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# EFFECT OF MATERNAL ASTHMA DURING PREGNANCY ON ASPECTS OF PLACENTAL IMMUNE FUNCTION

A thesis submitted for the degree of Doctor of Philosophy

The University of Newcastle, Australia

May 2011

## DECLARATION

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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. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

Signed.....

Naomi M Scott

May 2011

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## PUBLICATION LIST

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### JOURNAL PUBLICATIONS

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**Scott NM**, Hodyl, NA, Osei-Kumah A, Stark MJ, Smith R, Clifton, VL, *The presence of maternal asthma during pregnancy suppresses the placental pro-inflammatory response to an immune challenge in vitro.* Placenta, 2011 Jun;32(6):454-61.

### ABSTRACT PUBLICATIONS

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Hodyl NA, **Scott N**, Osei-Kumah A, Path N, Stark MJ, Clifton VL. *Glucocorticoid receptor chaperone molecules and sex-specific sensitivity to cortisol in the human placenta.* 52<sup>nd</sup> Annual Scientific Meeting of the Endocrine Society of Australia, 23-26 August 2009.

Hodyl NA, Wyper H, **Scott N**, Osei-Kumah A, Murphy VE, Smith R, Clifton VL. *Relationship between placental glucocorticoid receptor expression and fetal growth in pregnancies complicated by asthma.* 51<sup>st</sup> Annual Scientific Meeting of the Endocrine Society of Australia, 25<sup>th</sup>-28<sup>th</sup> August 2008

**Scott NM**, Stark MJ, Wright IMR, Hodgson DM, Smith R, Clifton VL. *Placental inflammatory response in pregnancies complicated by preterm delivery or asthma.* Journal of Paediatrics and Child Health Apr 2007 43 (Supp 1): A92

**Scott NM**, Stark MJ, Wright IMR, Hodgson DM, Smith R, Clifton VL. *Response of placental explants from term and preterm pregnancies to an immune challenge.* Journal of Reproductive Sciences 2007 14 1(Supp): 192A

Clifton VL, Smith R, **Scott N**, Osei-Kumah A, Wyper H. *Alterations in the placental glucocorticoid receptor gene and protein function are associated with sex specific changes in neonatal birthweight.* Australian Health and Medical Research Congress 2006.

**Scott NM**, Stark MJ, Wright IMR, Hodgson DM, Smith R, Clifton VL. *Differences in tumour necrosis factor alpha production by term and preterm placentae.* Endocrine Journal 2006 53(Supp):104

Clifton VL, Wyper H, **Scott N**, Osei-Kumah A. *Identification and characterisation of macrophages in placentae from pregnancies complication by asthma.* Journal of the Society for Gynecologic Investigation 2006 13(Supp): 356A

**Scott NM**, Wyper HJ, Osei-Kumah A, Smith R, Murphy VE and Clifton VL *Sex specific differences in placental cytokine expression and their relationship to fetal*

*glucocorticoid exposure*. Annual Meeting of Society of Gynecologic Investigation, Toronto Canada, March 22-25 2006.

Wyper HJ, **Scott NM**, Osei-Kumah A, Smith R, Johnson RF, Clifton VL. *Placental glucocorticoid receptor expression in pregnancies complicated by asthma*. Endocrine Journal 2005 52(Supp):115.

**Scott NM**, Wyper HJ, Osei-Kumah A, Smith R, Hodgson D, Clifton VL. *Sex differences in placental cytokine expression and their relationship to fetal cortisol*. Endocrine Journal 2005 52(Supp):113

TABLE OF CONTENTS

---

Declaration..... ii

Acknowledgements..... iii

Publication List ..... iv

    JOURNAL PUBLICATIONS ..... iv

    ABSTRACT PUBLICATIONS..... iv

Table of Contents..... vii

Table of Figures..... xiv

Table of Tables ..... xvi

List of Abbreviations ..... xix

Abstract.....xxiii

1    Introduction ..... 1

    1.1    Asthma ..... 2

        1.1.1    Definition of Asthma ..... 2

        1.1.2    Diagnosis of asthma ..... 2

        1.1.3    Asthma exacerbations ..... 4

        1.1.4    Management of Asthma ..... 5

    1.2    Asthma and Pregnancy..... 7

        1.2.1    Effect of pregnancy on asthma ..... 7

1.2.2	The effect of asthma on pregnancy .....	10
1.2.3	The effect of asthma treatment on pregnancy outcomes .....	13
1.2.4	Outcomes of pregnancies complicated by asthma with respect to fetal sex	16
1.3	Fetal growth .....	17
1.3.1	Effect of low birth weight on early life .....	17
1.3.2	Effect of low birth weight on health in adulthood .....	18
1.3.3	The effect of asthma on birth weight .....	19
1.4	Regulation of fetal growth .....	21
1.4.1	Inflammatory mechanisms that affect fetal and maternal health and alter fetal growth .....	21
1.4.2	Effect of alterations in placenta function on fetal growth .....	24
1.5	Immune function of the placenta .....	31
1.5.1	Placental immune regulation.....	31
1.5.2	Effect of maternal disease on placental immune function .....	35
1.5.3	Intracellular pathways in the placenta involved in immune signalling .	41
1.6	Sex differences in immune function .....	49
1.7	Summary .....	50
2	Hypothesis and Aims.....	52
2.1	Hypothesis.....	53
2.2	Aims.....	53



3	Methods.....	54
3.1	Participants.....	55
3.2	Assessment of Asthma Severity .....	55
3.3	Placenta collection .....	56
3.4	Measurement of cytokines in the placenta by quantitative real time reverse transcription polymerase chain reaction.....	57
3.4.1	Principles of PCR .....	57
3.4.2	Materials .....	59
3.4.3	RNA Extraction .....	60
3.4.4	RNA Quantitation.....	61
3.4.5	Reverse Transcription .....	61
3.4.6	Primer Design.....	61
3.4.7	Real-time quantitative RT-PCR.....	63
3.5	Model of placental immune response by explant culture of placental villi .	64
3.5.1	Materials .....	64
3.5.2	Placenta collection and explant culture .....	64
3.5.3	LPS stimulation.....	65
3.6	Measurement of levels of IL-6, IL-8, TNF- $\alpha$ in culture supernatants by sandwich enzyme linked immunosorbent assay .....	66
3.6.1	Principles of enzyme linked immunosorbent assay (ELISA) .....	66
3.6.2	Materials .....	67

3.6.3	Preparation for the assay.....	68
3.6.4	Enzyme linked immunosorbent assay .....	69
3.7	Measurement of levels of IL-1 $\beta$ , IL-4, IL-6, IL-7, IL-8, IL-10, GM-CSF, and TNF- $\alpha$ in culture supernatants by luminex multiplex assay .....	70
3.7.1	Principles of luminex multiplex assay .....	70
3.7.2	Pilot analysis.....	71
3.7.3	Preparation for the assay.....	72
3.7.4	Luminex multiplex assay .....	73
3.8	Investigation of signalling pathways involved in inflammation in the placenta by western immunoblotting .....	74
3.8.1	Principles of western immunoblotting .....	74
3.8.2	Materials .....	74
3.8.3	Buffers and solutions .....	76
3.8.4	Protein Extraction .....	76
3.8.5	Protein Quantitation .....	77
3.8.6	Western Blotting.....	78
3.8.7	Western Blotting – p38.....	80
3.8.8	Western Blotting – Glucocorticoid Receptor.....	81
3.9	Statistical Analysis .....	82
4	Baseline cytokine mRNA in the placenta .....	84
4.1	Introduction.....	85

4.2	Maternal and fetal characteristics .....	88
4.3	Collection of placentae for analysis .....	92
4.4	Effect of asthma and its treatment on placental cytokine expression .....	92
4.5	Effect of fetal sex and asthma on placental cytokine expression.....	93
4.6	Effect of asthma severity on cytokine expression in placenta from male and female fetuses.....	94
4.7	Effect of fetal sex and inhaled glucocorticoid treatment on placental cytokine expression .....	96
4.8	Factors affecting placental cytokine expression .....	97
4.8.1	Cord Blood Cortisol .....	97
4.8.2	Cigarette use during pregnancy.....	98
4.8.3	Multivariate model of all factors contributing to placental cytokine mRNA levels .....	100
4.8.4	Multivariate model of factors contributing to birth weight centile ....	100
4.9	Summary and discussion.....	101
5	Optimisations for an explant model of an aspect of immune function in the placenta.....	106
5.1	Introduction.....	107
5.2	Method of explant culture optimisation.....	107
5.3	LPS concentration.....	109
5.4	Glucocorticoid Concentration .....	112

5.5	Comparison of different time points .....	113
5.6	Summary and Discussion .....	114
6	Effect of gestational age on placental cytokine responses to LPS.....	115
6.1	Introduction.....	116
6.2	Clinical Characteristics.....	116
6.3	Baseline production of cytokines by placental explants in relation to gestational age-group .....	117
6.4	Cytokine production from placental explants in response to endotoxin in relation to gestational age .....	119
6.5	Cytokine production from placental explants in response to endotoxin in relation to gestation and sex .....	120
6.6	Effect of cortisol and dexamethasone on cytokine production from LPS-treated placental explants in relation to gestational age-group and sex.....	123
6.7	Summary and Discussion .....	126
7	Placental response to LPS stimulation .....	127
7.1	Introduction.....	128
7.2	Clinical Characteristics.....	130
7.3	Placental studies .....	131
7.4	Baseline production of cytokines by placental explants.....	132
7.5	Effect of LPS on placental explants cytokine production in vitro in relation to asthma, fetal sex and timing of exposure.....	133

7.6	Cytokine production from placental explants in response to endotoxin in relation to asthma and sex .....	135
7.7	Effect of cortisol on cytokine production from LPS-treated placental explants in relation to asthma, fetal sex and time of exposure .....	137
7.8	Summary and Discussion .....	140
8	Cell pathways involved in inflammation.....	144
8.1	Introduction.....	145
8.2	Western blotting for phosphorylated p38, total p38, S211 phosphorylated glucocorticoid receptor, and glucocorticoid receptor .....	146
8.3	p38 MAPK expression in placental explants .....	147
8.4	Glucocorticoid receptor expression in placental explants.....	150
8.5	Summary and Discussion .....	152
9	Discussion.....	154
9.1	Sex specific effects of maternal asthma and its treatment on placental immune regulation .....	156
9.2	Response of placenta to an LPS stimulation .....	158
9.3	Conclusion .....	163
10	References .....	166
11	Appendix .....	184

## TABLE OF FIGURES

---

Figure 1.1: Simplified model of LPS signalling. ....	46
Figure 1.2: Maternal asthma may impact birth weight by altering cytokine balance in the placenta. Solid lines, known effects, dotted lines, effects still unknown. ....	51
Figure 3.1: SYBR Green. ....	59
Figure 3.2: Sandwich ELISA. ....	67
Figure 4.1: Cytokine mRNA expression in placenta from pregnancies of control women and women with mild or moderate to severe asthma delivering a female (top panel) or male neonate (lower panel). ....	95
Figure 4.2: Cytokine mRNA expression in placenta from women who used cigarettes during pregnancy relative to women who did not use cigarettes delivering either a female (upper panel) or male (lower panel) neonate. ....	99
Figure 5.1: hCG optimisation of explant experiment ....	109
Figure 5.2: Explant optimization. ....	111
Figure 5.3: Optimisation of LPS concentration in explants. ....	112
Figure 5.4: Comparison of different time points ....	113
Figure 6.1: Cytokine response following 24 h incubation with 1 ng/ml LPS (left panel) or 10 ng/ml LPS (right panel) in the presence of cortisol (top panel) or LPS in the presence of dexamethasone (lower panel). ....	124
Figure 6.2: Cytokine response following a 24 h incubation with 1 ng of LPS in the presence of cortisol (top panel) or LPS in the presence of dexamethasone (bottom panel) in placentae from females (left panel) and males (right panel). ....	125

Figure 7.1: Effect of LPS on placental explants cytokine production. ....	134
Figure 7.2: Effect of cortisol on placental explants cytokine production. ....	139
Figure 8.1: Western Blots for (A) phospho p38 with positive and negative control, (B) total p38 on same blot with positive control, (C) phospho GR increasing concentrations of control sample, (D) total GR with control. ....	146
Figure 8.2: Total p38 MAPKinase. ....	147
Figure 8.3: Phosphorylated p38 MAPKinase. ....	148
Figure 8.4: GR protein. ....	150
Figure 8.5: Phosphorylated GR protein. ....	151
Figure 9.1: Maternal asthma affects cytokine expression in the placenta with possible long term impact on adult health .....	164

## TABLE OF TABLES

---

Table 1.1: Classification of asthma severity.....	3
Table 1.2: Respiratory pathogens in hospitalized children who have asthma exacerbation .....	5
Table 1.3: The six-step asthma management plan .....	7
Table 1.4: Adverse pregnancy outcomes that have been associated with asthma in the literature .....	11
Table 1.5: Local cytokine expression during normal human pregnancy. ....	35
Table 1.6: Toll-like receptors and ligands. ....	44
Table 3.1: Primer sequences.....	63
Table 4.1: Maternal characteristics relative to fetal sex and asthma severity.....	90
Table 4.2: Characteristics of male and female neonates, born to control mothers and mothers with mild and moderate-severe asthma.....	91
Table 4.3: Mean log transformed cytokine mRNA abundance in placenta collected from pregnancies complicated by mild asthma divided by glucocorticoid intake.....	93
Table 4.4: Mean log transformed cytokine mRNA abundance in placenta collected from pregnancies complicated by moderate-severe asthma divided by glucocorticoid intake. ....	93
Table 4.5: Cytokine mRNA abundance in placenta collected from male and female control and asthmatic pregnancies and cord blood cortisol levels.....	94



Table 4.6: Mean log transformed cytokine mRNA abundance in placenta collected from pregnancies complicated by mild asthma divided by sex and glucocorticoid intake. ....	96
Table 4.7: Mean log transformed cytokine mRNA abundance in placenta collected from pregnancies complicated by moderate-severe asthma divided by sex and glucocorticoid intake.....	97
Table 4.8: Pearson’s correlation co-efficients between cytokine mRNA, cord blood cortisol and birth weight in placenta from male (n=60) and female (n=67) pregnancies.....	98
Table 6.1: Clinical characteristics of preterm and term control infants.....	117
Table 6.2: Baseline placental explant cytokine concentrations (pg/mg tissue) in the supernatant of un-stimulated placental explants from different gestational age groups incubated for 24 h.....	118
Table 6.3: Baseline placental explant cytokine concentrations (pg/mg tissue) in the supernatant of un-stimulated placental explants from different gestational age groups and sexes.....	119
Table 6.4: Placental explant cytokine production following LPS stimulation.....	120
Table 6.5: Sex differences in placental explant cytokine production following LPS stimulation. ....	122
Table 7.1: Maternal and neonatal clinical characteristics of the control and asthma groups. ....	131
Table 7.2: Baseline placental explant cytokine concentrations (pg/ml) corrected for placental weight (kg) in supernatant of un-stimulated placental explants incubated for 2 or 24 h. ....	132

Table 7.3: Sex differences in placental explant cytokine production following an LPS stimulation. ....	136
Table 8.1: Total, phosphorylated and the ratio of p38 MAPKinase. ....	149
Table 8.2: GR protein. ....	152

## LIST OF ABBREVIATIONS

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11 $\beta$ HSD 2	11beta-hydroxysteroid dehydrogenase
CCL18	chemokine(C-C motif) ligand 18
CD	cluster of differentiation
cDNA	complimentary DNA
CISH	chromogenic in-situ hybridisation
CMRL-1066	media developed by Connaught Medical Research Laboratories
CSF	colony stimulating factor
Ct	<i>chlamydia trachomatis</i>
CXCL5	chemokine (C-X-C motif) ligand 5
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide-triphosphate
dsDNA	double stranded DNA
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinas
FEV <sub>1</sub>	forced expiratory volume in one second
FVC	forced vital capacity
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GR	glucocorticoid receptor

hCG	human chorionic gonadotropin
HELLP	haemolysis, elevated liver enzyme and low platelet
HLA	human leukocyte antigens
HMGB1	high-mobility group box 1 also known as amphoterin
HRP	horseradish peroxidase
HSP	Heat shock protein
IDO	idolemine 2,3-dioxygenase
IFN	interferon
IKK	I $\kappa$ B kinase
IL	interleukin
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon response factor
ISRE	interferon-sensitive response element
IUGR	intrauterine growth restriction
JNK	cJun N-terminal kinase
LBP	lipopolysaccharide binding protein
LDH	lactate dehydrogenase
LIF	leukemia inhibitor factor
LPS	lipopolysaccharide
Mal	MyD88 adapter like
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MKK	mitogen activated protein kinase kinase
mRNA	messenger ribonucleic acid

MyD88	myeloid differentiation primary response gene (88)
n	number of samples
NF- $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B cells
PBMC	peripheral blood mononuclear cell
PEFR	peak expiratory flow rate
pGR211	GR phosphorylated at serine at location 211
PVDF	polyvinylidene fluoride
RANTES	regulated on activation, normal T expressed and secreted
RBC	red blood cells
RIP	receptor interacting protein
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
SEM	standard error of the mean
SGA	small for gestational age
SIGIRR	single immunoglobulin interleukin-1 receptor-related molecule
SLE	systemic lupus erythematosus
TAB	TAK-1 binding protein
TAK	transforming growth factor- $\beta$ -activated kinase
TBK	TANK-binding kinase
TGF	transforming growth factor
Th	T lymphocyte helper
TLR	toll-like receptor
TNF- $\alpha$	tumour necrosis factor alpha
TNFR	tumour necrosis factor receptor

TRAF6	tumour necrosis factor receptor-associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adaptor-inducing interferon- $\beta$
VC	vital capacity
VEGF	vascular endothelial growth factor
VUE	villities of unknown aetiology
WHO	world health organisation

## ABSTRACT

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In the presence of maternal asthma, reduced placental blood flow, decreased cortisol metabolism, and reductions in fetal growth have been reported in response to maternal asthma and asthma exacerbations. The mechanisms that contribute to adverse outcomes for the neonate in pregnancies complicated by asthma may be mediated via changes in aspects of placental immune function.

The influence of maternal asthma and its severity, maternal cigarette use, and fetal sex on placental cytokine mRNA expression was examined in a prospective cohort study of pregnant women with and without asthma. Placental expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and IL-5 mRNA were all increased significantly in placenta of female fetuses whose mothers had mild asthma, but no changes were observed in placenta of male fetuses. The pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were negatively correlated with female cord blood cortisol, but there were no such correlations in placenta from males. Multivariate analysis indicated the strongest predictor of both cytokine mRNA expression in the placenta and birth weight was fetal cortisol, but only in females. Placental cytokine mRNA levels were not significantly altered by inhaled glucocorticoid use, moderate-severe asthma, or male sex. These data suggested that placental basal cytokine mRNA expression is sex specifically regulated in pregnancies complicated by asthma, and interestingly these changes are more prevalent in mild rather than severe asthma.

The placental cytokine response *in vitro* was examined in an additional prospective cohort study of women with asthma, and controls. Placentae were collected immediately following delivery, and placental explants were exposed to LPS immune stimulation, in the presence and absence of glucocorticoids *in vitro*. Cytokines, glucocorticoid receptor  $\alpha$  (GR  $\alpha$ ) and p38 MAPKinase protein were measured. Placentae from pregnancies complicated by maternal asthma had a more rapid response to LPS than control placenta, regardless of fetal sex, with early production of the cytokines IL-6, IL-8 and IL-10, but did not sustain the enhanced cytokine response by 24 h relative to a control population. Cortisol inhibition of placental cytokine production was dependent on timing of exposure, fetal sex, and presence and absence of asthma. GR $\alpha$  and p38 MAPK protein expression did not appear to contribute to differences in response to endotoxin or cortisol. This data demonstrates that the placentae from pregnancies complicated by maternal asthma differ from control placentae in relation to the timing of the response to LPS stimulation, and the regulation of the response by cortisol.

This is the first study to examine the impact of maternal asthma during pregnancy on placental inflammatory pathways. The data has identified that asthma during pregnancy alters pro-inflammatory pathways in a sex specific manner. Female placentae readily control pro-inflammatory cytokine expression via the glucocorticoid pathway while male placentae appear glucocorticoid resistant. This data suggests that during a pro-inflammatory event such as an asthma exacerbation the female fetus may protect herself from the effects of this stress via cortisol. Males babies may be more at risk of a poor outcome due to an inability to regulate



inflammation via cortisol. Placentae from asthmatic pregnancies had an enhanced early cytokine response to LPS stimulation, suggesting previous exposure to inflammatory disease alters responsiveness of cytokines in the placenta to an LPS stimulation.