations of NAD (0, 1, 10, 100 and 1000 µM shown). The concentration of cold cortisol used in this experiment was 100 nM.

A cortisol saturation curve was obtained by incubating 150 µg protein with a range of concentrations of cortisol (10, 25, 50, 100, 250 and 500 nM, 1, 5 and 10 µM) and 1 mM NAD for 15 minutes. The cortisol saturation curve indicated that the enzyme was saturated at 5 µM (Figure 3.4). The data obtained was transformed into a Lineweaver-Burk plot (Figure 3.5) to determine enzyme kinetics. The Michaelis constant (K_m) or substrate concentration at which the enzyme reaction rate was half maximal was found to be 249 ± 57 nM (n=3), which is similar to that reported by other authors (542, 653-655).

A protein concentration dependence study indicated that the region where 11β-HSD2 activity increases linearly with enzyme concentration was between 100 and 200 µg/ml protein (Figure 3.6). In further experiments, 100, 150 and 200 µg/ml protein was always used to verify that the reaction was occurring in the linear range.

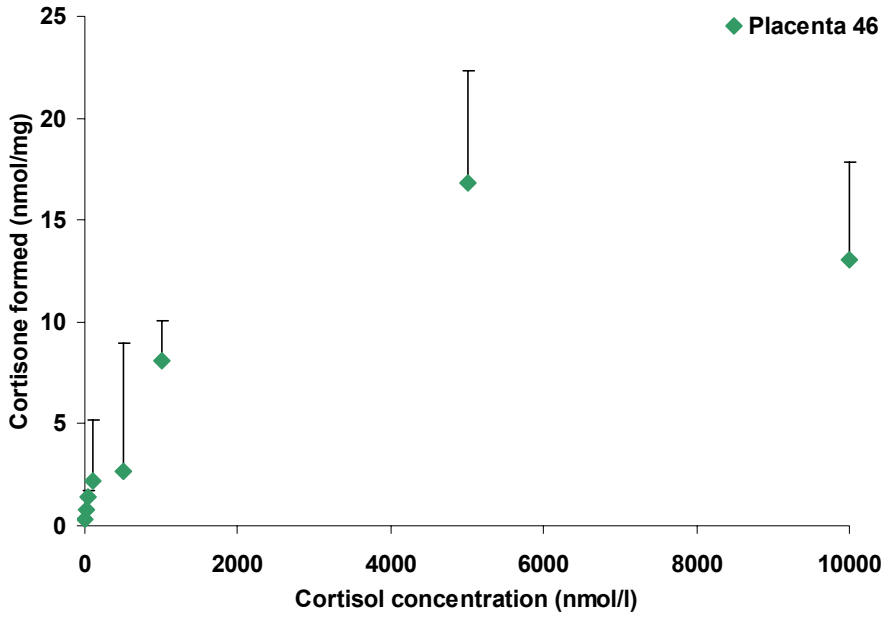

Figure 3.4 Cortisol saturation curve for 11β-HSD2

This figure shows a representative cortisol saturation curve for one placenta sample (tested in triplicate) measuring the amount of cortisone formed (nmol/mg) over increasing concentrations of cortisol (10, 25, 50, 100, 500 nM, 1, 5 and 10 μM).

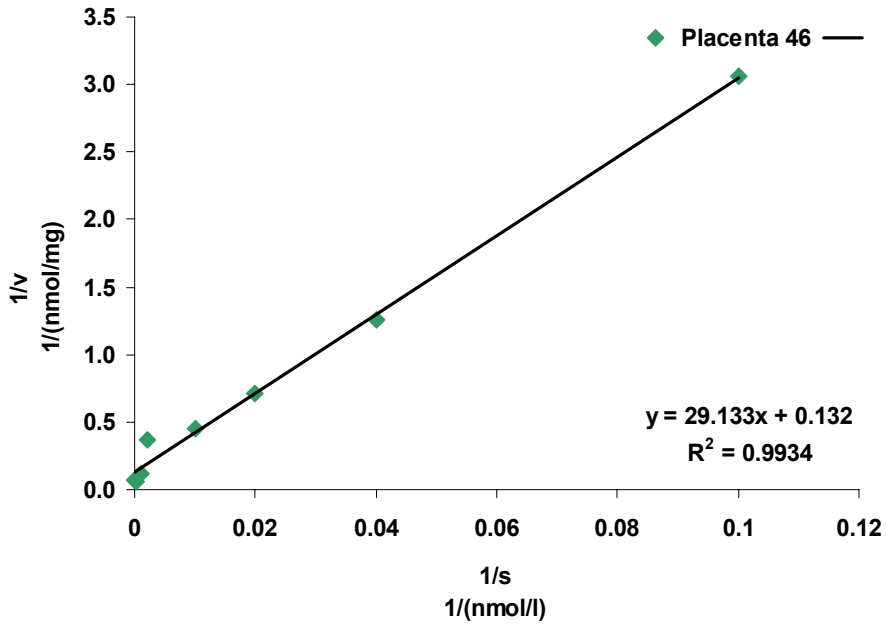

Figure 3.5 Lineweaver-Burk plot used to determine enzyme kinetics of 11β-HSD2

On this representative plot, the x-axis shows the reciprocal of substrate concentration (1/s = 1/[nmol/l]) used, and the y-axis shows the reciprocal of velocity (1/v = 1/[nmol cortisone per mg protein]). The K_m was determined as the negative reciprocal of the intercept on the x-axis. Three separate experiments were performed to determine the average K_m.

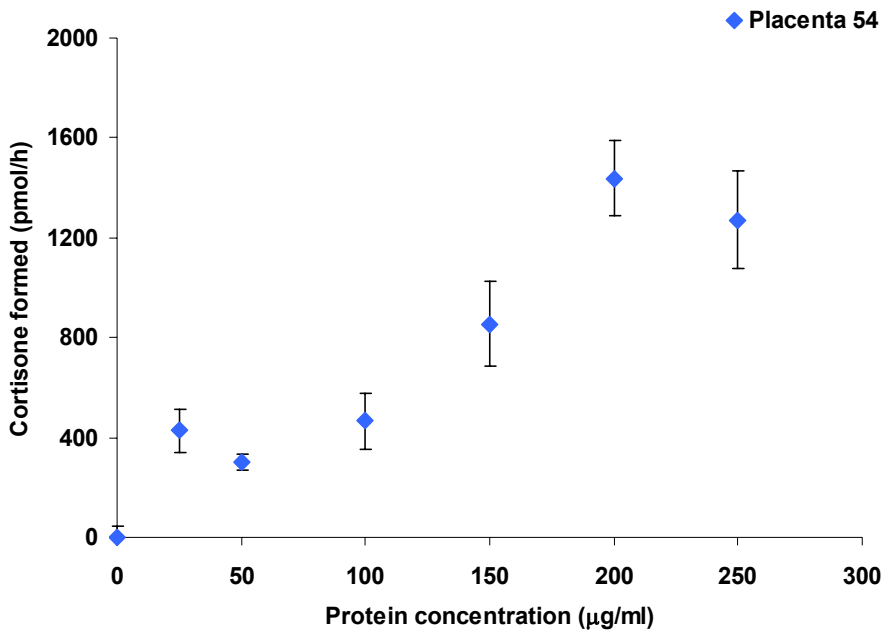

Figure 3.6 Protein concentration dependence of 11β-HSD2

This figure shows a representative protein concentration curve for 11β-HSD2, for one placenta (tested in triplicate) measuring the amount of cortisone formed (pmol/h) over a range of protein concentrations (0, 25, 50, 100, 150, 200 and 250 µg/ml).

Preliminary experiments were carried out in the presence of the 11β-HSD inhibitor, carbenoxolone to confirm that conversion of cortisol to cortisone was specifically due to 11β-HSD2 activity. In the presence of 100 nM cortisol, 1 mM NAD and 50 µg protein, there was 51% conversion of cortisol to cortisone over 15 minutes (average of three placentae tested in triplicate). However, at all concentrations of carbenoxolone tested (0.01, 0.1, 1 and 10 µM), conversion of cortisol to cortisone was reduced to <10%, indicating that the majority of the conversion was due to 11β-HSD2.

An internal standard of three pooled placentae was included in each assay of the asthmatic samples to allow direct comparison of results. The intra-assay variation was 19.4% and the inter-assay variation was 20.1%.

Measurement of 11β-HSD1 reductase activity in the placental samples was carried out in a similar manner to 11β-HSD2 oxidase activity. Placental microsomes were incubated at 37°C for 15 minutes with 100 nM cortisone, 1 mM NADH or NADPH and approximately 100000 cpm ^{3}H-cortisone. TLC separation and quantification occurred as for 11β-HSD2. ^{3}H-cortisone was generated in the laboratory following the method of Shaw and Quincey (656). ^{3}H-cortisol was oxidised with acetic acid/chromium trioxide

at room temperature for 20 minutes. Steroids were extracted with ethyl acetate, dried under high speed vacuum and re-constituted in 100 µl of ethyl acetate. Purification was by TLC as described previously. The cortisone band was scraped off the TLC plate and re-suspended in ethyl acetate. An aliquot was used to calculate yield which was found to be >90% and the remainder used in the conversion experiments. 11β-HSD1 reductase activity was very low in the placenta and was not easily measured above background.

3.9 Measurement of placental 11β-HSD2 protein expression by Western blotting

3.9.1 Principle of Western blotting

Western blotting is a technique used to detect and characterise proteins (657). Proteins are separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a solid support such as a nitrocellulose or polyvinylidene fluoride (PVDF) membrane (657). PVDF membranes are physically strong, chemically stable and have a high capacity for protein binding (657). Antibodies can subsequently be directed against proteins bound to the membrane and visualisation occurs via a secondary antibody linked to alkaline phosphatase, which produces an insoluble coloured product in the presence of a soluble substrate (657) such as nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). This staining allows semi-quantitative results to be generated by densitometric analysis.

3.9.2 Materials

Pre-cast Tris-glycine gels were from Novex (San Diego, CA, USA). PVDF membrane was from DuPont NEN (Boston, MA, USA). 11β-HSD2 antibody (sheep anti-human) was from The Binding Site (Birmingham, UK). Anti-sheep IgG was from Sigma (St Louis, MO, USA). NBT/BCIP was from Nalgene (Boston, MA, USA). Prestained SDS-PAGE standards (low range) were from Bio-Rad (Hercules, CA, USA). Buffer recipes are given in Appendix 8. Ethylene-glycol-bis(β-aminoethyl ether)-tetra-acetic acid (EGTA), glycerol, tween 20 and bromophenol blue were from Sigma (St Louis, MO, USA). Tris HCl, KCl, sodium dodecyl sulfate (SDS), sodium chloride (NaCl), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), disodium hydrogen phosphate

dodecahydrate ($Na_2HPO_4.12H_2O$) and methanol were from BDH Laboratory Supplies (Dorset, UK). β-mercaptoethanol was from ICN Biomedicals (Irvine, CA, USA).

3.9.3 Western blotting

Placental samples were homogenised and protein extracted as described above (Section 3.8.3). Placental samples (10 µg of protein) were suspended in reduced SDS sample buffer, heated for 3 minutes at 80°C and loaded in triplicate onto pre-cast 12% Tris-glycine gels. SDS-PAGE low range standards were loaded at either end of each gel. Placental proteins were separated by electrophoresis (658) at 100 V for 2 hours, in a Bio-Rad protein II gel electrophoresis tank (Bio-Rad, Hercules, CA, USA) containing lower electrode buffer and upper electrode buffer in the two chambers.

Proteins were transferred from the gel to a PVDF membrane using a transfer sandwich assembly, where the membrane is sandwiched between the gel, filter paper and support pads (657). PVDF membrane was briefly rinsed and activated with methanol and rinsed with distilled water. Gels, PVDF membrane and components of the transfer apparatus were pre-soaked in transfer buffer for 10-15 minutes prior to transfer. Air bubbles were removed by rolling with a 15 ml tube and the transfer sandwich was assembled with the membrane facing the anode. Proteins were transferred to the PVDF membrane, at 80 V for 2.5 hours at 4°C. The membrane was soaked in blocking buffer overnight at 4°C, to prevent non-specific binding prior to staining.

After washing in phosphate buffered saline (PBS) three times for 15 minutes each, the blots were incubated with 11β-HSD2 antibody (1:5000 dilution with Tris buffered saline (TBS)/tween) at 4°C overnight, on a rocking platform. Blots were washed three times with PBS (15 minutes each) and alkaline phosphatase conjugated second antibody (anti-sheep IgG, 1:10000 with TBS/tween) was added and incubated for 4 hours at room temperature. Staining solution (NBT/BCIP) was added and the container covered with foil for approximately 30 minutes during the development of the stain. The reaction was stopped by removing the NBT/BCIP solution and washing with distilled water. Membranes were dried between two pieces of filter paper and scanned for analysis with the computer assisted densitometry program, Scion Image (Release Beta 4.0.2, National Institutes of Health, Bethesda, MD, USA).

11β-HSD2 has a molecular weight of approximately 44 kDa (calculated from the amino acid sequence of accession number NP_00187, using the Expasy calculate pI/MW tool at www.us.expasy.org). The 11β-HSD2 band was close to the ovalbumin (49900 Da) standard (Figure 3.7). A band to which the second antibody bound in proportion to the total protein was used to adjust for loading. Preliminary studies demonstrated that the densitometry of this second band varied proportionally to the amount of total protein loaded. The identity of the band is unknown, but it corresponds to a molecular weight of approximately 29900 Da (soybean trypsin inhibitor on the prestained SDS-PAGE standards). This band is suitable for use as an internal control and as such is a more efficient assessment of loading than running two western blots, where there is the potential for larger inter-assay variation of loading.

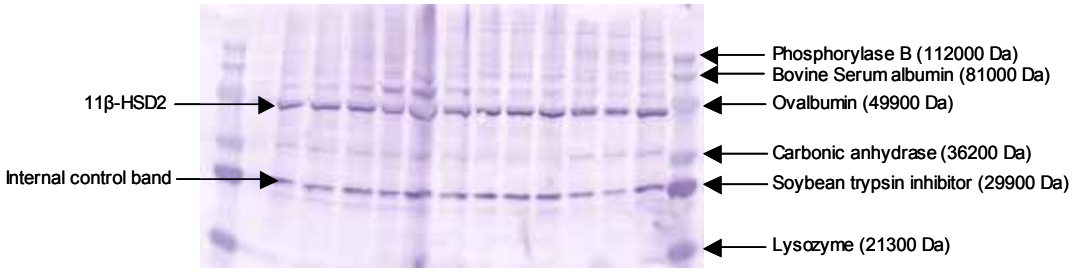

Figure 3.7 Western blot

This representative Western Blot shows the specific binding of 11β-HSD2, the internal control band used for loading adjustment and the molecular weight standards.

3.10 Measurement of placental gene expression by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

3.10.1 Principles of PCR

Polymerase chain reaction (PCR) is a technique used to amplify specific sequences of DNA in a gene (659). First, RNA extracted from tissue samples is converted by the enzyme reverse transcriptase into cDNA, which acts as a template for PCR. Amplification of the sequence of interest requires some knowledge of the surrounding sequence so that short oligonucleotide primers can be designed to hybridise specific sections of DNA. Following hybridisation, incubation with Taq polymerase, a heat stable DNA polymerase derived from thermophilic bacteria, results in extension of the

DNA sequence. The process is repeated throughout several cycles, controlled by changing temperature. The newly synthesised DNA strands serve as templates in further cycles, resulting in an exponential accumulation of PCR product (659, 660). The amounts of DNA produced can be monitored with fluorescence dyes such as SybrGreen. The major steps of RT-PCR are shown schematically in Figure 3.8.

Quantitative real-time RT-PCR is a relatively new technique which allows product amplification to be monitored in real-time, resulting in fast, accurate and sensitive assays (661, 662). The major advantage of the quantitative real-time PCR technology over previous PCR methods, is that gene expression is calculated in the early, linear phase of amplification, rather than at its maximum, when saturation and reaction plateaus are reached (663-665). Consequently, better comparisons between samples can be made and the measurement is faster and more accurate (666). Quantitative real-time RT-PCR has been verified against other methods including gene array (667), competitive RT-PCR (668), RNase protection assay (663) and nuclear run-on assay (669) and found to be much less variable than these conventional methods (670). The method is extremely sensitive, capable of detecting as few as 10 copies of target sequence in a sample (666), thus being able to identify low abundance mRNA and small changes in gene expression (671).

Fluorescence monitoring of PCR amplification is a direct detection method which removes the need for post-PCR analysis and the associated time, potential contamination and errors involved (664-666). The system used in my study employed SybrGreen as a fluorescent dye which is detected by the PCR machine during each amplification cycle. SybrGreen binds to the minor groove of double stranded DNA and is therefore a non-specific method of detecting PCR products. Although less sensitive, it is cheaper and simpler than using specifically designed probes as it can be used with any set of primers (666).

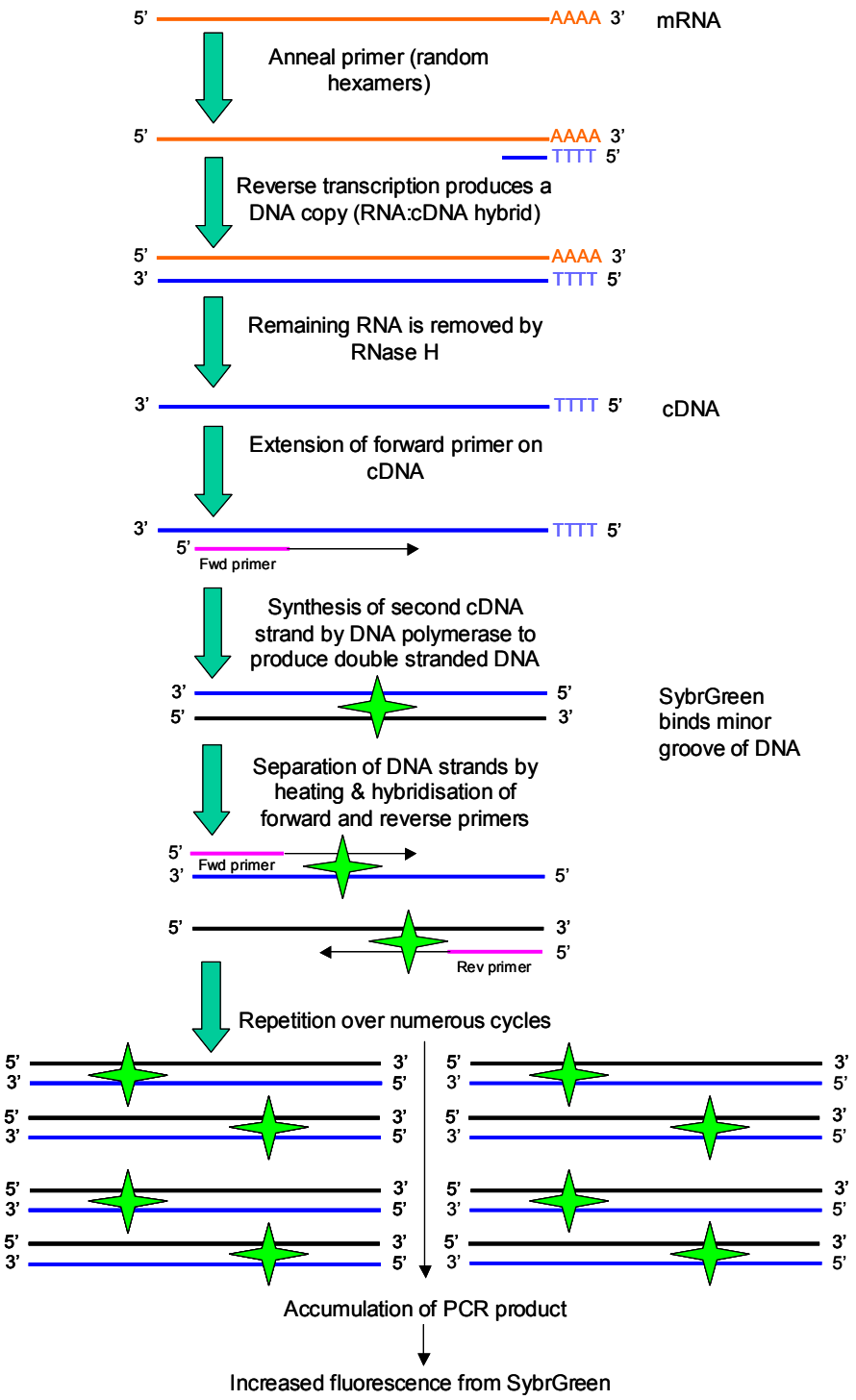

Figure 3.8 Quantitative RT-PCR using SybrGreen

This schematic diagram shows the major steps in the RT-PCR reaction. mRNA extracted from tissue is transformed into a DNA copy by reverse transcription. Any remaining RNA is removed from the mixture by the RNase H enzyme. The resulting first strand cDNA copy is converted to double stranded DNA by extension of the forward primer. Separation of the DNA strands by heating and subsequent hybridisation of forward and reverse primers coupled with extension by DNA polymerase leads to an accumulation of PCR product. The SybrGreen dye binds to the minor groove of DNA, emitting an increasing amount of fluorescence as the amount of PCR product increases. Diagram partially adapted from Alberts et al., 1994 (659) and Stryer, 1995 (660).

The thermal cycler and fluorescence detection system continuously measures the fluorescence spectra of each sample during the PCR reaction, thus monitoring the real-time accumulation of PCR product (665, 667). The DNA binding dye, SybrGreen has a low fluorescence when unbound, which increases dramatically when the dye binds to DNA (667, 670). The amount of fluorescence increases proportionally with the amount of DNA produced by the reaction (667, 672). The fluorescence of the SybrGreen dye (Rn^+) is continually normalised to that of a passive reference dye (Rn^-), giving calculated ΔRn values for each cycle, which increase exponentially above background once the detection threshold of the instrument is reached (673, 674). This method normalises each reaction for variability in the system's optics (674). Quantification of the PCR reaction is based on the concept of threshold cycle (C_T). At the threshold cycle, the fluorescence values (ΔRn) become significantly greater than background (661, 670). The C_T always occurs during the exponential phase of the reaction and consequently, is unaffected by limiting reagents in the plateau phase (670).

Performing a melting curve after PCR analysis allows an assessment of the specificity of the PCR reaction, since the melting temperature (T_m) of an amplicon depends on its sequence, size and nucleotide composition, particularly GC/AT ratio (667, 675). The fluorescence signal of the desired product can therefore be distinguished from undesired products, such as primer-dimer pairs, eliminating the need for gel electrophoresis (667, 675). At the amplicon's T_m, a characteristic peak will be observed, which is distinguishable from artefacts which melt at a lower temperature, in broader peaks (670).

Relative quantitation of the PCR requires the use of an internal control gene, or housekeeping gene, which is assumed to have equal expression in all tissues. β-actin has been used for this purpose by several studies (664, 668) as it is a ubiquitous cytoskeletal protein (670). Use of a housekeeping gene compensates for variations in input RNA amounts or RNA integrity and the efficiency of cDNA synthesis (664, 665). Typically, results are expressed as a ratio of mRNA abundance of the test gene compared to the housekeeping gene (664, 665).

With the use of SybrGreen, the specificity of the reaction is determined by primer design. Primers should be designed to cross intron-exon boundaries where possible, as this prevents false positive results from contaminating genomic DNA (670). When this

is not possible, the RNA can be treated with RNA-free DNase to remove any remaining DNA from the sample (670). Designing primers with less than three G or C nucleotides within the last five bases at the 3' end reduces stability at the 3' end, resulting in a lower likelihood of non-specific hybridisation and extension by DNA polymerase (670).

Appropriate controls were added to the PCR experiments described below. These included a sample which had been through the reverse transcription process without the addition of the reverse transcriptase enzyme (no RT or –RT control). There should be no PCR product amplified from this sample, confirming that the primers did not amplify contaminating genomic DNA (666). In addition, no template controls (NTC) were included for each primer used in each experiment, where water was substituted for sample.

3.10.2 Materials

Trizol, agarose and all designed primers were obtained from Life Technologies (Frederick, MD, USA). Isopropanol, chloroform and ethanol were from BDH Laboratory Supplies (Dorset, UK). RNeasy mini kit columns and RNase-free DNase kits were obtained from Qiagen Australia (Clifton Hill, Victoria, Australia). The Taqman RT kit was from Perkin Elmer (Branchburg, NJ, USA). The SuperScript™ first-strand synthesis system kit was from Invitrogen (Carlsbad, CA, USA). Agarose and ethidium bromide were from Sigma (St Louis, MO, USA). SybrGreen and MicroAmp Optical 96 well plates were from Perkin Elmer Applied Biosystems (Foster City, CA, USA). All PCR experiments used distilled water (RNase-free) which was obtained from a Milli Q ultrapure water system (Milli Q Synthesis A-10, Millipore Corporation, Billerica, MA, USA). Buffer recipes can be found in Appendix 8.

3.10.3 Primer design

Information about the primers used in this study is given in Table 3.3. Primers were designed using Primer Express version 1.0b6 (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and the published sequences for each gene found on the NCBI Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). Primer Express had several parameter preferences which were followed when designing primers. The melting temperature (T_m) of primers was set at 58-60°C (optimal T_m

59°C), the primer GC content was set at 20-80%, the primer length was between 9 and 40 bases (optimal 20 bases) and the maximum GGG repeat was 3 residues. The amplicon requirements included a T_m between 0 and 85°C and a minimum length of 50 bases. In addition to these, all primer options were checked to ensure there were no more than 3 G or C bases at the 3' end of the sequence. Where possible, primers were designed to overlap exon regions of the gene, to prevent co-amplification of potential contaminating genomic DNA. In general, amplicon lengths were kept short as this increases the efficiency of amplification (664).

All amplicon sequences were checked for specificity for the gene of interest by performing a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). GR-α and GR-β primers were designed to amplify sequences within exon 9, which was the only region which differed between these two mRNAs.

β-actin primers were kindly supplied by Dr Sam Mesiano and A/Prof Tamas Zakar (Mothers and Babies Research Centre, Newcastle). These amplify a sequence at open reading frame 878-1090 (676) and have been successfully used previously in our laboratory (669, 677). Primers for TNF-α, IL-1β, IL-6 and IL-8 were kindly supplied by Ms Annette Osei-Kumah (Mothers and Babies Research Centre, Newcastle).

Table 3.3 List of primers

Gene	Primer		Amplicon	
Sequence Reference	**Primer Name**	**Primer Sequence (5'-3')**	**Length (bases)**	**Melting Temp**
U27317	11β HSD2 fwd	TCAAGACAGAGTCAGTGAGAAACG	129	85.0°C
	11β HSD2 rev	GGAACTGCCCATGCAAGTG		
NM_005525	11β HSD1 fwd	GAATTCAGACCAGAGATGCTCCA	51	76.8°C
	11β HSD1 rev	GGCCCCTGTGACAATCACTTT		
V00571	CRH fwd	AGAAAGGCGGTCCGAGGA	67	81.9°C
	CRH rev	CAAGACTTCCCGGAGGAGGT		
X57025	IGF-I fwd	TGCCCAGCGCCACAC	99	80.8°C
	IGF-I rev	TCCTACATCCTGTAGTTCTTGTTTCCT		
J03242	IGF-II fwd	CCGTGCTTCCGGACAACTT	71	80.4°C
	IGF-II rev	GGACTGCTTCCAGGTGTCATATT		
M31145	IGFBP-1 fwd	CACAGGAGACATCAGGAGAAGAAA	81	75.2°C
	IGFBP-1 rev	ACTGTCTGCTGTGATAAAATCCATTC		
M10901	GR-α fwd	AGGTTGTGCAAATTAACAGTCCTAACT	90	79.0°C
	GR-α rev	TAGTCTTTTGCAACCATCATCCA		
XO3348	GR-β fwd	GGATAATTAGCATGGGATGAGCTC	78	81.1°C
	GR-β rev	GCTCCCTGCCTCTGAATTCTG		
M16801	MR fwd	CTCATGTCTAGGAGGAAATAGCAAAATA	90	74.0°C
	MR rev	CCTGAACATGAATGCTTGGTTG		
XM_034871	IL-4 fwd	CACAGGCACAAGCAGCTGA	62	82.2°C
	IL-4 rev	GCCAGGCCCCAGAGGTT		
XM_003778	IL-5 fwd	AGCTGCCTACGTGTATGCCA	68	79.3°C
	IL-5 rev	GTGCCAAGGTCTCTTTCACCA		
XM_001409	IL-10 fwd	GGTGATGCCCCAAGCTGA	63	80.3°C
	IL-10 rev	TCCCCCAGGGAGTTCACA		
M10988	TNF-α fwd	GGAGAAGGGTGACCGACTCA	69	82.0°C
	TNF-α rev	TGCCCAGACTCGGCAAAG		
XM_010760	IL-1β fwd	CCTCTGGATGGCGGCA	62	81.4°C
	IL-1β rev	TGCCTGAAGCCCTTGCTG		
M54894	IL-6 fwd	CCGCCCCACACAGACAG	70	79.3°C
	IL-6 rev	CCGTCGAGGATGTACCGAA		
XM_003501	IL-8 fwd	CGTGGCTCTCTTGGCAGC	73	80.6°C
	IL-8 rev	TTAGCACTCCTTGGCAAAACTG		
X00351	β-actin fwd	GGCCGCGGTGTACGCCAACACAGTGCTG	213	86.6°C
	β-actin rev	CCCGGGGCCGTCATACTCCTGCTTGCTG		

3.10.4 RNA extraction, purification and reverse transcription

Total RNA was extracted from frozen placental samples using the trizol method. Briefly, 1 ml trizol per 0.1 g tissue was added to crushed placental samples before homogenising (Polytron homogeniser, Kinematica AG, Switzerland). The homogenate was centrifuged at 7800 g for 10 minutes (J2-21 Centrifuge, Beckman Coulter, Palo Alto, CA, USA) and to the supernatant 0.2 ml chloroform/ml trizol was added. The mixture was shaken vigorously by hand for 15 seconds before centrifuging at 7800 g for

15 minutes. The supernatant was removed and 0.5 ml isopropanol per ml trizol was added. After standing at room temperature for 10 minutes, the solution was centrifuged at 7800 g for 10 minutes, the supernatant removed and 1 ml 75% ethanol/ml trizol added. The optical density of RNA dissolved in RNase-free water (1:50-1:200 dilution) was used to determine RNA concentration (Cary50 UV-Visible Spectrophotometer, Varian, Palo Alto, CA, USA). An absorbance of 1 unit at 260 nm corresponds to 40 µg RNA/ml. Purity was evaluated using the ratio between absorbance values at 260 nm and 280 nm which were ideally close to 1.8, but may range from 1.5-2.0 when dissolving in water (678).

RNA (60 µg/100 µl) was loaded onto RNeasy mini kit columns for purification. Buffers were provided with the kit and purification was carried out according to the manufacturer's instructions with few modifications. RNA was dissolved in 350 µl RLT buffer (denaturing lysis buffer with guanidine isothiocyanate) containing β-mercaptoethanol (10 µl/ml), and 250 µl ethanol and applied to the column which was centrifuged at 8000 g for 15 seconds (Biofuge pico microfuge, Heraeus, Hanau, Germany). The RNA bound to the membrane of the column, while contaminants were washed through. The sample was washed with 350 µl RW1 buffer (8000 g for 15 seconds) and treated with DNase I (10 µl stock in 70 µl RDD buffer) for 15 minutes at room temperature. This step removed any contaminating DNA, which was especially important, as some primers could not be designed to cross intron/exon boundaries (664). The RW1 wash was repeated, and 500 µl of RPE buffer added and columns centrifuged at 8000 g for 15 seconds. Another 500 µl of RPE buffer was added and centrifuged for 2 minutes at maximum speed (13000 rpm, Biofuge pico microfuge, Heraeus, Hanau, Germany) to dry the membrane. The RNA was eluted from the column with 60 µl of RNase-free water (30 µl added twice), with centrifugation at 8000 g for 1 minute after each addition. The RNA concentration and purity was again checked by measurement of optical density at 260 nm and 280 nm.

RNA (1 µg) was run on a 1.5% agarose gel in TBE buffer containing 0.5 µg/ml ethidium bromide (60 V, 1 hour), with λ*Hin*dIII markers (Life Technologies, Frederick, MD, USA). The presence of 18S and 28S rRNA bands was verified, indicating that the extracted RNA was intact (Figure 3.9).

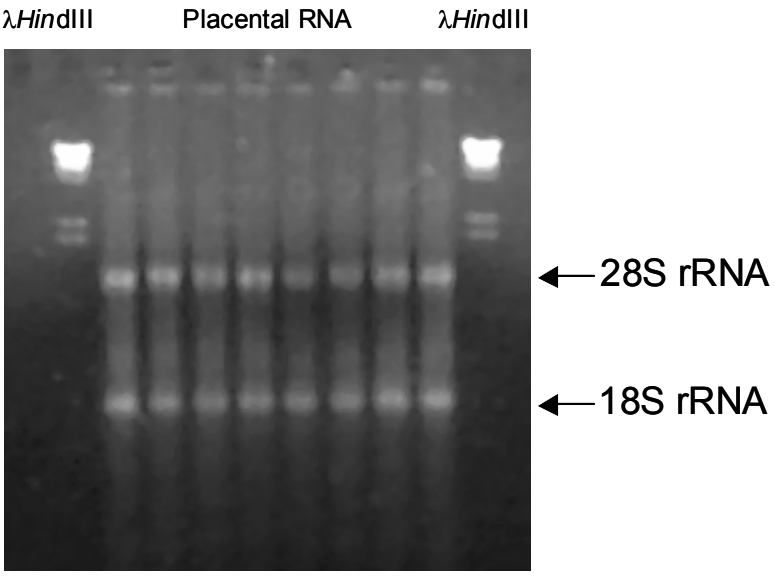

Figure 3.9 Agarose gel electrophoresis of extracted placental RNA

This representative gel shows column purified placental RNA which was run on a 1.5% agarose gel with λHindIII marker. The position of 28S and 18S rRNA bands is shown, verifying RNA integrity.

RNA was reverse transcribed using the Taqman RT kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) or the SuperScript™ first-strand synthesis system kit (Invitrogen, Carlsbad, CA, USA). Briefly, using the SuperScript™ kit, 2 µg of RNA was diluted to 8 µl with RNase-free water. To each 8 µl sample, 1 µl of random hexamers (50 ng/µl) and 1 µl of dNTP mix (dATP, dCTP, dGTP, dTTP, 10 mM each) was added and incubated for 5 minutes at 65°C using a 9600 GeneAmp PCR machine (Perkin Elmer Applied Biosystems, Foster City, CA, USA), then placed on ice. A master mix consisting of 10× RT buffer (200 mM Tris HCl, pH 8.4, 500 mM KCl), 25 mM $MgCl_2$, 0.1 M DTT and RNaseOUT (40 units/µl of recombinant ribonuclease inhibitor) was made up. One half was used for +RT samples (to be reverse transcribed), while the other half was used for the negative control –RT samples (not to be reverse transcribed). The –RT master mix also had water added (1 µl per sample). The appropriate master mix was added to the samples (9 µl to +RT, 11 µl to –RT) and incubated for 2 minutes at 25°C. Reverse transcription was catalysed by SuperScript II RNase H- Reverse Transcriptase (50 units per sample). The cycles used for reverse transcription were: 10 minutes at 25°C, 50 minutes at 42°C, and 15 minutes at 70°C. *Escherichia coli* RNase H (2 units per sample) was added to +RT samples only. All samples were incubated at 37°C for 20 minutes and stored at –20°C until further use.

cDNA was adjusted to a final concentration of 40 ng/2 µl, which was used in all PCR reactions.

3.10.5 Quantitative PCR

Quantitative real-time RT-PCR was used to determine mRNA abundance of several genes in placental samples, which were compared to levels of the constitutively expressed gene, β-actin. Primers were made up to a concentration of 100 pmol/µl with RNase-free water. The PCR reaction contained 40 ng of reverse transcribed sample (in 2 µl), 10 pmol of the appropriate primer mix and 12.5 µl of SybrGreen master mix made up to a total volume of 25 µl with RNase-free water in a MicroAmp Optical 96 well plate. The SybrGreen master mix contained all components necessary for the PCR reaction including optimised concentrations of $MgCl_2$ and other buffers (proprietary components), AmpliTaq Gold® DNA polymerase (designed to minimise primer-dimer and non-specific product formation), dNTPs with dUTP, SybrGreen dye and Passive Reference I, which normalises non-PCR fluctuation in fluorescence signal, minimising well to well variation (Applied Biosystems product information). Prior to analysis, a background run was performed on the ABI Prism 7700 sequence detector (Perkin Elmer Applied Biosystems, Foster City, CA, USA) to ensure no contamination of the wells. Exposure time was set to 25 when using PCR plates covered with caps, or 10 when using PCR plates covered with film. PCR analysis was performed on duplicate or triplicate samples under the following reaction conditions: 2 minutes at 50°C, 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A single or duplicate sample that had not been reverse transcribed was included as a control for each sample, along with single or duplicate no template controls (NTC) for each primer. At the end of the experiment, data was analysed using ABI Prism Sequence Detection Systems software version 1.9 (Applied Biosystems, Foster City, CA, USA). The baseline was set to 15 cycles (recommended), unless amplification began earlier, in which case the baseline was set at one or two cycles before amplification (Figure 3.10). The threshold line was adjusted to the early part of the exponential phase and C_T values (where C_T is the threshold cycle) for test gene and housekeeping gene exported. The comparative C_T method was used to derive a relative quantitative measure of the test gene expression compared to β-actin expression, using the formula: relative mRNA abundance = $2^{-\Delta CT}$, where $\Delta C_T = C_T$ (β-actin) – C_T (test gene).

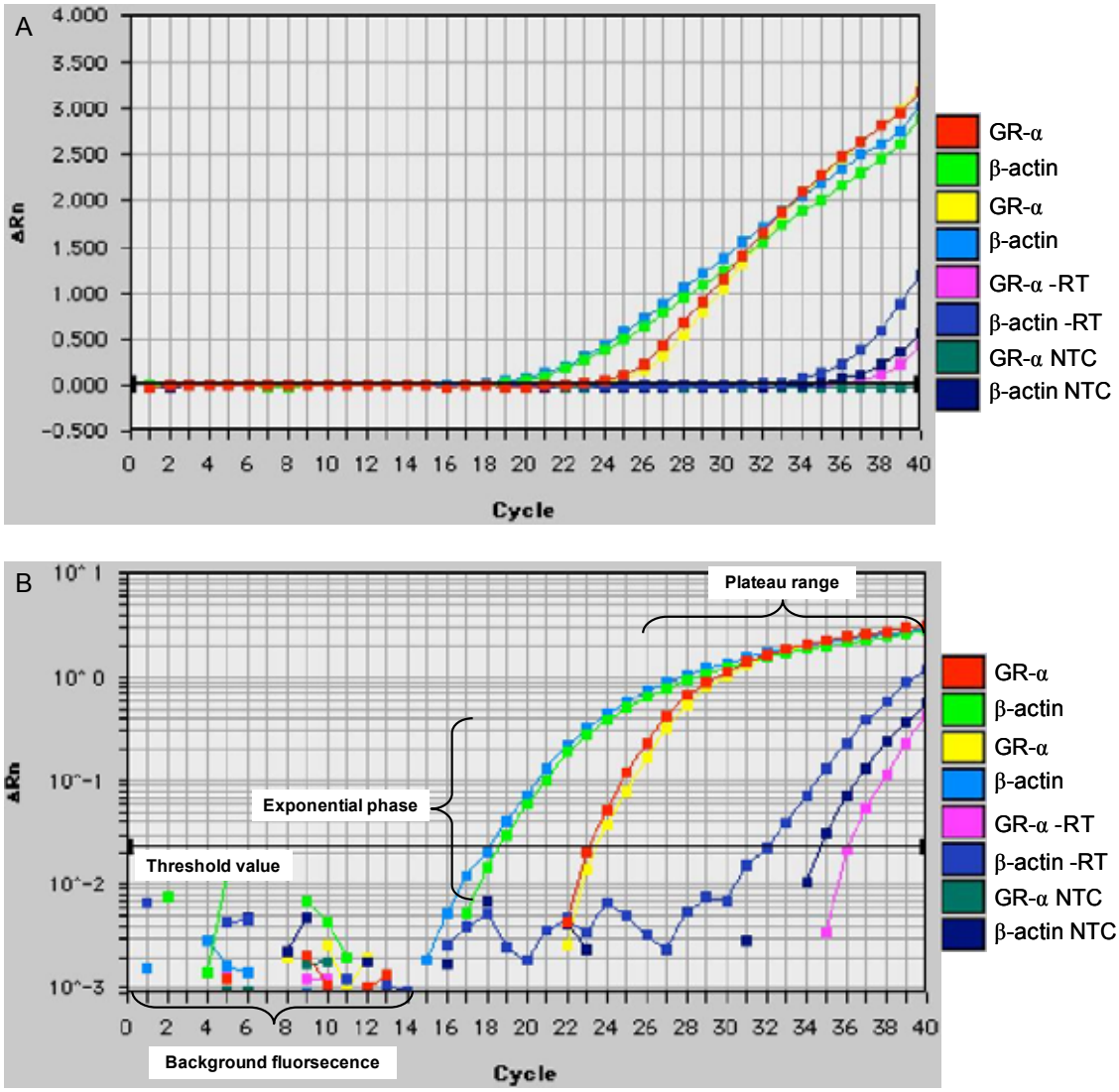

Figure 3.10 Amplification plots of the PCR reaction

An amplification plot for GR-α and β-actin is shown in linear view (Panel A) and exponential view (Panel B). Duplicate samples were amplified along with a –RT control and no template control (NTC).

Dissociation or melt curves were run subsequent to PCR on some samples to assess product purity. A product was considered pure if only one strong melting temperature was observed. Samples were melted at 95°C for 15 seconds, equilibrated at 60°C for 20 seconds, and re-heated (dissociated) to 95°C over 20 minutes. Melting temperatures were determined using the Dissociation Curves Software version 1.0 (Applied Biosystems, Foster City, CA, USA) and are given in Table 3.3. An example of the dissociation curves for 11β-HSD2 and 11β-HSD1 PCR products are shown in Figure 3.11.

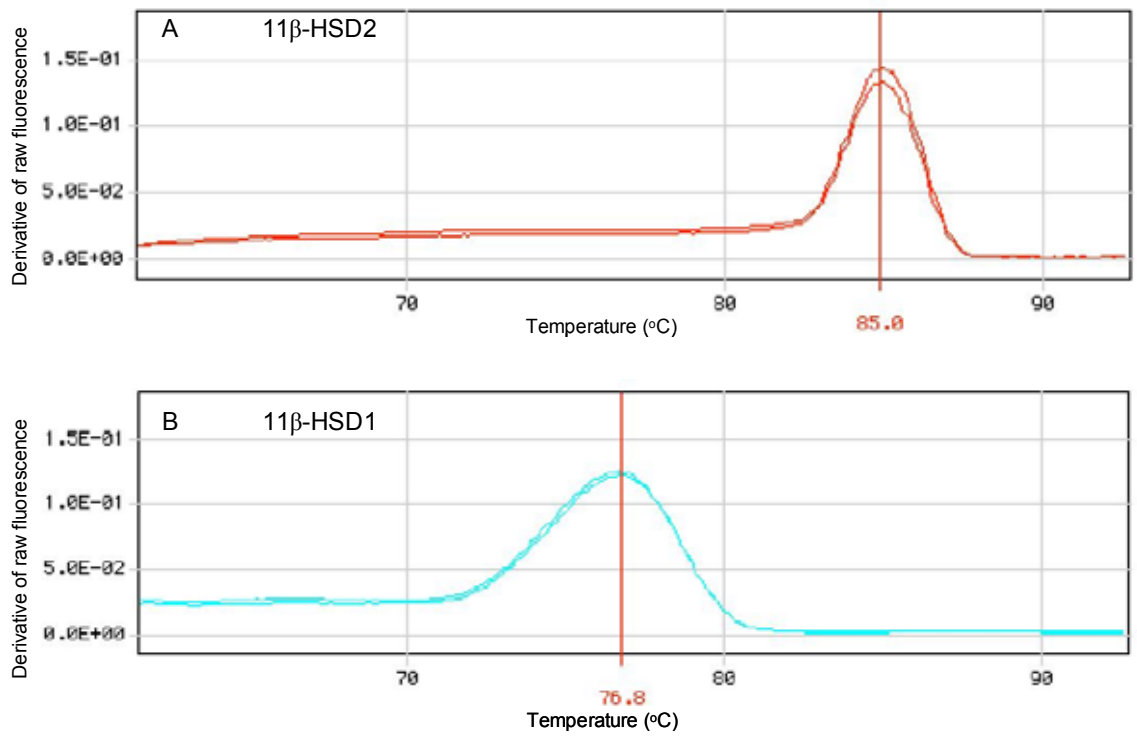

Figure 3.11 Dissociation curves of the PCR products

Dissociation or melt curves are shown for 11β-HSD2 PCR product (Panel A) and 11β-HSD1 PCR product (Panel B).

The intra-assay variation in C_T value was calculated from an initial experiment where six samples were tested in triplicate for three mRNAs (11β-HSD2, 11β-HSD1 and β-actin). The average intra-assay variation was found to be 1.5%. The inter-assay variation was calculated from preliminary experiments where the same sample was amplified with four sets of primers (IGF-I, IGF-II, IGFBP-1 and β-actin in duplicate) in three different assays. For this calculation, the threshold was set at a constant value for comparison. In all cases, the inter-assay variation was <2.2%, giving an average of 1.3%. These values are close to those reported by Pfaffl of <3.9% inter-assay variation and <2.2% intra-assay variation (671).

3.11 Cord blood hormone measurements

3.11.1 Principles of radioimmunoassay

Radioimmunoassay (RIA) is used to quantify concentrations of a substance in a sample by taking advantage of the specific interaction between antigens and antibodies. In an RIA, there is competition between a known amount of radioactively labelled antigen

and the unlabelled antigen (in the sample) for a fixed number of antibody binding sites (651). The distribution of the added antigen into bound and free forms depends upon the total concentration of antigen present in the sample, thereby allowing quantification of this total concentration (679). A standard curve is produced by adding increasing known amounts of unlabelled antigen in the presence of fixed concentrations of antibody and radiolabelled antigen (tracer). As the concentration of unlabelled antigen increases, the percentage of radiolabelled antigen which binds the antibody decreases (679).

3.11.2 Cortisol radioimmunoassay

Cortisol concentrations were measured directly in umbilical vein plasma collected from the placenta at delivery, using a commercial RIA kit (Orion Diagnostica, Espoo, Finland) following the manufacturer's instructions. The sensitivity of the cortisol assay was 4.7 nmol/l and cross-reactivity of the cortisol antiserum with most other steroids was <0.1%. Steroids with a cross-reactivity greater than 2% were 5α-dihydrocortisol (84%), 21-desoxycortisol (79%), prednisolone (45%), 5β-dihydrocortisol (12%), 6α-methylprednisolone (11%) and fludrocortisone (2.3%).

Umbilical vein plasma was thawed on ice prior to centrifugation at 1000 g for 15 minutes (J-6B centrifuge, Beckman Coulter, Palo Alto, CA, USA). Reagents were brought to room temperature. The RIA kit provided tubes coated with rabbit polyclonal cortisol antibody. Cortisol standards (0, 20, 50, 150, 500, 1000 and 2000 nmol/l) were reconstituted with 500 µl of distilled water. Standards and undiluted samples (20 µl) were added to the antibody-coated tubes. Cortisol tracer (500 µl of ^{125}I-cortisol) was added to all coated tubes and to duplicate uncoated tubes, used to calculate total counts. All tubes were mixed briefly by vortexing, before incubating in a 37°C water bath for 2 hours. The solutions were aspirated and washed with 1 ml of distilled water. The wash was aspirated and samples were counted in a gamma counter (Cobra II Auto-Gamma Counter, Packard Bioscience, Canberra, Australia) for 1 minute. The concentration of cortisol in each sample was calculated from the standard curve.

3.11.3 Unconjugated estriol radioimmunoassay

Estriol concentrations were measured directly in umbilical vein plasma collected from the placenta at delivery, using a commercial RIA kit for unconjugated estriol

(Diagnostic Systems Laboratories, Webster, TX, USA), following the manufacturer's instructions. The sensitivity of the estriol assay was 0.03 ng/ml. Cross-reactivity of the estriol antibody was <1% for all similar compounds except 16-epiestriol (3.7%).

Plasma was thawed on ice prior to centrifugation at 1000 g for 15 minutes (J-6B Centrifuge, Beckman Coulter, Palo Alto, CA, USA). Samples were diluted 1:10 and/or 1:50 with PBS. Reagents were allowed to reach room temperature and were mixed prior to use. The standards were 0 ng/ml (for estimating non specific binding) and 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10 and 20 ng/ml estriol (for the standard curve). Standard or sample (50 µl) and estriol tracer (200 µl of ^{125}I-estriol) was added to tubes, followed by 200 µl of estriol antiserum to all tubes, except those for estimating total counts and non-specific binding. Tubes were vortexed and incubated in a 37°C water bath for 30 minutes. Precipitating reagent was thoroughly mixed before use and 1 ml added to all tubes (except total counts), which were incubated at room temperature for 15 minutes. Samples were centrifuged at 1500 g for 30 minutes at 4°C. Liquid was aspirated from all tubes (except total counts) and counted in a gamma counter for 1 minute (Cobra II Auto-Gamma Counter, Packard Bioscience, Canberra, Australia). The concentration of estriol in each sample was calculated from the standard curve.

3.11.4 Corticotropin Releasing Hormone (CRH) radioimmunoassay

CRH concentrations were measured by RIA in umbilical vein plasma collected from the placenta at delivery. These assays were performed by Mrs Maria Bowman (Mothers and Babies Research Centre, Newcastle) and were based on previously published methods (680, 681).

Propan-1-ol, acetonitrile, sodium azide and chloramine T were from BDH Laboratory Supplies (Dorset, UK). Trifluoroacetic acid (TFA) and sodium metabisulfite were from Ajax Chemicals (Sydney, Australia). BSA and polypep (low viscosity) was from Sigma (St Louis, MO, USA). β-mercaptoethanol was from ICN Biomedicals (Irvine, CA, USA). Na^{125}I was from Australian Radioisotopes (Lucas Heights, Australia). Synthetic human CRH standard (Tyr-hCRH) was from Peninsula (Belmont, CA, USA). C-18 Sep-pak columns were from Millipore Corporation (Billerica, MA, USA).

The tracer was chloramine-T labelled [^{125}I]Tyr-hCRH, prepared by mixing 0.5 mCi Na^{125}I and 10 µg chloramine-T with 2.5 µg Tyr-hCRH in phosphate buffer (pH 7.5) to a total volume of 30 µL. After 30 seconds, the reaction was stopped by the addition of sodium metabisulfite (10 µg/20 µl) and 500 µl 0.1% TFA/0.01% BSA/0.01% polypep. The sample was partially purified on a C-18 Sep-pak column and 5 ml fractions were eluted with 0, 5, 10, 15, 20-30, 40 and 80% propan-1-ol (containing 0.1% TFA). Fractions 40 and 45 (containing the highest radioactivity and binding) were dried, refrigerated and further purified by HPLC using a reverse phase column (C18, 300 mm × 3.9 mm) from Activon (Thornleigh, NSW, Australia). Fractions were eluted with a 0-70% acetonitrile/0.1% TFA gradient and those with the highest radioactivity and binding were stored in an equal volume of assay buffer at 4°C.

Extraction of umbilical vein plasma samples was performed using silica glass powder (Vycor, Corning, NY, USA). Samples were lyophilized and reconstituted in 500 µl RIA buffer, which contained 0.1 M sodium phosphate (pH 7.45), 0.25% (w/v) BSA, 0.1% (v/v) β-mercaptoethanol and 0.02% (w/v) sodium azide.

Duplicate plasma samples (200 µl) were incubated with 50 µl CRH antiserum (Y_2B_0, a gift from Prof PJ Lowry and Dr E Linton, University of Reading, United Kingdom) at 4°C for 24 hours. Incubation with tracer (50 µl of [^{125}I]Tyr-hCRH, 5000-10000 cpm) was for 72 hours. Donkey anti-rabbit antibody-coated cellulose suspension (Sac-cel, Immuno Diagnostics, Tyne and Wear, England) was added (50 µl) to separate the antibody-bound tracer from unbound tracer. Samples were counted in a gamma counter (Cobra II Auto-Gamma Counter, Packard Bioscience, Canberra, Australia) and CRH concentrations determined from a standard curve.

3.11.5 IGFBP radioimmunoassays

IGFBP-1 and IGFBP-3 were measured in umbilical vein plasma collected from the placenta at delivery by Dr Robert Baxter (Kolling Medical Research Institute, Sydney, Australia). Cord blood IGFBP-1 was measured according to a previously published protocol (414). Briefly, samples were incubated in 0.5 ml assay buffer containing antiserum A2 (1:100000 final dilution) and binding protein tracer (10000 cpm, 90 pg) for 16-18 hours at 2°C. Bound radioactivity was precipitated using a secondary antibody

(414). Cord blood IGFBP-3 was also measured as previously described, using rabbit antiserum R-100 against human IGFBP-3 at a final dilution of 1:100000 (409).

3.12 Statistical Analysis

The majority of the results are presented as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Instat version 2.04a or 3.05 (GraphPad Software Inc., San Diego, CA, USA). Analysis of variance (ANOVA) and the non-parametric equivalent (Kruskal-Wallis non-parametric ANOVA) were used for comparing three or more groups, along with the appropriate post-hoc test (Tukey-Kramer multiple comparisons test for ANOVA or Dunn's multiple comparisons test for Kruskal-Wallis ANOVA). Instat used Bartlett's test to assess whether the standard deviations of the groups were equal and the method of Kolmogorov and Smirnov to test if the data were sampled from Gaussian (normal) distributions. ANOVA was used when both of these assumptions were met. If either the standard deviations of the groups were not equal or the data was not normally distributed, the Kruskal-Wallis non-parametric ANOVA was used. When comparing two groups with a normal distribution, the Student's t-test (unpaired t-test) was used to compare means, unless the standard deviations were not equal, in which case the unpaired t-test with Welch correction was used. The Mann Whitney test was used to compare medians of two groups where data was not normally distributed. Fisher's exact test was used to compare proportions, using a 2 × 2 contingency table. A P value of <0.05 was considered significant.

When investigating the relationship between two sets of data, the Pearson linear correlation and Spearman rank (non-parametric) correlation were used. A runs test was performed to verify that the data was linear.

For graphical purposes, normally distributed data (which may or may not have had equal variances) is presented as means ± SEM. Minitab statistical software release 12 (Minitab Inc, State College, PA, USA) was used to draw boxplots of data that was not normally distributed and GraphPad Prism version 4.02 (GraphPad Software Inc., San Diego, CA, USA) was used to draw scatter plots.

Multivariate analysis was performed on some data by Dr Andrew Bisits and Dr Vicki Clifton (Mothers and Babies Research Centre, Newcastle), using Stata version 7 (Stata Corporation, College Station, TX, USA). Generalised linear latent and mixed models

and generalised estimating equations were used (682, 683). Outcomes were adjusted for asthma severity, cumulative inhaled glucocorticoid intake, fetal sex and maternal smoking.

Power and sample size calculations were performed using the PS power and sample size program, version 2.1.30 (684).

Data in this thesis is used in multiple statistical comparisons between various sub-groups. In most cases, ANOVA or the non-parametric equivalent are used to analyse the data using multiple comparisons post-hoc tests. However, the results, especially where the *P* value is between 0.01 and 0.05, should be interpreted with caution given the multiple use of the data. Some data which is separated into male and female fetus sub-groups is underpowered to detect significant differences. Consequently, it would be appropriate to repeat some of the experiments described in this thesis, if a more thorough analysis of a particular aspect of this study is required.

3.13 Proteomics using Surface Enhanced Laser Desorption/Ionisation-Time of Flight Mass Spectrometry (SELDI-TOF MS)

Proteomics work was carried out as part of a collaboration between the Mothers and Babies Research Centre and Ferring Research Institute (FRI) in San Diego (CA, USA). Experiments were performed at FRI under the supervision of Dr Karen Akinsanya and Dr Yung-Chih Wang. Optimisation of the human plasma method was conducted with another PhD candidate, Ms Renee Johnson (Mothers and Babies Research Centre, Newcastle), as part of a separate commercially confidential project covered by a legal agreement between Ferring Research Institute Inc and The University of Newcastle Research Associates Limited. Details of these optimisations are not provided here, because they are not critical for understanding the final method chosen.

3.13.1 Principles of SELDI-TOF MS

Proteomics is a rapidly growing area for studying the protein products expressed by the genome of an organism, tissue or cell type (685-687). Unlike the genome, the proteome may change in response to developmental stage, environment or disease (685).

SELDI-TOF MS is a relatively new proteomic technique combining on-chip retentate chromatography with mass spectrometry (688). Proteins in crude biological samples such as serum, plasma, urine and other fluids, differentially bind to chip surfaces based upon their chemical and biochemical properties such as hydrophobicity or charge (689). The interaction between the protein and the chip surface is specific and based upon the amino acid sequence and structure of the protein and the detergent, salt concentration and pH of the binding buffer (690). The chip surfaces may interact with proteins in a general manner (reverse phase, cation exchange, anion exchange, normal phase), or they may be specifically designed for more complex interactions such as antibody/antigen, DNA/protein or receptor/ligand (688). The incubation times used in SELDI are fairly short which decreases low affinity binding and a series of wash steps removes any unbound material (690). Proteins which are retained are embedded in an energy absorbing molecule (EAM) or matrix (688). The EAM is ionised by a laser and the gaseous protein ions move through a vacuum towards a detector, with the time taken to reach the detector used to derive the mass to charge (m/z) ratio (688, 689). SELDI-TOF is particularly effective for detecting low mass proteins and peptides <20 kDa (689), but is also capable of detecting large proteins up to approximately 150 kDa. It is a more sensitive approach requiring less sample or sample preparation than two dimensional-polyacrylamide gel electrophoresis (2D-PAGE) (689, 691) while also detecting low abundance proteins and low mass proteins (<5 kDa), which usually cannot be visualised by 2D-PAGE (690, 692).

SELDI-TOF MS has many applications both in basic research and clinical proteomics. It is becoming widely used for biomarker discovery (688), particularly in the field of cancer research (690, 693, 694), where laser capture microdissection studies of diseased and healthy tissue (695) have been carried out along side studies of biological fluids such as serum (688). This technology can be used to identify diagnostic markers, monitor disease progression (695) and discover new therapeutic targets (693). The patterns of protein expression are of particular importance in diagnostic applications, since the actual identity of particular proteins is not essential and studying multiple proteins rather than individual proteins often allows better discrimination between patient groups (688, 696).

Very little proteomics work has been published in the field of pregnancy or asthma. Recent reviews suggest that the completion of the human genome project will allow

unique opportunities to use both genomics and proteomics to further understand numerous aspects of reproductive medicine (686, 697-699). To date, proteomic techniques have been used to identify markers of premature rupture of fetal membranes (700) and to characterise placental protein profiles of normal and complete hydatidiform mole in placental samples collected by laser capture microdissection (701). The application of proteomics to asthma research has been limited to studies of connective tissue biology carried out with bronchial biopsies using 2D-PAGE and matrix assisted laser desorption/ionisation (MALDI)-TOF MS (702, 703). The use of SELDI-TOF MS in the field of asthma and pregnancy research has the potential to further our understanding of the interactions occurring between the mother, placenta and fetus and may help to identify protein changes associated with reduced fetal growth.

3.13.2 Materials

ProteinChip® arrays, energy absorbing molecules (EAM), all-in-1 peptide molecular weight standard and all-in-1 protein standard were obtained from Ciphergen (Freemont, CA, USA). Three ProteinChip® arrays were used: WCX2 (weak cation exchange), SAX2 (strong anion exchange) and IMAC3 (immobilised metal affinity capture). The EAM or matrix molecules used were sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, SPA) and α-cyano-4-hydroxycinnamic acid (CHCA).

Urea and Ni-NTA agarose were from Invitrogen (Carlsbad, CA, USA). Ni-NTA agarose contained nickel-nitrilotriacetic acid and ethanol. Copper sulfate ($CuSO_4.5H_2O$), NaCl, glacial acetic acid, hydrochloric acid (HCl) and ammonium acetate were all obtained from Sigma (St Louis, MO, USA). 3-[(3-chloamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), Triton X-100 (TX-100), Tris HCl and trifluoroacetic acid (TFA) were from Pierce (Rockford, IL, USA). Sodium acetate was from Fisher Biotech (Pittsburg, PA, USA), and acetonitrile (HPLC grade) was from Fisher Chemicals (Fairlawn, NJ, USA).

Buffer recipes are given in Appendix 8. All buffers were syringe filtered (0.02 μm) before use.

3.13.3 Sample preparation

Protein profiling was carried out on maternal plasma collected at approximately 18 and 30 weeks gestation, umbilical vein plasma collected from the placenta following delivery and placental homogenates. Samples were collected from asthmatic and non-asthmatic women.

Crude placental homogenates were prepared by homogenising approximately 1.5-2 g of crushed placental tissue in 10 volumes of sodium phosphate buffer containing protease inhibitors (see section 3.8.3). Homogenates were centrifuged at 4°C at 1000 g for 10 minutes (J-6B centrifuge, Beckman Coulter, Palo Alto, CA, USA), and the supernatant removed. Protein concentration was determined by Bradford assay (652) as described in Section 3.8.3. Aliquots were stored at –80°C prior to transportation on dry ice to FRI in San Diego, CA (World Courier, Sydney, Australia).

Some samples were denatured prior to binding on the ProteinChip® arrays. This involved adding 80 µl of buffer (50 mM Tris pH 9 + 9 M urea + 2% CHAPS) for every 20 µl plasma, vortexing at room temperature for 30 minutes and loading 10 µl onto the chips.

SELDI-TOF analysis was conducted with the Ciphergen Protein Biology System IIc and Ciphergen ProteinChip® Software version 3.1.1, biomarker edition (Ciphergen, Freemont, CA, USA). There were eight spots per ProteinChip® array and a bioprocessor was used which allowed 12 arrays to be processed simultaneously (Figure 3.12). Each spot contained a single sample. Mass analysis was performed using TOF-MS in the Ciphergen Protein Biology System IIc (Figure 3.13) at laser intensities 195 and 220 (for low and high mass proteins respectively) using SPA and at laser intensity 170 for CHCA (low mass peptides only). For some optimisations, laser intensities of 230 for high mass (SPA), or 175 for low mass (CHCA) were used. The low and high mass spot protocols used are listed in Appendix 9. The method was found to be particularly useful for detecting low mass peptides from 1.5 to 20 kDa and was also able to detect proteins up to approximately 150 kDa (Figure 3.14).

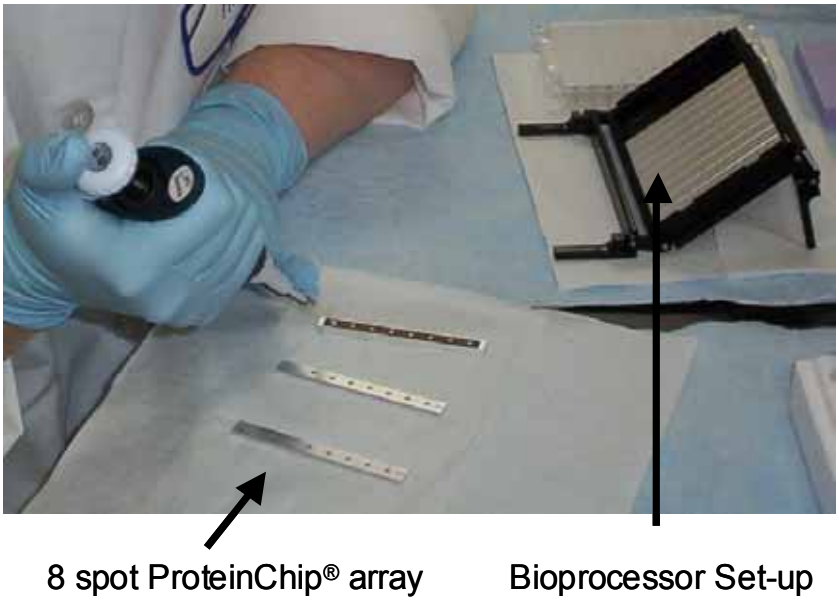

Figure 3.12 Bioprocessor and eight spot ProteinChip® arrays

Photograph of the bioprocessor set-up and eight spot ProteinChip® arrays used for proteomic analysis.

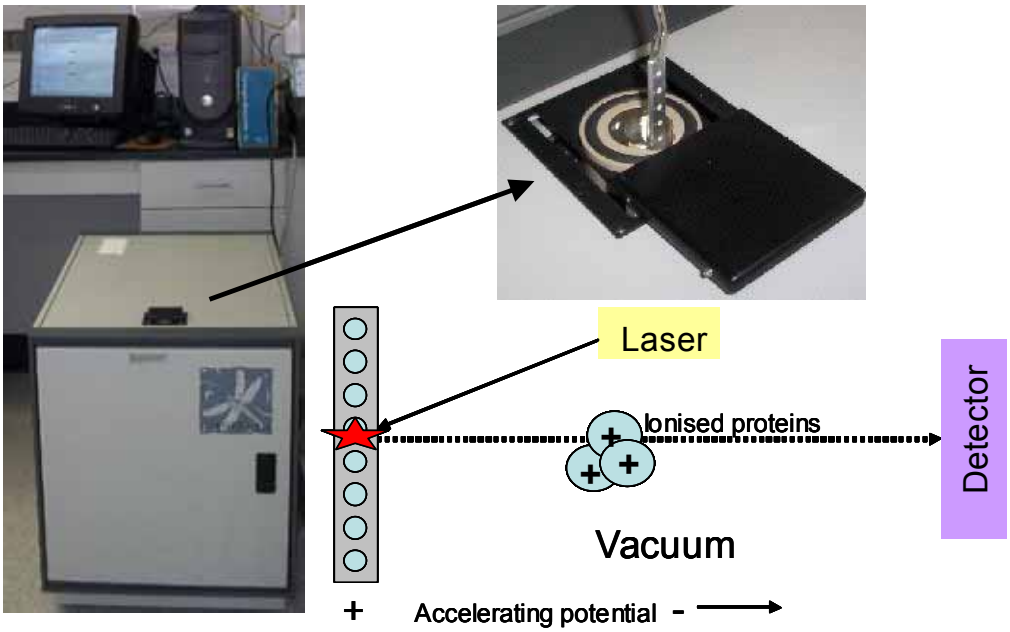

Figure 3.13 Ciphergen Protein Biology System IIc

Photograph of the SELDI machine used for proteomic analysis. The diagram shows how the laser irradiates the sample, resulting in the liberation of gaseous ions from the array. The velocity of these ions through the vacuum to the detector is used to derive their mass to charge (m/z) ratio (diagram adapted from Issaq et al., 2002)(689).

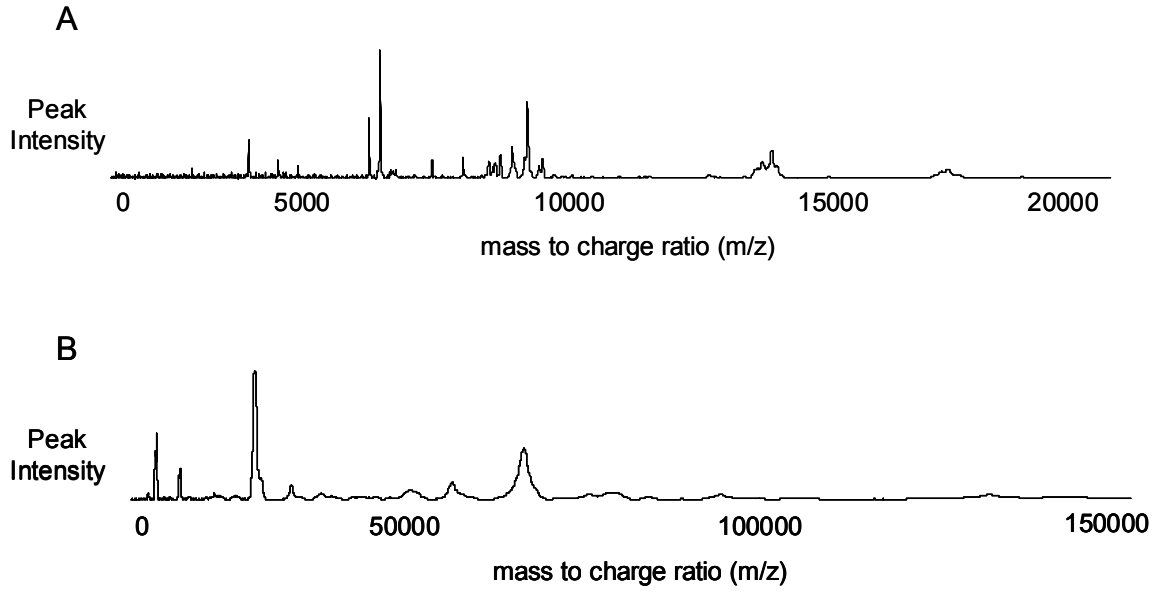

Figure 3.14 Low and high mass spectra

Panel A shows a representative low mass spectrum obtained using 30 week pregnant plasma on a SAX chip (pH 9) with SPA matrix. Panel B shows a representative high mass spectrum obtained using 30 week pregnant plasma on a SAX chip (pH 9) with SPA matrix.

3.13.4 Calibration

Low and high mass regions were calibrated daily using all-in-1 peptide molecular weight standards and all-in-1 protein standard respectively, which were made up according to the manufacturer's instructions. Briefly, the lyophilised peptide standards were warmed to room temperature, and reconstituted in 25 µl of resuspension solution (containing 10 mM ammonium acetate, 25% acetonitrile and 1.25% TFA). This solution was mixed well and transferred to a microcentrifuge tube. A further 25 µl of resuspension solution was used to wash the original vial and this solution was left to stand at room temperature for 10 minutes before pooling with the first wash. The pooled standards were distributed into 10 µl aliquots which were stored at –80°C for later use. One aliquot was combined with 10 µl of a saturated solution of SPA (acetonitrile/1% TFA) and spotted onto each of the eight spots of a WCX chip, which had been pre-wetted with water. The low mass peptide standard chip was read using the low mass calibration spot protocol (Appendix 9). To prepare the all-in-1 protein standard, a 5 µl aliquot was thawed on ice and diluted five-fold with a saturated solution of SPA. This solution was spotted onto each of the eight spots of a pre-wet IMAC chip and completely air dried before reading, using the high mass spot protocol (Appendix 9).

The peptide standard consisted of Arg-vasopressin (1084.2 Da), somatostatin (1637.9 Da), bovine insulin β-chain (3495.9 Da), human insulin (5807.7 Da) and hirudin (7033.6 Da). The protein standard consisted of equine cytochrome C (12360.2 Da), equine cardiac myoglobin (16951.5 Da), rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 35688.0 Da), bovine serum albumin (66433 Da) and *E. coli* β-galactosidase (116351 Da). Calibration was carried out using at least four of the five standards in each case. Examples of these calibrations are shown in Figure 3.15.

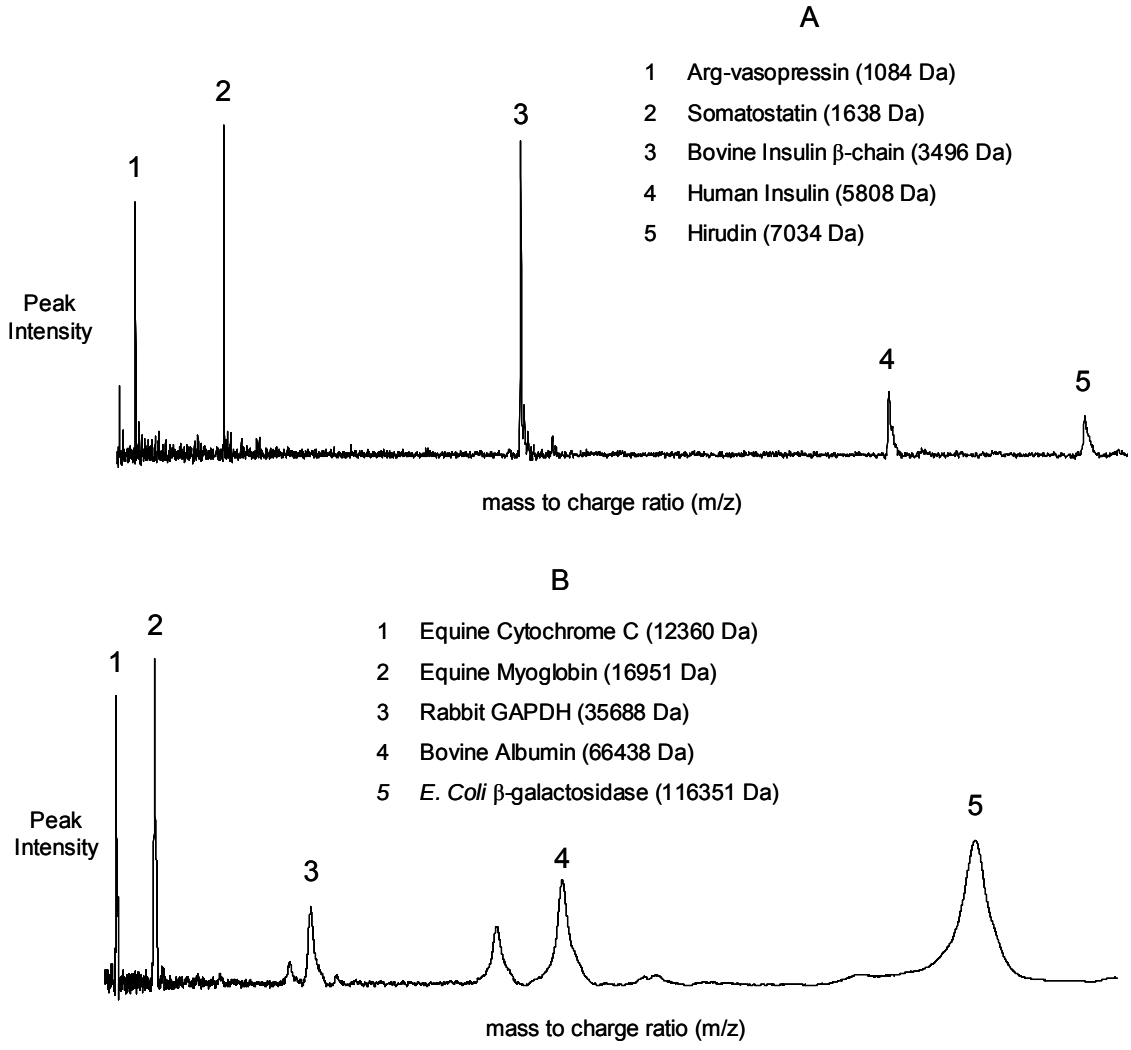

Figure 3.15 Low and high mass calibration spectra

These representative calibration spectra were obtained using the low mass calibration spot protocol (Panel A) and the high mass spot protocol (Panel B).

3.13.5 Plasma protein profiling method

Maternal and cord blood (umbilical vein) plasma samples were thawed once on ice, centrifuged and aliquoted into 100 µl portions which were stored at –80°C for later use in SELDI experiments. On the day of the experiment, aliquots were thawed on ice and centrifuged at 8000 g at 4°C for 10 minutes in a table-top centrifuge (Microfuge R, Beckman Coulter, Palo Alto, CA, USA). ProteinChip® arrays were activated, neutralised and equilibrated before the addition of sample. Each day, only one chip type was used and experiments for each individual chip surface were completed in a single run.

IMAC chips were pre-activated with 50 µl of $CuSO_4$ per spot. WCX chips were activated by adding 100 µl of 10 mM HCl per spot. SAX chips were not activated. Following 15 minutes on a shaker, wells of the bioprocessor were rinsed with distilled water. IMAC chips were neutralised with 100 µl of 50 mM sodium acetate (pH 4). WCX chips were neutralised with 100 µl of 100 mM ammonium acetate (pH 6). 100 µl of 50 mM Tris (pH 9) was added to SAX chips at this stage. The bioprocessor was shaken for 5 minutes. Wells were rinsed with distilled water. Spots were equilibrated by shaking twice with 150 µl of low stringency binding buffer (pH 4 for WCX, pH 7 for IMAC and pH 9 for SAX, Appendix 8) for 5 minutes. Following the second binding buffer wash, 100 µl of fresh binding buffer was added to each spot. Plasma sample (2.5 µl neat, native protein) was added to the binding buffer on each spot of the ProteinChip® array. The bioprocessor was covered with parafilm and incubated at room temperature for 30 minutes on a shaking platform.

The sample was removed from the bioprocessor wells, and the wells were washed three times with appropriate binding buffer (200 µl per well, shaken for 5 minutes each wash). After two further washes with distilled water (200 µl per well, shaken for 5 minutes each wash), individual chips were removed from the bioprocessor and air-dried. Fresh saturated SPA and CHCA solutions were made up daily as required. To one tube of SPA or CHCA (approximately 5 mg per tube), 100 µl of acetonitrile and 100 µl of 1% TFA was added. Solutions were vortexed for 3 minutes and centrifuged at 8000 g for 3 minutes at room temperature (Microfuge R, Beckman Coulter, Palo Alto, CA, USA). Matrix (0.5 µl) was added twice to each spot and allowed to fully air-dry between applications (Figure 3.12).

Initial condition optimisations were performed using non-pregnant and pregnant plasma. The suitability of the method for use with pregnant plasma was validated by the tentative identification of CRH in pregnant, but not in non-pregnant plasma. The CRH peptide has a molecular weight of 4758.5 (based on amino acids 154-194 of accession number A30327) and a theoretical isoelectric point (pI) of 5.09 (calculated on us.expasy.org). Based on this information, CRH would be expected to bind to a strong anion exchange surface at pH 9, since this pH is greater than the pI of the peptide, resulting in a negative charge under these conditions. A peak at m/z 4758 was observed using a SAX chip at pH 9, particularly in denatured pregnant plasma, but not in neat or denatured non-pregnant plasma (Figure 3.16). This is consistent with previous studies using HPLC or RIA methods where CRH could not be detected in non-pregnant or first trimester pregnant plasma (704, 705) and confirmed that the SELDI method could be used to identify a known peptide in pregnant plasma.

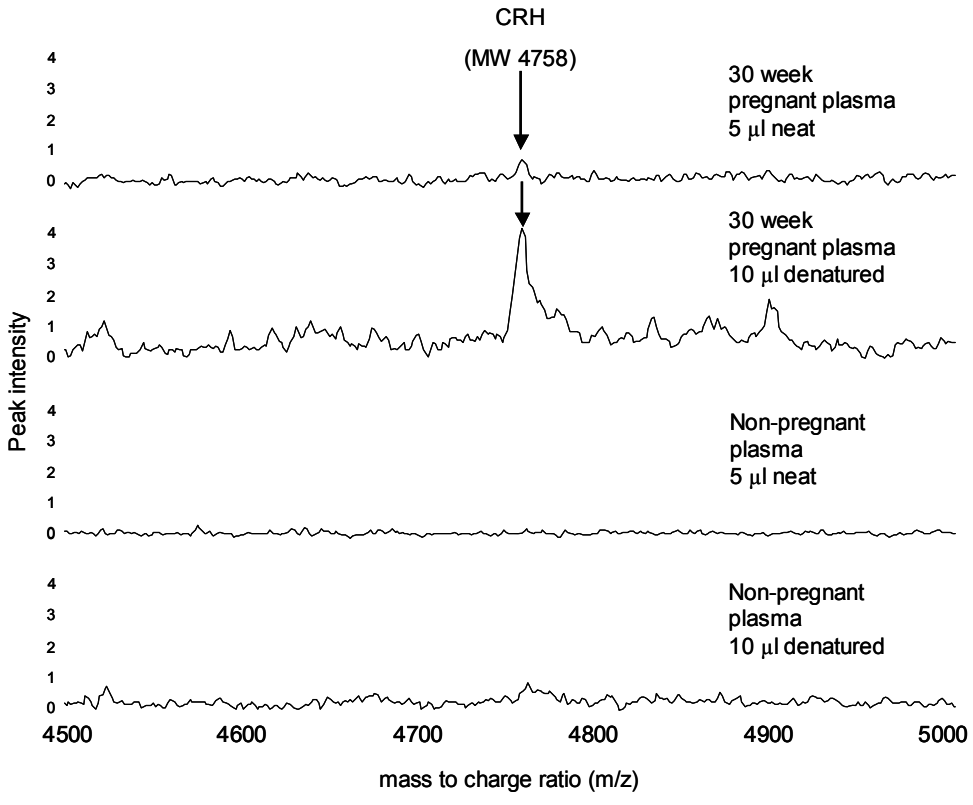

Figure 3.16 Detection of CRH in pregnant plasma

Samples of neat pregnant and non-pregnant plasma (5 µl) were compared. Plasma was denatured and compared between pregnant and non-pregnant individuals. A peak which potentially represents CRH with a m/z of 4758, was observed on SAX at pH 9 (SPA matrix) in neat and denatured pregnant plasma samples, but was not observed in non-pregnant plasma.

3.13.6 Placental tissue protein profiling

Placental homogenates were thawed on ice and centrifuged at 8000 g at 4°C for 10 minutes prior to use (Microfuge R, Beckman Coulter, Palo Alto, CA, USA). Placental samples (10 µg equivalents) were incubated on IMAC, WCX and SAX chips under low and high stringency conditions (Appendix 8), according to the standard protocol used for plasma samples.

Removal of hemoglobin with Ni-NTA Agarose

Placental samples contained hemoglobin, as evidenced by peaks at 7.5 and 15 kDa (Figure 3.17). Consequently, IMAC chips were not suitable for use as hemoglobin occupied most of the binding sites due to its metal affinity. An attempt to remove hemoglobin from the samples was made using nickel beads. The Ni-NTA agarose suspension (50% suspension in 30% ethanol) was vortexed and 20 µl was added to a 200 µg equivalent of placental homogenate. Samples were vortexed for 2 hours at 4°C. After 2 hours, the Ni beads had turned red (previously blue), indicating that some hemoglobin had been removed. The slurry was centrifuged at 700 g for 2 minutes (Microfuge R, Beckman Coulter, Palo Alto, CA, USA) and the supernatant removed. A 10 µg equivalent was added to the chips and incubated for 30 minutes at room temperature on a rocking platform, under high and low stringency binding conditions. It was noted that under low stringency conditions (WCX pH 6), hemoglobin still dominated the spectrum, even after Ni-purification (Figure 3.17). However, under high stringency conditions (pH 9), in both neat placental homogenate (10 µg) and Ni-purified placental homogenate (10 µg equivalent) samples, the major peaks from hemoglobin were no longer evident (Figure 3.17). The spectra were slightly different between neat and Ni-purified placenta. However, no added advantage was evident with Ni-purification and due to the extra time required, this method was not continued. However, the high stringency conditions were implemented for use with the placental samples since they were suitable for minimising spectral interference from hemoglobin.

Final placenta profiling conditions

The final placenta profiling optimisation experiment compared neat placental homogenates (10 µg), denatured placental homogenate (80 µg diluted to 40 µl total volume with 50 mM Tris pH 9 and incubated for 30 minutes at room temperature with

an equal volume of urea buffer, 10 µl added to chips), Ni-NTA purified placental homogenate (neat, 10 µg equivalent added to chips) and Ni-NTA purified placental homogenate (denatured, 10 µg equivalent added to chips). The following conditions were tested: SAX pH 9 (low stringency) and pH 4 (high stringency) and WCX pH 4 (low stringency) and pH 9 (high stringency), each using SPA and CHCA matrix.

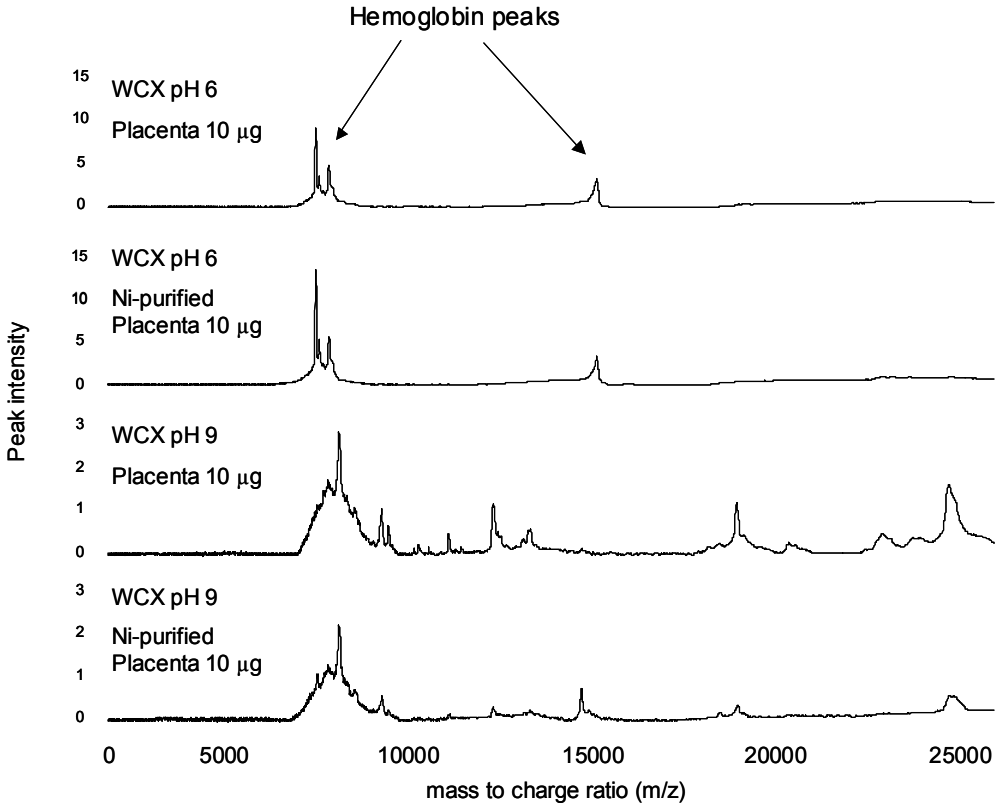

Figure 3.17 WCX pH 6 and pH 9 spectra for placental homogenates

Placental homogenates were incubated neat (10 µg) or following partial removal of hemoglobin with Ni-NTA agarose (10 µg equivalents) on WCX chips under low (pH 6) and high (pH 9) stringency binding conditions. The peak intensity of the hemoglobin peaks was greatly reduced under high stringency conditions (pH 9).

Various regions of each spectra were examined to decide the final conditions to be used. For WCX chips, there was a noticeable improvement in the detection of peaks on the spectra when pH 9 binding buffer was used, compared to pH 4, due to the fact that hemoglobin did not bind under these conditions. Neat placental homogenates were found to give the best overall result, especially in the high mass region. SPA was found to be the most suitable matrix. The final WCX conditions used for the placenta were therefore neat homogenates (10 µg), with pH 9 buffer and SPA matrix.

For SAX chips, there was a large amount of noise on the spectra when the high stringency buffer (pH 4) was used. On the pH 9 spectra, neat and Ni-NTA neat were considered the best conditions; however, some peaks were not visible when Ni-NTA samples were used. The use of CHCA matrix was found to result in peaks which were not present on the SPA spectra. This was considered a better choice, despite the sacrifice of the high mass region when using this matrix, since the high mass region was dominated by high abundance proteins such as hemoglobin, albumin and transferrin (Dr James LeBlanc, Ciphergen Field Scientist, personal communication). The final SAX conditions used for the placenta were therefore neat homogenates (10 µg), with pH 9 buffer and CHCA matrix.

3.13.7 Data Analysis

All protein profiles (spectra) to be compared were placed into a new experiment using the Ciphergen ProteinChip® Software and normalised for total ion current before proceeding. For low mass spectra, normalisation began at 1500 Da, while for high mass spectra, normalisation began at 10000 Da, as the regions prior to these were considered noise. The Biomarker Wizard was used to find new clusters of peaks. On the first pass, peaks with a signal/noise ratio >5 were selected and on the second pass peaks with a signal/noise ratio >2 were selected. The minimum peak threshold was set at 20%, signifying that 20% of all spectra must contain that peak in order for it to be considered a valid cluster. The cluster mass window was set at 0.3% of mass, signifying that all peaks considered to be from the same cluster must be within 0.3% of each other's mass. Statistical differences in protein peaks between groups were determined using the Biomarker Wizard Software, with $P<0.05$ considered significant. The software used one of the following statistical tests as appropriate: the parametric two tailed t-test, the non-parametric Mann Whitney U test (for comparing two groups), the parametric single factor/one way ANOVA, or the non-parametric Kruskal-Wallis H test (for comparing more than two groups).

All peaks were visually checked for outliers, possible chemical modifications, consistency between individuals (the same area of the spectrum labelled in each case), and for their relationship to other peaks, using the reference lines. For example, where possible, the reference lines were used as a guide to indicate the presence of a doubly or triply charged version of another peak. A category (A, B or C) was assigned to each

peak and was based generally on the visual checks and indicated the quality of each result. A category A peak was considered the best for follow-up. A category B peak was considered good for follow-up, but may have been related to, or a modification of another peak on the spectrum. A category C peak was felt to be of less quality, either due to being particularly flat or broad, or close to the noise region. Peaks that were significantly different between groups according to the Biomarker Wizard program, but did not pass the visual check were excluded.

The possible identity of peaks of interest was investigated by searching the Swiss-Prot protein database (http://us.expasy.org/tools/tagident.html). The molecular weight (MW) was estimated to be the m/z with 0.5% error. An estimate of the pI of the protein was made, based upon its binding to the chip surfaces. If the peak was found on a WCX chip at pH 4, the pI was assumed to be approximately ≥ 4. If the peak was found on a SAX chip at pH 9, the pI was assumed to be approximately ≤ 9. Peaks which bound to IMAC at pH 7 were assumed to have any possible pI. The search was narrowed to include proteins from *homo sapiens* only. The known functions of the matching proteins were examined using the NiceProt View (http://us.expasy.org/cgi-bin/niceprot).

Results/Discussion

Chapter 4 Fetal Growth in Asthmatic Pregnancies

4.1 Maternal characteristics

Pregnant women with asthma (n=138) and pregnant women without asthma (control, n=44) were recruited for the study. Asthmatic women were classified according to asthma severity, as mild (n=62), moderate (n=28) or severe (n=48). Groups were further divided based upon fetal sex. Clinical characteristics of these groups are shown in Table 4.1 for women pregnant with a female fetus and Table 4.2 for women pregnant with a male fetus. ANOVA was used to compare the maternal characteristics of age, height, early pregnancy weight, early pregnancy BMI and weight gain during pregnancy. No significant differences were found (P>0.05). The Kruskal-Wallis non-parametric ANOVA was used to compare the gravidity and parity of the groups and no significant differences were found (P>0.05). Early pregnancy blood pressure was measured at the first antenatal visit at approximately 8-15 weeks. There were no significant differences in systolic or diastolic blood pressure in early pregnancy between any groups (Kruskal-Wallis ANOVA, P>0.05).

Asthmatic women were also classified according to their inhaled glucocorticoid intake during pregnancy as no glucocorticoid (n=46) or glucocorticoid (n=92). Groups were further divided based upon fetal sex and the glucocorticoid group was also divided according to dose of inhaled glucocorticoid used (low n=22, moderate n=43 or high n=27). The low dose glucocorticoid group was a mixed group. Only seven of the 22 subjects used inhaled glucocorticoids throughout pregnancy. Two subjects commenced glucocorticoid therapy in the second trimester and continued use to the end of the pregnancy. Two subjects used inhaled glucocorticoids in the second trimester only, and 11 subjects used inhaled glucocorticoids in the third trimester only. For much of the data analysis the low, moderate and high groups were combined into one group (glucocorticoid) and in the tables, data for all four groups (low, moderate, high and glucocorticoid combined) is shown.

Table 4.1 Clinical characteristics of women pregnant with a female fetus and classified by asthma severity

Female Fetus	Classification of Asthma Severity During Pregnancy			
	Control	Mild	Moderate	Severe
Total Number of Subjects	21	32	10	27
Glucocorticoid Intake		18 No Glucocorticoid	0 No Glucocorticoid	4 No Glucocorticoid
		3 Low Dose	1 Low Dose	2 Low Dose
		7 Moderate Dose	4 Moderate Dose	10 Moderate Dose
		4 High Dose	5 High Dose	11 High Dose
Maternal Age (years)	28 ± 1 (n=21) *(22 – 37)*	25 ± 1 (n=32) *(16 – 36)*	26 ± 2 (n=10) *(19 – 35)*	27 ± 1 (n=27) *(17 – 39)*
Maternal Height (cm)	164.7 ± 1.3 (n=15) *(156 – 173)*	165.2 ± 1.1 (n=30) *(152 – 177)*	164.6 ± 1.3 (n=10) *(157 – 171)*	165.9 ± 1.4 (n=23) *(153.5 – 180)*
Maternal Early Pregnancy Weight (kg)	66.4 ± 4.0 (n=16) *(47.3 – 97.5)*	72.0 ± 2.9 (n=31) *(44.4 – 110.7)*	74.5 ± 6.8 (n=10) *(49 – 117)*	71.7 ± 3.8 (n=23) *(51 – 125)*
Maternal Pregnancy Weight Gain (kg)	12.3 ± 2.2 (n=10) *(0.6 – 26.6)*	12.6 ± 1.1 (n=24) *(1.5 – 24.6)*	8.7 ± 1.4 (n=9) *(2.4 – 15.7)*	11.1 ± 0.8 (n=22) *(1 – 15.1)*
Maternal Early Pregnancy BMI	24.5 ± 1.5 (n=15) *(19.4 – 35.4)*	26.4 ± 1.0 (n=30) *(19.2 – 41.7)*	27.3 ± 2.2 (n=10) *(19.9 – 41.2)*	26.4 ± 1.4 (n=21) *(19.9 – 44.3)*
Maternal Early Pregnancy Systolic Blood Pressure (mm Hg)	111 ± 3 (n=15) *(100 – 130)*	115 ± 2 (n=32) *(95 – 140)*	114 ± 5 (n=10) *(90 – 135)*	110 ± 2 (n=27) *(90 – 140)*
Maternal Early Pregnancy Diastolic Blood Pressure (mm Hg)	65 ± 2 (n=15) *(50 – 80)*	67 ± 2 (n=32) *(20 – 90)*	70 ± 2 (n=10) *(60 – 80)*	63 ± 2 (n=27) *(20 – 80)*
Gravidity	2.9 ± 0.6 (n=21) *(1 – 10)*	2.1 ± 0.2 (n=32) *(1 – 5)*	2.7 ± 0.3 (n=10) *(1 – 4)*	2.8 ± 0.3 (n=27) *(1 – 8)*
Parity	1.3 ± 0.3 (n=21) *(0 – 6)*	0.6 ± 0.1 (n=32) *(0 – 3)*	0.9 ± 0.3 (n=10) *(0 – 3)*	1.1 ± 0.3 (n=27) *(0 – 5)*
	Values given are Mean ± Standard Error of the Mean *(Range)*			

Table 4.2 Clinical characteristics of women pregnant with a male fetus and classified by asthma severity

Male Fetus	Classification of Asthma Severity During Pregnancy			
	Control	**Mild**	**Moderate**	**Severe**
Total Number of Subjects	**23**	**30**	**18**	**21**
Glucocorticoid Intake		**16 No Glucocorticoid**	**5 No Glucocorticoid**	**3 No Glucocorticoid**
		7 Low Dose	**3 Low Dose**	**6 Low Dose**
		7 Moderate Dose	**9 Moderate Dose**	**6 Moderate Dose**
		0 High Dose	**1 High Dose**	**6 High Dose**
Maternal Age (years)	**30 ± 1 (n=23)** *(19 – 42)*	**26 ± 1 (n=30)** *(18 – 36)*	**27 ± 2 (n=18)** *(18 – 40)*	**27 ± 1 (n=21)** *(18 – 39)*
Maternal Height (cm)	**164.9 ± 2.3 (n=17)** *(131 – 174)*	**163.7 ± 1.3 (n=27)** *(150.5 – 180)*	**162.7 ± 1.6 (n=18)** *(146 – 173)*	**162.9 ± 1.4 (n=20)** *(154 – 176)*
Maternal Early Pregnancy Weight (kg)	**76.2 ± 4.6 (n=17)** *(55.8 – 117.5)*	**74.4 ± 4.1 (n=28)** *(52.5 – 134)*	**70.2 ± 4.5 (n=17)** *(46.8 – 112.5)*	**81.8 ± 5.5 (n=21)** *(52 – 131.6)*
Maternal Pregnancy Weight Gain (kg)	**10.0 ± 1.5 (n=12)** *(1.6 – 17.2)*	**12.9 ± 1.3 (n=22)** *(1.1 – 29.7)*	**10.7 ± 1.1 (n=16)** *(3.9 – 16.5)*	**9.2 ± 1.8 (n=16)** *(-3.2 – 21.9)*
Maternal Early Pregnancy BMI	**28.1 ± 1.7 (n=17)** *(19.5 – 42.8)*	**27.1 ± 1.3 (n=25)** *(19.8 – 43.7)*	**26.5 ± 1.6 (n=17)** *(17.3 – 40.3)*	**31.1 ± 2.2 (n=20)** *(16.9 – 51.4)*
Maternal Early Pregnancy Systolic Blood Pressure (mm Hg)	**119 ± 3 (n=17)** *(91 – 150)*	**111 ± 2 (n=28)** *(90 – 125)*	**112 ± 5 (n=16)** *(90 – 150)*	**115 ± 3 (n=19)** *(90 – 160)*
Maternal Early Pregnancy Diastolic Blood Pressure (mm Hg)	**69 ± 2 (n=17)** *(60 – 85)*	**63 ± 2 (n=28)** *(50 – 78)*	**69 ± 3 (n=16)** *(55 – 90)*	**68 ± 3 (n=19)** *(60 – 110)*
Gravidity	**2.9 ± 0.4 (n=23)** *(1 – 8)*	**2.3 ± 0.3 (n=30)** *(1 – 9)*	**1.9 ± 0.3 (n=18)** *(1 – 5)*	**2.9 ± 0.4 (n=21)** *(1 – 9)*
Parity	**1.5 ± 0.3 (n=23)** *(0 – 6)*	**0.8 ± 0.2 (n=30)** *(0 – 4)*	**0.6 ± 0.2 (n=18)** *(0 – 3)*	**1.1 ± 0.2 (n=21)** *(0 – 3)*
	Values given are Mean ± Standard Error of the Mean ***(Range)***			

Clinical characteristics of the groups classified by glucocorticoid intake are shown in Table 4.3 for women pregnant with a female fetus and Table 4.4 for women pregnant with a male fetus. Clinical characteristics unrelated to asthma were not significantly different between the control, no glucocorticoid and glucocorticoid groups (ANOVA or Kruskal-Wallis ANOVA, $P>0.05$). Maternal weight gain was significantly different between women pregnant with a female fetus in the low, moderate and high dose glucocorticoid groups (ANOVA, $P=0.034$), with women in the low dose group gaining more weight during pregnancy than those in the moderate and high dose groups (Tukey-Kramer multiple comparisons post-test, $P<0.05$).

The no glucocorticoid and glucocorticoid groups contained different proportions of mild, moderate and severe asthmatics (Table 4.3, Table 4.4 and Figure 4.1). In the no glucocorticoid group, 82% of women pregnant with a female fetus were mild asthmatics, while 67% of those pregnant with a male fetus were mild asthmatics. In the glucocorticoid group, 49% of women pregnant with a female fetus were severe asthmatics while 40% of women pregnant with a male fetus were severe asthmatics.

Information about maternal asthma including FEV_1 and FVC (at the first visit to the AMS), the number of subjects who required periodic oral steroids during pregnancy and the inhaled glucocorticoid drug used by the subjects was collected. Results for the groups classified by asthma severity are shown in Table 4.5. Some non-asthmatic women did not attend the AMS or donated their placenta only and consequently, data on FEV_1 and FVC was not available for the entire group. Results for the groups classified by glucocorticoid intake are shown in Table 4.6. In these tables, data from women pregnant with male and female fetuses has been combined.

Table 4.3 Clinical characteristics of women pregnant with a female fetus and classified by glucocorticoid intake

Female Fetus	Classification of Glucocorticoid Intake During Pregnancy					
	Control	No Glucocorticoid	Low	Moderate	High	Glucocorticoid
Total Number of Subjects	21	22	6	21	20	47
Asthma Severity		18 Mild	3 Mild	7 Mild	4 Mild	14 Mild
		0 Moderate	1 Moderate	4 Moderate	5 Moderate	10 Moderate
		4 Severe	2 Severe	10 Severe	11 Severe	23 Severe
Maternal Age (years)	28 ± 1 (n=21) *(22 – 37)*	26 ± 1 (n=22) *(18 – 36)*	24 ± 3 (n=6) *(16 – 32)*	27 ± 1 (n=21) *(17 – 36)*	26 ± 1 (n=20) *(17 – 39)*	26 ± 1 (n=47) *(16 – 39)*
Maternal Height (cm)	164.7 ± 1.3 (n=15) *(156 – 173)*	166.5 ± 1.1 (n=21) *(152 – 173)*	167.2 ± 3.4 (n=6) *(160 – 180)*	164.2 ± 1.3 (n=19) *(155 – 177)*	164.7 ± 1.7 (n=17) *(153.5 – 177)*	164.8 ± 1.0 (n=42) *(153.5 – 180)*
Maternal Early Pregnancy Weight (kg)	66.4 ± 4.0 (n=16) *(47.3 – 97.5)*	69.7 ± 3.0 (n=22) *(44.4 – 108.4)*	63.9 ± 4.3 (n=5) *(52.4 – 78.6)*	71.5 ± 4.5 (n=19) *(47 – 110.7)*	78.6 ± 4.9 (n=18) *(54.4 – 125)*	73.6 ± 3.0 (n=42) *(47 – 125)*
Maternal Pregnancy Weight Gain (kg)	12.3 ± 2.2 (n=10) *(0.6 – 26.6)*	12.8 ± 1.1 (n=17) *(6.3 – 24.6)*	15.6 ± 2.0 (n=5)* *(11 – 22.7)*	10.1 ± 1.0 (n=17) *(1.5 – 15.7)*	9.9 ± 1.1 (n=16) *(1 – 20.9)*	10.7 ± 0.7 (n=38) *(1 – 22.7)*
Maternal Early Pregnancy BMI	24.5 ± 1.5 (n=15) *(19.4 – 35.4)*	25.2 ± 1.0 (n=21) *(19.2 – 36.9)*	23.6 ± 1.5 (n=5) *(20.4 – 29.2)*	26.4 ± 1.5 (n=18) *(19.6 – 41.7)*	29.3 ± 1.7 (n=17) *(20.5 – 44.3)*	27.3 ± 1.0 (n=41) *(19.6 – 44.3)*
Maternal Early Pregnancy Systolic Blood Pressure (mm Hg)	111 ± 3 (n=15) *(100 - 130)*	115 ± 3 (n=22) *(90 – 140)*	106 ± 6 (n=6) *(90 – 130)*	111 ± 3 (n=21) *(95 – 135)*	115 ± 3 (n=20) *(90 – 140)*	112 ± 2 (n=47) *(90 – 140)*
Maternal Early Pregnancy Diastolic Blood Pressure (mm Hg)	65 ± 2 (n=15) *(50 – 80)*	66 ± 3 (n=22) *(20 – 82)*	63 ± 2 (n=6) *(60 – 70)*	64 ± 2 (n=21) *(50 – 85)*	68 ± 3 (n=20) *(20 – 90)*	66 ± 2 (n=47) *(20 – 90)*
Gravidity	2.9 ± 0.6 (n=21) *(1 – 10)*	2.1 ± 0.2 (n=22) *(1 – 5)*	2.2 ± 0.5 (n=6) *(1 – 4)*	2.4 ± 0.2 (n=21) *(1 – 4)*	3.0 ± 0.4 (n=20) *(1 – 8)*	2.6 ± 0.2 (n=47) *(1 – 8)*
Parity	1.3 ± 0.3 (n=21) *(0 – 6)*	0.6 ± 0.2 (n=22) *(0 – 3)*	0.7 ± 0.5 (n=6) *(0 – 3)*	0.9 ± 0.2 (n=21) *(0 – 3)*	1.2 ± 0.3 (n=20) *(0 – 5)*	1.0 ± 0.2 (n=47) *(0 – 5)*
	Values given are Mean ± Standard Error of the Mean *(Range)*					

* Compared to moderate and high (ANOVA, $P<0.05$)

Table 4.4 Clinical characteristics of women pregnant with a male fetus and classified by glucocorticoid intake

Male Fetus	Classification of Glucocorticoid Intake During Pregnancy					
	Control	No Glucocorticoid	Low	Moderate	High	Glucocorticoid
Total Number of Subjects	23	24	16	22	7	45
Asthma Severity		16 Mild	7 Mild	7 Mild	0 Mild	14 Mild
		5 Moderate	3 Moderate	9 Moderate	1 Moderate	13 Moderate
		3 Severe	6 Severe	6 Severe	6 Severe	18 Severe
Maternal Age (years)	30 ± 1 (n=23) *(19 – 42)*	25 ± 1 (n=24) *(18 – 33)*	27 ± 2 (n=16) *(18 – 39)*	28 ± 1 (n=22) *(19 – 40)*	26 ± 2 (n=7) *(19 – 32)*	28 ± 1 (n=45) *(18 – 40)*
Maternal Height (cm)	164.9 ± 2.3 (n=17) *(131 – 174)*	163.9 ± 1.3 (n=24) *(154 – 180)*	164.4 ± 1.6 (n=15) *(156.5 – 176)*	161.2 ± 1.7 (n=19) *(146 – 173)*	163.4 ± 1.7 (n=7) *(157 – 168)*	162.8 ± 1.0 (n=41) *(146 – 176)*
Maternal Early Pregnancy Weight (kg)	76.2 ± 4.6 (n=17) *(55.8 – 117.5)*	77.1 ± 5.6 (n=22) *(46.8 – 134)*	70.7 ± 4.6 (n=16) *(52 – 120.5)*	75.4 ± 4.9 (n=21) *(52.1 – 130)*	83.5 ± 6.6 (n=7) *(66.1 – 112.5)*	75.0 ± 3.2 (n=44) *(52 – 130)*
Maternal Pregnancy Weight Gain (kg)	10.0 ± 1.5 (n=12) *(1.6 – 17.2)*	13.3 ± 1.1 (n=17) *(5.5 – 21.8)*	12.7 ± 2.0 (n=12) *(4.6 – 29.7)*	10.1 ± 1.2 (n=18) *(1.1 – 17.6)*	6.1 ± 2.9 (n=7) *(-3.2 – 21.5)*	10.2 ± 1.1 (n=37) *(-3.2 – 29.7)*
Maternal Early Pregnancy BMI	28.1 ± 1.7 (n=17) *(19.5 – 42.8)*	28.5 ± 2.0 (n=22) *(17.3 – 51.4)*	26.1 ± 1.8 (n=15) *(16.9 – 43.7)*	28.6 ± 1.7 (n=18) *(18.7 – 43.7)*	31.1 ± 2.2 (n=7) *(23.4 – 40.3)*	28.1 ± 1.1 (n=40) *(16.9 – 43.7)*
Maternal Early Pregnancy Systolic Blood Pressure (mm Hg)	119 ± 3 (n=17) *(91 – 150)*	110 ± 3 (n=22) *(90 – 150)*	115 ± 3 (n=15) *(100 – 150)*	111 ± 4 (n=19) *(90 – 160)*	120 ± 4 (n=7) *(110 – 130)*	114 ± 2 (n=41) *(90 – 160)*
Maternal Early Pregnancy Diastolic Blood Pressure (mm Hg)	69 ± 2 (n=17) *(60 – 85)*	64 ± 2 (n=22) *(50 – 80)*	65 ± 2 (n=15) *(50 – 80)*	68 ± 3 (n=19) *(50 – 110)*	73 ± 3 (n=7) *(60 – 85)*	68 ± 2 (n=41) *(50 – 110)*
Gravidity	2.9 ± 0.4 (n=23) *(1 – 8)*	2.8 ± 0.4 (n=24) *(1 – 9)*	2.3 ± 0.5 (n=16) *(1 – 9)*	1.9 ± 0.2 (n=22) *(1 – 5)*	2.7 ± 0.4 (n=7) *(1 – 4)*	2.1 ± 0.2 (n=45) *(1 – 9)*
Parity	1.5 ± 0.3 (n=23) *(0 – 6)*	1.0 ± 0.2 (n=24) *(0 – 4)*	0.7 ± 0.3 (n=16) *(0 – 3)*	0.6 ± 0.2 (n=22) *(0 – 2)*	1.6 ± 0.3 (n=7) *(0 – 2)*	0.8 ± 0.1 (n=45) *(0 – 3)*
	Values given are Mean ± Standard Error of the Mean *(Range)*					

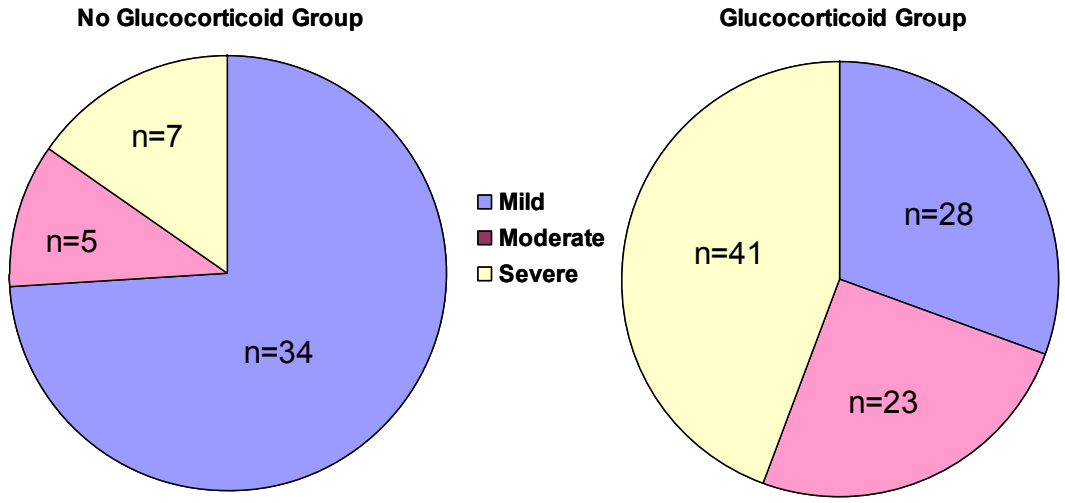

Figure 4.1 Distribution of mild, moderate and severe asthmatics within the no glucocorticoid and glucocorticoid groups

These pie charts show the proportion of women with mild, moderate and severe asthma in the no glucocorticoid and glucocorticoid groups (women pregnant with male and female fetuses combined). Most women in the combined no glucocorticoid group had mild asthma, while most women in the combined glucocorticoid group had moderate or severe asthma.

Table 4.5 Maternal asthma characteristics for women classified by asthma severity

	Classification of Asthma Severity During Pregnancy			
	Control	**Mild**	**Moderate**	**Severe**
Maternal FEV_1 (l)	**3.25 ± 0.10 (n=24)**	**3.19 ± 0.07 (n=55)**	**3.01 ± 0.07 (n=28)**	**2.98 ± 0.08 (n=47)**
Maternal FVC (l)	**3.82 ± 0.09 (n=24)**	**3.85 ± 0.07 (n=55)**	**3.82 ± 0.07 (n=28)**	**3.75 ± 0.08 (n=43)**
Maternal FEV_1:FVC Ratio	**0.85 ± 0.02 (n=24)**	**0.83 ± 0.01 (n=55)**	**0.79 ± 0.02 (n=28)***	**0.80 ± 0.01 (n=43)**
Periodic Oral Steroid Use (% of subjects)	**0%**	**0%**	**12%**	**29%**
Inhaled Glucocorticoid Drug Used				
Beclomethasone		**19.7%**	**32.1%**	**22.2%**
Budesonide		**13.1%**	**21.4%**	**26.7%**
Fluticasone		**11.5%**	**28.6%**	**35.6%**
	Values given are Mean ± Standard Error of the Mean			

*** Compared to control (ANOVA, *P*<0.05)**

Table 4.6 Maternal asthma characteristics for women classified by glucocorticoid intake

	Classification of Glucocorticoid Intake During Pregnancy					
	Control	**No Glucocorticoid**	**Low**	**Moderate**	**High**	**Glucocorticoid**
Maternal FEV_1 (l)	**3.25 ± 0.10 (n=24)**	**3.18 ± 0.07 (n=42)**	**3.06 ± 0.10 (n=21)**	**3.05 ± 0.08 (n=41)**	**2.94 ± 0.11 (n=26)**	**3.02 ± 0.05 (n=88)**
Maternal FVC (l)	**3.82 ± 0.09 (n=24)**	**3.81 ± 0.07 (n=41)**	**3.80 ± 0.11 (n=20)**	**3.81 ± 0.09 (n=38)**	**3.80 ± 0.14 (n=22)**	**3.81 ± 0.06 (n=80)**
Maternal FEV_1:FVC Ratio	**0.85 ± 0.02 (n=24)**	**0.84 ± 0.01 (n=41)**	**0.81 ± 0.02 (n=20)**	**0.79 ± 0.10 (n=38) ***	**0.80 ± 0.02 (n=22)**	**0.80 ± 0.01 (n=80)**
Periodic Oral Steroid Use (% of subjects)	**0%**	**0%**	**5%**	**9%**	**38%**	**16%**
Inhaled Glucocorticoid Drug Used						
Beclomethasone			**80.0%**	**33.3%**	**3.9%**	**35.2%**
Budesonide			**20.0%**	**26.2%**	**42.3%**	**29.6%**
Fluticasone			**0.0%**	**40.5%**	**53.8%**	**35.2%**
	Values given are Mean ± Standard Error of the Mean					

*** Compared to control (ANOVA, *P*<0.05)**

Maternal FEV_1 was not significantly different between the control, mild, moderate or severe asthma groups (Table 4.5, Kruskal-Wallis ANOVA, P=0.254) or the control, no glucocorticoid and glucocorticoid groups (Table 4.6, ANOVA, P=0.070). There were no significant differences in FVC between any groups (ANOVA, P>0.05).

The maternal FEV_1:FVC ratio was also used as an assessment of lung function. Maternal FEV_1:FVC was significantly different between the control group and the groups classified according to asthma severity (ANOVA, P=0.021, Tukey-Kramer multiple comparisons post-test P<0.05, control vs moderate asthma). The FEV_1:FVC ratio was significantly different between the control group and the groups classified according to inhaled glucocorticoid intake (ANOVA, P=0.018, Tukey-Kramer multiple comparisons post-test, P<0.05 control vs moderate dose glucocorticoid).

Of the women with mild asthma, 56% did not use any inhaled glucocorticoids for treatment, while 18% of women with moderate asthma did not use glucocorticoids (Table 4.5). 16% of women with severe asthma did not use inhaled glucocorticoids for treatment, contrary to medical advice (Sr Philippa Talbot, Research Nurse, Mothers and Babies Research Centre, Newcastle, personal communication).

Of the asthmatic women who used a low dose of inhaled glucocorticoid during pregnancy, 80% used beclomethasone dipropionate for treatment and 5% were prescribed periodic oral steroids at some time during pregnancy (Table 4.6). No women on a low dose of inhaled glucocorticoids used fluticasone propionate. Of the asthmatic women who used a moderate dose of inhaled glucocorticoids, 9% required oral steroids at some time during their pregnancy, while 38% of asthmatic women who used a high dose of inhaled glucocorticoids during pregnancy required oral steroids during pregnancy. Less than 4% of these women used beclomethasone dipropionate, while 54% used fluticasone propionate. Overall, 16% of asthmatic women in the glucocorticoid group required oral steroids during pregnancy and there was approximately an even distribution of women using each of the three inhaled glucocorticoid medications (Table 4.6).

4.2 Fetal growth during gestation

Fetal growth was examined by ultrasound at approximately 18 and 30 weeks gestation by Prof Warwick Giles. Measurements were made of head circumference, biparietal dimeter, femur length and abdominal circumference. The head circumference to abdominal circumference ratio (HC:AC) was also calculated, as an increase in HC:AC may give an indication of asymmetric growth restriction (640, 706, 707). A summary of the results for groups classified according to asthma severity is given in Table 4.7 and Table 4.8 and for groups classified according to glucocorticoid intake in Table 4.9 and Table 4.10.

Fetal growth in the asthmatic groups (mild, moderate and severe) was compared to growth in the control group, with male and female fetal growth analysed separately. From 18 to 30 weeks, the mean HC:AC ratio decreased in all groups, as found previously (706). There were no significant differences in the HC:AC ratio between the asthma groups and the control group (Kruskal-Wallis ANOVA, $P>0.05$) or in any other growth parameters at 18 or 30 weeks (ANOVA or Kruskal-Wallis ANOVA, $P>0.05$).

When data was analysed based upon inhaled glucocorticoid intake, no significant differences were found in any fetal growth parameters measured at 18 and 30 weeks gestation (ANOVA or Kruskal-Wallis ANOVA, $P>0.05$). There were no significant differences in the HC:AC ratio between any groups at 18 weeks (Kruskal-Wallis ANOVA, $P>0.05$). However, at 30 weeks, there was a significant reduction in HC:AC in the glucocorticoid female group compared to the control female group (Kruskal-Wallis ANOVA, $P=0.039$, Dunn's multiple comparisons test, $P<0.05$).

Table 4.7 Fetal growth measurements at 18 and 30 weeks gestation for women pregnant with a female fetus and classified by asthma severity

Female Fetus	Classification of Asthma Severity During Pregnancy			
	Control	Mild	Moderate	Severe
Gestational Age at 18 Week Ultrasound (weeks)	18.7 ± 0.3 (n=16)	18.9 ± 0.2 (n=25)	18.6 ± 0.5 (n=10)	18.9 ± 0.4 (n=17)
18 Week Biparietal Diameter (mm)	43.6 ± 1.2 (n=13)	43.9 ± 0.8 (n=23)	43.8 ± 1.3 (n=10)	45.6 ± 1.5 (n=16)
18 Week Femur Length (mm)	28.8 ± 1.0 (n=13)	29.4 ± 0.8 (n=23)	29.9 ± 1.2 (n=10)	30.4 ± 1.3 (n=16)
18 Week Head Circumference (mm)	155.9 ± 4.3 (n=13)	158.7 ± 2.8 (n=23)	158.8 ± 5.1 (n=10)	162.8 ± 5.1 (n=16)
18 Week Abdominal Circumference (mm)	135.1 ± 3.9 (n=16)	140.2 ± 3.0 (n=25)	136.5 ± 5.7 (n=10)	141.5 ± 4.6 (n=17)
18 Week HC:AC Ratio	1.16 ± 0.02 (n=13)	1.14 ± 0.01 (n=23)	1.17 ± 0.01 (n=10)	1.15 ± 0.01 (n=16)
Gestational Age at 30 Week Ultrasound (weeks)	29.9 ± 0.3 (n=14)	30.0 ± 0.3 (n=24)	30.4 ± 0.5 (n=10)	30.1 ± 0.2 (n=21)
30 Week Biparietal Diameter (mm)	78.4 ± 0.7 (n=12)	77.6 ± 0.9 (n=21)	79.4 ± 0.9 (n=10)	76.6 ± 0.8 (n=17)
30 Week Femur Length (mm)	58.9 ± 0.6 (n=12)	58.0 ± 0.9 (n=21)	58.5 ± 1.1 (n=10)	57.2 ± 0.6 (n=17)
30 Week Head Circumference (mm)	279.5 ± 3.8 (n=12)	278.9 ± 3.0 (n=21)	278.4 ± 3.6 (n=10)	272.9 ± 2.5 (n=17)
30 Week Abdominal Circumference (mm)	253.6 ± 4.6 (n=14)	262.6 ± 3.7 (n=24)	259.9 ± 4.3 (n=10)	260.0 ± 3.6 (n=21)
30 Week HC:AC Ratio	1.09 ± 0.01 (n=12)	1.07 ± 0.01 (n=21)	1.07 ± 0.01 (n=10)	1.06 ± 0.01 (n=17)
	Values given are Mean ± Standard Error of the Mean			

Table 4.8 Fetal growth measurements at 18 and 30 weeks gestation for women pregnant with a male fetus and classified by asthma severity

Male Fetus	Classification of Asthma Severity During Pregnancy			
	Control	Mild	Moderate	Severe
Gestational Age at 18 Week Ultrasound (weeks)	18.9 ± 0.3 (n=15)	19.1 ± 0.3 (n=19)	18.4 ± 0.3 (n=14)	19.4 ± 0.3 (n=14)
18 Week Biparietal Diameter (mm)	44.2 ± 0.9 (n=12)	46.0 ± 1.0 (n=18)	43.4 ± 1.2 (n=13)	46.4 ± 1.1 (n=14)
18 Week Femur Length (mm)	29.3 ± 0.8 (n=12)	30.7 ± 1.0 (n=18)	28.5 ± 0.8 (n=13)	31.2 ± 1.0 (n=14)
18 Week Head Circumference (mm)	154.8 ± 2.2 (n=12)	162.3 ± 3.4 (n=18)	161.5 ± 4.9 (n=13)	166.9 ± 4.2 (n=14)
18 Week Abdominal Circumference (mm)	140.7 ± 4.1 (n=15)	143.6 ± 3.7 (n=19)	129.7 ± 3.8 (n=14)*	148.8 ± 3.5 (n=14)
18 Week HC:AC Ratio	1.13 ± 0.01 (n=12)	1.14 ± 0.01 (n=18)	1.25 ± 0.05 (n=13)**	1.12 ± 0.01 (n=14)
Gestational Age at 30 Week Ultrasound (weeks)	30.0 ± 0.3 (n=13)	30.4 ± 0.2 (n=21)	30.4 ± 0.2 (n=16)	30.1 ± 0.3 (n=19)
30 Week Biparietal Diameter (mm)	79.2 ± 0.8 (n=12)	78.7 ± 0.8 (n=18)	81.2 ± 1.1 (n=13)	77.6 ± 1.2 (n=15)
30 Week Femur Length (mm)	60.1 ± 1.9 (n=12)	58.1 ± 0.7 (n=18)	57.2 ± 2.4 (n=13)	56.2 ± 1.0 (n=15)
30 Week Head Circumference (mm)	280.8 ± 2.3 (n=12)	281.8 ± 3.1 (n=18)	289.3 ± 3.4 (n=13)	273.5 ± 3.2 (n=15)
30 Week Abdominal Circumference (mm)	261.7 ± 2.8 (n=13)	260.6 ± 3.5 (n=21)	267.4 ± 4.1 (n=16)	266.2 ± 5.2 (n=19)
30 Week HC:AC Ratio	1.07 ± 0.01 (n=12)	1.08 ± 0.01 (n=18)	1.08 ± 0.01 (n=13)	1.05 ± 0.02 (n=15)
	Values given are Mean ± Standard Error of the Mean			
* Compared to severe (ANOVA, $P<0.05$), ** Compared to severe (Kruskal-Wallis ANOVA, $P<0.05$)				

Table 4.9 Fetal growth measurements at 18 and 30 weeks gestation for women pregnant with a female fetus and classified by glucocorticoid intake

Female Fetus	Classification of Glucocorticoid Intake During Pregnancy					
	Control	No Glucocorticoid	Low	Moderate	High	Glucocorticoid
Gestational Age at 18 Week Ultrasound (weeks)	18.7 ± 0.3 (n=16)	18.7 ± 0.3 (n=20)	18.8 ± 0.5 (n=4)	19.0 ± 0.4 (n=14)	19.0 ± 0.4 (n=14)	19.0 ± 0.2 (n=32)
18 Week Biparietal Diameter (mm)	43.6 ± 1.2 (n=13)	43.6 ± 1.1 (n=18)	42.3 ± 1.9 (n=4)	45.3 ± 1.2 (n=14)	45.4 ± 1.4 (n=13)	44.9 ± 0.8 (n=31)
18 Week Femur Length (mm)	28.8 ± 1.0 (n=13)	28.9 ± 1.1 (n=18)	28.0 ± 0.9 (n=4)	30.6 ± 1.0 (n=14)	30.7 ± 1.2 (n=13)	30.3 ± 0.7 (n=31)
18 Week Head Circumference (mm)	155.9 ± 4.3 (n=13)	156.2 ± 3.7 (n=18)	160.8 ± 7.2 (n=4)	163.6 ± 4.9 (n=14)	161.4 ± 4.5 (n=13)	162.3 ± 3.0 (n=31)
18 Week Abdominal Circumference (mm)	135.1 ± 3.9 (n=16)	138.2 ± 3.8 (n=20)	133.8 ± 7.9 (n=4)	144.0 ± 5.0 (n=14)	140.1 ± 3.9 (n=14)	141.0 ± 2.9 (n=32)
18 Week HC:AC Ratio	1.16 ± 0.02 (n=13)	1.14 ± 0.01 (n=18)	1.20 ± 0.02 (n=4)	1.14 ± 0.01 (n=14)	1.16 ± 0.01 (n=13)	1.15 ± 0.01 (n=31)
Gestational Age at 30 Week Ultrasound (weeks)	29.9 ± 0.3 (n=14)	29.9 ± 0.3 (n=17)	31.0 ± 1.0 (n=4)	30.3 ± 0.2 (n=16)	30.1 ± 0.3 (n=18)	30.2 ± 0.2 (n=38)
30 Week Biparietal Diameter (mm)	78.4 ± 0.7 (n=12)	76.8 ± 1.1 (n=17)	80.5 ± 2.8 (n=4)	78.2 ± 0.6 (n=13)	77.2 ± 0.9 (n=14)	78.0 ± 0.6 (n=31)
30 Week Femur Length (mm)	58.9 ± 0.6 (n=12)	57.9 ± 0.9 (n=17)	59.8 ± 1.6 (n=4)	58.5 ± 0.9 (n=13)	56.5 ± 0.9 (n=14)	57.8 ± 0.6 (n=31)
30 Week Head Circumference (mm)	279.5 ± 3.8 (n=12)	274.6 ± 3.3 (n=17)	287.5 ± 6.7 (n=4)	277.4 ± 2.9 (n=13)	275.4 ± 3.0 (n=14)	277.8 ± 2.1 (n=31)
30 Week Abdominal Circumference (mm)	253.6 ± 4.6 (n=14)	256.9 ± 3.6 (n=17)	262.8 ± 6.9 (n=4)	265.6 ± 4.0 (n=16)	260.7 ± 4.5 (n=18)	263.0 ± 2.8 (n=38)
30 Week HC:AC Ratio	1.09 ± 0.01 (n=12)	1.07 ± 0.01 (n=17)	1.10 ± 0.03 (n=4)	1.06 ± 0.03 (n=13)	1.06 ± 0.01 (n=14)	1.06 ± 0.01 (n=31)*
	Values given are Mean ± Standard Error of the Mean					

*Compared to control (Kruskal-Wallis ANOVA, $P<0.05$)

Table 4.10 Fetal growth measurements at 18 and 30 weeks gestation for women pregnant with a male fetus and classified by glucocorticoid intake

Male Fetus	Classification of Glucocorticoid Intake During Pregnancy					
	Control	No Glucocorticoid	Low	Moderate	High	Glucocorticoid
Gestational Age at 18 Week Ultrasound (weeks)	18.9 ± 0.3 (n=15)	18.9 ± 0.3 (n=16)	18.9 ± 0.3 (n=12)	18.9 ± 0.3 (n=15)	19.3 ± 0.6 (n=4)	18.9 ± 0.2 (n=31)
18 Week Biparietal Diameter (mm)	44.2 ± 0.9 (n=12)	44.7 ± 1.3 (n=15)	46.4 ± 1.1 (n=12)	45.0 ± 1.2 (n=14)	46.0 ± 1.7 (n=4)	45.7 ± 0.7 (n=30)
18 Week Femur Length (mm)	29.3 ± 0.8 (n=12)	30.4 ± 1.1 (n=15)	30.8 ± 1.2 (n=12)	29.4 ± 0.9 (n=14)	30.8 ± 2.1 (n=4)	30.1 ± 0.7 (n=30)
18 Week Head Circumference (mm)	154.8 ± 2.2 (n=12)	166.1 ± 5.2 (n=15)	163.2 ± 3.6 (n=12)	161.0 ± 3.8 (n=14)	163.5 ± 6.4 (n=4)	162.2 ± 2.4 (n=30)
18 Week Abdominal Circumference (mm)	140.7 ± 4.1 (n=15)	141.6 ± 4.5 (n=16)	141.5 ± 4.5 (n=12)	138.5 ± 4.4 (n=15)	146.3 ± 5.3 (n=4)	140.7 ± 2.8 (n=31)
18 Week HC:AC Ratio	1.13 ± 0.01 (n=12)	1.20 ± 0.04 (n=15)	1.16 ± 0.04 (n=12)	1.15 ± 0.02 (n=14)	1.12 ± 0.01 (n=4)	1.15 ± 0.01 (n=30)
Gestational Age at 30 Week Ultrasound (weeks)	30.0 ± 0.3 (n=13)	30.2 ± 0.2 (n=18)	30.9 ± 0.3 (n=15)	30.2 ± 0.2 (n=18)	29.8 ± 0.5 (n=5)	30.4 ± 0.2 (n=38)
30 Week Biparietal Diameter (mm)	79.2 ± 0.8 (n=12)	77.8 ± 1.1 (n=15)	79.8 ± 1.1 (n=13)	79.3 ± 1.1 (n=14)	80.3 ± 2.1 (n=4)	79.6 ± 0.7 (n=31)
30 Week Femur Length (mm)	60.1 ± 1.9 (n=12)	55.2 ± 2.0 (n=15)	59.3 ± 0.9 (n=13)	57.5 ± 0.9 (n=14)	57.0 ± 1.5 (n=4)	58.2 ± 0.6 (n=31)
30 Week Head Circumference (mm)	280.8 ± 2.3 (n=12)	279.6 ± 3.8 (n=15)	281.9 ± 2.6 (n=13)	282.5 ± 4.7 (n=14)	280.8 ± 6.2 (n=4)	282.0 ± 2.4 (n=31)
30 Week Abdominal Circumference (mm)	261.7 ± 2.8 (n=13)	261.7 ± 3.9 (n=18)	268.4 ± 5.1 (n=15)	261.2 ± 4.5 (n=18)	274.2 ± 7.7 (n=5)	265.8 ± 3.1 (n=38)
30 Week HC:AC Ratio	1.07 ± 0.01 (n=12)	1.08 ± 0.01 (n=15)	1.05 ± 0.02 (n=13)	1.09 ± 0.02 (n=14)	1.06 ± 0.01 (n=4)	1.07 ± 0.01 (n=31)
	Values given are Mean ± Standard Error of the Mean					

4.3 Neonatal size at birth

At birth, data on head circumference, length and weight were collected. Four subjects were excluded from this analysis. One asthmatic woman (severe asthma, moderate glucocorticoid use) was pregnant with twins and data on fetal and neonatal growth was omitted. One woman in the control group was induced due to a fetal abnormality at 20 weeks, one asthmatic woman (moderate asthma, moderate glucocorticoid use) had a still-birth at 40 weeks and one asthmatic woman (severe asthma, low glucocorticoid use) had a still-birth at 35 weeks. These pregnancies that resulted in a fetal death were not included in the analysis of neonatal size at birth.

A summary of neonatal growth parameters and centiles is given in Table 4.11 and Table 4.12 for groups classified according to asthma severity and in Table 4.13 and Table 4.14 for groups classified according to glucocorticoid intake.

There were no significant differences in gestational age at birth between any groups (Kruskal-Wallis ANOVA, $P>0.05$). However, in order to correct for this potential confounder, centiles for birth weight, head circumference and length were determined based on gestational age at delivery using the John Hunter Hospital intrauterine growth charts (Appendix 7). Overall, there were six preterm deliveries (<37 completed weeks gestation) among the 181 singleton pregnancies. Preterm neonates included one male neonate from the control group (33 weeks), one female neonate from the mild asthma group (28 weeks), one female neonate from the moderate asthma group (32 weeks) and three male neonates from the severe asthma group (delivered at 33, 34 and 36 weeks). Of these three male neonates, one asthmatic mother did not used inhaled glucocorticoids for treatment (36 week delivery), while one used a moderate dose of inhaled glucocorticoids (33 week delivery) and one used a high dose of inhaled glucocorticoids (34 week delivery). Post-term deliveries were defined as those at 41.5 weeks gestation or greater (708). More male neonates were born post-term (six in total) than female neonates (two in total). The sample numbers were not sufficient for statistical analysis of differences in preterm or post-term deliveries between asthmatic and non-asthmatic pregnancies.

There were no significant differences in weight, length, head circumference or ponderal index at birth between male or female fetuses from the control group and any of the

groups classified by asthma severity (ANOVA or Kruskal-Wallis ANOVA, $P>0.05$). There were no significant differences in weight, length, head circumference or ponderal index at birth between male or female fetuses from the control group and any of the groups classified by asthma severity (ANOVA or Kruskal-Wallis ANOVA, $P>0.05$). However, when groups were classified based upon glucocorticoid intake, significant differences in neonatal size were found. Statistical differences between the control, no glucocorticoid and glucocorticoid groups are discussed.

The birth weight of female neonates in the no glucocorticoid group was significantly reduced compared to females in the control group (Table 4.13 and Figure 4.2, ANOVA, $P=0.019$, Tukey-Kramer multiple comparisons test, $P<0.05$, control vs no glucocorticoid), while the birth weight of female neonates of the glucocorticoid group was similar to that of control female neonates (Figure 4.2). No differences in birth weight of male neonates was found between the groups (Table 4.14, ANOVA, $P>0.05$). The birth weight centile (BWC) of female neonates was significantly reduced in the no glucocorticoid group compared to females in the control group (ANOVA, $P=0.022$, Tukey-Kramer multiple comparisons test, $P<0.05$, control vs no glucocorticoid). Within the no glucocorticoid group there was a high proportion of small for gestational age (<10th centile) female neonates (18.2%) compared to the control group (0%) and the glucocorticoid group (10.6%, Fisher's exact test, $P>0.05$).

Most women in the no glucocorticoid group who had a female fetus were mild asthmatics (82%). Mild asthmatics who did not use inhaled glucocorticoids had significantly smaller female neonates compared to mild asthmatics who did use inhaled glucocorticoids and control subjects, while mild asthmatics who did use inhaled glucocorticoids had similar sized female neonates to the control group (Figure 4.3, ANOVA, $P=0.004$, Tukey Kramer multiple comparisons test, control vs mild no glucocorticoid $P<0.01$, control vs mild glucocorticoid $P>0.05$, mild no glucocorticoid vs mild glucocorticoid $P<0.05$).

Table 4.11 Neonatal growth parameters for women pregnant with a female fetus and classified by asthma severity

Female Fetus	Classification of Asthma Severity During Pregnancy			
	Control	**Mild**	**Moderate**	**Severe**
Gestational Age at Birth (weeks)	**39.9 ± 0.2 (n=20)** *(38 – 41)*	**38.8 ± 0.4 (n=32)** *(28 – 41)*	**38.9 ± 0.9 (n=10)** *(28 – 38.4)*	**39.6 ± 0.2 (n=27)** *(38 – 41.7)*
Birth Weight (g)	**3600.3 ± 101.2 (n=20)** *(2890 – 4700)*	**3244.7 ± 96.3 (n=32)** *(1040 – 4300)*	**3121.0 ± 269.5 (n=10)** *(1900 – 4780)*	**3395.9 ± 112.7 (n=27)** *(2170 – 5160)*
Birth Weight Centile	**57 ± 6 (n=20)** *(10 – 99)*	**45 ± 4 (n=32)** *(4 – 99)*	**39 ± 11 (n=10)** *(3 – 99)*	**46 ± 5 (n=27)** *(3 – 99)*
Birth Length (cm)	**51.9 ± 0.4 (n=18)** *(49 – 55.5)*	**50.3 ± 0.4 (n=31)** *(47 – 55)*	**50.6 ± 1.5 (n=10)** *(42 – 59)*	**50.8 ± 0.5 (n=24)** *(47 – 56)*
Length Centile	**78 ± 3 (n=18)** *(55 – 99)*	**61 ± 5 (n=31)** *(10 – 99)*	**61 ± 12 (n=10)** *(1 – 99)*	**66 ± 5 (n=24)** *(30 – 99)*
Head Circumference at Birth (cm)	**34.6 ± 0.3 (n=20)** *(33 – 37)*	**34.1 ± 0.3 (n=31)** *(31 – 37)*	**33.4 ± 0.5 (n=10)** *(31 – 37)*	**34.4 ± 0.3 (n=26)** *(32 – 37)*
Head Circumference Centile	**46 ± 6 (n=20)** *(10 – 98)*	**41 ± 5 (n=31)** *(1 – 97)*	**35 ± 10 (n=10)** *(3 – 90)*	**44 ± 5 (n=26)** *(3 – 92)*
Ponderal Index	**2.56 ± 0.06 (n=18)** *(2.06 – 3.06)*	**2.62 ± 0.05 (n=31)** *(2.03 – 3.24)*	**2.60 ± 0.13 (n=10)** *(2.30 – 3.32)*	**2.59 ± 0.06 (n=24)** *(1.90 – 3.08)*
Ponderal Index <2.2 (No. of subjects)	**2**	**2**	**0**	**3**
Delivery <37 weeks (No. of subjects)	**0**	**1**	**1**	**0**
Delivery ≥ 41.5 weeks (No. of subjects)	**0**	**0**	**1**	**1**
	Values given are Mean ± Standard Error of the Mean ***(Range)***			

Table 4.12 Neonatal growth parameters for women pregnant with a male fetus and classified by asthma severity

Male Fetus	Classification of Asthma Severity During Pregnancy			
	Control	Mild	Moderate	Severe
Gestational Age at Birth (weeks)	39.6 ± 0.4 (n=22) *(33 – 42)*	39.9 ± 0.2 (n=30) *(37 – 42.3)*	39.5 ± 0.3 (n=17) *(28 – 39)*	38.8 ± 0.5 (n=19) *(33 – 42.4)*
Birth Weight (g)	3643.6 ± 136.0 (n=22) *(2000 – 4900)*	3707.5 ± 87.5 (n=30) *(2840 – 4980)*	3406.5 ± 122.4 (n=17) *(2700 – 4500)*	3473.3 ± 125.6 (n=18) *(2160 – 4120)*
Birth Weight Centile	62 ± 6 (n=22) *(15 – 99)*	66 ± 4 (n=30) *(15 – 99)*	50 ± 7 (n=17) *(5 – 97)*	59 ± 6 (n=18) *(2 – 92)*
Birth Length (cm)	52.3 ± 0.6 (n=21) *(48 – 58)*	52.5 ± 0.4 (n=29) *(47 – 56)*	51.4 ± 0.8 (n=15) *(46 – 56)*	51.6 ± 0.6 (n=17) *(48 – 55)*
Length Centile	79 ± 4 (n=21) *(45 – 99)*	82 ± 4 (n=29) *(40 – 99)*	72 ± 7 (n=15) *(15 – 99)*	79 ± 6 (n=17) *(25 – 99)*
Head Circumference at Birth (cm)	34.8 ± 0.3 (n=22) *(32 – 37)*	35.0 ± 0.3 (n=29) *(32 – 38)*	34.7 ± 0.3 (n=17) *(33 – 37)*	34.8 ± 0.4 (n=18) *(31 – 37)*
Head Circumference Centile	55 ± 6 (n=22) *(10 – 90)*	57 ± 5 (n=29) *(2 – 97)*	53 ± 6 (n=17) *(30 – 90)*	59 ± 7 (n=18) *(2 – 97)*
Ponderal Index	2.60 ± 0.05 (n=21) *(2.33 – 2.97)*	2.53 ± 0.04 (n=29) *(2.09 – 2.94)*	2.47 ± 0.06 (n=15) *(2.09 – 2.96)*	2.58 ± 0.07 (n=17) *(2.12 – 3.21)*
Ponderal Index <2.2 (No. of subjects)	0	3	3	2
Delivery <37 weeks (No. of subjects)	1	0	0	3
Delivery ≥ 41.5 weeks (No. of subjects)	2	3	0	1
	Values given are Mean ± Standard Error of the Mean *(Range)*			

Table 4.13 Neonatal growth parameters for women pregnant with a female fetus and classified by glucocorticoid intake

Female Fetus	Classification of Glucocorticoid Intake During Pregnancy					
	Control	No Glucocorticoid	Low	Moderate	High	Glucocorticoid
Gestational Age at Birth (weeks)	39.9 ± 0.2 (n=20) *(38 – 41)*	39.0 ± 0.6 (n=22) *(28 – 41.7)*	39.4 ± 0.4 (n=6) *(32 – 39)*	39.2 ± 0.3 (n=21) *(37 – 42)*	39.1 ± 0.5 (n=20) *(32 – 41.4)*	39.2 ± 0.2 (n=47) *(32 – 42)*
Birth Weight (g)	3600.3 ± 101.2 (n=20) *(2890 – 4700)*	3094.5 ± 120.0 (n=22)* *(1040 – 3750)*	3653.3 ± 339.2 (n=6) *(2360 – 5160)*	3384.3 ± 121.7 (n=21) *(2170 – 4380)*	3283.0 ± 137.1 (n=20) *(1900 – 4780)*	3375.5 ± 89.9 (n=47) *(1900 – 5160)*
Birth Weight Centile	57 ± 6 (n=20) *(10 – 99)*	35 ± 5 (n=22)* *(4 – 80)*	55 ± 12 (n=6) *(9 – 99)*	51 ± 6 (n=21) *(3 – 99)*	45 ± 6 (n=20) *(3 – 99)*	49 ± 4 (n=47) *(3 – 99)*
Birth Length (cm)	51.9 ± 0.4 (n=18) *(49 – 55.5)*	50.4 ± 0.6 (n=21) *(47 – 56)*	49.3 ± 0.5 (n=5) *(48 – 51)*	51.3 ± 0.5 (n=19) *(48 – 56)*	50.0 ± 0.8 (n=18) *(42 – 59)*	50.5 ± 0.4 (n=42) *(42 – 59)*
Length Centile	78 ± 3 (n=18) *(55 – 99)*	61 ± 6 (n=21) *(10 – 99)*	51 ± 7 (n=5) *(35 – 70)*	72 ± 6 (n=19) *(15 – 99)*	59 ± 6 (n=18) *(1 – 99)*	64 ± 4 (n=42) *(1 – 99)*
Head Circumference at Birth (cm)	34.6 ± 0.3 (n=20) *(33 – 37)*	33.9 ± 0.3 (n=21) *(31 – 36)*	35.0 ± 0.4 (n=5) *(34 – 36)*	34.3 ± 0.3 (n=21) *(31.5 – 37)*	34.1 ± 0.3 (n=20) *(31 – 37)*	34.3 ± 0.2 (n=46) *(31 – 37)*
Head Circumference Centile	46 ± 6 (n=20) *(10 – 98)*	34 ± 5 (n=21) *(1 – 85)*	59 ± 10 (n=5) *(35 – 90)*	43 ± 7 (n=21) *(3 – 97)*	43 ± 7 (n=20) *(3 – 92)*	45 ± 4 (n=46) *(3 – 97)*
Ponderal Index	2.56 ± 0.06 (n=18) *(2.06 – 3.06)*	2.51 ± 0.08 (n=21) *(1.90 – 3.16)*	2.79 ± 0.10 (n=5) *(2.31 – 3.08)*	2.59 ± 0.06 (n=19) *(2.22 – 3.24)*	2.68 ± 0.06 (n=18) *(2.30 – 3.32)*	2.65 ± 0.04 (n=42) *(2.22 – 3.32)*
Delivery <37 weeks (No. of subjects)	0	1	0	0	1	1
Delivery ≥ 41.5 weeks (No. of subjects)	0	1	0	1	0	1
	Values given are Mean ± Standard Error of the Mean *(Range)*					

* Compared to control (ANOVA, $P<0.05$)

Table 4.14 Neonatal growth parameters for women pregnant with a male fetus and classified by glucocorticoid intake

Male Fetus	Classification of Glucocorticoid Intake During Pregnancy					
	Control	No Glucocorticoid	Low	Moderate	High	Glucocorticoid
Gestational Age at Birth (weeks)	39.6 ± 0.4 (n=22) *(33 – 42)*	39.8 ± 0.3 (n=24) *(36 – 42.3)*	39.6 ± 0.3 (n=15) *(37 – 41.4)*	39.3 ± 0.5 (n=20) *(33 – 42.4)*	38.8 ± 0.9 (n=7) *(32 – 39)*	39.3 ± 0.3 (n=42) *(32 – 42.4)*
Birth Weight (g)	3643.6 ± 136.0 (n=22) *(2000 – 4900)*	3701.5 ± 88.9 (n=24) *(3140 – 4980)*	3544.0 ± 135.7 (n=15) *(2500 – 4500)*	3474.7 ± 123.4 (n=19) *(2700 – 4540)*	3377.1 ± 242.1 (n=7) *(2160 – 3960)*	3483.4 ± 84.4 (n=41) *(2160 – 4540)*
Birth Weight Centile	62 ± 6 (n=22) *(15 – 99)*	63 ± 5 (n=24) *(18 – 99)*	64 ± 7 (n=15) *(2 – 97)*	52 ± 7 (n=19) *(5 – 99)*	61 ± 8 (n=7) *(31 – 85)*	58 ± 4 (n=41) *(2 – 99)*
Birth Length (cm)	52.3 ± 0.6 (n=21) *(48 – 58)*	52.6 ± 0.5 (n=22) *(49 – 56)*	52.1 ± 0.6 (n=15) *(48.5 – 56)*	51.4 ± 0.7 (n=19) *(46 – 56)*	51.3 ± 0.9 (n=5) *(48 – 53)*	51.6 ± 0.4 (n=39) *(46 – 56)*
Length Centile	79 ± 4 (n=21) *(45 – 99)*	84 ± 4 (n=22) *(40 – 99)*	81 ± 5 (n=15) *(48 – 99)*	71 ± 7 (n=19) *(15 – 99)*	79 ± 9 (n=5) *(45 – 92)*	76 ± 4 (n=39) *(15 – 99)*
Head Circumference at Birth (cm)	34.8 ± 0.3 (n=22) *(32 – 37)*	34.9 ± 0.3 (n=23) *(32 – 37)*	34.7 ± 0.4 (n=15) *(31 – 37)*	34.9 ± 0.4 (n=19) *(33 – 38)*	35.1 ± 0.7 (n=7) *(32 – 37)*	34.9 ± 0.2 (n=41) *(31 – 38)*
Head Circumference Centile	55 ± 6 (n=22) *(10 – 90)*	57 ± 6 (n=23) *(2 – 94)*	54 ± 7 (n=15) *(2 – 90)*	54 ± 7 (n=19) *(10 – 97)*	66 ± 9 (n=7) *(30 – 97)*	56 ± 4 (n=41) *(2 – 97)*
Ponderal Index	2.60 ± 0.05 (n=21) *(2.33 – 2.97)*	2.49 ± 0.05 (n=22) *(2.12 – 2.86)*	2.50 ± 0.06 (n=15) *(2.12 – 2.81)*	2.57 ± 0.07 (n=19) *(2.09 – 2.50)*	2.67 ± 0.05 (n=5) *(2.53 – 2.82)*	2.55 ± 0.04 (n=39) *(2.09 – 2.82)*
Ponderal Index <2.2 (No. of subjects)	0	1	2	4	0	6
Delivery <37 weeks (No. of subjects)	1	1	0	1	1	2
Delivery ≥ 41.5 weeks (No. of subjects)	2	2	0	2	0	2
	Values given are Mean ± Standard Error of the Mean *(Range)*					

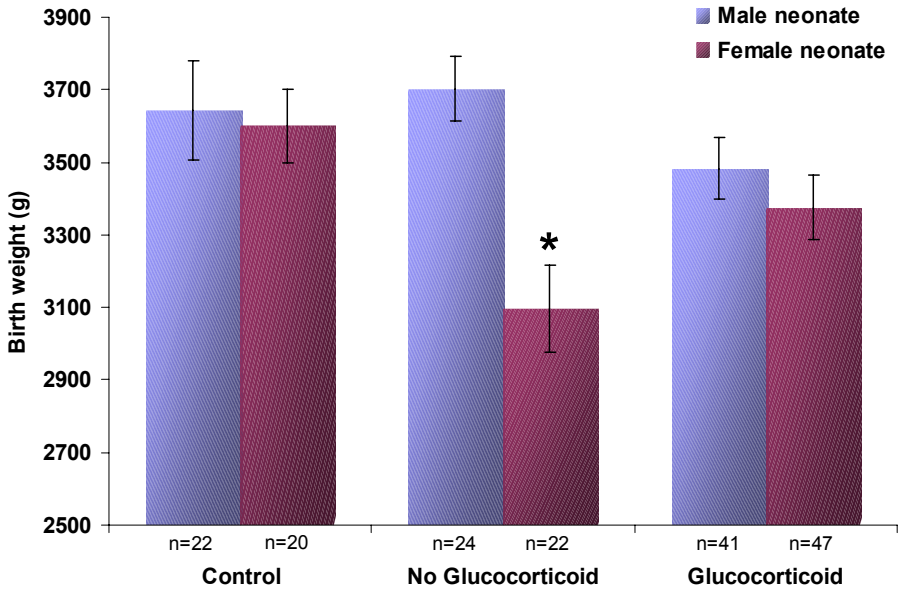

Figure 4.2 Birth weight of male and female neonates in asthmatic and non-asthmatic pregnancies

*The mean birth weight (g) ± SEM for male and female neonates is shown for women classified by inhaled glucocorticoid intake (control non-asthmatic, no glucocorticoid, glucocorticoid). * indicates P<0.02 (ANOVA, control female vs no glucocorticoid female).*

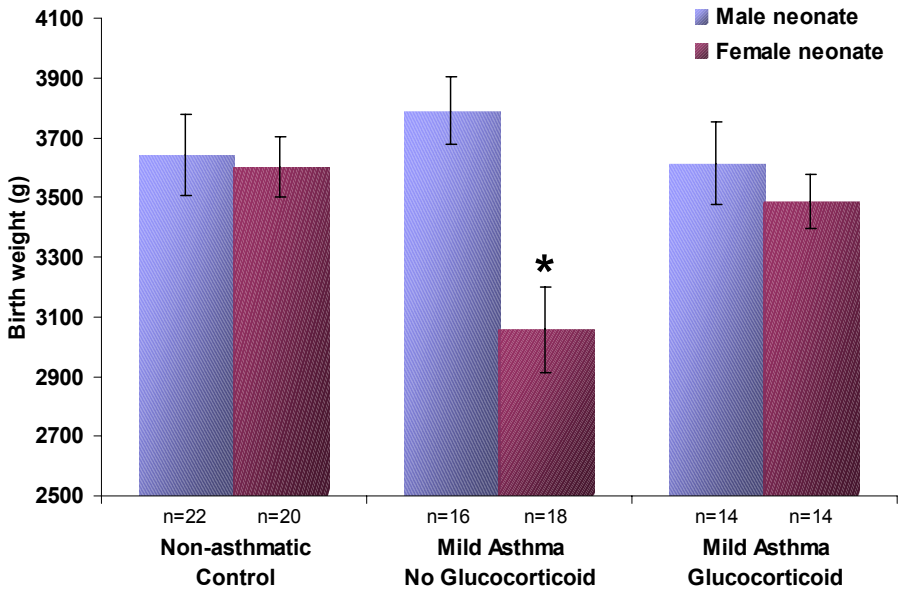

Figure 4.3 Birth weight of male and female neonates of women with mild asthma according to glucocorticoid use

*The mean birth weight (g) ± SEM for male and female neonates is shown for non-asthmatic women (control) and women with mild asthma who did not use inhaled glucocorticoids and women with mild asthma who did use inhaled glucocorticoids. * indicates P=0.004 (ANOVA, control female vs mild asthma no glucocorticoid female vs mild asthma glucocorticoid female).*

Female neonates in the no glucocorticoid group had a head circumference centile (33.9 ± 0.3, n=21), which was similar to their BWC (34.0 ± 5.4, n=21), but this was not significantly different from the head circumference centile of females in the control group or the glucocorticoid group (ANOVA, *P*=0.278).

Ponderal index gives an indication of appropriate growth, with a value <2.2 suggestive of IUGR (84), particularly asymmetric growth restriction (85). Ponderal index and length were not significantly different between groups (ANOVA, females: *P*=0.147 and males: *P*=0.132) and ponderal index was on average >2.2 in all groups. The proportion of subjects with a ponderal index <2.2 was not different between groups (Fisher's exact test, *P*>0.05). Length centiles tended to be higher than birth weight or head circumference centiles, possibly due to inaccuracy in measuring length in newborn infants. This may have altered the accuracy of the ponderal index, which was calculated from birth weight and length. Schatz *et al.* previously reported a significantly greater incidence of low ponderal index (<2.2) in asthmatic women whose FEV_1 was in the lowest quartile (83). I analysed my data in a similar manner and found that when divided into quartiles, those with lung function in the lowest quartile also had the lowest incidence of ponderal index <2.2 (6.5%) compared to those women with lung function in the second, third or fourth quartiles, where the incidence of ponderal index <2.2 was 12.5%, 14.8% and 11.1% respectively.

In summary, there was a 40% reduction in birth weight centile, a 27% reduction in head circumference centile and a 22% reduction in length centile in female neonates from the no glucocorticoid group compared to female neonates of the control non-asthmatic group.

A number of asthmatic women in the glucocorticoid group commenced therapy either in the second or third trimester of pregnancy, meaning that they used no glucocorticoids in the first trimester or in the first and second trimester. The effect of timing of commencement of inhaled glucocorticoid therapy on female birth weight was assessed. Asthmatic women who commenced treatment in the second trimester had a similar mean birth weight (3502.9 ± 277.4 g, n=7) to asthmatic women who used inhaled glucocorticoids throughout pregnancy (3380.6 ± 99.7 g, n=35, unpaired t-test, *P*=0.633). Although numbers were small and variation large, there was a trend for women who commenced glucocorticoid therapy in the third trimester to have smaller babies

(3162.0 ± 320.1 g, n=5) than women who used glucocorticoid therapy throughout pregnancy (3380.6 ± 99.7 g, n=35), which were of similar size to the no glucocorticoid group (3094.5 ± 120.0 g, n=22).

There was no effect of maternal asthma or its treatment on male fetal growth. Male birth weight, length, head circumference and ponderal index were not significantly different between the control group, the no glucocorticoid group or the glucocorticoid group (Figure 4.2, ANOVA, $P>0.05$). Male neonates from mild asthmatics were of similar size regardless of glucocorticoid use (ANOVA, $P=0.635$, Figure 4.3).

The inhaled steroid drug used (budesonide, beclomethasone or fluticasone) did not have any significant impact on birth weight or birth weight centile of male or female neonates in the glucocorticoid group with neonates of all glucocorticoid users being of similar size to the control group (Table 4.15, ANOVA, $P>0.05$). Similarly, the periodic use of oral steroids by asthmatic mothers in the glucocorticoid group did not affect male or female birth weight (Table 4.16, ANOVA, $P=0.639$) or BWC (ANOVA, $P=0.216$). There was no relationship between average glucocorticoid dose during pregnancy, or third trimester glucocorticoid dose and birth weight of female or male neonates from the glucocorticoid group (data not shown).

Some women in the study were smokers (approximately 28% of asthmatics), and smoking has been reported to contribute to low birth weight (300). However, there were no significant differences in birth weight between smokers and non-smokers within any groups. When examining female neonates of smoking mothers, those from the no glucocorticoid group had a birth weight of 3033.3 ± 118.6 g (n=6), while female neonates of smoking mothers from the glucocorticoid group had a birth weight of 3240.0 ± 184.8 g (n=14). This difference was not significant (Mann Whitney test, $P=0.444$). Female neonates from non-smoking mothers had a birth weight of 3117.5 ± 160.7 g (n=16) in the no glucocorticoid group and a birth weight of 3433.0 ± 101.5 g (n=33) in the glucocorticoid group, indicating that the absence of glucocorticoid use in asthmatics was associated with reduced female birth weight regardless of maternal smoking. Using multivariate analysis, there was a significant reduction in female fetal growth in women using no glucocorticoid ($P=0.037$) and mild asthmatics ($P=0.020$). There was no significant effect of smoking or asthma severity on birth weight and fetal sex still remained the determining factor.

Table 4.15 Neonatal birth weight according to inhaled glucocorticoid drug used by asthmatic mothers

Female Fetus	Inhaled Glucocorticoid Drug Used During Pregnancy		
	Budesonide	Beclomethasone	Fluticasone
Birth Weight (g)	3394.0 ± 147.3 (n=15)	3479.0 ± 227.5 (n=10)	3330.5 ± 150.7 (n=19)
Birth Weight Centile	52.7 ± 6.7 (n=15)	50.5 ± 9.1 (n=10)	46.6 ± 7.3 (n=19)
Male Fetus	**Budesonide**	**Beclomethasone**	**Fluticasone**
Birth Weight (g)	3333.3 ± 189.4 (n=9)	3531.5 ± 106.5 (n=20)	3495.5 ± 201.5 (n=11)
Birth Weight Centile	50.7 ± 9.9 (n=9)	57.2 ± 6.7 (n=20)	65.3 ± 6.4 (n=11)
	Values given are Mean ± Standard Error of the Mean		

Table 4.16 Neonatal birth weight according to the use of oral steroids by asthmatic mothers

Female Fetus	Oral Steroid Use During Pregnancy	
	No Oral Steroids	Periodic Oral Steroids
Birth Weight (g)	3343.6 ± 100.0 (n=39)	3536.3 ± 206.3 (n=8)
Birth Weight Centile	46.6 ± 4.4 (n=39)	60.8 ± 10.2 (n=8)
Male Fetus	**No Oral Steroids**	**Periodic Oral Steroids**
Birth Weight (g)	3501.6 ± 90.4 (n=37)	3420.0 ± 209.6 (n=5)
Birth Weight Centile	59.3 ± 4.4 (n=37)	55.4 ± 16.5 (n=5)
	Values given are Mean ± Standard Error of the Mean	

4.4 Fetal Growth - Discussion

In pregnant women with asthma there was reduced female fetal growth when no inhaled glucocorticoids were used for treatment. This occurred regardless of asthma severity or maternal smoking and was not due to differences in maternal height, BMI or gestational weight gain, nor due to a significantly shortened gestational length. However, there were no significant differences in growth of female fetuses of the no glucocorticoid group observed by ultrasound at 18 and 30 weeks, suggesting a late gestation alteration in fetal growth. In addition, there was no evidence of asymmetric growth restriction *in utero* as demonstrated by normal ultrasound HC:AC ratios in this group. Female birth weight and head circumference were both reduced to approximately the 35th percentile, and ponderal index was normal, suggesting symmetrical growth restriction. There was a 14% reduction in female birth weight, which equated to an approximately 500 g mean difference in size compared to female neonates from non-asthmatic mothers. This is far greater than fetal growth reductions previously reported for smoking mothers, which average 150-200 g (297, 300, 308-310, 313).

Schatz *et al.* have previously demonstrated that poor maternal lung function in asthmatics, indicated by lower maternal FEV_1 was associated with a greater incidence of birth weights in the lower quartile and asymmetric growth restriction (83). No association between low birth weight or low ponderal index and steroid use was found (83). In this study, 12.5% of mothers with an FEV_1 in the lowest quartile (<83% predicted) had infants with a ponderal index <2.2, while those with an FEV_1 in the highest quartile (>99% predicted) had a low incidence (4.4%) of infants with a ponderal index <2.2 (83). In my study there were no significant differences in the incidence of a low ponderal index within any group, no correlations between neonatal ponderal index and maternal FEV_1 and no relationship between low maternal FEV_1 and low ponderal index. In fact, women with a low FEV_1, had the lowest incidence of neonates with ponderal index <2.2 compared to women with better lung function. In the study from Schatz *et al.* there were 370 subjects examined and 56% of these used no medication or intermittent bronchodilators (83). In my study, lung function and ponderal index were examined in 117 subjects and only 33% would be similarly classified as users of intermittent bronchodilators only. This difference in patient severity distribution may have contributed to the different results with regard to asymmetric growth restriction. In

addition, Schatz *et al.* did not divide their subjects based upon fetal sex and this also may have affected the results (83) and contributed to the differences between studies.

Many asthmatic women avoid the use of medication due to concern about harming their unborn fetus. This has been documented in an analysis of women's perceptions of their asthma during pregnancy which indicated that 44% were worried about the effects of asthma attacks and asthma treatment on the baby (208). In this study, 43% of responders said they would take medication as a strategy to control asthma during pregnancy, and yet despite the fact that 41% had worsening asthma, only 17% actually used preventative asthma medication while pregnant (208). A recent study of 501 asthmatic women of child-bearing age found that 82% of women who currently used inhaled glucocorticoids were concerned about continuing to use them while pregnant (211). In women who had previously been pregnant, 39% revealed that they had discontinued or reduced their medication while pregnant, and one third of these had done so without consulting their doctor (211). Clearly, the results of my study show that the use of inhaled glucocorticoid medication when appropriate is beneficial for fetal growth, particularly that of the female fetus. Other studies would support this claim, although the majority have focussed on women with severe asthma (59, 171, 172).

Oral steroid use has been shown to reduce birth weight; however, in one study, the women used steroids throughout pregnancy (192). In my study, 16% of asthmatic women who used inhaled glucocorticoids also required periodic oral steroids during pregnancy, but this was not found to contribute to any changes in male or female birth weight. Similarly, the use of beclomethasone, budesonide or fluticasone inhalers for asthma treatment during pregnancy did not result in reduced fetal growth. Others have also demonstrated that there is no effect of inhaled glucocorticoid use for asthma treatment on birth weight (178, 202) and although inhaled fluticasone use in pregnant women with asthma has not previously been examined, a study in women with rhinitis showed no adverse effect of the fluticasone nasal spray on fetal growth (205).

Stenius-Aarniala *et al.* found that inhaled steroid use reduced the incidence of asthma exacerbations during pregnancy (147). Greenberger *et al.* demonstrated that in women with severe asthma requiring emergency therapy in hospital, mean birth weight was reduced by approximately 300 g compared to women with severe asthma who did not have exacerbations requiring hospitalisation, indicating that severe uncontrolled asthma

may contribute to reduced fetal growth (172). Mabie *et al*. and Jana *et al*. also found an association between moderate or severe asthma requiring hospitalisation during pregnancy and reduced fetal growth (57, 59).

The results of my study show that severe asthma does not contribute to reduced birth weight of either male or female neonates of asthmatic mothers. In my study, all asthmatic women were actively managed during pregnancy and most women with severe asthma used inhaled and sometimes oral glucocorticoids. The study design incorporated education, better patient self-management and close follow up by the respiratory specialist when required and this may have prevented acute asthma attacks in most women. During the study there was one patient who was admitted to hospital with severe asthma at approximately 30 weeks gestation. Her female baby was born at 39 weeks gestation and was 2170 g, with a birth weight centile of <5, possibly due to the severe asthma attacks. It is likely that in most cases, the careful monitoring of women with severe asthma and avoidance of uncontrolled asthma attacks in this study led to no observed effect of severe asthma on mean birth weight. Although severe asthmatics were encouraged to use inhaled glucocorticoids, several subjects did not, possibly due to concerns for their baby. However, exclusion of these severe asthmatics who did not use inhaled glucocorticoids from the analysis still demonstrated a significant effect of no glucocorticoid use on female birth weight in mild asthmatics alone (Figure 4.3).

It was surprising that reduced birth weight was observed in asthmatic women with mild asthma. These patients were considered so mild that they did not require inhaled glucocorticoids for effective asthma control. In my study, 56% of women with mild asthma did not use inhaled glucocorticoids and their lung function (FEV_1 or FEV_1:FVC) was not significantly different from the control group. Despite this, women with mild asthma who did not use inhaled glucocorticoids had significantly smaller female neonates than women with mild asthma who did use inhaled glucocorticoids. Previous studies have reported an effect of reducing asthma medication use during pregnancy on birth weight (201). In this study by Olesen *et al.*, asthmatic women who changed their medication use from inhaled steroids before pregnancy to inhaled β_2-agonists during pregnancy had babies with lower weights (mean difference 219 g) and shorter lengths than women who remained on inhaled steroids during pregnancy (201). The severity of the asthmatic women in this study was not known (201). Lao and Huengsburg found an

increased rate of low birth weight among asthmatic mothers who did not use treatment for asthma (56). No other studies have demonstrated changes in birth weight in relation to mild asthma. In fact, many studies have not included mild asthmatics in their analyses (49). One recent study demonstrated that women who had not been diagnosed as asthmatic by a physician but were experiencing asthma symptoms, were at increased risk of IUGR (64). This supports the possibility raised from my work, that a low grade inflammatory disease, either undiagnosed, or not appearing to warrant inhaled glucocorticoid therapy, may contribute to alterations in fetal growth.

Other inflammatory diseases have previously been shown to be associated with low birth weight. These include rheumatoid arthritis (94-96), inflammatory bowel disease (101-103) and systemic lupus erythematosus (100). The association between active inflammation and low birth weight suggests a role for inflammatory pathways in the mechanism of reduced fetal growth. Asthma-associated inflammation will be examined in Chapter 5.

A novel finding of this study was that only the birth weight of the female fetus was affected by maternal asthma. This finding has not previously been reported. One study found that maternal asthma and male fetal sex were associated with low birth weight; however, in this study, maternal smoking and living in an industrialised town were also found to be confounders (66). However, there have been reports of an increased incidence of preterm delivery and pre-eclampsia in asthmatic women pregnant with a female fetus (49). The mechanisms contributing to this particular susceptibility of the female fetus for these problems during asthmatic pregnancies are unknown.

Nine out of 20 studies reviewed in Chapter 1 (Table 1.1) found asthmatic women to be at increased risk of low birth weight. Jana *et al.* found that low birth weight occurred in asthmatics requiring hospitalisation for severe asthma only (59), while Perlow *et al.* found that low birth weight was associated with asthmatics who used steroids (41). However, some of the discrepancies between studies were due to the fact that asthmatic women were not characterised based upon disease severity, treatment or fetal sex. My study followed standard asthma management guidelines to clinical manage and classify pregnant women with asthma based on both severity and treatment. Therefore, this work represents the most comprehensive analysis to find reduced fetal growth in asthmatic women, specifically identifying female neonates to be at risk when their

asthmatic mother does not use inhaled glucocorticoid medication. It must be noted, however, that the mean birth weight of these female neonates was 3095 g which in itself, does not constitute "low birth weight" or IUGR. Despite this, previous studies have demonstrated that only a 100 g mean difference in birth weight between two groups is associated with a clinically relevant improvement in neonatal morbidity and mortality (227, 228). I found that there was a strong trend towards an increase in the prevalence of female SGA infants among the no glucocorticoid group, and with increased study numbers this may become significant. However, the finding of reduced birth weight in a sub-group of female neonates of asthmatic mothers does allow further investigation into the maternal and placental mechanisms which control fetal growth in asthmatic pregnancies, which has not been carried out in other studies.

4.5 Fetal Growth - Summary

In summary, maternal asthma that is not treated with inhaled glucocorticoids is associated with reduced growth of the female fetus (Figure 4.4). The possible mechanisms contributing to low birth weight in female neonates of asthmatic mothers will be examined in the following chapters.

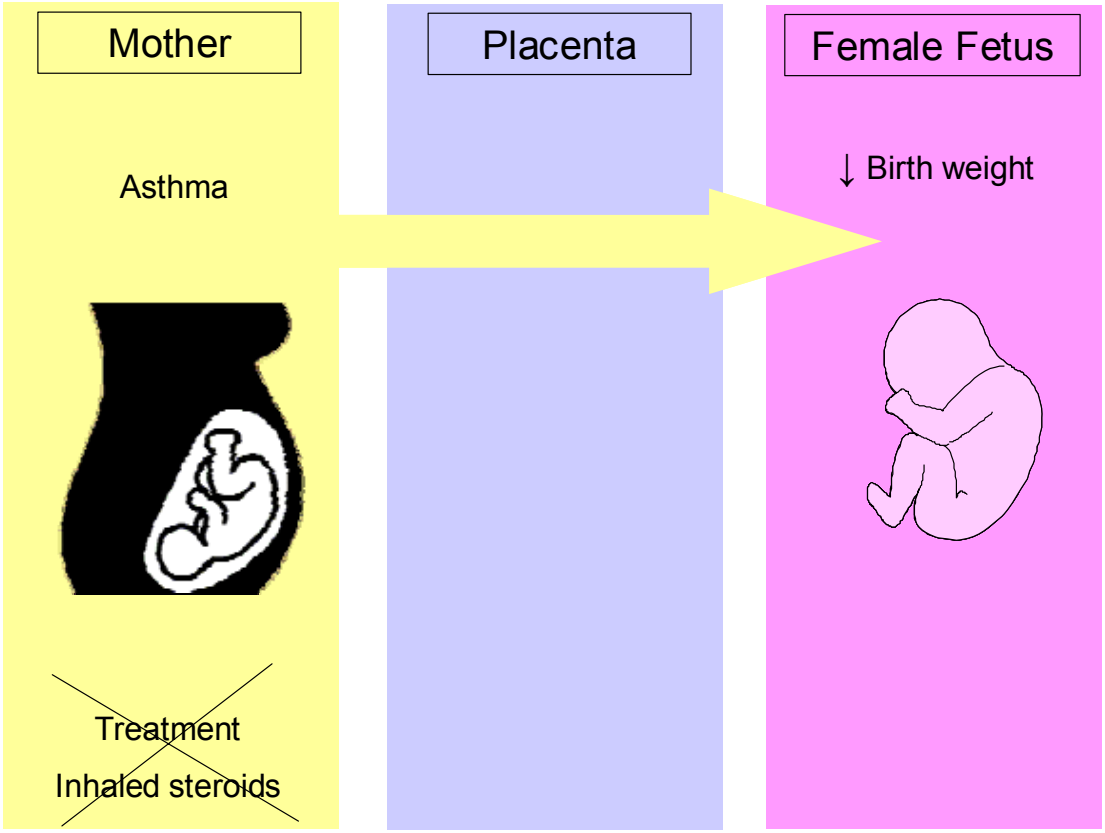

Figure 4.4 Alterations in fetal growth in asthmatic pregnancies

In the presence of maternal asthma which is not treated with inhaled glucocorticoids, female birth weight is significantly reduced.

Chapter 5 **The Mother**

The mother has an important role to play in the regulation of fetal growth. In asthmatic women, the progression of the asthmatic disease during pregnancy may affect fetal growth, through alterations in inflammatory pathways associated with asthma or through changes in lung function.

5.1 Maternal asthma during pregnancy

Asthmatic women were assessed several times throughout pregnancy in the Asthma Management Service (AMS), to ensure that they received adequate asthma education and to adjust their inhaled glucocorticoid requirements if necessary. The AMS education program has previously been demonstrated to improve asthma control and management skills in our non-pregnant population and to reduce re-admission rate in adults with acute severe asthma (635). Since the number of visits to the AMS by each woman varied, data was only analysed for the first and last visit to the AMS made by each individual. The first visit occurred at an average of 23.0 ± 0.6 weeks gestation (n=153) and the last visit at 32.2 ± 0.4 weeks gestation (n=111), while 42 asthmatic women only visited the AMS once during pregnancy. Data on asthma symptoms, skills and management collected during the first and last AMS visits are shown in Table 5.1 and Table 5.2 respectively.

In general, there was an improvement in asthmatic women's skills in using their inhalers, their knowledge of medications and an increase in the number of women with an asthma action plan as pregnancy progressed. The trend was for women to report fewer symptoms, and to be taking less reliever medication at their last visit. This is likely to have been a direct result of the asthma education they received which resulted in improved inhaler technique and increased knowledge about what their medications were used for and when to take them.

In most groups >85% of women had optimal or adequate inhaler technique at the first visit. However, in the no glucocorticoid (male fetus) group, 31% of women had inadequate inhaler technique at the first visit. This was significantly improved by the last AMS visit, when 100% of women in this group had optimal or adequate inhaler technique (Fisher's exact test, P=0.016).

Table 5.1 Maternal asthma assessment at the first AMS visit

First AMS Visit	No Glucocorticoid		Glucocorticoid	
	Female Fetus	Male Fetus	Female Fetus	Male Fetus
Gestational Age (weeks)	21.7 ± 1.1 (n=29)	23.3 ± 1.4 (n=29)	23.5 ± 1.1 (n=50)	23.0 ± 0.9 (n=45)
Night Symptoms (days/week)	1.0 ± 0.4 (n=29)	1.1 ± 0.4 (n=29)	2.3 ± 0.4 (n=50)	2.1 ± 0.4 (n=45)
Morning Symptoms (days/week)	0.5 ± 0.2 (n=29)	1.5 ± 0.5 (n=29)	2.6 ± 0.4 (n=50)	2.3 ± 0.4 (n=45)
Activity Limitation (days/week)	0.3 ± 0.3 (n=29)	0.8 ± 0.4 (n=29)	1.6 ± 0.4 (n=50)	1.5 ± 0.4 (n=45)
Symptom Free (% of subjects)	66%	48%	28%	29%
Reliever Medication Use (times/week)	4.6 ± 2.5 (n=29)	6.0 ± 2.0 (n=28)	19.8 ± 4.0 (n=50)*	13.4 ± 2.3 (n=45)*
Optimal Inhaler Technique (% of subjects)	42%	38%	68%	42%
Adequate Inhaler Technique (% of subjects)	46%	31%	24%	44%
Inadequate Inhaler Technique (% of subjects)	12%	31%	8%	14%
Medications Knowledge (% of subjects)	36%	50%	72%	42%
Action Plan Received (% of subjects)	14%	17%	25%	19%
	Values given are Mean ± Standard Error of the Mean			

* Compared to the no glucocorticoid group (Mann Whitney test, $P<0.05$)

Table 5.2 Maternal asthma assessment at the last AMS visit

Last AMS Visit	No Glucocorticoid		Glucocorticoid	
	Female Fetus	Male Fetus	Female Fetus	Male Fetus
Gestational Age (weeks)	32.9 ± 0.8 (n=18)	30.5 ± 1.0 (n=21)	32.4 ± 0.6 (n=40)	32.6 ± 0.6 (n=32)
Night Symptoms (days/week)	0.3 ± 0.2 (n=18)	0.8 ± 0.4 (n=20)	1.4 ± 0.3 (n=40)	0.8 ± 0.3 (n=32)**
Morning Symptoms (days/week)	1.0 ± 0.5 (n=18)	1.0 ± 0.5 (n=20)	2.0 ± 0.4 (n=40)	1.6 ± 0.5 (n=32)
Activity Limitation (days/week)	0.2 ± 0.2 (n=18)	0.5 ± 0.4 (n=20)	2.2 ± 0.5 (n=40)	0.8 ± 0.4 (n=31)
Symptom Free (% of subjects)	61%	60%	28%	59%*
Reliever Medication Use (times/week)	1.9 ± 0.6 (n=18)	2.5 ± 1.2 (n=20)	11.9 ± 2.2 (n=40)***	8.3 ± 1.4 (n=32)***
Optimal Inhaler Technique (% of subjects)	42%	31%	74%	57%
Adequate Inhaler Technique (% of subjects)	58%	69%	26%	29%
Inadequate Inhaler Technique (% of subjects)	0%	0%*	0%	14%
Medications Knowledge (% of subjects)	90%*	88%*	100%*	95%*
Action Plan Received (% of subjects)	80%*	57%*	80%*	61%*
	Values given are Mean ± Standard Error of the Mean			

*Compared to first AMS Visit (Fisher's exact test, $P<0.05$), **Compared to first AMS visit (Mann Whitney test, $P<0.05$), ***Compared to no glucocorticoid group (Mann Whitney test, $P<0.05$)

The proportion of asthmatic women with medication knowledge and an action plan significantly increased from first to last AMS visit in all four groups (Fisher's exact test, $P<0.021$).

The number of days per week that asthmatic women were affected by night-time symptoms did not change significantly from first to last visit in the no glucocorticoid groups, or the glucocorticoid female group (Mann Whitney test, $P>0.05$). However, in the glucocorticoid group, women pregnant with a male fetus had a significant decrease in the number of days per week that they were affected by night-time asthma symptoms (Mann Whitney test, $P=0.035$). There were no significant differences in morning symptoms and activity limitation from first to last visit in any group (Mann Whitney test, $P>0.05$).

The percentage (%) of subjects who were symptom free at each visit was examined, as an indicator of asthma control. Subjects were considered symptom free if they reported no night-time or morning symptoms and no activity limitation as a result of asthma. There was a rise in the % of subjects who were symptom free from first to last visit only in asthmatic women who were pregnant with a male fetus. In the no glucocorticoid group, this was not significant (48% at first visit to 60% at last visit, Fisher's exact test, $P=0.562$) and in the glucocorticoid group, this increase was significant (29% at first visit to 59% at last visit, Fisher's exact test, $P=0.010$, Table 5.1 and Table 5.2). These data suggest that the presence of a male fetus is associated with an improvement in asthma symptoms throughout pregnancy. In asthmatic women pregnant with a female fetus, there was either no change in the symptom free rate (glucocorticoid group, 28% symptom free at first and last AMS visit), or a decrease in the proportion of subjects who were symptom free (no glucocorticoid group, 66% to 61% from first to last AMS visit, Fisher's exact test, $P=0.766$). These data suggest no improvement in asthma symptoms in women pregnant with a female fetus, despite their improved knowledge and inhaler technique.

Asthmatic women in the glucocorticoid group used significantly more β_2-agonist (reliever) medication than women in the no glucocorticoid group, as expected from the greater proportion of severe asthmatics within this group (Table 5.1 and Table 5.2, Mann Whitney test, $P<0.05$). There was no difference in β_2-agonist use between women pregnant with a male or female fetus in either the no glucocorticoid or glucocorticoid

groups (Mann Whitney test, $P>0.05$) and no significant alteration in absolute level of β_2-agonist use (number of puffs per week) as pregnancy progressed in any group (Mann Whitney test, $P>0.05$).

The use of β_2-agonists at any time by women who did not use inhaled glucocorticoids was associated with significantly lower female birth weights than those who never reported using β_2-agonists (Figure 5.1, unpaired t-test, $P=0.049$). Male birth weight was not affected by β_2-agonist use (Figure 5.1, unpaired t-test, $P=0.513$). The asthmatic women who did not use inhaled glucocorticoids or report any β_2-agonist use during pregnancy had female babies of similar size (3507.5 ± 165.8 g, n=8) to the control non-asthmatic group (3600.3 ± 101.2 g, n=20, unpaired t-test, $P=0.632$). This data suggests the possibility that within the no glucocorticoid group, there was a sub-group of women whose asthma may have been in remission, such that it did not require any medications and although asthma had been previously diagnosed in these women, this did not affect female birth weight. The other sub-group who did use β_2-agonists may have had slightly worse asthma, which was not severe enough to warrant the prescription of inhaled glucocorticoids, but did have an effect on female birth weight, despite the use of β_2-agonists for symptom control. However, caution should be used in interpreting these results due to the small number of subjects within each group. The power to detect a 400 g difference in the birth weight of male neonates of mothers using β_2-agonists compared to mothers not using β_2-agonists, with these study numbers is 0.6.

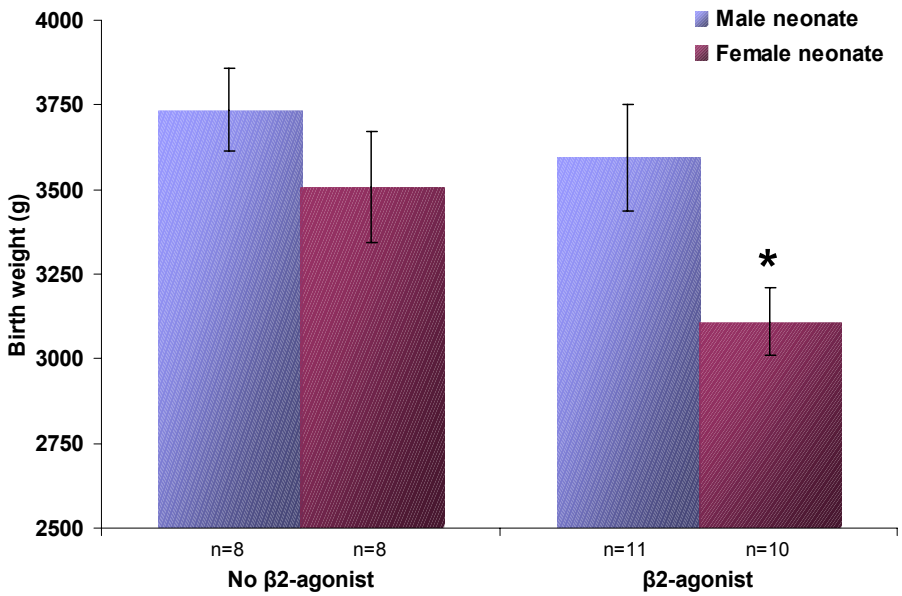

Figure 5.1 Birth weight in the no glucocorticoid group according to maternal use of β_2-agonists during pregnancy

*Mean birth weight (g) ± SEM is shown for male and female neonates of the no glucocorticoid group, according to maternal reported use of β_2-agonists during pregnancy. * indicates $P<0.05$ (unpaired t-test).*

Asthma remission occurs most often in childhood or during puberty (709) and may also be observed in adult asthmatics (710, 711). Remission, defined as no medication use or exacerbations within the previous 2 years, was found to occur in 46% of previously diagnosed asthmatics in a large Italian study (712). A follow up study of asthmatics diagnosed 25 years earlier found that 11% of subjects were no longer considered asthmatic as they had no evidence of bronchial hyperresponsiveness, an FEV_1 >90% predicted and no reported pulmonary symptoms (710).

In order to investigate the possibility of remission further, symptom reporting and lung function testing was examined in women pregnant with a female fetus who did not use inhaled glucocorticoids. There was no significant difference in lung function in women who may have been in remission (FEV_1:FVC = 0.873 ± 0.017, n=8) compared to women considered not in remission (FEV_1:FVC = 0.838 ± 0.028, n=9, unpaired t-test, P=0.306), although the trend was for better lung function in women thought to be in remission. However, lung functions in both these asthmatic groups were also not significantly different from the control (female) group (FEV_1:FVC = 0.878 ± 0.034, n=11, ANOVA, P=0.574). The reporting of symptoms by asthmatic women of the no

glucocorticoid group who did or did not report β_2-agonist use and were pregnant with a female fetus was examined. Women considered in remission (n=8) reported no night-time or morning symptoms due to asthma during pregnancy. Only one woman in this group reported activity limitation (1 day per week) at the first AMS visit. In addition, the asthma history in this group confirmed remission in one woman who had no problems with asthma in the previous 10 years. However, in the group considered not in remission (n=10), four women reported night symptoms during pregnancy (7 times per week in three women), seven women reported morning symptoms and one woman reported activity limitation at both the first (2 days per week) and last (4 days per week) AMS visit. These data suggest that asthma remission may be a real phenomenon in some women with mild asthma and that asthma remission is not associated with adverse effects on female birth weight. This suggests that women who experience mild asthma symptoms during pregnancy, may benefit from low dose inhaled glucocorticoid therapy during pregnancy, which by controlling symptoms and systemic inflammation may contribute to improved female fetal growth. However, the numbers here are small and larger groups will be required to investigate this further.

Male and female birth weight was also assessed in the no glucocorticoid group in relation to whether the asthmatic mother had received an asthma action plan at any of the AMS visits. Female birth weight was significantly lower than male birth weight regardless of whether the mother had received an action plan (unpaired t-test, $P<0.05$). Male birth weight was normal even when the mothers had not received an asthma action plan, supporting the concept that the male fetus is less affected by maternal asthma. Mean female birth weight was higher in mothers who received an action plan compared to mothers who did not receive an action plan, but this difference was not statistically significant (Figure 5.2, unpaired t-test, $P=0.274$). Greater study numbers are required to assess this more thoroughly. However, this preliminary evidence suggests that asthma education and a knowledge of how to alter medication use for proper asthma control in the event of an exacerbation are useful skills that could improve female fetal growth in asthmatic pregnancies.

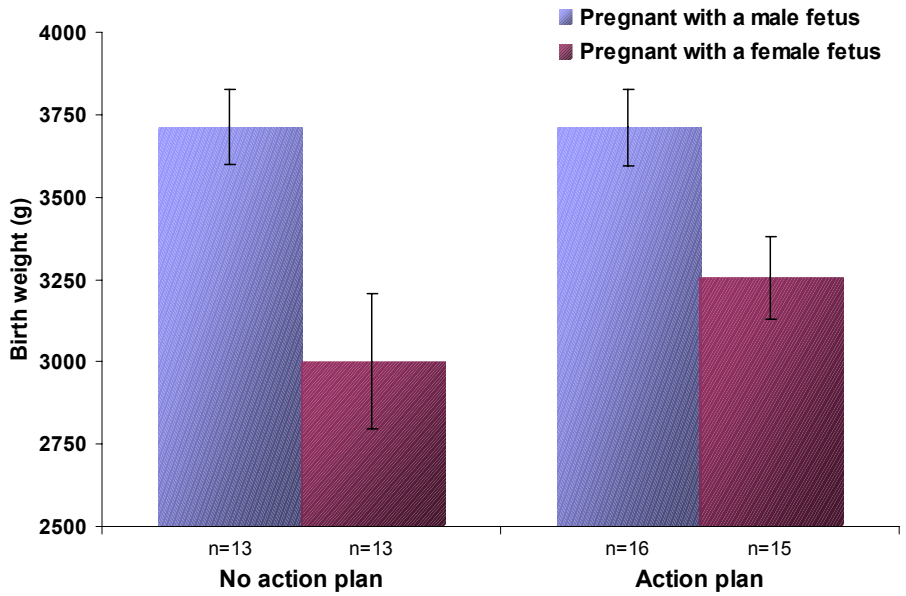

Figure 5.2 Birth weight in the no glucocorticoid group according to the provision of asthma action plans

Mean birth weight (g) ± SEM is shown for male and female neonates of the no glucocorticoid group, according to whether the mother received an asthma action plan during pregnancy.

Overall, these data demonstrate that education is an important part of monitoring asthma in pregnant women as it leads to a reduction in symptoms and improved inhaler technique and medication knowledge. Studies of asthmatic women of child-bearing age show that many women have concerns about the effect their asthma may have on the fetus and many would like more medical care, support and education (208, 211). However, in one study, only 19% of women made decisions about the management of their asthma while pregnant by consultation with a physician (211). In non-pregnant adults, randomised trials have demonstrated the effectiveness of asthma education in reducing re-admission rates and emergency room visits, reducing oral corticosteroid use and improving knowledge and self-management (713-716). The asthma management technique employed in this study has previously been demonstrated to be effective in reducing re-admission rates in non-pregnant adults who had recent severe asthma exacerbations (635). Similar education programs in pregnant asthmatic women have the potential to greatly improve the outcome for both mother and baby. Despite the education provided in our study, there was reduced fetal growth in mild asthmatic women pregnant with a female fetus, who did not use glucocorticoids during pregnancy and were not in remission. The following sections will outline changes in maternal

asthma which occur in the presence of a female fetus, which may contribute to reduced fetal growth.

5.2 Maternal lung function during pregnancy

Maternal lung function was assessed by spirometry during the AMS visits. Maternal lung function data collected in early pregnancy (average 23 weeks gestation) was compared to lung function data collected in late pregnancy (average 32 weeks gestation). While 73% of asthmatic women attended the AMS twice, only a small number had their lung function assessed on both occasions. The FEV_1:FVC ratio was used as an indicator of lung function, since previous studies have shown that this relates to the degree of bronchospasm (115).

There was a significant decrease in FEV_1:FVC as pregnancy progressed in asthmatic women pregnant with a female fetus, in both the no glucocorticoid group (Figure 5.3A, unpaired t-test with Welch correction, P=0.015) and the glucocorticoid group (Figure 5.3B, unpaired t-test, P=0.018). There was no change in maternal lung function during pregnancy when the woman was pregnant with a male fetus (Figure 5.3, no glucocorticoid, unpaired t-test, P=0.936, glucocorticoid, unpaired t-test, P=0.364). Results were similar when only the smaller number of subjects who had two assessments were included (data not shown). Women in the glucocorticoid group had significantly worse lung function compared to women in the no glucocorticoid group, when pregnant with a female fetus, both at the first AMS visit (unpaired t-test, P=0.018) and last AMS visit (unpaired t-test with Welch correction, P=0.024), as expected from the greater proportion of women with severe asthma in the glucocorticoid group. These data indicate that fetal sex has an effect on maternal lung function regardless of glucocorticoid therapy, with decreased lung function during pregnancy observed in the presence of a female fetus.

The relationship between lung function (first AMS visit) and female birth weight was examined. There was a significant positive correlation between maternal lung function, expressed as the FEV_1:FVC ratio and neonatal birth weight for females in the no glucocorticoid group only (Figure 5.4A, Pearson linear correlation, r = 0.458, n=26, P=0.019, excluding one pre-term delivery). This relationship was not observed for

females in the glucocorticoid group (Figure 5.4B, Pearson linear correlation, r = 0.033, n=36, *P*=0.848), or for male neonates (*P*>0.05, data not shown).

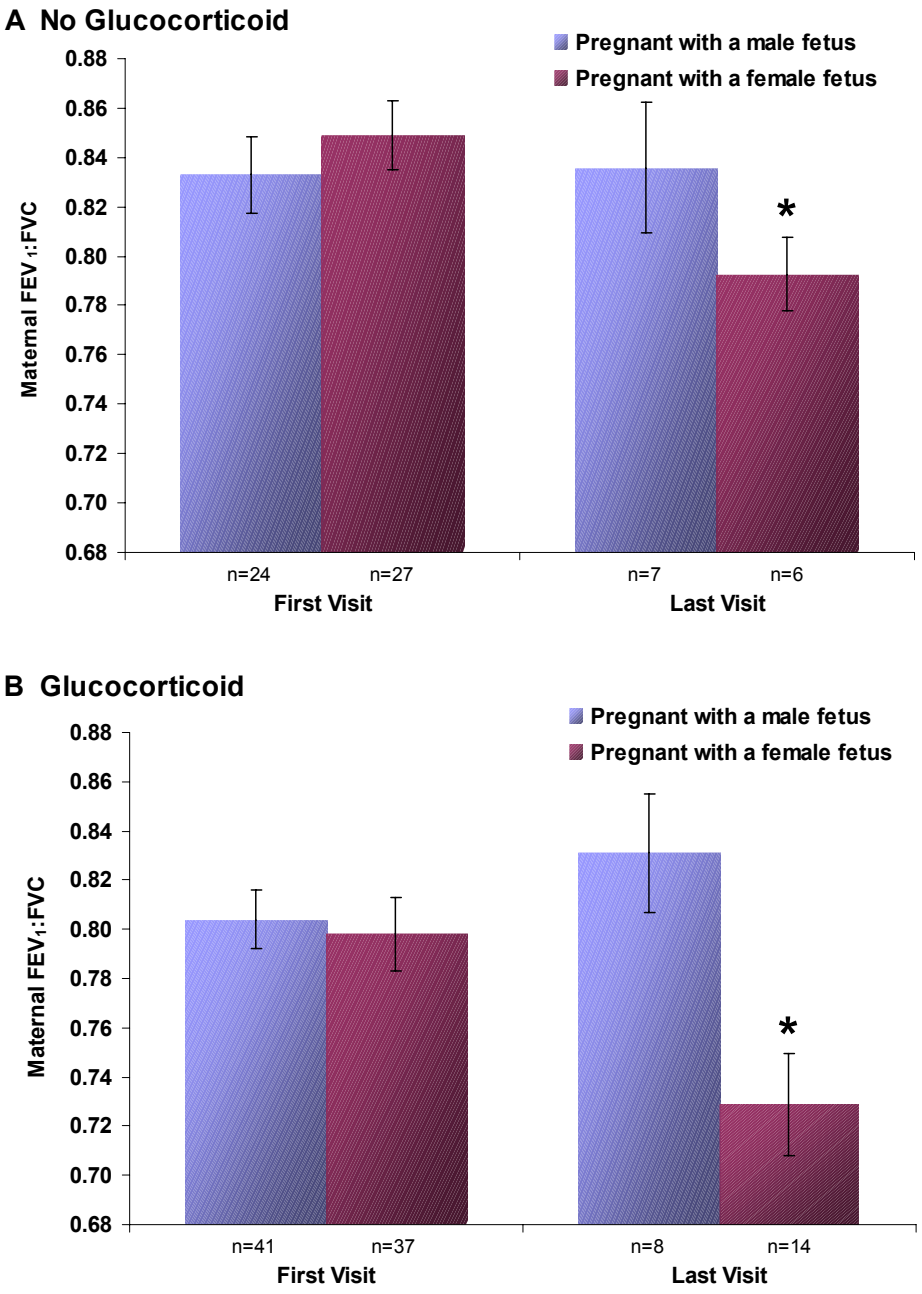

Figure 5.3 Maternal lung function during pregnancy in asthmatic women

*Maternal lung function is represented by the FEV_1:FVC ratio and is shown for women pregnant with a male and female fetus, from the no glucocorticoid group (Panel A) and the glucocorticoid group (Panel B). * indicates P<0.05 (unpaired t-test, first AMS visit vs last AMS visit in women pregnant with a female fetus).*

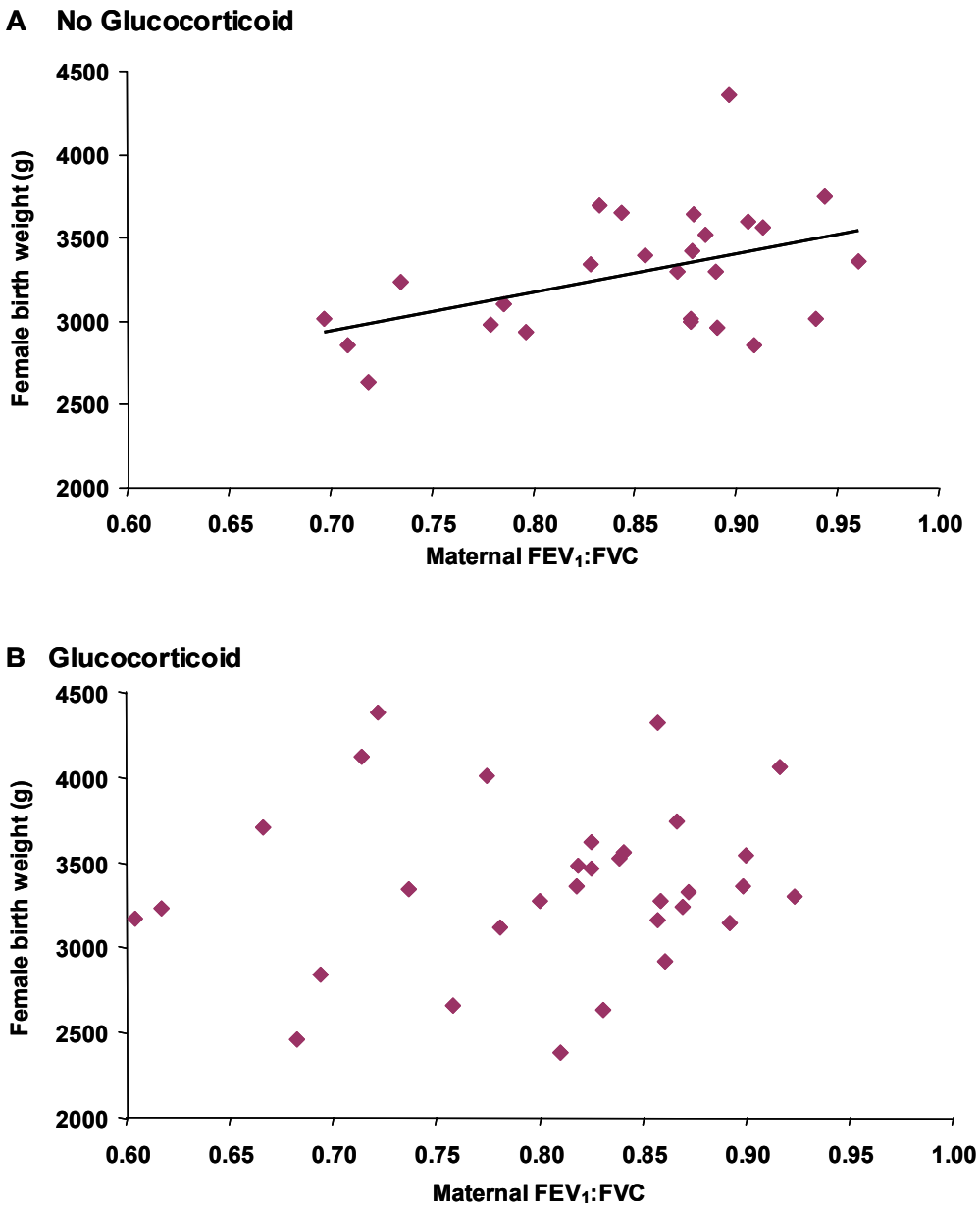

Figure 5.4 Relationship between maternal lung function and female birth weight

The relationship between maternal FEV_1:FVC and female birth weight is shown for women in the no glucocorticoid group (Panel A) and the glucocorticoid group (Panel B). The correlation was significant in the no glucocorticoid group (Pearson linear correlation, r = 0.458, n=26, P=0.019).

5.3 Maternal glucocorticoid use during pregnancy

The alterations in female fetal growth may be due to maternal inflammatory pathways associated with asthma, since asthmatic women who used anti-inflammatory glucocorticoid medication had female neonates of similar size to the non-asthmatic control group. To investigate changes in inflammation in asthmatic women, two methods were employed. In the glucocorticoid group, inhaled glucocorticoid use during each trimester of pregnancy was examined. In both the no glucocorticoid and glucocorticoid groups, maternal circulating white blood cells were examined (Section 5.4).

In asthmatic women treated with moderate or high doses of inhaled glucocorticoids who were pregnant with a female fetus (n=41), there was a significant increase in the inhaled glucocorticoid requirement during pregnancy, which increased from 917 ± 99 μg/day in the first trimester to 1350 ± 111 μg/day in the third trimester (Figure 5.5, non-parametric repeated measures ANOVA, P=0.0002, Dunn's multiple comparisons test, P<0.05, first trimester vs third trimester). In asthmatics using moderate or high doses of inhaled glucocorticoids who were pregnant with a male fetus (n=29) the mean inhaled glucocorticoid requirement was 945 ± 108 μg/day in the first trimester and 1100 ± 105 μg/day in the third trimester (Figure 5.5, non-parametric repeated measures ANOVA, P=0.039, Dunn's multiple comparisons test, P>0.05). Although the mean inhaled glucocorticoid dose also increased in women pregnant with a male fetus, this was not statistically or clinically significant, and the power to detect a 400 μg/day increase, such as that observed in women pregnant with a female fetus was 0.97.

These data suggest that in the presence of a female fetus, there is a rise in inflammatory pathways, which leads to a greater requirement for inhaled glucocorticoids in these women. In asthmatic women who do not use inhaled glucocorticoid medication, this alteration in inflammation would not be prevented and may contribute to alterations in placental function and fetal growth.

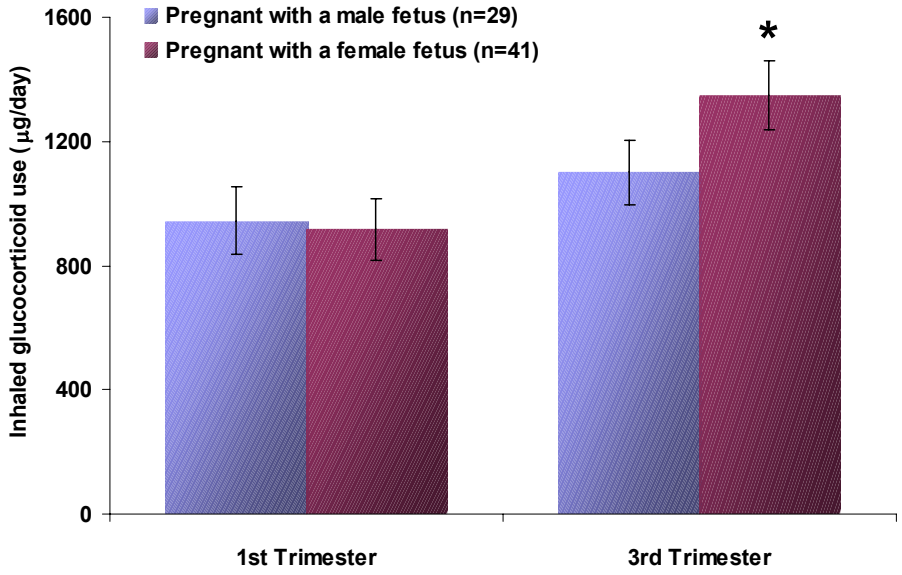

Figure 5.5 Inhaled glucocorticoid intake during pregnancy in asthmatic women

*The inhaled glucocorticoid dose (µg/day) in the first and third trimester is shown for asthmatic women using moderate and high doses of inhaled glucocorticoids who were pregnant with male or female fetuses. * indicates P<0.0002 (non-parametric repeated measures ANOVA, first trimester to third trimester change in women pregnant with a female fetus).*

5.4 Maternal inflammation during pregnancy

To further investigate inflammatory pathways, maternal white blood cell counts were examined in early pregnancy (<20 weeks, average 11.4 ± 0.3 weeks, n=119) and late pregnancy (>30 weeks, average 36.1 ± 0.4 weeks, n=52).

The maternal monocyte count significantly increased from early to late gestation in asthmatic women in the no glucocorticoid group who were pregnant with a female fetus (Figure 5.6, unpaired t-test, *P*=0.020). In addition, the percentage of white blood cells which were monocytes significantly increased from early to late pregnancy (6.2 ± 0.4%, n=22 to 7.6 ± 0.4%, n=7) in the no glucocorticoid group in women pregnant with a female fetus (Mann Whitney test, *P*=0.020). There was no significant change from early to late pregnancy in maternal monocyte count in any other group including the asthmatic women who did not use glucocorticoids and were pregnant with a male fetus (unpaired t-test, *P*=0.370).

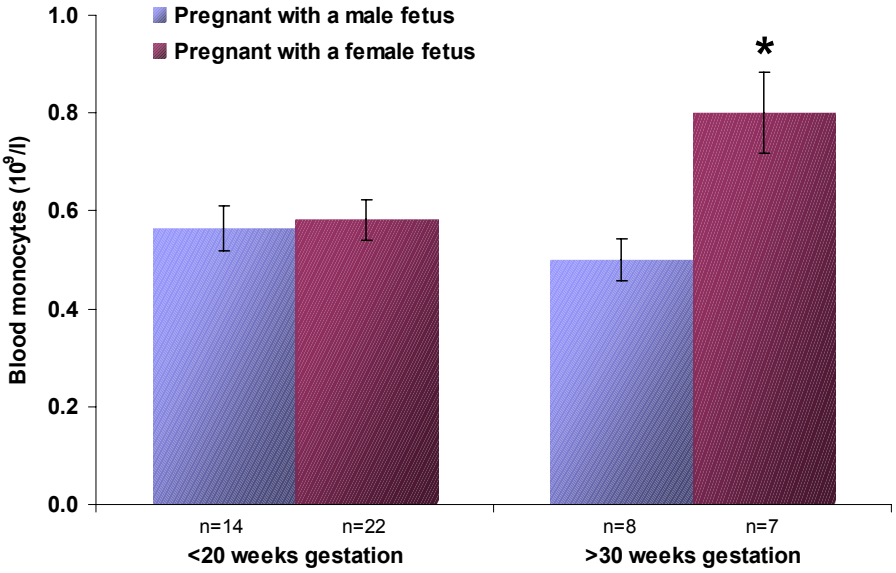

Figure 5.6 Circulating monocytes in asthmatic women during pregnancy

*Circulating monocyte levels (10^9/l) in early pregnancy (<20 weeks gestation) and late pregnancy (>30 weeks gestation) are shown for women pregnant with a male and female fetus in the no glucocorticoid group. * indicates P=0.02 (unpaired t-test).*

The total white blood cell count and lymphocyte, neutrophil, eosinophil and basophil counts were also examined (Table 5.3 and Table 5.4). There were no significant differences in total white blood cells, neutrophils or basophils between women pregnant with males or females of any group, and no significant alterations from early to late pregnancy (Kruskal-Wallis ANOVA, $P>0.05$).

Eosinophil counts in early pregnancy were significantly higher in the asthmatic groups (no glucocorticoid and glucocorticoid) compared to the control group and in the glucocorticoid group compared to the no glucocorticoid group (Figure 5.7, Kruskal-Wallis ANOVA, $P<0.0001$, Dunn's multiple comparisons test, control vs no glucocorticoid, $P<0.01$, control vs glucocorticoid $P<0.001$, no glucocorticoid vs glucocorticoid, $P<0.05$). However, there was no significant difference in eosinophil counts between the groups in late pregnancy (Kruskal-Wallis ANOVA, $P=0.117$). There was a trend towards a decrease in eosinophil counts in the no glucocorticoid group from early pregnancy ($0.23 \pm 0.03\ 10^9$/l, n=35) to late pregnancy ($0.13 \pm 0.03\ 10^9$/l, n=15, Mann Whitney test, $P=0.057$). In the glucocorticoid group, eosinophil counts significantly decreased from early ($0.34 \pm 0.03\ 10^9$/l, n=54) to late pregnancy ($0.16 \pm 0.02\ 10^9$/l, n=30, Mann Whitney test, $P<0.0001$). This significant decrease in

eosinophils during pregnancy occurred both in women pregnant with a male fetus (Mann Whitney test, *P*=0.007) and women pregnant with a female fetus (Mann Whitney test, *P*=0.001).

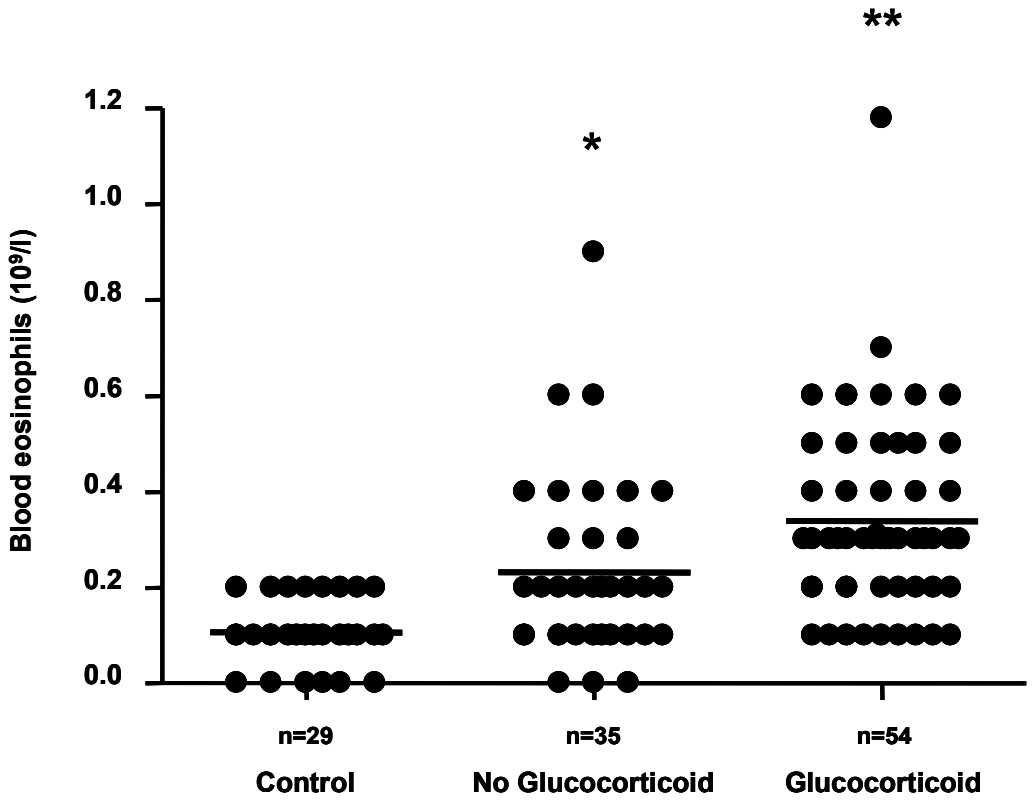

Figure 5.7 Circulating eosinophils in asthmatic women during pregnancy

*Circulating eosinophil levels (10^9/l) are shown for women in the control, no glucocorticoid and glucocorticoid groups in early pregnancy (<20 weeks gestation). * indicates P<0.01 (Kruskal-Wallis ANOVA, control vs no glucocorticoid). ** indicates P<0.05 (Kruskal-Wallis ANOVA, glucocorticoid vs no glucocorticoid and control). On this scatter plot, individual points are shown and the group median is given by the horizontal bar.*

Table 5.3 Circulating white blood cells in non-asthmatic and asthmatic women pregnant with a female fetus

Female Fetus	Circulating blood count (10^9/l)	Classification of Glucocorticoid Intake During Pregnancy		
		Control	No Glucocorticoid	Glucocorticoid
Early Pregnancy	White Blood Cell count	9.6 ± 0.3 (n=18)	9.5 ± 0.4 (n=22)	10.1 ± 0.5 (n=28)
	Neutrophil count	7.04 ± 0.24 (n=18)	6.50 ± 0.32 (n=22)	6.95 ± 0.43 (n=28)
	Lymphocyte count	1.86 ± 0.12 (n=18)	2.10 ± 0.13 (n=22)	2.15 ± 0.12 (n=28)
	Monocyte count	0.53 ± 0.04 (n=18)	0.58 ± 0.04 (n=22)	0.61 ± 0.03 (n=28)
	Eosinophil count	0.11 ± 0.02 (n=18)	0.25 ± 0.03 (n=22)	0.38 ± 0.04 (n=28)
	Basophil count	0.011 ± 0.008 (n=18)	0.016 ± 0.009 (n=19)	0.020 ± 0.010 (n=24)
Late Pregnancy	White Blood Cell count	11.4 ± 1.3 (n=3)	10.6 ± 1.0 (n=7)	11.3 ± 0.5 (n=15)
	Neutrophil count	8.96 ± 0.95 (n=3)	7.98 ± 0.81 (n=7)	8.42 ± 0.38 (n=15)
	Lymphocyte count	1.87 ± 0.35 (n=3)	1.61 ± 0.17 (n=7)	1.91 ± 0.13 (n=15)
	Monocyte count	0.53 ± 0.09 (n=3)	0.80 ± 0.08 (n=7)*	0.70 ± 0.05 (n=15)
	Eosinophil count	0 ± 0 (n=3)	0.17 ± 0.05 (n=7)	0.16 ± 0.03 (n=15)**
	Basophil count	0 ± 0 (n=3)	0.014 ± 0.014 (n=7)	0.036 ± 0.013 (n=14)
		Values given are Mean ± Standard Error of the Mean		

*Compared to early pregnancy (unpaired t-test, *P*=0.02), **Compared to early pregnancy (Mann Whitney test, *P*=0.001)

Table 5.4 Circulating white blood cells in non-asthmatic and asthmatic women pregnant with a male fetus

Male Fetus	Circulating blood count (10^9/l)	Classification of Glucocorticoid Intake During Pregnancy		
		Control	No Glucocorticoid	Glucocorticoid
Early Pregnancy	White Blood Cell count	9.0 ± 0.4 (n=11)	9.9 ± 0.4 (n=14)	9.6 ± 0.4 (n=26)
	Neutrophil count	6.05 ± 0.25 (n=11)	7.61 ± 0.78 (n=14)	6.58 ± 0.32 (n=26)
	Lymphocyte count	2.23 ± 0.22 (n=11)	1.54 ± 0.16 (n=14)*	2.13 ± 0.10 (n=26)
	Monocyte count	0.57 ± 0.04 (n=11)	0.56 ± 0.05 (n=14)	0.58 ± 0.04 (n=26)
	Eosinophil count	0.11 ± 0.03 (n=11)	0.21 ± 0.07 (n=13)	0.29 ± 0.03 (n=26)
	Basophil count	0.027 ± 0.014 (n=11)	0.008 ± 0.008 (n=12)	0.031 ± 0.009 (n=26)
Late Pregnancy	White Blood Cell count	9.0 ± 1.7 (n=3)	9.2 ± 0.6 (n=8)	11.1 ± 0.8 (n=16)
	Neutrophil count	6.30 ± 1.55 (n=3)	6.90 ± 0.53 (n=8)	8.63 ± 0.76 (n=16)
	Lymphocyte count	1.93 ± 0.17 (n=3)	1.69 ± 0.14 (n=8)	1.69 ± 0.15 (n=16)
	Monocyte count	0.57 ± 0.07 (n=3)	0.50 ± 0.05 (n=8)	0.63 ± 0.07 (n=16)
	Eosinophil count	0.17 ± 0.03 (n=3)	0.10 ± 0.02 (n=8)	0.16 ± 0.03 (n=16)**
	Basophil count	0.067 ± 0.033 (n=3)	0 ± 0 (n=8)	0.006 ± 0.006 (n=3)
		Values given are Mean ± Standard Error of the Mean		

*Compared to glucocorticoid group (ANOVA, $P<0.05$), **Compared to early pregnancy (Mann Whitney test, $P<0.01$)

5.5 Maternal plasma proteins during pregnancy

Maternal plasma proteins were profiled at 18 and 30 weeks gestation using SELDI-TOF MS. Comparisons were made between asthmatics and non-asthmatics, and between women pregnant with a female fetus and women pregnant with a male fetus. Changes in plasma proteins from 18 to 30 weeks gestation were examined specifically in asthmatic women pregnant with a female fetus from the no glucocorticoid group. The aim was to find proteins which may be involved in the mechanism of reduced female fetal growth, or may be altered as a consequence of changes in maternal asthma.

5.5.1 The effect of asthma on maternal plasma proteins during pregnancy

At 18 weeks gestation, there were 91 peaks which were significantly different (P<0.05) between plasma of asthmatic (n=20) and non-asthmatic women (n=13). Only 12 of these were identified on chips using CHCA as a matrix. The remainder were identified on chips using SPA as a matrix and represented 32% of the total number of protein clusters identified on these chips. Of the 91 significant differences between asthmatics and controls at 18 weeks gestation, 28 peaks were considered highly suitable for follow-up (classified as category A peaks).

At 30 weeks gestation, there were 51 peaks which were significantly different (P<0.05) between plasma of asthmatic (n=19) and non-asthmatic (n=10) women. Using CHCA matrix, 19 peak differences were identified, while using SPA matrix identified 32 peak differences. Where SPA matrix was used, 12% of the total number of protein clusters identified were significantly different between the two groups. Thus, the number of plasma protein differences between asthmatic and non-asthmatic women was high but decreased from early to late pregnancy.

One peak was identified on the SAX chip at pH 9, using SPA matrix, which was 1.6 times higher in asthmatics compared to controls at both 18 and 30 weeks (t-test, P=0.005 at 18 weeks and P=0.009 at 30 weeks). The relative peak intensities were similar at both time points, indicating that this is an asthma-associated peptide, the levels of which do not change with pregnancy (Figure 5.8). This peak had a m/z of 6444 and was category A. Searching the Swiss-Prot database (MW 6444 Da ± 0.5%, pI 5 ± 5)

revealed a total of three possible matches. They were pre-T/NK cell associated protein (accession number Q13412), glycophorin E precursor (P15421) and metallothionein-IV (P47944). T cells and natural killer (NK) cells are involved in inflammatory processes, while glycophorin has not previously been associated with asthma. Metallothionein is an intracellular storage molecule for Zn and Cu ions (717). Nitrogen monoxide is thought to liberate Zn from metallothionein, which may protect against asthma by preventing a Th2 cytokine shift (717). Further characterisation is required to confirm that this is not a novel protein.

One peak was identified on the IMAC chip at pH 7 using CHCA matrix which was approximately four to five-fold lower in asthmatics compared to controls at both 18 and 30 weeks gestation (t-test, P=0.010 at 18 weeks, P=0.008 at 30 weeks, category B). At 30 weeks gestation, the peak appeared to be absent in all but one asthmatic subject. This peak had a m/z of 1846. A search of the Swiss-Prot database (MW 1846 Da ± 0.5%, pI 7 ± 10) revealed one possible match (protachykinin 1 precursor, P20366). Members of the tachykinin family have various roles as neurotransmitters, vasoactive peptides (698) and contractors of smooth muscle and may play an important role in uterine contraction during pregnancy (718, 719). The significance of their absence in pregnant asthmatic plasma is unclear.

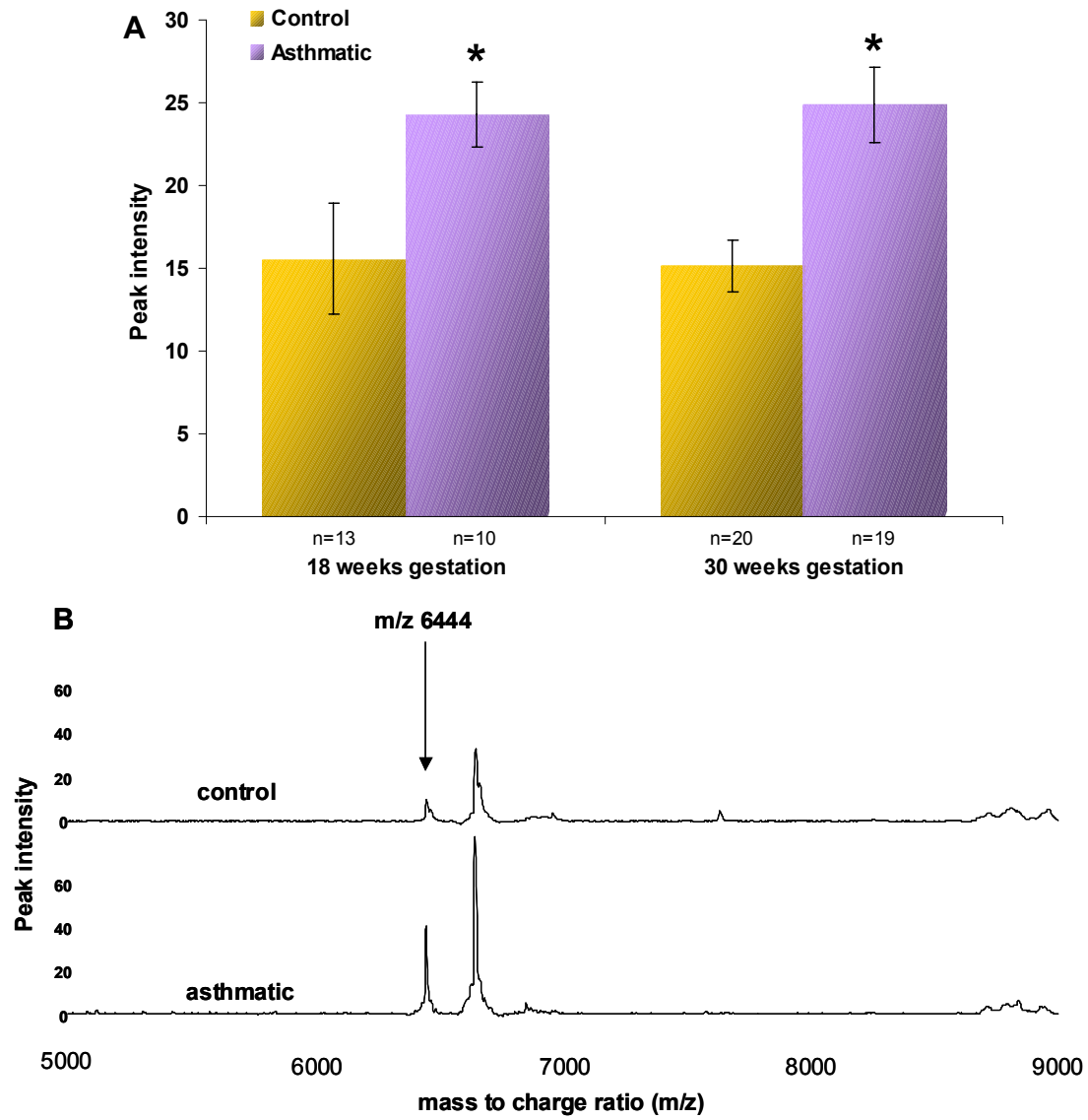

Figure 5.8 Asthma associated peptide in maternal plasma

*Panel A shows the mean peak intensity ± SEM of a maternal plasma peak with m/z 6444 identified using a SAX chip, pH 9 with SPA matrix. * indicates P<0.05 (t-test). Panel B shows a representative spectrum from a control and an asthmatic woman around the region 5-9 kDa.*

5.5.2 The effect of fetal sex on maternal plasma proteins during pregnancy

At 18 weeks gestation, there were 29 peaks which were significantly different between the plasma of women pregnant with a male fetus (n=17) and women pregnant with a female fetus (n=16). Of these, 11 were considered category A peaks.

At 30 weeks gestation, there were 46 significant differences between the plasma of women pregnant with a male fetus (n=15) and women pregnant with a female fetus

(n=14). Of these, 13 were considered category A peaks. None of the same protein peaks found to be significantly related to fetal sex during pregnancy were identified at both 18 and 30 weeks gestation.

Figure 5.9 shows a protein peak which was significantly increased in maternal plasma from women pregnant with a female fetus compared to maternal plasma from women pregnant with a male fetus at 18 weeks gestation (IMAC, pH 7, SPA matrix). However, at 30 weeks there was no significant difference in the intensity of this peak between women pregnant with male or female fetuses.

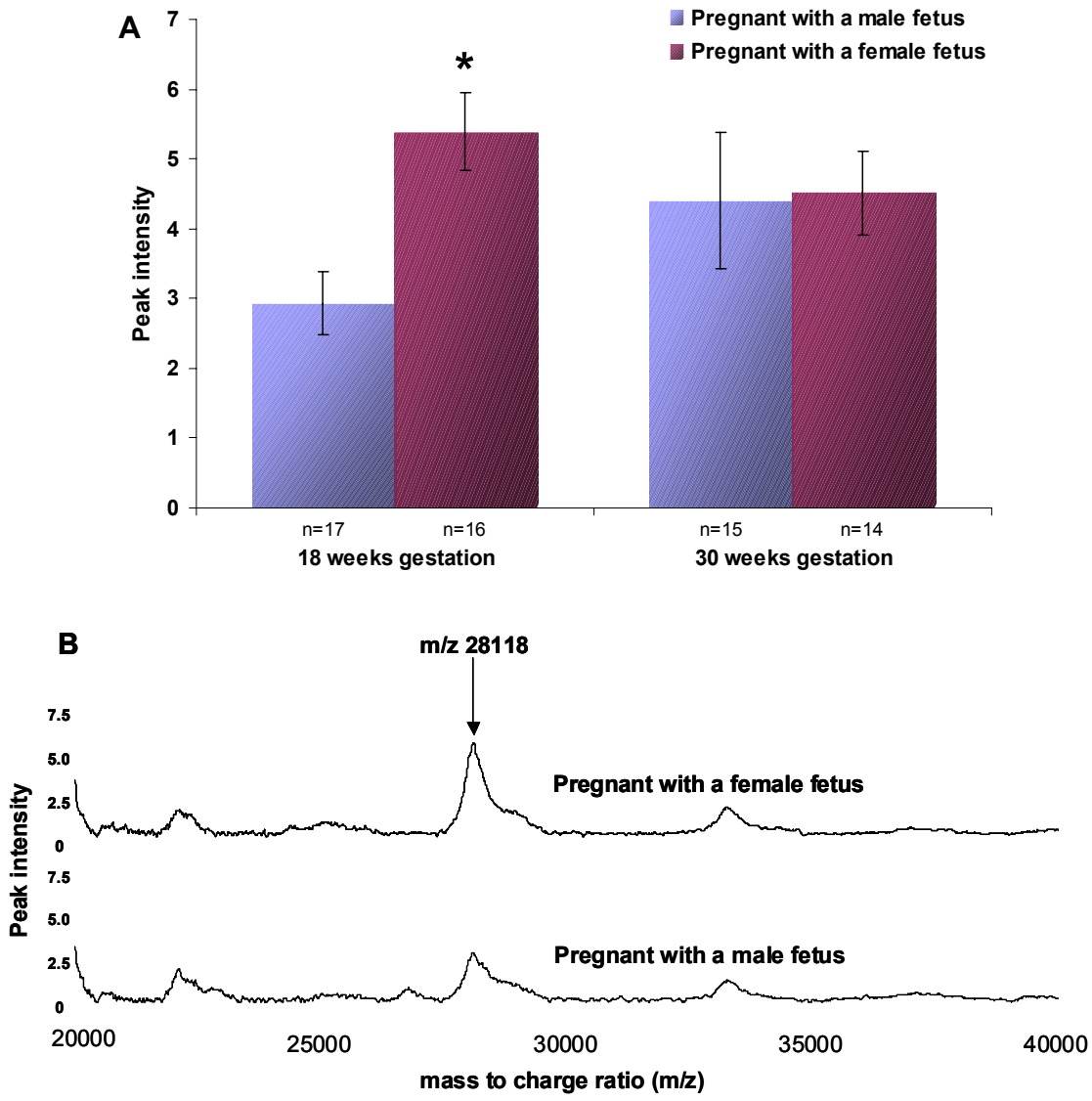

Figure 5.9 Female fetus associated protein in maternal plasma

*Panel A shows the mean peak intensity ± SEM of a maternal plasma peak with m/z 28118 identified using an IMAC chip, pH 7 with SPA matrix. * indicates P<0.05 (t-test). Panel B shows representative spectra from a woman pregnant with a female fetus and a woman pregnant with a male fetus, in the high mass region 20-40 kDa.*

5.5.3 The effect of asthma and fetal sex on maternal plasma proteins during pregnancy

The progression of asthma during pregnancy is unpredictable, although in general one third of asthmatic women have an improvement, one third have a worsening and one third remain unchanged (134). The data presented in this chapter indicate that female fetal sex results in worsening asthma, regardless of treatment with inhaled glucocorticoids. In the presence of a male fetus, the data presented in this chapter suggests that there is no change or an improvement in maternal asthma. In light of this information, maternal plasma proteins were analysed in the following way. The control group was compared to asthmatic women pregnant with a male fetus (asthma male) and asthmatic women pregnant with a female fetus (asthma female), with the glucocorticoid and no glucocorticoid groups combined. In addition, the asthma male and asthma female groups were compared with each other. Peaks which were significantly and uniquely different for either the asthma male group or the asthma female group were selected. It was hypothesised that there may be more differences in the plasma protein profile of asthmatic women pregnant with a female fetus than asthmatic women pregnant with a male fetus when compared to the control group, reflecting more asthma associated changes in plasma proteins.

At 18 weeks gestation, nine peaks were specifically increased in asthmatic women pregnant with a female fetus, while four peaks were specifically increased in asthmatic women pregnant with a male fetus. At 30 weeks gestation, this number increased to 15 peaks which were specifically increased in asthmatic women pregnant with a female fetus and eight peaks which were specifically increased in asthmatic women pregnant with a male fetus. All the peaks represented different proteins. This pattern indicates that as pregnancy progresses, the plasma protein profile of asthmatic women becomes less like that of the non-asthmatic control group, particularly when pregnant with a female fetus (Figure 5.10). Further identification of these peaks, especially those which differed at 30 weeks gestation, may improve our understanding of the changes in maternal asthma which occur during pregnancy, and specifically those changes which are influenced by fetal sex. Although the identity of these peaks is unknown, it is speculated that the peaks which are associated with being asthmatic and pregnant with a female fetus may be pro-inflammatory, thus promoting a worsening of asthma, while

those associated with being asthmatic and pregnant with a male fetus may be anti-inflammatory, thus contributing to a possible improvement in asthma during pregnancy.

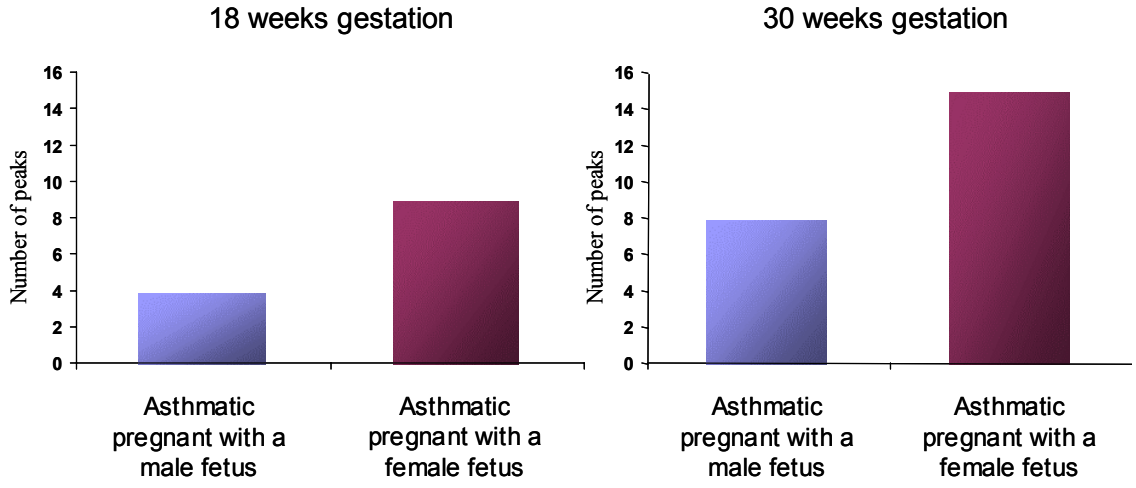

Figure 5.10 Changes in maternal plasma protein profiles in the presence of asthma according to fetal sex

This graph shows that the number of peaks at 18 and 30 weeks gestation which were uniquely different in asthmatics pregnant with a female fetus increased from nine to 15 through pregnancy, while the number of peaks which were uniquely different in asthmatics pregnant with a male fetus increased from four to eight from 18 to 30 weeks gestation.

To examine possible mechanisms associated with reduced female fetal growth in the no glucocorticoid group, protein peaks which were significantly increased or decreased in this group were examined. The aim was to gain further insight into the mechanisms involved in fetal growth restriction in asthmatic pregnancies. There were three peaks which were more highly expressed in the no glucocorticoid female group, and four peaks which were less highly expressed in the no glucocorticoid female group compared to the other groups. For example, one 18 week peak identified on the SAX chip at pH 9 with CHCA matrix was significantly lower in the no glucocorticoid female group compared to the other groups (ANOVA, P=0.022, category B). The m/z of this peak was 22205. A peak with a similar m/z (22231) and a similar pattern of expression at 18 weeks was also identified using the SAX chip at pH 9, with SPA as a matrix (ANOVA, P=0.0005, category B). The mean peak intensities of these two peaks in all groups are shown in Figure 5.11. Searching the Swiss-Prot database for a MW 22231 ± 0.5% or 22205 ± 0.5%, with pI 5 ± 5 revealed 29 common matches. One of these matches was superoxide dismutase (SOD), an enzyme which protects against superoxide induced damage. There is much interest in the role of oxidative stress in asthma (720-722) and

further investigation into the role of SOD in pregnant women with asthma could be of interest. It is also possible that these two peaks do not represent the same protein, but have different identities.

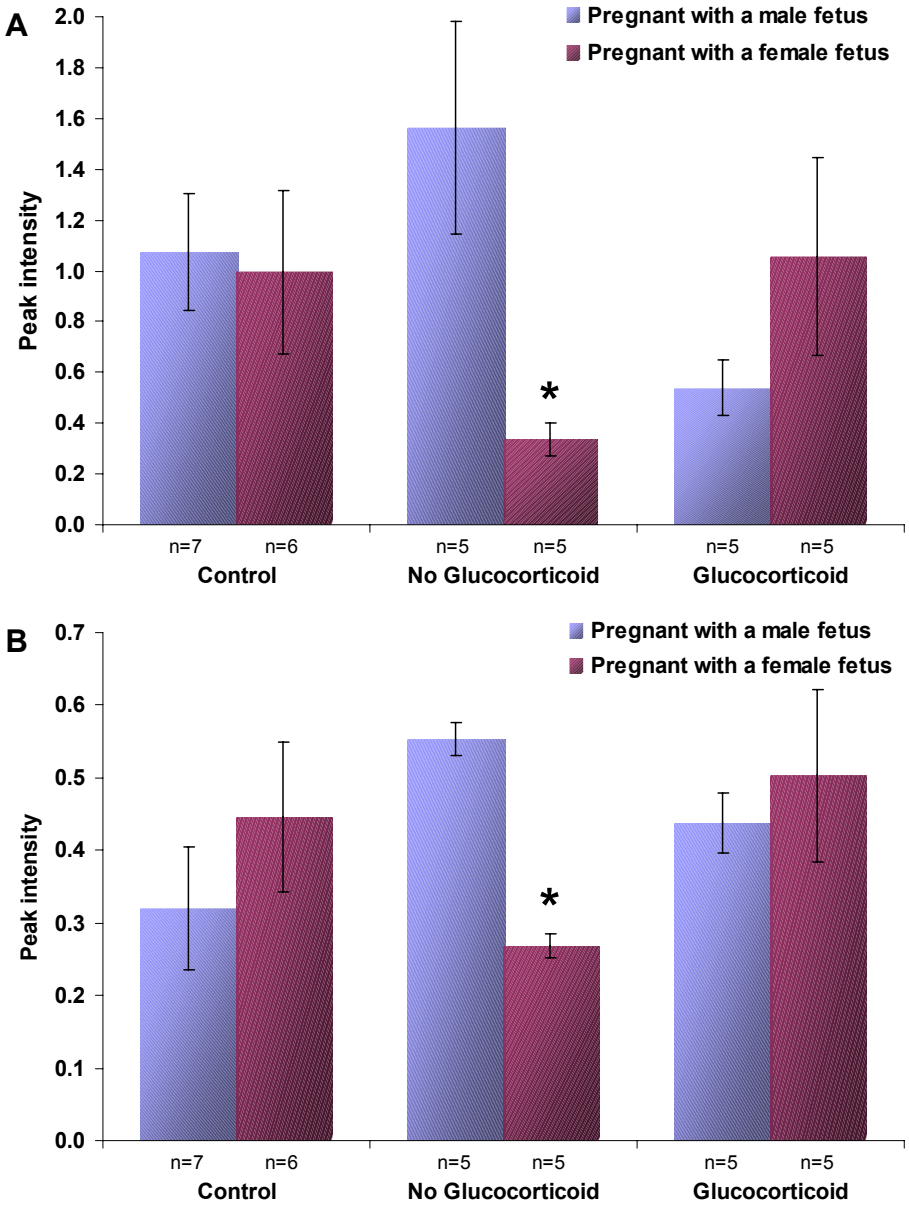

Figure 5.11 Maternal plasma peak 22205 and 22231

*There was a significant decrease in 18 week maternal plasma mean peak intensities of peak 22205 (Panel A, SAX, pH 9, CHCA matrix) and peak 22231 (Panel B, SAX, pH 9, SPA matrix) in women pregnant with a female fetus from the no glucocorticoid group. * indicates P<0.05 (ANOVA).*

In addition, maternal plasma proteins which increased from 18 to 30 weeks gestation in the no glucocorticoid female group were examined. One peak, with m/z 6556, identified using WCX pH 4, was found to significantly increase from 18 to 30 weeks gestation

only in the no glucocorticoid group, when women were pregnant with a female fetus (Figure 5.12A, ANOVA, *P*=0.016). This peak may be a chemical alteration, possibly an oxidised derivative of another peak on the spectrum with a m/z of 6640 Da (Figure 5.12B). Further identification of these peaks and characterisation of their function is required to fully understand their role in asthma during pregnancy. However, this peptide may be involved in the worsening of maternal asthma observed in the no glucocorticoid group as gestation progresses or it may contribute to alterations in placental function.

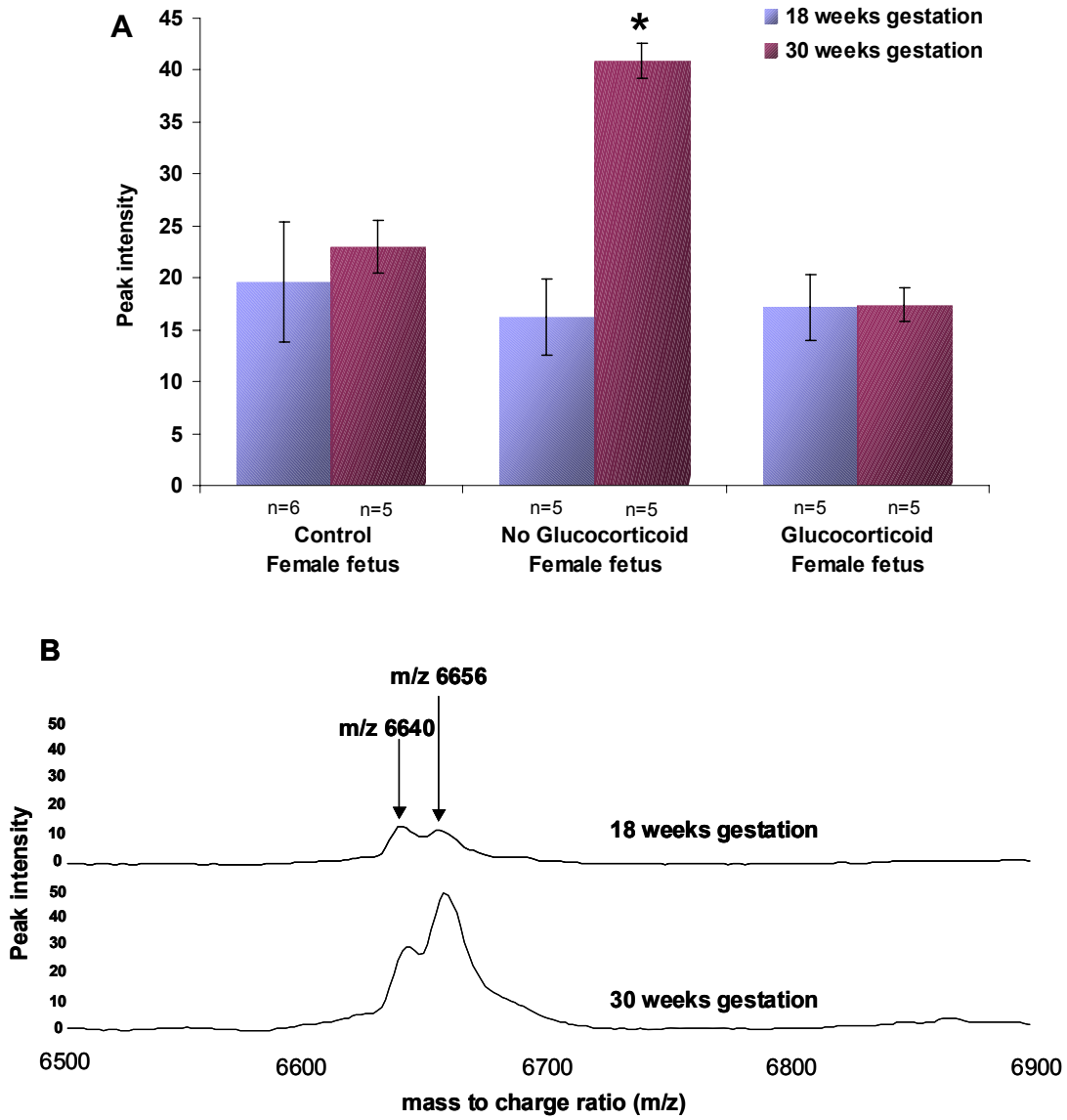

Figure 5.12 Maternal plasma peak 6556 at 18 and 30 weeks gestation

*Panel A shows the mean peak intensity ± SEM of a peak with m/z 6556 identified using a WCX chip, pH 4 with SPA matrix. * indicates P<0.05 (ANOVA). Panel B shows matched 18 and 30 week spectra from an asthmatic woman (no glucocorticoid) pregnant with a female fetus in the region 6500-6900 Da.*

5.6 Pregnancy outcomes for women with asthma

Previous epidemiological evidence suggests that asthmatic women are more at risk of poor pregnancy outcomes including preterm labour or delivery, pre-eclampsia, PIH, caesarean section and neonatal complications. Data was extracted from the medical records on maternal blood pressure in late pregnancy, the type of labour (spontaneous, induced, augmented or no labour), the total duration of labour, the duration of ruptured membranes, blood loss during delivery, the presence of meconium staining and the type of delivery (vaginal or C section delivery). The frequency of PIH or pre-eclampsia (combined) in each group was determined. Results are presented in Table 5.5 for groups classified according to asthma severity and in Table 5.6 for groups classified according to glucocorticoid intake. Data was also analysed separately for women pregnant with male and female fetuses (data not shown), but due to the small numbers, statistical analysis was performed on the combined male/female groups only.

Women with mild and severe asthma were found to have significantly higher systolic blood pressure in late gestation compared to non-asthmatic women (Kruskal-Wallis ANOVA, P=0.007, Dunn's multiple comparisons test, P<0.05). In the control group, 7% of women had PIH or pre-eclampsia diagnosed during pregnancy. However in the asthmatic groups, the frequency was higher, with 16% of women with mild asthma, 11% of women with moderate asthma and 13% of women with severe asthma having PIH or pre-eclampsia. However, these rates were not significantly different from the control group (Fisher's exact test, P>0.05).

The rates of augmented labour, vaginal delivery, emergency caesarean section and meconium staining did not differ significantly between the groups (Fisher's exact test, P>0.05). However, the rate of spontaneous labour onset was found to be significantly lower in the moderate asthma group (57%) compared to the control group (83%, Fisher's exact test, P=0.037). A recent multi-centre prospective study found an increased rate of caesarean delivery in women with moderate and severe asthma (65).

Table 5.5 Pregnancy outcomes for combined groups classified by asthma severity

	Classification of Asthma Severity During Pregnancy			
	Control	Mild	Moderate	Severe
Number of Subjects	43	62	27	46
Maternal Systolic Blood Pressure (mm Hg)	115 ± 2 (n=32)	123 ± 2 (n=61)*	117 ± 2 (n=27)	124 ± 2 (n=45)*
Maternal Diastolic Blood Pressure (mm Hg)	70 ± 2 (n=32)	74 ± 1 (n=61)	71 ± 2 (n=27)	73 ± 2 (n=45)
Gestational Age at Blood Pressure Measurement (weeks)	37.8 ± 0.6 (n=32)	38.4 ± 0.3 (n=61)	37.7 ± 0.6 (n=27)	38.5 ± 0.3 (n=45)
PIH or Pre-eclampsia (% of subjects)	7%	16%	11%	13%
Spontaneous Labour Onset (% of subjects)	83%	78%	57%*	71%
Induced Labour Onset (% of subjects)	17%	22%	43%	29%
Augmented Labour (% of subjects)	10%	16%	22%	17%
Total Duration of Labour (hours)	6.3 ± 1.0 (n=21)	6.3 ± 0.5 (n=37)	6.1 ± 0.9 (n=18)	6.5 ± 0.8 (n=27)
Duration of Ruptured Membranes (hours)	11.1 ± 2.5 (n=9)	7.5 ± 1.5 (n=20)	14.1 ± 4.4 (n=10)	8.0 ± 2.9 (n=10)
Vaginal Delivery (% of subjects)	88%	79%	74%	78%
C Section Delivery (% of subjects)	12%	21%	26%	22%
Emergency C Section Delivery (% of subjects)	7%	10%	11%	11%
Meconium Staining (% of subjects)	16%	15%	11%	22%
Blood Loss (ml)	344 ± 54 (n=42)	342 ± 29 (n=61)	426 ± 87 (n=27)	359 ± 35 (n=45)
	Values given are Mean ± Standard Error of the Mean			

*Compared to control (ANOVA, $P<0.05$), *Compared to control (Fisher's exact test, $P<0.05$)

Table 5.6 Pregnancy outcomes for combined groups classified by glucocorticoid intake

	Classification of Glucocorticoid Intake During Pregnancy					
	Control	No Glucocorticoid	Low	Moderate	High	Glucocorticoid
Number of Subjects	43	46	21	41	27	89
Maternal Systolic Blood Pressure (mm Hg)	115 ± 2 (n=32)	123 ± 2 (n=45)*	122 ± 3 (n=21)	119 ± 2 (n=40)	125 ± 2 (n=27)*	122 ± 1 (n=88)
Maternal Diastolic Blood Pressure (mm Hg)	70 ± 2 (n=32)	73 ± 2 (n=45)	73 ± 3 (n=21)	72 ± 2 (n=40)	75 ± 2 (n=27)	73 ± 1 (n=88)
Gestational Age at Blood Pressure Measurement (weeks)	37.8 ± 0.6 (n=32)	38.3 ± 0.3 (n=45)	38.9 ± 0.2 (n=21)	38.2 ± 0.5 (n=40)	37.8 ± 0.6 (n=27)	38.2 ± 0.3 (n=88)
PIH or Pre-eclampsia (% of subjects)	7%	11%	19%	10%	22%	16%
Spontaneous Labour Onset (% of subjects)	83%	78%	67%	76%	57%**	68%
Induced Labour Onset (% of subjects)	17%	22%	33%	24%	43%	32%
Augmented Labour (% of subjects)	10%	20%	22%	22%	4%	16%
Total Duration of Labour (hours)	6.3 ± 1.0 (n=21)	6.6 ± 0.8 (n=29)	6.8 ± 0.8 (n=14)	5.7 ± 0.7 (n=24)	6.3 ± 0.9 (n=15)	6.2 ± 0.5 (n=53)
Duration of Ruptured Membranes (hours)	11.1 ± 2.5 (n=9)	8.5 ± 2.9 (n=17)	10.3 ± 1.8 (n=9)	9.4 ± 3.1 (n=8)	9.6 ± 3.4 (n=6)	9.8 ± 1.7 (n=23)
Vaginal Delivery (% of subjects)	88%	78%	76%	73%	85%	77%
C Section Delivery (% of subjects)	12%	22%	24%	27%	15%	23%
Emergency C Section Delivery (% of subjects)	7%	11%	10%	17%	0%	10%
Meconium Staining (% of subjects)	16%	20%	10%	20%	15%	15%
Blood Loss (ml)	344 ± 54 (n=42)	294 ± 18 (n=45)	374 ± 69 (n=21)	434 ± 65 (n=41)	370 ± 43 (n=26)	401 ± 36 (n=88)
	Values given are Mean ± Standard Error of the Mean					

*Compared to control (Kruskal-Wallis ANOVA, $P<0.05$), **Compared to control (Fisher's exact test, $P<0.05$)

When data was analysed based upon inhaled glucocorticoid intake classification, women in the no glucocorticoid and high dose glucocorticoid groups were found to have significantly higher systolic blood pressure in late gestation than women in the control group (Kruskal-Wallis ANOVA, P=0.010, Dunn's multiple comparisons test, P<0.05). Diastolic blood pressure was not significantly different between groups (Kruskal-Wallis ANOVA, P=0.537). There was a trend towards an increase in the number of women with PIH or pre-eclampsia in the high dose glucocorticoid group (22%) compared to the control group (7%, Fisher's exact test, P=0.079). Interestingly, in the high dose group, three out of seven subjects (43%) pregnant with a male fetus had PIH or pre-eclampsia, while only three out of 20 subjects (15%) pregnant with a female fetus had PIH or pre-eclampsia. In the no glucocorticoid group, however, there was a higher rate of PIH and pre-eclampsia in women pregnant with a female fetus (18%) compared to women pregnant with a male fetus (4%). Due to the small numbers, these trends could not be verified statistically.

The rate of spontaneous labour onset was found to be significantly lower in the high dose glucocorticoid group (57%) compared to the control group (83%, Fisher's exact test, P=0.037). However, the rates of augmented labour, vaginal delivery, emergency caesarean section and meconium staining did not differ significantly between the groups (Fisher's exact test, P>0.05).

There were no significant differences in the total duration of labour (ANOVA, P=0.921), duration of ruptured membranes (ANOVA, P=0.974), or blood loss during delivery (Kruskal-Wallis ANOVA, P=0.179) between groups classified according to glucocorticoid intake.

5.7 The Mother - Discussion

Previous studies have demonstrated that there is no change in lung function parameters such as FEV_1, FEV_1:FVC or PEFR across each trimester of pregnancy in asthmatic or non-asthmatic women (43, 81, 115, 116). These earlier studies in pregnant women did not take fetal sex into account when examining maternal lung function. However, my data shows that in asthmatic women pregnant with a female fetus, there is a decrease in FEV_1:FVC from early to late pregnancy regardless of inhaled glucocorticoid use. This significant reduction in maternal lung function was observed between approximately 23 and 32 weeks gestation, which precedes the onset of reduced female fetal growth in the no glucocorticoid group. This is the first report of changes in lung function as measured by FEV_1:FVC during pregnancy in relation to fetal sex.

Schatz *et al.* have previously demonstrated that poor maternal lung function, indicated by lower maternal FEV_1 was associated with a greater incidence of birth weights in the lower quartile and asymmetric growth restriction (83). In their study, FEV_1 was measured several times during pregnancy (3-25 times) and the mean percent predicted FEV_1 was used in the data analysis. I also found a relationship between maternal lung function (FEV_1:FVC at the first AMS visit) and neonatal birth weight among females from the no glucocorticoid group, suggesting that reduced lung function may be a contributing factor to reduced birth weight in this group. However, the lung function of mothers in the glucocorticoid group was significantly worse than lung function in the no glucocorticoid group (with a female fetus present), and yet no changes in female fetal growth were observed in this group and no correlation between lung function and birth weight was found. In addition, the use of anti-inflammatory inhaled glucocorticoids by women with mild asthma was associated with female birth weights comparable to control non-asthmatics. Together, the data suggest that alterations in maternal inflammatory pathways rather than alterations in lung function alone, may be a major component of the mechanism contributing to low birth weight in asthmatic pregnancies.

Maternal hypoxia may not be a direct contributor to low birth weight in pregnancies complicated by mild asthma. Although maternal oxygenation was not directly measured in this study, the women who had smaller babies were mild asthmatics who reported few symptoms or exacerbations of asthma during their pregnancies. Mild asthmatics are

not thought to be hypoxic (723). Valente *et al.* studied a group of mild asthmatics with basal PO_2 levels which were slightly lower than healthy controls (borderline statistical significance), but were within the normal range and increased with β_2-agonist inhalation (724). Gries *et al.* found no difference in overnight oxygen saturation in asthmatics who did not use any medications compared to healthy controls (725). However, it is possible that PO_2 in the placental circulation may be unrelated to PO_2 at the level of the lungs. Conversely, patients with acute severe asthma requiring hospitalisation have reduced PO_2 (89, 723) sometimes requiring oxygen therapy (90). Hypoxia is a feature of asthma exacerbations (88), but unlikely to be significant in the women who were classified as mild asthmatics and did not require inhaled glucocorticoids. A study of high altitude pregnancies in Saudi Arabia did find reduced birth weight in asthmatic women (62). They suggested that the hypoxic environment may have contributed to this outcome; however, all of the asthmatics in their study had severe asthma, as they were selected based on visits to the emergency room for asthma during pregnancy (62). The hypoxic high altitude environment is associated with reduced late gestation fetal growth (293), which was evident by ultrasound from 25 weeks gestation, in a study of over 400 women, using regression models (320). In my study, no differences in fetal growth were observed by ultrasound up to 30 weeks gestation, suggesting that the overall reduction in fetal growth occurs after this point. Previous work in our population of asthmatics demonstrated altered placental blood flow in women with moderate and severe asthma, but not in women with mild asthma or women who did not use inhaled glucocorticoids (111). This suggests that altered placental blood flow, often a consequence of maternal hypoxia (324), may not be contributing to the reduced female birth weight in mild asthmatics observed in this study.

The use of β_2-agonists by women with mild asthma who did not use inhaled glucocorticoids is unlikely to contribute to low birth weight. Although within the no glucocorticoid group, women who used β_2-agonists had lower female birth weights than those who did not use β_2-agonists, this is likely to be related to asthma symptoms and asthma-associated inflammation, rather than the drug use itself, since the β_2-agonist was used less than two times per week, compared to an average of 12 times per week in the glucocorticoid group. Schatz *et al*. found no effect of β_2-agonist use on birth weight or any other fetal outcomes in 259 subjects, compared to 101 asthmatics who did not use β_2-agonists and 295 control non-asthmatic subjects (183). In my study, there was

evidence of two sub-groups of asthmatic women in the no glucocorticoid group: those who were symptomatic and those who were in remission. Those asthmatics considered in remission had normal female birth weights compared to the control group, while the presence of very mild symptomatic asthma contributed to reduced birth weight in asthmatic pregnancies. The possible beneficial effect of low dose inhaled glucocorticoids in symptomatic women with mild asthma will need to be investigated in a future randomised trial.

Asthma remission may protect against changes in female fetal growth through reduced inflammation as a result of improved clinical status. Noma *et al.* found that adolescents in remission from atopic asthma, had significant reductions in spontaneous IL-1α secretion, antigen-stimulated IL-1α or IL-1β secretion and the proliferative response to IL-5 in peripheral blood mononuclear cells compared to adolescents with active asthma (726, 727). However, airway obstruction (728) or bronchial hyperresponsiveness may still be present in individuals in remission (729). In addition, similar exhaled NO levels in patients in remission and with current asthma suggest that inflammation of the airway persists, despite remission of clinical symptoms (730). One study described increased eosinophils, T cells, mast cells and IL-5 in airway mucosa, as well as increased blood eosinophil counts in subjects in remission who had experienced no symptoms and used no medication for at least 12 months, compared to healthy controls (731). It is possible that inflammatory factors which are improved during clinical remission, contribute to reduced female fetal growth in symptomatic mild asthmatics who do not use inhaled glucocorticoids.

Recent data in asthmatic pregnancies suggests that women pregnant with a female fetus have increasing asthma severity as gestation progresses (164, 165), and an increase in the incidence of complications such as pre-eclampsia or preterm delivery (49). As reviewed previously, the possibility of an association between fetal sex and maternal asthma severity was first raised in the 1930s, but in these earlier studies the effect of fetal sex varied between individual women and only small numbers of patients were studied (140, 166, 170). One recent publication found no significant association between fetal sex and changes in maternal asthma during pregnancy (142). In addition, this study examined many other factors such as season of delivery, maternal smoking and maternal body weight and only found that changes in rhinitis symptoms were significantly associated with changes in maternal asthma during pregnancy (142). I

found that overall, inhaled glucocorticoid intake by asthmatic women using moderate or high doses significantly increased in late pregnancy when women were pregnant with a female fetus, suggesting an up-regulation of inflammation associated with asthma as gestation progressed. Conversely, no significant increase in glucocorticoid requirements was found in asthmatic women pregnant with a male fetus. This finding may also have been related to the decrease in lung function found in asthmatics pregnant with a female fetus, leading to an increase in glucocorticoid therapy in those asthmatic women already using these medications. Previous work has shown that up to 42% of asthmatic women require more therapy during pregnancy (55). However, the studies reported by Beecroft *et al.* and Dodds *et al.* did not examine changes in asthma treatment with increasing gestation (164, 165). Beecroft *et al.* reported that mothers of girls were more likely to have a worsening of asthma symptoms in the second trimester, while those pregnant with boys were more likely to improve (164). These results are similar to my finding that there was an increase in the number of asthmatic women pregnant with a male fetus who were symptom free by late pregnancy, while in asthmatic women pregnant with a female fetus there was no change in the number who were symptom free. In addition, I found that women in the glucocorticoid group who were pregnant with a male fetus had a significant reduction in reported night-time asthma symptoms. These data support the hypothesis that alterations in maternal asthma during pregnancy differ with fetal sex.

In the asthmatic women who were medically advised not to use inhaled glucocorticoids because they were assessed as having a very mild disease, a significant rise in the number of circulating monocytes was observed as gestation progressed when they were pregnant with a female fetus. This supports the concept that increased maternal inflammation is associated with reduced female fetal growth in this group. Changes in maternal systemic inflammation in the presence of a female fetus may also be involved in the increased risk of developing pre-eclampsia or preterm labour in asthmatic pregnancies, via a common pathway such as phenotypic activation of maternal monocytes which has previously been observed in women with pre-eclampsia and women in preterm labour (732, 733). In previous studies an increased risk for developing pre-eclampsia or PIH has been observed in women with asthma (42, 49-51, 55, 61, 62). Although numbers are small in my study, there was also a trend towards a greater incidence of hypertension in Australian asthmatic women. Liu *et al.* observed an increased risk for pre-eclampsia in asthmatic women pregnant with a female fetus (49).

In this study, asthma was identified using a coded administrative database, which the authors claim may not have included women with mild or intermittent asthma. In my preliminary analysis of pregnancy outcomes, there appeared to be an increased risk for pre-eclampsia in women pregnant with a male fetus if they used high doses of inhaled glucocorticoids, and women pregnant with a female fetus who did not use inhaled glucocorticoids. Further investigation into the mechanisms contributing to fetal sex-related differences in pre-eclampsia risk in the presence of maternal asthma will be required using larger sample sizes.

Monocytes, the precursors to macrophages, are important inflammatory mediators in asthma, via their interactions with Th2 lymphocytes, eosinophils and mast cells within the asthmatic airway. Alveolar monocytes/macrophages from patients with mild asthma are highly activated (734), as demonstrated by the presence of cell wall antigens required for recognition by $CD4^+$ lymphocytes (735). Previous studies have demonstrated that co-culture of $CD4^+$ T cells with peripheral blood monocytes from atopic asthmatic subjects results in enhanced production of IL-4 and IL-5 (736, 737). In addition, monocytes interact with airway smooth muscle cells *in vitro*, inducing collagen degradation through the induction of matrix metalloproteinase 1, 2 and 9 (738). Monocytes release numerous cytokines including TNF-α (739-742), IL-1β (739, 740, 742), IL-6 (742) and granulocyte macrophage-colony stimulating factor (GM-CSF) (740, 741). In my study, both the number and the percentage of monocytes in the maternal circulation of asthmatics who did not use glucocorticoids and were pregnant with a female fetus, increased during gestation, suggesting that there was a specific up-regulation of this leukocyte, rather than simply an overall increase in white blood cell numbers.

Both the number (743) and function (744-747) of monocytes can be inhibited by glucocorticoid use, thus supporting their specific role in pregnant asthmatic women who do not use inhaled glucocorticoids. Budesonide inhibits GM-CSF production from mononuclear blood cells (748). Steer *et al.* showed a reduction in peripheral blood lymphocytes and monocytes following methylprednisolone injection along with a decreased release of TNF-α from monocytes after 4 hours (749). Brohee *et al.* found that intravenous cortisol led to monocytopenia within 1 hour, while neutrophilia and eosinopenia were observed after 6 hours (750). Similar results were reported in patients on oral dexamethasone or prednisone therapy (751, 752). Increased monocytes may

represent a specific response to worsening asthma in the presence of a female fetus, which is inhibited by glucocorticoid use.

Eosinophil numbers were also higher in early pregnancy in asthmatic mothers compared to non-asthmatics, which is in agreement with previous studies in non-pregnant adults (753). Eosinophilic inflammation has been found to predominate in exacerbations of mild asthma (754). However, many individuals with persistent asthma display a non-eosinophilic pattern of airway inflammation (755), which may be associated with neutrophil activation (756). Although inflammation of the airway was not directly examined in my study, the available evidence from circulating white blood cells suggests that eosinophilic inflammation may not be the primary inflammatory mechanism operating during asthmatic pregnancies. Unlike the monocyte count, the eosinophil count did not differ significantly between mothers pregnant with a male and female fetus in the no glucocorticoid group, nor did it increase as gestation progressed. In fact, eosinophil numbers significantly decreased in the glucocorticoid group as gestation progressed in asthmatic women pregnant with male and female fetuses. These data suggest that in the absence of inhaled glucocorticoid use and in the presence of a female fetus, cytokines derived from circulating monocytes, rather than eosinophils may be important for alterations in placental function which contribute to reduced female fetal growth.

Active maternal inflammation may contribute to low birth weight in asthmatic pregnancies. In women with rheumatoid arthritis, only those with active disease (rather than disease in remission) had reduced birth weights compared to a control group (95). Furthermore, women with inflammatory bowel disease had an increased risk for low birth weight (103), particularly when exacerbations occurred during pregnancy (101). Asthmatic women pregnant with a female fetus showed signs of increased maternal inflammation during pregnancy, with increased circulating monocytes, which may have contributed to reduced fetal growth.

Proteomic analysis indicated that there were a large number of plasma protein differences between asthmatic and non-asthmatic pregnant women. The number of differences decreased during pregnancy, but at each time point, a different set of protein differences was represented. Despite this, there was one asthma associated peptide identified which was significantly higher in asthmatics compared to non-asthmatics at

both 18 and 30 weeks gestation. It is possible that this peptide is a novel protein, which could be a potential target for future drug therapies for asthma. The protein profile of asthmatic women in the no glucocorticoid group also showed at least one peak which increased as gestation progressed and may be a marker of protein modification, possibly oxidation. This will need to be verified with further SELDI studies.

The objective measurements of lung function and circulating white blood cells during asthmatic pregnancies suggest that the female fetus has an effect on maternal physiology. It is possible that factors derived from the female fetus cause changes in inflammation in the mother. It is also possible that factors derived from the male fetus protect the mother against pregnancy-induced alterations in asthma. Fetal DNA (757) as well as mRNA of placental origin (758) has been found to circulate in women for as many as 27 years post-partum (757). This evidence suggests that fetally-derived factors may circulate in pregnant women where they could potentially alter maternal physiology. Preliminary evidence from the proteomic profiling shows that protein expression patterns in the mother also differ depending on the sex of the fetus. In addition, these patterns change during the course of pregnancy, suggesting that there is a dynamic interaction between mother and fetus during pregnancy. Plasma proteins may contribute to changes in maternal inflammatory pathways in asthmatic pregnancies and further work is required to identify these proteins and characterise their role during pregnancy.

5.8 The Mother – Summary

The data outlined in this chapter indicate that there is an interaction between the fetus and the mother (Figure 5.13). The presence of a female fetus resulted in a decrease in lung function as pregnancy progressed, an increasing requirement for inhaled glucocorticoids, and an increase in the circulating levels of monocytes. Factors derived from the fetus may cause changes in inflammation in the mother. The placenta, being vitally important in the control of fetal growth, may represent an additional link between the mother and fetus. The next chapter will examine various aspects of placental function, which may contribute to the mechanism of reduced fetal growth in asthmatic pregnancies.

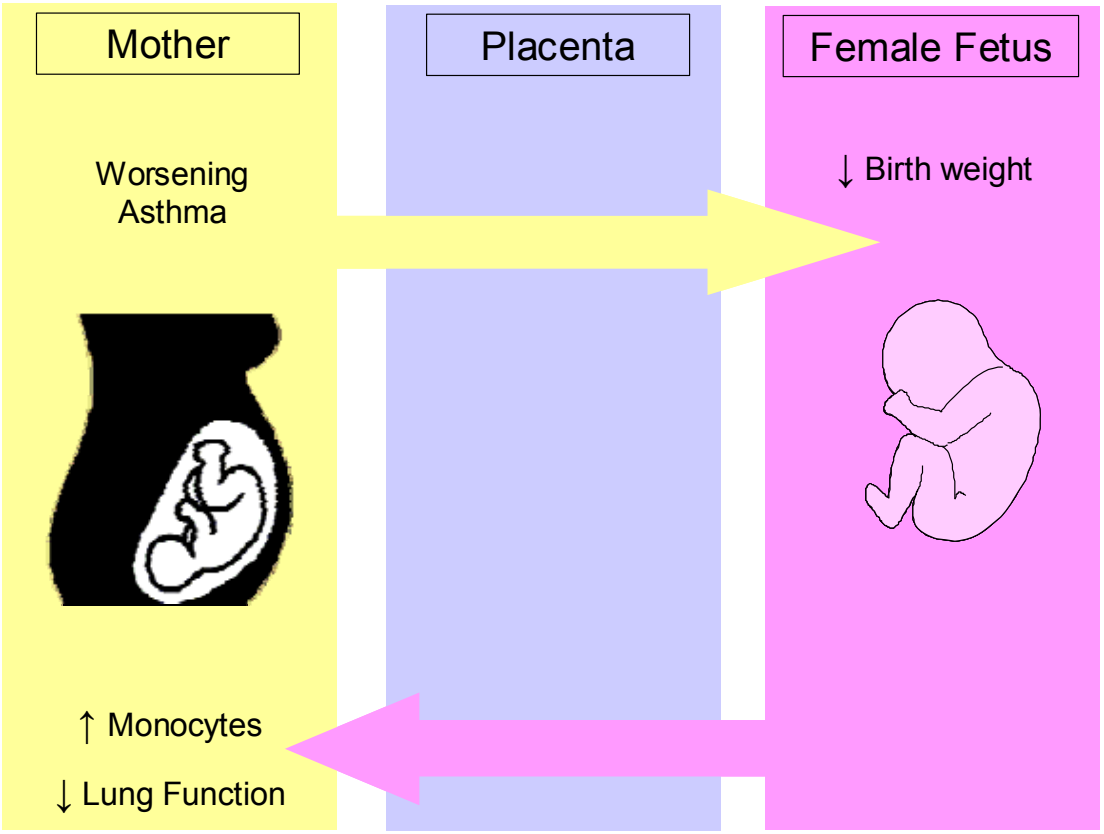

Figure 5.13 The interaction between mother and fetus in pregnancies complicated by asthma

In the presence of a female fetus, maternal asthma worsens during pregnancy, as demonstrated by a significant rise in circulating monocytes and a significant reduction in lung function. These alterations in maternal asthma in the absence of glucocorticoid therapy are associated with significantly reduced female birth weight.

Chapter 6 **The Placenta**

6.1 Placental characteristics

Placental weight and birth weight to placental weight ratios for groups classified by asthma severity are shown in Table 6.1 and for groups classified by inhaled glucocorticoid intake in Table 6.2. When analysed by maternal asthma severity classification, there were no significant differences in any female or male placental characteristics (ANOVA, $P>0.05$).

Despite reduced birth weight in females from the no glucocorticoid group, there was no significant reduction in placental weight in this group compared to females from the control and combined glucocorticoid group (Kruskal-Wallis ANOVA, $P=0.663$). The birth weight to placental weight ratio was slightly lower on average in females from the no glucocorticoid group due to lower birth weight, but was not significantly different from females of the other groups (Kruskal-Wallis ANOVA, $P=0.369$). There was no significant difference in placental weights or birth weight to placental weight ratios between females or males of any group (Kruskal-Wallis ANOVA, $P=0.949$).

SD ratios were determined using Doppler ultrasound measurements made by Prof Warwick Giles and were used to assess umbilical artery flow *in utero*. The SD ratio is normally higher in early pregnancy, decreasing due to increased diastolic flow as a result of fetal development (759, 760). There were no significant differences between groups in the 18 or 30 week gestation SD ratio in females (Kruskal-Wallis ANOVA, $P>0.05$) or males (Kruskal-Wallis ANOVA, $P>0.05$).

Despite normal placental size and umbilical artery blood flow, it is possible that there are other alterations in placental function which contribute to changes in female fetal growth in asthmatic pregnancies.

Table 6.1 Placental characteristics for groups classified by asthma severity

	Classification of Asthma Severity During Pregnancy			
Female Fetus	**Control**	**Mild**	**Moderate**	**Severe**
Placental Weight (g)	607.6 ± 31.8 (n=17)	627.2 ± 27.9 (n=21)	670.3 ± 191.3 (n=2)*	607.6 ± 32.0 (n=16)
Birth Weight: Placental Weight Ratio	5.85 ± 0.21 (n=17)	5.68 ± 0.21 (n=21)	5.26 ± 0.29 (n=2)*	5.77 ± 0.29 (n=16)
SD Ratio at 18 Weeks Gestation	4.15 ± 0.44 (n=8)	4.95 ± 0.35 (n=18)	4.45 ± 0.35 (n=2)*	4.96 ± 0.41 (n=15)
SD Ratio at 30 Weeks Gestation	2.96 ± 0.10 (n=24)	3.18 ± 0.14 (n=28)	3.20 ± 0.23 (n=9)	2.89 ± 0.13 (n=23)
Change in SD Ratio (18 to 30 Weeks)	1.35 ± 0.32 (n=8)	1.89 ± 0.41 (n=17)	0.80 ± 0.50 (n=2)*	1.98 ± 0.38 (n=15)
Male Fetus	**Control**	**Mild**	**Moderate**	**Severe**
Placental Weight (g)	653.0 ± 46.9 (n=16)	673.4 ± 37.4 (n=19)	635.6 ± 39.5 (n=11)	641.1 ± 57.3 (n=7)
Birth Weight: Placental Weight Ratio	5.79 ± 0.33 (n=15)	6.00 ± 0.23 (n=19)	5.68 ± 0.27 (n=11)	5.73 ± 0.34 (n=7)
SD Ratio at 18 Weeks Gestation	4.47 ± 0.26 (n=9)	4.08 ± 0.28 (n=16)	4.23 ± 0.35 (n=8)	4.24 ± 0.59 (n=5)
SD Ratio at 30 Weeks Gestation	2.78 ± 0.08 (n=20)	2.73 ± 0.10 (n=24)	2.74 ± 0.08 (n=16)	2.78 ± 0.10 (n=16)
Change in SD Ratio (18 to 30 Weeks)	1.64 ± 0.24 (n=8)	1.43 ± 0.29 (n=16)	1.50 ± 0.35 (n=8)	1.16 ± 0.59 (n=5)
	Values given are Mean ± Standard Error of the Mean			

*Data not included in statistical analysis due to small number of subjects

Table 6.2 Placental characteristics for groups classified by glucocorticoid intake

	Classification of Glucocorticoid Intake During Pregnancy		
Female Fetus	**Control**	**No Glucocorticoid**	**Glucocorticoid**
Placental Weight (g)	607.6 ± 31.8 (n=17)	639.7 ± 19.3 (n=21)	615.3 ± 35.1 (n=21)
Birth Weight: Placental Weight Ratio	5.85 ± 0.21 (n=17)	5.42 ± 0.17 (n=21)	5.85 ± 0.27 (n=21)
SD Ratio at 18 Weeks Gestation	4.15 ± 0.44 (n=8)	5.14 ± 0.36 (n=19)	4.67 ± 0.34 (n=16)
SD Ratio at 30 Weeks Gestation	2.96 ± 0.10 (n=24)	3.21 ± 0.15 (n=24)	3.00 ± 0.11 (n=36)
Change in SD Ratio (18 to 30 Weeks)	1.35 ± 0.32 (n=8)	2.08 ± 0.41 (n=18)	1.62 ± 0.33 (n=16)
Male Fetus	**Control**	**No Glucocorticoid**	**Glucocorticoid**
Placental Weight (g)	653.0 ± 46.9 (n=16)	664.6 ± 36.1 (n=18)	659.8 ± 31.9 (n=21)
Birth Weight: Placental Weight Ratio	5.79 ± 0.33 (n=15)	5.84 ± 0.21 (n=17)	5.82 ± 0.23 (n=21)
SD Ratio at 18 Weeks Gestation	4.47 ± 0.26 (n=9)	3.83 ± 0.20 (n=14)	4.44 ± 0.33 (n=15)
SD Ratio at 30 Weeks Gestation	2.78 ± 0.08 (n=20)	2.69 ± 0.11 (n=21)	2.76 ± 0.06 (n=36)
Change in SD Ratio (18 to 30 Weeks)	1.64 ± 0.24 (n=8)	1.11 ± 0.25 (n=14)	1.67 ± 0.32 (n=15)
	Values given are Mean ± Standard Error of the Mean		

6.2 Placental 11β-HSD

Previous studies have demonstrated that placental 11β-HSD2 activity and mRNA is reduced in pregnancies complicated by IUGR (554, 558). In addition, cortisol itself and synthetic glucocorticoids are known to have anti-mitogenic effects during pregnancy (486, 503).

6.2.1 Placental 11β-HSD2 activity

Placental 11β-HSD2 activity was found to be significantly lower overall when the fetus was male than when the fetus was female (3.67 ± 0.35 nmol/mg/h, n=26 male vs 5.82 ± 0.497 nmol/mg/h, n=39 female, unpaired t-test with Welch correction, P=0.0007).

When data was analysed according to maternal asthma severity, there was no significant difference in 11β-HSD2 activity in placentae collected from males or females in any group compared to the male or female control non-asthmatic group (ANOVA, females: P=0.818, males: P=0.985).

Placental 11β-HSD2 enzyme activity was lower in placentae collected from female neonates of the no glucocorticoid group (2.60 ± 0.33 nmol/mg/h, n=7) compared to placentae collected from females of the control group (4.96 ± 1.02 nmol/mg/h, n=6) and the combined glucocorticoid group (6.88 ± 0.59 nmol/mg/h, n=26, Kruskal-Wallis ANOVA, P=0.0007, Dunn's multiple comparisons test, no glucocorticoid vs glucocorticoid P<0.05, Figure 6.1). Placental 11β-HSD2 activity was not significantly different between males in the control, no glucocorticoid or glucocorticoid groups (Figure 6.1, ANOVA, P=0.420). Placental 11β-HSD2 activity was significantly lower in placentae collected from males compared to females both within the glucocorticoid group (Mann Whitney test, P=0.0016) and when the control and glucocorticoid groups were combined (Mann Whitney test, P=0.001), but activity was not significantly different between males and females from the no glucocorticoid group (Mann Whitney test, P=0.456).

Within the glucocorticoid group, there was no significant difference in 11β-HSD2 activity between the low, moderate and high dose groups (ANOVA, P=0.968). Activity was significantly reduced in the no glucocorticoid group (Kruskal-Wallis ANOVA, P=0.006) compared to females from the moderate (P<0.05) and high dose groups

(Dunn's multiple comparisons test, $P<0.01$), but was not significantly different from the low dose group ($P>0.05$).

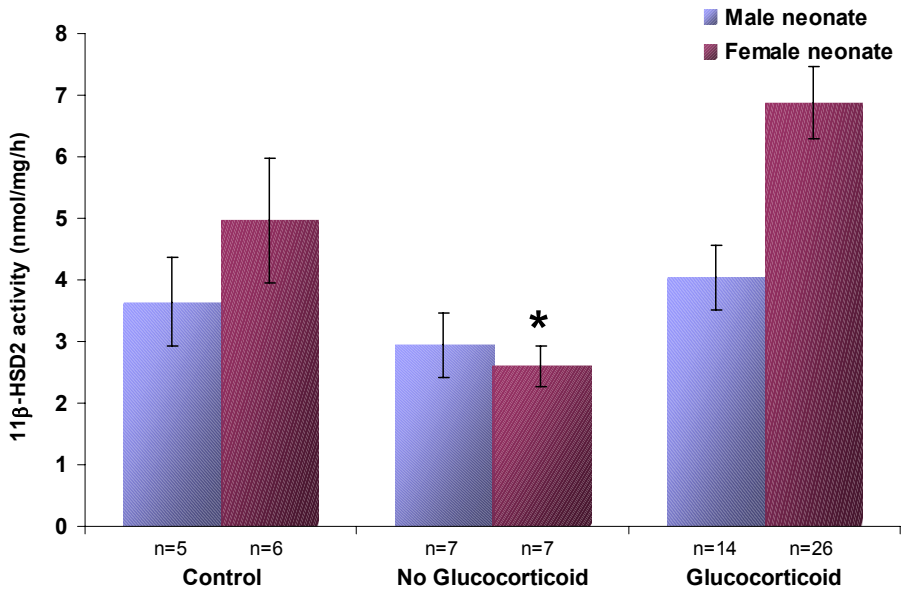

Figure 6.1 Placental 11β-HSD2 activity according to glucocorticoid intake classification

*Mean 11β-HSD2 activity (nmol/mg/h) ± SEM for placentae collected from male and female neonates of the control, no glucocorticoid and glucocorticoid groups. * indicates P<0.05 (Kruskal-Wallis ANOVA, no glucocorticoid female vs glucocorticoid female group).*

Placental 11β-HSD2 activity was not significantly altered by maternal smoking. 11β-HSD2 activity for smokers was 4.63 ± 0.76 nmol/mg/h (n=16) compared to 5.06 ± 0.40 nmol/mg/h (n=49) for non-smokers (unpaired t-test, $P=0.597$). There was no significant difference in placental 11β-HSD2 activity between smokers and non-smokers within any of the no glucocorticoid or glucocorticoid male/female sub-groups (data not shown). This result is consistent with *in vitro* studies showing no effect of nicotine on 11β-HSD2 activity in placental cell cultures (761).

Multivariate analysis verified that 11β-HSD2 activity was associated with fetal sex and maternal glucocorticoid intake ($P=0.008$), but was not affected by maternal asthma severity or smoking.

6.2.2 Placental 11β-HSD2 protein

Placental 11β-HSD2 protein was not significantly related to fetal sex (1.91 ± 0.22, n=25 male, vs 1.90 ± 0.18, n=32 female, unpaired t-test, $P=0.982$).

When placental 11β-HSD2 protein was analysed according to maternal asthma severity, there were no significant differences between the groups (Kruskal-Wallis ANOVA, females: *P*=0.375, males: *P*=0.522).

When placental 11β-HSD2 protein was examined in relation to inhaled glucocorticoid intake, there were no significant differences between the groups (Figure 6.2, Kruskal-Wallis ANOVA, females: *P*=0.575, males: *P*=0.201).

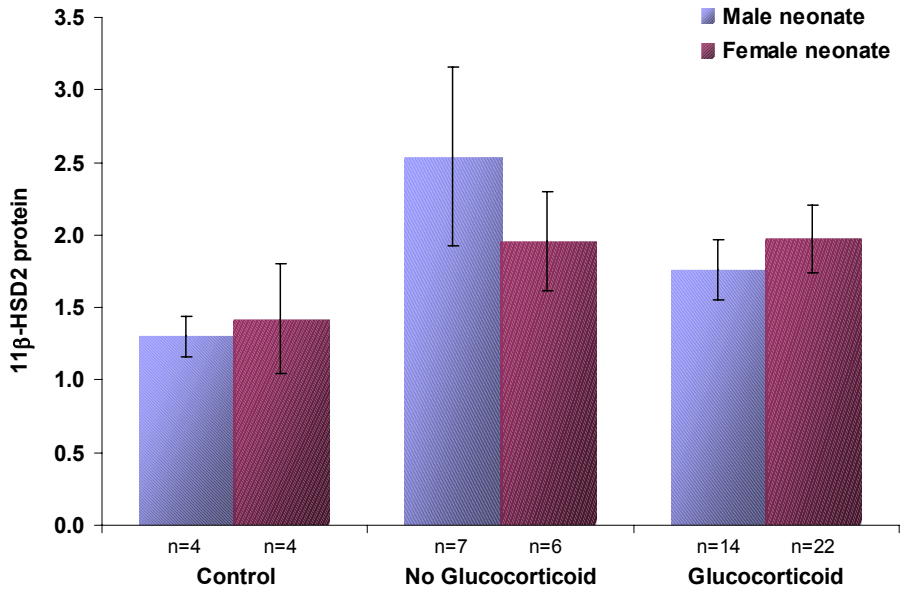

Figure 6.2 Placental 11β-HSD2 protein according to glucocorticoid intake classification

Placental 11β-HSD2 protein was measured by Western blotting. The figure shows mean 11β-HSD2 protein (arbitrary densitometric units) ± SEM for placentae collected from male and female neonates of the control, no glucocorticoid and glucocorticoid groups.

6.2.3 Placental 11β-HSD2 mRNA

Placental 11β-HSD2 mRNA abundance was not significantly different between placentae collected from male (0.654 ± 0.050, n=37) and female neonates (0.630 ± 0.050, n=51, unpaired t-test, *P*=0.734).

11β-HSD2 mRNA in placentae collected from males was not significantly different between the groups classified by maternal asthma severity (ANOVA, *P*=0.1034). However, there was a significant reduction in placental 11β-HSD2 mRNA in females from the severe asthma group compared to the control group (ANOVA, *P*=0.036, Tukey-Kramer multiple comparisons test, *P*<0.05).

Placental 11β-HSD2 mRNA was not significantly different between the groups classified by inhaled glucocorticoid intake (Figure 6.3, ANOVA, females: *P*=0.111, males: *P*=0.408).

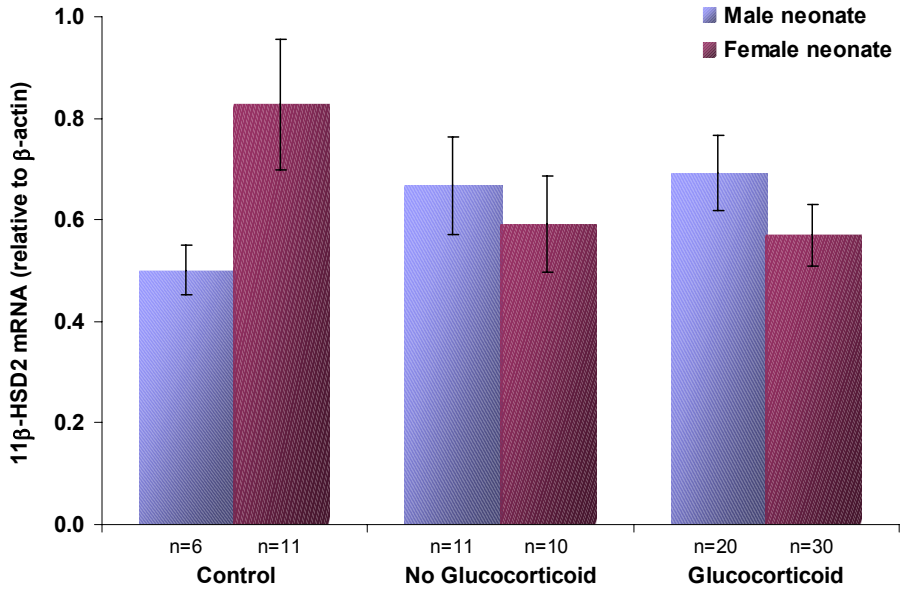

Figure 6.3 Placental 11β-HSD2 mRNA according to glucocorticoid intake classification

Placental 11β-HSD2 mRNA abundance was measured by quantitative real-time RT-PCR. The figure shows mean 11β-HSD2 mRNA abundance (relative to β-actin mRNA) ± SEM for placentae collected from male and female neonates of the control, no glucocorticoid and glucocorticoid groups.

6.2.4 Placental 11β-HSD1 mRNA

Placental 11β-HSD1 and 11β-HSD2 mRNA abundance were both determined by quantitative RT-PCR in 88 placental samples. Two of these samples contained more 11β-HSD1 mRNA than 11β-HSD2 mRNA, while in the remainder, the level of expression of 11β-HSD1 was much lower than that of 11β-HSD2. On average the level of 11β-HSD2 mRNA was 36 times higher than that of 11β-HSD1 mRNA (range 0.6 to 117). This was expected since 11β-HSD1 is found only in the endothelium of placental villous tissue (521), while 11β-HSD2 is found in the syncytiotrophoblast (521, 526). 11β-HSD1 reductase activity was found to be very low in the placenta, and was not easily measurable above background (data not shown).

There was no difference in 11β-HSD1 mRNA between placentae collected from male (0.030 ± 0.005, n=34) and female neonates (0.029 ± 0.003, n=52, Mann Whitney test, *P*=0.519).

Placental 11β-HSD1 mRNA abundance was not significantly different between the groups classified according to asthma severity (ANOVA, males: *P*=0.718, Kruskal-Wallis ANOVA, females: *P*=0.634) or glucocorticoid intake (Kruskal-Wallis ANOVA, females: *P*=0.297, ANOVA, males: *P*=0.860, data not shown).

6.3 Placental CRH

CRH gene expression in the placenta is positively regulated by glucocorticoids (762, 763) and it was hypothesised that exogenous glucocorticoids such as those used by women for asthma treatment, or endogenous glucocorticoids crossing the placenta due to decreased 11β-HSD2 activity, may influence placental CRH mRNA.

There was one outlier in the CRH mRNA data. This subject was a moderate asthmatic using a high dose of inhaled glucocorticoids who had a preterm delivery of a female neonate and had been administered antenatal corticosteroids for fetal lung maturation. As expected, CRH mRNA relative to β-actin in this placenta was more than eight times greater than the control group average. This data point was removed from further analysis.

There was no significant difference between CRH mRNA in placentae collected from male neonates (0.751 ± 0.098, n=37) and placentae collected from female neonates (0.818 ± 0.111, n=42, Mann Whitney test, *P*=0.783).

When combining male and female data (due to small numbers in the control group), there was no significant difference between the control group and the asthma groups classified by asthma severity (Kruskal-Wallis ANOVA, *P*=0.827) or by inhaled glucocorticoid intake (Figure 6.4, Kruskal-Wallis ANOVA, *P*=0.730).

No correlation was found between average pregnancy dose of inhaled glucocorticoids and placental CRH mRNA or the third trimester dose of inhaled glucocorticoids and placental CRH mRNA, suggesting that even high doses of inhaled glucocorticoids used for asthma treatment during pregnancy, do not have an effect on the placenta.

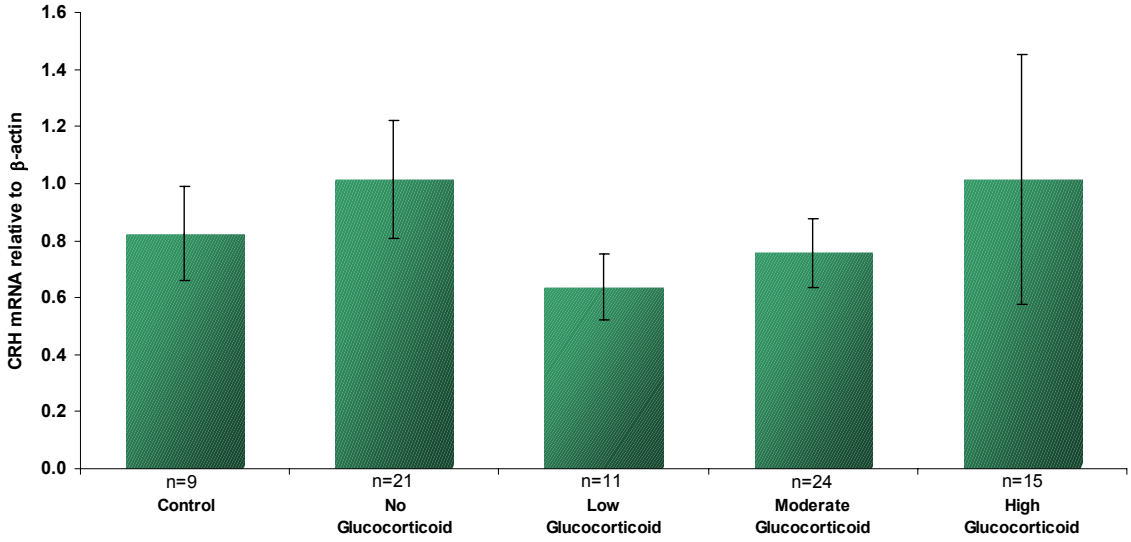

Figure 6.4 Placental CRH mRNA according to glucocorticoid intake classification

Placental CRH mRNA was measured by quantitative real-time RT-PCR. The figure shows mean CRH mRNA abundance (relative to β-actin mRNA) ± SEM for placentae collected from the control, no glucocorticoid, low, moderate and high dose glucocorticoid groups (male and female neonates combined).

6.4 Placental growth factors

Insulin-like growth factors are important growth regulators during pregnancy, both for the fetus and the placenta. IGF-I, IGF-II and IGFBP-1 were possible candidates involved in fetal growth regulation in asthmatic pregnancies and mRNA was measured in the placenta by quantitative real time RT-PCR, comparing abundance to β-actin mRNA.

There was no difference between overall male and female placental expression of IGF-I mRNA (Mann Whitney test, P=0.884), IGF-II mRNA (Mann Whitney test, P=0.436) or IGFBP-1 mRNA (Mann Whitney test, P=0.319).

When data was analysed according to maternal asthma severity, there was a significant reduction in IGF-I mRNA in placentae from females from the severe asthma group compared to the placentae from females from the control group (Kruskal-Wallis ANOVA, P=0.008, Dunn's multiple comparisons test, P<0.05). This was also the group which had a significant reduction in 11β-HSD2 mRNA compared to the control female group. There were no significant differences in IGF-I mRNA between the groups in placentae from male neonates (Kruskal-Wallis ANOVA, P=0.573).

There were no significant differences in placental IGF-II mRNA or IGFBP-1 mRNA in males or females from the mild, moderate or severe asthma groups, compared to males or females from the control group (Kruskal-Wallis ANOVA, $P>0.05$).

When data was analysed according to inhaled glucocorticoid intake classification, there was a significant reduction in placental IGF-I mRNA in females from the glucocorticoid group compared to the control group (Table 6.3, Kruskal-Wallis ANOVA, $P=0.010$, Dunn's multiple comparisons test, $P<0.01$). This may be related to the reduction observed in the severe asthmatic group. There were no significant differences in IGF-I mRNA between males of the control, no glucocorticoid or glucocorticoid groups (Kruskal-Wallis ANOVA, $P=0.423$).

There were no significant differences between the control, no glucocorticoid and glucocorticoid groups in either male or female placental IGF-II mRNA or IGFBP-1 mRNA (Kruskal-Wallis ANOVA, $P>0.05$).

Table 6.3 Placental IGF-I, IGF-II and IGFBP-1 mRNA according to glucocorticoid intake classification

	Classification of Glucocorticoid Intake During Pregnancy		
Female Fetus	**Control**	**No Glucocorticoid**	**Glucocorticoid**
Placental IGF-I mRNA	0.128 ± 0.024 (n=13)	0.161 ± 0.063 (n=13)	0.054 ± 0.020 (n=23)*
Placental IGF-II mRNA	3.84 ± 0.79 (n=13)	4.37 ± 0.88 (n=13)	4.75 ± 0.83 (n=23)
Placental IGFBP-1 mRNA	0.055 ± 0.012 (n=13)	0.079 ± 0.026 (n=13)	0.076 ± 0.017 (n=22)
	Values given are Mean ± Standard Error of the Mean		
*Compared to control (Kruskal-Wallis ANOVA, $P<0.05$)			
Male Fetus	**Control**	**No Glucocorticoid**	**Glucocorticoid**
Placental IGF-I mRNA	0.242 ± 0.119 (n=9)	0.088 ± 0.042 (n=12)	0.094 ± 0.030 (n=17)
Placental IGF-II mRNA	4.12 ± 0.89 (n=9)	5.10 ± 1.12 (n=12)	4.62 ± 0.79 (n=17)
Placental IGFBP-1 mRNA	0.104 ± 0.039 (n=9)	0.037 ± 0.016 (n=12)	0.104 ± 0.049 (n=17)
	Values given are Mean ± Standard Error of the Mean		

6.5 Placental cytokines

Local inflammatory pathways were examined within the placenta by measurement of mRNA abundance for various cytokines including TNF-α, (a representative Th1 cytokine), IL-4 and IL-5 (Th2 cytokines important in asthma), IL-1β, IL-6, IL-8 and IL-10. The Th2:Th1 cytokine mRNA ratio was examined, as asthma is predominantly a Th2 disease (2) and the relative expression of these cytokine types was of interest. The variation in cytokine mRNA between individuals was large, but this was partially overcome when examining ratios.

There was no significant difference between the control group and the mild, moderate or severe asthma groups, separated by fetal sex, for any of the cytokines measured (Kruskal-Wallis ANOVA, $P>0.05$).

When groups were classified by glucocorticoid intake and fetal sex, there were no significant differences between any groups (Table 6.4, Kruskal-Wallis ANOVA, $P>0.05$).

The Th2:Th1 cytokine ratio was assessed in the placenta by measuring TNF-α (Th1) and IL-5, IL-4 or IL-10 (Th2) mRNA. The IL-10:TNF-α ratio was always >1, ranging from 2-83, reflecting the relative Th2 environment of pregnancy, and specifically the placenta. The IL-5:TNF-α ratio was >1 in 83% of all samples tested (range 1-45) and the IL-4:TNF-α ratio was >1 in 77% of samples tested (range 1-17).

There was no difference in the Th2:Th1 cytokine ratios (IL5:TNF-α, IL-4:TNF-α or IL-10:TNF-α) in placentae from females or males of the control, mild, moderate or severe asthma groups (Kruskal-Wallis ANOVA, $P>0.05$).

Table 6.4 Placental cytokine mRNA abundance according to glucocorticoid intake classification

	Classification of Glucocorticoid Intake During Pregnancy		
Female Fetus	**Control**	**No Glucocorticoid**	**Glucocorticoid**
Placental IL-1β mRNA	0.026 ± 0.016 (n=8)	0.020 ± 0.009 (n=10)	0.031 ± 0.014 (n=17)
Placental IL-4 mRNA	0.0028 ± 0.0014 (n=8)	0.0032 ± 0.0013 (n=10)	0.0017 ± 0.0003 (n=20)
Placental IL-5 mRNA	0.0065 ± 0.0029 (n=8)	0.0078 ± 0.0032 (n=11)	0.0022 ± 0.0007 (n=18)
Placental IL-6 mRNA	0.0052 ± 0.0018 (n=6)	0.0025 ± 0.0010 (n=7)	0.0057 ± 0.0011 (n=16)
Placental IL-8 mRNA	0.022 ± 0.008 (n=8)	0.021 ± 0.009 (n=11)	0.043 ± 0.035 (n=20)
Placental IL-10 mRNA	0.0179 ± 0.0076 (n=6)	0.0081 ± 0.0028 (n=8)	0.0071 ± 0.0017 (n=18)
Placental TNF-α mRNA	0.0028 ± 0.0018 (n=8)	0.0013 ± 0.0006 (n=11)	0.0011 ± 0.0003 (n=16)
Male Fetus	**Control**	**No Glucocorticoid**	**Glucocorticoid**
Placental IL-1β mRNA	0.019 ± 0.007 (n=9)	0.017 ± 0.011 (n=6)	0.021 ± 0.009 (n=14)
Placental IL-4 mRNA	0.0028 ± 0.0008 (n=9)	0.0018 ± 0.0005 (n=9)	0.0015 ± 0.0003 (n=16)
Placental IL-5 mRNA	0.0048 ± 0.0016 (n=9)	0.0041 ± 0.0020 (n=9)	0.0026 ± 0.0009 (n=15)
Placental IL-6 mRNA	0.0038 ± 0.0011 (n=6)	0.0047 ± 0.0026 (n=5)	0.0070 ± 0.0023 (n=13)
Placental IL-8 mRNA	0.013 ± 0.004 (n=9)	0.038 ± 0.026 (n=9)	0.016 ± 0.005 (n=13)
Placental IL-10 mRNA	0.0086 ± 0.0037 (n=6)	0.0100 ± 0.0044 (n=8)	0.0073 ± 0.0012 (n=15)
Placental TNF-α mRNA	0.0011 ± 0.0003 (n=9)	0.0012 ± 0.0006 (n=6)	0.0016 ± 0.0007 (n=14)
	Values given are Mean ± Standard Error of the Mean		

Placentae collected from females of the no glucocorticoid group had higher Th2:Th1 cytokine ratios than females of the control or glucocorticoid group. This was evidenced by significantly higher IL-5:TNF-α in this group (Kruskal-Wallis ANOVA, P=0.0003, Dunn's multiple comparisons test, no glucocorticoid vs glucocorticoid, P<0.001). In placentae collected from males, there was a significant difference between the groups, attributable to a lower ratio in the glucocorticoid group (Kruskal-Wallis ANOVA, P=0.032, Dunn's multiple comparisons test, P<0.05, no glucocorticoid vs glucocorticoid). Figure 6.5 shows the IL5:TNF-α ratio in the groups classified by inhaled glucocorticoid intake. For the IL-5:TNF-α ratio, the no glucocorticoid female group contained five of the 10 highest values across all samples tested. Values within this group ranged from 1.8 to 44.8; hence the large standard error bars (all groups were normally distributed).

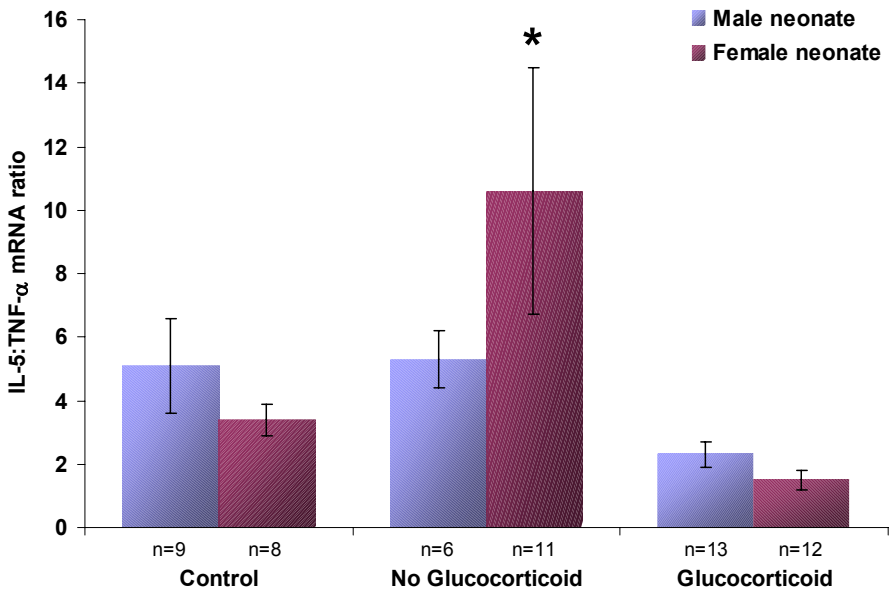

Figure 6.5 Placental IL5:TNF-α mRNA ratio according to glucocorticoid intake classification

*The figure depicts the IL-5:TNF-α (Th2:Th1) cytokine mRNA ratio in the control, no glucocorticoid and glucocorticoid groups for placentae collected from male and female neonates (mean ± SEM). * indicates P<0.05 (Kruskal-Wallis ANOVA, no glucocorticoid female vs glucocorticoid female).*

The IL-10:TNF-α ratio was higher in placentae from females of the no glucocorticoid group compared to females of the glucocorticoid group (Kruskal-Wallis ANOVA, P=0.003, Dunn's multiple comparisons test, no glucocorticoid vs glucocorticoid

P<0.01). However, the IL-4:TNF-α ratio was not significantly different between the groups (Kruskal-Wallis ANOVA, *P*=0.072 for females, *P*=0.717 for males).

Alterations in placental inflammation may be linked to changes in placental 11β-HSD2 activity, either as the cause of decreased 11β-HSD2 activity or as a consequence of this change. There was no relationship between placental Th2:Th1 ratio and 11β-HSD2 activity in male neonates (n=19, r = –0.240, *P*=0.322). However, there was a significant inverse correlation between placental Th2:Th1 ratio and 11β-HSD2 activity in female neonates (Spearman rank correlation, n=20, r = –0.588, *P*=0.006). The data did not differ significantly from linearity (runs test, *P*=0.159). One female data point related to a very high cytokine ratio and when this point was removed, the significant inverse linear correlation between placental Th2:Th1 cytokine ratio and 11β-HSD2 activity in female neonates remained (Figure 6.6, Pearson linear correlation, n=19, *P*=0.016, runs test, *P*=0.834).

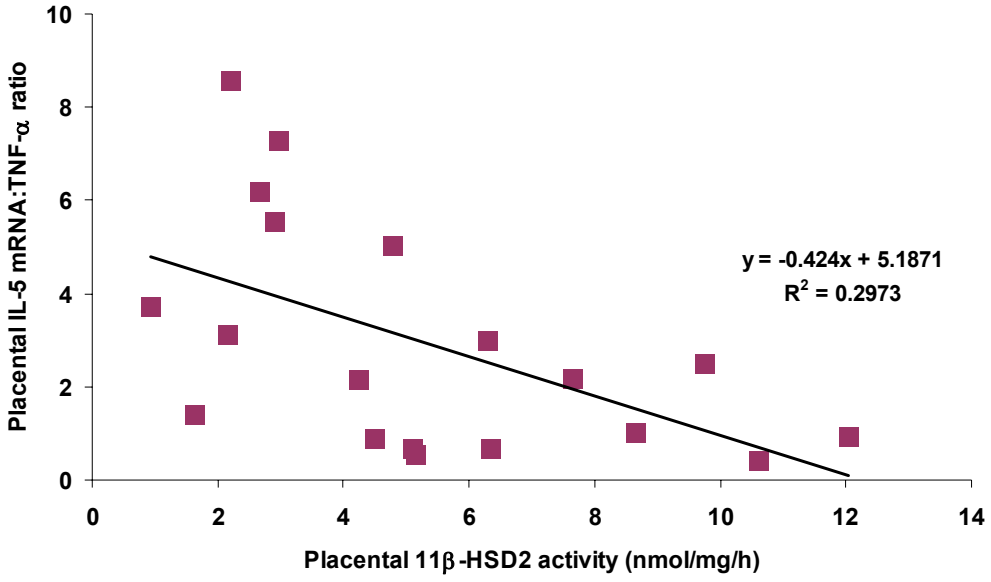

Figure 6.6 The relationship between the Th2:Th1 cytokine ratio and placental 11β-HSD2 activity

The figure shows the significant inverse linear correlation between 11β-HSD2 activity (nmol/mg/h) and IL-5:TNF-α mRNA ratio in placentae collected from female neonates (Pearson linear correlation, P<0.05, n=19).

6.6 Placental glucocorticoid receptors

The actions of cortisol are primarily mediated through the glucocorticoid receptor (GR) and sometimes the mineralocorticoid receptor (MR). There are two isoforms of the GR which determine tissue sensitivity to glucocorticoids. GR-α is the main ligand binding form, while the alternatively spliced GR-β is a dominant negative inhibitor of ligand binding and GR-α action (764). GR-β does not alter the affinity of GR-α for its ligand, but may compete for GRE target sites on DNA sequences of glucocorticoid target genes (764). The mRNAs contain the same first eight exons, but differ in splicing of exon 9 into 9-α or 9-β. GR-β replaces the 50 amino acid C-terminus of GR-α with 15 different amino acids, thus making it unable to bind glucocorticoids (764). Driver *et al.* localised the GR and MR within placental bed biopsies and cultured trophoblast cells (542) and suggested that some of the effects of cortisol within the trophoblast may be mediated via the MR (542).

The data presented so far suggests that the male fetus is relatively insensitive to the growth-restricting effects of cortisol, since the levels of 11β-HSD2 activity were the same in males and females from the no glucocorticoid group and yet only the female had reduced mean birth weight. Male neonates are known to be more prone to developing RDS when delivered preterm, possibly due to differences in cortisol-mediated lung maturation (70, 765). Some authors report that prevention of RDS with betamethasone is more effective in females than males (69, 766), suggesting greater glucocorticoid sensitivity in females. GR expression may give an indication of sensitivity to cortisol. Previous studies have shown that there is increased cytokine-induced expression of GR-β in peripheral blood of glucocorticoid insensitive asthmatics (767). In the placenta of a male fetus, reduced sensitivity to cortisol may be mediated via increased expression of GR-β. Reduced glucocorticoid sensitivity may protect the male fetus from alterations in fetal growth. In the placenta of a female fetus, greater sensitivity to cortisol may be due to "normal" expression of GR-α and GR-β, or to increased GR-α expression. Greater sensitivity to cortisol in the female fetus, when accompanied by reduced placental 11β-HSD2 activity may result in reduced fetal growth (Figure 6.7).

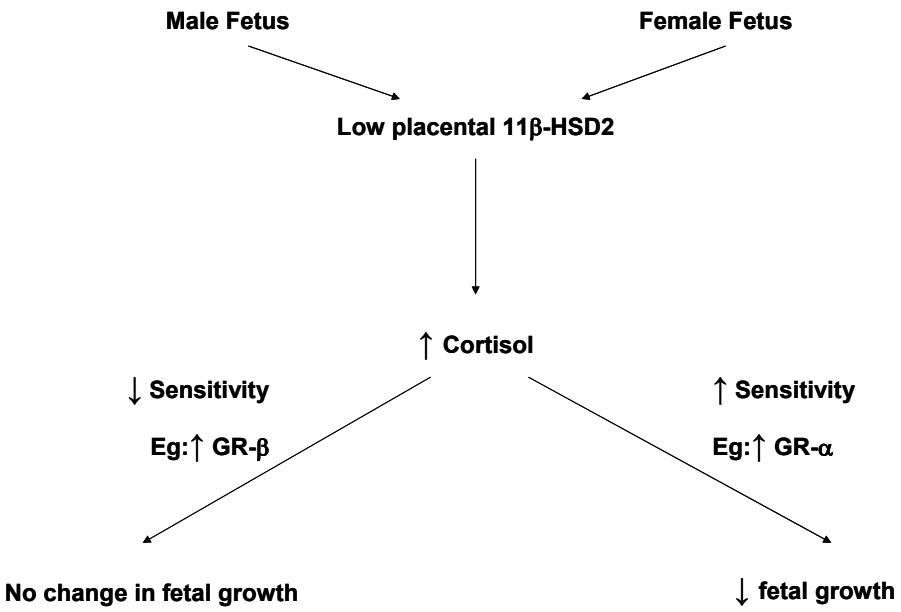

Figure 6.7 Proposed model of altered glucocorticoid sensitivity of male and female fetuses

Low 11β-HSD2 activity in male and female fetuses leads to an increase in the amount of cortisol crossing the placenta from mother to fetus. Decreased sensitivity to cortisol, possibly mediated by increased GR-β expression could account for the lack of change in fetal growth in males. Conversely, increased sensitivity to cortisol, possibly mediated by increased GR-α expression, may account for the decrease in fetal growth observed in female fetuses in the no glucocorticoid group.

The mRNA abundance of GR-α, GR-β and MR were measured in the placenta by quantitative RT-PCR. The GR-α: GR-β ratio was calculated as a relative measure of the active GR expression.

When GR-α, GR-β and MR mRNA abundance and GR-α:GR-β mRNA ratio were examined based upon asthma severity classification, there were no significant differences found between the groups in either placentae from female neonates or male neonates (Kruskal-Wallis ANOVA, $P>0.05$).

Placentae collected from females of the no glucocorticoid group had significantly lower GR-α (Figure 6.8A, Kruskal-Wallis ANOVA, $P=0.019$) and GR-β (Figure 6.8B Kruskal-Wallis ANOVA, $P=0.010$) compared to females from the control and glucocorticoid groups (Dunn's multiple comparisons test, $P<0.05$). The GR-α:GR-β ratio was slightly higher in the female no glucocorticoid group compared to the female control and female glucocorticoid groups, but this was not significant (Figure 6.9, Kruskal-Wallis ANOVA, $P=0.211$). Placentae collected from females of the no

glucocorticoid group had a trend towards lower MR mRNA abundance compared to females from the control group (Figure 6.10, Kruskal-Wallis ANOVA, P=0.054).

Placentae collected from males of the no glucocorticoid group had significantly higher GR-α mRNA compared to males from the control group (Figure 6.8, Kruskal-Wallis ANOVA, P=0.028, Dunn's multiple comparisons test, P<0.05). There were no significant differences in GR-β, GR-α:GR-β ratio or MR mRNA between males of the control, no glucocorticoid and glucocorticoid groups (Kruskal-Wallis ANOVA, P>0.05).

Placentae from males of the no glucocorticoid group had significantly higher GR-α (Kruskal-Wallis ANOVA, P=0.007, Dunn's multiple comparisons test, P<0.05) and GR-β (Kruskal-Wallis ANOVA, P=0.011, Dunn's multiple comparisons test, P<0.01) than placentae from females of the no glucocorticoid group (Figure 6.8).

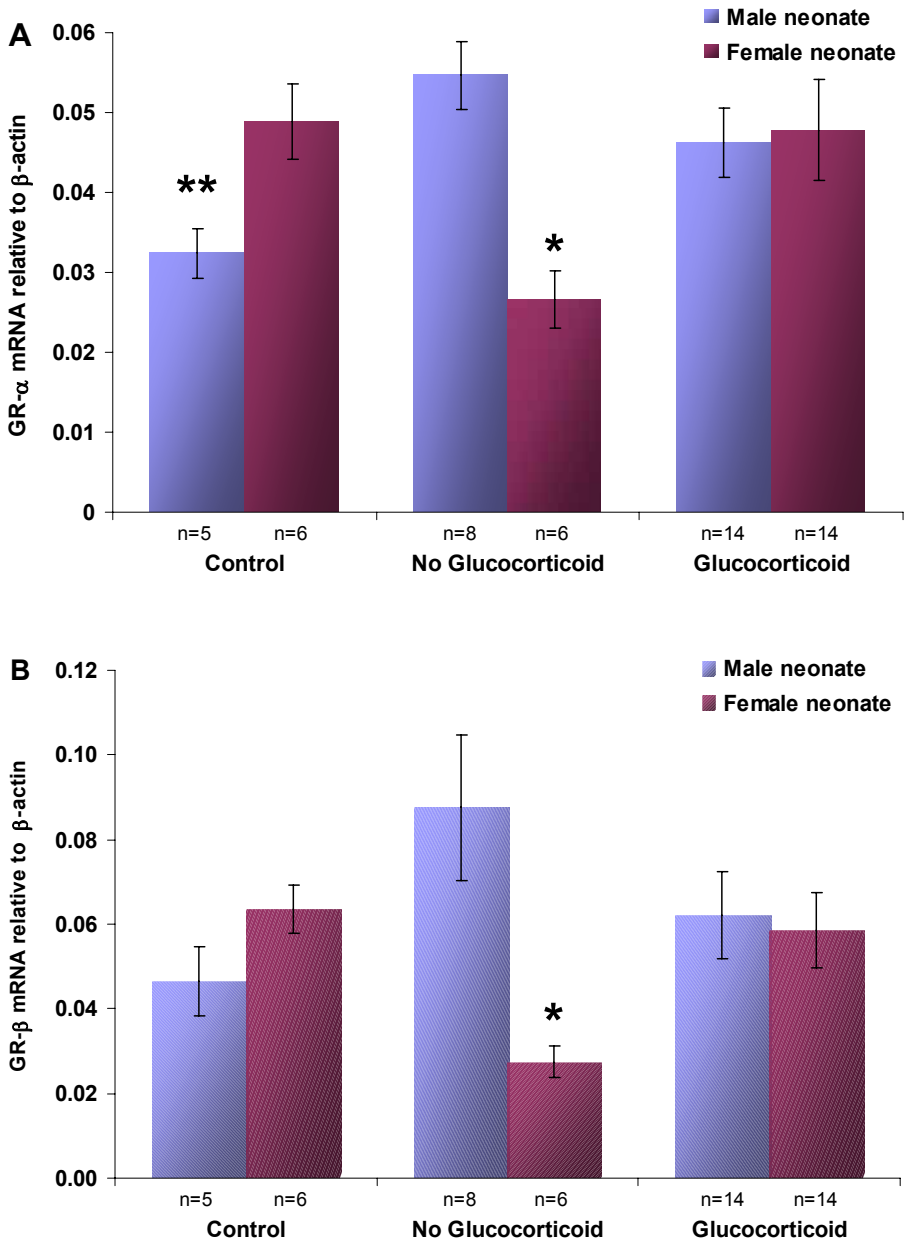

Figure 6.8 Placental GR-α mRNA and GR-β mRNA according to glucocorticoid intake classification

Placental GR-α (Panel A) and GR-β (Panel B) mRNA abundance relative to β-actin is shown for males and females of the control, no glucocorticoid and glucocorticoid groups. For both GR-α and GR-β, there were significant differences between females of the no glucocorticoid group and males of the no glucocorticoid group as well as control females and glucocorticoid females (P<0.05, Kruskal-Wallis ANOVA). For GR-α, males of the control group had significantly lower expression than males of the no glucocorticoid group (** P<0.05, Kruskal-Wallis ANOVA).*

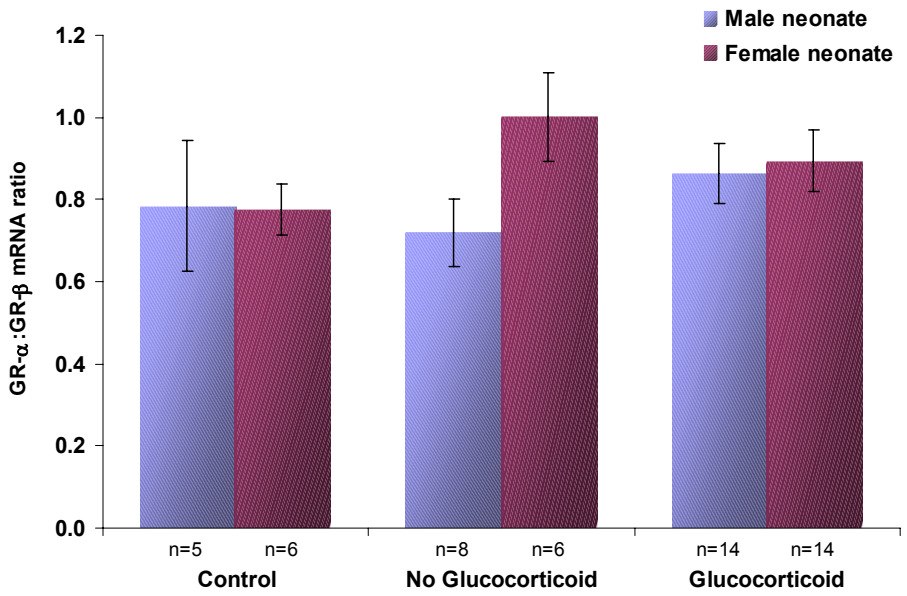

Figure 6.9 Placental GR-α:GR-β mRNA ratio according to glucocorticoid intake classification

The figure depicts the GR-α:GR-β mRNA ratio in the control, no glucocorticoid and glucocorticoid groups for placentae collected from male and female neonates (mean ± SEM).

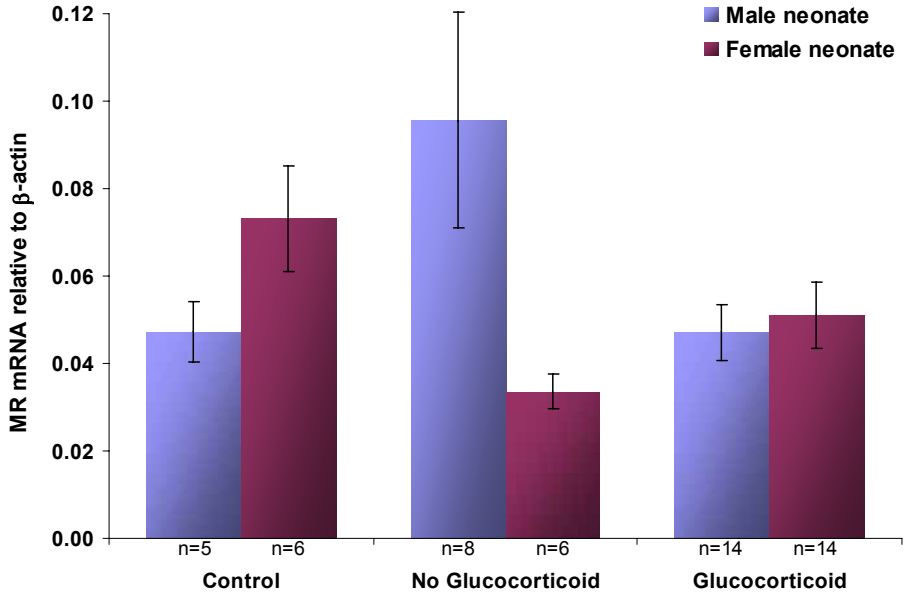

Figure 6.10 Placental MR mRNA abundance according to glucocorticoid intake classification

The figure depicts MR mRNA abundance (relative to β-actin) in the control, no glucocorticoid and glucocorticoid groups for placentae collected from male and female neonates (mean ± SEM).

6.7 Placental protein profile

6.7.1 The effect of maternal asthma on placental proteins

Protein profiling of placental homogenates was carried out using SELDI-TOF MS to further investigate changes in placental function which occur in the presence of maternal asthma. Comparisons were made between asthmatics (n=20) and non-asthmatics (n=10), and between women pregnant with a female fetus (n=15) and women pregnant with a male fetus (n=15).

There were seven proteins more highly expressed and seven proteins less highly expressed in asthmatic placentae compared to non-asthmatic placentae. An example is shown in Figure 6.11 of a peak with m/z 2944. This peak did not match any other peptides in the Swiss-Prot database.

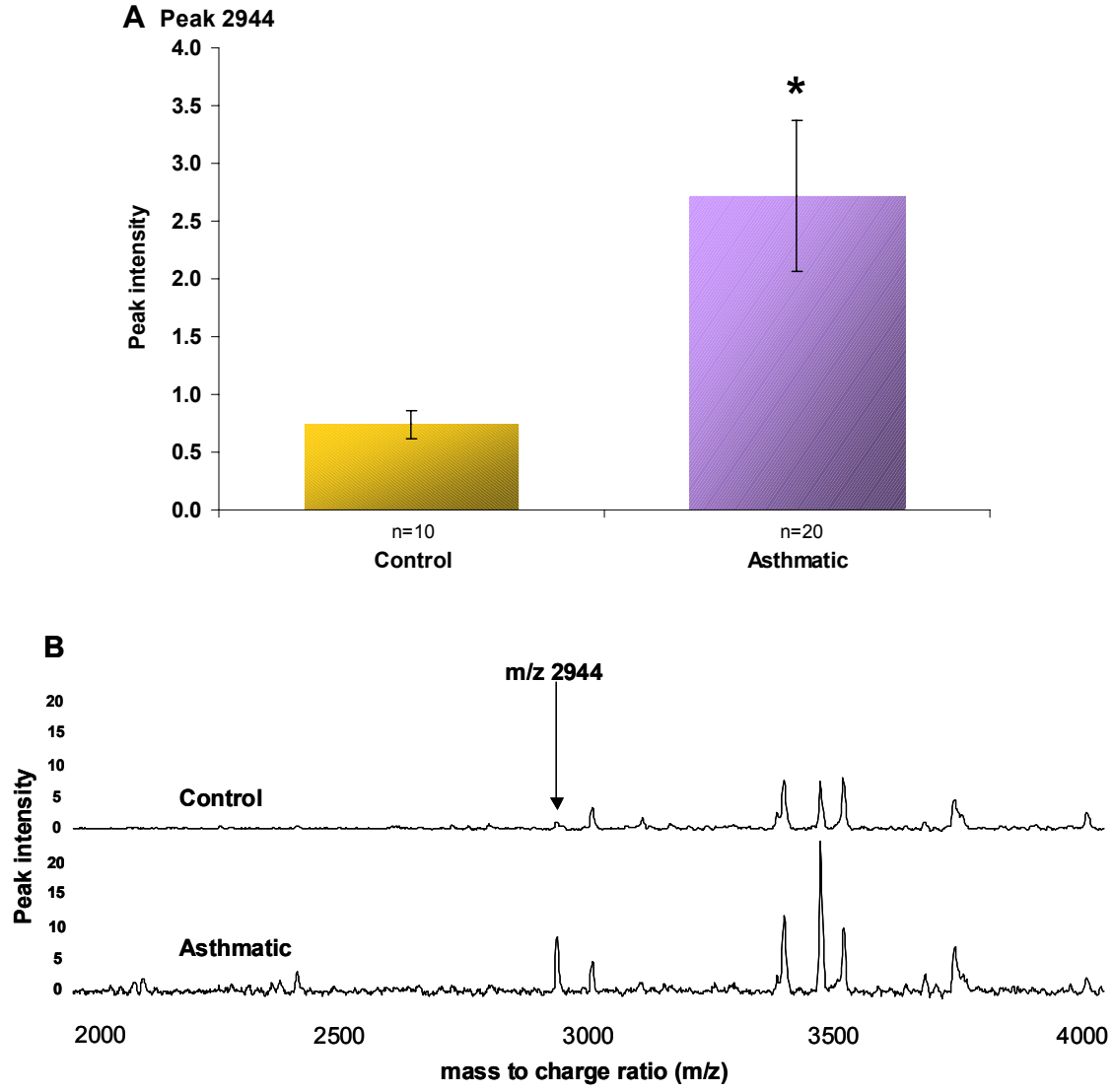

Figure 6.11 Protein 2944 in asthmatic and non-asthmatic placentae

*Panel A shows the mean peak intensity ± SEM of a placental peak with m/z 2944 identified using a WCX chip, pH 9 with SPA matrix. * indicates P<0.05 (t-test). Panel B shows representative spectra from placentae collected from control non-asthmatic and asthmatic women in the region 2-4 kDa.*

6.7.2 The effect of fetal sex on placental proteins

There were four placental proteins which differed significantly between samples collected from male or female neonates. An example is shown in Figure 6.12 of a peak with m/z 3109. This peak matched no other peptides in the Swiss-Prot database.

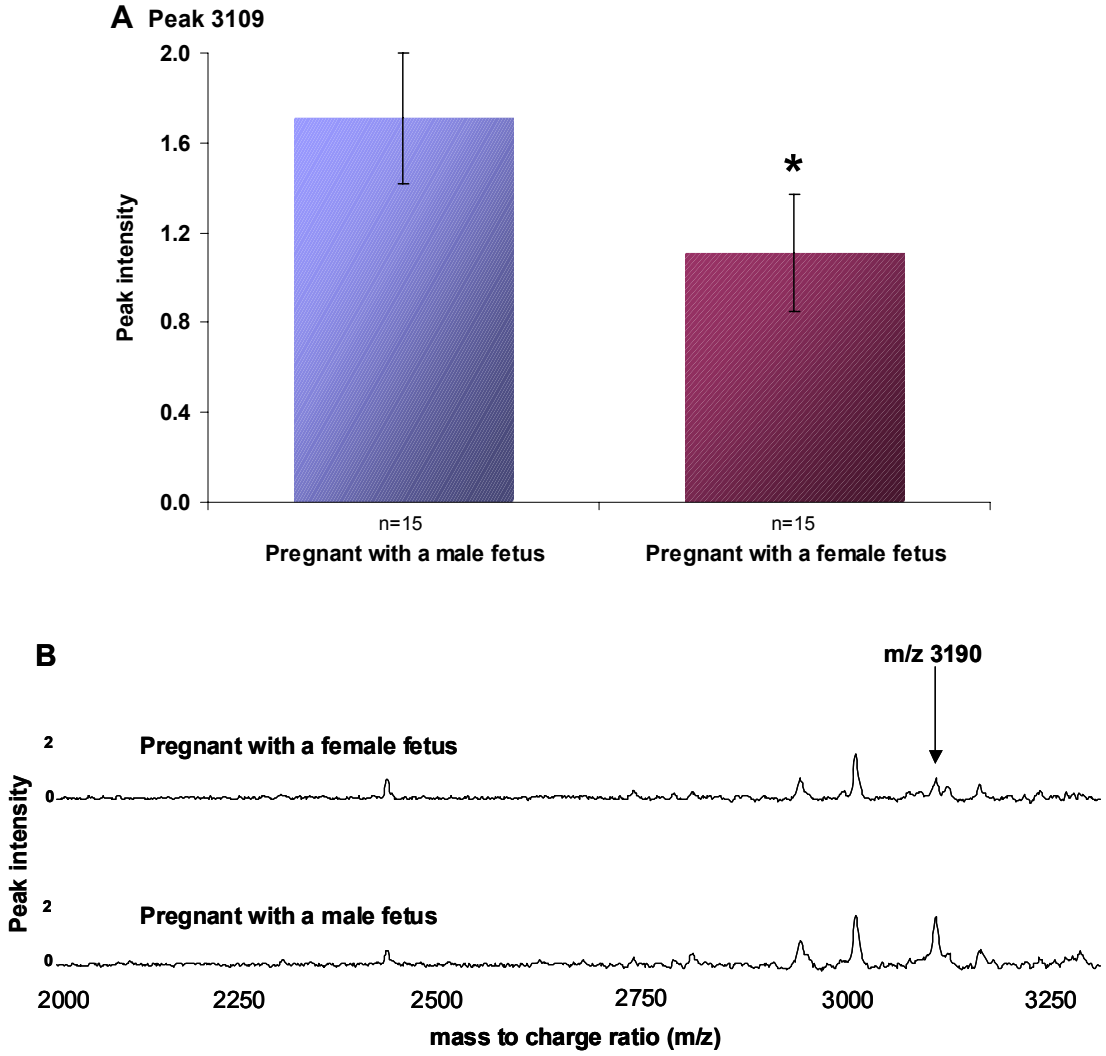

Figure 6.12 Protein 3109 in placentae from male and female neonates

*Panel A shows the mean peak intensity ± SEM of a placental peak with m/z 3109 identified using a WCX chip, pH 9 with SPA matrix. * indicates P<0.05 (t-test). Panel B shows representative spectra from placentae collected from male and female neonates in the region 2-3.3 kDa.*

6.7.3 The effect of maternal asthma and fetal sex on placental proteins

Peaks which were specifically increased or decreased in placentae from the no glucocorticoid female group were examined. These differences may provide information about the placental mechanisms involved in fetal growth restriction in asthmatic pregnancies. Three peaks were found to be significantly different in placentae from asthmatic mothers pregnant with a female fetus from the no glucocorticoid group. A peak with m/z 22251 (SAX pH 9, CHCA, category B) was significantly lower in placentae from females of the no glucocorticoid group compared to the other groups (ANOVA, P=0.040). A search of the Swiss-Prot protein database revealed 31 matches, including lactogen (accession number P01243), suppressor of cytokine signalling-2 (SOCS-2, O14508), superoxide dismutase (P04179) and placenta specific growth hormone (P01242). A peak with similar properties was previously found to be significantly lower in maternal plasma from the no glucocorticoid female group (Figure 5.11), and may represent a link between mother and placenta.

A peak with m/z 3973 (WCX pH 9, SPA, category B) was significantly higher in females of the no glucocorticoid group compared to males (t-test, P=0.047). There was only one match in the Swiss-Prot database to protachykinin 1 precursor/neuropeptide K (P20366). A peak with m/z of 18174 (WCX pH 9, SPA high mass, category B) was significantly higher in females compared to males of the no glucocorticoid group (t-test, P=0.028). There were 18 matches for this peak in the Swiss-Prot database, including interleukin 17B (Q9UHF5), interleukin 18 (Q14116), endothelial monocyte activating protein (Q12904) and T cell surface glycoprotein CD3 gamma (P09693). Further work is required to positively identify these peaks and to determine their role in placental function in asthmatic pregnancies.

6.7.4 Human defensins in the placenta

Defensins are anti-microbial peptides which play an important role in the immune response. The α-defensins or human neutrophil peptides (HNP 1-4) are expressed by many immune cells including neutrophils (768), CD8 lymphocytes (769), natural killer cells (768), B cells (768) and monocytes and macrophages (768). Defensin peptides damage the membrane of bacteria (769) and have the potential to modulate

inflammatory responses through regulation of cytokine production in monocytes and endothelial cells (770) and recruitment of monocytes by neutrophils into inflammatory sites (771). Human neutrophil defensin mRNA was previously identified in the placenta by Svinarich *et al.* using PCR amplification and Southern analysis (772).

Human defensin proteins were tentatively identified in the placenta for the first time using SELDI-TOF MS. A previous study from Zhang *et al.* profiled culture supernatants with SELDI-TOF using WCX chips at pH 4.5 and found a typical pattern of three peaks which upon further characterisation were identified to be the α-defensins 2, 1 and 3 (769). I found a similar pattern of peaks using WCX pH 9 (SPA matrix) at m/z 3383 (α-defensin 2), 3454 (α-defensin 1) and 3498 (α-defensin 3, Figure 6.13), which also matched entries for these peptides in the Swiss-Prot database. No significant differences were observed between expression in placentae from males or females (t-test, $P>0.05$) or between any other groups (unpaired t-test, $P>0.05$). There was a tendency for the α-defensin peak intensities to be higher in placentae from females of the no glucocorticoid group, but these were not significantly different from placentae from the female control group or female glucocorticoid group (Kruskal-Wallis ANOVA, $P=0.110$, α-defensin 2, $P=0.205$, α-defensin 1, $P=0.758$, α-defensin 3). Further investigation is required to determine if these immune mediators have a role to play in asthmatic placentae.

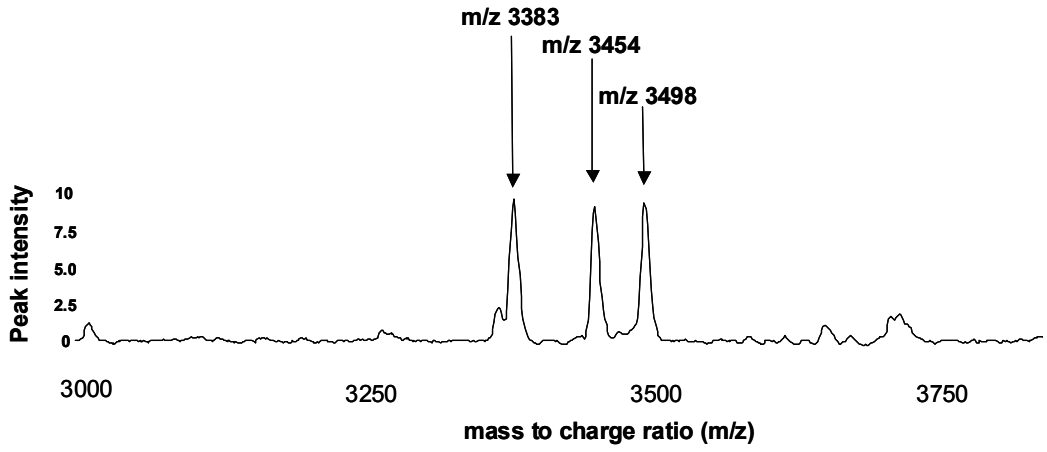

Figure 6.13 Human defensin proteins in the placenta

The figure shows a representative spectra showing the expected positions of α-defensin 2 (m/z 3383), α-defensin 1 (m/z 3454) and α-defensin 3 (m/z 3498) in the placenta.

6.8 The Placenta - Discussion

11β-HSD2 activity, but not mRNA or protein, was significantly reduced in placentae from female neonates of asthmatic mothers who did not use inhaled glucocorticoids. These female neonates also had reduced mean birth weight compared to female neonates of non-asthmatic mothers, suggesting that changes in female fetal growth in asthmatic pregnancies may be mediated by decreased placental 11β-HSD2 activity and the anti-mitogenic effects of cortisol. Fowden *et al.* described that the cortisol surge towards late gestation in sheep was coincident with the slowing down of growth, reflected by a decrease in the increment of crown-rump length growth (503). Placental 11β-HSD2 is an important regulator of the passage of cortisol from mother to fetus in humans. Significant reductions in placental 11β-HSD2 activity have previously been observed in IUGR placentae (554) and fetal plasma cortisol levels are higher in SGA fetuses compared to normally grown controls (582). Multiple doses of betamethasone, a steroid that is not metabolised by placental 11β-HSD2, administered to women at risk of preterm delivery, resulted in a 9% reduction in neonatal birth weight and 4% reduction in neonatal head circumference (486). I found a 14% reduction in female birth weight in the no glucocorticoid group, compared to female neonates from non-asthmatic mothers. These data support the hypothesis that decreased placental 11β-HSD2 activity, which may result in increased circulating concentrations of bioactive cortisol in the fetus, contributes to symmetrically reduced growth of the female fetus.

Previous studies have also demonstrated reductions in mRNA levels of placental 11β-HSD2 in pregnancies complicated by IUGR (554, 558). I did not find a relationship between 11β-HSD2 mRNA or protein levels and reduced birth weight in asthmatic pregnancies, suggesting a post-transcriptional regulation of 11β-HSD2 enzyme activity. Many regulators of 11β-HSD2 enzyme activity and mRNA have previously been identified in placental cells, including the prostaglandins, PGE_2 and $PGF_{2\alpha}$, the leukotriene, LTB_4 and calcium, which inhibit enzyme activity but not mRNA expression (594, 597). Other studies have indicated that placental 11β-HSD2 activity and mRNA can be inhibited by progesterone, while estradiol inhibits activity but not mRNA (590). Hypoxia may also regulate 11β-HSD2 with low levels of O_2 inhibiting protein expression and activity in placental explants or cell cultures (595, 596). Studies of 11β-HSD2 enzyme activity in other cell types has suggested the possibility of

regulation by inflammatory cytokines such as TNF-α and IL-1β (604). Many of these factors are potentially associated with asthma, particularly prostaglandins, leukotrienes, cytokines and hypoxia. However, the regulation of placental 11β-HSD2 by these factors in the context of maternal asthma was not further examined in my study.

Previous studies have demonstrated increased 11β-HSD2 in the presence of synthetic glucocorticoids (556, 603). Kajantie *et al.* found increased placental 11β-HSD2 activity in women who received betamethasone 24-72 hours before birth for fetal lung maturation in preterm infants (556). My data showed that there was a tendency for increased 11β-HSD2 activity in placentae from asthmatic women who used high doses of inhaled glucocorticoids, only when pregnant with a female fetus, but this did not reach statistical significance. An effect of high dose inhaled glucocorticoid use on placental function is possible, since systemic side effects, such as HPA axis suppression (773-775) and long-term alterations in bone metabolism (776), have been observed in adults using more than 1 mg/day of these medications (773-775). However, in my study, the use of high doses of inhaled glucocorticoids by pregnant women had no significant effect on placental CRH mRNA expression or placental 11β-HSD2 activity. Marinoni *et al.* found that betamethasone treatment to pregnant women in preterm labour resulted in an increase in CRH immunostaining in placental syncytiotrophoblast as well as an increase in maternal and umbilical cord concentrations of CRH (777). Together my data suggest that inhaled glucocorticoid medications used for the treatment of asthma do not reach the placenta in an active form.

Sexual dimorphism of 11β-HSD2 has previously been demonstrated in the kidney of hypopituitary patients and healthy adults (778-780), mouse kidney and colon (781) and mouse placenta (634). Raven and Taylor found that there was a higher excretion ratio of 11-oxo to 11-hydroxy cortisol metabolites in women compared to men, signifying greater conversion of cortisol to cortisone (778). In the mouse placenta, Montano *et al.* demonstrated greater transplacental metabolism of corticosterone from the maternal blood in female fetuses compared to males (634). I found a similar result, with placentae collected from female neonates having higher placental 11β-HSD2 activity than those collected from males, which would be expected to result in higher levels of maternally-derived cortisol crossing the placenta to the male fetus. This finding may be related to the relative glucocorticoid insensitivity in the male fetus, demonstrated by normal growth in the presence of maternal asthma and low placental 11β-HSD2 and shown

previously by reduced responsiveness to glucocorticoid treatment for fetal lung maturation compared to the female fetus (69). This is the first demonstration of fetal sex-specific effects on human placental 11β-HSD2 activity and suggests that the fetus may exert some control over the passage of cortisol from the mother.

Placental IGF-I, IGF-II or IGFBP-1 mRNA were not found to be altered in females of reduced birth weight and therefore were not primarily involved in the alterations in female fetal growth observed in asthmatic women who did not use inhaled glucocorticoids for treatment. Previous studies have shown that immunostaining of IGF-I in the placenta is increased in IUGR, possibly as a compensatory mechanism (428, 429), while placental IGF-II mRNA has been shown to correlate with placental weight (433). I did not measure IGF-I, IGF-II or IGFBP-1 protein expression, nor did I examine phosphorylation of placental IGFBP-1, which is known to be an important regulatory mechanism for controlling IGFBP-1 action during pregnancy (419). Circulating IGFBPs are also important components of the IGF axis in fetal growth control and will be examined in Chapter 7.

Detection of cytokine protein levels can be difficult in tissue samples; however, the use of quantitative RT-PCR is a useful and very sensitive technique for the detection of cytokine mRNA (664-668). The results of my study demonstrate that there is a wide variability in placental cytokine mRNA expression between individuals. No significant differences in cytokine mRNA in females from the no glucocorticoid group were found to be related either to alterations in maternal asthma or to the mechanism of reduced fetal growth in this group. Hahn-Zoric *et al.* studied placental cytokine mRNA in IUGR and found that compared to normally grown controls, there was a decrease in IL-10 and an increase in IL-8 mRNA in IUGR, but no alteration in IL-1α, IL-1β, transforming growth factor (TGF)-β or TNF-α (109). Rivera *et al.* also implicated IL-10 as having a role in fetal growth regulation, by demonstrating that co-administration of IL-10 and lipopolysaccharide (LPS) to pregnant rats reduced the LPS-associated fetal death rate and improved fetal growth (782). I did not find any evidence for the involvement of IL-10 or any other individual cytokine at the mRNA level, in fetal growth regulation in human pregnancies complicated by asthma.

However, there were interesting changes in the ratio of Th2 type cytokines to Th1 type cytokines in females from the no glucocorticoid group, with a significant increase in the

placental IL-5:TNF-α and IL-10:TNF-α mRNA ratios, compared to the control and glucocorticoid female groups. Th2 type cytokines are involved in the pathogenesis of asthma (2) and successful pregnancy is also considered to be the result of a Th2 bias (155-158). The mRNA data clearly supported the concept of a Th2 bias in the placenta, and also suggested that asthmatic women who do not use inhaled glucocorticoids and are pregnant with a female fetus have an enhanced Th2 environment in the placenta. In my study, the placental Th2:Th1 cytokine mRNA ratio was inversely correlated with placental 11β-HSD2 activity in samples collected from female neonates. Thus, an increase in the relative production of Th2 and Th1 cytokines may be involved in reducing placental 11β-HSD2 activity. Alternatively, changes in the Th2:Th1 cytokine ratio may occur as a result of reduced local cortisol metabolism across the placenta.

Several studies have shown that glucocorticoids can alter the cytokine balance towards Th2 dominance *in vitro* (783-787). Agarwal and Marshall demonstrated that dexamethasone inhibited IFN-γ (Th1) production from human peripheral blood mononuclear cells, and increased IL-4 and IL-10 (Th2) production (788). These effects may be mediated via decreased IL-12 production in the presence of glucocorticoids, as Blotta *et al.* found that this mechanism contributed to a reduced capacity of monocytes to produce IFN-γ and an increased capacity for IL-4 production in T cells (789). These data may explain the inverse correlation between Th2:Th1 mRNA and 11β-HSD2 activity in female placentae and may have implications for fetal programming of allergic disease in the children of asthmatic mothers. *In vivo*, mice treated with an 11β-HSD2 inhibitor, glycyrrhetinic acid, have reduced capability to fight infection, as a result of a skewed cytokine balance in their spleen (790). This is very strong evidence for the role of 11β-HSD2 in modulating the response of T cells to the effects of cortisol *in vivo*. It has recently been suggested that increased cortisol secretion associated with maternal stress during pregnancy may affect the developing fetal immune system and T cell differentiation (791). The data presented in this section demonstrate that reduced 11β-HSD2 activity, and hence greater cortisol availability, is associated with an increased ratio of Th2:Th1 cytokines in the placenta of female neonates. It is possible that altered 11β-HSD2 activity in the presence of maternal asthma could further increase the risk of the female neonate becoming atopic by altering the Th2:Th1 cytokine balance of the placenta and possibly the fetus itself.

These studies are important because they suggest a possible mechanism for priming of the neonatal immune system *in utero*, which may contribute to the escalating prevalence of asthma worldwide. Maternal atopy is known to be a greater risk factor than paternal atopy for allergic sensitisation in children (792) and high maternal IgE, but not paternal IgE was found to correlate with cord blood and infant IgE levels and infant atopy (74). One study found that cord blood IgE levels were elevated in female neonates only, of mothers with asthma compared to neonates of non-atopic parents (73). These studies suggest that the *in utero* environment may have a role in determining childhood atopy and that the female fetus may be particularly susceptible to changes in this environment. Transplacental priming of the immune system may be involved in altering the cytokine profile of neonates to the Th2 allergic type (793). It has previously been hypothesised that atopic mothers have a greater Th2 dominance at the maternal-fetal interface than non-atopic mothers and this may contribute to altered neonatal immune function (794, 795). My study demonstrates that at the mRNA level, there is a dominance of the Th2 cytokine, IL-5 compared to the Th1 cytokine, TNF-α in the placenta of women with untreated asthma, when a female fetus is present. It has been suggested that the placenta may have a role to play in maintaining the cytokine environment through most of pregnancy, with the fetus and/or mother taking on a greater role only later in pregnancy (794).

Cytokine levels in the feto-placental unit may be influenced by hypoxia. Benyo *et al.* demonstrated increased production of TNF-α and IL-1β by placental explants cultured in low (2%) O_2 (796). Pierce *et al.* found that placental perfusion with hyperoxic solution resulted in elevated IL-6 and TNF-α within 4 hours, compared to perfusion with hypoxic solution (797). Although I did not measure cytokine production by the placenta, it is unlikely that the placentae from the no glucocorticoid asthmatics were hypoxic, since no significant changes in individual cytokine mRNA were found.

Several studies have previously used TNF-α as a representative Th1 cytokine in studies of immune function (798) and pregnancy (799, 800). However, there are other cytokines which could be used and may be more suitable, such as IFN-γ. Future studies would also examine IFN-γ production as an indicator of Th1 cytokine activity. Further work is required to determine if there are alterations in cytokine protein production in the placenta from females of the no glucocorticoid group. Preliminary analysis of crude protein extracts demonstrated that TNF-α could not be measured in these placental

samples by enzyme linked immunosorbent assay (ELISA). Some studies have stimulated placental cytokine production using LPS in order for concentrations to be measurable (801, 802). Placental explant cultures stimulated with LPS may be suitable for an analysis of placental cytokine production by ELISA in asthmatic and non-asthmatic placentae.

Few studies have examined Th2 cytokine production in the human placenta. My work showed the presence of IL-4, IL-5 and IL-10 mRNA. Immunohistochemistry has previously been used to detect IL-4 and IL-4 receptors in some samples of syncytiotrophoblast and placental macrophages (803). In addition, mRNA for IL-4 and IL-4 receptor was also detected by RT-PCR in placental villous tissue (803). To my knowledge, no previous studies have demonstrated the presence of IL-5 mRNA or protein in the human placenta. I found that placental IL-5 mRNA was expressed at similar levels to IL-4, IL-6 and TNF-α. Since this cytokine has an important role in asthma, it may also have a function in the placenta from asthmatic women. My results showed a clear predominance of Th2 cytokines over Th1 cytokines in the human placenta, which is in agreement with the hypothesis that a Th2 environment is required for successful pregnancy (804).

Alterations in placental GR expression may be important for determining fetal sensitivity to the effects of cortisol and synthetic glucocorticoids. The data suggest that the female fetus is particularly sensitive to the effects of maternally derived cortisol, when the mother has asthma. This was demonstrated by the fact that although placentae from both males and females of the no glucocorticoid group had similar levels of 11β-HSD2 activity, only the female neonates of this group were smaller. In addition, only the females of this group had alterations in fetal HPA axis function, which will be discussed in the following chapter (section 7.2.2). These changes in sensitivity to cortisol may be mediated via altered GR expression or function. My data indicates that placentae from females of the control group have significantly higher GR-α expression than placentae from males of the control group, possibly mediating the greater glucocorticoid sensitivity in the female fetus. In addition, placental 11β-HSD2 activity was higher overall in females than males and together these data suggest that the female fetus is more sensitive to glucocorticoids than the male fetus.

In the no glucocorticoid group, placental GR-α and GR-β mRNA were both significantly decreased in females, possibly due to down-regulation by locally elevated cortisol concentrations, as a consequence of reduced 11β-HSD2 activity. Down-regulation of the GR by cortisol and synthetic glucocorticoids has been demonstrated previously (805-810). Korn *et al.* treated bronchial epithelial cells with the synthetic glucocorticoid budesonide and found a dose-dependent decrease in both GR-α and GR-β (810, 811). In asthmatic patients treated with methyl prednisolone for 10 days, Vachier *et al.* found that there was a significant reduction in GR mRNA in isolated monocytes (812). Down-regulation of the MR in placentae from females of the no glucocorticoid group may be occurring through a similar mechanism, since *in vitro* studies have also demonstrated reduced MR number and binding following synthetic glucocorticoid treatment (813). Hypoxia in the no glucocorticoid group is unlikely to be contributing to these changes in receptor mRNA, since Jenq *et al.* found reduced MR but increased GR mRNA in human renal cortex epithelial cells cultured under mild hypoxic conditions (814). The higher GR-α:GR-β mRNA ratio in female placentae from the no glucocorticoid group may result in a greater ability of local cortisol to exert its effects through the transcriptionally active GR-α, possibly explaining the increased glucocorticoid sensitivity in this group.

A small number of differences in placental protein expression were found between asthmatics and non-asthmatics using the proteomics technique, SELDI-TOF MS. These proteins may have important roles in modulating placental function in asthmatic pregnancies and further identification and characterisation is required to explore this. However, these data provide leads for further investigation into the placental mechanisms contributing to reduced fetal growth in pregnancies complicated by asthma.

Only one study has previously used the SELDI technique to examine protein expression in the placenta. This study examined differences between normal placentae and those with complete hydatidiform mole (701). Hydatidiform mole is a gestational trophoblastic disease where a mass of tumour forms within the uterus at the beginning of pregnancy, which may develop into the malignant choriocarcinoma (701). Batorfi *et al.* used laser capture microdissection and SELDI-TOF MS to characterise proteins present in normal and complete mole placenta samples obtained around 10 weeks gestation. They successfully found three proteins with significantly higher expression in normal placenta using the IMAC chip at pH 7.2 with SPA matrix and hope that further

identification will contribute to a better understanding of the mechanisms involved in gestational trophoblastic disease (701).

In my study, defensin proteins were tentatively identified in the human placenta for the first time. This was possible because of a previous report identifying defensin proteins in culture supernatants of stimulated $CD8^+$ T lymphocytes from patients with HIV, which used similar SELDI-TOF conditions (769). In this publication, the profiles obtained using WCX chips and pH 4.5 binding conditions were published and my results obtained for human placenta samples on WCX chips at pH 9 show much similarity in the profile of these three peaks. In addition, the molecular weights obtained from SELDI-TOF MS were consistent with those of the defensin peptides in the Swiss-Prot database. In the study from Zhang *et al.* the identity of these three peaks was investigated by numerous methods. The culture supernatants which had been enriched for these proteins were reduced with DTT to test for the existence of disulfide bonds. The reduced material was analysed on a normal phase chip by SELDI and comparison with non-reduced material showed a shift in molecular weight of 6 Da for all three peaks, suggesting the presence of three internal disulfide bridges, known to be present in defensins (769). Substantial further characterisation was performed, including incubation of T cell culture supernatants with beads coated with biotinylated monoclonal antibody specific for human α-defensin which resulted in an elimination of the three peaks of interest on WCX chips without affecting other peptide peaks. A trypsin digest of enriched protein produced a 1060 Da fragment which was further fragmented by collision associated MS-MS into seven unique ions which were used to search the Swiss-Prot and NCBI databases. This result matched the conserved region of human α-defensin 1, 2 and 3, which was confirmed by amino acid sequencing (769).

Placental defensin peptides may play a wider role in all pregnancies, protecting the fetus from infectious agents. Amniotic fluid defensins are higher in patients with subclinical intrauterine infection and increase further with increasing severity of histologic chorioamnionitis (815). Mid-gestation bacterial vaginal infection and leukocytes in vaginal fluid are associated with the presence of neutrophil defensins (816, 817) and women with high defensin levels were found to be at increased risk of delivery prior to 32 weeks compared to women with undetectable vaginal fluid defensins (817). The role of placental defensin peptides in the context of infection during pregnancy is unknown.

Further analysis of circulating defensins in pregnant women with asthma will be important to determine whether they may play a role in the inflammatory changes which occur during asthmatic pregnancies. It is of particular interest that α-defensins have monocyte chemoattractant properties and are found in increased amounts in patients with other inflammatory lung diseases such as cystic fibrosis (818). However, the current data was only able to examine their expression in the placenta and no significant differences between groups were found.

6.9 The Placenta - Summary

The placenta has a major role to play in the mechanisms controlling fetal growth in asthmatic pregnancies. In particular, activity of the placental enzyme, 11β-HSD2 is important for preventing the passage of cortisol from mother to fetus, where it may have anti-mitogenic effects. Alterations in placental 11β-HSD2 also has other downstream effects such as altering the Th2:Th1 cytokine ratio and decreasing GR and MR expression. Figure 6.14 summarises the information gained so far about the interactions between the asthmatic mother, placenta and female fetus.

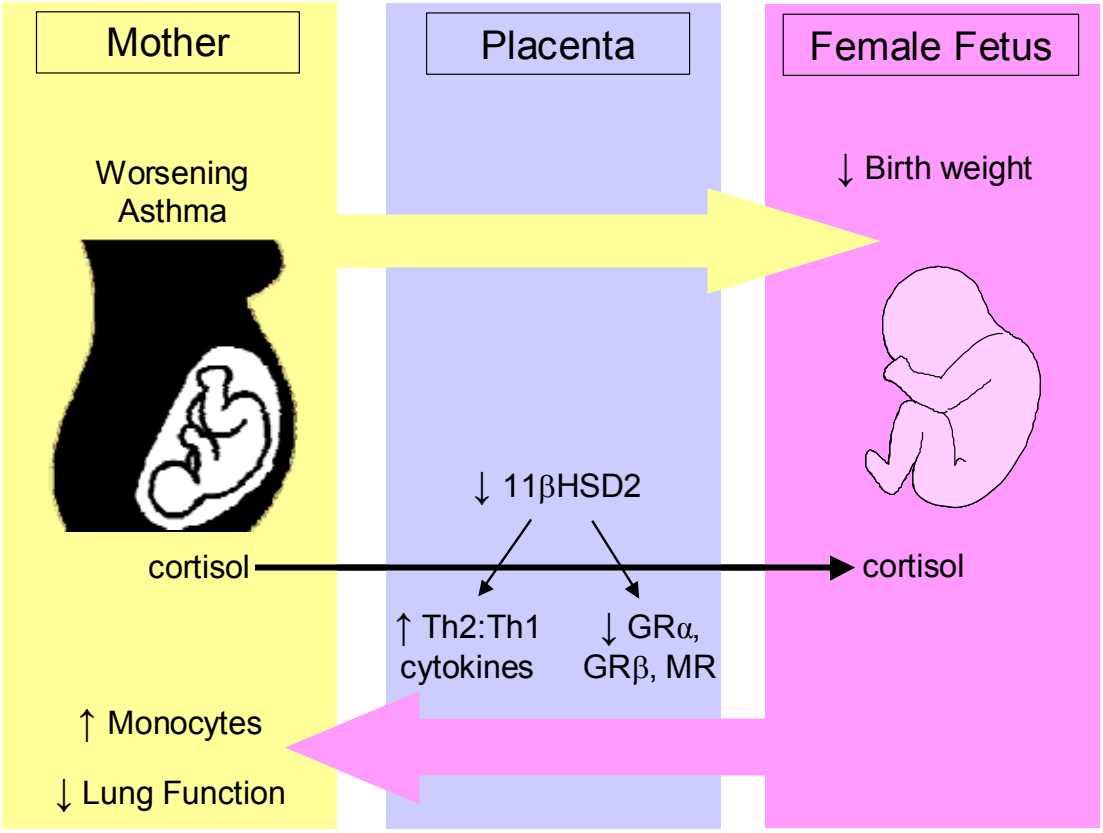

Figure 6.14 The interactions between mother, placenta and fetus in pregnancies complicated by asthma (part 1)

In the presence of a female fetus, maternal asthma worsens during pregnancy, as demonstrated by a significant rise in circulating monocytes and a significant reduction in lung function. These alterations in maternal asthma in the absence of glucocorticoid therapy are associated with significantly reduced female birth weight and changes in placental function. Placental 11β-HSD2 activity is significantly reduced, which allows more maternally-derived cortisol to reach the female fetus. Further changes in placental function which may be due to the decrease in 11β-HSD2 activity include a rise in the local Th2:Th1 cytokine mRNA ratio and decreased GR and MR expression.

Chapter 7 **The Fetus**

7.1 Neonatal characteristics

Female neonates from asthmatic mothers who did not use inhaled glucocorticoids were smaller than female neonates of non-asthmatic mothers. However, whether there were any other effects of maternal asthma on the fetus was unclear. Data was collected on the presence of fetal heart rate decelerations during labour and delivery and neonatal Apgar score at 1 minute and 5 minutes after birth. A summary is presented in Table 7.1 for asthmatics classified by severity and in Table 7.2 for asthmatics classified by glucocorticoid intake.

There was a tendency for more female neonates from the no glucocorticoid group to have low Apgar scores (<7) at 1 minute and for fewer female neonates of this group to have perfect Apgar scores (10) at 5 minutes. In addition, both male and female neonates from the no glucocorticoid group were more likely to have fetal heart rate decelerations during delivery than the other groups, but again, this was not statistically significant (Fisher's exact test, $P>0.05$).

Table 7.1 Fetal and neonatal characteristics for groups classified by asthma severity

	Classification of Asthma Severity During Pregnancy			
Female Fetus	**Control**	**Mild**	**Moderate**	**Severe**
Apgar Score at 1 minute	8 ± 1 (n=20)	8 ± 1 (n=32)	8 ± 1 (n=10)	8 ± 1 (n=27)
Apgar Score at 5 minutes	9 ± 1 (n=20)	9 ± 1 (n=32)	10 ± 1 (n=10)	9 ± 1 (n=27)
Apgar <7 at 1 minute (% of subjects)	10%	16%	30%	15%
Apgar = 10 at 5 minutes (% of subjects)	30%	34%	50%	19%
Heart Rate Decelerations (% of subjects)	15%	22%	30%	19%
Male Fetus	**Control**	**Mild**	**Moderate**	**Severe**
Apgar Score at 1 minute	8 ± 1 (n=21)	8 ± 1 (n=30)	8 ± 1 (n=17)	8 ± 1 (n=19)
Apgar Score at 5 minutes	9 ± 1 (n=22)	9 ± 1 (n=29)	9 ± 1 (n=17)	9 ± 1 (n=19)
Apgar <7 at 1 minute (% of subjects)	5%	3%	17%	21%
Apgar = 10 at 5 minutes (% of subjects)	38%	21%	53%	21%
Heart Rate Decelerations (% of subjects)	9%	20%	18%	21%
	Values given are Mean ± Standard Error of the Mean			

Table 7.2 Fetal and neonatal characteristics for groups classified by glucocorticoid intake

	Classification of Glucocorticoid Intake During Pregnancy		
Female Fetus	**Control**	**No Glucocorticoid**	**Glucocorticoid**
Apgar Score at 1 minute	8 ± 1 (n=20)	8 ± 1 (n=22)	8 ± 1 (n=47)
Apgar Score at 5 minutes	9 ± 1 (n=20)	9 ± 1 (n=22)	9 ± 1 (n=47)
Apgar <7 at 1 minute (% of subjects)	10%	18%	15%
Apgar = 10 at 5 minutes (% of subjects)	30%	23%	34%
Heart Rate Decelerations (% of subjects)	15%	27%	19%
Male Fetus	**Control**	**No Glucocorticoid**	**Glucocorticoid**
Apgar Score at 1 minute	8 ± 1 (n=21)	8 ± 1 (n=24)	8 ± 1 (n=42)
Apgar Score at 5 minutes	9 ± 1 (n=22)	9 ± 1 (n=23)	9 ± 1 (n=42)
Apgar <7 at 1 minute (% of subjects)	5%	8%	12%
Apgar = 10 at 5 minutes (% of subjects)	38%	25%	26%
Heart Rate Decelerations (% of subjects)	9%	35%	17%
	Values given are Mean ± Standard Error of the Mean		

7.2 Fetal HPA axis development

7.2.1 Umbilical vein cortisol

Cortisol concentrations were measured in the umbilical vein plasma at delivery to ascertain whether changes in placental 11β-HSD2 activity had any significant effect on circulating cortisol levels crossing the placenta to the fetus.

Cortisol concentrations were examined in groups classified by maternal asthma severity and fetal sex. There were no significant differences between males or females of the control, mild, moderate or severe asthma groups (Kruskal-Wallis ANOVA, P=0.229 and P=0.682 respectively).

Cortisol concentrations according to maternal glucocorticoid intake and fetal sex are shown in Figure 7.1. Mean fetal cortisol concentrations in the umbilical vein at delivery were similar in male and female fetuses from the no glucocorticoid group, as expected from low placental 11β-HSD2 activity in these groups. However, female cortisol values in the no glucocorticoid group were not significantly different from values in females of the control or glucocorticoid groups (Kruskal-Wallis ANOVA, P=0.951). Male cortisol concentrations were also not significantly different between the control, no glucocorticoid and glucocorticoid groups (Kruskal-Wallis ANOVA, P=0.472).

The relationship between placental 11β-HSD2 activity and umbilical vein cortisol was examined in all samples. There was a trend towards an inverse correlation; however, this was not statistically significant (Pearson linear correlation, n=49, r = -0.248, P=0.086).

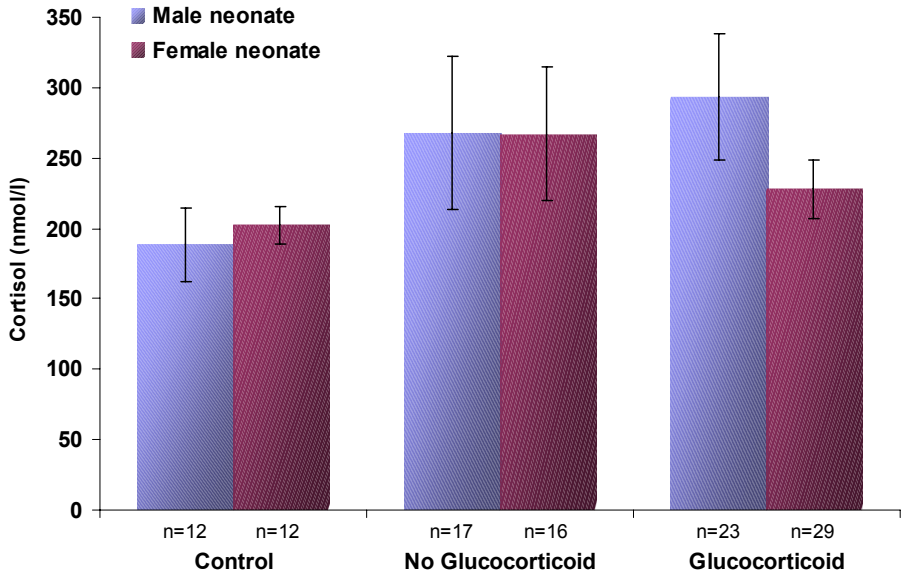

Figure 7.1 Umbilical vein cortisol according to glucocorticoid intake classification

Cortisol concentrations were measured by RIA. The figure shows mean umbilical vein cortisol (nmol/l) ± SEM in male and female fetuses of the control, no glucocorticoid and glucocorticoid groups.

7.2.2 Umbilical vein estriol

Estriol is a derivative of fetal adrenal dehydroepiandrosterone sulfate (DHEA-S), produced by the placenta (819). A 16-hydroxylated DHEA-S precursor from the fetal liver is aromatised to estriol in the placenta (819). Less than 10% of estriol circulating during pregnancy is derived from the mother and hence estriol concentrations are used as an indicator of fetal adrenal function (819, 820) and have been proposed as a marker of fetal well-being (821, 822). Previous studies have shown decreased maternal salivary or urinary estriol levels following betamethasone administration (823, 824). I hypothesised that reduced placental 11β-HSD2 activity in females from the no glucocorticoid group, would result in increased maternally-derived cortisol reaching the fetus, which may suppress fetal adrenal function via negative feedback (Figure 7.2).

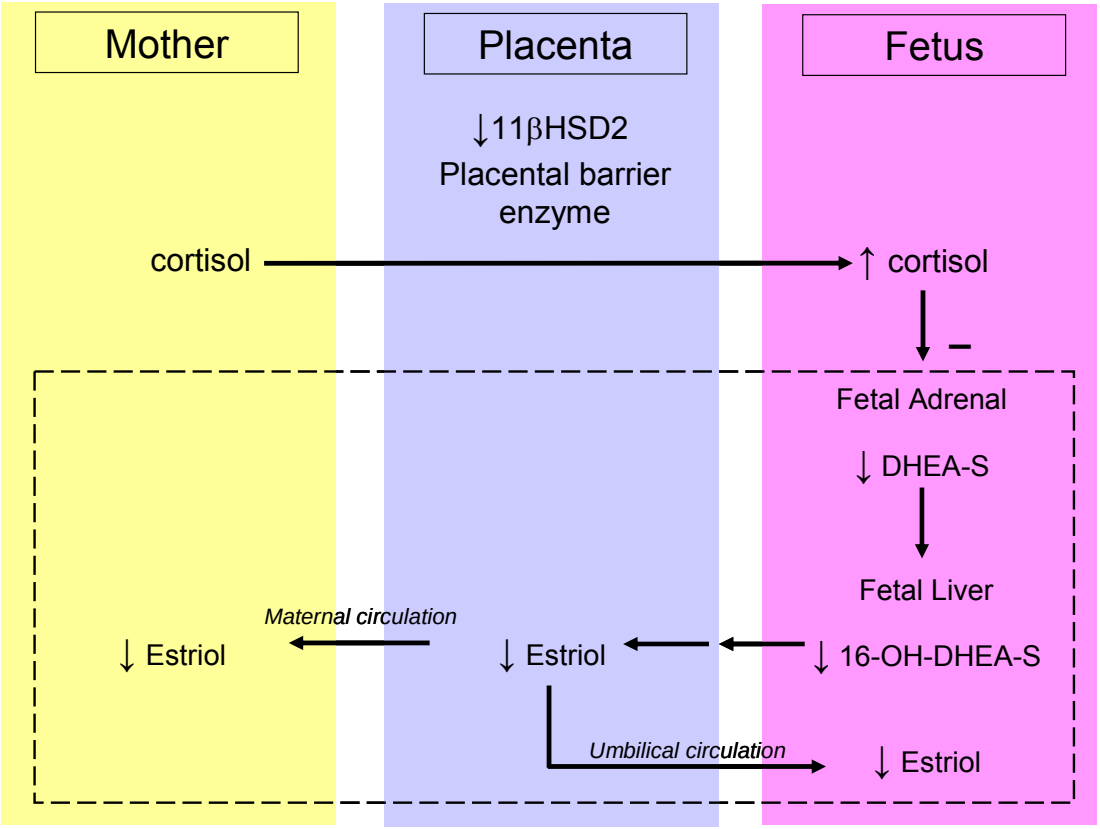

Figure 7.2 Production of estriol and proposed negative feedback loop

Estriol is produced from a fetal adrenal precursor, DHEA-S, which is 16-hydroxylated in the fetal liver (16-OH-DHEA-S) and converted to estriol in the placenta. This conversion occurs through several steps, including removal of the sulfate to give 16-OH-DHEA, and formation of estriol via androstenetriol and 16α-hydroxytestosterone derivatives (819). From the placenta estriol enters the maternal and umbilical circulations and can be measured from around 10 weeks gestation. Maternally derived cortisol, which passes the placental enzyme barrier, 11β-HSD2, may have a negative feedback effect on fetal HPA function.

When analysed according to maternal asthma severity, there was no difference in estriol concentrations between males or females of the control, mild, moderate or severe asthma groups (Kruskal-Wallis ANOVA, P=0.735 and P=0.980 respectively).

Estriol concentrations according to glucocorticoid intake and fetal sex are shown in Figure 7.3. Fetal estriol concentrations were significantly reduced in females from the no glucocorticoid group compared to females from glucocorticoid group (Kruskal-Wallis ANOVA, P=0.007, Dunn's multiple comparisons test, P<0.01). There was no significant difference in male estriol concentrations between groups (Kruskal-Wallis non-parametric ANOVA, P=0.308).

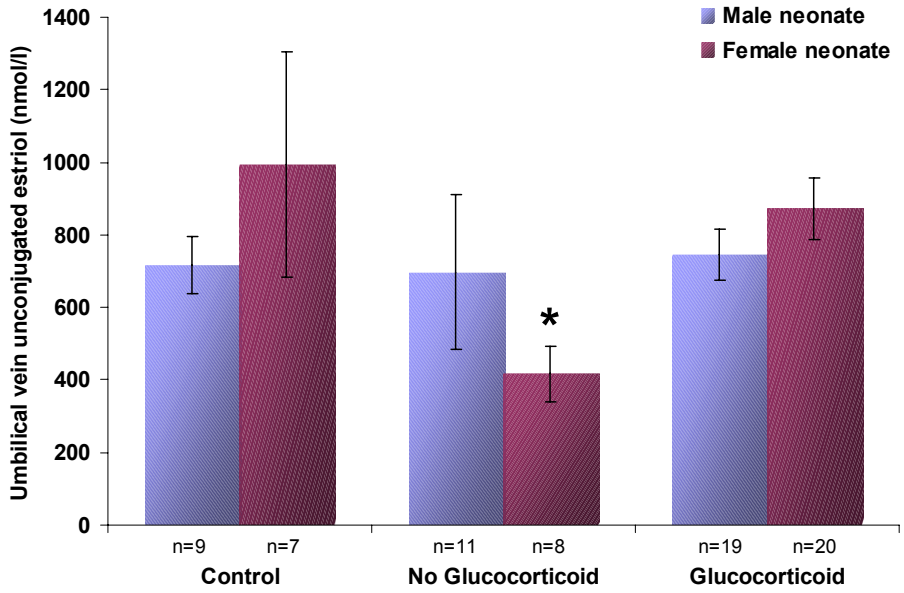

Figure 7.3 Unconjugated estriol concentrations in the umbilical vein according to glucocorticoid intake classification

*Mean umbilical vein unconjugated estriol (nmol/l) ± SEM in male and female fetuses of the control, no glucocorticoid and glucocorticoid groups. * indicates P<0.05 (Kruskal-Wallis ANOVA, no glucocorticoid female vs glucocorticoid female).*

There was a significant inverse correlation between umbilical vein cortisol and umbilical vein estriol in all samples collected (Figure 7.4, Pearson linear correlation, n=74, r = -0.248, *P*=0.033), supporting the hypothesis that reduced estriol is associated with increased cortisol crossing from mother to fetus.

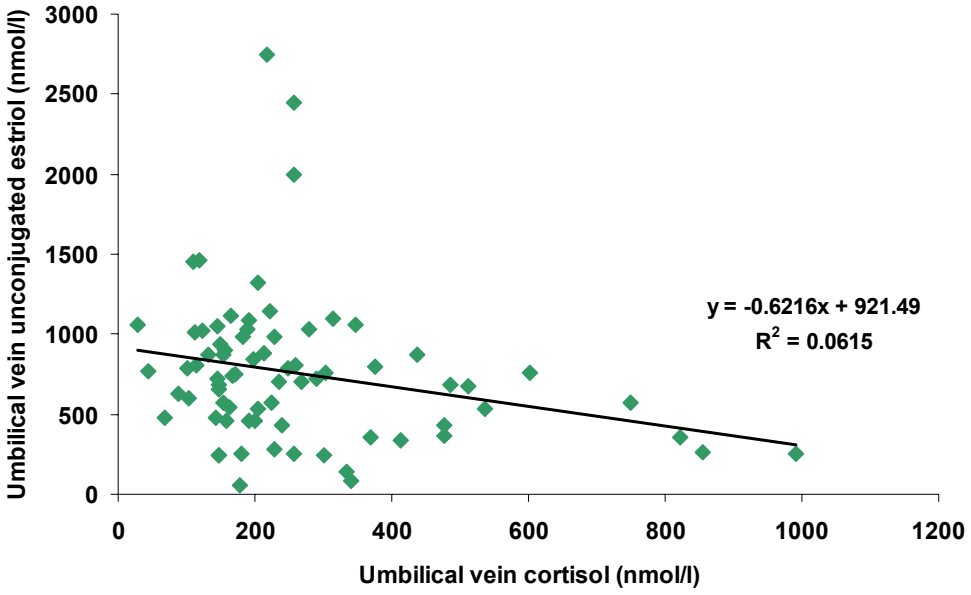

Figure 7.4 Correlation between umbilical vein cortisol and estriol

This figure shows the significant inverse linear correlation between umbilical vein cortisol (nmol/l) and umbilical vein unconjugated estriol (nmol/l, $P<0.05$, Pearson linear correlation, $n=74$), indicating that as cortisol levels increase, estriol levels decrease.

7.2.3 Umbilical vein CRH

There was no significant difference in male umbilical vein concentrations of CRH between the control, mild, moderate and severe asthma groups (Kruskal-Wallis ANOVA, P=0.702) or in female cord blood concentrations of CRH between the groups (Kruskal-Wallis ANOVA, P=0.033, Dunn's multiple comparisons test, P>0.05). There was no significant difference in CRH concentrations between males or females of the control, no glucocorticoid and glucocorticoid groups (Kruskal-Wallis ANOVA, P=0.460 and P=0.165 respectively, data not shown).

7.3 Umbilical vein IGFBP-1 and IGFBP-3

The actions of IGF-I and IGF-II on fetal growth are regulated by a series of binding proteins. IGFBP-3 is a major reservoir for IGFs in the circulation, with 75% of IGF-I circulating complexed with IGFBP-3 and an acid-labile subunit (408). Approximately one quarter of IGF-I circulates with other binding proteins, while 1% circulates as free IGF-I (825). IGFBP-1 is thought to have an important role particularly during pregnancy in regulating the amount of IGF-I which is able to bind to its receptor (419).

There may be alterations in the IGF system in pregnancies complicated by asthma, which contribute to the reduced growth of female neonates in this population. The expression of placental IGF-I, IGF-II and IGFBP-1 mRNA was described previously in Section 6.4 and expression was not altered with maternal asthma. Cord blood levels of IGFBP-1 and IGFBP-3 were measured by RIA by a collaborator, Dr Robert Baxter.

There were no significant differences in male or female cord blood IGFBP-1 or IGFBP-3 between asthmatics and non-asthmatics when classified by asthma severity or by inhaled glucocorticoid intake (Table 7.3, Kruskal-Wallis ANOVA, $P>0.05$).

IGFBP-1 was significantly higher in male fetuses compared to female fetuses in all groups (Figure 7.5A, Mann Whitney test, $P=0.009$). Cord blood IGFBP-1 inversely correlated with birth weight in female neonates only (Figure 7.5B, Spearman rank correlation, n=43, r = -0.375, $P=0.013$).

Table 7.3 Cord blood IGFBP-1, IGFBP-3 and cortisol concentrations according to glucocorticoid intake classification

	Classification of Glucocorticoid Intake During Pregnancy		
Female Fetus	**Control**	**No Glucocorticoid**	**Glucocorticoid**
Cord blood IGFBP-1 (ng/ml)	81.8 ± 24.6 (n=10)	77.2 ± 14.1 (n=13)	130.4 ± 40.9 (n=20)
Cord blood IGFBP-3 (µg/ml)	2.21 ± 0.16 (n=10)	2.47 ± 0.21 (n=13)	2.46 ± 0.11 (n=20)
Cord blood cortisol	202.6 ± 13.2 (n=12)	267.1 ± 47.5 (n=16)	227.9 ± 20.4 (n=29)
Male Fetus	**Control**	**No Glucocorticoid**	**Glucocorticoid**
Cord blood IGFBP-1 (ng/ml)	156.3 ± 59.7 (n=8)	150.8 ± 36.2 (n=11)	121.4 ± 18.5 (n=15)
Cord blood IGFBP-3 (µg/ml)	2.29 ± 0.24 (n=8)	1.94 ± 0.11 (n=11)	1.98 ± 0.16 (n=15)
Cord blood cortisol	188.4 ± 25.8 (n=12)	267.9 ± 54.4 (n=17)	293.4 ± 44.6 (n=23)
	Values given are Mean ± Standard Error of the Mean		

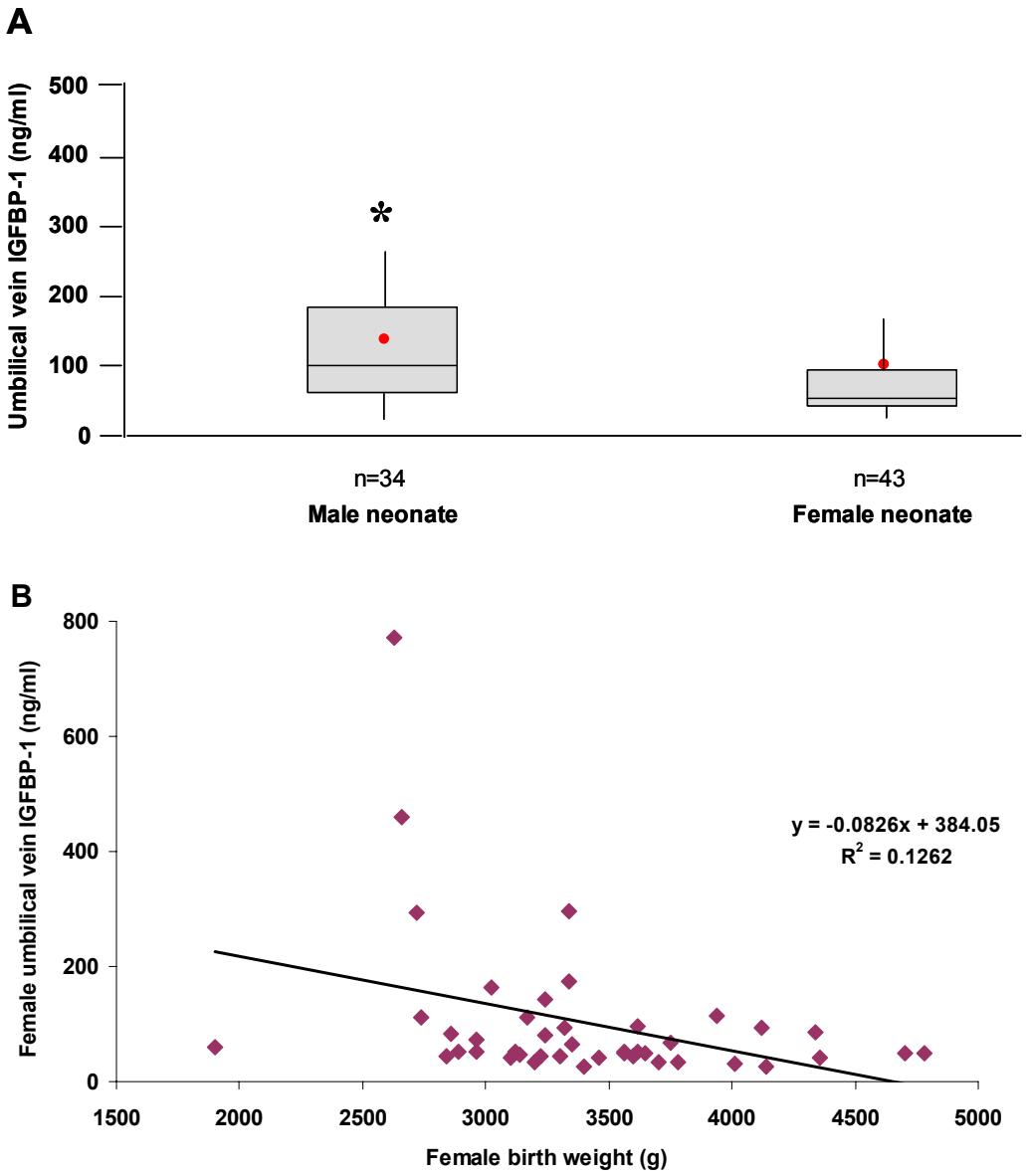

Figure 7.5 Cord blood IGFBP-1 according to fetal sex and its correlation with birth weight

*Panel A shows a boxplot of cord blood IGFBP-1 concentration (ng/ml) for all male and female neonates. The red dot gives the mean value, while the median is given by the horizontal bar. The outsides of the box represent the upper and lower quartiles and the range is indicated by the vertical lines. * indicates P=0.009 (Mann Whitney test). Panel B shows the significant inverse correlation between cord blood IGFBP-1 concentration (ng/ml) and birth weight (g) in all female neonates (P<0.05, Spearman rank correlation, n=43).*

Overall, IGFBP-3 was significantly higher in female fetuses than males (Figure 7.6A, Mann Whitney test, *P*=0.007). This was the case in all asthmatic groups, but not the control group (Table 7.3). Overall, IGFBP-3 correlated positively with birth weight in male neonates only (Figure 7.6B, Pearson linear correlation, n=34, r = 0.537, *P*=0.001).

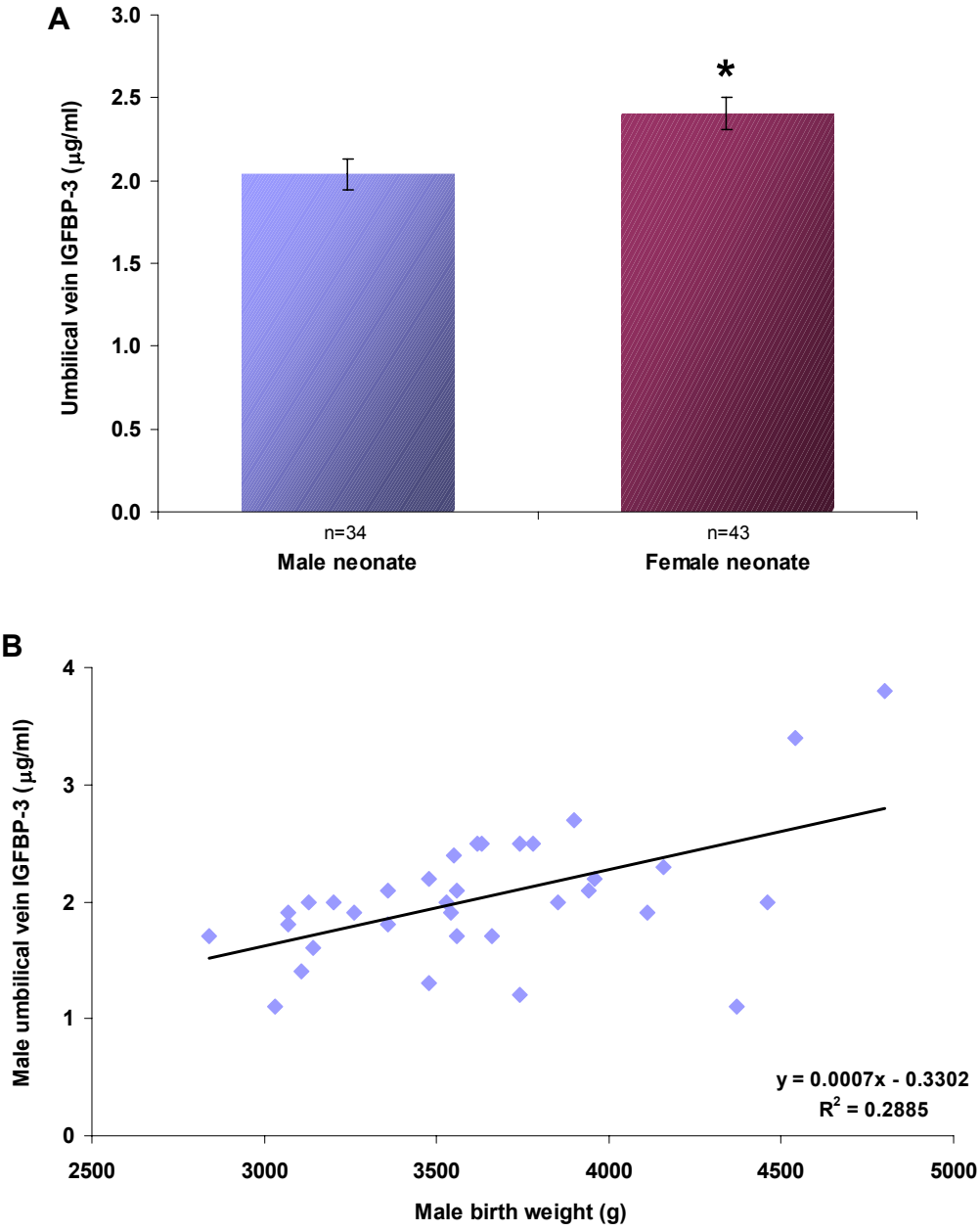

Figure 7.6 Cord blood IGFBP-3 according to fetal sex and its correlation with birth weight

*Panel A shows the mean cord blood IGFBP-3 concentration (µg/ml) ± SEM for all male and female neonates. * indicates P=0.007 (Mann Whitney test). Panel B shows the significant direct correlation between cord blood IGFBP-3 concentration and birth weight (g) in male neonates (P<0.05, Pearson linear correlation, n=34).*

Cord blood IGFBP-3 correlated directly with placental weight in both males (Pearson linear correlation, n=25, r = 0.392, *P*=0.053) and females (Pearson linear correlation, n=30, r= 0.359, *P*=0.051). When males and females were combined, the correlation

between IGFBP-3 and placental weight was significant (Pearson linear correlation, n=55, r = 0.333, *P*=0.013).

Cord blood cortisol concentrations positively correlated with cord blood IGFBP-1 in females (Figure 7.7, Spearman rank correlation, n=43, r = 0.335, *P*=0.028), but not in males (Pearson linear correlation, n=34, r = –0.017, *P*=0.923). However, exclusion of two outliers which were more than 3 standard deviations from the mean resulted in a non-significant relationship (Spearman rank correlation, n=41, r = 0.241, *P*=0.129). There was no correlation between IGFBP-3 and cortisol in either male or female neonates (*P*>0.05).

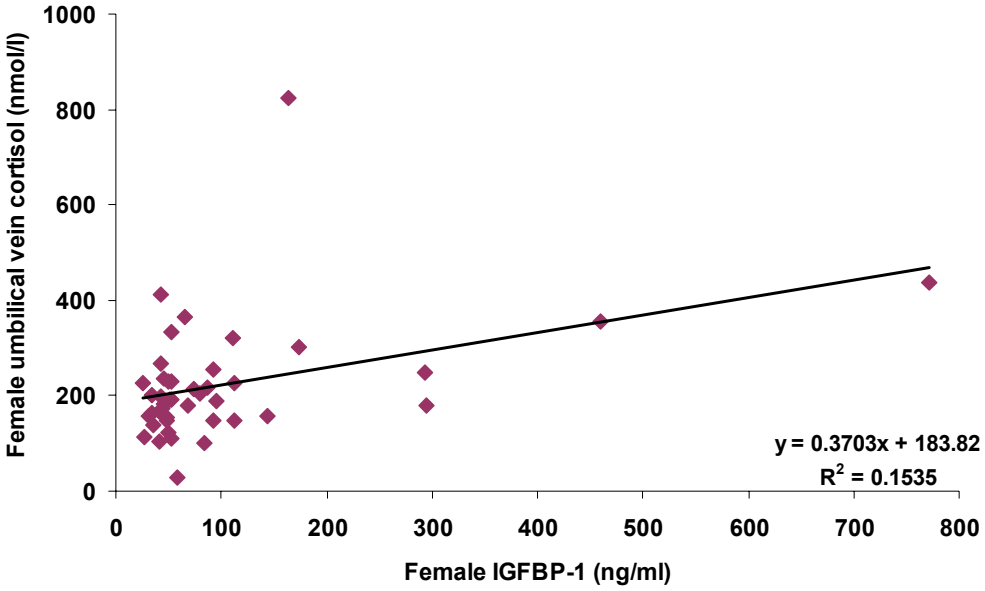

Figure 7.7 The relationship between cortisol and IGFBP-1 in female cord blood

The significant direct correlation between cord blood cortisol (nmol/l) and cord blood IGFBP-1 (ng/ml) in female neonates (P<0.05, Spearman rank correlation, n=43).

7.4 Cord blood protein profile

7.4.1 The effect of maternal asthma on cord blood proteins

Protein profiling of umbilical vein plasma was carried out using SELDI-TOF MS. There were 10 peaks which differed significantly between cord blood samples taken from asthmatics (n=20) and non-asthmatics (n=10). Five of these were considered highly suitable for follow up (category A). An example is shown in Figure 7.8. This peak

matched one other in the Swiss-Prot database (islet amyloid polypeptide precursor or diabetes associated protein, P10997).

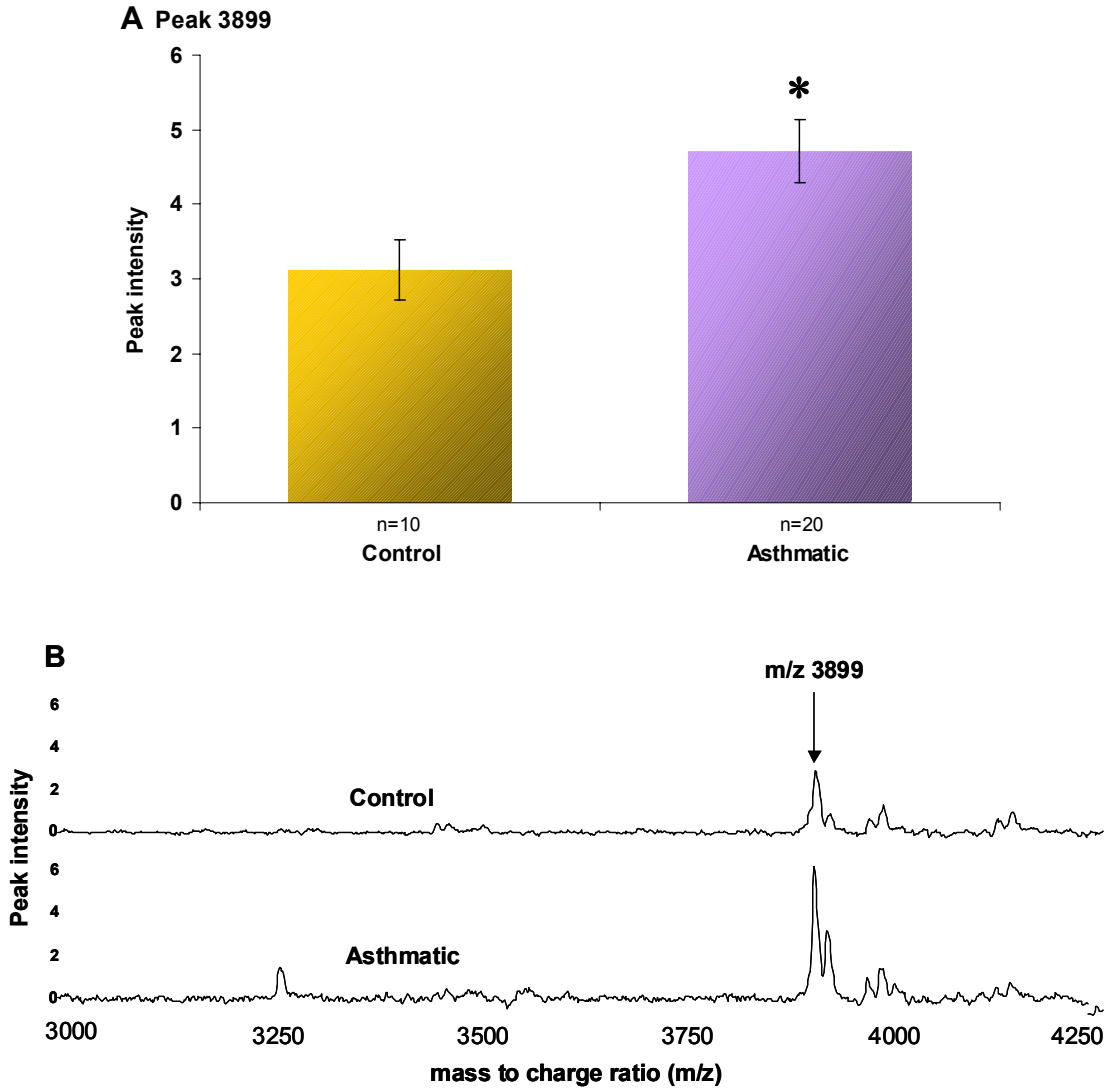

Figure 7.8 Cord blood peak 3899 in asthmatic and non-asthmatic pregnancies

*Panel A shows the mean peak intensity ± SEM in non-asthmatic (control) and asthmatics of a cord blood peak with m/z 3899 identified using an IMAC chip, pH 7 with SPA matrix. * indicates P<0.05 (t-test). Panel B shows representative spectra from asthmatic and non-asthmatic cord blood in the region 3-4.3 kDa.*

7.4.2 The effect of fetal sex on cord blood proteins

The cord blood protein profile of all female fetuses (n=15) and all male fetuses (n=15) was compared and there were only three peaks which differed significantly between male and female fetuses. The peak intensity of one peak, assigned a category B (m/z 37305), was influenced by very high levels in the no glucocorticoid female group. The mean levels of the other two peaks were less than 1.8 times different between groups and were considered category C (m/z 8045 and 72973). These data suggest that there are few differences in cord blood plasma proteins in males and females at the time of delivery, as measured by SELDI-TOF MS.

7.4.3 The effect of maternal asthma and fetal sex on cord blood proteins

Peaks which were significantly increased or decreased in asthmatic women who did not use inhaled glucocorticoids and were pregnant with a female fetus were examined. Six peaks found to be significantly different in asthmatic mothers pregnant with a female fetus from the no glucocorticoid group compared to the other groups. An example is shown in Figure 7.9. A search was conducted of the Swiss-Prot protein database to determine possible identities of this peak (m/z 8701). There were five matches, including pulmonary surfactant associated protein (P07988), monocyte chemotactic protein 1 (MCP-1, P13500) and small inducible cytokine A14 (Q16627), which has weak activities on human monocytes.

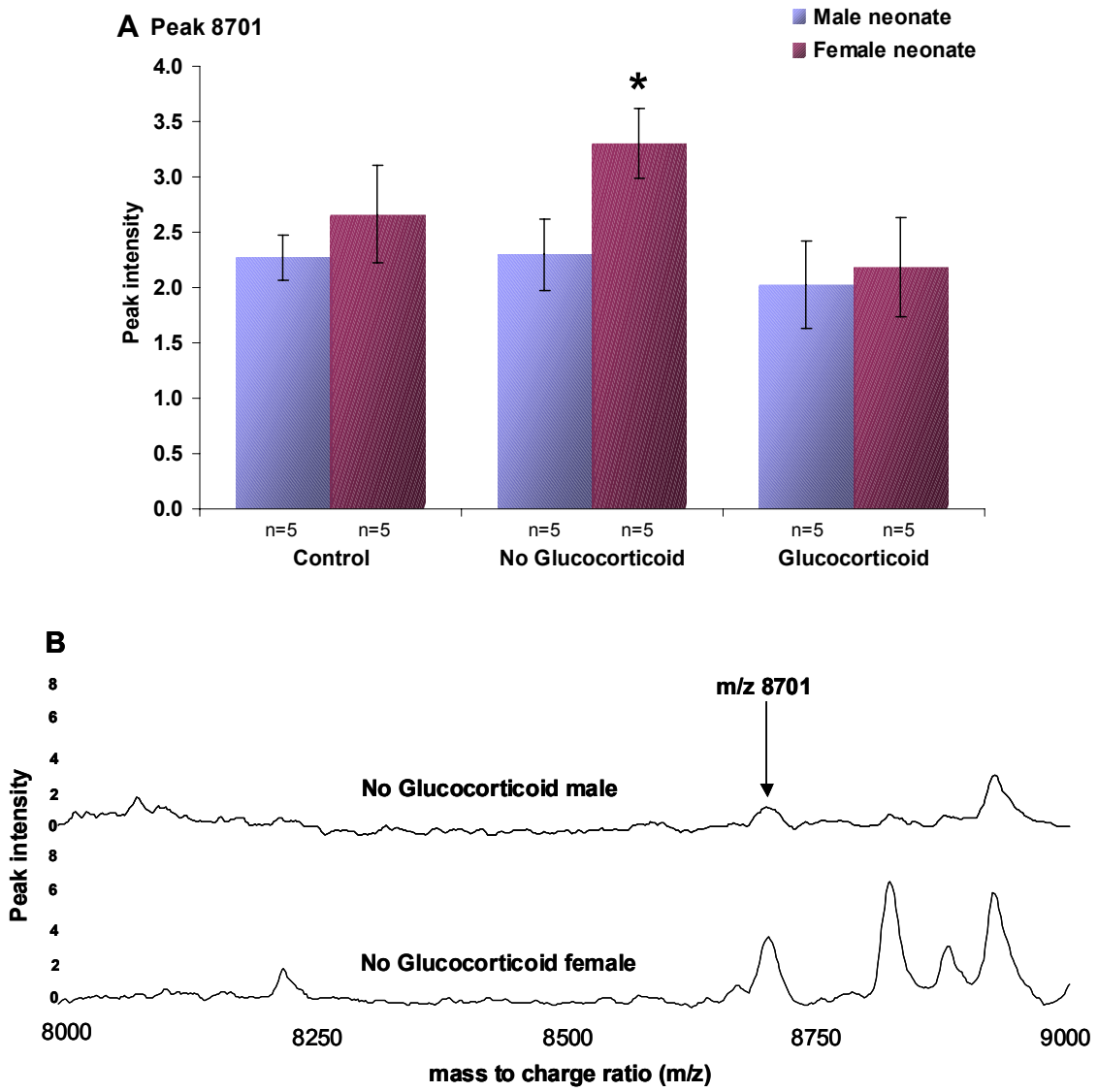

Figure 7.9 Cord blood peak 8701 in asthmatic and non-asthmatic pregnancies

*Panel A shows the mean peak intensity ± SEM of a cord blood peak with m/z 8701 identified using a SAX chip, pH 9 with SPA matrix. * indicates P<0.05 (t-test, no glucocorticoid male vs no glucocorticoid female). Panel B shows representative spectra from cord blood collected from male and female fetuses of the no glucocorticoid group in the region 8-9 kDa.*

7.5 The Fetus - Discussion

Increased fetal exposure to glucocorticoids in animal models has previously been associated with alterations in fetal HPA axis development and long-term changes into adulthood. Pregnant guinea pigs exposed to synthetic glucocorticoids have altered female fetal HPA function associated with increased hippocampal MR and GR expression (576). In rats, inhibition of placental 11β-HSD2 activity is associated with altered stress responses in offspring (574) and long-term changes such as delays in the development of puberty in females (573). My human study showed suppression of the female fetal HPA axis in the presence of maternal asthma and reduced placental 11β-HSD2 activity, as demonstrated by reduced umbilical vein concentrations of estriol, a derivative of fetal adrenal DHEA-S (819). This data suggests that despite similar levels of cortisol in cord blood from males and females of asthmatic mothers not treated with glucocorticoids, the females of this group are more sensitive to changes in placental cortisol metabolism. This observation supports previous clinical data demonstrating a greater response to synthetic glucocorticoid treatment for lung maturation in female fetuses at risk of preterm delivery (69, 766) and supports the suggestion raised in the previous chapter that the female fetus may be more sensitive to changes in cortisol concentration.

A previous study has shown that maternal infection is associated with significantly increased levels of the estriol precursor, DHEA-S in umbilical cord serum (826). Similar findings have been reported in amniotic fluid in the context of maternal infection (827). These data are in contrast to my results and those of others, which show that in situations of maternal or fetal stress (828) associated with conditions including miscarriage, IUGR, PIH or fetal distress, there are reduced umbilical cord concentrations of estriol or DHEA-S (822, 829). In addition, administration of the synthetic glucocorticoid betamethasone to pregnant women has been reported to significantly decrease urinary estriol excretion from as early as 24 hours after betamethasone treatment, with decreased excretion persisting for up to 3 weeks (823). DHEA-S is also reduced in cord blood samples 24 hours following betamethasone treatment (830, 831). In contrast, no significant association between high altitude hypoxia during pregnancy and maternal estriol concentrations could be demonstrated in one study (321). I found a significant inverse correlation between umbilical vein cortisol

and estriol, suggesting that a rise in cortisol crossing the placenta from mother to fetus has a negative effect on the production of estriol, which is derived from fetal adrenal DHEA-S. These data indicate that maternally derived or exogenous glucocorticoids have a significant effect on fetal adrenal function.

It was expected that umbilical vein cortisol concentrations would be correlated with placental 11β-HSD2 activity, since this enzyme regulates the amount of cortisol which passes the placenta from mother to fetus. However, in my study, no significant correlation between placental 11β-HSD2 activity and fetal cortisol concentrations was found (P=0.086). Kajantie *et al.* measured placental 11β-HSD2 activity and cord vein cortisol and cortisone in small preterm infants (556). They also did not find any correlation between placental 11β-HSD2 activity rate and cord vein cortisol or cortisone, but did demonstrate that low birth weight was associated with reduced 11β-HSD2 activity and reduced cord vein cortisone concentrations (556). Most of my cord blood samples were collected following vaginal delivery and it is possible that individual variation in the stress of the process of labour contributed to some variation in cord blood cortisol levels, such that they were not significantly different between any asthma groups and did not significantly correlate with the amount of enzyme activity measured in the placenta. Nonetheless, my data clearly show that 11β-HSD2 activity is reduced in placentae from females of the no glucocorticoid group, and the downstream effects of this reduction in transplacental cortisol inactivation were observed in the form of reduced placental GR and MR expression, increased placental Th2:Th1 cytokine mRNA and reduced umbilical vein estriol.

The IGF system is not primarily responsible for the alterations in female fetal growth observed in asthmatic pregnancies, when no inhaled glucocorticoids are used for treatment. Animal and human studies clearly demonstrate the importance of the IGF system in the control of fetal and placental growth (384, 385, 435). Most previous studies in animal models or human twins, have examined induced or spontaneous IUGR. However, I examined a group where growth was reduced on average to the 35th centile and only a small subset were SGA. This may explain the negative findings with regard to the importance of fetal and placental IGF-I and IGFBP-1 in growth regulation in the asthmatic population.

Fetal hypoxia is associated with increased IGFBP-1 concentrations in the umbilical artery (832). In fetal distress, demonstrated by meconium staining or abnormal fetal heart rate, an inverse correlation between umbilical arterial or venous concentrations of IGFBP-1 and PO_2 was found (833). *In vitro*, hypoxic conditions increase IGFBP-1 mRNA in fetal hepatocytes (834). In my study neither placental IGFBP-1 mRNA or fetal circulating IGFBP-1 was elevated in the no glucocorticoid female group, suggesting that local placental or fetal hypoxia is not present in these asthmatic pregnancies.

The regulation of fetal growth by IGFBPs may differ according to the sex of the fetus. In females, cord blood IGFBP-1 was inversely correlated with birth weight while there was no correlation between cord blood IGFBP-1 and male birth weight. However, for cord blood IGFBP-3, there was a significant positive correlation with birth weight in males only. These data are intriguing and suggest that the mechanisms controlling fetal growth are dependent upon the sex of the fetus. Previous studies have demonstrated positive correlations between IGFBP-3 and birth weight, but these studies did not separate data based upon fetal sex (446, 459). My study also found that the absolute levels of IGFBP-1 and IGFBP-3 in cord blood differed between males and females. Males had significantly higher levels of IGFBP-1, while females had significantly higher levels of IGFBP-3. A similar trend in fetal sex-related differences in IGFBPs was previously reported but not found to be statistically significant (445). However, a recent study examining sexual dimorphism in the growth hormone (GH)-IGF axis in cord blood, supported my finding that cord blood IGFBP-3 was reduced in male neonates compared to female neonates (629). In addition, this group also found reduced IGF-I and increased GH in male neonates compared to female neonates, but no difference in cord blood IGF-II. They suggest that this is evidence for the early establishment of the sexually dimorphic pattern of GH secretion and IGF production found in children (629).

Studies have demonstrated that cortisol is an important indirect regulator of fetal growth through its actions on IGFs and their binding proteins. Cianfarani *et al.* found that there was a positive correlation between IGFBP-1 and cortisol in human cord blood from appropriately grown fetuses (835). However, they could not demonstrate any relationship in IUGR fetuses (835). Other studies also described increased plasma IGFBP-1 following cortisol infusions in adults (836) and increased cord blood IGFBP-1 following the relative stress of labour and delivery (837). In my study, there was a

significant positive correlation between cord blood cortisol and cord blood IGFBP-1 in females only. As previously described, reduced cortisol metabolism across the placenta as a result of decreased 11β-HSD2 activity was associated with reduced female fetal growth and adrenal function in asthmatic pregnancies. These data suggest that as well as its direct effect on fetal growth, cortisol levels may also indirectly control female fetal growth through the regulation of IGFBP-1. In female fetuses, increased circulating IGFBP-1 may result in increased binding of free IGF-I, which would prevent IGF-I from exerting its effects on the fetus through the IGF-I receptor. It is known that phosphorylation of IGFBP-1 is an important component of its regulation and action during pregnancy (417). The measurement of IGFBP-1 phosphorylation was beyond the scope of this thesis, but may be an important component of its action in fetal growth regulation during human pregnancy and worth investigating in the future.

IGFs and their binding proteins may not be the principle regulators of fetal growth in pregnancies complicated by asthma. However, the correlations between cord blood IGFBP-1 or IGFBP-3 and birth weight were different in males and females, suggesting that the mechanisms controlling fetal growth during pregnancy may differ depending upon the sex of the fetus.

Preliminary proteomic analysis of the cord blood protein profile demonstrated several differences due to maternal asthma, but few differences attributable to fetal sex. The presence of maternal asthma may alter cord blood proteins and some of these could be important in establishing immune function of the neonate. Further identification of the protein differences is required to examine this prospect. It is also possible that fetally-derived protein factors alter maternal physiology during asthmatic pregnancies, and further identification of the cord blood protein profile may lead to a better understanding of the mechanisms involved in pregnancy-induced alterations in maternal asthma.

7.6 The Fetus - Summary

In this chapter I have demonstrated that alterations in maternal asthma and placental function during pregnancy have down-stream effects on the female fetus, such as suppressed adrenal function, demonstrated by reduced concentrations of estriol in cord blood. This may have implications for later life and could represent a programming effect. This data confirms that alterations in placental 11β-HSD2 enzyme activity has a functional effect on the development of the fetal HPA axis in humans. Figure 7.10 summarises the interactions between female fetus, asthmatic mother and placenta.

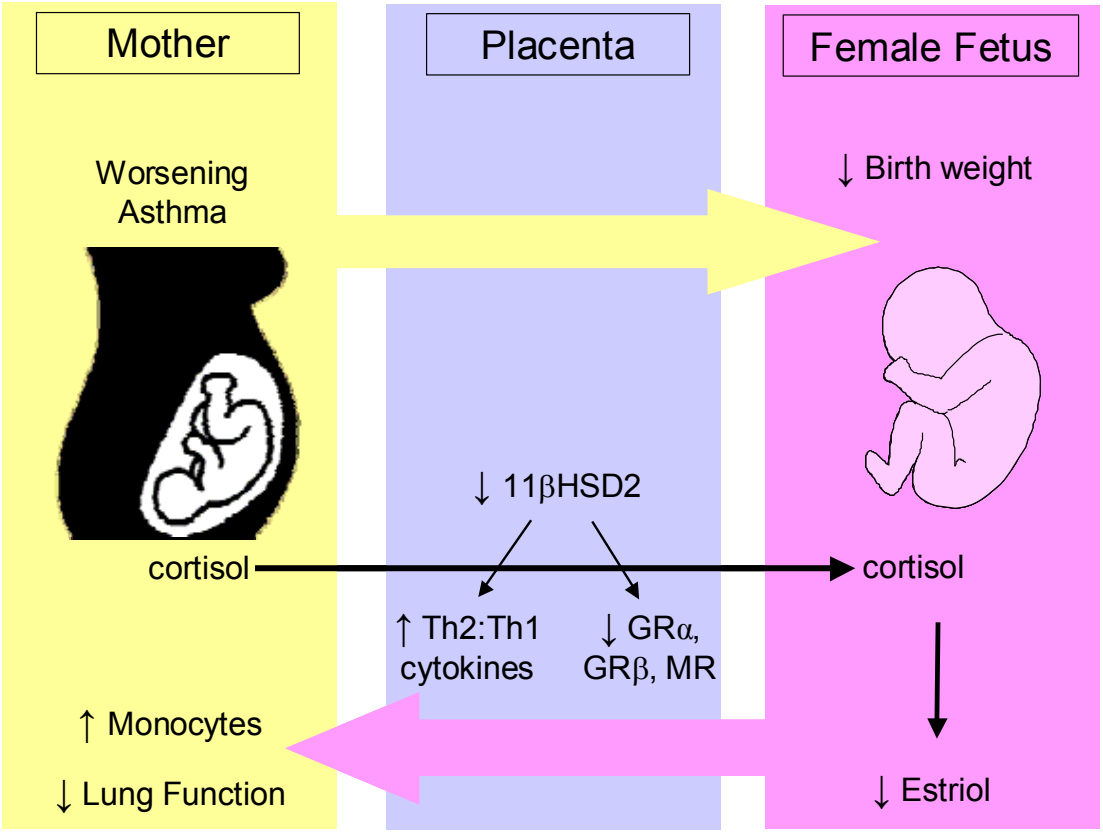

Figure 7.10 Interactions between mother, placenta and fetus in pregnancies complicated by asthma (part 2)

In the presence of a female fetus, maternal asthma worsens during pregnancy, as demonstrated by a significant rise in circulating monocytes and a significant reduction in lung function. These alterations in maternal asthma in the absence of glucocorticoid therapy are associated with significantly reduced female birth weight and changes in placental function. Placental 11β-HSD2 activity is significantly reduced, which allows more maternally derived cortisol to reach the female fetus. Further changes in placental function which may be due to the decrease in 11β-HSD2 activity include a rise in the local Th2:Th1 cytokine mRNA ratio and decreased glucocorticoid and mineralocorticoid receptor expression. The changes in placental cortisol metabolism contribute to changes in the fetus, reducing growth in late gestation, and suppressing fetal HPA axis function, as demonstrated by significantly reduced estriol concentrations in female cord blood.

Chapter 8 **Linking the Mother, Placenta and Fetus**

8.1 Final summary

The key findings of this work were as follows:

1. Maternal asthma, which is not treated with inhaled glucocorticoids is associated with reduced female fetal growth in late gestation.
2. Inhaled glucocorticoids used for asthma treatment have no adverse effects on male or female fetal growth or HPA development.
3. Asthma education improves asthma management in pregnant women.
4. The presence of a female fetus is associated with worsening maternal asthma during pregnancy, indicated by reduced lung function and an increase in inflammatory pathways. In women not using inhaled glucocorticoids, there was an increase in the maternal monocyte count as pregnancy progressed, while women who did use inhaled glucocorticoids, significantly increased their dose as gestation progressed, only when pregnant with a female fetus.
5. Reduced lung function is associated with reduced female birth weight in the absence of glucocorticoid therapy.
6. Placental 11β-HSD2 activity is reduced in females from asthmatic mothers who do not use inhaled glucocorticoids and may contribute to reduced growth by allowing more maternally-derived cortisol to reach the fetus.
7. Alterations in the transplacental passage of cortisol in the presence of maternal asthma is associated with increased placental Th2:Th1 cytokine mRNA ratios and reduced expression of glucocorticoid and mineralocorticoid receptors.
8. A decrease in cortisol inactivation by placental 11β-HSD2 is associated with downstream effects of excess cortisol on the female fetus, such as a reduction in fetal adrenal function.
9. Protein profiling demonstrated that there are alterations in several maternal, placental and cord blood proteins in the presence of maternal asthma which may also be influenced by fetal sex.

8.2 Final discussion

This study was the first to simultaneously examine the effects of asthma on the endocrine and immune relationships between the mother, placenta and fetus and to assess their role in the control of fetal growth during human pregnancy. The study used a standard asthma management protocol designed to maximise asthma control in individual patients. Subjects were classified based on both disease severity and treatment, allowing a comprehensive analysis of the effect of both these factors on fetal growth. This work uniquely contributes to the literature and proposes maternal and placental mechanisms which lead to decreased female fetal growth in pregnant women with asthma.

The mother, placenta and fetus all played a role in alterations in fetal growth in pregnancies complicated by asthma. The fetus influenced maternal asthma, alterations in maternal asthma affected placental function, and changes in placental function ultimately had consequences for fetal growth and development.

The study found that female fetal growth was reduced in asthmatic women who did not use inhaled glucocorticoids for treatment and had relatively mild asthma. Unlike many previous analyses, this study classified pregnant asthmatic women based upon disease severity independently of treatment. As a result, I was able to show that mild asthma, which was not recommended for inhaled glucocorticoid therapy contributes to reduced female fetal growth in late gestation. In addition, no previous studies have examined fetal growth by ultrasound during asthmatic pregnancies. However, I found no differences in fetal size as measured by ultrasound at 18 or 30 weeks, but a significant reduction in birth weight and a similar reduction in head size at birth, suggesting a late gestation symmetrical growth reduction in female neonates.

Severe asthma was not found to be a major contributor to the alterations in placental function or fetal growth in asthmatic pregnancies. In addition, the use of high doses of inhaled glucocorticoids did not contribute to significant changes in placental function or fetal development. Surprisingly, women with mild asthma, who were medically advised that they did not require glucocorticoid therapy for asthma control, had alterations in maternal inflammation, placental function and female fetal growth.

This was the first study to identify that changes in fetal growth in asthmatic pregnancies are fetal sex-specific. I found that the female fetus was particularly susceptible to reduced growth, and in addition, the female fetus had a different effect from the male fetus on the maternal immune system during pregnancy, with an up-regulation of inflammatory pathways and a decrease in lung function observed in asthmatic women pregnant with a female fetus. Previous reports had suggested the possibility that female fetal sex leads to a worsening of maternal asthma, while male fetal sex was associated with an improvement in symptoms and less drug use during pregnancy (164, 165). However, my study is the first to report both a worsening of asthma in the presence of a female fetus as well as reduced female fetal growth in the same group of asthmatic mothers with very mild asthma. This provides evidence of a strong link between mother and fetus in asthmatic pregnancies. Alterations in maternal asthma in the presence of a female fetus may also be involved in the observed changes in placental function, which included a reduction in placental 11β-HSD2 activity and a trend towards increased cord blood cortisol. These changes in placental function were associated with reduced fetal growth and suppressed fetal adrenal function in females.

Maternal inflammation, rather than maternal hypoxia, may be the key to alterations in female fetal growth in this setting, since the use of anti-inflammatory inhaled glucocorticoids by pregnant women with mild asthma were protective. Moreover, changes previously observed in the context of maternal hypoxia, such as reduced abdominal circumference by 25 weeks gestation (320), altered uteroplacental blood flow (324) and changes in the expression of placental cytokines (796) and glucocorticoid receptors (814), were not observed in the no glucocorticoid group (111) in my study. Nonetheless, further investigation into maternal and fetal hypoxia in the context of maternal asthma will be required in future studies. The male fetus appeared to be insensitive to the effects of inflammation in the mother, with no changes in placental function or growth observed in male fetuses. In conclusion, the female fetus has an adverse effect on maternal asthma, which when not treated with inhaled glucocorticoids results in reduced fetal growth.

A further aspect of my study was that the asthmatic women were actively managed during pregnancy, as was similarly performed in the many prospective studies carried out by Schatz and colleagues (53, 83, 134, 148). However, this also included patient education to improve self-management of asthma, which was found to increase

knowledge of medications and inhaler technique and equipped women to better manage their asthma in the event of an exacerbation by providing an asthma action plan. This education program is part of the strategy to improve the health outcomes of mothers with asthma and their babies.

The placental enzyme 11β-HSD2, which prevents large amounts of the anti-mitogenic hormone cortisol from reaching the fetus in an active form, was found to be important in the mechanism of reduced fetal growth, since decreased enzyme activity was observed in placentae from females of the no glucocorticoid group. Previous studies have identified placental 11β-HSD2 to be an important controller of human fetal growth in relation to IUGR (554) and pre-eclampsia (563, 595). Although placental 11β-HSD2 activity can be altered by hypoxia (595), its regulation by inflammatory mediators has not yet been investigated in the placenta.

No studies from other research groups have investigated changes in placental function in relation to maternal asthma. My work has contributed to an understanding of the placental mechanisms contributing to reduced female fetal growth in asthmatics who do not use inhaled glucocorticoids for treatment. In addition to reduced placental 11β-HSD2 activity, there were further alterations in placental expression of cytokines and glucocorticoid receptors. The placental cytokine Th2:Th1 mRNA ratio was increased and mRNA abundance of GR-α, GR-β and MR was reduced in females from the no glucocorticoid group. Such changes are likely to be a consequence of altered 11β-HSD2 activity resulting in increased local cortisol concentrations, as suggested by the correlation between Th2:Th1 cytokines and enzyme activity, and may ultimately have effects on the developing fetal immune system and fetal glucocorticoid sensitivity which will need to be specifically addressed in future studies.

It is possible that the increased maternal monocyte count in asthmatic women pregnant with a female fetus contributed to alterations in the placental IL-5:TNF-α (Th2:Th1) cytokine ratio. A recent study demonstrated that IL-5 gene transcription was up-regulated in the presence of human monocytes (838). In cultures of $CD4^+$ T cells, IL-5 production was enhanced by the addition of monocytes from atopic asthmatics but not by monocytes from non-atopic non-asthmatic subjects (736). Placental monocyte infiltration in malaria has been associated with low birth weight (839). In pregnant women with asthma, maternal monocytes may influence placental function, particularly

Th2 and Th1 cytokine mRNA expression. This hypothesis will need to be tested in future experiments.

Reduced transplacental metabolism of cortisol may have contributed to changes in fetal HPA axis development, as a significant decrease in cord blood estriol was found specifically in females from the no glucocorticoid group, and there was an inverse correlation between cord blood cortisol and estriol. Previous studies in humans have demonstrated that the levels of estriol or its precursor, DHEA-S, are reduced with maternal or fetal stress (822, 829) and maternal betamethasone administration (823, 830, 831). Animal studies have shown that glucocorticoid exposure *in utero*, or maternal stress during pregnancy can lead to fetal programming of the HPA axis and altered stress responses in offspring later in life (576, 578, 579). Female fetal HPA function appeared to be significantly altered in the presence of maternal asthma not treated with inhaled glucocorticoids and this could potentially contribute to changes in HPA function in neonatal or later life. Future follow-up studies will examine this possibility in the offspring of asthmatic mothers.

The results of my study indicated that the female fetus was particularly susceptible to alterations in fetal growth in the context of asthmatic pregnancies. Previous reports have described an increased likelihood of reduced male fetal growth in the presence of maternal smoking (313, 631) or caffeine use (633). On the other hand, the female fetus is more likely to be affected by growth restriction associated with maternal hypertension (632). Various other aspects of pregnancy outcome are also related to fetal sex. Placental dysfunction is more likely to occur in pregnancies with a male fetus compared to pregnancies with a female fetus (840) and women pregnant with a male fetus are more at risk of placenta previa (841). Eogan *et al.* found that women who went into spontaneous labour and were pregnant with a male fetus were more likely to require an assisted delivery or to have complications during labour and delivery, such as oxytocin augmentation, epidural analgesia or fetal distress, than women pregnant with a female fetus (842). In addition, maternal nutrient intake has been shown to differ depending on the sex of the fetus, with women pregnant with a male fetus having a higher energy intake than women pregnant with a female fetus (630). These studies and my work establish that there is a strong link between mother and fetus related to fetal sex.

These fetal sex differences may be due to variation in circulating sex hormones or other factors such as proteins which differ between the male and female fetus. However, previous data on fetal sex-specific hormonal variation during pregnancy has been conflicting and dependent upon gestational age. Abramovich and Rowe found that cord blood concentrations of testosterone were significantly higher in males than females between 12 to 18 weeks gestation, but were not different at term (843). Reyes *et al.* found that fetal serum testosterone was higher in males than females between 9 and 25 weeks gestation, while follicle stimulating hormone (FSH) in fetal serum was significantly higher in females than in males (844). Other authors have reported that after 17 weeks gestation, circulating testosterone in males declines and by term is the same as circulating levels in females (845). However, Ketupanya and Wiest reported higher levels of testosterone in amniotic fluid from male fetuses throughout gestation, even from 31-40 weeks (846). Robinson *et al.* showed that between 14 and 20 weeks gestation, amniotic fluid levels of testosterone and androstenedione were higher in males, while estradiol was higher in females (847). To investigate the possibility of mid-gestation testosterone and FSH being used for sex determination, amniotic fluid levels were measured by Belisle *et al.* (848). They found that in the majority of cases, testosterone was higher in males, while FSH levels in males were 10 times lower than those in females (848). Forest *et al.* found no differences between the sexes in amniotic fluid levels of progesterone, estrone, cortisol and cortisone (849). They did, however, note an increase in testosterone and androstenedione in males between 12 and 19 weeks and in estradiol between 15 and 19 weeks in females, probably due to fetal gonadal activity at this time (849). Several progesterone metabolites including 17-hydroxyprogesterone have been reported to be elevated in females compared to males (849), while another study found no sex differences in the levels of progesterone itself, or cortisol, estradiol and estriol in the umbilical vein between 28 and 40 weeks gestation (850). Riley *et al.* showed that inhibin B, a pituitary hormone which suppresses the secretion of pituitary FSH, was detectable in fetal cord serum from males but not females (851). Placental explants have been shown to produce more hCG if from females compared to males (852). Based on this information, testosterone, androstenedione, estradiol and FSH appear to differ between males and females at some times during gestation, but these differences were rarely observed in later gestation, when changes in maternal asthma and fetal growth occurred in asthmatic women.

Few studies have identified fetal sex-specific hormonal differences using maternal blood. However, given that fetal cells and placental mRNA can be detected in the maternal circulation (757, 758), it is not unlikely that sex-specific products originating from the fetus may circulate in the mother and alter her immune system. Previous studies indicate that female sex hormones alter cytokine release from macrophages (853). Eosinophil function can also be altered by sex hormones, with estradiol and progesterone causing an increase in adhesion of eosinophils to mucosal endothelial cells and subsequent eosinophil degranulation, while testosterone caused a reduction of adhesion and viability of eosinophils (854). Fetally-derived factors may also be involved in an alteration of monocyte/macrophage phenotype or function in the mother. This possibility has not previously been examined in relation to fetal sex or maternal asthma. An up-regulation of maternal inflammation may be directly involved in the reduction of placental cortisol metabolism by 11β-HSD2 and ultimately in altering female fetal growth and development.

In this study, I have used the SELDI-TOF proteomics technique to examine proteins expressed in maternal plasma, the placenta and cord blood. Preliminary data analysis suggests that this method could be useful for identifying novel interactions between the mother, placenta and fetus in asthmatic pregnancies. There is a need for further replication and verification of this data. However, the initial results suggest a complex interplay between mother and fetus during pregnancy which contributes to changes in maternal plasma proteins associated with asthma as pregnancy progresses.

In-depth statistical analysis of the SELDI data with principal component analysis (PCA) would be beneficial, as alterations in the pattern of protein peaks, rather than individual protein changes could be extremely important in the altered physiology of asthma during pregnancy. PCA is a technique which reduces the complexity of data with a large number of interrelated variables, by transforming the data into principal components or key variables, allowing visualisation of the data set (855-857). This method has been used previously to analyse data from gene expression studies using cDNA arrays (858-860) and could also be useful for proteomic data analysis. Such an approach, coupled with further identification and characterisation of protein peaks would facilitate data interpretation.

An unknown factor derived from the female fetus may alter maternal immune function in asthmatic women. This factor could be a novel sex steroid hormone, or a novel protein originating from the fetus. My preliminary proteomics studies suggested that fetal sex alters the pattern of maternal, placental and cord blood proteins. However, only three protein differences were identified between male and female cord blood at term. Work from our laboratory has also demonstrated that there are several differences in unknown steroid compounds in term cord blood analysed by HPLC (Dr Vicki Clifton and Dr Pawel Zarzycki, unpublished data). These fetally-derived factors may play a role in altering maternal physiology during asthmatic pregnancies.

The SELDI technique has not previously been used to study plasma protein profiles in asthma or pregnancy. Further identification of these proteins will increase our understanding of the factors which are exchanged between mother and fetus and how they are involved in the alteration of maternal asthma during pregnancy and the alteration of fetal growth in the female fetus.

8.3 Clinical and scientific implications

This study has several important clinical and scientific implications. The results have demonstrated that the use of inhaled glucocorticoids by women with mild asthma was beneficial for the growth of female fetuses, possibly by controlling maternal systemic inflammation. In addition, the female fetus influenced the course of maternal asthma through pregnancy. This has implications for the management of pregnant women with asthma, as the use of low dose inhaled glucocorticoids for mild symptomatic asthmatics may be warranted during pregnancy. A recent randomised clinical trial investigated low dose budesonide use (400 μg/day) in adults who had developed mild asthma within the previous two years (861). The study showed that budesonide decreased the requirement for systemic glucocorticoids to treat severe exacerbations and increased prebronchodilator FEV_1 by 2.24% after 1 year (861). In addition, the number of symptom free days significantly increased and there was a significant decrease in hospital and emergency department visits for asthma (862). Preliminary results from the same trial demonstrated that in 198 pregnant women (217 pregnancies) there were no adverse effects of low dose budesonide treatment in mild asthmatics (204), which confirmed similar epidemiological data from the Swedish medical birth registry (202).

Low dose budesonide treatment may be beneficial for pregnant women with mild asthma who have not previously used inhaled glucocorticoids. Further research into the type of airway inflammation in pregnant women with asthma and an understanding of the mechanisms by which pregnancy alters asthma will improve the treatment of these pregnant women, which ultimately improves the health of their baby both in the short-term and long-term.

Scientifically, this study has contributed to an understanding of the mechanisms regulating fetal growth in human pregnancy. Placental 11β-HSD2 activity is a key component of this mechanism, through its control of active cortisol concentrations reaching the fetus. The female fetus was particularly sensitive to alterations in cortisol exposure and the downstream effects of this included reduced fetal adrenal function and reduced growth. These changes in fetal HPA axis development and growth potentially expose these female neonates to an increased risk of developing diseases in later life through altered fetal programming. By examining the endocrine and immune relationships between mother, placenta and fetus during asthmatic pregnancies, this study has provided evidence for a detrimental effect of maternal inflammation on placental function and female fetal growth and development.

8.4 Future work

8.4.1 Regulation of placental 11β-HSD2

The results of this study showed that the reduction in female fetal growth is associated with reduced activity of the placental enzyme 11β-HSD2, which regulates the passage of cortisol from mother to fetus. In addition, there was an increased mRNA ratio of Th2:Th1 cytokines in the placenta, and 11β-HSD2 activity was inversely correlated with the Th2:Th1 mRNA ratio. Whether the placental enzyme 11β-HSD2 is regulated by inflammatory factors remains unknown and has not previously been examined by any other groups. In osteosarcoma cells, TNF-α and IL-1β inhibited 11β-HSD2 activity and mRNA (604) and these or other cytokines may also be involved in placental 11β-HSD2 activity regulation. In addition, the balance of Th2:Th1 cytokines may be crucial for determining placental 11β-HSD2 activity. Future work would examine the regulation of placental 11β-HSD2 by inflammatory factors in a placental explant model and in primary syncytiotrophoblast cells.

8.4.2 Maternal inflammatory pathways in asthma

Data from this study indicate that the fetus can influence how a mother's asthma progresses through pregnancy. Specifically, in the presence of a female fetus, asthmatic women (both those treated and those not treated with inhaled glucocorticoids) have a worsening of their lung function from early to late pregnancy. In addition, there is an increase in the percentage and number of circulating monocytes in the mother from early to late pregnancy, in women not using inhaled glucocorticoids for treatment who are pregnant with a female fetus. Eosinophils are often considered the major inflammatory cell in asthma (754, 863). However, my data suggests that alternative inflammatory pathways involving monocytes may play a more important role such that, during pregnancy, a non-eosinophilic mechanism of asthma may be occurring. Future studies will use flow cytometry to examine whether there are changes in the activation phenotype of eosinophils, monocytes and neutrophils or in their production of cytokines during pregnancies complicated by asthma. This may help improve treatment options for pregnant asthmatic women.

In my study, no markers of airway inflammation were specifically examined. The measurement of NO in exhaled breath condensate is a non-invasive method which could be extremely useful for monitoring airway inflammation in pregnant asthmatics. Studies have shown that exhaled NO reflects airway hyperresponsiveness, is reduced by inhaled glucocorticoid use and is a useful marker for airway inflammation in mild asthmatics (864, 865). Future studies will examine exhaled NO in pregnant asthmatic and non-asthmatic women as a marker of airway inflammation and determine whether it is altered by pregnancy itself, fetal sex or maternal glucocorticoid use. A marker such as exhaled NO could be used as a management tool, to observe changes in maternal airway inflammation during pregnancy, and alter treatment as required.

8.4.3 Maternal and fetal oxygenation in asthmatic pregnancies

Although much of the available evidence suggests that maternal hypoxia is not likely to contribute to reduced birth weight and altered placental function in pregnant women with mild asthma, maternal and fetal oxygenation were not directly measured in this study. It is important to confirm that women with mild asthma who do not use inhaled

glucocorticoids have normal arterial oxygenation and that there are no adverse effects of maternal asthma on fetal oxygenation. Fetal oxygenation could be investigated by measuring the hematocrit or erythropoietin levels in cord blood, which if increased, are indicators of hypoxia (866-869).

8.4.4 Identification and characterisation of maternal, placental and fetal proteins

The results of the proteomics studies have built up a complex picture of the changes in plasma proteins which occur during pregnancy, highlighting the large number of plasma protein differences between pregnant women with and without asthma. The findings support the concept of a dynamic interaction between fetus and mother during pregnancy and with further information the data may also lead to a greater understanding about the effect pregnancy itself has on the progression of asthmatic disease in these women.

There are several approaches to protein identification and characterisation which could be carried out in future studies. The SELDI data gives an estimate of the protein's MW and an estimate of pI can be obtained from the known binding properties of the protein. Using this information, a purification technique could be designed utilising size exclusion and/or ion exchange column chromatography, HPLC and possibly 2D-PAGE for proteins >10 kDa (690, 692). Repeat examination of the purified fractions by SELDI would be necessary to ensure the correct peak was being isolated. The purified protein would be subject to trypsin digest and the fragments produced by the trypsin digest and subsequent collision-induced dissociation in MS-MS would produce a unique set of ions, which could be matched with theoretical fragments available in protein databases (769). Amino acid sequencing would be performed to confirm the sequence. Other methods such as reduction with DTT to probe for the existence of disulfide bonds and binding to antibodies, with subsequent SELDI analysis to visualise changes in peptide structure, could be performed to confirm the identity of the proteins (769).

Some of the factors to be identified may be novel proteins, which potentially could be used for the diagnosis or treatment of asthma during pregnancy. These discoveries could improve the health care of mothers and their babies by improving the management of pregnant women with asthma.

8.5 Final conclusion

This thesis has presented a comprehensive study of the endocrine and immune interactions between mother, placenta and fetus in pregnancies complicated by asthma (Figure 8.1). It has addressed the question of whether fetal growth is reduced in asthmatic pregnancies and has proposed a placental mechanism for the change in growth of the female fetus. Female fetal growth is reduced in women with mild asthma who do not used inhaled glucocorticoids, through altered placental 11β-HSD2 activity. In addition, the results of this study indicate that inhaled glucocorticoids have no effect on placental function, fetal HPA axis development or fetal growth in pregnancies complicated by asthma. This work has added considerably to the existing literature on asthma and pregnancy by examining the mechanisms involved in alterations in the mother's asthma during pregnancy as well as the mechanisms contributing to reduced fetal growth in asthmatic pregnancies. This study has provided strong evidence both for the detrimental effects of maternal inflammation on placental function and female fetal growth and development, and for the role of the fetus in influencing maternal health during pregnancy.

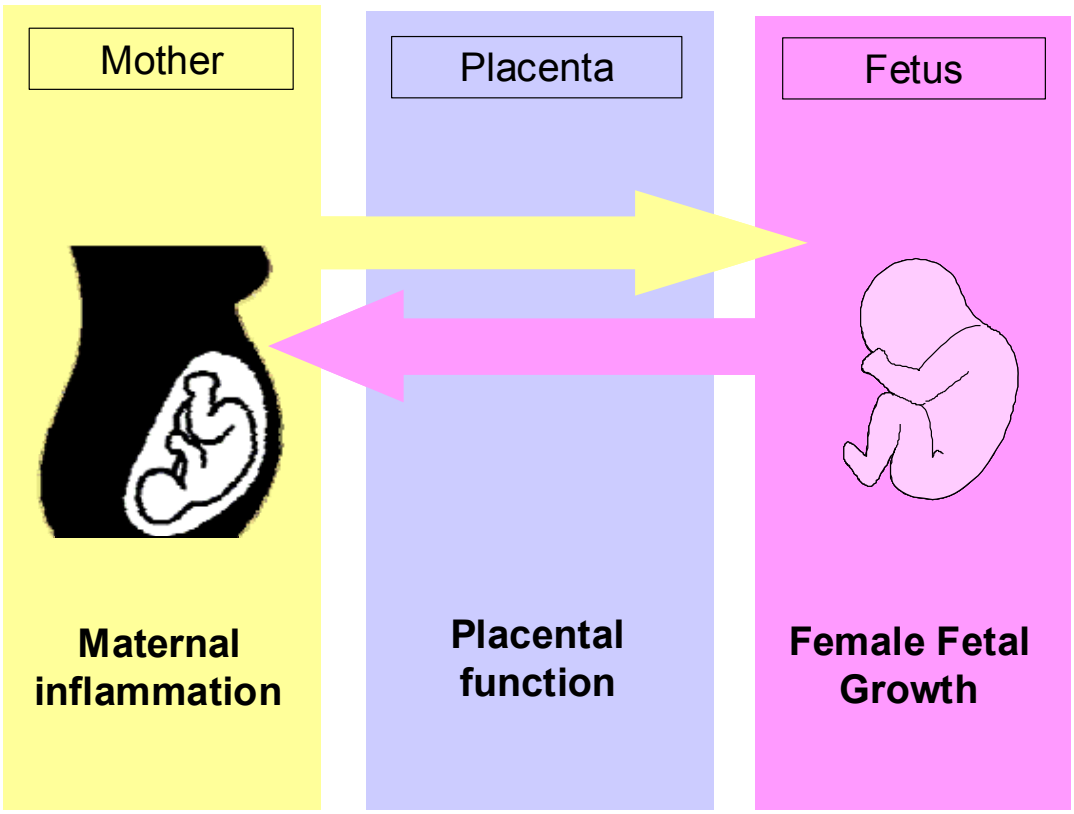

Figure 8.1 Summary of the interactions between mother, placenta and fetus in asthmatic pregnancies

This study has examined the endocrine and immune relationships between mother, placenta and fetus in pregnancies complicated by asthma. The female fetus is able to influence the course of maternal asthma during pregnancy. Maternal inflammation may be important in altering placental function, which ultimately influences female fetal growth.

References

1. **Guyton AC, Hall JE** 1996 *Textbook of medical physiology*. W.B. Saunders Company, Philadelphia
2. **Mazzarella G, Bianco A, Catena E, De Palma R, Abbate GF** 2000 *Th1/Th2 lymphocyte polarization in asthma*. Allergy 55 Suppl 61:6-9
3. **Thien F** 2000 *Immunology of asthma: the role of allergy*. In: Walls RS, Jenkins CR (eds) Understanding asthma. MacLennan and Petty, Sydney, pp 51-61
4. 2002 *Asthma Management Handbook*. National Asthma Council Australia Ltd, South Melbourne
5. **Gore CJ, Crockett AJ, Pederson DG, Booth ML, Bauman A, Owen N** 1995 *Spirometric standards for healthy adult lifetime nonsmokers in Australia*. Eur Respir J 8:773-82
6. **Pierce R, Johns DP** 1995 *Spirometry: the measurement and interpretation of ventilatory function in clinical practice*. National Asthma Campaign Ltd, Melbourne
7. **Laitinen LA, Heino M, Laitinen A, Kava T, Haahtela T** 1985 *Damage of the airway epithelium and bronchial reactivity in patients with asthma*. Am Rev Respir Dis 131:599-606
8. **Chu HW, Halliday JL, Martin RJ, Leung DY, Szefler SJ, Wenzel SE** 1998 *Collagen deposition in large airways may not differentiate severe asthma from milder forms of the disease*. Am J Respir Crit Care Med 158:1936-44
9. **Fish JE, Peters SP** 1999 *Asthma severity: histopathologic correlations*. Drugs Today (Barc) 35:585-94
10. **Beasley R, Roche WR, Roberts JA, Holgate ST** 1989 *Cellular events in the bronchi in mild asthma and after bronchial provocation*. Am Rev Respir Dis 139:806-17
11. **Barnes PJ, Chung KF, Page CP** 1998 *Inflammatory mediators of asthma: an update*. Pharmacol Rev 50:515-96
12. **Renauld JC** 2001 *New insights into the role of cytokines in asthma*. J Clin Pathol 54:577-89
13. **Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL** 1986 *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins*. J Immunol 136:2348-57
14. **Wierenga EA, Snoek M, de Groot C, Chretien I, Bos JD, Jansen HM, Kapsenberg ML** 1990 *Evidence for compartmentalization of functional subsets of CD2+ T lymphocytes in atopic patients*. J Immunol 144:4651-6
15. **Del Prete GF, De Carli M, Mastromauro C, Biagiotti R, Macchia D, Falagiani P, Ricci M, Romagnani S** 1991 *Purified protein derivative of Mycobacterium tuberculosis and excretory-secretory antigen(s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production*. J Clin Invest 88:346-50
16. **Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB** 1992 *Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma*. N Engl J Med 326:298-304
17. **Vercelli D, Jabara HH, Arai K, Geha RS** 1989 *Induction of human IgE synthesis requires interleukin 4 and T/B cell interactions involving the T cell receptor/CD3 complex and MHC class II antigens*. J Exp Med 169:1295-307

18. **Gascan H, Gauchat JF, Roncarolo MG, Yssel H, Spits H, de Vries JE** 1991 *Human B cell clones can be induced to proliferate and to switch to IgE and IgG4 synthesis by interleukin 4 and a signal provided by activated CD4+ T cell clones*. J Exp Med 173:747-50
19. **Seder RA, Paul WE** 1994 *Acquisition of lymphokine-producing phenotype by CD4+ T cells*. Annu Rev Immunol 12:635-73
20. **Rankin JA, Picarella DE, Geba GP, Temann UA, Prasad B, DiCosmo B, Tarallo A, Stripp B, Whitsett J, Flavell RA** 1996 *Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity*. Proc Natl Acad Sci U S A 93:7821-5
21. **Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K** 1997 *Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production*. J Exp Med 186:1737-47
22. **McKenzie GJ, Fallon PG, Emson CL, Grencis RK, McKenzie AN** 1999 *Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses*. J Exp Med 189:1565-72
23. **Kay AB** 1991 *T lymphocytes and their products in atopic allergy and asthma*. Int Arch Allergy Appl Immunol 94:189-93
24. **Yamaguchi Y, Hayashi Y, Sugama Y, Miura Y, Kasahara T, Kitamura S, Torisu M, Mita S, Tominaga A, Takatsu K** 1988 *Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor*. J Exp Med 167:1737-42
25. **Dent LA, Strath M, Mellor AL, Sanderson CJ** 1990 *Eosinophilia in transgenic mice expressing interleukin 5*. J Exp Med 172:1425-31
26. **Kopf M, Brombacher F, Hodgkin PD, Ramsay AJ, Milbourne EA, Dai WJ, Ovington KS, Behm CA, Kohler G, Young IG, Matthaei KI** 1996 *IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses*. Immunity 4:15-24
27. **Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG** 1996 *Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model*. J Exp Med 183:195-201
28. **Chung F** 2001 *Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon-gamma*. Mediators Inflamm 10:51-9
29. **Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A** 1991 *IL-10 inhibits cytokine production by activated macrophages*. J Immunol 147:3815-22
30. **Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G, Romagnani S** 1993 *Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells*. J Exp Med 177:1199-204
31. **Leonard C, Tormey V, Burke C, Poulter LW** 1997 *Allergen-induced cytokine production in atopic disease and its relationship to disease severity*. Am J Respir Cell Mol Biol 17:368-75
32. **Bentley AM, Hamid Q, Robinson DS, Schotman E, Meng Q, Assoufi B, Kay AB, Durham SR** 1996 *Prednisolone treatment in asthma. Reduction in the numbers of eosinophils, T cells, tryptase-only positive mast cells, and modulation of IL-4, IL-5, and interferon-gamma cytokine gene expression within the bronchial mucosa*. Am J Respir Crit Care Med 153:551-6

33. **Naseer T, Minshall EM, Leung DY, Laberge S, Ernst P, Martin RJ, Hamid Q** 1997 *Expression of IL-12 and IL-13 mRNA in asthma and their modulation in response to steroid therapy*. Am J Respir Crit Care Med 155:845-51
34. **Borish L, Aarons A, Rumbyrt J, Cvietusa P, Negri J, Wenzel S** 1996 *Interleukin-10 regulation in normal subjects and patients with asthma*. J Allergy Clin Immunol 97:1288-96
35. **Schandene L, Alonso-Vega C, Willems F, Gerard C, Delvaux A, Velu T, Devos R, de Boer M, Goldman M** 1994 *B7/CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10*. J Immunol 152:4368-74
36. **Peat JK** 2000 *Epidemiology and the changing prevalence of asthma*. In: Walls RS, Jenkins CR (eds) Understanding asthma. MacLennan and Petty, Sydney, pp 11-19
37. **Alexander S, Dodds L, Armson BA** 1998 *Perinatal outcomes in women with asthma during pregnancy*. Obstet Gynecol 92:435-40
38. **Demissie K, Marcella SW, Breckenridge MB, Rhoads GG** 1998 *Maternal asthma and transient tachypnea of the newborn*. Pediatrics 102:84-90
39. **Kwon HL, Belanger K, Bracken MB** 2003 *Asthma prevalence among pregnant and childbearing-aged women in the United States: estimates from national health surveys*. Ann Epidemiol 13:317-24
40. **Gordon M, Niswander KR, Berendes H, Kantor AG** 1970 *Fetal morbidity following potentially anoxigenic obstetric conditions. VII. Bronchial asthma*. Am J Obstet Gynecol 106:421-9
41. **Perlow JH, Montgomery D, Morgan MA, Towers CV, Porto M** 1992 *Severity of asthma and perinatal outcome*. Am J Obstet Gynecol 167:963-7
42. **Demissie K, Breckenridge MB, Rhoads GG** 1998 *Infant and maternal outcomes in the pregnancies of asthmatic women*. Am J Respir Crit Care Med 158:1091-5
43. **Beckmann CA** 2003 *The effects of asthma on pregnancy and perinatal outcomes*. J Asthma 40:171-80
44. 2001 *From the Centers for Disease Control and Prevention. Self-reported asthma prevalence among adults--United States, 2000*. JAMA 286:1571-2
45. 1998 *Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee*. Lancet 351:1225-32
46. **Kurinczuk JJ, Parsons DE, Dawes V, Burton PR** 1999 *The relationship between asthma and smoking during pregnancy*. Women Health 29:31-47
47. **Schaefer G, Silverman F** 1961 *Pregnancy complicated by asthma*. Am J Obstet Gynecol 82:182-91
48. **Williams DA** 1967 *Asthma and pregnancy*. Acta Allergol 22:311-23
49. **Liu S, Wen SW, Demissie K, Marcoux S, Kramer MS** 2001 *Maternal asthma and pregnancy outcomes: A retrospective cohort study*. Am J Obstet Gynecol 184:90-96
50. **Wen SW, Demissie K, Liu S** 2001 *Adverse outcomes in pregnancies of asthmatic women: results from a Canadian population*. Ann Epidemiol 11:7-12
51. **Mihrshahi S, Belousova E, Marks GB, Peat JK** 2003 *Pregnancy and birth outcomes in families with asthma*. J Asthma 40:181-7
52. **Fisher ES, Whaley FS, Krushat WM, Malenka DJ, Fleming C, Baron JA, Hsia DC** 1992 *The accuracy of Medicare's hospital claims data: progress has been made, but problems remain*. Am J Public Health 82:243-8

53. **Schatz M, Zeiger RS, Hoffman CP, Harden K, Forsythe A, Chilingar L, Saunders B, Porreco R, Sperling W, Kagnoff M, et al.** 1995 *Perinatal outcomes in the pregnancies of asthmatic women: a prospective controlled analysis*. Am J Respir Crit Care Med 151:1170-4
54. **Bahna SL, Bjerkedal T** 1972 *The course and outcome of pregnancy in women with bronchial asthma*. Acta Allergol 27:397-406
55. **Stenius-Aarniala B, Piirila P, Teramo K** 1988 *Asthma and pregnancy: a prospective study of 198 pregnancies*. Thorax 43:12-8
56. **Lao TT, Huengsburg M** 1990 *Labour and delivery in mothers with asthma*. Eur J Obstet Gynecol Reprod Biol 35:183-90
57. **Mabie WC, Barton JR, Wasserstrum N, Sibai BM** 1992 *Clinical observations on asthma and pregnancy*. J Matern Fetal Med 1:45-50
58. **Doucette JT, Bracken MB** 1993 *Possible role of asthma in the risk of preterm labor and delivery*. Epidemiology 4:143-50
59. **Jana N, Vasishta K, Saha SC, Khunnu B** 1995 *Effect of bronchial asthma on the course of pregnancy, labour and perinatal outcome*. J Obstet Gynaecol 21:227-32
60. **Minerbi-Codish I, Fraser D, Avnun L, Glezerman M, Heimer D** 1998 *Influence of asthma in pregnancy on labor and the newborn*. Respiration 65:130-5
61. **Kallen B, Rydhstroem H, Aberg A** 2000 *Asthma during pregnancy--a population based study*. Eur J Epidemiol 16:167-71
62. **Sobande AA, Archibong EI, Akinola SE** 2002 *Pregnancy outcome in asthmatic patients from high altitudes*. Int J Gynaecol Obstet 77:117-21
63. **Sorensen TK, Dempsey JC, Xiao R, Frederick IO, Luthy DA, Williams MA** 2003 *Maternal asthma and risk of preterm delivery*. Ann Epidemiol 13:267-72
64. **Bracken MB, Triche EW, Belanger K, Saftlas A, Beckett WS, Leaderer BP** 2003 *Asthma symptoms, severity, and drug therapy: a prospective study of effects on 2205 pregnancies*. Obstet Gynecol 102:739-52
65. **Dombrowski MP, Schatz M, Wise R, Momirova V, Landon M, Mabie W, Newman RB, McNellis D, Hauth JC, Lindheimer M, Caritis SN, Leveno KJ, Meis P, Miodovnik M, Wapner RJ, Paul RH, Varner MW, O'Sullivan MJ, Thurnau GR, Conway DL** 2004 *Asthma during pregnancy*. Obstet Gynecol 103:5-12
66. **Corchia C, Bertollini R, Forastiere F, Pistelli R, Perucci C** 1995 *Is maternal asthma a risk factor for low birth weight? Results of an epidemiologic survey*. Eur J Epidemiol 11:627-31
67. **Lehrer S, Stone J, Lapinski R, Lockwood CJ, Schachter BS, Berkowitz R, Berkowitz GS** 1993 *Association between pregnancy-induced hypertension and asthma during pregnancy*. Am J Obstet Gynecol 168:1463-6
68. **Shohat M, Levy G, Levy I, Schonfeld T, Merlob P** 1989 *Transient tachypnoea of the newborn and asthma*. Arch Dis Child 64:277-9
69. **Papageorgiou AN, Colle E, Farri-Kostopoulos E, Gelfand MM** 1981 *Incidence of respiratory distress syndrome following antenatal betamethasone: role of sex, type of delivery, and prolonged rupture of membranes*. Pediatrics 67:614-7
70. **Torday JS, Nielsen HC, Fencl Mde M, Avery ME** 1981 *Sex differences in fetal lung maturation*. Am Rev Respir Dis 123:205-8

71. **Schatz M, Zeiger RS, Hoffman CP, Saunders BS, Harden KM, Forsythe AB** 1991 *Increased transient tachypnea of the newborn in infants of asthmatic mothers*. Am J Dis Child 145:156-8
72. **Sears MR, Holdaway MD, Flannery EM, Herbison GP, Silva PA** 1996 *Parental and neonatal risk factors for atopy, airway hyper-responsiveness, and asthma*. Arch Dis Child 75:392-8
73. **Johnson CC, Ownby DR, Peterson EL** 1996 *Parental history of atopic disease and concentration of cord blood IgE*. Clin Exp Allergy 26:624-9
74. **Liu CA, Wang CL, Chuang H, Ou CY, Hsu TY, Yang KD** 2003 *Prenatal prediction of infant atopy by maternal but not paternal total IgE levels*. J Allergy Clin Immunol 112:899-904
75. **Weinstein RE, Gurvitz M, Greenberg D, Weinstein A, Solomon W, Subbaiah P, Pieper DR** 1992 *Altered cerebral dominance in atopy and in children of asthmatic mothers*. Ann N Y Acad Sci 650:25-9
76. **Kelly YJ, Brabin BJ, Milligan P, Heaf DP, Reid J, Pearson MG** 1995 *Maternal asthma, premature birth, and the risk of respiratory morbidity in schoolchildren in Merseyside*. Thorax 50:525-30
77. **Schatz M, Harden K, Kagnoff M, Zeiger RS, Chilingar L** 2001 *Developmental follow-up in 15-month-old infants of asthmatic vs. control mothers*. Pediatr Allergy Immunol 12:149-53
78. **Szczeklik A, Milner PC, Birch J, Watkins J, Martin JF** 1986 *Prolonged bleeding time, reduced platelet aggregation, altered PAF-acether sensitivity and increased platelet mass are a trait of asthma and hay fever*. Thromb Haemost 56:283-7
79. **Szczeklik A, Schmitz-Schumann M, Krzanowski M, Virchow C, Sr.** 1991 *Delayed generation of thrombin in clotting blood of atopic patients with hayfever and asthma*. Clin Exp Allergy 21:411-5
80. **Apter AJ, Greenberger PA, Patterson R** 1989 *Outcomes of pregnancy in adolescents with severe asthma*. Arch Intern Med 149:2571-5
81. **Brancazio LR, Laifer SA, Schwartz T** 1997 *Peak expiratory flow rate in normal pregnancy*. Obstet Gynecol 89:383-6
82. **Kramer MS, Coates AL, Michoud MC, Dagenais S, Moshonas D, Davis GM, Hamilton EF, Nuwayhid B, Joshi AK, Papageorgiou A** 1995 *Maternal asthma and idiopathic preterm labor*. Am J Epidemiol 142:1078-1088
83. **Schatz M, Zeiger RS, Hoffman CP** 1990 *Intrauterine growth is related to gestational pulmonary function in pregnant asthmatic women. Kaiser-Permanente Asthma and Pregnancy Study Group*. Chest 98:389-92
84. **Miller HC, Hassanein K** 1971 *Diagnosis of impaired fetal growth in newborn infants*. Pediatrics 48:511-22
85. **Brar HS, Rutherford SE** 1988 *Classification of intrauterine growth retardation*. Semin Perinatol 12:2-10
86. **Bertrand JM, Riley SP, Popkin J, Coates AL** 1985 *The long-term pulmonary sequelae of prematurity: the role of familial airway hyperreactivity and the respiratory distress syndrome*. N Engl J Med 312:742-5
87. **Chan KN, Noble-Jamieson CM, Elliman A, Bryan EM, Aber VR, Silverman M** 1988 *Airway responsiveness in low birthweight children and their mothers*. Arch Dis Child 63:905-10
88. **Tai E, Read J** 1967 *Blood-gas tensions in bronchial asthma*. Lancet 1:644-6
89. **Karetzky MS** 1975 *Blood studies in untreated patients with acute asthma*. Am Rev Respir Dis 112:607-13

90. **Rudolf M, Riordan JF, Grant BJ, Maberly DJ, Saunders KB** 1980 *Arterial blood gas tensions in acute severe asthma*. Eur J Clin Invest 10:55-62
91. **Bureau MA, Shapcott D, Berthiaume Y, Monette J, Blouin D, Blanchard P, Begin R** 1983 *Maternal cigarette smoking and fetal oxygen transport: a study of P50, 2,3-diphosphoglycerate, total hemoglobin, hematocrit, and type F hemoglobin in fetal blood*. Pediatrics 72:22-6
92. **Beck SA** 2001 *Asthma in the female: hormonal effect and pregnancy*. Allergy Asthma Proc 22:1-4
93. **McClure JH, James JM** 1960 *Oxygen administration to the mother and its relation to blood oxygen in the newborn infant*. Am J Obstet Gynecol 80:554-6
94. **Skomsvoll JF, Ostensen M, Irgens LM, Baste V** 1998 *Obstetrical and neonatal outcome in pregnant patients with rheumatic disease*. Scand J Rheumatol Suppl 107:109-12
95. **Bowden AP, Barrett JH, Fallow W, Silman AJ** 2001 *Women with inflammatory polyarthritis have babies of lower birth weight*. J Rheumatol 28:355-9
96. **Skomsvoll JF, Baste V, Irgens LM, Ostensen M** 2002 *The recurrence risk of adverse outcome in the second pregnancy in women with rheumatic disease*. Obstet Gynecol 100:1196-202
97. **Brabin BJ** 1983 *An analysis of malaria in pregnancy in Africa*. Bull World Health Organ 61:1005-16
98. **Moormann AM, Sullivan AD, Rochford RA, Chensue SW, Bock PJ, Nyirenda T, Meshnick SR** 1999 *Malaria and pregnancy: placental cytokine expression and its relationship to intrauterine growth retardation*. J Infect Dis 180:1987-93
99. **Sullivan AD, Nyirenda T, Cullinan T, Taylor T, Harlow SD, James SA, Meshnick SR** 1999 *Malaria infection during pregnancy: intrauterine growth retardation and preterm delivery in Malawi*. J Infect Dis 179:1580-3
100. **Aggarwal N, Sawhney H, Vasishta K, Chopra S, Bambery P** 1999 *Pregnancy in patients with systemic lupus erythematosus*. Aust N Z J Obstet Gynaecol 39:28-30
101. **Fedorkow DM, Persaud D, Nimrod CA** 1989 *Inflammatory bowel disease: a controlled study of late pregnancy outcome*. Am J Obstet Gynecol 160:998-1001
102. **Baird DD, Narendranathan M, Sandler RS** 1990 *Increased risk of preterm birth for women with inflammatory bowel disease*. Gastroenterology 99:987-94
103. **Fonager K, Sorensen HT, Olsen J, Dahlerup JF, Rasmussen SN** 1998 *Pregnancy outcome for women with Crohn's disease: a follow-up study based on linkage between national registries*. Am J Gastroenterol 93:2426-30
104. **McGaw T** 2002 *Periodontal disease and preterm delivery of low-birth-weight infants*. J Can Dent Assoc 68:165-9
105. **Teng YT, Taylor GW, Scannapieco F, Kinane DF, Curtis M, Beck JD, Kogon S** 2002 *Periodontal health and systemic disorders*. J Can Dent Assoc 68:188-92
106. **Fried M, Muga RO, Misore AO, Duffy PE** 1998 *Malaria elicits type 1 cytokines in the human placenta: IFN-g and TNF-a associated with pregnancy outcomes*. J Immunol 160:2523-2530
107. **Ida A, Tsuji Y, Muranaka J, Kanazawa R, Nakata Y, Adachi S, Okamura H, Koyama K** 2000 *IL-18 in pregnancy; the elevation of IL-18 in maternal peripheral blood during labour and complicated pregnancies*. J Reprod Immunol 47:65-74

108. **Hayashi M, Ohkura T** 2002 *Elevated levels of serum macrophage colony-stimulating factor in normotensive pregnancies complicated by intrauterine fetal growth restriction*. Exp Hematol 30:388-93
109. **Hahn-Zoric M, Hagberg H, Kjellmer I, Ellis J, Wennergren M, Hanson LA** 2002 *Aberrations in placental cytokine mRNA related to intrauterine growth retardation*. Pediatr Res 51:201-6
110. **Kumar RM, Uduman SA, Khurranna AK** 1995 *Impact of maternal HIV-1 infection on perinatal outcome*. Int J Gynaecol Obstet 49:137-43
111. **Clifton VL, Giles WB, Smith R, Bisits AT, Hempenstall PA, Kessell CG, Gibson PG** 2001 *Alterations of placental vascular function in asthmatic pregnancies*. Am J Respir Crit Care Med 164:546-53
112. **Cugell DW, Frank NR, Gaensler EA, Badger TL** 1953 *Pulmonary function in pregnancy. I. Serial observations in normal women*. Am Rev Tuberc 67:568-97
113. **Gazioglu K, Kaltreider NL, Rosen M, Yu PN** 1970 *Pulmonary function during pregnancy in normal women and in patients with cardiopulmonary disease*. Thorax 25:445-50
114. **Milne JA, Mills RJ, Howie AD, Pack AI** 1977 *Large airways function during normal pregnancy*. Br J Obstet Gynaecol 84:448-51
115. **Sims CD, Chamberlain GV, de Swiet M** 1976 *Lung function tests in bronchial asthma during and after pregnancy*. Br J Obstet Gynaecol 83:434-7
116. **Juniper EF, Daniel EE, Roberts RS, Kline PA, Hargreave FE, Newhouse MT** 1989 *Improvement in airway responsiveness and asthma severity during pregnancy. A prospective study*. Am Rev Respir Dis 140:924-31
117. **Prowse CM, Gaensler EA** 1965 *Respiratory and acid-base changes during pregnancy*. Anesthesiology 26:381-92
118. **Knuttgen HG, Emerson K, Jr.** 1974 *Physiological response to pregnancy at rest and during exercise*. J Appl Physiol 36:549-53
119. **Cousins L** 1999 *Fetal oxygenation, assessment of fetal well-being, and obstetric management of the pregnant patient with asthma*. J Allergy Clin Immunol 103:S343-9
120. **McAuliffe F, Kametas N, Krampl E, Ernsting J, Nicolaides K** 2001 *Blood gases in pregnancy at sea level and at high altitude*. BJOG 108:980-5
121. **D'Alonzo GE** 1990 *The pregnant asthmatic patient*. Semin Perinatol 14:119-29
122. **Cydulka RK, Emerman CL, Schreiber D, Molander KH, Woodruff PG, Camargo CAJ** 1999 *Acute asthma among pregnant women presenting to the emergency department*. Am J Respir Crit Care Med 160:887-892
123. **Seyal AM** 2001 *Asthma in the hospitalized obstetrical patient*. Clin Rev Allergy Immunol 20:327-39
124. **Garcia-Rio F, Pino JM, Gomez L, Alvarez-Sala R, Villasante C, Villamor J** 1996 *Regulation of breathing and perception of dyspnea in healthy pregnant women*. Chest 110:446-53
125. **Blaiss MS** 2003 *Management of rhinitis and asthma in pregnancy*. Ann Allergy Asthma Immunol 90:16-22
126. **Nelson-Piercy C** 2001 *Asthma in pregnancy*. Thorax 56:325-8
127. **Lyons HA, Antonio R** 1959 *The sensitivity of the respiratory center in pregnancy and after the administration of progesterone*. Trans Assoc Am Physic 72:173-80
128. **Regensteiner JG, Woodard WD, Hagerman DD, Weil JV, Pickett CK, Bender PR, Moore LG** 1989 *Combined effects of female hormones and metabolic rate on ventilatory drives in women*. J Appl Physiol 66:808-13

129. **Templeton A, Kelman GR** 1976 *Maternal blood-gases, (PAO2--PaO2), physiological shunt and VD/VT in normal pregnancy*. Br J Anaesth 48:1001-4
130. **Awe RJ, Nicotra B, Newsom T, Viles R** 1979 *Arterial oxygenation and alveolar-arterial gradients in term pregnancy*. Obstet Gynecol 53:182-6
131. **Schatz M** 1992 *Asthma during pregnancy: interrelationships and management*. Ann Allergy 68:123-33
132. **Sachs BP, Brown RS, Yeh J, Acker D, Niaraki M** 1987 *Is maternal alkalosis harmful to the fetus?* Int J Gynaecol Obstet 25:65-8
133. **Gluck JC, Gluck PA** 1976 *The effects of pregnancy on asthma: a prospective study*. Ann Allergy 37:164-168
134. **Schatz M, Harden K, Forsythe A, Chilingar L, Hoffman C, Sperling W, Zeiger RS** 1988 *The course of asthma during pregnancy, post partum, and with successive pregnancies: a prospective analysis*. J Allergy Clin Immunol 81:509-17
135. **Fein BT, Kamin PB** 1964 *Management of Allergy in Pregnancy*. Ann Allergy 22:341-8
136. **Gibbs CJ, Coutts, II, Lock R, Finnegan OC, White RJ** 1984 *Premenstrual exacerbation of asthma*. Thorax 39:833-6
137. **Gelber M, Sidi Y, Gassner S, Ovadia Y, Spitzer S, Weinberger A, Pinkhas J** 1984 *Uncontrollable life-threatening status asthmaticus--an indicator for termination of pregnancy by cesarean section*. Respiration 46:320-2
138. **Shanies HM, Venkataraman MT, Peter T** 1997 *Reversal of intractable acute severe asthma by first-trimester termination of pregnancy*. J Asthma 34:169-72
139. **Juniper EF, Daniel EE, Roberts RS, Kline PA, Hargreave FE, Newhouse MT** 1991 *Effect of pregnancy on airway responsiveness and asthma severity. Relationship to serum progesterone*. Am Rev Respir Dis 143:S78
140. **Green B** 1934 *Bronchial asthma as a complication of pregnancy*. JAMA 102:360-63
141. **Friedman A, Solomons E** 1957 *Asthma in pregnancy; a report of seven cases*. Am J Obstet Gynecol 74:318-23
142. **Kircher S, Schatz M, Long L** 2002 *Variables affecting asthma course during pregnancy*. Ann Allergy Asthma Immunol 89:463-6
143. **Braunstahl GJ, Hellings PW** 2003 *Allergic rhinitis and asthma: the link further unraveled*. Curr Opin Pulm Med 9:46-51
144. **Henderson CE, Ownby DR, Trumble A, DerSimonian R, Kellner LH** 2000 *Predicting asthma severity from allergic sensitivity to cockroaches in pregnant inner city women*. J Reprod Med 45:341-4
145. **Gandevia B** 1953 *A note on the course of bronchial asthma and vasomotor rhinitis during pregnancy*. Royal Melbourne Hospital Clinical Reports 23:72-74
146. **Wendel PJ, Ramin SM, Barnett-Hamm C, Rowe TF, Cunningham FG** 1996 *Asthma treatment in pregnancy: a randomized controlled study*. Am J Obstet Gynecol 175:150-4
147. **Stenius-Aarniala BS, Hedman J, Teramo KA** 1996 *Acute asthma during pregnancy*. Thorax 51:411-4
148. **Schatz M, Dombrowski MP, Wise R, Thom EA, Landon M, Mabie W, Newman RB, Hauth JC, Lindheimer M, Caritis SN, Leveno KJ, Meis P, Miodovnik M, Wapner RJ, Paul RH, Varner MW, O'Sullivan M J, Thurnau GR, Conway D, McNellis D** 2003 *Asthma morbidity during pregnancy can be predicted by severity classification*. J Allergy Clin Immunol 112:283-8

149. **Clark SL** 1993 *Asthma in pregnancy. National Asthma Education Program Working Group on Asthma and Pregnancy. National Institutes of Health, National Heart, Lung and Blood Institute*. Obstet Gynecol 82:1036-40
150. **Lye SJ, Porter DG** 1978 *Demonstration that progesterone 'blocks' uterine activity in the ewe in vivo by a direct action on the myometrium*. J Reprod Fertil 52:87-94
151. **Tan KS, Thomson NC** 2000 *Asthma in pregnancy*. Am J Med 109:727-33
152. **Tan KS, McFarlane LC, Lipworth BJ** 1997 *Paradoxical down-regulation and desensitization of beta2-adrenoceptors by exogenous progesterone in female asthmatics*. Chest 111:847-51
153. **Vrieze A, Postma DS, Kerstjens HA** 2003 *Perimenstrual asthma: a syndrome without known cause or cure*. J Allergy Clin Immunol 112:271-82
154. **Tan KS** 2001 *Premenstrual asthma: epidemiology, pathogenesis and treatment*. Drugs 61:2079-86
155. **Wegmann TG, Lin H, Guilbert L, Mosmann TR** 1993 *Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon?* Immunol Today 14:353-6
156. **Saito S, Sakai M, Sasaki Y, Tanebe K, Tsuda H, Michimata T** 1999 *Quantitative analysis of peripheral blood Th0, Th1, Th2 and the Th1:Th2 cell ratio during normal human pregnancy and preeclampsia*. Clin Exp Immunol 117:550-5
157. **Saito S** 2000 *Cytokine network at the feto-maternal interface*. J Reprod Immunol 47:87-103
158. **Margni RA, Zenclussen AC** 2001 *During pregnancy, in the context of a Th2-type cytokine profile, serum IL-6 levels might condition the quality of the synthesized antibodies*. Am J Reprod Immunol 46:181-7
159. **Lapa e Silva JR, Possebon da Silva MD, Lefort J, Vargaftig BB** 2000 *Endotoxins, asthma, and allergic immune responses*. Toxicology 152:31-35
160. **Chaouat G, Zourbas S, Ostojic S, Lappree-Delage G, Dubanchet S, Ledee N, Martal J** 2002 *A brief review of recent data on some cytokine expressions at the materno-foetal interface which might challenge the classical Th1/Th2 dichotomy*. J Reprod Immunol 53:241-56
161. **Ostensen M, Aune B, Husby G** 1983 *Effect of pregnancy and hormonal changes on the activity of rheumatoid arthritis*. Scand J Rheumatol 12:69-72
162. **Ostensen M, Villiger PM** 2002 *Immunology of pregnancy-pregnancy as a remission inducing agent in rheumatoid arthritis*. Transpl Immunol 9:155-60
163. **Rubio Ravelo L, Gago Rodriguez B, Almirall Collazo JJ, Bell Heredia L, Fernandez Fernandez L** 1988 *Comparative study of progesterone, estradiol and cortisol concentrations in asthmatic and non-asthmatic women*. Allergol Immunopathol (Madr) 16:263-6
164. **Beecroft N, Cochrane GM, Milburn HJ** 1998 *Effect of sex of fetus on asthma during pregnancy: blind prospective study*. BMJ 317:856-7
165. **Dodds L, Armson BA, Alexander S** 1999 *Use of asthma drugs is less among women pregnant with boys rather than girls*. BMJ 318:1011
166. **Derbes VJ, Sodeman WA** 1946 *Reciprocal influences of bronchial asthma and pregnancy*. Am J Med 1:367-75
167. **Lami L** 1937 *Sopra due casi di asma bronchiale in gravidanza*. Riforma Med 53:1202
168. **Dorn JH, Sugarman EI** 1932 *A method for the prediction of sex in the unborn: preliminary report*. JAMA 99:1659

169. **Maietta AL** 1955 *The management of the allergic patient during pregnancy*. Ann Allergy 13:516-22
170. **Williamson AC** 1930 *Pregnancy concomitant with asthma or hay fever*. Am J Obstet Gynecol 20:192-7
171. **Fitzsimons R, Greenberger PA, Patterson R** 1986 *Outcome of pregnancy in women requiring corticosteroids for severe asthma*. J Allergy Clin Immunol 78:349-53
172. **Greenberger PA, Patterson R** 1988 *The outcome of pregnancy complicated by severe asthma*. Allergy Proc 9:539-43
173. **Seale P** 2000 *Use of bronchodilators*. In: Wells RS, Jenkins CR (eds) Understanding asthma. MacLennan and Petty, Sydney, pp 211-216
174. **Dombrowski MP, Bottoms SF, Boike GM, Wald J** 1986 *Incidence of preeclampsia among asthmatic patients lower with theophylline*. Am J Obstet Gynecol 155:265-7
175. **Stenius-Aarniala B, Riikonen S, Teramo K** 1995 *Slow-release theophylline in pregnant asthmatics*. Chest 107:642-7
176. **Gardner MJ, Schatz M, Cousins L, Zeiger R, Middleton E, Jusko WJ** 1987 *Longitudinal effects of pregnancy on the pharmacokinetics of theophylline*. Eur J Clin Pharmacol 32:289-95
177. **Demoly P, Piette V, Daures JP** 2003 *Asthma therapy during pregnancy*. Expert Opin Pharmacother 4:1019-23
178. **Schatz M, Zeiger RS, Harden K, Hoffman CC, Chilingar L, Petitti D** 1997 *The safety of asthma and allergy medications during pregnancy*. J Allergy Clin Immunol 100:301-6
179. **Arwood LL, Dasta JF, Friedman C** 1979 *Placental transfer of theophylline: two case reports*. Pediatrics 63:844-6
180. **Labovitz E, Spector S** 1982 *Placental theophylline transfer in pregnant asthmatics*. JAMA 247:786-8
181. **Aranda JV, Sitar DS, Parsons WD, Loughnan PM, Neims AH** 1976 *Pharmacokinetic aspects of theophylline in premature newborns*. N Engl J Med 295:413-6
182. **Lulich KM, Goldie RG, Paterson JW** 1988 *Beta-adrenoceptor function in asthmatic bronchial smooth muscle*. Gen Pharmacol 19:307-11
183. **Schatz M, Zeiger RS, Harden KM, Hoffman CP, Forsythe AB, Chilingar LM, Porreco RP, Benenson AS, Sperling WL, Saunders BS, et al.** 1988 *The safety of inhaled beta-agonist bronchodilators during pregnancy*. J Allergy Clin Immunol 82:686-95
184. **Rayburn WF, Atkinson BD, Gilbert K, Turnbull GL** 1994 *Short-term effects of inhaled albuterol on maternal and fetal circulations*. Am J Obstet Gynecol 171:770-3
185. **Mann RD, Kubota K, Pearce G, Wilton L** 1996 *Salmeterol: a study by prescription-event monitoring in a UK cohort of 15,407 patients*. J Clin Epidemiol 49:247-50
186. **Jenkins CR** 2000 *Preventative medications*. In: Walls RS, Jenkins CR (eds) Understanding asthma. MacLennan and Petty, Sydney, pp 217-225
187. **Jackson LD, Polygenis D, McIvor RA, Worthington I** 1999 *Comparative efficacy and safety of inhaled corticosteroids in asthma*. Can J Clin Pharmacol 6:26-37
188. **Windholz M** 1983 *The Merck Index*. Merck & Co, Rahway, NJ
189. 1995 *MIMS Annual*. Tien Wah Press, Singapore

190. **O'Byrne PM, Pedersen S** 1998 *Measuring efficacy and safety of different inhaled corticosteroid preparations*. J Allergy Clin Immunol 102:879-86
191. **Warrell DW, Taylor R** 1968 *Outcome for the foetus of mothers receiving prednisolone during pregnancy*. Lancet 1:117-8
192. **Reinisch JM, Simon NG, Karow WG, Gandelman R** 1978 *Prenatal exposure to prednisone in humans and animals retards intrauterine growth*. Science 202:436-8
193. **Smith KD, Steinberger E, Rodriguez-Rigau LJ** 1979 *Prednisone therapy and birth weight*. Science 206:96-7
194. **Lee F, Nelson N, Faiman C, Choi NW, Reyes FI** 1982 *Low-dose corticoid therapy for anovulation: effect upon fetal weight*. Obstet Gynecol 60:314-7
195. **New MI, Carlson A, Obeid J, Marshall I, Cabrera MS, Goseco A, Lin-Su K, Putnam AS, Wei JQ, Wilson RC** 2001 *Prenatal diagnosis for congenital adrenal hyperplasia in 532 pregnancies*. J Clin Endocrinol Metab 86:5651-7
196. **Schatz M, Patterson R, Zeitz S, O'Rourke J, Melam H** 1975 *Corticosteroid therapy for the pregnant asthmatic patient*. JAMA 233:804-7
197. **Snyder RD, Snyder D** 1978 *Corticosteroids for asthma during pregnancy*. Ann Allergy 41:340-1
198. **Rodriguez-Pinilla E, Martinez-Frias ML** 1998 *Corticosteroids during pregnancy and oral clefts: a case-control study*. Teratology 58:2-5
199. **Greenberger PA, Patterson R** 1983 *Beclomethasone diproprionate for severe asthma during pregnancy*. Ann Intern Med 98:478-80
200. **Dombrowski MP, Brown CL, Berry SM** 1996 *Preliminary experience with triamcinolone acetonide during pregnancy*. J Matern Fetal Med 5:310-3
201. **Olesen C, Thrane N, Nielsen GL, Sorensen HT, Olsen J** 2001 *A population-based prescription study of asthma drugs during pregnancy: changing the intensity of asthma therapy and perinatal outcomes*. Respiration 68:256-61
202. **Norjavaara E, de Verdier MG** 2003 *Normal pregnancy outcomes in a population-based study including 2,968 pregnant women exposed to budesonide*. J Allergy Clin Immunol 111:736-42
203. **Kallen B, Rydhstroem H, Aberg A** 1999 *Congenital malformations after the use of inhaled budesonide in early pregnancy*. Obstet Gynecol 93:392-5
204. **Silverman M, Sheffer A, Woolcock A, Diaz-Amor P, Lindmark B, Radner F, Broddene M, Pedersen S, Pauwels R** 2003 *Prospective pregnancy outcome data in patients with recent onset, mild persistent asthma treated with once-daily budesonide: results from the START study [abstract]*. Am J Respir Crit Care Med 167:A772
205. **Ellegard EK, Hellgren M, Karlsson NG** 2001 *Fluticasone propionate aqueous nasal spray in pregnancy rhinitis*. Clin Otolaryngol 26:394-400
206. **Burdon JG, Goss G** 1994 *Asthma and pregnancy*. Aust N Z J Med 24:3-4
207. **Lipson A** 1994 *Asthma and pregnancy--misleading and incorrect recommendation on the effect of medication on the foetus--and a remedy*. Aust N Z J Med 24:407-8
208. **Beckmann CA** 2002 *A descriptive study of women's perceptions of their asthma during pregnancy*. MCN Am J Matern Child Nurs 27:98-102
209. **Barron WM, Leff AR** 1993 *Asthma in pregnancy*. Am Rev Respir Dis 147:510-1
210. **Patterson R, Greenberger PA, Frederiksen MC** 1990 *Asthma and pregnancy: responsibility of physicians and patients*. Ann Allergy 65:469-72

211. **Chambers K** 2003 *Asthma education and outcomes for women of childbearing age*. Case Manager 14:58-61
212. **Liccardi G, Cazzola M, Canonica GW, D'Amato M, D'Amato G, Passalacqua G** 2003 *General strategy for the management of bronchial asthma in pregnancy*. Respir Med 97:778-89
213. **Burton J, Reyes M** 2001 *Breathe in breathe out. Controlling asthma during pregnancy*. AWHONN Lifelines 5:24-30
214. 1961 *Public health aspects of low birth weight: Third report of the expert committee on maternal and child health*. WHO Tech Rep Ser 217:3-16
215. **de Onis M, Blossner M, Villar J** 1998 *Levels and patterns of intrauterine growth retardation in developing countries*. Eur J Clin Nutr 52 Suppl 1:S5-15
216. **Lu TH, Sung FC, Li CY** 2003 *Demographic characteristics and trends in the prevalence of low birth weight from singleton pregnancies in Taiwan, 1978-1997*. J Formos Med Assoc 102:313-8
217. **Kramer MS** 2003 *The epidemiology of adverse pregnancy outcomes: an overview*. J Nutr 133:1592S-1596S
218. **Gould JB, Madan A, Qin C, Chavez G** 2003 *Perinatal outcomes in two dissimilar immigrant populations in the United States: a dual epidemiologic paradox*. Pediatrics 111:e676-82
219. **Peleg D, Kennedy CM, Hunter SK** 1998 *Intrauterine growth restriction: identification and management*. Am Fam Physician 58:453-60, 466-7
220. **Smith GC, Smith MF, McNay MB, Fleming JE** 1998 *First-trimester growth and the risk of low birth weight*. N Engl J Med 339:1817-22
221. **Gruenwald P** 1963 *Chronic fetal distress and placental insufficiency*. Biol Neonate 33:215-65
222. **Battaglia FC** 1970 *Intrauterine growth retardation*. Am J Obstet Gynecol 106:1103-14
223. **Yerushalmy J** 1970 *Relation of birth weight, gestational age, and the rate of intrauterine growth to perinatal mortality*. Clin Obstet Gynecol 13:107-29
224. **McCormick MC** 1985 *The contribution of low birth weight to infant mortality and childhood morbidity*. N Engl J Med 312:82-90
225. **Petrou S** 2003 *Economic consequences of preterm birth and low birthweight*. BJOG 110 Suppl 20:17-23
226. **Shrimpton R** 2003 *Preventing low birthweight and reduction of child mortality*. Trans R Soc Trop Med Hyg 97:39-42
227. **Ashworth A** 1998 *Effects of intrauterine growth retardation on mortality and morbidity in infants and young children*. Eur J Clin Nutr 52 Suppl 1:S34-41; discussion S41-2
228. **Ceesay SM, Prentice AM, Cole TJ, Foord F, Weaver LT, Poskitt EM, Whitehead RG** 1997 *Effects on birth weight and perinatal mortality of maternal dietary supplements in rural Gambia: 5 year randomised controlled trial*. BMJ 315:786-90
229. **Friedlander Y, Paltiel O, Deutsch L, Knaanie A, Massalha S, Tiram E, Harlap S** 2003 *Birthweight and relationship with infant, child and adult mortality in the Jerusalem perinatal study*. Paediatr Perinat Epidemiol 17:398-406
230. **Bard H** 1970 *Intrauterine growth retardation*. Clin Obstet Gynecol 13:511-25
231. **Kramer MS, Olivier M, McLean FH, Willis DM, Usher RH** 1990 *Impact of intrauterine growth retardation and body proportionality on fetal and neonatal outcome*. Pediatrics 86:707-13

232. **Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM** 1993 *Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth*. Diabetologia 36:62-7
233. **Barker DJ, Osmond C, Simmonds SJ, Wield GA** 1993 *The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life*. BMJ 306:422-6
234. **Gale CR, Ashurst HE, Hall NF, MacCallum PK, Martyn CN** 2002 *Size at birth and carotid atherosclerosis in later life*. Atherosclerosis 163:141-7
235. **Law CM, Egger P, Dada O, Delgado H, Kylberg E, Lavin P, Tang GH, von Hertzen H, Shiell AW, Barker DJ** 2000 *Body size at birth and blood pressure among children in developing countries*. Int J Epidemiol 29:52-9
236. **Barker DJ, Lackland DT** 2003 *Prenatal influences on stroke mortality in England and Wales*. Stroke 34:1598-602
237. **Barker DJ, Osmond C, Golding J, Kuh D, Wadsworth ME** 1989 *Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease*. BMJ 298:564-7
238. **Barker DJ, Winter PD, Osmond C, Margetts B, Simmonds SJ** 1989 *Weight in infancy and death from ischaemic heart disease*. Lancet 2:577-80
239. **Sayer AA, Cooper C, Evans JR, Rauf A, Wormald RP, Osmond C, Barker DJ** 1998 *Are rates of ageing determined in utero?* Age Ageing 27:579-83
240. **Barker DJ, Osmond C** 1986 *Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales*. Lancet 1:1077-81
241. **Barker DJ, Osmond C** 1987 *Death rates from stroke in England and Wales predicted from past maternal mortality*. Br Med J (Clin Res Ed) 295:83-6
242. **Whincup PH, Cook DG, Shaper AG** 1989 *Early influences on blood pressure: a study of children aged 5-7 years*. BMJ 299:587-91
243. **Law CM, de Swiet M, Osmond C, Fayers PM, Barker DJ, Cruddas AM, Fall CH** 1993 *Initiation of hypertension in utero and its amplification throughout life*. BMJ 306:24-7
244. **Barker DJ, Bull AR, Osmond C, Simmonds SJ** 1990 *Fetal and placental size and risk of hypertension in adult life*. BMJ 301:259-62
245. **Barker DJ, Godfrey KM, Osmond C, Bull A** 1992 *The relation of fetal length, ponderal index and head circumference to blood pressure and the risk of hypertension in adult life*. Paediatr Perinat Epidemiol 6:35-44
246. **Folkow B** 1987 *Structure and function of the arteries in hypertension*. Am Heart J 114:938-48
247. **Safar ME, Levy BI, Laurent S, London GM** 1990 *Hypertension and the arterial system: clinical and therapeutic aspects*. J Hypertens Suppl 8:S113-9
248. **Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, Winter PD** 1991 *Fetal and infant growth and impaired glucose tolerance at age 64*. BMJ 303:1019-22
249. **Law CM, Gordon GS, Shiell AW, Barker DJ, Hales CN** 1995 *Thinness at birth and glucose tolerance in seven-year-old children*. Diabet Med 12:24-9
250. **Robinson S, Walton RJ, Clark PM, Barker DJ, Hales CN, Osmond C** 1992 *The relation of fetal growth to plasma glucose in young men*. Diabetologia 35:444-6
251. **Phipps K, Barker DJ, Hales CN, Fall CH, Osmond C, Clark PM** 1993 *Fetal growth and impaired glucose tolerance in men and women*. Diabetologia 36:225-8

252. **Barker DJ, Eriksson JG, Forsen T, Osmond C** 2002 *Fetal origins of adult disease: strength of effects and biological basis*. Int J Epidemiol 31:1235-9
253. **Osmond C, Barker DJ, Winter PD, Fall CH, Simmonds SJ** 1993 *Early growth and death from cardiovascular disease in women*. BMJ 307:1519-24
254. **Eriksson JG, Forsen T, Tuomilehto J, Winter PD, Osmond C, Barker DJ** 1999 *Catch-up growth in childhood and death from coronary heart disease: longitudinal study*. BMJ 318:427-31
255. **Yajnik CS, Fall CH, Coyaji KJ, Hirve SS, Rao S, Barker DJ, Joglekar C, Kellingray S** 2003 *Neonatal anthropometry: the thin-fat Indian baby. The Pune Maternal Nutrition Study*. Int J Obes Relat Metab Disord 27:173-80
256. **Campbell DM, Hall MH, Barker DJ, Cross J, Shiell AW, Godfrey KM** 1996 *Diet in pregnancy and the offspring's blood pressure 40 years later*. Br J Obstet Gynaecol 103:273-80
257. **Shiell AW, Campbell-Brown M, Haselden S, Robinson S, Godfrey KM, Barker DJ** 2001 *High-meat, low-carbohydrate diet in pregnancy: relation to adult blood pressure in the offspring*. Hypertension 38:1282-8
258. **Ravelli AC, van der Meulen JH, Michels RP, Osmond C, Barker DJ, Hales CN, Bleker OP** 1998 *Glucose tolerance in adults after prenatal exposure to famine*. Lancet 351:173-7
259. **Roseboom TJ, Van Der Meulen JH, Ravelli AC, Osmond C, Barker DJ, Bleker OP** 2003 *Perceived health of adults after prenatal exposure to the Dutch famine*. Paediatr Perinat Epidemiol 17:391-7
260. **Lackland DT, Bendall HE, Osmond C, Egan BM, Barker DJ** 2000 *Low birth weights contribute to high rates of early-onset chronic renal failure in the Southeastern United States*. Arch Intern Med 160:1472-6
261. **Thompson C, Syddall H, Rodin I, Osmond C, Barker DJ** 2001 *Birth weight and the risk of depressive disorder in late life*. Br J Psychiatry 179:450-5
262. **Martyn CN, Gale CR, Jespersen S, Sherriff SB** 1998 *Impaired fetal growth and atherosclerosis of carotid and peripheral arteries*. Lancet 352:173-8
263. **Dempsey JC, Williams MA, Luthy DA, Emanuel I, Shy K** 2003 *Weight at birth and subsequent risk of preeclampsia as an adult*. Am J Obstet Gynecol 189:494-500
264. **Lagerstrom M, Bremme K, Eneroth P, Magnusson D** 1990 *Behavior at 10 and 13 years of age for children with low birth weight*. Percept Mot Skills 71:579-94
265. **Breslau N, DelDotto JE, Brown GG, Kumar S, Ezhuthachan S, Hufnagle KG, Peterson EL** 1994 *A gradient relationship between low birth weight and IQ at age 6 years*. Arch Pediatr Adolesc Med 148:377-83
266. **Rona RJ, Gulliford MC, Chinn S** 1993 *Effects of prematurity and intrauterine growth on respiratory health and lung function in childhood*. BMJ 306:817-20
267. **Lercher P, Schmitzberger R** 1997 *Birth weight, education, environment, and lung function at school age: a community study in an alpine area*. Eur Respir J 10:2502-7
268. **Shaheen SO, Sterne JA, Montgomery SM, Azima H** 1999 *Birth weight, body mass index and asthma in young adults*. Thorax 54:396-402
269. **Joseph CL, Ownby DR, Peterson EL, Johnson CC** 2002 *Does low birth weight help to explain the increased prevalence of asthma among African-Americans?* Ann Allergy Asthma Immunol 88:507-12

270. **Mai XM, Gaddlin PO, Nilsson L, Finnstrom O, Bjorksten B, Jenmalm MC, Leijon I** 2003 *Asthma, lung function and allergy in 12-year-old children with very low birth weight: A prospective study*. Pediatr Allergy Immunol 14:184-192
271. **Dezateux C, Lum S, Hoo AF, Hawdon J, Costeloe K, Stocks J** 2004 *Low birth weight for gestation and airway function in infancy: exploring the fetal origins hypothesis*. Thorax 59:60-66
272. **Godfrey KM, Barker DJ, Osmond C** 1994 *Disproportionate fetal growth and raised IgE concentration in adult life*. Clin Exp Allergy 24:641-8
273. **Yuan W, Basso O, Sorensen HT, Olsen J** 2002 *Fetal growth and hospitalization with asthma during early childhood: a follow-up study in Denmark*. Int J Epidemiol 31:1240-5
274. **Barker DJ, Godfrey KM, Fall C, Osmond C, Winter PD, Shaheen SO** 1991 *Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease*. BMJ 303:671-5
275. **Stein CE, Kumaran K, Fall CH, Shaheen SO, Osmond C, Barker DJ** 1997 *Relation of fetal growth to adult lung function in south India*. Thorax 52:895-9
276. **Lopuhaa CE, Roseboom TJ, Osmond C, Barker DJ, Ravelli AC, Bleker OP, van der Zee JS, van der Meulen JH** 2000 *Atopy, lung function, and obstructive airways disease after prenatal exposure to famine*. Thorax 55:555-61
277. **Harding R, Tester ML, Moss TJ, Davey MG, Louey S, Joyce B, Hooper SB, Maritz G** 2000 *Effects of intra-uterine growth restriction on the control of breathing and lung development after birth*. Clin Exp Pharmacol Physiol 27:114-9
278. **Joyce BJ, Louey S, Davey MG, Cock ML, Hooper SB, Harding R** 2001 *Compromised respiratory function in postnatal lambs after placental insufficiency and intrauterine growth restriction*. Pediatr Res 50:641-9
279. **Thomson AM, Billewicz WZ, Hytten FE** 1968 *The assessment of fetal growth*. J Obstet Gynaecol Br Commonw 75:903-16
280. **Cogswell ME, Yip R** 1995 *The influence of fetal and maternal factors on the distribution of birthweight*. Semin Perinatol 19:222-40
281. **Brooks AA, Johnson MR, Steer PJ, Pawson ME, Abdalla HI** 1995 *Birth weight: nature or nurture?* Early Hum Dev 42:29-35
282. **Shoham-Vardi I, Leiberman JR, Kopernik G** 1994 *The association of primiparity with intrauterine growth retardation*. Eur J Obstet Gynecol Reprod Biol 53:95-101
283. **Mohsin M, Wong F, Bauman A, Bai J** 2003 *Maternal and neonatal factors influencing premature birth and low birth weight in Australia*. J Biosoc Sci 35:161-74
284. **Hunscher HA, Tompkins WT** 1970 *The influence of maternal nutrition on the immediate and long-term outcome of pregnancy*. Clin Obstet Gynecol 13:130-44
285. **Picciano MF** 2003 *Pregnancy and lactation: physiological adjustments, nutritional requirements and the role of dietary supplements*. J Nutr 133:1997S-2002S
286. **Lechtig A, Habicht JP, Delgado H, Klein RE, Yarbrough C, Martorell R** 1975 *Effect of food supplementation during pregnancy on birthweight*. Pediatrics 56:508-20
287. **Godfrey K, Robinson S, Barker DJ, Osmond C, Cox V** 1996 *Maternal nutrition in early and late pregnancy in relation to placental and fetal growth*. BMJ 312:410-4

288. **Christian P, Khatry SK, Katz J, Pradhan EK, LeClerq SC, Shrestha SR, Adhikari RK, Sommer A, West KP, Jr.** 2003 *Effects of alternative maternal micronutrient supplements on low birth weight in rural Nepal: double blind randomised community trial*. BMJ 326:571
289. **Langer O, Levy J, Brustman L, Anyaegbunam A, Merkatz R, Divon M** 1989 *Glycemic control in gestational diabetes mellitus--how tight is tight enough: small for gestational age versus large for gestational age?* Am J Obstet Gynecol 161:646-53
290. **Leguizamon G, von Stecher F** 2003 *Third trimester glycemic profiles and fetal growth*. Curr Diab Rep 3:323-6
291. **Yip R** 1987 *Altitude and birth weight*. J Pediatr 111:869-76
292. **Unger C, Weiser JK, McCullough RE, Keefer S, Moore LG** 1988 *Altitude, low birth weight, and infant mortality in Colorado*. JAMA 259:3427-32
293. **Moore LG, Rounds SS, Jahnigen D, Grover RF, Reeves JT** 1982 *Infant birth weight is related to maternal arterial oxygenation at high altitude*. J Appl Physiol 52:695-9
294. **Xiao R, Sorensen TK, Williams MA, Luthy DA** 2003 *Influence of pre-eclampsia on fetal growth*. J Matern Fetal Neonatal Med 13:157-62
295. **Godfrey KM, Redman CW, Barker DJ, Osmond C** 1991 *The effect of maternal anaemia and iron deficiency on the ratio of fetal weight to placental weight*. Br J Obstet Gynaecol 98:886-91
296. **Allen LH** 2001 *Biological mechanisms that might underlie iron's effects on fetal growth and preterm birth*. J Nutr 131:581S-589S
297. **de Haas JH** 1975 *Parental smoking. Its effects on fetus and child health*. Eur J Obstet Gynecol Reprod Biol 5:283-96
298. **Lumley J** 1987 *Stopping smoking*. Br J Obstet Gynaecol 94:289-92
299. **Newnham JP, Patterson L, James I, Reid SE** 1990 *Effects of maternal cigarette smoking on ultrasonic measurements of fetal growth and on Doppler flow velocity waveforms*. Early Hum Dev 24:23-36
300. **Andres RL, Day MC** 2000 *Perinatal complications associated with maternal tobacco use*. Semin Neonatol 5:231-41
301. **Maloni JA** 2001 *Preventing low birth weight. How smoking cessation counseling can help*. AWHONN Lifelines 5:32-5
302. **Larroque B, Kaminski M, Lelong N, Subtil D, Dehaene P** 1993 *Effects of birth weight of alcohol and caffeine consumption during pregnancy*. Am J Epidemiol 137:941-50
303. **Pastrakuljic A, Derewlany LO, Koren G** 1999 *Maternal cocaine use and cigarette smoking in pregnancy in relation to amino acid transport and fetal growth*. Placenta 20:499-512
304. **Bateman DA, Chiriboga CA** 2000 *Dose-response effect of cocaine on newborn head circumference*. Pediatrics 106:E33
305. **Kaiser LL, Allen L** 2002 *Position of the American Dietetic Association: nutrition and lifestyle for a healthy pregnancy outcome*. J Am Diet Assoc 102:1479-90
306. **Simpson WJ** 1957 *A preliminary report on cigarette smoking and the incidence of prematurity*. Am J Obstet Gynecol 73:807-15
307. **MacMahon B, Alpert M, Salber EJ** 1965 *Infant weight and parental smoking habits*. Am J Epidemiol 82:247-61
308. **Lowe CR** 1959 *Effect of mothers' smoking habits on birth weight of their children*. Br Med J No 5153:673-6

309. **Astrup P, Olsen HM, Trolle D, Kjeldsen K** 1972 *Effect of moderate carbon-monoxide exposure on fetal development*. Lancet 2:1220-2
310. **Butler NR, Goldstein H, Ross EM** 1972 *Cigarette smoking in pregnancy: its influence on birth weight and perinatal mortality*. Br Med J 2:127-30
311. **Haddon W, Jr., Nesbitt RE, Garcia R** 1961 *Smoking and pregnancy: carbon monoxide in blood during gestation and at term*. Obstet Gynecol 18:262-7
312. **Lehtovirta P, Forss M** 1978 *The acute effect of smoking on intervillous blood flow of the placenta*. Br J Obstet Gynaecol 85:729-31
313. **Zaren B, Lindmark G, Bakketeig L** 2000 *Maternal smoking affects fetal growth more in the male fetus*. Paediatr Perinat Epidemiol 14:118-26
314. **Sexton M, Hebel JR** 1984 *A clinical trial of change in maternal smoking and its effect on birth weight*. JAMA 251:911-5
315. **Mayhew TM, Jackson MR, Haas JD** 1990 *Oxygen diffusive conductances of human placentae from term pregnancies at low and high altitudes*. Placenta 11:493-503
316. **Giussani DA, Phillips PS, Anstee S, Barker DJ** 2001 *Effects of altitude versus economic status on birth weight and body shape at birth*. Pediatr Res 49:490-4
317. **Jensen GM, Moore LG** 1997 *The effect of high altitude and other risk factors on birthweight: independent or interactive effects?* Am J Public Health 87:1003-7
318. **McCullough RE, Reeves JT** 1977 *Fetal growth retardation and increased infant mortality at high altitude*. Arch Environ Health 32:36-9
319. **Moore LG** 2003 *Fetal growth restriction and maternal oxygen transport during high altitude pregnancy*. High Alt Med Biol 4:141-56
320. **Krampl E, Lees C, Bland JM, Espinoza Dorado J, Moscoso G, Campbell S** 2000 *Fetal biometry at 4300 m compared to sea level in Peru*. Ultrasound Obstet Gynecol 16:9-18
321. **Coussons-Read ME, Mazzeo RS, Whitford MH, Schmitt M, Moore LG, Zamudio S** 2002 *High altitude residence during pregnancy alters cytokine and catecholamine levels*. Am J Reprod Immunol 48:344-54
322. **Moore LG, Brodeur P, Chumbe O, D'Brot J, Hofmeister S, Monge C** 1986 *Maternal hypoxic ventilatory response, ventilation, and infant birth weight at 4,300 m*. J Appl Physiol 60:1401-6
323. **Clapp JF, 3rd** 2003 *The effects of maternal exercise on fetal oxygenation and feto-placental growth*. Eur J Obstet Gynecol Reprod Biol 110 Suppl 1:S80-5
324. **Zamudio S, Palmer SK, Droma T, Stamm E, Coffin C, Moore LG** 1995 *Effect of altitude on uterine artery blood flow during normal pregnancy*. J Appl Physiol 79:7-14
325. **Moore LG, Zamudio S, Zhuang J, Sun S, Droma T** 2001 *Oxygen transport in Tibetan women during pregnancy at 3,658 m*. Am J Phys Anthropol 114:42-53
326. **Tissot van Patot M, Grilli A, Chapman P, Broad E, Tyson W, Heller DS, Zwerdlinger L, Zamudio S** 2003 *Remodelling of uteroplacental arteries is decreased in high altitude placentae*. Placenta 24:326-35
327. **Aherne W** 1975 *Morphometry*. In: Gruenwald P (ed) The placenta and its maternal supply line. University Park Press, Baltimore, pp 80-97
328. **Kinare AS, Natekar AS, Chinchwadkar MC, Yajnik CS, Coyaji KJ, Fall CH, Howe DT** 2000 *Low midpregnancy placental volume in rural Indian women: A cause for low birth weight?* Am J Obstet Gynecol 182:443-8
329. **Heinonen S, Taipale P, Saarikoski S** 2001 *Weights of placentae from small-for-gestational age infants revisited*. Placenta 22:399-404

330. **Sherwood L** 1993 *Human physiology: from cells to systems*, Second ed. West Publishing Company, Minneapolis/St.Paul
331. **Rockwell LC, Vargas E, Moore LG** 2003 *Human physiological adaptation to pregnancy: inter- and intraspecific perspectives*. Am J Human Biol 15:330-41
332. **Lyall F, Bulmer JN, Duffie E, Cousins F, Theriault A, Robson SC** 2001 *Human trophoblast invasion and spiral artery transformation: the role of PECAM-1 in normal pregnancy, preeclampsia, and fetal growth restriction*. Am J Pathol 158:1713-21
333. **Kliman HJ** 2000 *Uteroplacental blood flow. The story of decidualization, menstruation, and trophoblast invasion*. Am J Pathol 157:1759-68
334. **Sheppard BL, Bonnar J** 1974 *The ultrastructure of the arterial supply of the human placenta in early and late pregnancy*. J Obstet Gynaecol Br Commonw 81:497-511
335. **Khong TY, De Wolf F, Robertson WB, Brosens I** 1986 *Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants*. Br J Obstet Gynaecol 93:1049-59
336. **Carbillon L, Challier JC, Alouini S, Uzan M, Uzan S** 2001 *Uteroplacental circulation development: Doppler assessment and clinical importance*. Placenta 22:795-9
337. **Brosens I, Dixon HG, Robertson WB** 1977 *Fetal growth retardation and the arteries of the placental bed*. Br J Obstet Gynaecol 84:656-63
338. **Lang U, Baker RS, Braems G, Zygmunt M, Kunzel W, Clark KE** 2003 *Uterine blood flow--a determinant of fetal growth*. Eur J Obstet Gynecol Reprod Biol 110 Suppl 1:S55-61
339. **Ueland K** 1976 *Maternal cardiovascular dynamics. VII. Intrapartum blood volume changes*. Am J Obstet Gynecol 126:671-7
340. **Thaler I, Manor D, Itskovitz J, Rottem S, Levit N, Timor-Tritsch I, Brandes JM** 1990 *Changes in uterine blood flow during human pregnancy*. Am J Obstet Gynecol 162:121-5
341. **Palmer SK, Zamudio S, Coffin C, Parker S, Stamm E, Moore LG** 1992 *Quantitative estimation of human uterine artery blood flow and pelvic blood flow redistribution in pregnancy*. Obstet Gynecol 80:1000-6
342. **Lunell NO, Nylund LE, Lewander R, Sarby B** 1982 *Uteroplacental blood flow in pre-eclampsia measurements with indium-113m and a computer-linked gamma camera*. Clin Exp Hypertens B 1:105-17
343. **Zygmunt M, Herr F, Keller-Schoenwetter S, Kunzi-Rapp K, Munstedt K, Rao CV, Lang U, Preissner KT** 2002 *Characterization of human chorionic gonadotropin as a novel angiogenic factor*. J Clin Endocrinol Metab 87:5290-6
344. **Zygmunt M, Herr F, Munstedt K, Lang U, Liang OD** 2003 *Angiogenesis and vasculogenesis in pregnancy*. Eur J Obstet Gynecol Reprod Biol 110 Suppl 1:S10-8
345. **Teasdale F** 1984 *Idiopathic intrauterine growth retardation: histomorphometry of the human placenta*. Placenta 5:83-92
346. **Jackson MR, Walsh AJ, Morrow RJ, Mullen JB, Lye SJ, Ritchie JW** 1995 *Reduced placental villous tree elaboration in small-for-gestational-age pregnancies: relationship with umbilical artery Doppler waveforms*. Am J Obstet Gynecol 172:518-25
347. **Laurin J, Lingman G, Marsal K, Persson PH** 1987 *Fetal blood flow in pregnancies complicated by intrauterine growth retardation*. Obstet Gynecol 69:895-902

348. **Ferrazzi E, Rigano S, Bozzo M, Bellotti M, Giovannini N, Galan H, Battaglia FC** 2000 *Umbilical vein blood flow in growth-restricted fetuses*. Ultrasound Obstet Gynecol 16:432-8
349. **Barbera A, Galan HL, Ferrazzi E, Rigano S, Jozwik M, Battaglia FC, Pardi G** 1999 *Relationship of umbilical vein blood flow to growth parameters in the human fetus*. Am J Obstet Gynecol 181:174-9
350. **Di Naro E, Raio L, Ghezzi F, Franchi M, Romano F, Addario VD** 2002 *Longitudinal umbilical vein blood flow changes in normal and growth-retarded fetuses*. Acta Obstet Gynecol Scand 81:527-33
351. **Philipps AF, Holzman IR, Teng C, Battaglia FC** 1978 *Tissue concentrations of free amino acids in term human placentas*. Am J Obstet Gynecol 131:881-7
352. **Economides DL, Nicolaides KH, Gahl WA, Bernardini I, Evans MI** 1989 *Plasma amino acids in appropriate- and small-for-gestational-age fetuses*. Am J Obstet Gynecol 161:1219-27
353. **Cetin I** 2003 *Placental transport of amino acids in normal and growth-restricted pregnancies*. Eur J Obstet Gynecol Reprod Biol 110 Suppl 1:S50-4
354. **Johnson LW, Smith CH** 1988 *Neutral amino acid transport systems of microvillous membrane of human placenta*. Am J Physiol 254:C773-80
355. **Jansson T** 2001 *Amino acid transporters in the human placenta*. Pediatr Res 49:141-7
356. **Ayuk PT, Sibley CP, Donnai P, D'Souza S, Glazier JD** 2000 *Development and polarization of cationic amino acid transporters and regulators in the human placenta*. Am J Physiol Cell Physiol 278:C1162-71
357. **Loy GL, Quick AN, Jr., Hay WW, Jr., Meschia G, Battaglia FC, Fennessey PV** 1990 *Fetoplacental deamination and decarboxylation of leucine*. Am J Physiol 259:E492-7
358. **Jansson T, Scholtbach V, Powell TL** 1998 *Placental transport of leucine and lysine is reduced in intrauterine growth restriction*. Pediatr Res 44:532-7
359. **Cetin I, Marconi AM, Bozzetti P, Sereni LP, Corbetta C, Pardi G, Battaglia FC** 1988 *Umbilical amino acid concentrations in appropriate and small for gestational age infants: a biochemical difference present in utero*. Am J Obstet Gynecol 158:120-6
360. **Cetin I, Corbetta C, Sereni LP, Marconi AM, Bozzetti P, Pardi G, Battaglia FC** 1990 *Umbilical amino acid concentrations in normal and growth-retarded fetuses sampled in utero by cordocentesis*. Am J Obstet Gynecol 162:253-61
361. **Paolini CL, Marconi AM, Ronzoni S, Di Noio M, Fennessey PV, Pardi G, Battaglia FC** 2001 *Placental transport of leucine, phenylalanine, glycine, and proline in intrauterine growth-restricted pregnancies*. J Clin Endocrinol Metab 86:5427-32
362. **Mahendran D, Donnai P, Glazier JD, D'Souza SW, Boyd RD, Sibley CP** 1993 *Amino acid (system A) transporter activity in microvillous membrane vesicles from the placentas of appropriate and small for gestational age babies*. Pediatr Res 34:661-5
363. **Harrington B, Glazier J, D'Souza S, Sibley C** 1999 *System A amino acid transporter activity in human placental microvillous membrane vesicles in relation to various anthropometric measurements in appropriate and small for gestational age babies*. Pediatr Res 45:810-4
364. **Jansson T, Wennergren M, Illsley NP** 1993 *Glucose transporter protein expression in human placenta throughout gestation and in intrauterine growth retardation*. J Clin Endocrinol Metab 77:1554-62

365. **Marconi AM, Paolini C, Buscaglia M, Zerbe G, Battaglia FC, Pardi G** 1996 *The impact of gestational age and fetal growth on the maternal-fetal glucose concentration difference*. Obstet Gynecol 87:937-42
366. **Nicolini U, Hubinont C, Santolaya J, Fisk NM, Coe AM, Rodeck CH** 1989 *Maternal-fetal glucose gradient in normal pregnancies and in pregnancies complicated by alloimmunization and fetal growth retardation*. Am J Obstet Gynecol 161:924-7
367. **Economides DL, Nicolaides KH** 1989 *Blood glucose and oxygen tension levels in small-for-gestational-age fetuses*. Am J Obstet Gynecol 160:385-9
368. **Barros LF, Yudilevich DL, Jarvis SM, Beaumont N, Baldwin SA** 1995 *Quantitation and immunolocalization of glucose transporters in the human placenta*. Placenta 16:623-33
369. **Challis DE, Pfarrer CD, Ritchie JW, Koren G, Adamson SL** 2000 *Glucose metabolism is elevated and vascular resistance and maternofetal transfer is normal in perfused placental cotyledons from severely growth-restricted fetuses*. Pediatr Res 47:309-15
370. **Kainulainen H, Jarvinen T, Heinonen PK** 1997 *Placental glucose transporters in fetal intrauterine growth retardation and macrosomia*. Gynecol Obstet Invest 44:89-92
371. **Jansson T, Ylven K, Wennergren M, Powell TL** 2002 *Glucose transport and system A activity in syncytiotrophoblast microvillous and basal plasma membranes in intrauterine growth restriction*. Placenta 23:392-9
372. **Larque E, Demmelmair H, Koletzko B** 2002 *Perinatal supply and metabolism of long-chain polyunsaturated fatty acids: importance for the early development of the nervous system*. Ann N Y Acad Sci 967:299-310
373. **Haggarty P** 2002 *Placental regulation of fatty acid delivery and its effect on fetal growth--a review*. Placenta 23 Suppl A:S28-38
374. **Jumpsen J, van Aerde J, Clandinin MT** 1997 *Fetal lipid requirements: implications in fetal growth retardation*. In: Battaglia FC (ed) Placental function and fetal nutrition. Vevey/Lippincott-Raven Publishers, Philadelphia, pp 157-67
375. **Garnica AD, Chan WY** 1996 *The role of the placenta in fetal nutrition and growth*. J Am Coll Nutr 15:206-22
376. **Campbell FM, Bush PG, Veerkamp JH, Dutta-Roy AK** 1998 *Detection and cellular localization of plasma membrane-associated and cytoplasmic fatty acid-binding proteins in human placenta*. Placenta 19:409-15
377. **Pardi G, Marconi AM, Cetin I** 2002 *Placental-fetal interrelationship in IUGR fetuses--a review*. Placenta 23 Suppl A:S136-41
378. **Crawford MA, Costeloe K, Ghebremeskel K, Phylactos A, Skirvin L, Stacey F** 1997 *Are deficits of arachidonic and docosahexaenoic acids responsible for the neural and vascular complications of preterm babies?* Am J Clin Nutr 66:1032S-1041S
379. **Rinderknecht E, Humbel RE** 1978 *The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin*. J Biol Chem 253:2769-76
380. **Zapf J, Rinderknecht E, Humbel RE, Froesch ER** 1978 *Nonsuppressible insulin-like activity (NSILA) from human serum: recent accomplishments and their physiologic implications*. Metabolism 27:1803-28
381. **Ashton IK, Spencer EM** 1983 *Effect of partially purified human somatomedin on human fetal and postnatal cartilage in vitro*. Early Hum Dev 8:135-40

382. **Kniss DA, Shubert PJ, Zimmerman PD, Landon MB, Gabbe SG** 1994 *Insulinlike growth factors. Their regulation of glucose and amino acid transport in placental trophoblasts isolated from first-trimester chorionic villi*. J Reprod Med 39:249-56
383. **DeChiara TM, Efstratiadis A, Robertson EJ** 1990 *A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting*. Nature 345:78-80
384. **Baker J, Liu JP, Robertson EJ, Efstratiadis A** 1993 *Role of insulin-like growth factors in embryonic and postnatal growth*. Cell 75:73-82
385. **Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A** 1993 *Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r)*. Cell 75:59-72
386. **Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C, Reik W** 2002 *Placental-specific IGF-II is a major modulator of placental and fetal growth*. Nature 417:945-8
387. **Tycko B, Efstratiadis A** 2002 *Genomic imprinting: piece of cake*. Nature 417:913-4
388. **Crossey PA, Pillai CC, Miell JP** 2002 *Altered placental development and intrauterine growth restriction in IGF binding protein-1 transgenic mice*. J Clin Invest 110:411-8
389. **Fujita-Yamaguchi Y, LeBon TR, Tsubokawa M, Henzel W, Kathuria S, Koyal D, Ramachandran J** 1986 *Comparison of insulin-like growth factor I receptor and insulin receptor purified from human placental membranes*. J Biol Chem 261:16727-31
390. **Chernausek SD, Jacobs S, Van Wyk JJ** 1981 *Structural similarities between human receptors for somatomedin C and insulin: analysis by affinity labeling*. Biochemistry 20:7345-50
391. **Massague J, Czech MP** 1982 *The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor*. J Biol Chem 257:5038-45
392. **Grizzard JD, D'Ercole AJ, Wilkins JR, Moats-Staats BM, Williams RW** 1984 *Affinity-labeled somatomedin-C receptors and binding proteins from the human fetus*. J Clin Endocrinol Metab 58:535-43
393. **Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, Henzel W, Le Bon T, Kathuria S, Chen E, et al.** 1986 *Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity*. Embo J 5:2503-12
394. **LeBon TR, Jacobs S, Cuatrecasas P, Kathuria S, Fujita-Yamaguchi Y** 1986 *Purification of insulin-like growth factor I receptor from human placental membranes*. J Biol Chem 261:7685-9
395. **Germain-Lee EL, Janicot M, Lammers R, Ullrich A, Casella SJ** 1992 *Expression of a type I insulin-like growth factor receptor with low affinity for insulin-like growth factor II*. Biochem J 281 (Pt 2):413-7
396. **Tally M, Enberg G, Li CH, Hall K** 1987 *The specificity of the human IGF-2 receptor*. Biochem Biophys Res Commun 147:1206-12
397. **Morgan DO, Edman JC, Standring DN, Fried VA, Smith MC, Roth RA, Rutter WJ** 1987 *Insulin-like growth factor II receptor as a multifunctional binding protein*. Nature 329:301-7

398. **Woods KA, Camacho-Hubner C, Savage MO, Clark AJ** 1996 *Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene*. N Engl J Med 335:1363-7
399. **Abuzzahab MJ, Schneider A, Goddard A, Grigorescu F, Lautier C, Keller E, Kiess W, Klammt J, Kratzsch J, Osgood D, Pfaffle R, Raile K, Seidel B, Smith RJ, Chernausek SD** 2003 *IGF-I receptor mutations resulting in intrauterine and postnatal growth retardation*. N Engl J Med 349:2211-22
400. **Gargosky SE, Owens PC, Walton PE, Owens JA, Robinson JS, Wallace JC, Ballard FJ** 1991 *Most of the circulating insulin-like growth factors-I and -II are present in the 150 kDa complex during human pregnancy*. J Endocrinol 131:491-7
401. **Wilson DM, Bennett A, Adamson GD, Nagashima RJ, Liu F, DeNatale ML, Hintz RL, Rosenfeld RG** 1982 *Somatomedins in pregnancy: a cross-sectional study of insulin-like growth factors I and II and somatomedin peptide content in normal human pregnancies*. J Clin Endocrinol Metab 55:858-61
402. **Leger J, Oury JF, Noel M, Baron S, Benali K, Blot P, Czernichow P** 1996 *Growth factors and intrauterine growth retardation. I. Serum growth hormone, insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein 3 levels in normally grown and growth-retarded human fetuses during the second half of gestation*. Pediatr Res 40:94-100
403. **Leger J, Noel M, Limal JM, Czernichow P** 1996 *Growth factors and intrauterine growth retardation. II. Serum growth hormone, insulin-like growth factor (IGF) I, and IGF-binding protein 3 levels in children with intrauterine growth retardation compared with normal control subjects: prospective study from birth to two years of age*. Pediatr Res 40:101-7
404. **Hernandez-Valencia M, Zarate A, Ochoa R, Fonseca ME, Amato D, De Jesus Ortiz M** 2001 *Insulin-like growth factor I, epidermal growth factor and transforming growth factor beta expression and their association with intrauterine fetal growth retardation, such as development during human pregnancy*. Diabetes Obes Metab 3:457-62
405. **Giudice LC, Martina NA, Crystal RA, Tazuke S, Druzin M** 1997 *Insulin-like growth factor binding protein-1 at the maternal-fetal interface and insulin-like growth factor-I, insulin-like growth factor-II, and insulin-like growth factor binding protein-1 in the circulation of women with severe preeclampsia*. Am J Obstet Gynecol 176:751-7; discussion 757-8
406. **Bang P, Westgren M, Schwander J, Blum WF, Rosenfeld RG, Stangenberg M** 1994 *Ontogeny of insulin-like growth factor-binding protein-1, -2, and -3: quantitative measurements by radioimmunoassay in human fetal serum*. Pediatr Res 36:528-36
407. **Lee PD, Conover CA, Powell DR** 1993 *Regulation and function of insulin-like growth factor-binding protein-1*. Proc Soc Exp Biol Med 204:4-29
408. **Baxter RC, Martin JL** 1989 *Structure of the Mr 140,000 growth hormone-dependent insulin-like growth factor binding protein complex: determination by reconstitution and affinity-labeling*. Proc Natl Acad Sci U S A 86:6898-902
409. **Baxter RC, Martin JL** 1986 *Radioimmunoassay of growth hormone-dependent insulin-like growth factor binding protein in human plasma*. J Clin Investig 78:1504-12
410. **Coulter CL, Han VK** 1996 *Expression of insulin-like growth factor-II and IGF-binding protein-1 mRNAs in term rhesus monkey placenta: comparison with human placenta*. Horm Res 45:167-71

411. **Han VK, Bassett N, Walton J, Challis JR** 1996 *The expression of insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) genes in the human placenta and membranes: evidence for IGF-IGFBP interactions at the feto-maternal interface*. J Clin Endocrinol Metab 81:2680-93
412. **Drop SL, Kortleve DJ, Guyda HJ** 1984 *Isolation of a somatomedin-binding protein from preterm amniotic fluid. Development of a radioimmunoassay*. J Clin Endocrinol Metab 59:899-907
413. **Drop SL, Kortleve DJ, Guyda HJ, Posner BI** 1984 *Immunoassay of a somatomedin-binding protein from human amniotic fluid: levels in fetal, neonatal, and adult sera*. J Clin Endocrinol Metab 59:908-15
414. **Baxter RC, Martin JL, Wood MH** 1987 *Two immunoreactive binding proteins for insulin-like growth factors in human amniotic fluid: relationship to fetal maturity*. J Clin Endocrinol Metab 65:423-31
415. **Busby WH, Jr., Klapper DG, Clemmons DR** 1988 *Purification of a 31,000-dalton insulin-like growth factor binding protein from human amniotic fluid. Isolation of two forms with different biologic actions*. J Biol Chem 263:14203-10
416. **Koistinen R, Kalkkinen N, Huhtala ML, Seppala M, Bohn H, Rutanen EM** 1986 *Placental protein 12 is a decidual protein that binds somatomedin and has an identical N-terminal amino acid sequence with somatomedin-binding protein from human amniotic fluid*. Endocrinology 118:1375-8
417. **Westwood M** 1999 *Role of insulin-like growth factor binding protein 1 in human pregnancy*. Rev Reprod 4:160-7
418. **Jones JI, D'Ercole AJ, Camacho-Hubner C, Clemmons DR** 1991 *Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: effects on affinity for IGF-I*. Proc Natl Acad Sci U S A 88:7481-5
419. **Westwood M, Gibson JM, Davies AJ, Young RJ, White A** 1994 *The phosphorylation pattern of insulin-like growth factor-binding protein-1 in normal plasma is different from that in amniotic fluid and changes during pregnancy*. J Clin Endocrinol Metab 79:1735-41
420. **Abbas A, Johnson M, Chard T, Nicolaides KH** 1995 *Maternal plasma concentrations of insulin-like growth factor binding protein-1 and placental protein 14 in multifetal pregnancies before and after fetal reduction*. Hum Reprod 10:207-10
421. **Westwood M, Gibson JM, White A** 1997 *Purification and characterization of the insulin-like growth factor-binding protein-1 phosphoform found in normal plasma*. Endocrinology 138:1130-6
422. **Wang HS, Perry LA, Kanisius J, Iles RK, Holly JM, Chard T** 1991 *Purification and assay of insulin-like growth factor-binding protein-1: measurement of circulating levels throughout pregnancy*. J Endocrinol 128:161-8
423. **Fant M, Munro H, Moses AC** 1986 *An autocrine/paracrine role for insulin-like growth factors in the regulation of human placental growth*. J Clin Endocrinol Metab 63:499-505
424. **Hamilton GS, Lysiak JJ, Han VK, Lala PK** 1998 *Autocrine-paracrine regulation of human trophoblast invasiveness by insulin-like growth factor (IGF)-II and IGF-binding protein (IGFBP)-1*. Exp Cell Res 244:147-56

425. **Giudice LC, Mark SP, Irwin JC** 1998 *Paracrine actions of insulin-like growth factors and IGF binding protein-1 in non-pregnant human endometrium and at the decidual-trophoblast interface*. J Reprod Immunol 39:133-48
426. **Fang J, Furesz TC, Lurent RS, Smith CH, Fant ME** 1997 *Spatial polarization of insulin-like growth factor receptors on the human syncytiotrophoblast*. Pediatr Res 41:258-65
427. **Fang J, Furesz TC, Smith CH, Fant ME** 1999 *IGF binding protein-1 (IGFBP-1) is preferentially associated with the fetal-facing basal surface of the syncytiotrophoblast in the human placenta*. Growth Horm IGF Res 9:438-44
428. **Dalcik H, Yardimoglu M, Vural B, Dalcik C, Filiz S, Gonca S, Kokturk S, Ceylan S** 2001 *Expression of insulin-like growth factor in the placenta of intrauterine growth-retarded human fetuses*. Acta Histochem 103:195-207
429. **Sheikh S, Satoskar P, Bhartiya D** 2001 *Expression of insulin-like growth factor-I and placental growth hormone mRNA in placentae: a comparison between normal and intrauterine growth retardation pregnancies*. Mol Hum Reprod 7:287-92
430. **Heffner LJ, Bromley BS, Copeland KC** 1992 *Secretion of prolactin and insulin-like growth factor I by decidual explant cultures from pregnancies complicated by intrauterine growth retardation*. Am J Obstet Gynecol 167:1431-6
431. **Heffner LJ, Benoit LA, Clemmons DR, Copeland KC** 1998 *The secretion of insulin-like growth factor I, prolactin and insulin-like growth factor binding protein 1 by the decidua as predictors of human fetal growth*. Growth Horm IGF Res 8:33-8
432. **Sorem KA, Siler-Khodr TM** 1998 *Placental IGF-I in severe intrauterine growth retardation*. J Matern Fetal Med 7:1-7
433. **Liu YJ, Tsushima T, Onoda N, Minei S, Sanaka M, Nagashima T, Yanagisawa K, Omori Y** 1996 *Expression of messenger RNA of insulin-like growth factors (IGFs) and IGF binding proteins (IGFBP1-6) in placenta of normal and diabetic pregnancy*. Endocr J 43 Suppl:S89-91
434. **Bajoria R, Wigglesworth J, Fisk NM** 1995 *Angioarchitecture of monochorionic placentas in relation to the twin-twin transfusion syndrome*. Am J Obstet Gynecol 172:856-63
435. **Bajoria R, Gibson MJ, Ward S, Sooranna SR, Neilson JP, Westwood M** 2001 *Placental regulation of insulin-like growth factor axis in monochorionic twins with chronic twin-twin transfusion syndrome*. J Clin Endocrinol Metab 86:3150-6
436. **Verhaeghe J, Loos R, Vlietinck R, Herck EV, van Bree R, Schutter AM** 1996 *C-peptide, insulin-like growth factors I and II, and insulin-like growth factor binding protein-1 in cord serum of twins: genetic versus environmental regulation*. Am J Obstet Gynecol 175:1180-8
437. **Westwood M, Gibson JM, Sooranna SR, Ward S, Neilson JP, Bajoria R** 2001 *Genes or placenta as modulator of fetal growth: evidence from the insulin-like growth factor axis in twins with discordant growth*. Mol Hum Reprod 7:387-95
438. **Yu J, Iwashita M, Kudo Y, Takeda Y** 1998 *Phosphorylated insulin-like growth factor (IGF)-binding protein-1 (IGFBP-1) inhibits while non-phosphorylated IGFBP-1 stimulates IGF-I-induced amino acid uptake by cultured trophoblast cells*. Growth Horm IGF Res 8:65-70

439. **Bajoria R, Sooranna SR, Ward S, Hancock M** 2002 *Placenta as a link between amino acids, insulin-IGF axis, and low birth weight: evidence from twin studies*. J Clin Endocrinol Metab 87:308-15
440. **Gluckman PD, Johnson-Barrett JJ, Butler JH, Edgar BW, Gunn TR** 1983 *Studies of insulin-like growth factor -I and -II by specific radioligand assays in umbilical cord blood*. Clin Endocrinol (Oxf) 19:405-13
441. **Bennett A, Wilson DM, Liu F, Nagashima R, Rosenfeld RG, Hintz RL** 1983 *Levels of insulin-like growth factors I and II in human cord blood*. J Clin Endocrinol Metab 57:609-12
442. **Fant M, Salafia C, Baxter RC, Schwander J, Vogel C, Pezzullo J, Moya F** 1993 *Circulating levels of IGFs and IGF binding proteins in human cord serum: relationships to intrauterine growth*. Regul Pept 48:29-39
443. **Ostlund E, Bang P, Hagenas L, Fried G** 1997 *Insulin-like growth factor I in fetal serum obtained by cordocentesis is correlated with intrauterine growth retardation*. Hum Reprod 12:840-4
444. **Klauwer D, Blum WF, Hanitsch S, Rascher W, Lee PD, Kiess W** 1997 *IGF-I, IGF-II, free IGF-I and IGFBP-1, -2 and -3 levels in venous cord blood: relationship to birthweight, length and gestational age in healthy newborns*. Acta Paediatr 86:826-33
445. **Ong K, Kratzsch J, Kiess W, Costello M, Scott C, Dunger D** 2000 *Size at birth and cord blood levels of insulin, insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-1 (IGFBP-1), IGFBP-3, and the soluble IGF-II/mannose-6-phosphate receptor in term human infants. The ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood*. J Clin Endocrinol Metab 85:4266-9
446. **Orbak Z, Darcan S, Coker M, Goksen D** 2001 *Maternal and fetal serum insulin-like growth factor-I (IGF-I) IGF binding protein-3 (IGFBP-3), leptin levels and early postnatal growth in infants born asymmetrically small for gestational age*. J Pediatr Endocrinol Metab 14:1119-27
447. **Christou H, Connors JM, Ziotopoulou M, Hatzidakis V, Papathanassoglou E, Ringer SA, Mantzoros CS** 2001 *Cord Blood Leptin and Insulin-Like Growth Factor Levels are Independent Predictors of Fetal Growth*. J Clin Endocrinol Metab 86:935-938
448. **Shibata A, Harris DT, Billings PR** 2002 *Concentrations of estrogens and IGFs in umbilical cord blood plasma: a comparison among Caucasian, Hispanic, and Asian-American females*. J Clin Endocrinol Metab 87:810-5
449. **Vatten LJ, Nilsen ST, Odegard RA, Romundstad PR, Austgulen R** 2002 *Insulin-like growth factor I and leptin in umbilical cord plasma and infant birth size at term*. Pediatrics 109:1131-5
450. **Boyne MS, Thame M, Bennett FI, Osmond C, Miell JP, Forrester TE** 2003 *The relationship among circulating insulin-like growth factor (IGF)-I, IGF-binding proteins-1 and -2, and birth anthropometry: a prospective study*. J Clin Endocrinol Metab 88:1687-91
451. **Ashton IK, Zapf J, Einschenk I, MacKenzie IZ** 1985 *Insulin-like growth factors (IGF) 1 and 2 in human foetal plasma and relationship to gestational age and foetal size during midpregnancy*. Acta Endocrinol (Copenh) 110:558-63
452. **Davidson S, Shtaif B, Gil-Ad I, Maayan R, Sulkes J, Weizman A, Merlob P** 2001 *Insulin, insulin-like growth factors-I and -II and insulin-like growth factor binding protein-3 in newborn serum: association with normal fetal head growth and head circumference*. J Pediatr Endocrinol Metab 14:151-8

453. **Lassarre C, Hardouin S, Daffos F, Forestier F, Frankenne F, Binoux M** 1991 *Serum insulin-like growth factors and insulin-like growth factor binding proteins in the human fetus. Relationships with growth in normal subjects and in subjects with intrauterine growth retardation*. Pediatr Res 29:219-25
454. **Langford KS, Nicolaides KH, Jones J, Abbas A, McGregor AM, Miell JP** 1995 *Serum insulin-like growth factor-binding protein-3 (IGFBP-3) levels and IGFBP-3 protease activity in normal, abnormal, and multiple human pregnancy*. J Clin Endocrinol Metab 80:21-7
455. **Nieto-Diaz A, Villar J, Matorras-Weinig R, Valenzuela-Ruiz P** 1996 *Intrauterine growth retardation at term: association between anthropometric and endocrine parameters*. Acta Obstet Gynecol Scand 75:127-31
456. **Cianfarani S, Germani D, Rossi P, Rossi L, Germani A, Ossicini C, Zuppa A, Argiro G, Holly JM, Branca F** 1998 *Intrauterine growth retardation: evidence for the activation of the insulin-like growth factor (IGF)-related growth-promoting machinery and the presence of a cation-independent IGF binding protein-3 proteolytic activity by two months of life*. Pediatr Res 44:374-80
457. **Halhali A, Tovar AR, Torres N, Bourges H, Garabedian M, Larrea F** 2000 *Preeclampsia is associated with low circulating levels of insulin-like growth factor I and 1,25-dihydroxyvitamin D in maternal and umbilical cord compartments*. J Clin Endocrinol Metab 85:1828-33
458. **Diaz E, Halhali A, Luna C, Diaz L, Avila E, Larrea F** 2002 *Newborn birth weight correlates with placental zinc, umbilical insulin-like growth factor I, and leptin levels in preeclampsia*. Arch Med Res 33:40-7
459. **Ochoa R, Zarate A, Hernandez M, Galvan R** 2001 *Serum leptin and somatotropin components correlate with neonatal birth weight*. Gynecol Obstet Invest 52:243-7
460. **Verhaeghe J, Van Bree R, Van Herck E, Laureys J, Bouillon R, Van Assche FA** 1993 *C-peptide, insulin-like growth factors I and II, and insulin-like growth factor binding protein-1 in umbilical cord serum: correlations with birth weight*. Am J Obstet Gynecol 169:89-97
461. **Wang HS, Lee CL, Chard T** 1993 *Levels of insulin-like growth factor-I and insulin-like growth factor-binding protein-1 in pregnancy with preterm delivery*. Br J Obstet Gynaecol 100:472-5
462. **Verhaeghe J, Van Herck E, Billen J, Moerman P, Van Assche FA, Giudice LC** 2003 *Regulation of insulin-like growth factor-I and insulin-like growth factor binding protein-1 concentrations in preterm fetuses*. Am J Obstet Gynecol 188:485-91
463. **Iwashita M, Sakai K, Kudo Y, Takeda Y** 1998 *Phosphoisoforms of insulin-like growth factor binding protein-1 in appropriate-for-gestational-age and small-for-gestational-age fetuses*. Growth Horm IGF Res 8:487-93
464. **Larsen T, Main K, Andersson AM, Juul A, Greisen G, Skakkebaek NE** 1996 *Growth hormone, insulin-like growth factor I and its binding proteins 1 and 3 in last trimester intrauterine growth retardation with increased pulsatility index in the umbilical artery*. Clin Endocrinol (Oxf) 45:315-9
465. **Holmes R, Montemagno R, Jones J, Preece M, Rodeck C, Soothill P** 1997 *Fetal and maternal plasma insulin-like growth factors and binding proteins in pregnancies with appropriate or retarded fetal growth*. Early Hum Dev 49:7-17
466. **McIntyre HD, Serek R, Crane DI, Veveris-Lowe T, Parry A, Johnson S, Leung KC, Ho KK, Bougoussa M, Hennen G, Igout A, Chan FY, Cowley D,**

Cotterill A, Barnard R 2000 *Placental growth hormone (GH), GH-binding protein, and insulin-like growth factor axis in normal, growth-retarded, and diabetic pregnancies: correlations with fetal growth*. J Clin Endocrinol Metab 85:1143-50

467. **Bhatia S, Faessen GH, Carland G, Balise RL, Gargosky SE, Druzin M, El-Sayed Y, Wilson DM, Giudice LC** 2002 *A longitudinal analysis of maternal serum insulin-like growth factor I (IGF-I) and total and nonphosphorylated IGF-binding protein-1 in human pregnancies complicated by intrauterine growth restriction*. J Clin Endocrinol Metab 87:1864-70
468. **Verhaeghe J, Pintiaux A, Van Herck E, Hennen G, Foidart JM, Igout A** 2002 *Placental GH, IGF-I, IGF-binding protein-1, and leptin during a glucose challenge test in pregnant women: relation with maternal body weight, glucose tolerance, and birth weight*. J Clin Endocrinol Metab 87:2875-82
469. **Smith ID, Shearman RP** 1974 *Fetal plasma steroids in relation to parturition. I. The effect of gestational age upon umbilical plasma corticosteroid levels following vaginal delivery*. J Obstet Gynaecol Br Commonw 81:11-5
470. **Liggins GC** 1968 *Premature parturition after infusion of corticotrophin or cortisol into foetal lambs*. J Endocrinol 42:323-9
471. **Liggins GC** 1969 *Premature delivery of foetal lambs infused with glucocorticoids*. J Endocrinol 45:515-23
472. **Liggins GC** 1976 *Adrenocortical-related maturational events in the fetus*. Am J Obstet Gynecol 126:931-41
473. **Liggins GC** 1994 *The role of cortisol in preparing the fetus for birth*. Reprod Fertil Dev 6:141-50
474. **Schwartz AL, Rall TW** 1975 *Hormonal regulation of incorporation of alanine-U-14C into glucose in human fetal liver explants. Effect of dibutyryl cyclic AMP, glucagon, insulin, and triamcinolone*. Diabetes 24:650-7
475. **Zanardo V, Giacobbo F, Zambon P, Trevisanuto D, Griffith P, Grella P, Zacchello G** 1990 *Antenatal aminophylline and steroid exposure: effects on glomerular filtration rate and renal sodium excretion in preterm newborns*. J Perinat Med 18:283-8
476. **Gonzales LW, Ertsey R, Ballard PL, Froh D, Goerke J, Gonzales J** 1990 *Glucocorticoid stimulation of fatty acid synthesis in explants of human fetal lung*. Biochim Biophys Acta 1042:1-12
477. **Liggins GC, Howie RN** 1972 *A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants*. Pediatrics 50:515-25
478. **Block MF, Kling OR, Crosby WM** 1977 *Antenatal glucocorticoid therapy for the prevention of respiratory distress syndrome in the premature infant*. Obstet Gynecol 50:186-90
479. **Spellacy WN, Buhi WC, Riggall FC, Holsinger KL** 1973 *Human amniotic fluid lecithin-sphingomyelin ratio changes with estrogen or glucocorticoid treatment*. Am J Obstet Gynecol 115:216-8
480. **Caspi E, Schreyer P, Weinraub Z, Bukovsky I, Tamir I** 1975 *Changes in amniotic fluid lecithin-sphingomyelin ratio following maternal dexamethasone administration*. Am J Obstet Gynecol 122:327-31
481. **Elimian A, Verma U, Canterino J, Shah J, Visintainer P, Tejani N** 1999 *Effectiveness of antenatal steroids in obstetric subgroups*. Obstet Gynecol 93:174-9

482. **Bloom SL, Sheffield JS, McIntire DD, Leveno KJ** 2001 *Antenatal dexamethasone and decreased birth weight*. Obstet Gynecol 97:485-90
483. **Banks BA, Cnaan A, Morgan MA, Parer JT, Merrill JD, Ballard PL, Ballard RA** 1999 *Multiple courses of antenatal corticosteroids and outcome of premature neonates. North American Thyrotropin-Releasing Hormone Study Group*. Am J Obstet Gynecol 181:709-17
484. **Guinn DA, Atkinson MW, Sullivan L, Lee M, MacGregor S, Parilla BV, Davies J, Hanlon-Lundberg K, Simpson L, Stone J, Wing D, Ogasawara K, Muraskas J** 2001 *Single vs weekly courses of antenatal corticosteroids for women at risk of preterm delivery: A randomized controlled trial*. Jama 286:1581-7
485. **Lee MJ, Davies J, Guinn D, Sullivan L, Atkinson MW, McGregor S, Parilla BV, Hanlon-Lundberg K, Simpson L, Stone J, Wing D, Ogasawara K, Muraskas J** 2004 *Single versus weekly courses of antenatal corticosteroids in preterm premature rupture of membranes*. Obstet Gynecol 103:274-81
486. **French NP, Hagan R, Evans SF, Godfrey M, Newnham JP** 1999 *Repeated antenatal corticosteroids: size at birth and subsequent development*. Am J Obstet Gynecol 180:114-21
487. **Chin SO, Brodsky NL, Bhandari V** 2003 *Antenatal steroid use is associated with increased gastroesophageal reflux in neonates*. Am J Perinatol 20:205-13
488. **Rotmensch S, Liberati M, Vishne TH, Celentano C, Ben-Rafael Z, Bellati U** 1999 *The effect of betamethasone and dexamethasone on fetal heart rate patterns and biophysical activities. A prospective randomized trial*. Acta Obstet Gynecol Scand 78:493-500
489. **Subtil D, Tiberghien P, Devos P, Therby D, Leclerc G, Vaast P, Puech F** 2003 *Immediate and delayed effects of antenatal corticosteroids on fetal heart rate: a randomized trial that compares betamethasone acetate and phosphate, betamethasone phosphate, and dexamethasone*. Am J Obstet Gynecol 188:524-31
490. **Clifton VL, Wallace EM, Smith R** 2002 *Short-term effects of glucocorticoids in the human fetal-placental circulation in vitro*. J Clin Endocrinol Metab 87:2838-42
491. **Potter SM, Dennedy MC, Morrison JJ** 2002 *Corticosteroids and fetal vasculature: effects of hydrocortisone, dexamethasone and betamethasone on human umbilical artery*. BJOG 109:1126-31
492. **Sloboda DM, Newnham JP, Challis JR** 2000 *Effects of repeated maternal betamethasone administration on growth and hypothalamic-pituitary-adrenal function of the ovine fetus at term*. J Endocrinol 165:79-91
493. **Kerzner LS, Stonestreet BS, Wu KY, Sadowska G, Malee MP** 2002 *Antenatal dexamethasone: effect on ovine placental 11beta-hydroxysteroid dehydrogenase type 2 expression and fetal growth*. Pediatr Res 52:706-12
494. **Ikegami M, Jobe AH, Newnham J, Polk DH, Willet KE, Sly P** 1997 *Repetitive prenatal glucocorticoids improve lung function and decrease growth in preterm lambs*. Am J Respir Crit Care Med 156:178-84
495. **Benediktsson R, Lindsay RS, Noble J, Seckl JR, Edwards CR** 1993 *Glucocorticoid exposure in utero: new model for adult hypertension*. Lancet 341:339-41
496. **Barrada MI, Blomquist CH, Kotts C** 1980 *The effects of betamethasone on fetal development in the rabbit*. Am J Obstet Gynecol 136:234-8

497. **Johnson JW, Mitzner W, Beck JC, London WT, Sly DL, Lee PA, Khouzami VA, Cavalieri RL** 1981 *Long-term effects of betamethasone on fetal development*. Am J Obstet Gynecol 141:1053-64
498. **Dean F, Yu C, Lingas RI, Matthews SG** 2001 *Prenatal glucocorticoid modifies hypothalamo-pituitary-adrenal regulation in prepubertal guinea pigs*. Neuroendocrinology 73:194-202
499. **Gumbinas M, Oda M, Huttenlocher P** 1973 *The effects of corticosteroids on myelination of the developing rat brain*. Biol Neonate 22:355-66
500. **Uno H, Lohmiller L, Thieme C, Kemnitz JW, Engle MJ, Roecker EB, Farrell PM** 1990 *Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques. I. Hippocampus*. Brain Res Dev Brain Res 53:157-67
501. **Huang WL, Beazley LD, Quinlivan JA, Evans SF, Newnham JP, Dunlop SA** 1999 *Effect of corticosteroids on brain growth in fetal sheep*. Obstet Gynecol 94:213-8
502. **Graf R, Gossrau R, Frank HG** 1989 *Placental toxicity in rats after administration of synthetic glucocorticoids. A morphological, histochemical and immunohistochemical investigation*. Anat Embryol (Berl) 180:121-30
503. **Fowden AL, Szemere J, Hughes P, Gilmour RS, Forhead AJ** 1996 *The effects of cortisol on the growth rate of the sheep fetus during late gestation*. J Endocrinol 151:97-105
504. **Mosier HD, Jr., Dearden LC, Jansons RA, Roberts RC, Biggs CS** 1982 *Disproportionate growth of organs and body weight following glucocorticoid treatment of the rat fetus*. Dev Pharmacol Ther 4:89-105
505. **Mosier HD, Jr., Spencer EM, Dearden LC, Jansons RA** 1987 *The effect of glucocorticoids on plasma insulin-like growth factor I concentration in the rat fetus*. Pediatr Res 22:92-5
506. **Bro-Rasmussen F, Buus O, Trolle D** 1962 *Ratio of cortisone/cortisol in mother and infant at birth*. Acta Endocrinology 40:579-583
507. **Predine J, Merceron L, Barrier G, Sureau C, Milgrom E** 1979 *Unbound cortisol in umbilical cord plasma and maternal plasma: a reinvestigation*. Am J Obstet Gynecol 135:1104-8
508. **Gitau R, Cameron A, Fisk NM, Glover V** 1998 *Fetal exposure to maternal cortisol*. Lancet 352:707-8
509. **Tannin GM, Agarwal AK, Monder C, New MI, White PC** 1991 *The human gene for 11 beta-hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization*. J Biol Chem 266:16653-8
510. **Albiston AL, Obeyesekere VR, Smith RE, Krozowski ZS** 1994 *Cloning and tissue distribution of the human 11 beta-hydroxysteroid dehydrogenase type 2 enzyme*. Mol Cell Endocrinol 105:R11-7
511. **Bujalska IJ, Kumar S, Stewart PM** 1997 *Does central obesity reflect "Cushing's disease of the omentum"?* Lancet 349:1210-3
512. **Bujalska I, Shimojo M, Howie A, Stewart PM** 1997 *Human 11 beta-hydroxysteroid dehydrogenase: studies on the stably transfected isoforms and localization of the type 2 isozyme within renal tissue*. Steroids 62:77-82
513. **Brown RW, Chapman KE, Edwards CR, Seckl JR** 1993 *Human placental 11 beta-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform*. Endocrinology 132:2614-21
514. **Krozowski Z** 1992 *11 beta-hydroxysteroid dehydrogenase and the short-chain alcohol dehydrogenase (SCAD) superfamily*. Mol Cell Endocrinol 84:C25-31

515. **Stewart PM, Krozowski ZS** 1999 *11 beta-Hydroxysteroid dehydrogenase*. Vitam Horm 57:249-324
516. **Agarwal AK, Rogerson FM, Mune T, White PC** 1995 *Gene structure and chromosomal localization of the human HSD11K gene encoding the kidney (type 2) isozyme of 11 beta-hydroxysteroid dehydrogenase*. Genomics 29:195-9
517. **Ricketts ML, Verhaeg JM, Bujalska I, Howie AJ, Rainey WE, Stewart PM** 1998 *Immunohistochemical localization of type 1 11beta-hydroxysteroid dehydrogenase in human tissues*. J Clin Endocrinol Metab 83:1325-35
518. **Schleimer RP** 1991 *Potential regulation of inflammation in the lung by local metabolism of hydrocortisone*. Am J Respir Cell Mol Biol 4:166-73
519. **Monder C, Lakshmi V** 1990 *Corticosteroid 11 beta-dehydrogenase of rat tissues: immunological studies*. Endocrinology 126:2435-43
520. **Lopez Bernal A, Anderson AB, Parry DM, Turnbull AC** 1980 *Evidence that fetal membranes are not involved in cortisol metabolism: study of dichorionic twin pregnancies*. Am J Obstet Gynecol 138:1168-72
521. **Sun K, Yang K, Challis JR** 1997 *Differential expression of 11 beta-hydroxysteroid dehydrogenase types 1 and 2 in human placenta and fetal membranes*. J Clin Endocrinol Metab 82:300-5
522. **Patel FA, Sun K, Challis JR** 1999 *Local modulation by 11beta-hydroxysteroid dehydrogenase of glucocorticoid effects on the activity of 15-hydroxyprostaglandin dehydrogenase in human chorion and placental trophoblast cells*. J Clin Endocrinol Metab 84:395-400
523. **Challis JRG, Matthews SG, Gibb W, Lye SJ** 2000 *Endocrine and paracrine regulation of birth at term and preterm*. Endocr Rev 21:514-50
524. **Jenkins JS, Sampson PA** 1966 *The conversion of cortisone to cortisol and prednisone to prednisolone in man*. Proc R Soc Med 59:603-4
525. **Jenkins JS** 1966 *The metabolism of cortisol by human extra-hepatic tissues*. J Endocrinol 34:51-6
526. **Krozowski Z, Maguire JA, Stein-Oakley AN, Dowling J, Smith RE, Andrews RK** 1995 *Immunohistochemical localization of the 11 beta-hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta*. J Clin Endocrinol Metab 80:2203-9
527. **Burton AF, Anderson FH** 1983 *Inactivation of corticosteroids in intestinal mucosa by 11 beta-hydroxysteroid: NADP oxidoreductase (EC 1.1.1.146)*. Am J Gastroenterol 78:627-31
528. **Osinski PA** 1960 *Steroid 11beta-ol dehydrogenase in human placenta*. Nature 187:777
529. **Edwards CR, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS, de Kloet ER, Monder C** 1988 *Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific protector of the mineralocorticoid receptor*. Lancet 2:986-9
530. **Funder JW, Pearce PT, Smith R, Smith AI** 1988 *Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated*. Science 242:583-5
531. **Krozowski ZS, Funder JW** 1983 *Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity*. Proc Natl Acad Sci U S A 80:6056-60
532. **Ulick S, Levine LS, Gunczler P, Zanconato G, Ramirez LC, Rauh W, Rosler A, Bradlow HL, New MI** 1979 *A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol*. J Clin Endocrinol Metab 49:757-64

533. **Stewart PM, Wallace AM, Valentino R, Burt D, Shackleton CH, Edwards CR** 1987 *Mineralocorticoid activity of liquorice: 11-beta-hydroxysteroid dehydrogenase deficiency comes of age*. Lancet 2:821-4
534. **Wilson RC, Harbison MD, Krozowski ZS, Funder JW, Shackleton CH, Hanauske-Abel HM, Wei JQ, Hertecant J, Moran A, Neiberger RE, et al.** 1995 *Several homozygous mutations in the gene for 11 beta-hydroxysteroid dehydrogenase type 2 in patients with apparent mineralocorticoid excess*. J Clin Endocrinol Metab 80:3145-50
535. **Wilson RC, Krozowski ZS, Li K, Obeyesekere VR, Razzaghy-Azar M, Harbison MD, Wei JQ, Shackleton CH, Funder JW, New MI** 1995 *A mutation in the HSD11B2 gene in a family with apparent mineralocorticoid excess*. J Clin Endocrinol Metab 80:2263-6
536. **Benediktsson R, Calder AA, Edwards CR, Seckl JR** 1997 *Placental 11 beta-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure*. Clin Endocrinol (Oxf) 46:161-6
537. **Walker BR, Connacher AA, Webb DJ, Edwards CR** 1992 *Glucocorticoids and blood pressure: a role for the cortisol/cortisone shuttle in the control of vascular tone in man*. Clin Sci (Colch) 83:171-8
538. **Hult M, Jornvall H, Oppermann UC** 1998 *Selective inhibition of human type 1 11beta-hydroxysteroid dehydrogenase by synthetic steroids and xenobiotics*. FEBS Lett 441:25-8
539. **Clifton VL, Fittock R, Murphy VE, Smith R, Zarzycki P** 2003 *Metabolism of semi-synthetic glucocorticoids by the human placenta [abstract]*. Placenta 24:A43
540. **Hirasawa G, Takeyama J, Sasano H, Fukushima K, Suzuki T, Muramatu Y, Darnel AD, Kaneko C, Hiwatashi N, Toyota T, Nagura H, Krozowski Z** 2000 *11beta-hydroxysteroid dehydrogenase type II and mineralocorticoid receptor in human placenta*. J Clin Endocrinol Metab 85:1306-1309
541. **Driver PM, Rauz S, Walker EA, Hewison M, Kilby MD, Stewart PM** 2003 *Characterization of human trophoblast as a mineralocorticoid target tissue*. Mol Hum Reprod 9:793-8
542. **Driver PM, Kilby MD, Bujalska I, Walker EA, Hewison M, Stewart PM** 2001 *Expression of 11beta-hydroxysteroid dehydrogenase isozymes and corticosteroid hormone receptors in primary cultures of human trophoblast and placental bed biopsies*. Mol Hum Reprod 7:357-63
543. **Sun K, Adamson SL, Yang K, Challis JR** 1999 *Interconversion of cortisol and cortisone by 11beta-hydroxysteroid dehydrogenases type 1 and 2 in the perfused human placenta*. Placenta 20:13-9
544. **Dodds HM, Taylor PJ, Johnson LP, Mortimer RH, Pond SM, Cannell GR** 1997 *Cortisol metabolism and its inhibition by glycyrrhetinic acid in the isolated perfused human placental lobule*. J Steroid Biochem Mol Biol 62:337-43
545. **Beitins IZ, Bayard F, Ances IG, Kowarski A, Migeon CJ** 1973 *The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term*. Pediatr Res 7:509-19
546. **Murphy BE, Clark SJ, Donald IR, Pinsky M, Vedady D** 1974 *Conversion of maternal cortisol to cortisone during placental transfer to the human fetus*. Am J Obstet Gynecol 118:538-41
547. **Giannopoulos G, Jackson K, Tulchinsky D** 1982 *Glucocorticoid metabolism in human placenta, decidua, myometrium and fetal membranes*. J Steroid Biochem 17:371-4

548. **Blasco MJ, Lopez Bernal A, Turnbull AC** 1986 *11 beta-Hydroxysteroid dehydrogenase activity of the human placenta during pregnancy*. Horm Metab Res 18:638-41
549. **Tanswell AK, Worthington D, Smith BT** 1977 *Human amniotic membrane corticosteroid 11-oxidoreductase activity*. J Clin Endocrinol Metab 45:721-5
550. **Alfaidy N, Li W, MacIntosh T, Yang K, Challis J** 2003 *Late gestation increase in 11beta-hydroxysteroid dehydrogenase 1 expression in human fetal membranes: a novel intrauterine source of cortisol*. J Clin Endocrinol Metab 88:5033-8
551. **Lopez Bernal A, Anderson AB, Turnbull AC** 1982 *The lack of influence of labor on human placental 11 beta- hydroxysteroid dehydrogenase activity*. J Clin Endocrinol Metab 54:1251-4
552. **Murphy VE, Clifton VL** 2003 *Alterations in human placental 11beta-hydroxysteroid dehydrogenase type 1 and 2 with gestational age and labour*. Placenta 24:739-44
553. **Muneyyirci-Delale O, Lakshmi V, McCalla CO, Karacan M, Neil G, Camilien L** 1996 *Variations in human placental 11 beta-dehydrogenase and 11-oxoreductase activities of 11 beta-hydroxysteroid dehydrogenase enzyme during pregnancy*. Early Pregnancy 2:201-6
554. **Shams M, Kilby MD, Somerset DA, Howie AJ, Gupta A, Wood PJ, Afnan M, Stewart PM** 1998 *11Beta-hydroxysteroid dehydrogenase type 2 in human pregnancy and reduced expression in intrauterine growth restriction*. Hum Reprod 13:799-804
555. **Schoof E, Girstl M, Frobenius W, Kirschbaum M, Repp R, Knerr I, Rascher W, Dotsch J** 2001 *Course of placental 11beta-hydroxysteroid dehydrogenase type 2 and 15-hydroxyprostaglandin dehydrogenase mRNA expression during human gestation*. Eur J Endocrinol 145:187-92
556. **Kajantie E, Dunkel L, Turpeinen U, Stenman UH, Wood PJ, Nuutila M, Andersson S** 2003 *Placental 11beta-hydroxysteroid dehydrogenase-2 and fetal cortisol/cortisone shuttle in small preterm infants*. J Clin Endocrinol Metab 88:493-500
557. **Sampath-Kumar R, Matthews SG, Yang K** 1998 *11beta-hydroxysteroid dehydrogenase type 2 is the predominant isozyme in the guinea pig placenta: decreases in messenger ribonucleic acid and activity at term*. Biol Reprod 59:1378-84
558. **McTernan CL, Draper N, Nicholson H, Chalder SM, Driver P, Hewison M, Kilby MD, Stewart PM** 2001 *Reduced placental 11beta-hydroxysteroid dehydrogenase type 2 mRNA levels in human pregnancies complicated by intrauterine growth restriction: an analysis of possible mechanisms*. J Clin Endocrinol Metab 86:4979-4983
559. **Stewart PM, Rogerson FM, Mason JI** 1995 *Type 2 11 beta-hydroxysteroid dehydrogenase messenger ribonucleic acid and activity in human placenta and fetal membranes: its relationship to birth weight and putative role in fetal adrenal steroidogenesis*. J Clin Endocrinol Metab 80:885-90
560. **Rogerson FM, Kayes KM, White PC** 1997 *Variation in placental type 2 11beta-hydroxysteroid dehydrogenase activity is not related to birth weight or placental weight*. Mol Cell Endocrinol 128:103-9
561. **Hofmann M, Pollow K, Bahlmann F, Casper F, Steiner E, Brockerhoff P** 2001 *11 beta-hydroxysteroid dehydrogenase (11 beta-HSD-II) activity in human*

placenta: its relationship to placental weight and birth weight and its possible role in hypertension. J Perinat Med 29:23-30

562. **McCalla CO, Nacharaju VL, Muneyyirci-Delale O, Glasgow S, Feldman JG** 1998 *Placental 11 beta-hydroxysteroid dehydrogenase activity in normotensive and pre-eclamptic pregnancies*. Steroids 63:511-5
563. **Schoof E, Girstl M, Frobenius W, Kirschbaum M, Dorr HG, Rascher W, Dotsch J** 2001 *Decreased gene expression of 11beta-hydroxysteroid dehydrogenase type 2 and 15-hydroxyprostaglandin dehydrogenase in human placenta of patients with preeclampsia*. J Clin Endocrinol Metab 86:1313-7
564. **Kitanaka S, Tanae A, Hibi I** 1996 *Apparent mineralocorticoid excess due to 11 beta-hydroxysteroid dehydrogenase deficiency: a possible cause of intrauterine growth retardation*. Clin Endocrinol (Oxf) 44:353-9
565. **Stewart PM, Krozowski ZS, Gupta A, Milford DV, Howie AJ, Sheppard MC, Whorwood CB** 1996 *Hypertension in the syndrome of apparent mineralocorticoid excess due to mutation of the 11 beta-hydroxysteroid dehydrogenase type 2 gene*. Lancet 347:88-91
566. **Krozowski ZS, Stewart PM, Obeyesekere VR, Li K, Ferrari P** 1997 *Mutations in the 11 beta-hydroxysteroid dehydrogenase type II enzyme associated with hypertension and possibly stillbirth*. Clin Exp Hypertens 19:519-29
567. **Levitt NS, Lindsay RS, Holmes MC, Seckl JR** 1996 *Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat*. Neuroendocrinology 64:412-8
568. **Lindsay RS, Lindsay RM, Edwards CR, Seckl JR** 1996 *Inhibition of 11-beta-hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the offspring*. Hypertension 27:1200-4
569. **Lindsay RS, Lindsay RM, Waddell BJ, Seckl JR** 1996 *Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: studies with the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone*. Diabetologia 39:1299-305
570. **Saegusa H, Nakagawa Y, Liu YJ, Ohzeki T** 1999 *Influence of placental 11beta-hydroxysteroid dehydrogenase (11beta-HSD) inhibition on glucose metabolism and 11beta-HSD regulation in adult offspring of rats*. Metabolism 48:1584-8
571. **Langley-Evans SC, Phillips GJ, Benediktsson R, Gardner DS, Edwards CR, Jackson AA, Seckl JR** 1996 *Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat*. Placenta 17:169-72
572. **Bertram C, Trowern AR, Copin N, Jackson AA, Whorwood CB** 2001 *The maternal diet during pregnancy programs altered expression of the glucocorticoid receptor and type 2 11beta-hydroxysteroid dehydrogenase: potential molecular mechanisms underlying the programming of hypertension in utero*. Endocrinology 142:2841-53
573. **Smith JT, Waddell BJ** 2000 *Increased fetal glucocorticoid exposure delays puberty onset in postnatal life*. Endocrinology 141:2422-8
574. **Welberg LA, Seckl JR, Holmes MC** 2000 *Inhibition of 11beta-hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring*. Eur J Neurosci 12:1047-54

575. **Lesage J, Blondeau B, Grino M, Breant B, Dupouy JP** 2001 *Maternal undernutrition during late gestation induces fetal overexposure to glucocorticoids and intrauterine growth retardation, and disturbs the hypothalamo-pituitary adrenal axis in the newborn rat*. Endocrinology 142:1692-702
576. **Dean F, Matthews SG** 1999 *Maternal dexamethasone treatment in late gestation alters glucocorticoid and mineralocorticoid receptor mRNA in the fetal guinea pig brain*. Brain Res 846:253-9
577. **Owen D, Matthews SG** 2003 *Glucocorticoids and sex-dependent development of brain glucocorticoid and mineralocorticoid receptors*. Endocrinology 144:2775-84
578. **McCabe L, Marash D, Li A, Matthews SG** 2001 *Repeated antenatal glucocorticoid treatment decreases hypothalamic corticotropin releasing hormone mRNA but not corticosteroid receptor mRNA expression in the fetal guinea-pig brain*. J Neuroendocrinol 13:425-31
579. **Weinstock M, Matlina E, Maor GI, Rosen H, McEwen BS** 1992 *Prenatal stress selectively alters the reactivity of the hypothalamic-pituitary adrenal system in the female rat*. Brain Res 595:195-200
580. **McCormick CM, Smythe JW, Sharma S, Meaney MJ** 1995 *Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats*. Brain Res Dev Brain Res 84:55-61
581. **Liu L, Li A, Matthews SG** 2001 *Maternal glucocorticoid treatment programs HPA regulation in adult offspring: sex-specific effects*. Am J Physiol Endocrinol Metab 280:E729-39
582. **Economides DL, Nicolaides KH, Linton EA, Perry LA, Chard T** 1988 *Plasma cortisol and adrenocorticotropin in appropriate and small for gestational age fetuses*. Fetal Ther 3:158-64
583. **Phillips DI, Walker BR, Reynolds RM, Flanagan DE, Wood PJ, Osmond C, Barker DJ, Whorwood CB** 2000 *Low birth weight predicts elevated plasma cortisol concentrations in adults from 3 populations*. Hypertension 35:1301-6
584. **Reynolds RM, Walker BR, Syddall HE, Andrew R, Wood PJ, Whorwood CB, Phillips DI** 2001 *Altered control of cortisol secretion in adult men with low birth weight and cardiovascular risk factors*. J Clin Endocrinol Metab 86:245-50
585. **Kajantie E, Phillips DI, Andersson S, Barker DJ, Dunkel L, Forsen T, Osmond C, Tuominen J, Wood PJ, Eriksson J** 2002 *Size at birth, gestational age and cortisol secretion in adult life: foetal programming of both hyper- and hypocortisolism?* Clin Endocrinol (Oxf) 57:635-41
586. **Kajantie E, Eriksson J, Barker DJ, Forsen T, Osmond C, Wood PJ, Andersson S, Dunkel L, Phillips DI** 2003 *Birthsize, gestational age and adrenal function in adult life: studies of dexamethasone suppression and ACTH1-24 stimulation*. Eur J Endocrinol 149:569-75
587. **Clark PM, Hindmarsh PC, Shiell AW, Law CM, Honour JW, Barker DJ** 1996 *Size at birth and adrenocortical function in childhood*. Clin Endocrinol (Oxf) 45:721-6
588. **van Os J, Selten JP** 1998 *Prenatal exposure to maternal stress and subsequent schizophrenia. The May 1940 invasion of The Netherlands*. Br J Psychiatry 172:324-6

589. **Lou HC, Hansen D, Nordentoft M, Pryds O, Jensen F, Nim J, Hemmingsen R** 1994 *Prenatal stressors of human life affect fetal brain development*. Dev Med Child Neurol 36:826-32
590. **Sun K, Yang K, Challis JR** 1998 *Regulation of 11beta-hydroxysteroid dehydrogenase type 2 by progesterone, estrogen, and the cyclic adenosine 5′-monophosphate pathway in cultured human placental and chorionic trophoblasts*. Biol Reprod 58:1379-84
591. **Pepe GJ, Albrecht ED** 1984 *Comparison of cortisol-cortisone interconversion in vitro by the human and baboon placenta*. Steroids 44:229-40
592. **Sun K, Yang K, Challis JR** 1997 *Differential regulation of 11 beta-hydroxysteroid dehydrogenase type 1 and 2 by nitric oxide in cultured human placental trophoblast and chorionic cell preparation*. Endocrinology 138:4912-20
593. **Pasquarette MM, Stewart PM, Ricketts ML, Imaishi K, Mason JI** 1996 *Regulation of 11 beta-hydroxysteroid dehydrogenase type 2 activity and mRNA in human choriocarcinoma cells*. J Mol Endocrinol 16:269-75
594. **Hardy DB, Pereria LE, Yang K** 1999 *Prostaglandins and leukotriene B4 are potent inhibitors of 11beta- hydroxysteroid dehydrogenase type 2 activity in human choriocarcinoma JEG-3 cells*. Biol Reprod 61:40-5
595. **Alfaidy N, Gupta S, DeMarco C, Caniggia I, Challis JR** 2002 *Oxygen regulation of placental 11 beta-hydroxysteroid dehydrogenase 2: physiological and pathological implications*. J Clin Endocrinol Metab 87:4797-805
596. **Hardy DB, Yang K** 2002 *The expression of 11 beta-hydroxysteroid dehydrogenase type 2 is induced during trophoblast differentiation: effects of hypoxia*. J Clin Endocrinol Metab 87:3696-701
597. **Hardy DB, Dixon SJ, Narayanan N, Yang K** 2001 *Calcium inhibits human placental 11beta-hydroxysteroid dehydrogenase type 2 activity*. Biochem Biophys Res Commun 283:756-61
598. **Sarkar S, Tsai SW, Nguyen TT, Plevyak M, Padbury JF, Rubin LP** 2001 *Inhibition of placental 11beta-hydroxysteroid dehydrogenase type 2 by catecholamines via alpha-adrenergic signaling*. Am J Physiol Regul Integr Comp Physiol 281:R1966-74
599. **Yang K, Hardy DB, Doumouras MA, van Beek JP, Rocha E** 2002 *ATP stimulates human placental 11beta-hydroxysteroid dehydrogenase type 2 activity by a novel mechanism independent of phosphorylation*. J Cell Biochem 84:295-300
600. **Tremblay J, Hardy DB, Pereira LE, Yang K** 1999 *Retinoic acid stimulates the expression of 11beta-hydroxysteroid dehydrogenase type 2 in human choriocarcinoma JEG-3 cells*. Biol Reprod 60:541-5
601. **Quinkler M, Johanssen S, Grossmann C, Bahr V, Muller M, Oelkers W, Diederich S** 1999 *Progesterone metabolism in the human kidney and inhibition of 11beta- hydroxysteroid dehydrogenase type 2 by progesterone and its metabolites*. J Clin Endocrinol Metab 84:4165-71
602. **Heiniger CD, Kostadinova RM, Rochat MK, Serra A, Ferrari P, Dick B, Frey BM, Frey FJ** 2003 *Hypoxia causes down-regulation of 11 beta-hydroxysteroid dehydrogenase type 2 by induction of Egr-1*. FASEB J 17:917-9
603. **Suzuki S, Koyama K, Darnel A, Ishibashi H, Kobayashi S, Kubo H, Suzuki T, Sasano H, Krozowski ZS** 2003 *Dexamethasone upregulates 11beta-hydroxysteroid dehydrogenase type 2 in BEAS-2B cells*. Am J Respir Crit Care Med 167:1244-9

604. **Cooper MS, Bujalska I, Rabbitt E, Walker EA, Bland R, Sheppard MC, Hewison M, Stewart PM** 2001 *Modulation of 11beta-hydroxysteroid dehydrogenase isozymes by proinflammatory cytokines in osteoblasts: an autocrine switch from glucocorticoid inactivation to activation*. J Bone Miner Res 16:1037-44
605. **Murphy BE** 1981 *Ontogeny of cortisol-cortisone interconversion in human tissues: a role for cortisone in human fetal development*. J Steroid Biochem 14:811-7
606. **Coulter CL, Smith RE, Stowasser M, Sasano H, Krozowski ZS, Gordon RD** 1999 *Expression of 11beta-hydroxysteroid dehydrogenase type 2 (11betaHSD-2) in the developing human adrenal gland and human adrenal cortical carcinoma and adenoma*. Mol Cell Endocrinol 154:71-7
607. **Stewart PM, Murry BA, Mason JI** 1994 *Type 2 11 beta-hydroxysteroid dehydrogenase in human fetal tissues*. J Clin Endocrinol Metab 78:1529-32
608. **Stewart PM, Whorwood CB, Mason JI** 1995 *Type 2 11 beta-hydroxysteroid dehydrogenase in foetal and adult life*. J Steroid Biochem Mol Biol 55:465-71
609. **Takahashi K, Sasano H, Fukushima K, Hirasawa G, Miura H, Sasaki I, Matsuno S, Krozowski ZS, Nagura H** 1998 *11 beta-hydroxysteroid dehydrogenase type II in human colon: a new marker of fetal development and differentiation in neoplasms*. Anticancer Res 18:3381-8
610. **Condon J, Gosden C, Gardener D, Nickson P, Hewison M, Howie AJ, Stewart PM** 1998 *Expression of type 2 11beta-hydroxysteroid dehydrogenase and corticosteroid hormone receptors in early human fetal life*. J Clin Endocrinol Metab 83:4490-7
611. **Hirasawa G, Sasano H, Suzuki T, Takeyama J, Muramatu Y, Fukushima K, Hiwatashi N, Toyota T, Nagura H, Krozowski ZS** 1999 *11Beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor in human fetal development*. J Clin Endocrinol Metab 84:1453-8
612. **Midgley PC, Holownia P, Smith J, Moore M, Russell K, Oates N, Shaw JC, Honour JW** 2001 *Plasma cortisol, cortisone and urinary glucocorticoid metabolites in preterm infants*. Biol Neonate 79:79-86
613. **Sara VR, Hall K, Misaki M, Fryklund L, Christensen N, Wetterberg L** 1983 *Ontogenesis of somatomedin and insulin receptors in the human fetus*. J Clin Invest 71:1084-94
614. **Shifren JL, Osathanondh R, Yeh J** 1993 *Human fetal ovaries and uteri: developmental expression of genes encoding the insulin, insulin-like growth factor I, and insulin-like growth factor II receptors*. Fertil Steril 59:1036-40
615. **Sara VR, Carlsson-Skwirut C** 1986 *The biosynthesis of somatomedins and their role in the fetus*. Acta Endocrinol Suppl (Copenh) 279:82-5
616. **Hill DJ, Crace CJ, Strain AJ, Milner RD** 1986 *Regulation of amino acid uptake and deoxyribonucleic acid synthesis in isolated human fetal fibroblasts and myoblasts: effect of human placental lactogen, somatomedin-C, multiplication-stimulating activity, and insulin*. J Clin Endocrinol Metab 62:753-60
617. **Han VK, Hill DJ, Strain AJ, Towle AC, Lauder JM, Underwood LE, D'Ercole AJ** 1987 *Identification of somatomedin/insulin-like growth factor immunoreactive cells in the human fetus*. Pediatr Res 22:245-9
618. **Mesiano S, Mellon SH, Jaffe RB** 1993 *Mitogenic action, regulation, and localization of insulin-like growth factors in the human fetal adrenal gland*. J Clin Endocrinol Metab 76:968-76

619. **D'Ercole AJ, Hill DJ, Strain AJ, Underwood LE** 1986 *Tissue and plasma somatomedin-C/insulin-like growth factor I concentrations in the human fetus during the first half of gestation*. Pediatr Res 20:253-5
620. **Scott J, Cowell J, Robertson ME, Priestley LM, Wadey R, Hopkins B, Pritchard J, Bell GI, Rall LB, Graham CF, et al.** 1985 *Insulin-like growth factor-II gene expression in Wilms' tumour and embryonic tissues*. Nature 317:260-2
621. **Hill DJ** 1990 *Relative abundance and molecular size of immunoreactive insulin-like growth factors I and II in human fetal tissues*. Early Hum Dev 21:49-58
622. **Han VK, D'Ercole AJ, Lund PK** 1987 *Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus*. Science 236:193-7
623. **Hill DJ, Clemmons DR, Wilson S, Han VK, Strain AJ, Milner RD** 1989 *Immunological distribution of one form of insulin-like growth factor (IGF)-binding protein and IGF peptides in human fetal tissues*. J Mol Endocrinol 2:31-8
624. **Pannier EM, Irwin JC, Giudice LC** 1994 *Insulin-like growth factor-binding proteins in the human fetus: tissue-specific protein secretion, immunologic characterization, and gene expression*. Am J Obstet Gynecol 171:746-52
625. **Han VK, Matsell DG, Delhanty PJ, Hill DJ, Shimasaki S, Nygard K** 1996 *IGF-binding protein mRNAs in the human fetus: tissue and cellular distribution of developmental expression*. Horm Res 45:160-6
626. **Karn MN, Penrose LS** 1951 *Birth weight and gestation time in relation to maternal age, parity and infant survival*. Ann Eugenics 16:147-64
627. **Thomas P, Peabody J, Turnier V, Clark RH** 2000 *A new look at intrauterine growth and the impact of race, altitude, and gender*. Pediatrics 106:E21
628. **Williams RL, Creasy RK, Cunningham GC, Hawes WE, Norris FD, Tashiro M** 1982 *Fetal growth and perinatal viability in California*. Obstet Gynecol 59:624-32
629. **Geary MP, Pringle PJ, Rodeck CH, Kingdom JC, Hindmarsh PC** 2003 *Sexual dimorphism in the growth hormone and insulin-like growth factor axis at birth*. J Clin Endocrinol Metab 88:3708-14
630. **Tamimi RM, Lagiou P, Mucci LA, Hsieh CC, Adami HO, Trichopoulos D** 2003 *Average energy intake among pregnant women carrying a boy compared with a girl*. BMJ 326:1245-6
631. **Wertelecki W, Hoff C, Zansky S** 1987 *Maternal smoking: greater effect on males, fetal tobacco syndrome?* Teratology 35:317-20
632. **Spinillo A, Capuzzo E, Nicola S, Colonna L, Iasci A, Zara C** 1994 *Interaction between fetal gender and risk factors for fetal growth retardation*. Am J Obstet Gynecol 171:1273-7
633. **Vik T, Bakketeig LS, Trygg KU, Lund-Larsen K, Jacobsen G** 2003 *High caffeine consumption in the third trimester of pregnancy: gender-specific effects on fetal growth*. Paediatr Perinat Epidemiol 17:324-31
634. **Montano MM, Wang MH, vom Saal FS** 1993 *Sex differences in plasma corticosterone in mouse fetuses are mediated by differential placental transport from the mother and eliminated by maternal adrenalectomy or stress*. J Reprod Fertil 99:283-90
635. **Gibson PG, Wilson AJ** 1996 *The use of continuous quality improvement methods to implement practice guidelines in asthma*. J Qual Clin Pract 16:87-102

636. **Woolcock A, Rubinfeld AR, Seale JP, Landau LL, Antic R, Mitchell C, Rea HH, Zimmerman P** 1989 *Thoracic society of Australia and New Zealand. Asthma management plan, 1989*. Med J Aust 151:650-3
637. 1996 *National Asthma Campaign: Asthma management handbook*. National Asthma Council Australia, Sydney
638. 1997 *National Institute of Health: Guidelines for the diagnosis and management of asthma*. National Heart, Lung and Blood Institute, Bethesda, MD
639. **Barnes NC, Marone G, Di Maria GU, Visser S, Utama I, Payne SL** 1993 *A comparison of fluticasone propionate, 1 mg daily, with beclomethasone dipropionate, 2 mg daily, in the treatment of severe asthma. International Study Group*. Eur Respir J 6:877-85
640. **Crane JP, Kopta MM** 1980 *Comparative newborn anthropometric data in symmetric versus asymmetric intrauterine growth retardation*. Am J Obstet Gynecol 138:518-22
641. **Kitchen WH, Robinson HP, Dickinson AJ** 1983 *Revised intrauterine growth curves for an Australian hospital population*. Aust Paediatr J 19:157-61
642. **Apgar V** 1953 *A proposal for a new method of evaluation of the newborn infant*. Curr Res Anesth Analg 32:260-7
643. **Casey BM, McIntire DD, Leveno KJ** 2001 *The continuing value of the Apgar score for the assessment of newborn infants*. N Engl J Med 344:467-71
644. **Bernal AL, Flint AP, Anderson AB, Turnbull AC** 1980 *11 beta-Hydroxysteroid dehydrogenase activity (E.C. 1.1.1.146) in human placenta and decidua*. J Steroid Biochem 13:1081-7
645. **Lakshmi V, Nath N, Muneyyirci-Delale O** 1993 *Characterization of 11 beta-hydroxysteroid dehydrogenase of human placenta: evidence for the existence of two species of 11 beta-hydroxysteroid dehydrogenase*. J Steroid Biochem Mol Biol 45:391-7
646. **Oldham KG** 1992 *Radiometric assays*. In: Eisenthal R, Danson MJ (eds) Enzyme assays: a practical approach. Oxford University Press, Oxford, pp 93-122
647. **Tipton KF** 1992 *Principles of enzyme assay and kinetic studies*. In: Eisenthal R, Danson MJ (eds) Enzyme assays: a practical approach. Oxford University Press, Oxford, pp 1-58
648. **Murphy BE** 1981 *Specificity of human 11 beta-hydroxysteroid dehydrogenase*. J Steroid Biochem 14:807-9
649. **Skoog DA, West DM, Holler FJ** 1996 *Fundamentals of analytical chemistry*, Seventh ed. Saunders College Publishing, Fort Worth
650. **Brady JE, Holum JR** 1993 *Chemistry: the study of matter and its changes*. John Wiley & Sons, Inc, New York
651. **Jones A, Reed R, Weyers J** 1994 *Practical skills in biology*. Longman Scientific & Technical, Essex
652. **Bradford MM** 1976 *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. Anal Biochem 72:248-54
653. **Stewart PM, Murry BA, Mason JI** 1994 *Human kidney 11 beta-hydroxysteroid dehydrogenase is a high affinity nicotinamide adenine dinucleotide-dependent enzyme and differs from the cloned type I isoform*. J Clin Endocrinol Metab 79:480-4

654. **Diederich S, Hanke B, Oelkers W, Bahr V** 1997 *Metabolism of dexamethasone in the human kidney: nicotinamide adenine dinucleotide-dependent 11beta-reduction*. J Clin Endocrinol Metab 82:1598-602
655. **Gomez-Sanchez EP, Ganjam V, Chen YJ, Liu Y, Clark SA, Gomez-Sanchez CE** 2001 *The 11beta hydroxysteroid dehydrogenase 2 exists as an inactive dimer*. Steroids 66:845-8
656. **Shaw DA, Quincey RV** 1963 *The preparation of tritium-labelled cortisol metabolites of high specific activity*. J Endocrinol 26:577-8
657. **Kurien BT, Scofield RH** 2003 *Protein blotting: a review*. J Immunol Methods 274:1-15
658. **Robinson PJ** 1992 *Differential stimulation of protein kinase C activity by phorbol ester or calcium/phosphatidylserine in vitro and in intact synaptosomes*. J Biol Chem 267:21637-44
659. **Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD** 1994 *Molecular biology of the cell*, Third ed. Garland Publishing Inc, New York
660. **Stryer L** 1995 *Biochemistry*, Fourth Edition ed. W.H. Freeman and Company, New York
661. **Gibson UE, Heid CA, Williams PM** 1996 *A novel method for real time quantitative RT-PCR*. Genome Res 6:995-1001
662. **Heid CA, Stevens J, Livak KJ, Williams PM** 1996 *Real time quantitative PCR*. Genome Res 6:986-94
663. **Wang T, Brown MJ** 1999 *mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection*. Anal Biochem 269:198-201
664. **Overbergh L, Valckx D, Waer M, Mathieu C** 1999 *Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR*. Cytokine 11:305-12
665. **Takahashi M, Funato T, Ishii KK, Kaku M, Sasaki T** 2001 *Measurement of tumor necrosis factor-alpha messenger RNA in synovial fibroblasts by real-time quantitative reverse transcriptase-polymerase chain reaction*. J Lab Clin Med 137:101-6
666. **Yin JL, Shackel NA, Zekry A, McGuinness PH, Richards C, Putten KV, McCaughan GW, Eris JM, Bishop GA** 2001 *Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I*. Immunol Cell Biol 79:213-21
667. **Ramos-Payan R, Aguilar-Medina M, Estrada-Parra S, Gonzalez YMJA, Favila-Castillo L, Monroy-Ostria A, Estrada-Garcia IC** 2003 *Quantification of cytokine gene expression using an economical real-time polymerase chain reaction method based on SYBR Green I*. Scand J Immunol 57:439-45
668. **Blaschke V, Reich K, Blaschke S, Zipprich S, Neumann C** 2000 *Rapid quantitation of proinflammatory and chemoattractant cytokine expression in small tissue samples and monocyte-derived dendritic cells: validation of a new real-time RT-PCR technology*. J Immunol Methods 246:79-90
669. **Johnson RF, Mitchell CM, Giles WB, Walters WA, Zakar T** 2002 *The in vivo control of prostaglandin H synthase-2 messenger ribonucleic acid expression in the human amnion at parturition*. J Clin Endocrinol Metab 87:2816-23
670. **Bustin SA** 2000 *Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays*. J Mol Endocrinol 25:169-93

671. **Pfaffl MW** 2001 *A new mathematical model for relative quantification in real-time RT-PCR*. Nucleic Acids Res 29:E45-5
672. **Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP** 1997 *Continuous fluorescence monitoring of rapid cycle DNA amplification*. Biotechniques 22:130-1, 134-8
673. **Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C** 2001 *An overview of real-time quantitative PCR: applications to quantify cytokine gene expression*. Methods 25:386-401
674. **Ginzinger DG** 2002 *Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream*. Exp Hematol 30:503-12
675. **Ririe KM, Rasmussen RP, Wittwer CT** 1997 *Product differentiation by analysis of DNA melting curves during the polymerase chain reaction*. Anal Biochem 245:154-60
676. **Ponte P, Ng SY, Engel J, Gunning P, Kedes L** 1984 *Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA*. Nucleic Acids Res 12:1687-96
677. **Johnson RF, Mitchell CM, Giles WB, Walters WA, Zakar T** 2003 *The control of prostaglandin endoperoxide H-Synthase-2 expression in the human chorion laeve at term*. J Soc Gynecol Investig 10:222-30
678. **Okamoto T, Okabe S** 2000 *Ultraviolet absorbance at 260 and 280 nm in RNA measurement is dependent on measurement solution*. Int J Mol Med 5:657-9
679. **Chard T** 1989 *An introduction to radioimmunoassay and related techniques*, Third ed. Elsevier, Amsterdam
680. **Smith R, Cubis J, Brinsmead M, Lewin T, Singh B, Owens P, Chan EC, Hall C, Adler R, Lovelock M, Hurt D, Rowley M, Nolan M** 1990 *Mood changes, obstetric experience and alterations in plasma cortisol, beta-endorphin and corticotrophin releasing hormone during pregnancy and the puerperium*. J Psychosom Res 34:53-69
681. **Smith R, Chan EC, Bowman ME, Harewood WJ, Phippard AF** 1993 *Corticotropin-releasing hormone in baboon pregnancy*. J Clin Endocrinol Metab 76:1063-8
682. **Burton P, Gurrin L, Sly P** 1998 *Extending the simple linear regression model to account for correlated responses: an introduction to generalized estimating equations and multi-level mixed modelling*. Stat Med 17:1261-91
683. **Senn S, Stevens L, Chaturvedi N** 2000 *Repeated measures in clinical trials: simple strategies for analysis using summary measures*. Stat Med 19:861-77
684. **Dupont WD, Plummer WD** 1997 *PS power and sample size program available for free on the internet*. Control Clin Trials 18:274
685. **Williams KL** 1999 *Genomes and proteomes: towards a multidimensional view of biology*. Electrophoresis 20:678-88
686. **Brewis IA** 1999 *Proteomics in reproductive research: the potential importance of proteomics to research in reproduction*. Hum Reprod 14:2927-9
687. **Gaston B** 2003 *Functional genomics and proteomics in control of breathing*. Respir Physiol Neurobiol 135:231-8
688. **Weinberger SR, Dalmasso EA, Fung ET** 2002 *Current achievements using ProteinChip Array technology*. Curr Opin Chem Biol 6:86-91
689. **Issaq HJ, Veenstra TD, Conrads TP, Felschow D** 2002 *The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification*. Biochem Biophys Res Commun 292:587-92

690. **Wulfkuhle JD, Liotta LA, Petricoin EF** 2003 *Proteomic applications for the early detection of cancer*. Nat Rev Cancer 3:267-75
691. **Merchant M, Weinberger SR** 2000 *Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry*. Electrophoresis 21:1164-77
692. **Caputo E, Moharram R, Martin BM** 2003 *Methods for on-chip protein analysis*. Anal Biochem 321:116-24
693. **Srinivas PR, Srivastava S, Hanash S, Wright GL, Jr.** 2001 *Proteomics in early detection of cancer*. Clin Chem 47:1901-11
694. **Wulfkuhle JD, Paweletz CP, Steeg PS, Petricoin EF, 3rd, Liotta L** 2003 *Proteomic approaches to the diagnosis, treatment, and monitoring of cancer*. Adv Exp Med Biol 532:59-68
695. **Paweletz CP, Gillespie JW, Ornstein DK, Simone NL, Brown MR, Cole KA, Wang QH, Huang J, Hu N, Yip TT, Rich WE, Kohn EC, Linehan WM, Weber T, Taylor P, Emmert-Buck MR, Liotta LA, Petricoin EF** 2000 *Rapid protein display profiling of cancer progression directly from human tissue using a protein biochip*. Drug Dev Res 49:34-42
696. **Petricoin EF, Paweletz CP, Liotta LA** 2002 *Clinical applications of proteomics: proteomic pattern diagnostics*. J Mammary Gland Biology and Neoplasia 7:433-40
697. **Romero R, Kuivaniemi H, Tromp G** 2002 *Functional genomics and proteomics in term and preterm parturition*. J Clin Endocrinol Metab 87:2431-4
698. **Page NM, Kemp CF, Butlin DJ, Lowry PJ** 2002 *Placental peptides as markers of gestational disease*. Reproduction 123:487-95
699. **Kalionis B, Moses E** 2003 *Advanced molecular techniques in pregnancy research: proteomics and genomics--a workshop report*. Placenta 24 Suppl A:S119-22
700. **Vuadens F, Benay C, Crettaz D, Gallot D, Sapin V, Schneider P, Bienvenut WV, Lemery D, Quadroni M, Dastugue B, Tissot JD** 2003 *Identification of biologic markers of the premature rupture of fetal membranes: proteomic approach*. Proteomics 3:1521-5
701. **Batorfi J, Ye B, Mok SC, Cseh I, Berkowitz RS, Fulop V** 2003 *Protein profiling of complete mole and normal placenta using ProteinChip analysis on laser capture microdissected cells*. Gynecol Oncol 88:424-8
702. **Westergren-Thorsson G, Malmstrom J, Marko-Varga G** 2001 *Proteomics -- the protein expression technology to study connective tissue biology*. J Pharm Biomed Anal 24:815-24
703. **Malmstrom J, Tufvesson E, Lofdahl CG, Hansson L, Marko-Varga G, Westergren-Thorsson G** 2003 *Activation of platelet-derived growth factor pathway in human asthmatic pulmonary-derived mesenchymal cells*. Electrophoresis 24:276-85
704. **Sasaki A, Liotta AS, Luckey MM, Margioris AN, Suda T, Krieger DT** 1984 *Immunoreactive corticotropin-releasing factor is present in human maternal plasma during the third trimester of pregnancy*. J Clin Endocrinol Metab 59:812-4
705. **Goland RS, Wardlaw SL, Stark RI, Brown LS, Jr., Frantz AG** 1986 *High levels of corticotropin-releasing hormone immunoactivity in maternal and fetal plasma during pregnancy*. J Clin Endocrinol Metab 63:1199-203

706. **Campbell S, Thoms A** 1977 *Ultrasound measurement of the fetal head to abdomen circumference ratio in the assessment of growth retardation*. Br J Obstet Gynaecol 84:165-74
707. **Crane JP, Kopta MM** 1979 *Prediction of intrauterine growth retardation via ultrasonically measured head/abdominal circumference ratios*. Obstet Gynecol 54:597-601
708. **McLean M, Bisits A, Davies J, Woods R, Lowry P, Smith R** 1995 *A placental clock controlling the length of human pregnancy*. Nat Med 1:460-3
709. **Martin AJ, McLennan LA, Landau LI, Phelan PD** 1980 *The natural history of childhood asthma to adult life*. Br Med J 280:1397-1400
710. **Panhuysen CI, Vonk JM, Koeter GH, Schouten JP, van Altena R, Bleecker ER, Postma DS** 1997 *Adult patients may outgrow their asthma: a 25-year follow-up study*. Am J Respir Crit Care Med 155:1267-72
711. **Ronmark E, Jonsson E, Lundback B** 1999 *Remission of asthma in the middle aged and elderly: report from the Obstructive Lung Disease in Northern Sweden study*. Thorax 54:611-3
712. **De Marco R, Locatelli F, Cerveri I, Bugiani M, Marinoni A, Giammanco G** 2002 *Incidence and remission of asthma: a retrospective study on the natural history of asthma in Italy*. J Allergy Clin Immunol 110:228-35
713. **Charlton I, Charlton G, Broomfield J, Mullee MA** 1990 *Evaluation of peak flow and symptoms only self management plans for control of asthma in general practice*. BMJ 301:1355-9
714. **Bolton MB, Tilley BC, Kuder J, Reeves T, Schultz LR** 1991 *The cost and effectiveness of an education program for adults who have asthma*. J Gen Intern Med 6:401-7
715. **Yoon R, McKenzie DK, Bauman A, Miles DA** 1993 *Controlled trial evaluation of an asthma education programme for adults*. Thorax 48:1110-6
716. **Osman LM, Abdalla MI, Beattie JA, Ross SJ, Russell IT, Friend JA, Legge JS, Douglas JG** 1994 *Reducing hospital admission through computer supported education for asthma patients. Grampian Asthma Study of Integrated Care (GRASSIC)*. BMJ 308:568-71
717. **Sprietsma JE** 1999 *Modern diets and diseases: NO-zinc balance. Under Th1, zinc and nitrogen monoxide (NO) collectively protect against viruses, AIDS, autoimmunity, diabetes, allergies, asthma, infectious diseases, atherosclerosis and cancer*. Med Hypotheses 53:6-16
718. **Patak EN, Pennefather JN, Story ME** 2000 *Effects of tachykinins on uterine smooth muscle*. Clin Exp Pharmacol Physiol 27:922-7
719. **Patak EN, Ziccone S, Story ME, Fleming AJ, Lilley A, Pennefather JN** 2000 *Activation of neurokinin NK(2) receptors by tachykinin peptides causes contraction of uterus in pregnant women near term*. Mol Hum Reprod 6:549-54
720. **Vachier I, Chanez P, Le Doucen C, Damon M, Descomps B, Godard P** 1994 *Enhancement of reactive oxygen species formation in stable and unstable asthmatic patients*. Eur Respir J 7:1585-92
721. **Vachier I, Damon M, Le Doucen C, de Paulet AC, Chanez P, Michel FB, Godard P** 1992 *Increased oxygen species generation in blood monocytes of asthmatic patients*. Am Rev Respir Dis 146:1161-6
722. **Kinnula VL, Crapo JD** 2003 *Superoxide dismutases in the lung and human lung diseases*. Am J Respir Crit Care Med 167:1600-19

723. **Wagner PD, Hedenstierna G, Rodriguez-Roisin R** 1996 *Gas exchange, expiratory flow obstruction and the clinical spectrum of asthma*. Eur Respir J 9:1278-82
724. **Valente S, De Rosa M, Corbo GM, Carlucci A, Fumagalli G, Ciappi G** 1998 *Slow compartment features and gas exchange impairment in mild asthma: effects of beta agonist inhalation*. Respiration 65:40-8
725. **Gries RE, Brooks LJ** 1996 *Normal oxyhemoglobin saturation during sleep. How low does it go?* Chest 110:1489-92
726. **Noma T, Ichikawa K, Yoshizawa I, Aoki K, Kawano Y, Baba M** 1998 *Reduced IL-1 production in adolescents with mite antigen asthma in remission*. Clin Exp Immunol 113:10-6
727. **Noma T, Hayashi M, Kawano Y, Yoshizawa I, Ishikawa Y, Saeki T, Aoki K, Matsuura N** 1999 *Functional interleukin-5 activity in peripheral blood mononuclear cells from adolescents with mite antigen asthma in remission*. Clin Exp Allergy 29:780-5
728. **Boulet LP, Turcotte H, Brochu A** 1994 *Persistence of airway obstruction and hyperresponsiveness in subjects with asthma remission*. Chest 105:1024-31
729. **Obase Y, Shimoda T, Kawano T, Saeki S, Tomari S, Izaki K, Fukushima C, Matsuse H, Kohno S** 2003 *Bronchial hyperresponsiveness and airway inflammation in adolescents with asymptomatic childhood asthma*. Allergy 58:213-20
730. **van Den Toorn LM, Prins JB, Overbeek SE, Hoogsteden HC, de Jongste JC** 2000 *Adolescents in clinical remission of atopic asthma have elevated exhaled nitric oxide levels and bronchial hyperresponsiveness*. Am J Respir Crit Care Med 162:953-7
731. **van den Toorn LM, Overbeek SE, de Jongste JC, Leman K, Hoogsteden HC, Prins JB** 2001 *Airway inflammation is present during clinical remission of atopic asthma*. Am J Respir Crit Care Med 164:2107-13
732. **Gervasi MT, Chaiworapongsa T, Naccasha N, Blackwell S, Yoon BH, Maymon E, Romero R** 2001 *Phenotypic and metabolic characteristics of maternal monocytes and granulocytes in preterm labor with intact membranes*. Am J Obstet Gynecol 185:1124-9
733. **Gervasi MT, Chaiworapongsa T, Pacora P, Naccasha N, Yoon BH, Maymon E, Romero R** 2001 *Phenotypic and metabolic characteristics of monocytes and granulocytes in preeclampsia*. Am J Obstet Gynecol 185:792-7
734. **Burke LA, Wilkinson JR, Howell CJ, Lee TH** 1991 *Interactions of macrophages and monocytes with granulocytes in asthma*. Eur Respir J Suppl 13:85s-90s
735. **Mazzarella G, Grella E, D'Auria D, Paciocco G, Perna F, Petillo O, Peluso G** 2000 *Phenotypic features of alveolar monocytes/macrophages and IL-8 gene activation by IL-1 and TNF-alpha in asthmatic patients*. Allergy 55 Suppl 61:36-41
736. **Tang C, Rolland JM, Li X, Ward C, Bish R, Walters EH** 1998 *Alveolar macrophages from atopic asthmatics, but not atopic nonasthmatics, enhance interleukin-5 production by CD4+ T cells*. Am J Respir Crit Care Med 157:1120-6
737. **Tang C, Rolland JM, Ward C, Thien F, Li X, Gollant S, Walters EH** 1998 *Differential regulation of allergen-specific T(H2)- but not T(H1)-type responses by alveolar macrophages in atopic asthma*. J Allergy Clin Immunol 102:368-75

738. **Zhu YK, Liu X, Wang H, Kohyama T, Wen FQ, Skold CM, Rennard SI** 2001 *Interactions between monocytes and smooth-muscle cells can lead to extracellular matrix degradation*. J Allergy Clin Immunol 108:989-96
739. **Borish L, Mascali JJ, Rosenwasser LJ** 1991 *IgE-dependent cytokine production by human peripheral blood mononuclear phagocytes*. J Immunol 146:63-7
740. **Ackerman V, Marini M, Vittori E, Bellini A, Vassali G, Mattoli S** 1994 *Detection of cytokines and their cell sources in bronchial biopsy specimens from asthmatic patients. Relationship to atopic status, symptoms, and level of airway hyperresponsiveness*. Chest 105:687-96
741. **Lim S, John M, Seybold J, Taylor D, Witt C, Barnes PJ, Chung KF** 2000 *Increased interleukin-10 and macrophage inflammatory protein-1 alpha release from blood monocytes ex vivo during late-phase response to allergen in asthma*. Allergy 55:489-95
742. **Mathy NL, Scheuer W, Lanzendorfer M, Honold K, Ambrosius D, Norley S, Kurth R** 2000 *Interleukin-16 stimulates the expression and production of pro-inflammatory cytokines by human monocytes*. Immunology 100:63-9
743. **Dayyani F, Belge KU, Frankenberger M, Mack M, Berki T, Ziegler-Heitbrock L** 2003 *Mechanism of glucocorticoid-induced depletion of human CD14+CD16+ monocytes*. J Leukoc Biol 74:33-9
744. **Gin W, Shaw RJ, Kay AB** 1985 *Airways reversibility after prednisolone therapy in chronic asthma is associated with alterations in leukocyte function*. Am Rev Respir Dis 132:1199-203
745. **Gin W, Kay AB** 1985 *The effect of corticosteroids on monocyte and neutrophil activation in bronchial asthma*. J Allergy Clin Immunol 76:675-82
746. **Gosset P, Lamblin-Degros C, Tillie-Leblond I, Charbonnier AS, Joseph M, Wallaert B, Kochan JP, Tonnel AB** 2001 *Modulation of high-affinity IgE receptor expression in blood monocytes: opposite effect of IL-4 and glucocorticoids*. J Allergy Clin Immunol 107:114-22
747. **Terpigorev SA, Il'chenko VA, Vasilenko IA, Slinchenko OI, Stotskaya TV, Markina TR, Paleev NR** 2003 *Correlation between the results of glucocorticoid therapy and in vitro effect of glucocorticoids on monocytes in asthma*. Bull Exp Biol Med 135:582-5
748. **Larsson S, Lofdahl CG, Linden M** 1999 *IL-2 and IL-4 counteract budesonide inhibition of GM-CSF and IL-10, but not of IL-8, IL-12 or TNF-alpha production by human mononuclear blood cells*. Br J Pharmacol 127:980-6
749. **Steer JH, Ma DT, Dusci L, Garas G, Pedersen KE, Joyce DA** 1998 *Altered leucocyte trafficking and suppressed tumour necrosis factor alpha release from peripheral blood monocytes after intra-articular glucocorticoid treatment*. Ann Rheum Dis 57:732-7
750. **Brohee D, Vanhaeverbeek M, Kennes B, Neve P** 1990 *Leukocyte and lymphocyte subsets after a short pharmacological stress by intravenous epinephrine and hydrocortisone in healthy humans*. Int J Neurosci 53:53-62
751. **Fauci AS** 1976 *Mechanisms of corticosteroid action on lymphocyte subpopulations. II. Differential effects of in vivo hydrocortisone, prednisone and dexamethasone on in vitro expression of lymphocyte function*. Clin Exp Immunol 24:54-62
752. **Norris DA, Fine R, Weston WL, Spector S** 1978 *Monocyte cellular function in asthmatic patients on alternate-day steroid therapy*. J Allergy Clin Immunol 61:255-60

753. **Louis R, Bury T, Corhay JL, Radermecker MF** 1994 *Acute bronchial and hematologic effects following inhalation of a single dose of PAF. Comparison between asthmatics and normal subjects*. Chest 106:1094-9
754. **Chang AB, Harrhy VA, Simpson J, Masters IB, Gibson PG** 2002 *Cough, airway inflammation, and mild asthma exacerbation*. Arch Dis Child 86:270-5
755. **Turner MO, Hussack P, Sears MR, Dolovich J, Hargreave FE** 1995 *Exacerbations of asthma without sputum eosinophilia*. Thorax 50:1057-61
756. **Gibson PG, Simpson JL, Saltos N** 2001 *Heterogeneity of airway inflammation in persistent asthma : evidence of neutrophilic inflammation and increased sputum interleukin-8*. Chest 119:1329-36
757. **Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA** 1996 *Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum*. Proc Natl Acad Sci U S A 93:705-8
758. **Ng EK, Tsui NB, Lau TK, Leung TN, Chiu RW, Panesar NS, Lit LC, Chan KW, Lo YM** 2003 *mRNA of placental origin is readily detectable in maternal plasma*. Proc Natl Acad Sci U S A 100:4748-53
759. **Thompson RS, Trudinger BJ, Cook CM, Giles WB** 1988 *Umbilical artery velocity waveforms: normal reference values for A/B ratio and Pourcelot ratio*. Br J Obstet Gynaecol 95:589-91
760. **Hendricks SK, Sorensen TK, Wang KY, Bushnell JM, Seguin EM, Zingheim RW** 1989 *Doppler umbilical artery waveform indices--normal values from fourteen to forty-two weeks*. Am J Obstet Gynecol 161:761-5
761. **Benediktsson R, Magnusdottir EM, Seckl JR** 1997 *Lack of effect of nicotine or ethanol on the activity of 11beta-hydroxysteroid dehydrogenase type 2*. J Steroid Biochem Mol Biol 63:303-7
762. **Robinson BG, Emanuel RL, Frim DM, Majzoub JA** 1988 *Glucocorticoid stimulates expression of corticotropin-releasing hormone gene in human placenta*. Proc Natl Acad Sci U S A 85:5244-8
763. **Cheng YH, Nicholson RC, King B, Chan EC, Fitter JT, Smith R** 2000 *Glucocorticoid stimulation of corticotropin-releasing hormone gene expression requires a cyclic adenosine 3',5'-monophosphate regulatory element in human primary placental cytotrophoblast cells*. J Clin Endocrinol Metab 85:1937-45
764. **Bamberger CM, Bamberger AM, de Castro M, Chrousos GP** 1995 *Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans*. J Clin Invest 95:2435-41
765. **Fleisher B, Kulovich MV, Hallman M, Gluck L** 1985 *Lung profile: sex differences in normal pregnancy*. Obstet Gynecol 66:327-30
766. 1981 *Effect of antenatal dexamethasone administration on the prevention of respiratory distress syndrome*. Am J Obstet Gynecol 141:276-87
767. **Leung DY, Hamid Q, Vottero A, Szefler SJ, Surs W, Minshall E, Chrousos GP, Klemm DJ** 1997 *Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta*. J Exp Med 186:1567-74
768. **Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L, Kiessling R, Jornvall H, Wigzell H, Gudmundsson GH** 2000 *The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations*. Blood 96:3086-93
769. **Zhang L, Yu W, He T, Yu J, Caffrey RE, Dalmasso EA, Fu S, Pham T, Mei J, Ho JJ, Zhang W, Lopez P, Ho DD** 2002 *Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor*. Science 298:995-1000

770. **Chaly YV, Paleolog EM, Kolesnikova TS, Tikhonov, II, Petratchenko EV, Voitenok NN** 2000 *Neutrophil alpha-defensin human neutrophil peptide modulates cytokine production in human monocytes and adhesion molecule expression in endothelial cells*. Eur Cytokine Netw 11:257-66
771. **Territo MC, Ganz T, Selsted ME, Lehrer R** 1989 *Monocyte-chemotactic activity of defensins from human neutrophils*. J Clin Invest 84:2017-20
772. **Svinarich DM, Gomez R, Romero R** 1997 *Detection of human defensins in the placenta*. Am J Reprod Immunol 38:252-5
773. **Brown PH, Blundell G, Greening AP, Crompton GK** 1991 *Hypothalamo-pituitary-adrenal axis suppression in asthmatics inhaling high dose corticosteroids*. Respir Med 85:501-10
774. **Geddes DM** 1992 *Inhaled corticosteroids: benefits and risks*. Thorax 47:404-7
775. **Grebe SK, Feek CM, Durham JA, Kljakovic M, Cooke RR** 1997 *Inhaled beclomethasone dipropionate suppresses the hypothalamo-pituitary-adrenal axis in a dose dependent manner*. Clin Endocrinol (Oxf) 47:297-304
776. **Israel E, Banerjee TR, Fitzmaurice GM, Kotlov TV, LaHive K, LeBoff MS** 2001 *Effects of inhaled glucocorticoids on bone density in premenopausal women*. N Engl J Med 345:941-7
777. **Marinoni E, Korebrits C, Di Iorio R, Cosmi EV, Challis JR** 1998 *Effect of betamethasone in vivo on placental corticotropin-releasing hormone in human pregnancy*. Am J Obstet Gynecol 178:770-8
778. **Raven PW, Taylor NF** 1996 *Sex differences in the human metabolism of cortisol*. Endocr Res 22:751-5
779. **Raven PW, Taylor NF** 1998 *11beta-HSD and 17beta-HSD as biological markers of depression: sex differences and correlation with symptom severity*. Endocr Res 24:659-62
780. **Weaver JU, Taylor NF, Monson JP, Wood PJ, Kelly WF** 1998 *Sexual dimorphism in 11 beta hydroxysteroid dehydrogenase activity and its relation to fat distribution and insulin sensitivity; a study in hypopituitary subjects*. Clin Endocrinol (Oxf) 49:13-20
781. **Condon J, Ricketts ML, Whorwood CB, Stewart PM** 1997 *Ontogeny and sexual dimorphic expression of mouse type 2 11beta-hydroxysteroid dehydrogenase*. Mol Cell Endocrinol 127:121-8
782. **Rivera DL, Olister SM, Liu X, Thompson JH, Zhang XJ, Pennline K, Azuero R, Clark DA, Miller MJ** 1998 *Interleukin-10 attenuates experimental fetal growth restriction and demise*. FASEB J 12:189-97
783. **Daynes RA, Araneo BA** 1989 *Contrasting effects of glucocorticoids on the capacity of T cells to produce the growth factors interleukin 2 and interleukin 4*. Eur J Immunol 19:2319-25
784. **Ramirez F, Fowell DJ, Puklavec M, Simmonds S, Mason D** 1996 *Glucocorticoids promote a TH2 cytokine response by $CD4^+$ T cells in vitro*. J Immunol 156:2406-12
785. **Ramirez F** 1998 *Glucocorticoids induce a Th2 response in vitro*. Dev Immunol 6:233-43
786. **DeKruyff RH, Fang Y, Umetsu DT** 1998 *Corticosteroids enhance the capacity of macrophages to induce Th2 cytokine synthesis in $CD4^+$ lymphocytes by inhibiting IL-12 production*. J Immunol 160:2231-7
787. **Visser J, van Boxel-Dezaire A, Methorst D, Brunt T, de Kloet ER, Nagelkerken L** 1998 *Differential regulation of interleukin-10 (IL-10) and IL-12 by glucocorticoids in vitro*. Blood 91:4255-64

788. **Agarwal SK, Marshall GD, Jr.** 2001 *Dexamethasone promotes type 2 cytokine production primarily through inhibition of type 1 cytokines*. J Interferon Cytokine Res 21:147-55
789. **Blotta MH, DeKruyff RH, Umetsu DT** 1997 *Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4+ lymphocytes*. J Immunol 158:5589-95
790. **Hennebold JD, Mu HH, Poynter ME, Chen XP, Daynes RA** 1997 *Active catabolism of glucocorticoids by 11 beta-hydroxysteroid dehydrogenase in vivo is a necessary requirement for natural resistance to infection with Listeria monocytogenes*. Int Immunol 9:105-15
791. **von Hertzen LC** 2002 *Maternal stress and T-cell differentiation of the developing immune system: possible implications for the development of asthma and atopy*. J Allergy Clin Immunol 109:923-8
792. **Kuehr J, Karmaus W, Forster J, Frischer T, Hendel-Kramer A, Moseler M, Stephan V, Urbanek R, Weiss K** 1993 *Sensitization to four common inhalant allergens within 302 nuclear families*. Clin Exp Allergy 23:600-5
793. **Prescott SL, Holt PG** 1998 *Abnormalities in cord blood mononuclear cytokine production as a predictor of later atopic disease in childhood*. Clin Exp Allergy 28:1313-6
794. **Warner JA** 1999 *Primary sensitization in infants*. Ann Allergy Asthma Immunol 83:426-30
795. **Williams TJ, Jones CA, Miles EA, Warner JO, Warner JA** 2000 *Fetal and neonatal IL-13 production during pregnancy and at birth and subsequent development of atopic symptoms*. J Allergy Clin Immunol 105:951-9
796. **Benyo DF, Miles TM, Conrad KP** 1997 *Hypoxia stimulates cytokine production by villous explants from the human placenta*. J Clin Endocrinol Metab 82:1582-8
797. **Pierce BT, Napolitano PG, Pierce LM, Apodaca CC, Hume RF, Jr., Calhoun BC** 2001 *The effects of hypoxia and hyperoxia on fetal-placental vascular tone and inflammatory cytokine production*. Am J Obstet Gynecol 185:1068-72
798. **Mollers M, Aries SP, Dromann D, Mascher B, Braun J, Dalhoff K** 2001 *Intracellular cytokine repertoire in different T cell subsets from patients with sarcoidosis*. Thorax 56:487-93
799. **Clark DA, Croitoru K** 2001 *TH1/TH2,3 imbalance due to cytokine-producing NK, gammadelta T and NK-gammadelta T cells in murine pregnancy decidua in success or failure of pregnancy*. Am J Reprod Immunol 45:257-65
800. **Kwak-Kim JY, Chung-Bang HS, Ng SC, Ntrivalas EI, Mangubat CP, Beaman KD, Beer AE, Gilman-Sachs A** 2003 *Increased T helper 1 cytokine responses by circulating T cells are present in women with recurrent pregnancy losses and in infertile women with multiple implantation failures after IVF*. Hum Reprod 18:767-73
801. **Reuben JM, Turpin JA, Lee BN, Doyle M, Gonik B, Jacobson R, Shearer WT** 1996 *Induction of inflammatory cytokines in placental monocytes of gravidae infected with the human immunodeficiency virus type 1*. J Interferon Cytokine Res 16:963-71
802. **Melendez J, Garcia V, Sanchez E, Delgado R, Torres G, Melendez-Guerrero LM** 2001 *Is decreased HIV-1 infectivity of placental macrophages caused by high levels of beta-chemokines?* Cell Mol Biol (Noisy-le-grand) 47 Online Pub:OL51-9

803. **de Moraes-Pinto MI, Vince GS, Flanagan BF, Hart CA, Johnson PM** 1997 *Localization of IL-4 and IL-4 receptors in the human term placenta, decidua and amniochorionic membranes*. Immunology 90:87-94
804. **Dealtry GB, O'Farrell MK, Fernandez N** 2000 *The Th2 cytokine environment of the placenta*. Int Arch Allergy Immunol 123:107-19
805. **Okret S, Poellinger L, Dong Y, Gustafsson JA** 1986 *Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone*. Proc Natl Acad Sci U S A 83:5899-903
806. **Dong Y, Poellinger L, Gustafsson JA, Okret S** 1988 *Regulation of glucocorticoid receptor expression: evidence for transcriptional and posttranslational mechanisms*. Mol Endocrinol 2:1256-64
807. **Silva CM, Powell-Oliver FE, Jewell CM, Sar M, Allgood VE, Cidlowski JA** 1994 *Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoid*. Steroids 59:436-42
808. **Shimojo M, Hiroi N, Yakushiji F, Ueshiba H, Yamaguchi N, Miyachi Y** 1995 *Differences in down-regulation of glucocorticoid receptor mRNA by cortisol, prednisolone and dexamethasone in HeLa cells*. Endocr J 42:629-36
809. **Knutsson PU, Bronnegard M, Marcus C, Stierna P** 1996 *Regulation of glucocorticoid receptor mRNA in nasal mucosa by local administration of fluticasone and budesonide*. J Allergy Clin Immunol 97:655-61
810. **Korn SH, Wouters EF, Wesseling G, Arends JW, Thunnissen FB** 1997 *In vitro and in vivo modulation of alpha- and beta-glucocorticoid-receptor mRNA in human bronchial epithelium*. Am J Respir Crit Care Med 155:1117-22
811. **Korn SH, Wouters EF, Wesseling G, Arends JW, Thunnissen FB** 1998 *Interaction between glucocorticoids and beta2-agonists: alpha and beta glucocorticoid-receptor mRNA expression in human bronchial epithelial cells*. Biochem Pharmacol 56:1561-9
812. **Vachier I, Roux S, Chanez P, Loubatiere J, Terouanne B, Nicolas JC, Godard P** 1996 *Glucocorticoids induced down-regulation of glucocorticoid receptor mRNA expression in asthma*. Clin Exp Immunol 103:311-5
813. **Hagley RD, Watlington CO** 1996 *Down-regulation of the mineralocorticoid receptor by dexamethasone in an amphibian kidney cell line (A6)*. Endocr Res 22:221-35
814. **Jenq W, Rabb H, Wahe M, Ramirez G** 1996 *Hypoxic effects on the expression of mineralocorticoid and glucocorticoid receptors in human renal cortex epithelial cells*. Biochem Biophys Res Commun 218:444-8
815. **Heine RP, Wiesenfeld H, Mortimer L, Greig PC** 1998 *Amniotic fluid defensins: potential markers of subclinical intrauterine infection*. Clin Infect Dis 27:513-8
816. **Balu RB, Savitz DA, Ananth CV, Hartmann KE, Miller WC, Thorp JM, Heine RP** 2002 *Bacterial vaginosis and vaginal fluid defensins during pregnancy*. Am J Obstet Gynecol 187:1267-71
817. **Balu RB, Savitz DA, Ananth CV, Hartmann KE, Miller WC, Thorp JM, Heine RP** 2003 *Bacterial vaginosis, vaginal fluid neutrophil defensins, and preterm birth*. Obstet Gynecol 101:862-8
818. **Soong LB, Ganz T, Ellison A, Caughey GH** 1997 *Purification and characterization of defensins from cystic fibrosis sputum*. Inflamm Res 46:98-102

819. **Siiteri PK, MacDonald PC** 1966 *Placental estrogen biosynthesis during human pregnancy*. J Clin Endocrinol Metab 26:751-61
820. **Tulchinsky D** 1973 *Placental secretion of unconjugated estrone, estradiol and estriol into the maternal and the fetal circulation*. J Clin Endocrinol Metab 36:1079-87
821. **Peter M, Dorr HG, Sippell WG** 1994 *Changes in the concentrations of dehydroepiandrosterone sulfate and estriol in maternal plasma during pregnancy: a longitudinal study in healthy women throughout gestation and at term*. Horm Res 42:278-81
822. **Yaron Y, Cherry M, Kramer RL, O'Brien JE, Hallak M, Johnson MP, Evans MI** 1999 *Second-trimester maternal serum marker screening: maternal serum alpha-fetoprotein, beta-human chorionic gonadotropin, estriol, and their various combinations as predictors of pregnancy outcome*. Am J Obstet Gynecol 181:968-74
823. **Ohrlander SA, Gennser GM, Grennert L** 1975 *Impact of betamethasone load given to pregnant women on endocrine balance of fetoplacental unit*. Am J Obstet Gynecol 123:228-36
824. **Hendershott CM, Dullien V, Goodwin TM** 1999 *Serial betamethasone administration: effect on maternal salivary estriol levels*. Am J Obstet Gynecol 180:S219-22
825. **Rajaram S, Baylink DJ, Mohan S** 1997 *Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions*. Endocr Rev 18:801-31
826. **Falkenberg ER, Davis RO, DuBard M, Parker CR, Jr.** 1999 *Effects of maternal infections on fetal adrenal steroid production*. Endocr Res 25:239-49
827. **Gravett MG, Hitti J, Hess DL, Eschenbach DA** 2000 *Intrauterine infection and preterm delivery: evidence for activation of the fetal hypothalamic-pituitary-adrenal axis*. Am J Obstet Gynecol 182:1404-13
828. **Goldkrand JW** 1978 *Unconjugated estriol and cortisol in maternal and cord serum and amniotic fluid in normal and abnormal pregnancy*. Obstet Gynecol 52:264-71
829. **Lechner W, Heim K, Zech J, Daxenbichler G, Marth C** 1987 *The relation between saliva estriol levels in pregnancy and infant birth weight*. Arch Gynecol Obstet 241:9-12
830. **Parker CR, Jr., Buchina ES, Barefoot TK** 1994 *Abnormal adrenal steroidogenesis in growth-retarded newborn infants*. Pediatr Res 35:633-6
831. **Parker CR, Jr., Atkinson MW, Owen J, Andrews WW** 1996 *Dynamics of the fetal adrenal, cholesterol, and apolipoprotein B responses to antenatal betamethasone therapy*. Am J Obstet Gynecol 174:562-5
832. **Tazuke SI, Mazure NM, Sugawara J, Carland G, Faessen GH, Suen LF, Irwin JC, Powell DR, Giaccia AJ, Giudice LC** 1998 *Hypoxia stimulates insulin-like growth factor binding protein 1 (IGFBP-1) gene expression in HepG2 cells: a possible model for IGFBP-1 expression in fetal hypoxia*. Proc Natl Acad Sci U S A 95:10188-93
833. **Verhaeghe J, Billen J, Giudice LC** 2001 *Insulin-like growth factor-binding protein-1 in umbilical artery and vein of term fetuses with signs suggestive of distress during labor*. J Endocrinol 170:585-90
834. **Popovici RM, Lu M, Bhatia S, Faessen GH, Giaccia AJ, Giudice LC** 2001 *Hypoxia regulates insulin-like growth factor-binding protein 1 in human fetal hepatocytes in primary culture: suggestive molecular mechanisms for in utero*

fetal growth restriction caused by uteroplacental insufficiency. J Clin Endocrinol Metab 86:2653-9

835. **Cianfarani S, Germani D, Rossi L, Argiro G, Boemi S, Lemon M, Holly JM, Branca F** 1998 *IGF-I and IGF-binding protein-1 are related to cortisol in human cord blood*. Eur J Endocrinol 138:524-9
836. **Conover CA, Divertie GD, Lee PD** 1993 *Cortisol increases plasma insulin-like growth factor binding protein-1 in humans*. Acta Endocrinol (Copenh) 128:140-3
837. **Hills FA, Crawford R, Harding S, Farkas A, Chard T** 1994 *The effects of labor on maternal and fetal levels of insulin-like growth factor binding protein-1*. Am J Obstet Gynecol 171:1292-5
838. **Ogawa K, Kaminuma O, Kikkawa H, Akiyama K, Mori A** 2003 *Interaction with monocytes enhances IL-5 gene transcription in peripheral T cells of asthmatic patients*. Int Arch Allergy Immunol 131 Suppl 1:20-5
839. **Rogerson SJ, Pollina E, Getachew A, Tadesse E, Lema VM, Molyneux ME** 2003 *Placental monocyte infiltrates in response to Plasmodium falciparum malaria infection and their association with adverse pregnancy outcomes*. Am J Trop Med Hyg 68:115-9
840. **Edwards A, Megens A, Peek M, Wallace EM** 2000 *Sexual origins of placental dysfunction*. Lancet 355:203-4
841. **Wen SW, Demissie K, Liu S, Marcoux S, Kramer MS** 2000 *Placenta praevia and male sex at birth: results from a population-based study*. Paediatr Perinat Epidemiol 14:300-4
842. **Eogan MA, Geary MP, O'Connell MP, Keane DP** 2003 *Effect of fetal sex on labour and delivery: retrospective review*. BMJ 326:137
843. **Abramovich DR, Rowe P** 1973 *Foetal plasma testosterone levels at mid-pregnancy and at term: relationship to foetal sex*. J Endocrinol 56:621-2
844. **Reyes FI, Boroditsky RS, Winter JS, Faiman C** 1974 *Studies on human sexual development. II. Fetal and maternal serum gonadotropin and sex steroid concentrations*. J Clin Endocrinol Metab 38:612-7
845. **Forest MG, Ances IG, Tapper AJ, Migeon CJ** 1971 *Percentage binding of testosterone, androstendione and dihydroisoandrosterone in plasma at the time of delivery*. J Clin Endocrinol Metab 32:417-25
846. **Ketupanya A, Wiest WG** 1978 *Amniotic fluid testosterone concentration as an index of fetal sex*. Pediatr Res 12:708-10
847. **Robinson JD, Judd HL, Young PE, Jones OW, Yen SS** 1977 *Amniotic fluid androgens and estrogens in midgestation*. J Clin Endocrinol Metab 45:755-61
848. **Belisle S, Fencl MM, Tulchinsky D** 1977 *Amniotic fluid testosterone and follicle-stimulating hormone in the determination of fetal sex*. Am J Obstet Gynecol 128:514-9
849. **Forest MG, de Peretti E, Lecoq A, Cadillon E, Zabot MT, Thoulon JM** 1980 *Concentration of 14 steroid hormones in human amniotic fluid of midpregnancy*. J Clin Endocrinol Metab 51:816-22
850. **Hercz P, Kazy Z, Siklos P, Ungar L** 1989 *Quantitative comparison of serum steroid and peptide hormone concentrations in male and female fetuses in the maternal-fetoplacental system during the 28th-40th weeks of pregnancy*. Eur J Obstet Gynecol Reprod Biol 30:201-4
851. **Riley SC, Leask R, Balfour C, Brennand JE, Groome NP** 2000 *Production of inhibin forms by the fetal membranes, decidua, placenta and fetus at parturition*. Hum Reprod 15:578-83

852. **Haning RV, Jr., Breault PH, DeSilva MV, Hackett RJ, Pouncey CL** 1988 *Effects of fetal sex, stage of gestation, dibutyryl cyclic adenosine monophosphate, and gonadotropin releasing hormone on secretion of human chorionic gonadotropin by placental explants in vitro*. Am J Obstet Gynecol 159:1332-7
853. **Chao TC, Van Alten PJ, Greager JA, Walter RJ** 1995 *Steroid sex hormones regulate the release of tumor necrosis factor by macrophages*. Cell Immunol 160:43-9
854. **Hamano N, Terada N, Maesako K, Numata T, Konno A** 1998 *Effect of sex hormones on eosinophilic inflammation in nasal mucosa*. Allergy Asthma Proc 19:263-9
855. **Friston K, Phillips J, Chawla D, Buchel C** 1999 *Revealing interactions among brain systems with nonlinear PCA*. Hum Brain Mapp 8:92-7
856. **Raychaudhuri S, Stuart JM, Altman RB** 2000 *Principal components analysis to summarize microarray experiments: application to sporulation time series*. Pac Symp Biocomput:455-66
857. **Yeung KY, Ruzzo WL** 2001 *Principal component analysis for clustering gene expression data*. Bioinformatics 17:763-74
858. **Wikman H, Kettunen E, Seppanen JK, Karjalainen A, Hollmen J, Anttila S, Knuutila S** 2002 *Identification of differentially expressed genes in pulmonary adenocarcinoma by using cDNA array*. Oncogene 21:5804-13
859. **Misra J, Schmitt W, Hwang D, Hsiao LL, Gullans S, Stephanopoulos G** 2002 *Interactive exploration of microarray gene expression patterns in a reduced dimensional space*. Genome Res 12:1112-20
860. **Tsunoda T, Koh Y, Koizumi F, Tsukiyama S, Ueda H, Taguchi F, Yamaue H, Saijo N, Nishio K** 2003 *Differential gene expression profiles and identification of the genes relevant to clinicopathologic factors in colorectal cancer selected by cDNA array method in combination with principal component analysis*. Int J Oncol 23:49-59
861. **Pauwels RA, Pedersen S, Busse WW, Tan WC, Chen YZ, Ohlsson SV, Ullman A, Lamm CJ, O'Byrne PM** 2003 *Early intervention with budesonide in mild persistent asthma: a randomised, double-blind trial*. Lancet 361:1071-6
862. **Sullivan SD, Buxton M, Andersson LF, Lamm CJ, Liljas B, Chen YZ, Pauwels RA, Weiss KB** 2003 *Cost-effectiveness analysis of early intervention with budesonide in mild persistent asthma*. J Allergy Clin Immunol 112:1229-36
863. **Holgate ST, Roche WR, Church MK** 1991 *The role of the eosinophil in asthma*. Am Rev Respir Dis 143:S66-70
864. **Hunt J** 2002 *Exhaled breath condensate: an evolving tool for noninvasive evaluation of lung disease*. J Allergy Clin Immunol 110:28-34
865. **Reid DW, Johns DP, Feltis B, Ward C, Walters EH** 2003 *Exhaled nitric oxide continues to reflect airway hyperresponsiveness and disease activity in inhaled corticosteroid-treated adult asthmatic patients*. Respirology 8:479-86
866. **Khalid ME, Ali ME, Ali KZ** 1997 *Full-term birth weight and placental morphology at high and low altitude*. Int J Gynaecol Obstet 57:259-65
867. **Rondo PH, Tomkins AM** 1999 *Maternal iron status and intrauterine growth retardation*. Trans R Soc Trop Med Hyg 93:423-6
868. **Maier RF, Gunther A, Vogel M, Dudenhausen JW, Obladen M** 1994 *Umbilical venous erythropoietin and umbilical arterial pH in relation to morphologic placental abnormalities*. Obstet Gynecol 84:81-7

869. **Jazayeri A, Tsibris JC, Spellacy WN** 1999 *Fetal erythropoietin levels in growth-restricted and appropriately grown neonates with and without abnormal fetal heart rate tracings: a comparison with cord blood gases and Apgar scores*. J Perinatol 19:255-9

Appendices

Appendix 1 **Participant information sheet and consent form (control subjects)**

WOULD YOU LIKE TO PARTICIPATE AS A CONTROL SUBJECT IN A STUDY OF ASTHMA AND PREGNANCY ?

PLEASE READ OUR INFORMATION SHEET

CHIEF INVESTIGATORS

Dr Vicki Clifton	**Dept of Endocrinology. Phone: 4921 4393, pager 5092**
Dr Peter Gibson	**Dept of Respiratory Medicine. Phone: 4921 3470**
Professor Warwick Giles	**Dept of Obstetrics and Gynaecology. Phone: 4921 4381**
Professor Roger Smith	**Dept of Endocrinology. Phone: 4921 4380**
Ms Vanessa Murphy	**Dept of Endocrinology. Phone: 4921 4380**
Ms Annette Osei-Kumah	**Dept of Endocrinology. Phone: 4921 4380**

Short Title: Effect of severe asthma during pregnancy on placental function and fetal outcome.

Patient Initials..... **Patient Number**.....

What is the purpose of the study?

We are investigating the effects of asthma on the baby's growth and placental function. Pregnancies complicated by severe asthma may be associated with preterm delivery and low birthweight babies. The researchers involved are Dr Vicki Clifton, Prof Warwick Giles, Prof Peter Gibson, Prof Roger Smith and PhD students, Ms Vanessa Murphy and Ms Annette Osei-Kumah at The University of Newcastle, under the supervision of Dr Vicki Clifton. This study will monitor the baby's growth throughout pregnancy in women who have mild, moderate or severe asthma and determine if there are any changes in growth associated with asthma. We also want to look at the placenta (the afterbirth) and examine if there are any changes in how the placenta functions in women with asthma. We will also collect cord blood from the placenta and examine the effect of the blood on the activity of different cells. This study will increase our understanding of pregnancy and asthma and may improve the treatment of asthma during pregnancy.

The samples we collect from the placenta may be used for future studies of asthma and pregnancy. We would like to compare the findings in asthmatic women to women who have normal healthy pregnancies (control subjects).

What does the study involve?

1. 3 × 10 ml blood samples at around 12, 18 and 30 weeks of pregnancy

2. 3 ultrasounds at 18, 30 and 36 weeks

3. collection of a morning sample of urine at 12, 18 and 30 weeks of pregnancy

4. the donation of your placenta after your delivery

At your first antenatal visit you usually give a blood sample and some extra blood will be taken with your permission for this study. Then at 18 weeks and 30 weeks we will take another blood sample. These samples are not normally taken during your pregnancy. The blood sample may hurt a little and there could be bruising but this doesn't happen very often.

We will ask you to take home a pot for a sample of urine from your first morning visit to the toilet, which is any time after 4.00 am. We would like to collect a urine sample at 12, 18 and 30 weeks of pregnancy. Our research nurse would contact you the day before you give the sample to ask you if it would be convenient for her to collect the sample the next morning. She would then collect the sample from your house at a convenient time of your choice. We would like to collect 2 mls of this urine to monitor hormones produced by you and the baby.

As part of your usual antenatal care, at around **18, 30 and 36 weeks** of your pregnancy, we will monitor your baby's growth using an ultrasound and record these findings in our study. One study has reported that ultrasound may be associated with a 30% increase in left-handedness which equates to 3 children in every 100 births. No harm has been demonstrated from ultrasound.

After your baby is delivered, if you agree, we will collect your placenta (the afterbirth) and determine whether there are any changes in how it functions. The placenta is delivered after the baby and our collection of it will not harm mother or baby. We will collect blood from the placental cord and use it in the laboratory to examine the effect blood from a male or female fetus has on the activity of different cells. Ms Murphy will conduct experiments on the placenta in our laboratory and the results will contribute to

her PhD thesis. Ms Annette Osei-Kumah will use the cord blood on myometrial and bronchial smooth muscle cells in our laboratory and the results will contribute to her PhD thesis. Some of the samples of blood, urine, cord blood and placenta will be stored and used in the future for further studies of asthma and pregnancy. The samples of blood, urine and placenta may also be used by students in the future as part of their research projects. All of the samples collected will be de-identified so that your privacy is maintained.

These samples will not be used for cloning or stem cell research.

If you agree to donate your placenta and cord blood for this study, there will be a note on your medical record to tell the midwife how to contact Dr Clifton to collect it. If you are able to remind the midwife after the birth of your baby we would be most appreciative.

What are the extra things I will have to do for this study?

Most of the information that we will collect from you are part of your routine antenatal care. The additional requests are:

1. 2 × 10 ml blood samples at 18 and 30 weeks of your pregnancy
2. 3 × morning urine samples at 12, 18 and 30 weeks of pregnancy
3. 2 extra ultrasounds at 30 and 36 weeks gestation

What do I need to tell the doctor before I participate?

We would like to know if you have any illnesses such as diabetes or hypertension. We would also like to know if you smoke and how many you smoke each day. Please tell us if you take any medicines including herbal medicines or medicines bought from the supermarket, chemist or health food shop.

Is the information collected confidential?

Any information we collect about your pregnancy and asthma will be kept confidential and you will not be identifiable in any reports of the study.

What if I change my mind?

Taking part in this study is completely voluntary and if you participate you are free to withdraw from the study at any time without giving a reason. Decisions you make regarding participation will not affect your access to care and services you would

normally receive. If you choose to withdraw from the study just let the doctor or nurse know at your next visit.

What if I have a complaint about this study?

Should you have a complaint concerning the manner this study is conducted it may be given to the doctor or write to the Professional Officer, Dr Nicole Gerrand, HAREC, c/o HAHS, Locked Bag No 1, New Lambton Heights, 2305, or telephone 049 21 4950.

Can I see the results of this study?

You can receive the results of this study by contacting Dr Vicki Clifton on 49214380 and leaving your name and address. Study results will then be posted to your home address.

CONSENT TO PARTICIPATE IN RESEARCH.

STUDY OF THE EFFECTS OF ASTHMA ON PREGNANCY, PLACENTAL FUNCTION AND FETAL OUTCOME

Chief Investigator to contact: Dr Warwick Giles, Dept Obstetrics and Gynaecology, phone 21 4381

This study will assess the effect of asthma on the growth of the baby and placental function and will be compared to pregnancies without asthma.

Women who present to the John Hunter Hospital Antenatal Clinic will be asked to participate in this study in the first trimester of their pregnancy. Participants will be asked to give 3 × 10 ml blood samples at around 12, 20 and 38 weeks of pregnancy, have 3 ultrasounds at 18, 30 and 36 weeks, have an assessment of their asthma and keep a record of the drugs they take for asthma, the donation of their placenta after delivery.

It is essential that you read and understand the information sheet which gives details of what will happen during the study. Please ask the doctor or nurse to answer any questions you have before signing the consent form. You may telephone the research team anytime.

All information gained from this study will remain confidential and personal identifying information will be deleted from all records when the study is complete.

CONSENT

I have been asked to participate in the above research project and give my free consent by signing this form. I understand that:

1. The research project will be carried out as described in the Information Sheet, a copy of which I have retained.

2. If I do not volunteer, or decide to withdraw, my decision will be accepted and my nonparticipation will not affect the treatment I am receiving

3. My consent to participate is voluntary and I may withdraw from the trial at any time. I do not have to give a reason for the withdrawal of my consent.

4. I have read and understood the information sheet and had my questions answered to my satisfaction.

5. If I wish to raise matters of concern or complaints I can contact the Professional Officer, Dr Nicole Gerrand, HAREC, c/o HAHS, Locked Bag No 1, New Lambton Heights, 2305, or telephone 049 21 4950.

SIGNATURE.....

DATE.....

Appendix 2 **Participant information sheet and consent form (asthmatic subjects)**

WOULD YOU LIKE TO PARTICIPATE IN A STUDY OF ASTHMA AND PREGNANCY ?

PLEASE READ OUR INFORMATION SHEET

CHIEF INVESTIGATORS

Dr Vicki Clifton	**Dept of Endocrinology. Phone: 4921 4393, pager 5092**
Dr Peter Gibson	**Dept of Respiratory Medicine. Phone: 4921 3470**
Professor Warwick Giles	**Dept of Obstetrics and Gynaecology. Phone: 4921 4381**
Professor Roger Smith	**Dept of Endocrinology. Phone: 4921 4380**
Ms Vanessa Murphy	**Dept of Endocrinology. Phone: 4921 4380**
Ms Annette Osei-Kumah	**Dept of Endocrinology. Phone: 4921 4380**

Short Title: Effect of severe asthma during pregnancy on placental function and fetal outcome.

Patient Initials..... **Patient Number**.....

What is the purpose of the study?

We are investigating the effects of asthma on the baby's growth and placental function. Pregnancies complicated by severe asthma may be associated with preterm delivery and low birthweight babies. The researchers involved are Dr Vicki Clifton, Prof Warwick Giles, Prof Peter Gibson, Prof Roger Smith and PhD students, Ms Vanessa Murphy and Ms Annette Osei-Kumah at The University of Newcastle, under the supervision of Dr Vicki Clifton. This study will monitor the baby's growth throughout pregnancy in women who have mild, moderate or severe asthma and determine if there are any changes in growth associated with asthma. We also want to look at the placenta (the afterbirth) and examine if there are any changes in how the placenta functions in women with asthma. We will also collect cord blood from the placenta and examine the effect of the blood on the activity of different cells. This study will increase our understanding of pregnancy and asthma and may improve the treatment of asthma during pregnancy.

The samples we collect from the placenta may be used for future studies of asthma and pregnancy.

What does the study involve?

1. 3 × 10 ml blood samples at around 12, 18 and 30 weeks of pregnancy

2. 3 ultrasounds at 18, 30 and 36 weeks

3. collection of a morning sample of urine at 12, 18 and 30 weeks of pregnancy

4. an assessment of your asthma at the beginning and the end of your pregnancy

5. the donation of your placenta after your delivery and collection of cord blood

At your first antenatal visit you usually give a blood sample and some extra blood will be taken with your permission for this study. The respiratory nurse will talk to you about what you take for your asthma and how often you get sick. We will look at your lung capacity using a peak flow monitor and we will ask you to record your peak flow every day for 2 weeks in a diary card. When you are sick with asthma we will ask you to record this in your diary. At the end of the pregnancy you will have another asthma assessment with the respiratory nurse.

We will ask you to take home a pot for a sample of urine from your first morning visit to the toilet which is any time after 4.00 am. We would like to collect a urine sample at 12, 18 and 30 weeks of pregnancy. Our research nurse would contact you the day before you give the sample to ask you if it would be convenient for her to collect the sample the next morning. She would then collect the sample from your house at a convenient time of your choice. We would like to collect 2 mls of this urine to monitor hormones produced by you and the baby.

At 18 weeks and 30 weeks we will take another blood sample. These samples are not normally taken during your pregnancy. The blood sample may hurt a little and there could be bruising but this doesn't happen very often.

At around 18, 30 and 36 weeks of your pregnancy we will monitor your baby's growth using an ultrasound. One study has reported that ultrasound may be associated with a 30% increase in left-handedness which equates to 3 children in every 100 births. No harm has been demonstrated from ultrasound.

You will be given a diary card to fill out to detail your asthma symptoms and the drugs you take during your pregnancy. We will also give you a peak flow meter to measure your lung function.

After your baby is delivered, if you agree, we will collect your placenta (the afterbirth) and determine whether there are any changes in how it functions. The placenta is delivered after the baby and our collection of it will not harm mother or baby. We will collect blood from the placental cord and use it in the laboratory to examine the effect blood from a male or female fetus has on the activity of different cells. Ms Murphy will conduct experiments on the placenta in our laboratory and the results will contribute to her PhD thesis. Ms Annette Osei-Kumah will use the cord blood on myometrial and bronchial smooth muscle cells in our laboratory and the results will contribute to her PhD thesis. Some of the samples of blood, urine, cord blood and placenta will be stored and used in the future for further studies of asthma and pregnancy. The samples of blood, urine and placenta may also be used by students in the future as part of their research projects. All of the samples collected will be de-identified so that your privacy is maintained.

These samples will not be used for cloning or stem cell research.

If you agree to donate your placenta and cord blood for this study, there will be a note on your medical record to tell the midwife how to contact Dr Clifton to collect it. If you are able to remind the midwife after the birth of your baby we would be most appreciative.

What are the extra things I will have to do for this study?

Most of the information that we will collect from you are part of your routine antenatal care. The additional requests are

1. 2 × 10 ml blood samples at 18 and 30 weeks of your pregnancy

2. 3 × morning urine samples at 12, 18 and 30 weeks of pregnancy

3. record your asthma symptoms, peak flow and the drugs you take during your pregnancy in a diary card

4. 2 extra ultrasounds at 30 and 36 weeks gestation

What if I have an asthma attack during my pregnancy?

If you are sick with asthma during your pregnancy, it is important that you follow your action plan and seek medical help from your usual doctor. In addition, please contact Dr Peter Gibson of Respiratory Medicine on 02 4921 3470. If you have a severe asthma attack and need to come to the emergency outpatients at the John Hunter Hospital, let the doctor know that you are part of this study. If you go to another hospital please note the visit and your treatment in the diary card and let us know by ringing 02 4921 3470.

What do I need to tell the doctor before I participate?

We would like to know if you have any other illnesses other than asthma such as diabetes or hypertension. We would also like to know if you smoke and how many you smoke each day. Please tell us if you take any other medicines including herbal medicines or medicines bought from the supermarket, chemist or health food shop.

Is the information collected confidential?

Any information we collect about your pregnancy and asthma will be kept confidential and you will not be identifiable in any reports of the study.

What if I change my mind?

Taking part in this study is completely voluntary and if you participate you are free to withdraw from the study at any time without giving a reason. Decisions you make regarding participation will not affect your access to care and services you would normally receive. If you choose to withdraw from the study just let the doctor or nurse know at your next visit.

What if I have a complaint about this study?

Should you have a complaint concerning the manner this study is conducted it may be given to the doctor or write to the Professional Officer, Dr Nicole Gerrand, HAREC, c/o HAHS, Locked Bag No 1, New Lambton Heights, 2305, or telephone 049 21 4950.

Can I see the results of this study?

You can receive the results of this study by contacting Dr Vicki Clifton on 49214380 and leaving your name and address. Study results will then be posted to your home address.

please place this form with my hospital notes

PLACENTA AND CORD BLOOD DONATION

I AM PARTICIPATING IN AN ASTHMA STUDY. I AM DONATING MY PLACENTA AND CORD BLOOD FOR RESEARCH TO THE DEPARTMENT OF ENDOCRINOLOGY AND OBSTETRICS AND GYNAECOLOGY.

COULD NURSING STAFF PLEASE CONTACT DR VICKI CLIFTON ON 49855641 OR 49431449 OR PAGER 5092 AT ANY TIME DAY OR NIGHT WITHIN 45 MINUTES OF THE DELIVERY

Signature.....

Date.....

Appendix 3 **Ethics approval**

THE UNIVERSITY OF NEWCASTLE

HE2:7/92

APPROVAL FOR RESEARCH PROJECT INVOLVING HUMAN SUBJECTS

Applicant Details H 390 1097

Chief Investigator/Project Supervisor: (First named in application)	Dr Vicki Clifton
Other Investigators:	Professor Roger Smith Dr Peter Gibson Professor Warwick Giles
Project Title:	*Effect of severe asthma during pregnancy on placental function and fetal outcome*
Administering Institution:	THE UNIVERSITY OF NEWCASTLE

Ethics Committee Use

Is it your opinion that this project complies with the provisions contained in the NH&MRC document 'Statement on Human Experimentation and Supplementary Notes'? YES ☑ NO ☐

Is it your opinion that this project complies with the requirements concerning experimentation on humans within this University? YES ☑ NO ☐

Comments, provisos or reservations:

Approved.

Name of responsible Ethics Committee: THE UNIVERSITY OF NEWCASTLE HUMAN RESEARCH ETHICS COMMITTEE	Ethics Monitoring: *Annually and/or upon completion*

Name of Ethics Committee representative:

Ms S J O'Connor Secretary to the Committee

Signature: [signature] Date: 15 October 1997

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Appendix 4 **Asthma Management Service scripting**

This section describes the type of information given and questions asked to subjects at the Asthma Management Service (Courtesy of Sr Philippa Talbot, Research Nurse, Mothers and Babies Research Centre, Newcastle).

TRIGGERS

Asthma is an oversensitive airway disease that can be easily triggered. The following are examples of some possible triggers: season, exercise, upper respiratory tract infection, fumes, stress, laughter, premenstrual asthma, pets (especially cats), food and aspirin. Please let me know if any of these apply to you.

Some triggers are unavoidable and you may need to implement your asthma management plan at these times.

Other triggers should be avoided where possible.

HISTORY PAST 2 YEARS

Over the past 2 years, have you ever presented to casualty and been sent home with asthma?

How many hospital admissions have you had for your asthma over the past 2 years?

During the past 2 years how many prednisone courses have you taken?

PATIENT'S BEST

Over the past 2 years, when your asthma is at its best, do you wake at night with asthma? If so, around how many nights per week?

Over the past 2 years when your asthma is at its best, do you have symptoms such as chest tightness, cough, wheeze or breathlessness when you wake in the morning?

Over the past 2 years when your asthma is at its best, how many times a day do you require your short acting beta-agonist such as ventolin?

When you are well and your asthma is at its best, how many times per day do you take your preventer or inhaled corticosteroid puffer?

Have you been monitoring your peak flows over the last 2 years? If so, what is your best peak flow reading?

MEDICATIONS

Over the past week what medications have you been taking for your asthma?

Note name and dosage of drug. Prednisone dose is the dose that was taken on that day.

ASTHMA CONTROL

How many nights in the past week have you woken from your sleep with asthma symptoms?

How many mornings over the last week have you had asthma symptoms when you wake?

How many days over the last week has your activity been limited due to your asthma?

KNOWLEDGE OF MEDICATION

Briefly explain how your reliever medication works.

Briefly explain how your preventer medication works.

Which inhaler would you carry with you if you were going shopping?

If you woke in the morning with no asthma symptoms and you felt your asthma was under control, which inhaler would you use?

If you had asthma symptoms which inhaler would you use?

ACTION PLANS

Do you know how to recognise worsening asthma? What are the early signs and symptoms of worsening asthma?

When would you begin to implement your asthma management plan?

How would you increase your treatment? Which medication would you increase? How long would you stay on your increased treatment?

When would you see your doctor?

Appendix 5 **Asthma action plan**

ASTHMA MANAGEMENT SERVICE
Dr P Gibson, Co-ordinator
Professor M J Hensley
Dr K Murree-Allen
Dr N Saltos
Dr L G Olson
Dr M Deacon
C Kessell RN
V McDonald RN
P Pratt - Med Technician
R Toneguzzi RN

DEPARTMENT OF RESPIRATORY MEDICINE

MRN
NAME
ADDRESS
DOB

ASTHMA ACTION PLAN

WHEN WELL

_____ Dose _____
_____ Dose _____
_____ Dose _____

Take _____ *10 minutes before exercise*

WHEN NOT WELL

If your peak flow reading does not reach _____ following your medication for a 24 hr period.
Or
If you are getting a cold.
Or
If you are waking at night due to your asthma or have symptoms when you wake in the morning.
Or
If you require your bronchodilator (_____) frequently and are not getting the same effect.

Then

Increase your _____
Have extra _____
Other _____

Continue this treatment for 2 weeks.
See your doctor if _____

FOR A SEVERE ATTACK

If your peak flows do not reach _____
If you have severe shortness of breath and can only speak in short sentences.
If you are having a severe attack of asthma and are frightened.
If you are needing your _____ more than 4 hourly and not gaining an effect.

Then

Take _____ repeat if you do not improve.
Take _____ of prednisone.
Seek medical attention immediately by calling an ambulance on 000.
Continue to use your _____ until help arrives.

Signature _____ Date _____

Version 22-Dec-97

NEWEY & BEATH PRINTERS

Appendix 6 Cumulative inhaled glucocorticoid use during pregnancy

The cumulative inhaled glucocorticoid use during pregnancy was calculated as follows:

T1 = Dose in first trimester

T2 = Dose in second trimester

T3 = Dose in third trimester

$a = (T1 \times 12) + (T2 \times 12) + (T3 \times 12)$

If using fluticasone, double a

Subjects classified as low dose glucocorticoid users if $a \leq 14400$

Subjects classified as moderate dose glucocorticoid users if $14400 \leq a \geq 54000$

Subjects classified as high dose glucocorticoid users if $a \geq 54000$

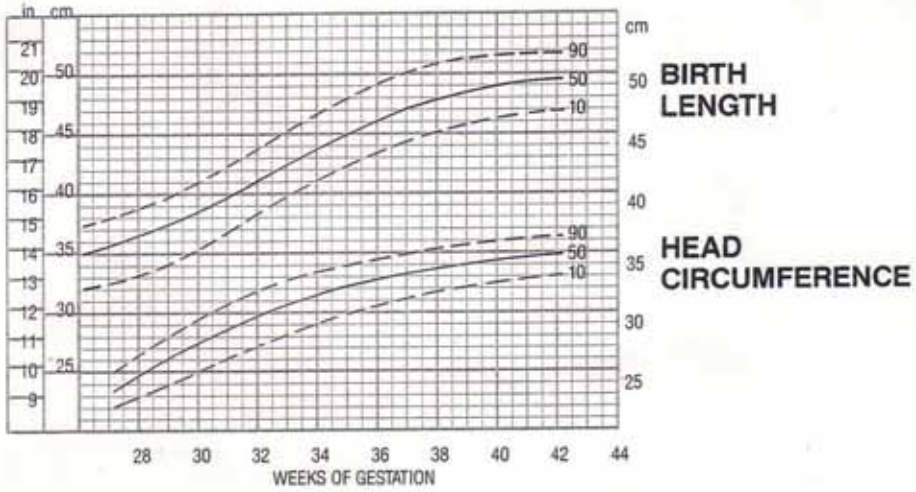
Appendix 7 **John Hunter Hospital intrauterine growth charts**

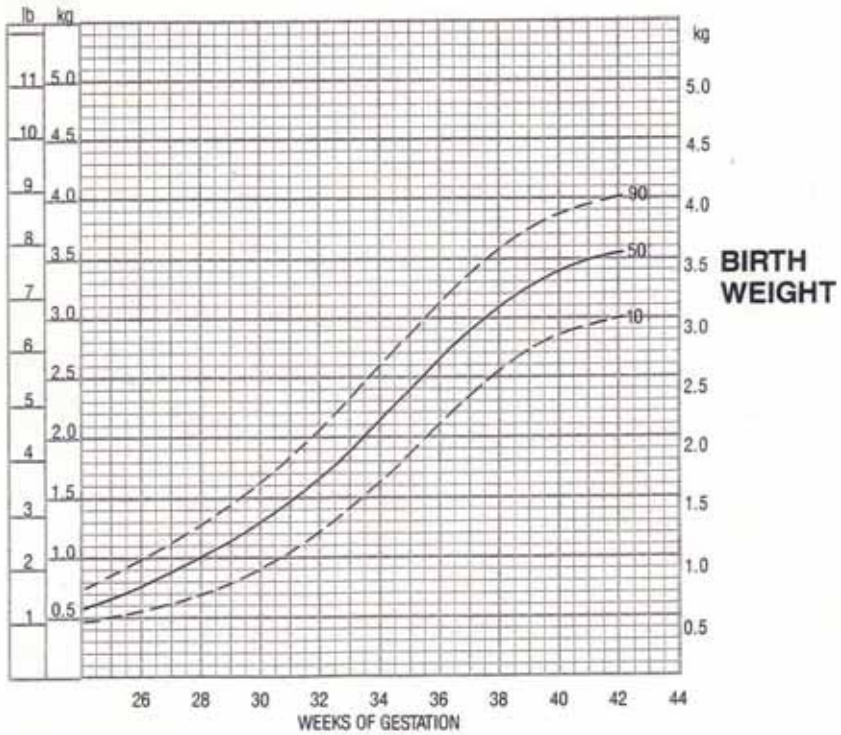
INTRAUTERINE GROWTH CURVES (COMPOSITE MALE/FEMALE)

Measuring techniques: (as for ages 0–36 months – see over page)

Additional Notes: Gestational ages are recorded in completed weeks from the first day of the mother's last menstrual period. Foetal growth is influenced by many factors including age, body weight, height, parity, ethnic origin of the mother and sex of the foetus. Corrections for some of these factors are found in the quoted reference.

Data Source: W.H. Kitchen et al Revised intrauterine growth curves for an Australian hospital population. Aust. Paediatr. J. (1983) 19:157–161.





Appendix 8 **Buffer recipes**

Sodium phosphate buffer with protease inhibitors

Component	Amount
$NaH_2PO_4.2H_2O$	1.48 g
$Na_2HPO_4.12H_2O$	14.5 g
EDTA	0.38 g
Protease inhibitor cocktail tablets	10 tablets
Trasylol	500 µl
Dithiothreitol	0.0075 g
Benzamidine	0.0784 g
Bacitracin	0.05 g
Pepstatin A	55.5 µl of 10 mg/ml
Distilled water	Up to 500 ml

Adjust pH to 7.4

Western blot buffers

SDS sample buffer

Component	Amount
Tris pH 6.8	40 ml
Glycerol	28 ml
6 mM EGTA	0.228 g
SDS	6 g
Bromophenol blue	0.07 g
Distilled water	19 ml
β-mercaptoethanol *	600 µl

* SDS sample buffer is stored at room temperature without β-mercaptoethanol. Make the working solution up as required with 4.4 ml SDS sample buffer and 0.6 ml β-mercaptoethanol.

Lower electrode buffer (10×)

Component	Amount
Tris pH 8.8	302.8 g
Distilled Water	Up to 5 L

Upper electrode buffer (10×)

Component	Amount
Tris pH 8.3	151 g
Glycine	720 g
10% SDS (100 g/L)	50 ml
Distilled Water	Up to 5 L

Transfer buffer

Component	Amount
Tris pH 8.3	15 g
Glycine	72 g
Methanol	1 L
Distilled Water	Up to 5 L

Blocking buffer

Component	Amount
Transfer Buffer	300 ml
BSA	1 g

Store at 4°C

Phosphate buffered saline (10×)

Component	Amount
NaCl	80 g
KCl	3 g
$Na_2HPO_4.12H_2O$	21.9 g
$NaH_2PO_4.2H_2O$	7.2 g

Dissolve all components except $NaH_2PO_4.2H_2O$ in 700 ml distilled water. Add $NaH_2PO_4.2H_2O$ slowly until pH reaches 7.3 and adjust total volume to 1 L.

Antibody dilution buffer

Component	Amount
PBS (1×)	100 ml
BSA	0.1 g
Sodium azide	0.0001 g

Store at 4°C

Tris buffered saline (TBS)/tween

Component	Amount
Tris pH 7.4	48.5 g
NaCl	360 g
Tween 20 (0.1%)	4 ml
Distilled Water	Up to 4 L

PCR buffers

TBE (Tris Borate EDTA) buffer (10×)

Component	Amount
Tris base	108 g
Boric acid	55 g
EDTA	40 ml of 0.5 M
Distilled Water	Up to 1 L

Adjust to pH 8.0

SELDI buffers

IMAC low stringency binding buffer

(PBS/0.5 M NaCl/0.1% TX-100, pH 7)

Component	Volume
10 × PBS	50 ml
5 M NaCl	50 ml
10% TX-100	5 ml
Distilled Water	395 ml

Adjust pH to 7 with 1N HCl

IMAC high stringency binding buffer

(0.1 M sodium acetate/0.5 M NaCl/0.1% TX-100, pH 4)

Component	Volume
100 mM sodium acetate	445 ml
5 M NaCl	50 ml
10% TX-100	5 ml

Adjust pH to 4 with 1N HCl

WCX low stringency/SAX high stringency binding buffer

(0.1 M ammonium acetate/0.1% TX-100, pH 4 or 6)

Component	Volume
100 mM ammonium acetate	480 ml
10% TX-100	5 ml

Adjust pH to 4 or 6 with glacial acetic acid (approximately 15 ml to pH 4)

WCX high stringency binding buffer

(0.1 M Tris/0.1% TX-100, pH 9)

Component	Volume
100 mM Tris	495 ml
10% TX-100	5 ml

Adjust pH to 9 with 1N HCl

SAX low stringency binding buffer

(0.05 M Tris/0.1% TX-100, pH 9)

Component	Volume
50 mM Tris	495 ml
10% TX-100	5 ml

Adjust pH to 9 with 1N HCl

Appendix 9 **SELDI spot protocols**

Low mass spot protocol

Step	Description
1	Set high mass to 30000 Da, optimised from 900 Da to 20000 Da
2	Set starting laser intensity to 195 (for SPA) or 170 (for CHCA)
3	Set starting detector sensitivity to 7
4	Focus mass at 10000 Da
5	Set mass deflector to 900 Da
6	Set data acquisition method to Seldi Quantitation
7	Set Seldi acquisition parameters 24 delta to 5 transients per to 5 ending position to 84
8	Set warming positions with 2 shots at intensity 200 and don't include warming shots
9	Process sample
10	Identify peaks using auto identify from 900 Da to 20000 Da

High mass spot protocol

Step	Description
1	Set high mass to 200000 Da, optimised from 10000 Da to 200000 Da
2	Set starting laser intensity to 220
3	Set starting detector sensitivity to 9
4	Focus mass at 16500 Da
5	Set mass deflector to 10000 Da
6	Set data acquisition method to Seldi Quantitation
7	Set Seldi acquisition parameters 22 delta to 5 transients per to 5 ending position to 82
8	Set warming positions with 2 shots at intensity 225 and don't include warming shots
9	Process sample
10	Identify peaks using auto identify from 10000 Da to 200000 Da

Low mass calibration spot protocol

Step	Description
1	Set high mass to 10000 Da, optimised from 900 Da to 7500 Da
2	Set starting laser intensity to 190
3	Set starting detector sensitivity to 8
4	Focus mass at 3500 Da
5	Set mass deflector to 400 Da
6	Set data acquisition method to Automatic Laser Adjustment
7	Set shots to collect to 25 shots
8	Set points on scale to accept to 3 points and on-scale intensity to 40
9	Set points off scale to reject to 2 points and off-scale intensity to 242
10	Increase laser intensity by 1 after 1 consecutive low shot
11	Decrease laser intensity by 3 after 1 consecutive high shot
12	Revive signal with increased laser after 8 consecutive shots without signal, boost intensity 5
13	Set minimum number of shots per fresh spot to 2 shots
14	Set maximum shots per position to 10 shots
15	Process sample