Role of Lycopene and Long Chain n-3 Polyunsaturated Fatty Acid Supplements in Airway Inflammation

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A thesis submitted for the degree of Doctor of Philosophy

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July, 2008
STATEMENT OF ORIGINALITY

This work 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.

Ahmad Saedi
ACKNOWLEDGMENTS

I would like to acknowledge the practical and emotional support of my family. Firstly, to my wife Soheila, my daughter Sarvin and my son Amir, I would like to say thank you for your support and understanding when my study interrupted our family life. To my father Ali and my mother Fahimeh who raised me to believe that higher education is a worthwhile pursuit, I thank you for always helping me with everything and encouraging me to take new challenges.

I would like to acknowledge my supervisors, Professor Manohar Garg, Dr. Lisa Wood, and Professor Peter Gibson, for their ongoing support, enthusiasm and friendship. I do appreciate their valuable advice and accessibility. I also would like to thank Dr. Peter Wark for his involvement and helpful advice on cell culture experiments.

I would like to acknowledge all of my friends in Respiratory Medicine, Hunter Medical Research Institute, and also Nutraceutical Research Group, School of Biomedical Science. In particular, I would like to acknowledge Terry Grissell, Glenda Walker, Robert Blake, Melinda Phang, Jodie Simpson, Rebecca Oldham, Alan Hsu, Deborah Hall, Michelle Gleeson, Kellie Fakes and Katie Baines for their friendly help.

I would like to sincerely thank the people who participated in the studies outlined in this thesis. In particular, I would like to acknowledge the involvement of asthmatic patients and their families.

Finally, I would like to acknowledge my sponsor of PhD program; Tehran University of Medical Sciences for their financial support during my study in the University of Newcastle. I would say that without their help I would not have been able to finish my study.
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List of abbreviations:

AA: arachidonic acid
AHR: airway hyper-responsiveness
BAL: bronchoalveolar lavage
BALF: bronco-alveolar-lavage fluid
BCDF: B cell differentiation factor
BHT: butylated hydroxytoluene
BMI: body mass index
COPD: chronic obstructive pulmonary disease
CRP: C-reactive protein
DHA: docosahexaenoic acid
DMSO: dimethylsulfoxide
DRI: dietary reference intake
EIA: exercise induced asthma
ELISA: enzyme linked immunosorbent assay
EPA: eicosapentaenoic
FCS/MEM: foetal calf serum/minimum essential medium
FEV$_1$: forced expiratory volume in 1 second
FVC: forced vital capacity
GC: gas chromatography
G-CSF: granulocyte colony stimulation factor
GINA: global initiative for asthma guidelines
GM-CSF: granulocyte-monocyte colony stimulation factor
GSHPx: glutathione peroxidise
HDL: high density lipoprotein
HPLC: high performance liquid chromatography
ICAM-1: intercellular adhesion molecule-1
ICS: inhaled corticosteroid (µg beclamethasone equivalent)
ICS: inhaled corticosteroids
IFN-γ: interferon-gamma
IgG: immunoglobulin G
IgM: immunoglobulin M
IL-1: interleukin-1
IL-2: interleukin-2
IL-3: interleukin-3
IL-4: interleukin-4
IL-5: interleukin-5
IL-6: interleukin-6
IL-8: interleukin-8
IL-12: interleukin-12
IL-17: interleukin-17
IP-10: interferon-gamma inducible protein-10
LCn-3PUFA: long chain n-3 polyunsaturated fatty acid
LDH: lactate dehydrogenase
LDL: low density lipoprotein
LPS: lipopolysaccharide
LTB: Leukotriene B
M-CSF: monocytes colony stimulation factor
MDA: malondialdehyde
MOI: multiplicity of infection (plaque forming unit (PFU)/the number of cells)
NF-κB: nuclear factor-κB
NO: nitric oxide
PEF: peak expiratory flow
PFU: plaque forming unit
PG: prostaglandin
PMN: polymorphonuclear
RDA: recommended dietary allowances
RER: relative expression ratio
ROS: reactive oxygen species
RT-PCR: reverse transcription polymerase chain reaction
RV: rhinovirus
SEM: standard error of mean
SOD: superoxide dismutase
TAC: total antioxidant capacity
TCID_{50}: tissue culture infective dose 50%
TDI: toluene diisocyanate
THF: tetrahydrofuran
TLR: toll-like receptor
TNF-α: tumour necrosis factor-α
TXA: thromboxane A
vRNA: viral ribonucleic acid
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Publications arising from this thesis:

Articles:


3. **Saedisomeolia A**, Wood LG, Garg ML, Gibson PG, Wark PAB. Supplementation of long chain n-3 polyunsaturated fatty acids increases the utilisation of lycopene in cultured airway epithelial cells. *Journal of Food Lipids* (Accepted 04/06/2008).

Abstracts:


Synopsis

In Western society, increased asthma prevalence over recent years has coincided with changes in dietary patterns, leading to the hypothesis that a Western diet increases susceptibility to asthma. Components of the diet that may be important are antioxidants (e.g. lycopene) and fatty acids. Lycopene and long chain n-3 polyunsaturated fatty acids (LCn-3PUFA) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have anti-inflammatory effects. As asthma is a disease linked to oxidative stress and inflammation, it was hypothesised that these nutrients may have a beneficial effect individually, and may have a synergistic anti-inflammatory effect when used in combination. The aim was to examine the ability of lycopene and/or LCn-3PUFAs to protect against virus-induced inflammation, as rhinovirus infection is the primary cause of asthma exacerbation.

The results presented demonstrate that both lycopene and DHA (but not EPA) individually decreased the inflammatory response of airway epithelial cells infected with rhinovirus. The results also showed that DHA supplementation increased the utilization of lycopene by cells. Furthermore, lycopene reduced rhinovirus replication. A combination of lycopene and DHA also reduced the inflammatory response of cells to rhinovirus infection, however, no synergistic anti-inflammatory effect was apparent. It is concluded that consumption of foods containing lycopene and DHA may exhibit a beneficial effect on the inflammatory response to rhinovirus infection. This may have important clinical implications, as increased dietary intake of foods rich in these nutrients may lead to a reduction in the frequency and severity of asthma exacerbations.
Chapter 1:

Introduction
1.1 Antioxidants in general
Antioxidants are substances that inhibit oxidation of other molecules in the body [1, 2] and they prevent various degenerative disease caused by oxidative stress [3]. Antioxidants exert this function via: decreasing the concentration of O$_2$ [4], catalytic metal ions [5-7], H$_2$O$_2$ [8-10] and also scavenging the radicals that initiate oxidative stress [11, 12], breaking the initiated sequence chain of oxidation [13-15], quenching the singlet oxygen [16-18] and repairing oxidative damage caused by radicals [19-22]. When the balance of antioxidant-oxidant interrupted and oxidants overwhelm the antioxidants, disease could appear [23-25].

Antioxidants are divided into two main groups of enzymatic and non-enzymatic [26]. Enzymatic antioxidants include superoxide dismutase (SOD) [27, 28], glutathione peroxidise (GSHPx) [28, 29] and catalase [27]. Non-enzymatic antioxidants include dietary ascorbic acid (vitamin C) [30-32], vitamin E [33-35], flavonoids [36-42] and carotenoids [43, 44]. Non-dietary non-enzymatic antioxidants include glutathione [45], pyruvate [46], plasma albumin [47, 48], ubiquinone [49, 50] and uric acid [51-53]. Studies show that dietary antioxidants are important to respiratory health. Dietary intake of fruit and vegetables, is inversely associated with wheeze [54, 55] and asthma prevalence [56, 57]. And they are positively associated with lung function [58, 59].

In this chapter, before discussion about asthma and inflammation, antioxidation properties of some selected antioxidants (ascorbic acid, catechin, quercetin) will be presented, then the most involved antioxidants and anti-inflammatory nutrients in this thesis (lycopene and long chain n-3 polyunsaturated fatty acids) will be presented in details.

1.1.1 Ascorbic acid:
Ascorbic acid (vitamin C) is a water soluble vitamin which is a white and odourless solid compound [60]. However, most animals can synthesize ascorbic acid from glucose, humans cannot, therefore there are completely dependent upon dietary intake to satisfy their needs [61]. Ascorbic acid is a reducing agent [60] and its molecular weight is 176. The molecular structure (C$_6$H$_8$O$_6$) of ascorbic acid is presented in figure 1.1.1.1.
Figure 1.1.1.1 Schematic structure of ascorbic acid molecule:

\[
\text{HO} \quad \text{HO} \quad \text{O} \quad \text{O} \\
\text{HO} \quad \text{HO} \quad \text{OH}
\]

*In vitro* studies show that ascorbic acid enters to the mammalian cells by sodium electrochemical gradient [62] which is believed to be blocked by presence of glucose [63]. *In vitro* studies also reported that adding ascorbic acid to the cell media modulates the proliferation of cells via promoting the cell growth [64, 65]. Supplementation of ascorbic acid with high concentrations caused ascorbic acid to be auto-oxidized in the cell culture media and show cytotoxicity properties [66, 67], most probably via H$_2$O$_2$ production [68-70].

Ascorbic acid plays beneficial roles in human body such as connective tissue integrity [71], neurotransmitter synthesis [72], enhancing iron absorption [73], carnitine biosynthesis [74], detoxification of toxic materials such as lead [75] or arsenic [76] and antioxidant properties [77-79]. Antioxidant property of ascorbic acid is well understood and it has been shown that ascorbic acid can scavenge free radicals in human body [80]. Briefly, after exposing to reactive oxygen species (ROS), ascorbic acid is oxidised to (1) dehydroascorbate (DHA) and (2) ascorbate free radical (AFR) [81-83]. AFR is relatively an un-reactive free radical. Therefore, ascorbic acid converts reversibly from highly reactive free radicals to poorly reactive ascorbate radicals [84]. Ascorbic acid can recycle other antioxidants such as glutathione and $\alpha$-tocopherol from their radical species forms to the antioxidant forms, also AFR can be recycled to their antioxidant form by NADH-dependent reductase [85, 86]. Ascorbic acid protects cell membrane lipids from peroxidation [87, 88] and also influences gene expression of anti-oxidative enzymes [89].
1.1.2 Flavonoids (quercetin):

Flavonoids are a group of polyphenolic compounds found in fruits, seeds, olive oil, tea and red wine [90]. They are regularly consumed via a normal diet by human [91]. Total amount of flavonoids consumed daily is estimated around 1g/day [91]. Flavonoid molecule include three-ring structure (A, B and C) [92]. Flavonoids provide colour, taste and texture in foods [93]. Previously, they were named vitamin P [94-96]. Flavonoids are divided to four sub-groups of (1) flavane (e.g. catechin), (2) flavanone (e.g. naringin), (3) flavone (e.g. apigenin) and (4) flavonol (e.g. quercetin, fisetin, morin, and kaempferol) [92]. Some of the biological roles of flavonoids are attributed to their metal-binding abilities (i.e. iron and copper) which support their antioxidant and UV screening actions [97].

Quercetin is one of 4000 phenolics isolated from the plants [93] and belongs to the flavonol sub-group of flavonoids [98]. It is a hydrophilic compound with molecular weight of 338 [99]. Quercetin is the most commonly consumed flavonoid in human diet [100, 101]. Onion leaves with 1.5g quercetin/kg [102] and onion with 284-486mg quercetin/kg [103] are among the best sources of quercetin, followed by kale, French beans and broccoli [103]. Among the fruits, apples have the highest quercetin content [103]. Quercetin is a non-toxic component [101, 104-106] and mostly presented as its glycoside form in the human diet [102, 107]. It has been shown that quercetin plays many roles in human body such as an antioxidant [108-110] and anti-inflammatory agent [92, 107, 111-114]. Antioxidant action of quercetin is attributed to its capability to scavenge the superoxide anions [110], singlet oxygen [115] and lipid peroxyl radicals [116]. The probable mechanisms [117] and pharmacological actions [118] of quercetin on inflammation are not completely understood. However, the most probable mechanism of anti-inflammatory property of quercetin is attributed to (1) its antioxidant and free-radical scavenging property [119, 120] and also (2) its effect on nuclear factor-κB (NF-κB) suppression that leads to decreased production of inflammatory biomarkers [118, 120-124], including interleukin-6 [125] and interleukin-8 [126]. Antioxidant and free-radical scavenging property of quercetin is investigated as its inhibitory effect on ROS production by human monocytes [127]. Moreover, the studies regarding to the effect of quercetin on NF-κB inactivation, confirmed the inhibitory effect of quercetin on chemokins [112, 120, 123, 127] and cytokine production [128].
Figure 1.1.2.1 Schematic molecular structure of quercetin:

1.1.3 Catechin:
Catechin is one of the most important flavan sub-group of flavonoids [92]. It constitutes more than 30% of dry weight of green tea and 9% of dry weight of black tea [129, 130]. It has been estimated that catechin constitutes 60-80% of total flavonoids in green tea and 6-24% of flavonoids in black tea [131]. Drinking green tea increases plasma catechin levels [132] and adding milk to the tea does not have any effect on catechin absorption [133]. The molecular weight of catechin is 287 [117]. Catechin shows antioxidant activity in vitro [134] and decreases ROS production by human monocytes [127]. The ranking of antioxidation activity of some selected flavonoids reportedly follows: pelargonidin > quercetin> oenin > querciwin > catechin > malvin >cyanidin > pelargonin > rutin > callistephin > peonidin> morin > apigenin [134]. Therefore, it seems that catechin is less potential antioxidant compared to the previously described quercetin.

Figure 1.1.3.1 Schematic molecular structure of catechin:
1.2 Carotenoids

1.2.1 What are carotenoids?

Carotenoids are natural fat-soluble pigments mainly found in plants [135]. They provide the red, orange and yellow colours of plant leaves, fruits and flowers [136], including the orange colour of carrots and citrus fruits and red colour of tomatoes and peppers [137]. Carotenoids are a subclass of phytochemicals called terpene [138, 139]. The vast variety of carotenoids are derived from a polyene molecule usually containing 40 carbon atoms [135]. Although, both the cis and trans isomers of carotenoids naturally exist, the most prevalent and stable form of each carotenoid is all-trans isomer [140]. Carotenoids can exist in many forms, for example β-carotene can form 272 different isomers and α-carotene can form 512 isomers [140]. Carotenoids are unpolar components and consequently interact with lipophilic parts of the cells; namely, membranes and lipid globules [140]. Acetone and alcohols can be used in order to extract carotenoids [139, 141]. Carotenoids are destroyed in the presence of extreme heat, light, acids and bases [139]. The maximum light absorbance of carotenoids ranges from 440-490nm [139-141], depending on the numbers of conjugated double bounds of molecules and the solvent used for extraction [140]. The most-used methods for separation and quantitation of carotenoids involve high-performance liquid chromatography (HPLC) on reverse-phase or straight-phase columns combined with spectrophotometric detection and peak integration [142]. Humans and some animals are not able to synthesis carotenoids and they obtain carotenoids from their food [143]. From almost 600 carotenoids isolated from natural sources, around 40 of them are found in the diet of western countries [144] and around 21 of them are found in human plasma [43, 145]. It has been reported that α-carotene, β-carotene, lutein, β-cryptoxanthin and lycopene are the most abundant carotenoids in human body [146, 147]. Carotenoids are poorly soluble in aqueous systems and it has been found that in the human small intestine their molecules are solubilised by bile salts and absorbed like other lipids where they are placed in the low density lipoprotein (LDL) and high density lipoprotein (HDL) fractions by the liver to be circulated via the bloodstream [148].

Structure of some common carotenoids:

Figure 1.2.1.1 shows the structure of some common carotenoids.
**Figure 1.2.1.1 Structure of common carotenoids:**

Canthaxanthin:

α-carotene:

β-carotene:

Lutein:

Lycopene:

Zeaxanthin:
1.2.2 Classification of carotenoids:

Generally, carotenoids are classified as two main groups of carotenes and xanthophylls. Xanthophylls include lutein, zeaxanthin, \(\alpha\)-cryptoxanthin and \(\beta\)-cryptoxanthin. Carotenes include \(\alpha\)-carotene, \(\beta\)-carotene and lycopene [43, 139]. Figure 1.2.2.1 shows the classification of carotenoids.
**Figure 1.2.2.1 Classification of carotenoids:**

![Carotenoids Diagram](image)

**1.2.3 Biological functions of carotenoids:**

*Pro-vitamin A activity:*

Carotenoids were initially studied as retinoid precursors [149] and their role as retinoid precursor is one of the most widely studied and well-understood nutritional roles of carotenoids [150]. Around 50 types of carotenoids can convert to vitamin A [151]. Splitting a carotenoid molecule into two molecules of vitamin A, utilises the NADPH-dependent carotenoid deoxygenase enzyme [140], the activity of which is dependant on factors such as genetics, carotenoid species and the quantity of carotenoids consumed [152]. The process of cleavage of β-carotene into vitamin A is shown in figure 1.2.3.1 [153]. The health benefits of vitamin A is one of the best understood fields in nutrition science [150]. It has been reported that vitamin A deficiency is one of the major causes of death among children in developing countries [154, 155].
Figure 1.2.3.1 Cleavage of β-carotene into vitamin A:

\[
\begin{align*}
\text{β-carotene} & \xrightarrow{\text{β-carotene dioxygenase}} \text{Retinaldehyde} & \xrightarrow{\text{Retinaldehyde reductase}} \text{Retinol} \\
\text{Bile, O}_2 & & \text{NAD(P)H}
\end{align*}
\]

**Antioxidant property of carotenoids:**

Large growing body of studies confirm the antioxidant effects of carotenoids [156-160]. However, some studies have demonstrated that while low-level supplementation (1-3µM) of carotenoids (e.g., β-carotene, lycopene and lutein) shows antioxidant effects, large dose supplementation (4-10µM) results in increased reactive oxygen species production (ROS) *in vitro* [161-165]. The reason for the pro-oxidant action of high level carotenoid supplementation is not fully understood, however, it has been attributed to carotenoid interaction with carotenoid radicals, a phenomenon named “carotenoid aggregation” [43]. It has also been reported that different types of carotenoids act as pro-oxidants in the presence of higher pressures of oxygen than are physiologically relevant [166-169]. Interestingly, carotenoids are not alone in this respect and some other antioxidants such as ascorbic acid and vitamin E show pro-oxidant activity in presence of high oxygen pressure [170, 171].

More research is needed to determine the exact mechanism by which carotenoids protect cells against oxidative damage, particularly in the presence of other antioxidants [172]. The antioxidant properties of carotenoids is mainly associated with (1) their singlet oxygen quenching properties [173, 174] and (2) their radical scavenging abilities [166, 175, 176]. Carotenoids scavenge peroxyl radicals via a number of processes including the “electron transfer”, “allylic hydrogen abstraction” and “addition” [135, 166, 177-180]. Following equations show these mechanisms:

\[
\begin{align*}
\text{CAR} + \text{ROO}^- \rightarrow \text{CAR}^\cdot + \text{ROO}^- & \quad \text{(Electron transfer)} \\
\text{CAR} + \text{ROO}^- \rightarrow \text{CAR}^\cdot + \text{ROOH} & \quad \text{(Hydrogen abstraction)} \\
\text{CAR} + \text{ROO}^- \rightarrow \text{ROOCAR}^\cdot & \quad \text{(Addition)}
\end{align*}
\]
The peroxyl radical-scavenging property of carotenoids via “addition” (ROOCAR•) is reported by Burton [166], and “electron transfer” and “hydrogen abstraction” are reported by Woodall et al [180]. Reactive oxygen species such as superoxide (O$_2$•$^-$) and hydroxyl radicals (•OH) are highly reactive free radicals and containing a single unpaired electron [181, 182]. Singlet oxygen interacts with carotenoids to form triplet oxygen, which releases its energy into the surroundings [135, 183].

The antioxidant activity of carotenoids can be ranked as follows: lycopene > α-carotene > β-cryptoxanthin > zeaxanthin = β-carotene > lutein [44]. Therefore lycopene is the most potent antioxidant among carotenoids. It is reported that a mixture of carotenoids is more effective than the single compounds and this synergistic effect is most described when lycopene or lutein is present at the same time [44]. Using results based on pulsed radiation techniques, it has been suggested that antioxidant protection of carotenoids has a synergistic effect with vitamins C [184, 185] and E [184, 186].

**Antioxidant property of lycopene:**
It has been found that lycopene is the most powerful antioxidant among carotenoids [44, 173, 187] and it is the key antioxidant in tomato and tomato products [188]. It has been shown that lycopene supplementation *in vivo* increases total antioxidant capacity (TAC) in patients with type 2 diabetes mellitus [189]. Studies have also reported that higher plasma lycopene concentration is associated with increased activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and also decreased concentrations of peroxidation biomarkers such as malondialdehyde (MDA) [190]. It has also been reported that lycopene prevents cutaneous damage [191] and nephrotoxicity due to gentamicin [157] and cisplatin [158]. Some studies investigating the beneficial effects of tomato and tomato products in preventing oxidative stress of lipoproteins *ex vivo*, found a strong protective effect [192, 193]. However, some others found that this protective effect is because of the tomato generally and is not solely related to its lycopene content [185, 194]. It has been proposed that other components in tomato have a synergistic effect with lycopene [195].

**Other functions of carotenoids:**
Carotenoids have been suggested to provide a wide variety of health benefits. Carotenoids decrease early markers of oxidative stress caused by alcohol [196]. They
also prevent muscle tissue from oxidative stress after exhaustive exercise [197]. Carotenoids are believed to play a key role in protecting the macular region of the human retina [198], improving lung function [199], improving the function of immune system [200], protecting from sunburn [201], inhibiting the development of certain types of cancers [202-206] and cataract [207], reducing blood pressure and risk of cardiovascular disease [208], inhibiting toxic effects of some toxic substances such as doxorubicine [209], preventing platelet aggregation and thrombosis [210], defending against some of the damaging effects of solar radiation (lutein) [211], reducing the development of pre-eclampsia [212] and rheumatoid arthritis [213], protecting against chronic bronchitis induced by long-term cigarette smoking [214], protecting the cells from DNA damage [159, 215] and even decreasing the risk of death among the people [216].

1.2.4 Bioavailability of carotenoids:
Our current knowledge about the bioavailability of carotenoids in foods is insufficient and difficult to interpret [217, 218]. The bioavailability of carotenoids ranges from less than 10%, in intact raw carrots, to more than 50% in oily solutions or in synthetic gelatine-based commercial preparations [219, 220]. It is believed that baseline carotenoids levels affect their absorbance and appearance in the blood [221]. Carotenoids are not destroyed during food processing and heat even improves their bioavailability [187, 222].

Previously, bioavailability of carotenoids has been assessed by analysing serum, following administration of (13) C-labelled carotenoids [218]. However, more recently, a less invasive method has been used, involving measurement of faecal carotenoid levels [223].

1.2.5 Food sources of carotenoids:
Generally, carotenoids are found in some fruits and dark green, yellow and orange vegetables [136]. Consumption of fruits and vegetables increases the concentration of circulating carotenoids [224]. The main sources of specific carotenoids found in the diet include [138]:

β-carotene: carrots, dried apricots, dried peach, tomatoes, spinach and dill
α-carotene: carrots and pumpkin
Lutein + Zeaxanthin: kale, parsley, spinach, green mustard, dill and celery
Lycopene: tomatoes: watermelon, guava, grapefruit and apricot

Table 1.2.5.1 provides carotenoid content of some selected foods [138].

Table 1.2.5.1 Carotenoid contents of some selected fruits and vegetables (µg/100g):

<table>
<thead>
<tr>
<th>Food</th>
<th>β-carotene</th>
<th>α-carotene</th>
<th>Lutein+Zeaxanthin</th>
<th>Lycopene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apricot</td>
<td>3524</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Banana</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Broccoli</td>
<td>700</td>
<td>1</td>
<td>1900</td>
<td>0</td>
</tr>
<tr>
<td>Carrot</td>
<td>7900</td>
<td>3600</td>
<td>260</td>
<td>0</td>
</tr>
<tr>
<td>Dill</td>
<td>4500</td>
<td>0</td>
<td>6700</td>
<td>0</td>
</tr>
<tr>
<td>Mango</td>
<td>1300</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nectarine</td>
<td>103</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Peach</td>
<td>99</td>
<td>1</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Pumpkin</td>
<td>3100</td>
<td>3800</td>
<td>1500</td>
<td>0</td>
</tr>
<tr>
<td>Potato, cooked</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spinach</td>
<td>4100</td>
<td>0</td>
<td>10200</td>
<td>0</td>
</tr>
<tr>
<td>Tomato paste</td>
<td>1700</td>
<td>NA</td>
<td>NA</td>
<td>6500</td>
</tr>
<tr>
<td>Tomato juice</td>
<td>900</td>
<td>NA</td>
<td>NA</td>
<td>8500</td>
</tr>
<tr>
<td>Tomato raw</td>
<td>520</td>
<td>NA</td>
<td>100</td>
<td>3100</td>
</tr>
<tr>
<td>Watermelon</td>
<td>230</td>
<td>1</td>
<td>14</td>
<td>4100</td>
</tr>
</tbody>
</table>

Food sources of lycopene:
Tomatoes are one of the most important dietary sources of lycopene and more than 85% of lycopene is consumed by tomato products [225, 226]. Circulating lycopene levels increase in a dose-dependent manner after consumption of tomato based foods [227, 228]. Some studies show the importance of how a food is prepared and consumed in determining the bioavailability of dietary carotenoids such as lycopene. For example, is has been shown that the absorption of lycopene is increased when it cooked in fats such as olive oil [229]. Recently it has been reported that passionfruit could be an alternative source of lycopene for those people who do not eat tomatoes and tomato products more often [230]. The carotenoid content of various foods are listed by USDA [231].
1.2.6 Carotenoid supplements available in the Australian market:
There are varieties of carotenoid supplements available in the Australian market. Table 1.2.6.1 shows examples of supplements including the name of the product, their doses and the price (all the information about the price of the products have been collected in 2007).

*Table 1.2.6.1 Carotenoid supplements available in Australian market:*

<table>
<thead>
<tr>
<th>Trade name of the product</th>
<th>Kind of supplement</th>
<th>Dosage (mg)</th>
<th>Price (in Australian dollars)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doctor’s trust</td>
<td>Capsule (of lycopene)</td>
<td>15</td>
<td>120 capsule / $14.40</td>
</tr>
<tr>
<td>Twin Lab</td>
<td>Soft gel (of lycopene)</td>
<td>10</td>
<td>60 capsule / $15.00</td>
</tr>
<tr>
<td>NOW foods</td>
<td>Soft gel (of lycopene)</td>
<td>10</td>
<td>60 capsule / $10.60</td>
</tr>
<tr>
<td>Pure Encapsulation</td>
<td>Soft gel (of lycopene)</td>
<td>5</td>
<td>100 capsule / $12.00</td>
</tr>
<tr>
<td>Lycopene plus</td>
<td>Capsule (of lycopene)</td>
<td>NA</td>
<td>60 capsule / $21.55</td>
</tr>
<tr>
<td>Nature’s way Lycopene</td>
<td>Soft gel (of lycopene)</td>
<td>NA</td>
<td>60 soft gel / $10.80</td>
</tr>
<tr>
<td>Lycomato (from VITA HEALTHCARE)</td>
<td>Capsule (of lycopene)</td>
<td>15</td>
<td>60 capsule / $9.99</td>
</tr>
<tr>
<td>Blackmore, β-carotene</td>
<td>Capsule (of β-carotene)</td>
<td>6 mg</td>
<td>50 capsule / $6.58</td>
</tr>
<tr>
<td>Nature’s own, β-carotene</td>
<td>Tablet (of β-carotene)</td>
<td>6 mg</td>
<td>75 tablet / $7.95</td>
</tr>
<tr>
<td>Pretorious, β-carotene</td>
<td>Tablet (of β-carotene)</td>
<td>NA</td>
<td>60 tablet / $30.00</td>
</tr>
<tr>
<td>Super Carotene Complete</td>
<td>Tablet (mixed carotenoids)</td>
<td>12.5 mg</td>
<td>60 tablet / $14.85</td>
</tr>
<tr>
<td>Blackmore, Lutein</td>
<td>Capsule (of lutein)</td>
<td>6mg</td>
<td>60 capsule / $19.85</td>
</tr>
</tbody>
</table>

1.2.7 Recommended daily amounts for carotenoids:
There are no daily recommended allowances or upper limits for carotenoids. Some researchers believe that it is better not to set upper limits for carotenoids, to urge people to use caution before taking carotenoid supplements in high doses and to recommend supplementation only to prevent or control a vitamin A deficiency [232]. The recommended dietary allowances (RDA) for vitamin A (for which β-carotene is the precursor) for children and adults (µg/day) is listed below (according to dietary
reference intakes (DRIs) of the Food and Nutrition Board, Institute of Medicine, National Academy, 2001).

Table 1.2.7.1 Recommended dietary allowances (RDA, USA) for vitamin A:

<table>
<thead>
<tr>
<th>Age group</th>
<th>Children</th>
<th>Men</th>
<th>Women</th>
<th>Pregnancy</th>
<th>Lactation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>300µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-8</td>
<td>400µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-13</td>
<td>600µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-18</td>
<td>900µg</td>
<td>700µg</td>
<td>750µg</td>
<td>1200µg</td>
<td></td>
</tr>
<tr>
<td>19+</td>
<td>900µg</td>
<td>700µg</td>
<td>770µg</td>
<td>1300µg</td>
<td></td>
</tr>
</tbody>
</table>

Note: RDI for vitamin A for Australian adults is 750 micrograms Retinol Equivalents per day.

1.2.8 The mean intake of carotenoids:
Table 1.2.8.1 shows the mean intake of some carotenoids found by different studies undertaken in different countries:

Table 1.2.8.1 Mean intake of some carotenoids found by different studies:

<table>
<thead>
<tr>
<th>Carotenoids:</th>
<th>Australian</th>
<th>Intake in Spain</th>
<th>Intake in USA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lycopene</td>
<td>3741µg/day [233]</td>
<td>780µg/day [234]</td>
<td>1040 (F)-1320 (M)µg/d [235]</td>
</tr>
<tr>
<td>β-carotene</td>
<td>7301µg/day [233]</td>
<td>2113µg/day [234]</td>
<td>2698µg/d [236]</td>
</tr>
<tr>
<td>α-carotene</td>
<td>2675µg/day [233]</td>
<td>245µg/day [234]</td>
<td></td>
</tr>
</tbody>
</table>

1.2.9 Toxic effects of carotenoids:
Carotenoids are micronutrients contained in a wide variety of fruit and vegetables and thus a regular low-dose intake from natural sources is normally assured. There has been no detectable toxicity found with carotenoid supplementation, even at very large doses among humans [237] and experimental animals [238, 239]. Due to (a) decreased carotenoids absorbance from the intestine when consumed in large amounts, and (b) decreased rate of carotenoid cleavage into vitamin A, when consumed in high doses, there is no serious problem accrued as a result of high dose intake of carotenoids, except
yellowish skin [240]. American Food and Drug Administration has recently approved lycopene as a safe natural coloring agent (GRAS Notice No. GRN 000185).

1.2.10 Circulating concentration of carotenoids:
It has been reported that the level of total carotenoids in the whole blood and plasma of healthy people are 821µg/L and 888µg/L respectively [241]. Plasma lycopene concentration is from 0.14µM/L [242], to 0.25µM/L [243]. That is equivalent to 0.8 to 1.34µg/mL.
1.3 Long chain n-3 polyunsaturated fatty acids (LCn-3PUFA):

1.3.1 Lipids and fatty acids in general:
Lipids are diverse group of chemical compounds, sharing the characteristic of being insoluble in water, but soluble in organic solvents [244]. Triglycerides (TG) are the most common type of lipids found in the body and food. Each TG molecule is comprised of a glycerol backbone with three fatty acids attached and are stored in the adipose tissue for use during dietary deficits [245]. Phospholipids are polar lipids that comprise integral part of cellular and tissue membranes and are involved in signal transduction [136]. Sterols are also classified as lipids and can exist in un-esterified form in the membranes or as esterified transport/storage form [136]. Fatty acids are the common component in TG, phospholipids and sterol esters but may also exist as free (unesterified) fatty acids [244]. These consist of carbon atoms, flanked by hydrogen atoms and carboxyl group [245]. However, unlike carbohydrates or proteins, triglycerides have more carbon and hydrogen relative to oxygen. Therefore they can supply more energy per gram when they are catabolised (37 kJoule/g lipids compared to 17 kJoule/g carbohydrates and proteins) [244]. Fatty acids generally have 4-24 (even numbers of) carbons. One end of the fatty acid (alpha end) consists of a carboxyl group and the other end (“omega” or “n” end) consists of a methyl group [136]. Fatty acids may be divided to saturated or unsaturated. Unsaturated fatty acids have one (monounsaturated) or more (polyunsaturated) double bonds, however, saturated fatty acids have no double bonds [245]. The degree of unsaturation influences the solidity of fats at room temperature. Generally, polyunsaturated vegetable oils are liquid and more saturated animal fats are harder at room temperature. However, not all vegetable oils are polyunsaturated. Palm and coconut oils are saturated even though they are from vegetable origins [246]. Unsaturated fatty acids are named according to the position of the last double bond from the methyl group (“omega” or “n” end). Omega (ω) is a Greek word that means end. Therefore, an ω-3 fatty acid (also called n-3 fatty acid) for example, refers to a fatty acid in which there is a double bond on the third carbon from the methyl end of the molecule [244].

*Essential fatty acids:*
The human body is able to synthesise saturated fatty acids, as well as n-7 and n-9 monounsaturated fatty acids. However, due to lack of delta (Δ)12-desaturase and Δ15-
desaturase enzymes, the human body is not able to synthesise n-3 and n-6 polyunsaturated fatty acids [247]. Therefore these fatty acids must be obtained from the diet to avoid deficiency. The essential fatty acids (EFA) are α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6). Essential fatty acid deficiency in humans is rare, except in those with severe, untreated fat malabsorption, or those suffering from severe famine. Symptoms of deficiency include dry, cracked, scaly and bleeding skin [245]. Essential fatty acids are precursors of the n-6 and n-3 families of fatty acids and they can be converted to Lcn-3PUFA such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) [244], via elongation and desaturation pathways. This process will be discussed in Section 1.3.4 “Body synthesis and dietary sources of Lcn-3PUFA”.

1.3.2 What are long chain n-3 polyunsaturated fatty acids (LCn-3PUFA)?

LCn-3PUFA refers to fatty acids such as (1) docosahexaenoic acid (22:6 n-3; DHA) and (2) eicosapentaenoic acid (20:5 n-3; EPA) [244]. EPA has 20 carbons and five double bonds (20:5); and DHA has 22 carbons and six double bonds (22:6) [246]. Figures 1.3.2.1 and 1.3.2.2 show the schematic structure of EPA and DHA.

*Figure 1.3.2.1 Schematic structure of Eicosapentaenoic acid (EPA):*
Figure 1.3.2.2 Schematic structure of Docosahexaenoic acid (DHA):

1.3.3 Biological functions of LCn-3PUFA:

In addition to being a source of energy in the human body, LCn-3PUFA play a key role in a wide variety of physiological functions in the human body [248]. LCn-3PUFA are found in high concentrations in the brain and retina of mammals [249-251], and may contribute to learning and memory (in the brain) and also to visual function (in the retina) [252, 253]. The benefit of LCn-3PUFA supplementation has been demonstrated in many studies [254], with possible beneficial effects in terms of coronary heart disease [255, 256], plasma lipid profiles [257-259], reduction of depression risk [260], increased arterial elasticity [261], decreased inflammation in cystic fibrosis [262] and asthma [263] and also probable physiological improvement in Alzheimer disease [264, 265], schizophrenia [266], rheumatoid arthritis [267], Crohn's disease [268] and different types of cancers [269-272]. It has also been reported that oral supplementation of LCn-3PUFA may help to improve weight loss programs [273, 274].

LCn-3PUFA are also converted to eicosanoids such as prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT), that play an important regulatory role on the human body [245]. Depending on the fatty acid substrate, different series of eicosanoids are produced [244]. It has been demonstrated that particularly EPA and to a lesser extent DHA [275] compete with AA, to produce eicosanoids that are less potent than those produced by AA and have less inflammatory and chemoattractive effects [276, 277]. As shown in figure 1.3.3.1, AA is converted to 2 series of PG and TX and also 4
series of LT. However, EPA converts to 3 series of PG and TX and 5 series of LT [245, 278]. The effect of increased incorporation of LCn-3PUFA in the cellular membrane is blocking the inflammatory pathway of AA, thus decreasing production of potent eicosanoids which have more inflammatory effects. Two pathways of lipoxygenase (LOX) and cyclooxygenase (COX) are presented briefly in figure 1.3.3.1 [245, 278, 279]. This figure also shows the pro-inflammatory activities of eicosanoids produced by AA.
Figure 1.3.3.1 LOX and COX pathways leading eicosanoid production from different fatty acids [245, 278, 279]:

1.3.4 Body synthesis and dietary sources of LCn-3PUFA:

EPA and DHA are synthesized from α-linolenic acid (figure 1.3.4.1) [244, 246, 280] which is an essential fatty acid. However, this synthesis may not be efficient in humans (with the conversion efficiency of 9%), therefore, consumption of preformed EPA and DHA may be preferable [275]. Besides synthesis of EPA and DHA from essential fatty acids *in vivo*, they can be obtained from the diet, in foods such as seafood (specially oily fish), human milk [246, 248], red meat and eggs [281]. Fish oil contains considerable amount of LCn-3PUFA (approximately 8-20% by weight) [282].

![Diagram of LOX and COX pathways leading eicosanoid production from different fatty acids](image-url)
1.3.5 Recommended daily amounts of LCn-3PUFA:
British Department of Health recommends 210mg of EPA and DHA per day. That could be obtained from 60g of fish [244].

1.3.6 LCn-3PUFA and inflammation:
The effects of LCn-3PUFA on inflammation can be presented as their effect on (a) eicosanoid production, (b) cytokine profile and (c) immune cell function.

Effects of LCn-3PUFA on inflammation via their effect on eicosanoids:
LCn-3PUFAs are converted to eicosanoids that play an important regulatory role in human body [244]. Eicosanoids of 2 series can mediate inflammation and chemoattraction of immune cells [277, 283]. It has been shown that supplementation of EPA and DHA decreases LTB₄ produced by leukocytes [284]. It has also been reported that DHA [285] and EPA [286] suppress the activation of cyclooxygenase-2 (COX-2), that converts AA to PG₂ and TX₂. The probable mechanism for this suppression is related to inactivation of toll-like receptor-2 (TLR2) [285, 287]. The net effect of increasing the LCn-3PUFA contents of cellular membrane is a reduction in the inflammatory pathway of AA [275, 277].
Effects of LCn-3PUFA on inflammation via their effect on cytokines profile:

There is evidence that increased levels of LCn-3PUFA in cellular membranes decreases cytokine production and also minimizes the response to pro-inflammatory cytokines [277, 288, 289]. It has been reported that oral supplementation of LCn-3PUFA in humans decreases the systemic inflammation, with reduced levels of acute inflammatory biomarkers such as CRP and IL-6 being observed [290]. This effect of LCn-3PUFA has been confirmed by an in vitro study of human mononuclear cells, in which LCn-3PUFA supplementation decreased the production of IL-6 [291]. In vivo studies in mice also showed decreased circulating levels of IL-6, IL-10, TNF-α [292-294], IL-12, IL-1β and IFN-γ [293] due to the consumption of high LCn-3PUFA. Interestingly, it has been shown that mice with a high intake of LCn-3PUFA have a decreased systemic response including IL-12 and IFN-γ, following infection with Listeria monocytogenes [293]. However, supplementation with LCn-3PUFA did not reduce in a change in IL-6 production in lipopolysaccharide (LPS) induced monocytes [295]. Conversely, it has also been found that n-6 fatty acids such as AA have a pro-inflammatory effect that is opposite to the effect of LCn-3PUFA such as EPA and DHA [296-298]. To some extent, the anti-inflammatory effects of LCn-3PUFA are is attributable to their potent effect on suppression of nuclear factor-κB (NF-κB) [286, 299-301], which has been shown to regulate a wide range of pro-inflammatory cytokines (reviewed in [124]) such as IL-6 and IL-8 [125, 126]. It has been reported that LCn-3PUFA suppresses NF-κB via inactivation of toll-like receptors (TLR2, 3, 4, 5, or 9) [285, 287, 301], blocking I-κB (the inhibitor of NF-κB) degradation and also blocking the mitogen-activated protein kinase (MAPK) [299]. Recently, it has been found that DHA is more potent than EPA in suppression of NF-κB [300].

Effects of LCn-3PUFA on inflammation via their effect on immune cell function:

It has been reported that the supplementation with fish oil (at levels where fish oil provides more than 20% of total fatty acids in the diet), decreases lymphocytes proliferation, natural killer cell and monocytes activity and neutrophil and monocyte chemotaxis [278, 288, 289, 291, 302, 303]. However, it has been shown that even at lower supplementation levels (in which EPA and DHA provided 4.4% of total fatty acids or 1.7% of dietary daily energy) some of these effects were still observed [304]. It has been demonstrated that these lower levels of EPA and DHA supplementation, which
are comparable to levels observed in a normal balanced human diet [244], inhibit lymphocyte proliferation. Furthermore, dietary EPA but not DHA, inhibits natural killer cell activity [305]. The anti-inflammatory mechanisms of LCn-3PUFA, which involve modification of eicosanoid production and suppression of NF-κB are summarised in Figure 1.3.6.1.

**Figure 1.3.6.1 Summary of the anti-inflammatory effect of LCn-3PUFA:**

**LCn-3PUFA and oxidative stress:**

While there is much evidence regarding the anti-inflammatory benefits of DHA and EPA [275], there is a growing concern that increased DHA and EPA in the cellular membrane results in increased susceptibility of the cells to lipid peroxidation [306] due to increased oxidizability of highly unsaturated fatty acids in the cell membrane [307]. The extent of this effect however is uncertain and it has been suggested that while this increased susceptibility may occur in *in vitro* studies, it may not occur in the human body which has complex and multi-factorial based immunological mechanisms [275]. It has been shown that while EPA decreases inflammation in rats with induced ulcerative colitis, it decreases the colonic antioxidant defences and promotes oxidative injury at the site of inflammation [308]. Conversely it has been shown that the concentration of
F₂-isoprostanes, which are a marker of lipid peroxidation, are decreased in vivo after high intake of DHA and EPA [309-311]. Thus the role of EPA and DHA in promoting oxidative stress in vivo remains unclear.

1.3.7 LC n-3 PUFA and asthma:
With the knowledge that n-6 fatty acids such as AA produce inflammatory mediators that are more biologically active than those produced by LCn-3PUFA [277, 278] and also the fact that asthma is a chronic inflammatory disease [312-314], it is logical to assume that supplementation with LCn-3PUFA may attenuate asthma symptoms. However, studies to date have produced variable results making it difficult to make firm recommendations on either supplementation or dietary modification. A number of clinical trials in asthma have been reported, but the results are inconsistent [315-317]. It has been shown that daily supplementation of LCn-3PUFA improved the asthma symptoms in asthmatic children [318]. However, other studies found that LCn-3PUFA did not have any beneficial effects on asthma symptoms [263], exhaled nitric oxide levels, asthma control and lung functions [319]. An immunomodulatory effect of the n-6:n-3 fatty acid ratio in the diet has been shown on the presence of childhood asthma [320]. However, Reisman et al, in a recent meta-analysis, reported that it is hard to show any beneficial effect of LCn-3PUFA on asthma, and further studies were needed to clarify the potential benefit of LCn-3PUFA supplementation in asthma [321].
1.4 Inflammation

1.4.1 Human immune system in brief:
The immune system in human body defends against infectious agents and foreign substances, via different mechanisms including physical barriers such as skin, protective substances in the blood and tissue fluids and the physiologic reactions of tissue to injury or infection [322]. Immunology deals with how the body distinguishes between what is self and what is non-self [323]. Our bodies have a specialised system to attack different infectious and toxic agents. This system is composed of certain blood leukocytes and tissues cells derived from the tissue leukocytes [324]. These cells work together in two ways to attack invaders: (a) via killing the invading pathogens (by phagocytosis) and (b) via forming antibodies and sensitized lymphocytes. One or both of these responses may destroy or inactivate the invaders [325].

Immunity may be innate (natural) or acquired (adaptive) [324, 326]. Innate immunity refers to those elements which an individual is born with, which include the external barriers such as skin and mucous membranes and also phagocytic cells which engulf and digest the microorganisms [327]. These kinds of mechanisms are not enhanced by previous exposure nor do they discriminate between most foreign substances [328]. The more specialized immune system is called specific acquired immunity (mainly involved with complement pathway) in which the initial contact with the foreign agent (immunization) triggers a chain of events that leads to the activation of certain cells (lymphocytes) and the synthesis of proteins which exhibit specific reactivity against the specific foreign invaders [323, 324, 327]. Weir and Stewart [328] compared innate and acquired immunity as shown in table 1.4.1.1.
**Table 1.4.1.1 Characteristics and determinants of innate and acquired immunity [328]:**

<table>
<thead>
<tr>
<th>Innate immunity</th>
<th>Acquired immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>● Non-specific</td>
<td>● Specific</td>
</tr>
<tr>
<td>● No change with repeated exposure</td>
<td>● Memory</td>
</tr>
<tr>
<td>● Mechanical barriers</td>
<td></td>
</tr>
<tr>
<td>● Bactericidal substances</td>
<td></td>
</tr>
<tr>
<td>● Natural flora</td>
<td></td>
</tr>
<tr>
<td><strong>Homoral</strong></td>
<td></td>
</tr>
<tr>
<td>● Acute phase proteins</td>
<td>● Antibody</td>
</tr>
<tr>
<td>● Lysosome</td>
<td></td>
</tr>
<tr>
<td>● Complement</td>
<td></td>
</tr>
<tr>
<td><strong>Cell-mediated</strong></td>
<td></td>
</tr>
<tr>
<td>● Natural killer cells</td>
<td>● T-lymphocytes</td>
</tr>
<tr>
<td>● Phagocytes</td>
<td></td>
</tr>
</tbody>
</table>

The bone marrow is the source of the precursor cells that increase the cellular constituents of the immune system, also the liver is a site of immune cell development [324]. There are four lines of defence against invading pathogens by neutrophils and macrophages; (1) phagocytic action of macrophages in the tissue, (2) neutrophil invasion, diapedesis and chemotaxis, (3) second macrophage invasion and (4) increased macrophages and granulocytes in the bone marrow [326]. Regarding the last defence line, inflammatory mediators such as cytokines, tumour necrosis factor-α (TNF-α), granulocyte colony stimulation factor (G-CSF), monocyte colony stimulation factor (M-CSF) and granulocyte-monocyte colony stimulation factor (GM-CSF) are released from inflammatory cells and theses inflammatory mediators can increase the production of granulocytes and monocytes in bone marrow [325]. T-lymphocytes also release cytokines such as IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF and IFN-γ that regulate the immune system via their effects on other cells of the immune system as well as bone marrow [325]. All of these events protect the human body against invading pathogens. Figure 1.4.1.2 schematically shows whether bone marrow controls granulocyte production in response to multiple growth factors released from activated macrophages in an inflamed tissue [325].
1.4.2 Inflammation and its physiology:
Inflammatory responses are triggered by infectious invaders and these responses are amplified by production of reactive oxygen species (ROS), lipid mediators and cytokines [329]. Inflammation is a complex process initiated by tissue damage caused by endogenous as well as exogenous factors, include mechanical (e.g. cut), physical (e.g. burn), chemical (e.g. exposure to a corrosive chemical) and biological injuries (e.g. hypersensitivity reactions) [323, 330]. Regardless of the type of tissue damage, multiple substances are released by the injured tissues causing dramatic secondary changes in the tissues [325].

The cells involved in the immune system are widely distributed throughout the body, but if an infection occurs it is necessary to concentrate them and their products at the site of infection [327]. Three major events occur during infection: (1) increased blood supply to the infected area, (2) increased capillary permeability and (3) increased leukocyte migration to the infected area [331]. These processes are initiated within
minutes but consequences can remain for weeks to months [330]. The inflammatory response constitutes an important part of both innate and acquired immunity and it has been developed as a protective response against injury and infection [327]. Although, in certain cases such as hypersensitivity, inflammation becomes a problem rather than a solution in infection [323]. As table 1.4.2.1 shows, inflammatory responses are mediated by the immune system can be divided into four categories, based on pathogenesis [332-334].

**Table 1.4.2.1 Categories of inflammation [332-334]:**

<table>
<thead>
<tr>
<th>Category</th>
<th>Immune recognition component</th>
<th>Soluble mediators</th>
<th>Time course</th>
<th>Cellular response</th>
<th>Clinical example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allergic (reaginic)</td>
<td>IgE</td>
<td>Mast cell/basophil products</td>
<td>Seconds to Minutes</td>
<td>Neutrophils, eosinophils; smooth muscle constriction</td>
<td>Atopy, asthma, Seasonal rhinitis</td>
</tr>
<tr>
<td>Viral infection</td>
<td>RIG-I/MDA5</td>
<td>RIG-1/MDA5</td>
<td>Minutes</td>
<td>Antiviral response</td>
<td>Common cold</td>
</tr>
<tr>
<td></td>
<td>TLR 3, 7, 8, 9</td>
<td>TLR 3, 7, 8, 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cytokines Chemokines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytotoxic antibody</td>
<td>IgG, IgM</td>
<td>Complement</td>
<td>Hours to 1 day</td>
<td>Acute inflammation (neutrophils); lysis or phagocytosis of antigens</td>
<td>Autoimmune haemolytic anemia, thrombocytopenia</td>
</tr>
<tr>
<td>Immune complex</td>
<td>IgG, IgM</td>
<td>Complement</td>
<td>Hours to 1 day</td>
<td>Accumulation of neutrophils, eosinophils, and macrophages</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Chronic inflammation</td>
<td>T cells</td>
<td>Lymphokines, monokines</td>
<td>2-3 days</td>
<td>Lymphocytes, macrophages</td>
<td>Granulomatous disease, tuberculosis</td>
</tr>
<tr>
<td>Delayed-Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypersensitivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After attacking the invading pathogen in the tissue, white blood cells enter the tissue spaces by diapedesis, move through by ameboid motion and attract to the inflamed tissue area by chemotaxis [325]. At the site of inflammation, neutrophils adhere to the walls of postcapillary venules and diapedese between the endothelial cells into the connective tissue matrix and exert their effects by releasing the content of their cytoplasmic granules, which contain hydrolytic enzymes and reactive oxygen species (ROS) [330]. Neutrophils are the phagocytic cells especially effective against bacteria.
Table 1.4.2.2 presents neutrophilic involvement in viral infections [335].

Table 1.4.2.2 Summary of neutrophilic involvement in viral infections [335]:

<table>
<thead>
<tr>
<th>Neutrophilic response after viral infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Increased neutrophil immigration to the site of infection due to increased chemokines</td>
</tr>
<tr>
<td>2) Up-regulation of adhesion molecules on neutrophils and epithelial cells</td>
</tr>
<tr>
<td>3) Increased epithelial cell damage</td>
</tr>
<tr>
<td>4) Increased neutrophils apoptosis to prevent further epithelial cell damage</td>
</tr>
</tbody>
</table>

Mast cells that located in perivascular connective tissue throughout the body, are most commonly known for their role in type I hypersensitivity reactions or immediate hypersensitivity [330]. Eosinophils are involved in allergic responses, as they are particularly active in parasitic infections [330]. The systemic inflammatory response to infection includes (1) the induction of swelling of the tissue cells and walling off the injured area from remaining tissues, (2) increased white blood cell accumulation in the injured area (especially neutrophils and macrophages) and their production by the bone marrow, (3) increased hydrocortisone, (4) increased adrenocorticoid hormone (ACTH), (5) increased C-reactive protein (CRP), (6) increased cytokines especially interleukins-1 (IL-1), interleukins-6 (IL-6) and tumour necrosis factor-α (TNF-α), (7) accumulation of fluids and leukocytic cells in injured areas, (8) vasodilatation, (9) increased permeability of the capillaries, (10) clotting of fluids, (11) migration of granulocytes and monocytes into the tissues and (12) fever [323, 325]. Increasing the production of some cytokines is dependant to the activation of nuclear factor-κB (NF-κB) which plays an important role in the production of wide range of inflammatory biomarkers (reviewed in [124]) including IL-6, and IL-8 [125, 126, 336]. Thus, NF-κB mediates immune response [337].

1.4.3 A closer look at neutrophils:
Neutrophils are the most abundant white blood cells; they consist of 70% of total white blood cells [330] and they are one of the most important compounds of the human immune system, which are normally found in the blood [332]. Neutrophils have a
diameter of 12-15 µm in peripheral blood smears [338] and an estimated life span of 6-8 hours in peripheral blood [339]. Neutrophils are produced via myeloid progenitor pathway from the stem cells [328]. The totipotent stem cells are the progenitors for all blood cells, including lymphocytes [339]. During the acute phase of inflammation, neutrophils leave the vasculature and migrate toward the site of inflammation in a process called chemotaxis [330]. The receptors on neutrophil surface are able to detect chemical gradients of chemotactic molecules such as interleukin-8 (IL-8), interferon-gamma (INF-γ) and complement component-5a (C5a) [340]. Neutrophils can release cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-8 (IL-8), which causes additional neutrophil recruitment [341]. It has been shown that IL-8 may associate with neutrophil recruitment and activation, in which there is a dose-dependent migration of neutrophils in response to IL-8 [342]. There are also other growth factors for bone marrow haematopoietic stem cells such as interleukin-1 (IL-1), interleukin-4 (IL-4) and interleukin-6 (IL-6). These biomarkers act preferentially on early progenitor cells and they stimulate cell differentiation and act synergistically with other molecules [343].

At the time of leaving the bone marrow, neutrophils are completely differentiated and equipped with the complete spectrum of surface receptors and intracytoplasmic granules with their secretions [343]. Neutrophils are active phagocytes, capable of ingesting the invading microorganisms or particles [332]. However, they can only execute one phagocytic event, expending all of their glucose reserves in an extremely vigorous "respiratory burst", which involves the activation of an NADPH oxidase enzyme, that produces large quantities of superoxide, which generally kills the ingested organism [340]. Superoxide anion is both a one-electron reductant and oxidant that can pass through cell membranes via anion channels [344]. It is reported that superoxide exerts its toxic effects on target cells by penetration to important sites where it subsequently is converted to other toxic products [332]. Neutrophils also result in increased production of many inflammatory mediators with the capacity to help injured tissues by amplifying the inflammatory response [339, 345, 346]. Table 1.4.3.1 briefly show the list of mediators that are produced or stimulated by neutrophils [332].
Table 1.4.3.1 Inflammatory mediators produced or stimulated by neutrophils:

<table>
<thead>
<tr>
<th>Mediators</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic enzymes</td>
<td>Serine elastase, cathepsin G, proteinase 3, collagenase, gelatinase</td>
</tr>
<tr>
<td>Microbicidal protein</td>
<td>Lactoferrin, defensine, bacterial permeability-increasing protein, lysosome</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>Lipid mediators</td>
<td>LTB₄, PAF, TXA₂</td>
</tr>
<tr>
<td>Oxygen radicals</td>
<td>Superoxide anion, hydrogen peroxide, radical hydroxyl</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IL-1β, IL-6, IL-8, TNF-α, TGF-β1</td>
</tr>
</tbody>
</table>

1.4.4 A closer look at the chemotaxis:
Chemotaxis is a directed migration along a concentration gradient, as opposed to chemokinesis or cytokinesis, which involve random cell movement [330]. Cells are diverted from the vascular system to sites of tissue damage laterally [347]. This is mainly due to the interaction of a chemotactic factor with a receptor on the surface of the leukocytes [348]. Many different chemical substances in the tissue cause both neutrophils and macrophages to move toward the source of the inflammation [332]. Some of these products include (a) bacterial or viral toxins, (b) degenerative products of the inflamed tissues themselves, (c) several reaction products of the complement complex such as C5a that is activated in inflamed tissue and (d) several reaction products caused by plasma clotting in the inflamed area [325, 330].

1.4.5 Inflammation in asthma:
Airway inflammation is the underlying pathophysiology of asthma [323, 349-351]. A wide range of mediators and immune cells are involved in the pathophysiology of asthma [351, 352]. A large growing body of studies have confirmed an increased number of inflammatory cells including eosinophils, mast cells, T lymphocytes, neutrophils and macrophages in the bronchoalveolar lavage (BAL) of the airways of asthmatics [352-356]. The role of these cells in chronic airway inflammation is well known, specifically their ability to produce inflammatory biomarkers, that may affect the airways directly [357, 358]. However, the precise mechanism by which
inflammatory cells and their mediators cause airway hyper-responsiveness in asthma remains incompletely understood [353]. Recently, the key role of neutrophils in asthma is added to our current knowledge of eosinophilic asthma [352, 354]. Further information about different phenotypes of asthma (neutrophilic and eosinophilic asthma) is provided in asthma section of current chapter. Eosinophils are one of the most abundant inflammatory cells found in epithelial and submucosal layers [351]. It has been reviewed that 50% of asthma cases are attributed to eosinophilic airway inflammation [354]. Activated eosinophils are recruited from the blood [358] and cause tissue damage in the airway layers [353]. They also produce cytokines [359], which result in further production of cytokines by epithelial cells [357].

It has been reported that mast cells are increased in the broncho-alveolar lavage (BAL) and they associate with asthma severity [358]. It has been also shown that mast cells are immuno-reactive to some inflammatory biomarkers such as IL-3, IL-4, IL-5, IL-6 and GM-CSF [360]. Recently it has been shown that increased mast cells are located within airway smooth muscles [355, 356]. Macrophages release cytokines such as TNF-α, IL-1, IL-6, IL-8, INF-γ and also remove the cellular debris from the site of inflammation [361]. They also produce ROS to attack the invader [361]. It has been proposed that airway inflammation can be induced by exposure to ROS, or alternatively ROS can be produced by epithelial cells, macrophages and/or neutrophils during inflammatory events [362].

1.4.6 Rhinovirus:
Rhinovirus (RV) is the most common cause of the common cold [363] and the major cause of an exacerbation in asthma among adults [364] and children [364, 365]. Over one hundred different RV serotypes have been identified [363]. RVs have single-stranded and linear RNA and they are divided into two subclasses; major and minor. Major group binds to intercellular adhesion molecule-1 (ICAM-1) receptor and minor group binds to the low density lipoprotein (LDL) receptor of target cells [366, 367]. This binding results in internalisation of virus particles into the cytoplasm of the cells [368], where the RV replicates [369]. RVs are transmitted via direct contact [370] and they target epithelial cells [371] and initiate innate immune responses [372, 373] locally and systemically [372]. The presence of viral RNA (vRNA) (due to replication of single stranded RNA) results in transcription of interferons which may induce apoptosis [374].
After RV infection, large numbers of neutrophils and lymphocytes are recruited to the airways [375]. As a result, epithelial cells produce various cytokines, chemokines and other inflammatory mediators that might contribute to the host defence and inflammatory responses [376]. Some of these inflammatory molecules include interleukin-1 (IL-1) [377], IL-6 [125, 371, 378], IL-8 [126, 371, 378-381], IL-11 [382], granulocyte-macrophage colony-stimulating factor (GM-CSF) [371, 381], RANTES [380, 383], intercellular adhesion molecule-1 (ICAM-1) [377, 384-386] and interferon-gamma induced protein-10 (IP-10) [376]. RV infection increases oxidative stress in airway epithelial cells most probably via increased production of ROS [337, 387]. Increased production of ROS activates nuclear factor-κB (NF-κB) [337], that is a protein transcription factor which mediates the transcription of inflammatory biomarkers [124] such as interleukin-6 (IL-6), and IL-8 [124-126]. There is an strong evidence regarding increased activation of NF-κB after RV infection [125, 126, 337].

1.4.7 Inflammatory mediators:
Many of the individual events in inflammation are controlled by cytokines or other small regulatory molecules [332]. Cytokines are soluble molecules that mediate cell-to-cell interactions and communications [332]. Commonly measured cytokines include IL-2, TNF-α and IFN-γ, IL-4, IL-5, IL-6, IL-8 and IL-10 [388]. Mediators may affect directly and/or indirectly via stimulation of other mediators [389]. Table 1.4.7.1 shows the main inflammatory mediators in human body [326]:
Table 1.4.7.1 Main inflammatory mediators in human body [326]:

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Main source</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>Mast cells, basophils</td>
<td>Vasodilation, increased vascular permeability, contraction of smooth muscle</td>
</tr>
<tr>
<td>Kinins (e.g. bradykinin)</td>
<td>Plasma</td>
<td>Vasodilation, increased vascular permeability, contraction of smooth muscle, pain</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>Neutrophils, eosinophils, monocytes, platelets</td>
<td>Vasodilation, increased vascular permeability, pain</td>
</tr>
<tr>
<td>Leukotrienes</td>
<td>Neutrophils, mast cells, basophils</td>
<td>Vasodilation, increased vascular permeability, contraction of smooth muscle, induce cell adherence and chemotaxis</td>
</tr>
<tr>
<td>Complement component (e.g. C3a, C5a)</td>
<td>Plasma</td>
<td>Cause mast cells to release mediators C5a as a chemotactic factor</td>
</tr>
<tr>
<td>Plasmin</td>
<td>Plasma</td>
<td>Break down fibrin, kinin formation</td>
</tr>
<tr>
<td>Cytokines and chemokines</td>
<td>Lymphocytes, macrophages, and epithelial cells</td>
<td>Chemotactic factors, colony stimulation factors, macrophage activation</td>
</tr>
</tbody>
</table>

**Interleukin-6:**
Interleukin-6 (IL-6) is a cytokine that acts as one of the central factors of growth and differentiation of immune cells [390-392]. IL-6 is an acute inflammatory response agent [373]. IL-6 is also known as interferon-2, IL-1-inducible 26 KD protein, hepatocyte stimulating factor, cytotoxic T cell differentiation factor, B cell differentiation factor (BCDF) and B cell stimulatory factor 2 (BSF2) [393]. IL-6 is produced by various cells such as macrophages, T cells, endothelial cells [394] and adipose tissue [395]. It may target many types of cells including B lymphoblasts, T cells and hepatocytes to induce an acute-phase reaction. A number of cytokines make up the IL-6 cytokine family, which is based on a helical cytokine structure and receptor subunit makeup [396, 397].
IL-6 functions as a growth factor for a wide variety of cells [398-402] and its production is generally correlated with cell immuno-activation [332].

**Interleukin-8:**
Interleukin-8 (IL-8) is a chemokine, which is considered as a “second-order” mediator that appears to be less poly-potent than “first-order” pro-inflammatory cytokines (such as IL-6), because it is not potent inducer of other cytokines and exhibits more specialized functions in inflammation and repair [394]. IL-8 is a potent neutrophil chemoattractant [403, 404], which causes the accumulation of neutrophils at the site of inflammation [404]. It is secreted by monocytes, neutrophils, fibroblast and epithelial cells in response to cytokines such as IL-1, IL-3, TNF-α and interferon-gamma costimulant [394]. The expression of the IL-8 and IL-6 genes is controlled by nuclear factor-κB (NF-κB) [124-126]. NF-κB starts transcription of these genes by being attached to DNA in the promoter regions of target genes [405].

**Tumour necrosis factor-α (TNF-α):**
TNF-α was discovered based on its ability to induce hemorrhagic necrosis in certain tumours *in vivo* [406]. TNF-α is a protein produced by activated macrophages and many other immune cells including T cells, B cells, endothelial cells and neutrophils [394]. The production of TNF-α is stimulated by variety of inflammatory biomarkers such as IL-1 [407], granulocyte-monocyte colony stimulation factor (GM-CSF) [408], interferon-gamma (IFN-γ) and TNF-α itself [394]. The action of TNF-α in the cells is comparable to the action of IL-1 [409], which they can increase the gene expression of NF-κB [124]. Neutrophils can react to TNF-α [410], however, TNF-α can be produced by neutrophils [403].

**C-reactive protein (CRP):**
C-reactive protein (CRP) was discovered by Tillet and Francis in 1930 [411]. CRP is an acute reactant inflammatory biomarker and its production increases generally in response to chronic and acute inflammation [412]. CRP is produced by the liver and its molecular weight is 1150D [413]. It has been reported that IL-1 can stimulate the production of CRP in the liver [414]. CRP binds to the cell wall of the invading organism and activates the classic complement pathway [415]. It has also been reported that CRP binds to neutrophils but reportedly it does not activate neutrophils [416].
Interferon-gamma induced protein-10 (IP-10):
IP-10 is a chemokine which is released by a wide variety of cells, particularly epithelial cells [417] in response to interferon-gamma (IFN-γ) [394]. IP-10 is a selective chemoattractant of natural killer cells, activated T cells [418] and monocytes [394]. The expression of IP-10 is associated with lymphocyte numbers [419]. IP-10 expression is reportedly high in the airway of asthmatics [420] and people suffering from chronic obstructive pulmonary disease (COPD) [418]. In vitro experiments show that rhinovirus infection can increase IP-10 expression and it is associated with viral titre [376]. However, the viral-replication-independent release of IP-10 has been reported previously [421]. IP-10 is also known as CXCL10 [376].

The role of NF-κB in cytokine production:
NF-κB is a nuclear protein transcription factor which is discovered in 1986 [422], as a factor that increases the transcription of wide range of genes such as growth factors, chemokines, cytokines and adhesion molecules [124]. NF-κB is activated by TNF-α, IL-1β, endotoxins and viral proteins [423]. NF-κB attaches to DNA in the promoter regions of target genes as a dimer composed of p50 and p65 [405, 424]. The activation of NF-κB is controlled by positive feedback loop (via IL-1β and TNF-α [425, 426]) as well as negative feedback (via the inhibitory effect of up-regulated IκB-α and p105 genes [427, 428]). IL-10 is also an extracellular inhibitor of NF-κB [429].

1.4.8 Nutrients and inflammation:
The nutritional status of the host plays an important role in the susceptibility of the body to the infection [430, 431]. There is a growing body of findings on the effect of antioxidant nutrients on the activation of nuclear factor-κB (NF-κB). NF-κB can increase the expression of selected cytokines and chemokines including IL-6 and IL-8, and promotes inflammation in the body [378, 432, 433]. Some of these antioxidant nutrients that have a demonstrated effect on NF-κB Lycopene [432, 434, 435], β-carotene [436] quercetin [434], vitamin C [437, 438] and vitamin E [437, 439, 440] are shown to decrease the inflammation via their probable redox-based effect on inactivation of NF-κB. It has also been found that there is a negative correlation between vitamin C and carotenoids with CRP and intercellular adhesion molecule-1 (ICAM-1) levels [441]. Studies show that inflammation considerably affects the level of
some nutrients in blood serum [442, 443]. During infection, the concentrations of retinol, tocopherol and carotenoids are decreased [444-446]. It has also been shown that the concentration of serum α-carotene, β-carotene and lycopene, but not lutein/zeaxanthin or β-cryptoxanthin is lower in inflammatory situations [445, 447]. It has been suggested that dietary supplementation with lycopene may be an effective approach to reduce the level of oxidative stress and improve the inflammatory status of colitis [448]. It has also been found that circulating levels of antioxidant nutrients have an inverse association with circulating IL-6 [449] and CRP concentrations [444, 450].

Carotenoids and inflammation:
Carotenoids are naturally found in vegetables and fruits [138]. In addition to be antioxidant [156-160], they are considered as immunomodulator, gap junction communicator [451] and activator of cell surface adhesion molecule genes [452]. Lycopene and β-carotene supplementation has been shown to be effective in reducing the level of oxidative stress and improving inflammatory status in colitis [448]. An inverse correlation also has been reported between β-carotene and inflammation [441, 444, 453]. A relationship between low β-carotene levels and increased burden of inflammation has been reported [450]. Other observed relationships include a strong and inverse association between serum β-carotene level and CRP levels [441, 454], as well as an inverse association between interleukin-6 and lycopene has been reported [449]. Another study demonstrated that oral supplementation of lutein/zeaxanthin decreases the effects of ultraviolet irradiation by reducing acute inflammatory responses and ultraviolet-induced hyper-proliferative rebound [211, 455, 456]. It has been also shown that low circulating carotenoid levels are associated with the inflammation in smokers [457]. Furthermore, serum lycopene has a positive association with allergen sensitization [458]. Lycopene as an anti-inflammatory agent [185, 448, 452, 459] reduces inflammatory biomarkers in vitro [432, 460] and in vivo [185]. Studies showed that dietary supplementation of lycopene may reduce oxidative stress and improve inflammatory status in colitis [448]. It has also been shown that lycopene reduces inflammatory signals in lateral prostate lobe [461] and inhibits the expression of inflammatory agents in hyperhomocysteinemic rats [462]. The mechanism by which carotenoids decrease the inflammation is related to their action on suppression of NF-κB. It has been found that β-carotene can inhibit NF-κB activity in LPS exposed cells. This effect of β-carotene on the redox-based NF-κB activation is attributed to the
antioxidant effect of β-carotene [436]. Lycopene has also been shown to suppress the NF-κB via decreased nuclear translocation of NF-κB p65 subunit in LPS-stimulated dendritic cells [432].

**Omega-3 fatty acids and inflammation:**
In 1980, Kromann and Green compared the prevalence of inflammatory disease among Eskimos in Greenland with age/gender-matched people from Denmark. They found a low prevalence of inflammatory disorders including coronary heart disease, asthma, multiple sclerosis and psoriasis among the people from Greenland [463]. Later this was attributed to anti-inflammatory effects of long chain n-3 polyunsaturated fatty acids (LCn-3PUFA) obtained from seafood dominated diets [464]. In the 1980s many studies confirmed the anti-inflammatory properties of LCn-3PUFA [277]. The essential roles of LCn-3PUFA in normal growth and development, as well as their preventive effects on coronary disease, cancer, arthritis and hypertension have been well described now [465]. It is believed that an imbalanced circulating ratio of n-6/n-3 long chain PUFA results in an overproduction of inflammatory biomarkers [466]. Simopoulos et al (2002) described how EPA competes with arachidonic acid (AA) to produce leukotriene and prostaglandin via lipoxygenase and cyclooxygenase pathways [277]. Leukotrienes produced by EPA (series-3) are less active compared to leukotriene produced by arachidonic acid (series-2) [283, 297]. It has also been reported that supplementation with LCn-3PUFA decreases interleukin-1 (IL-1) and tumour necrosis factor (TNF) produced by monocytes in vivo [467] and by macrophages in vitro [468, 469]. These cytokines have a key role in inflammatory functions of the human body [470]. One of the most probable mechanisms by which LCn-3PUFA can reduce the inflammation, is reportedly their potent effect on inactivation of nuclear factor-κB (NF-κB), which has a proven effect on the production of other cytokines such as IL-6 and IL-8 [125, 126]. It has been shown that EPA and DHA, but not arachidonic acid supplementation of stimulated endothelial cells decreased the production of IL-6 [471, 472], with the effect of EPA being more potent than DHA [471]. It has also been reported that LCn-3PUFA may decrease the inflammation in vitro via suppression of monocytes adherence to the endothelial cells [473].
1.5 Asthma

1.5.1 Definition of asthma:
Asthma has been known since ancient times (time of Hippocrates) and the term was derived from a Greek word (ασθµα), that means short-drawn breath [474]. Asthma is considered as a chronic inflammatory disease characterized by variable and reversible obstruction of airflow, bronchial hyper-responsiveness and excessive airway narrowing in response to a variety of different stimuli [312-314]. The significance of inflammation in asthma has been recently considered [475, 476]. And it has been shown that even in the newly diagnosed asthmatics, there is an increased inflammation in the airways, which has been defined as increased number of inflammatory cells such as eosinophil, mast cells and macrophages in the airways [477]. Besides being a single disease, asthma is thought to be a group of different disorders: (a) reversible airway obstruction leading to wheezing, breathlessness, chest tightness, and cough; (b) broncho-hyper-responsiveness (BHR), which is defined as an increased sensitivity to broncho-constrictors such as histamine or cholinergic agonists; and (c) airway inflammation [478]. Asthma can be classified as intermittent or persistent, and persistent asthma can be further classified as mild, moderate and severe, based on symptoms and degree of airway hyper-responsiveness (AHR) [474].

1.5.2 Prevalence of asthma:
In 1998 it was estimated that asthma affected 17.3 million people in the United States, and 130-150 million worldwide [349, 474], which represents approximately 2% of the world population [479]. According to the National Health Survey (NHS) from 2004-2005, 10-12% of Australian adults and 14-16% of Australian children reported they currently had asthma [480]. An study in 2006 shows that in Australia from 1990 to 2003, asthma prevalence increased from 7.3% to 14.6% in females and from 7.8% to 9.4% in males [481]. This increasing trend which has been shown in other countries as well [482-485], may be the result of number of factors including actual increased prevalence of asthma and heightened awareness of this condition [486]. Australia has one of the highest asthma prevalence rates in comparison to other countries in the world [474].
1.5.3 Aetiology of asthma:
Asthma arises as a result of interactions between multiple genetic and environmental factors [479]. Asthma is often aggravated by specific allergens such as indoor dust mites [487, 488], cockroaches and cockroach allergens in the home [488] (especially among children with asthma [489]), dogs and cats inside the home [490], fungi [491] and nonspecific stimuli such as cold air [492, 493], exercise [493], food additives, drugs such as β-blockers [493], tobacco smoking specially in closed area [494] and infectious triggers [495] such as viruses [364, 365, 496]. These stimuli act via several mechanisms including (1) the innate immune pathway (production of IL-8 and subsequent recruitment and activation of neutrophils) or via (2) the acquired immune pathway (production of IL-5 and subsequent recruitment and activation of eosinophils) [354, 497].

1.5.4. Eosinophilic and neutrophilic asthma:
Asthma can be classified according to the inflammatory cells that dominate the airways. Asthmatics with an eosinophilic phenotype are characterised by increased eosinophils in the airways [354], which correlates with the severity of asthma [498-500] and exacerbation [501]. However, an increased percentage of activated eosinophils can be presented in the blood without asthma [502]. After treatment by inhaled corticosteroids, the numbers of epithelial eosinophils decrease [499]. Thus, eosinophilic asthma can be identified by responsiveness to corticosteroids [503]. On the other hand, there is another type of asthma that is poorly-responsive to corticosteroid drugs called non-eosinophilic asthma which usually happens in severe asthma [354, 503]. A proportion of non-eosinophilic asthmatics have an increased number of neutrophils and their activation is increased in the airways [504] and during exacerbation [505]. Neutrophils can release cytokines which causes additional neutrophil recruitment, such as, tumour necrosis factor-α (TNF-α) and interleukin-8 (IL-8), both of which are likely to contribute to bronchial hyper-responsiveness in the airways [341]. Another subgroup of non-eosinophilic asthmatics has an absence of granulocytes in the airways and can be classified as “paucigranulocytic”. This subgroup displays symptoms, despite and absence of airway inflammatory cells [506].
Chapter 1

1.5.5 Asthma demographics

Age and gender:
Martinez and colleagues have found that the prevalence of asthma in children and teenagers is higher than the adults [507] and also it is higher in boys than girls [507]. It is also believed that the relationship of asthma with sex should be more investigated [312]. In Australia, National Health Survey (NHS, 2007) reported that the prevalence of asthma is almost similar among adult males (19.7%) and females (20.6%). However, it is higher in boys comparing to girls among children [480].

Place:
It is believed that asthma is more prevalent in English speaking countries. Geographically, asthma is increased in more “westernized” countries [312]. It has been demonstrated that migration to the western countries could increase asthma symptoms [508]. Among the states of Australia, Tasmania has the highest and NSW has the lowest prevalence of asthma [480].

Time:
The prevalence of asthma has increased in recent years [483, 509, 510]. In Australia the prevalence of asthma is double the prevalence 20 years ago [474]. However, it is generally accepted that a part of the increase is attributable to improved diagnosis [511].

Family size and history:
One of the most important findings of the descriptive epidemiology of allergies in childhood is related to family size or birth order. Children with no or few siblings have increased risk of developing asthma [512, 513]. Asthma prevalence is similar for all birth orders [513]. Children of asthmatic or allergic parents have more risk of developing asthma than the children of healthy parents, especially, for children with allergic mothers [312]. Study on twins indicated some evidences regarding the involving of genetics on onset of asthma [514].

1.5.6 Evaluation of asthma:

1.5.6.1 Clinical evaluation of asthma:
Asthma is diagnosed by reversible and variable obstruction of airflow, bronchial hyper-
responsiveness and excessive airway narrowing in response to a variety of different
stimuli [312-314]. There is no single satisfactory clinical diagnostic test for all
asthmatic patients [515]. However, some of the most comprehensive tests include:

**Respiratory function tests:**
These tests include non-invasive methods of peak expiratory flow (PEF) and forced
expiratory volume in 1 second (FEV₁) [515]. The use of spirometry by health staff is
now practicable and supported by a comprehensive range of devices, training courses
and reference materials. Systematic use of ventilation assessment both in the clinic and
with patient self monitoring of peak flow and FEV₁, has the capacity to improve patient
confidence in self management, understanding, and increase quality of life for those
with lung disease [516].

**Bronchial provocation tests:**
Bronchial provocation tests using histamine, methacholine and hypertonic saline are
widely used as safe methods with consistent results [517]. The bronchial provocation
test evaluates the sensitivity of the airways. Spirometry breathing test is undertaken
before and after inhalation of the challenge agent and this test indicates the presence of
airway hyper-responsiveness [515, 518]. Mannitol challenge is another recently
developed challenge agent for assessing airway hyper-responsiveness [518].

**Skin prick tests:**
This test is a simple and efficient method of introducing a small amount of allergen into
the body in order to identify allergic sensitization [515]. Studies show that it is useful in
the prediction of positive airway responses to house dust mites in people with
established asthma and dust mite sensitization [519].

**Exercise test:**
Exercise testing is a suitable test especially in children [515]. In this test spirometry is
performed before and after a standard exercise and decrease of ≥10% in FEV₁ after
exercise is considered as positive result [520].

**1.5.6.2 Biochemical evaluation of asthma:**
Generally, patients with asthma may have increased number of eosinophils in peripheral blood. The presence of large numbers of eosinophils in sputum is also useful for diagnosis [515]. It has been shown that eosinophilic inflammation of the airways is also correlated with the severity of asthma [498]. Neutrophilic inflammation in asthma, particularly, during exacerbation [505] has also been highlighted [521]. Neutrophils are recruited and activated by inflammatory biomarkers, and their activation result in airway obstruction [522], most probably via IL-8 activity [523]. Other biochemical tests that are useful in assessing the severity and activity of asthma include; exhaled nitric oxide [524, 525], exhaled carbon monoxide [526], 8-isoprostane [527], plasma interleukins such as IL-6 [528, 529], IL-13 [528, 530], IL-17 [528, 531], IL-18 [528], IL-4 [532, 533], IL-10, IL-12 [528, 534] and TNF-α [529]. These mentioned tests can be used for assess asthma activity, but not for diagnosis.

1.5.7 Treatment of asthma:

1.5.7.1 Treatment of asthma with drugs:
Although different therapeutic strategies are currently available for asthma, it can only be controlled currently, not cured [535]. Thus, prevention should be the primary focus [536]. Asthma drugs are ranging from those that dilate smooth muscle of the bronchial wall to those that have an anti-inflammatory action or inhibit release of chemical mediators from mast cells [515, 537].

1.5.7.2 Treatment of asthma with complementary treatments:
Treatment of asthma with complementary therapy could be divided into two categories of (a) non-dietary complementary treatments and (b) dietary complementary treatments.

1.5.7.2.1 Non-dietary complementary treatments of asthma:
Some of the non-dietary complementary therapies of asthma are: acupuncture (old traditional Chinese treatment) [538, 539], yoga training [540, 541] and massage [542]. National Asthma Council of Australia has provided a guide for asthma and complementary therapies for health professionals and it suggested that there is less information about the safety and effectiveness of these complementary therapies [543].

1.5.7.2.2 Dietary complementary treatments of asthma:
Some of the antioxidant nutrients have been shown to have a probable preventive or treating effect on asthma. McKeever and Briton reviewed the probable nutrients involving to the asthma. According to their results from cross-sectional, longitudinal and randomised clinical trial studies wide varieties of nutrients may have a beneficial effect on asthma improvement [544]. These nutrients include vitamin C [545-547], vitamin E [547, 548], vitamin A [549], lycopene [550], β-carotene [551], flavones and flavonoids [552, 553], n-3 fatty acids [554, 555], magnesium [556] and selenium [552].

1.5.8 Oxidative stress and asthma:
Oxidative stress describes cellular damage that occurs when antioxidant defences are overwhelmed by the excessive production of oxidative stress. Human cells are constantly exposed to the reactive oxidative species (ROS) [557], as physiologically normal products produced by immune cells [558], mainly due to aerobic metabolism [559]. Almost 2% of consumed oxygen by human body is converted into ROS [560]. The most common ROS include superoxide ion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxide radical (OH$^-$), which are highly reactive free radicals and containing single unpaired electron [181, 182, 561]. Seifried et al defined ROS as “double-edged sword” as they prevent disease by assisting the immune system and damage some of the molecules in cells [561].

Oxidative stress appears to play an important role in the pathophysiology of asthma [313, 562-564]. Studies also suggest that acute exacerbations of asthma are associated with increased oxidative stress [565]. In asthma, ROS are produced by inflammatory cells such as neutrophils and eosinophils when they are stimulated by triggers such as allergens, viruses and air pollution [566-568]. Free radical activity indicators are also increased in asthmatic children and adults [569, 570]. Moreover, it has been reported that in asthma oxidative stress is overwhelmed antioxidants [571]. ROS are cytotoxic to bronchial epithelium [572]. They may also explain some pathological changes such as hardening of the airways, bronchial hyper-reactivity and inflammation [573]. Increased ROS in the airways increases epithelial shedding [574], the contraction of airway smooth muscle cells [575], beta-adrenoceptors function impairment [576], pulmonary vasoconstriction [577], vascular permeability [578] and the formation of chemotactic factors from arachidonic acid of cellular membranes [579]. The activity of catalase and glutathione peroxidise (GSH-Px) are decreased in asthma [580, 581]. Moreover,
indicators of free radical activity are increased in adults and children with asthma [580]. Therefore, there is an evidence for an imbalance oxidants and antioxidants equilibrium in patients with stable and acute asthma [582].

1.5.9 Carotenoids and oxidative stress in asthma

Carotenoids are powerful antioxidants, obtained mainly from fruits and vegetables [156-160]. Studies have shown that carotenoid intake [241, 583, 584] and circulating carotenoid levels in blood [241, 585-587] are low in asthma. It has been proposed that increased antioxidant intake may help reduce the burden of severe asthma and improve asthma outcomes [241, 583]. It has been found that due to increased antioxidant intake, consumption of fruit and vegetables, is inversely associated with wheeze [54, 55] and asthma prevalence [56, 57] and is positively associated with lung function [58, 59]. Studies have shown that carotenoid intake is associated with reduced asthma risk [545]. Among carotenoids, lycopene which can be found in high concentrations in tomato and tomato products [188], is the most powerful antioxidant [44, 173, 187]. Lycopene intake has been shown to decrease pulmonary exacerbations [588] and increased lung function [589]. Supplementation studies have suggested that lycopene [550, 590] and β-carotene [551] exert a protective effect against exercise induced asthma (EIA) in some patients, most probably through an in vivo anti-oxidative effect [590]. Lycopene supplementation is shown to increase total antioxidant capacity in diabetic patients [189]. Another recent study demonstrated that lycopene supplementation decreased airway inflammation in asthma, measured by percentage of sputum neutrophils [591].
1.6 Conclusion:
Inflammation and oxidative stress play an important role in the pathology of asthma. The studies undertaken to date demonstrated that asthma patients commonly have elevated inflammation and reduced antioxidant defence. Some nutrients have been shown to have anti-inflammatory effect in some situations. However, it is not clear whether nutrients such as lycopene or long chain n-3 polyunsaturated fatty acids (LCn-3PUFA) decrease inflammation in asthma.

The aims of this thesis are:
1) To determine the therapeutic effects of lycopene supplementation on the systemic inflammatory biomarkers in asthmatics

2) To determine the best method of lycopene supplementation of cultured airway epithelial cells (Calu-3 cells) via comparing lycopene enrichment of cultured airway epithelial cells using liposomes (as a carrier) to tetrahydrofuran (as a solvent)

3) To determine whether lycopene supplementation affects inflammation of airway epithelial cells infected by rhinovirus or exposed to lipopolysaccharide

4) To determine whether long chain n-3 polyunsaturated fatty acids supplementation affects inflammation of airway epithelial cells infected by rhinovirus

5) To determine whether the combination of lycopene and long chain n-3 polyunsaturated fatty acid supplementation has synergistic anti-inflammatory effect on airway epithelial cells infected by rhinovirus.
Chapter 2

General Materials and Methods
2.1 Clinical assessments of asthma:

2.1.1 Anthropometry:
Height of the participants was measured using Holtain, Crymych, Dyfed stadiometer and their weight was recorded using GEC/Avery digital scale. Then using height and weight the body mass index of the participants (BMI) is calculated (weight in Kg/(height in m)^2). Age, sex and demographic details of participants also were recorded.

2.1.2 Blood collection:
Ten mL forearm blood samples were taken from patients (visiting respiratory clinic John Hunter Hospital, NSW, Australia) by a nurse. Whole blood samples were kept in EDTA coated vials and plasma samples prepared by centrifugation of the whole blood samples (3000rpm, 10min, at 4°C) (Heraeus Biofuge Sratos, USA). All of the whole blood and plasma samples were kept in -80°C freezer (Sanyo, Japan) until they were used in experiments.

2.1.3 Lung function:
Forced expiratory volume in one second (FEV$_1$) (that is the volume of air that is expired in the first second) and forced vital capacity (FVC) (that is total volume of expired air after a maximal inspiration) were measured by spirometer, (Koko, Pulmonary Data Service Instrumentation Inc. USA).

2.1.4 Asthma diagnosis:
Asthma was diagnosed on the basis of doctor’s diagnosis of asthma and hyper-responsiveness to hypertonic saline (that was defined as FEV$_1$% dropped by 15% or more) [592].

2.1.5 Asthma classification:
The clinical asthma pattern was categorised as intermittent, mild, moderate or severe persistent according to global initiative for asthma guidelines (GINA) [593]. Table 2.1.5.1 shows the classification of asthma according to GINA guidelines.
Table 2.1.5.1 GINA classification of asthma severity:

<table>
<thead>
<tr>
<th>Classification</th>
<th>Symptoms/Day</th>
<th>Symptoms/Night</th>
<th>PEF or FEV₁</th>
<th>PEF variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermittent</td>
<td>&lt; 1 time a week</td>
<td>&lt;= 2 times a month</td>
<td>&gt;= 80%</td>
<td>&lt; 20%</td>
</tr>
<tr>
<td></td>
<td>Asymptomatic and normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PEF between attacks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild Persistent</td>
<td>&gt; 1 time a week but &lt; 1 time a day</td>
<td>&gt; 2 times a month</td>
<td>&gt;= 80%</td>
<td>20-30%</td>
</tr>
<tr>
<td></td>
<td>Attacks may affect activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate Persistent</td>
<td>Daily</td>
<td>&gt; 1 time a week</td>
<td>60%-80%</td>
<td>&gt; 30%</td>
</tr>
<tr>
<td></td>
<td>Attacks affect activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe Persistent</td>
<td>Continuous</td>
<td>Frequent</td>
<td>&lt;= 60%</td>
<td>&gt; 30%</td>
</tr>
<tr>
<td></td>
<td>Limited physical activity</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: PEF=Peak Expiratory Flow, FEV₁= Forced Expiratory Volume in one second

2.2 Biochemical analysis:

2.2.1 Carotenoid analysis:

Concentrations of extracted carotenoids (lutein, β-cryptoxanthin, lycopene, alpha-carotene, β-carotene and total carotenoids) from plasma, cell culture media and cultured cell pellets were measured by reversed phase high performance liquid chromatography (HPLC). The method of analysis is the same for plasma, cell culture media and cell pellets. In order to measure the carotenoids, Barua’s method was employed [141]. This method will be presented briefly.

Carotenoid extraction from samples:

All work, during the extraction process, was carried out on ice and in the dark to minimize degradation of carotenoids in the samples. All chemical reagents were of HPLC grade and were filtered through nylon, 0.2µm filter, (Alltech, Australia) prior to use. All glassware was solvent washed (with dichloromethane). The internal standard used was Canthaxanthin (Carotenature, Switzerland) and this was diluted in ethyl acetate to give a final extracted concentration of 2.5µg/mL. The same concentration of internal standard was also used in the external standards for HPLC analysis.
Samples (500µL of plasma, cell culture media or cell pellets of 20 million) were initially transferred to polypropylene culture tubes for the extraction process. Tubes containing an internal standard control and one reagent blank were also added for each extraction. One mL of ethanol (Sigma, Australia) and 1mL of ethyl acetate (Sigma, Australia), which contained the internal standard, canthaxanthin, was added to each sample except for the reagent blank. In this tube the internal standard was replaced by 1mL of ethyl acetate. Samples were vortxed for 30 seconds and centrifuged at 3000rpm, at a temperature of 4°C, for 5 minutes (Heraeus Biofuge Stratos Centrifuge). Using a glass Pasteur pipette, each supernatant was decanted to a clean culture tube. The resulting pellet was washed twice with 1mL of ethyl acetate and then once with 1mL of hexane (BDH Laboratory Supplies, England). After each addition, the samples were vortexed and centrifuged as above. The supernatants were decanted to tubes containing the combined extracts previously collected. The sample pellets were then discarded and 1mL of ultra pure water (Millipore Inc, USA) was added to each of the supernatant tubes. These were vortexed and centrifuged as before, and the supernatants were transferred to glass culture tubes for evaporation under ultra pure nitrogen gas (Linde, Australia) using nitrogen evaporator (Organamation Associates, Inc, USA). Each dried sample extract was re-suspended in 100µL of prepared injection solvent (dichloromethane: methanol; 1:2 v/v) (BDH Laboratory Supplies, England), transferred to a high recovery glass insert in an amber glass vial and stored at -80°C until HPLC analysis.

Samples were analysed by high performance liquid chromatography (HPLC) using a 1200 Series machine (Agilent Technologies, USA). The column was a Hypersil ODS C18 which was 100µm in length, with an internal diameter of 2.1µm and a 5µm particle size (Thermo Electron Corporation, USA). The isocratic mobile phase was based on acetonitrile, with dichloromethane and methanol used as organic modifiers, and ammonium acetate (0.05%) was added to the methanol for buffering purposes (85:10:5v/v/v). The flow rate of the mobile phase was set to 0.3mL/min. A 5µL aliquot of each extracted sample was injected into the mobile phase. Carotenoids were detected by the diode array detector (DAD) (Agilent Technologies, USA) at a preset 450nm wavelength. External standards of known concentrations were used to create a standard curve, for each carotenoid of interest, from which a raw concentration could be
calculated. A final, corrected concentration was obtained by incorporating the dilution factor into the calculations.

**Preparing carotenoid standards:**
In order to prepare three sets of carotenoid standards, 100µL of each carotenoid (lutein, lycopene, α-carotene, β-carotene and β-cryptoxanthin) were mixed as carotenoid stock. Then according to the following table standards were prepared. Table 2.2.1.1 shows carotenoid content of each standard.

**Table 2.2.1.1 Carotenoid content of each standard:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Carotenoid standard 1 (µL)</th>
<th>Carotenoid standard 2 (µL)</th>
<th>Carotenoid standard 3 (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotenoid stock</td>
<td>25</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Canthaxathin</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Injection solvent</td>
<td>365</td>
<td>340</td>
<td>290</td>
</tr>
<tr>
<td>Total volume</td>
<td>400</td>
<td>400</td>
<td>400</td>
</tr>
</tbody>
</table>

Table 2.2.1.2 shows the final concentration of each carotenoid in each three carotenoid standards.

**Table 2.2.1.2 Final concentration of each carotenoid in each three carotenoid standards:**

<table>
<thead>
<tr>
<th>Carotenoids</th>
<th>Final concentration of standard 1 (µg/mL)</th>
<th>Final concentration of standard 2 (µg/mL)</th>
<th>Final concentration of standard 3 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lutein</td>
<td>1.25</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>β-cryptoxanthin</td>
<td>1.25</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Lycopene</td>
<td>1.25</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>α-carotene</td>
<td>1.25</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>β-carotene</td>
<td>6.25</td>
<td>12.5</td>
<td>25.0</td>
</tr>
<tr>
<td>Canthaxathin</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>
2.2.2 Fatty acids analysis:
The incorporation of fatty acids (including EPA, DHA and AA) into Calu-3 cells was analysed using gas chromatography (GC) [594]. The cell pellet was suspended in 2mL of a methanol/toluene mixture (4:1 v/v), containing C19:0 (0.02mg/mL) and BHT (0.12g/L) and vortexed vigorously. The samples were methylated by adding 200μL acetyl chloride drop-wise while vortexing, followed by heating to 100°C for 1 hr. After cooling, the reaction was stopped by adding 5mL of 6% K2CO3 followed by vigorous mixing by vortex. The sample was centrifuged at 3000 x G at 4°C for 10 min to facilitate separation of layers. The upper toluene layer containing the fatty acid methyl esters was transferred to a 2mL glass vial and crimp sealed with a teflon lined cap for analysis by gas chromatography (GC). GC analysis was conducted using a 30m x 0.25mm (DB-225) fused carbon-silica column, coated with cyanopropylphenyl (J & W Scientific, Folsom, CA). Both injector and detector port temperatures were set at 250°C. The oven temperature was 170°C for 2 min, increased 10°C/min to 190°C, held for 1 min, then increased 3°C/min up to 220°C and maintained to give a total run time of 30 min. A split ratio of 10:1 and an injection volume of 3μL were used. The chromatograph was equipped with a flame ionization detector, auto-sampler and auto-detector. Sample fatty acid methyl ester peaks were identified by comparing their retention times with those of a standard mixture of fatty acid methyl esters and quantified using a Hewlett Packard 6890 Series Gas Chromatograph with Chemstations Version A.04.02.

2.2.3 Analysis of inflammation biomarkers:
Selected cytokines [interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necroses factor-α (TNF-α), C-reactive protein (CRP) and interferon-gamma induced protein-10 (IP-10)] were analysed using enzyme linked immunosorbent assay (ELISA). All of the measurements were done by duplicated and the results with coefficient of variance (CV) > 0.05 were repeated. For cell culture media samples it was established that the cell culture media did not affect the ELISA assays for IL-6, IL-8 or IP-10, as the standard curves prepared using the cell culture media were equivalent to that prepared using assay buffer (figure 2.2.3.1, 2.2.3.2, and 2.2.3.3).
Figure 2.2.3.1 Standard curves of IL-6 ELISA assay using ELISA buffer and media:

![Standard curves of IL-6 using ELISA buffer and Media](image)

Note: Vertical axe shows different concentration of IL-6 standard.

Figure 2.2.3.2 Standard curves of IL-8 ELISA assay using ELISA buffer and media:

![Standard curves of IL-8 using ELISA buffer and Media](image)

Note: Vertical axe shows different concentration of IL-8 standard.
Figure 2.2.3.3 Standard curves of IP-10 ELISA assay using ELISA buffer and media:

Note: Vertical axe shows different concentration of IP-10 standard.

**IL-6 analysis:**

For analysing the concentration of IL-6, ELISA kit from *R and D Systems* was used. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a micro plate. Standards and samples were added into the wells and any IL-6 presented was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. After an incubation period, an amplifier solution was added to the wells and colour developed in proportion to the amount of IL-6 bound in the initial step. The colour development was stopped and the intensity of the colour in 492nm was measured by plate reader (Multiskan Ascent, Inc, USA) controlled by its software compatible with Microsoft Windows. After subtracting the optical density (OD) of the blank wells from all the standards and samples, ODs and concentrations of the standard were plotted and according to the standard curve equation the concentrations of the samples were calculated.

**IL-8 analysis:**
IL-8 was measured by ELISA kit purchased from Invitrogen Corporation (Carlsbad, USA). The sample addition, colour development and its amplification, as well as measurement were the same as what is presented in IL-6 section.

**TNF-α analysis:**
For analysing TNF-α, ELISA kit from *R and D Systems* (Minneapolis, USA) was used. The sample addition, colour development and its amplification, as well as measurement were the same as what is presented in IL-6 section.

**CRP analysis:**
In order to analyse the plasma C-reactive protein (CRP) concentration, ELISA kits were used. The kits were purchased from *MP Biomedicals, Orangeburg, NY USA*. The hs-CRP ELISA was based on the principle of a solid phase enzyme-linked immunosorbent assay. The sample addition, colour development and its amplification, as well as measurement were the same as what is presented in IL-6 section.

**IP-10 analysis:**
For analysing the interferon-gamma induced protein (IP-10), ELISA kit from *R and D Systems* was used. The sample addition, colour development and its amplification, as well as measurement were the same as what is presented in IL-6 section.

### 2.2.4 Measuring lactate dehydrogenase:
Lactate dehydrogenase (LDH) concentration in the media was measured by the Hunter Area Pathology Service (HAPS), John Hunter Hospital, NSW, Australia. Lactate dehydrogenase is assayed using enzymatic method on the Dade Behring RXL Dimension platform (Dade Behring Inc. USA). The assay had a coefficient of variation of 4.9% at 350 U/L.

### 2.3 Cell culture experiments:
#### 2.3.1 *In vitro* epithelial cell culture:
The human airway epithelial cell line Calu-3 (American Type Culture Collection, USA) was cultured in complete Minimum Essential Medium (MEM) containing 2.2g/L NaHCO₃, 100U/ml penicillin, 100μg/ml streptomycin, 1mM sodium pyruvate, 0.1μM non-essential amino acids, 2mM L-glutamine and 10% foetal bovine serum (FBS). All
media and additives were purchased from Invitrogen Corporation (Carlsbad, USA). Cells were maintained at 37°C in a humidified incubator containing 5% CO₂. To limit the rate of cell growth during supplementation of nutrients and virus infection treatment phases, cells were maintained in complete MEM with 1% FBS. Only sterile media was used and all cell culture was performed under sterile conditions within a Class II biohazard hood.

2.3.2 Preparing virus stock and TCID₅₀ assay:
Rhinovirus stocks of serotypes RV-43 and RV-1B were cultured using the susceptible cell line RD-ICAM-1, cultured in complete Dulbecco’s Modified Eagle Medium (DMEM) containing 2.2g/L NaHCO₃, 100U/ml penicillin, 100µg/ml streptomycin, 1mM sodium pyruvate, 20mM HEPES, 2mM L-glutamine and 5% foetal bovine serum (FBS). All media and additives were purchased from Invitrogen Corporation (Carlsbad, USA). Cells were maintained at 37°C in a humidified incubator containing 5% CO₂. Around 3-5 days following infection with either RV-43 or RV-1B, significant cytopathological effects in the RD-ICAM-1 cells were observed by microscope and the cell culture supernatant collected and centrifuged for 10min at 2000rpm. Each clarified supernatant stock was then aliquoted and stored at -80°C.

Viral titration assay:
TCID₅₀ experiments were performed using confluent RD-ICAM-1 cells seeded in 96-well tissue culture plates (NUNC, Roskilde, Denmark). Once the cells 80% confluence they were infected with either media alone or virus contained media as varying dilutions as outlines below. Serial ten-fold dilutions of the samples were prepared and four individual wells were infected with each dilution. For titration of samples 5 dilutions were prepared. Additionally, for every dilution 2 controls wells were prepared with media alone. After four days the plates were read for the TCID₅₀ calculation. Infected wells were score based on the cytopathic effect (CPE) seen, where there was >50% CPE demonstrated by light microscopy it was considered a positive result.

Viral titers of the samples determined by cell titration assay using RD-ICAM-1 cells and the viral titer was calculated and expressed as tissue culture infectious dose at 50% in log value (TCID₅₀ log₁₀) [595] and viral titres were calculated using the Karber formula for the tissue culture infective dose 50% (TCID₅₀) [596]:

57
Karber formula: \[ \text{Log (TCID}_{50} = a - D(\Sigma p - 0.5) \]

Where

- \(a\) = dilution index for last dilution where all wells are infected
- \(D\) = log of dilution factor (ie, for a dilution factor of 10: \(\log_{10} 1 = 1\))
- \(\Sigma p\) = Sum of \(p\) between \(x\) and \(y\)

where

- \(p\) = proportion of well infected
- \(x\) = greatest dilution at which all are infected
- \(y\) = lowest dilution at which none are infected

The Karber formula provides an estimate what dilution factor is required to cause infection in 50% of test wells. This gives a value to compare the relative strengths of viruses.

### 2.3.3 Preparing the medium containing lycopene liposome:

Lycopene is a fat soluble compound and in order to make it available to the cells it should be water soluble. For this reason lycopene containing liposome were made. The method can be found in the paper published by Xu and co-workers [597]. Briefly, in order to make 10mL of lycopene liposome containing cell medium, in the glass container, 13µL of L-\(\alpha\)-phosphatidylcholine (Sigma), 100µL of monoolein (Nu-chek Prep, Inc), 100µL of oleic acid (Nu-chek Prep, Inc) and 0, 100 and 200µL lycopene (Sigma) dissolved in the tetrahydrofuran (Sigma) (500µg/mL) as the stock solution were combined and the organic solvent was removed under a stream of nitrogen. Next, 10.76mg of sodium taurocholate (Merk) was added to 10mL of complete cell culture and the mixture was sonicated in water bath (Sanophon, Ultrasonic Industries, USA) at room temperature for 15 minutes to form liposome containing lycopene. The final concentration of each component in the solution was follows: 100\(\mu\)M monoolein, 33.3\(\mu\)M oleic acid, 2\(\mu\)M sodium taurocholate and 16.7\(\mu\)M L-\(\alpha\)-phosphatidylcholine. Lycopene concentrations in the cell medium were 0 (for control), 5 and 10\(\mu\)g/mL. Each 10mL batch of cell medium was sterilized by passage through a pre-sterilized 0.22\(\mu\)m filter (Sartorius). Lycopene containing liposome was made freshly everyday in darkened lab.

### 2.3.4 Preparing medium containing lycopene-THF:
1% FCS/MEM was prepared first (as described before). Then stock solution of lycopene/THF was prepared by dissolving 1mg of lycopene (Sigma) in 2mL of tetrahydrofuran (THF) (Sigma). The appropriate amounts of this stock solution was added to the 1% FCS/MEM to achieve 5%, 2%, 1% and 0.5% THF/lycopene containing medium. Then as a control, similar percentages of THF (with no lycopene) were added to 1% FCS/MEM (without lycopene).

2.3.5 Preparing medium containing omega-3 fatty acids:
To prepare the medium containing docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA), the method of Nair et al. is employed [598]. Final concentration of 0, 10, 200 and 400µM of each fatty acid (all were purchased from Sigma) was dissolved in 0.04% ethanol (400µL/L) and added to 10% foetal calf serum/minimum essential medium (10% FCS/MEM) containing 2.2g/L NaHCO₃, 100U/ml penicillin, 100µg/ml streptomycin, 1mM sodium pyruvate, 0.1µM non-essential amino acids, 2mM L-glutamine and 10% foetal bovine serum (FBS) all purchased from Invitrogen Corporation (Carlsbad, USA).

2.3.6 Supplementation of Calu-3 cells with the combination of lycopene, EPA and DHA:
Calu-3 cells were incubated with 10%FCS/MEM containing final concentrations of following supplements; lycopene (2.5µg lycopene dissolved in 0.5%THF), EPA (400µM dissolved in 0.04% ethanol), DHA (400µM dissolved in 0.04% ethanol) and the combinations of EPA-lycopene and DHA-lycopene. Control cells incubated with media with no supplementation.

2.4 Gene expression assay by quantitative RT-PCR assay:
Reverse transcription polymerase chain reaction (RT-PCR) assay was used to measure the relative transcription of IL-6, IL-8 and IP-10 mRNA in Calu-3 cells. After removing the media from the cells, 0.7mL of lysis solution (buffer RLT; Qiagen, Hilden, Germany) was added per well of a 12 well plate of Calu-3 cells for five minutes at room temperature. The lysates were then collected for RNA extraction using the RNeasy mini-kit (Qiagen) according to manufacturer’s instructions. Extracted RNA samples were reverse transcribed to total cDNA using random primers and the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). PCR primers and probes for
inflammatory mediator targets (IL-6, IL-8 and IL-10) were obtained and quantified against a stable comparator mammalian eukaryotic 18S ribosomal RNA (18S rRNA) in duplex real-time PCR (Applied Biosystems 7500; Applied Biosystems, Foster City, CA, USA). Relative quantitation of mRNA was determined by a modification of the method by Pfaffl, 2001[599], whereby the level of the target genes in samples are compared with that of a calibrator source and expressed as relative expression ratio (RER):

\[
\text{Relative Expression Ratio (RER)} = 2^{\frac{\Delta \text{mRNA (calibrator Ct - sample Ct)}}{\Delta \text{18S rRNA (calibrator Ct - sample Ct)}}}
\]

CT= Cycle threshold obtained when amplification of target peaks above the background fluorescence
2= Theoretical amplification efficiency of each assay within the PCR reaction

**2.5 Ethics approval:**

Project involving in human participants in this thesis has been approved by University of Newcastle Ethics Committee (Approval Reference No: H-783-0304) and Hunter Area Research Ethics Committee (Approval Reference No: 03/09/10/3.13). All of the participants gave written informed consent to participate in the study.
Chapter 3

The effect of lycopene supplementation on inflammatory biomarkers in asthmatics
3. 1. Introduction:
Asthma is a disease characterized by obstruction of airflow [313, 314, 600], and is known as a chronic inflammatory disease [323, 349, 350, 475, 476]. Developed countries and especially Australia have been shown to have a high prevalence of asthma [486]. Asthma can be divided to two groups based on the type of immune cells involved in airway inflammation; (1) eosinophilic and (2) non-eosinophilic (neutrophilic) type. It is believed that a major proportion of neutrophilic asthma, is triggered by environmental exposure to infections e.g. viral infection [354]. In neutrophilic asthma, neutrophil recruitment to the lung is one of the mechanisms that have been well studied. Studies identified a significantly large number of neutrophils in the airways compared to healthy people [503, 601, 602]. Figure 3.1.1 shows acquired and innate immune pathways mediated to eosinophilic and neutrophilic airway inflammation [125, 126, 354]:
Neutrophils are one of the major inflammatory cells in the sputum of asthmatics [521], particularly, during exacerbation [505]. Neutrophils are recruited and activated by inflammatory biomarkers and their activation results in airway obstruction [522] most probably via IL-8 activity [523]. These cells are short-lived phagocytic cells that contain enzyme-rich lysosomes which can facilitate destruction of pathogens [603]. Neutrophil activation can lead to oxidative stress, as neutrophils generate free radicals during the respiratory burst, then they attempt to destroy invading pathogens [604]. Neutrophils
also release cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-8 (IL-8), both known to cause additional neutrophil recruitment [403], possibly via stimulation of bronchial epithelial cells by TNF-α and also subsequent release of IL-8 [605]. TNF-α and interleukin-6 (IL-6) are produced by T-cells to stimulate immune response in inflammation [388, 606]. IL-8 (as a chemokine) also is secreted from macrophages and epithelial cells to attract neutrophils to the inflammation site (chemotaxis) [607]. C-reactive protein is a plasma protein which is synthesised and secreted by the liver [608]. IL-6 and CRP are known as activators of acute phase response [606]. Studies showed that the blood concentrations of these systemic inflammation biomarkers are elevated in asthmatics compared to healthy controls. For example, IL-6 [609], IL-8, TNF-α [610, 611] and CRP (in allergic asthma, but not in non-allergic asthma) are higher in the asthmatics compared to healthy controls [612].

Recently, Wood et al have shown that manipulation of dietary antioxidant levels affects neutrophilic airway inflammation in asthmatics. This study demonstrated that as antioxidants are withdrawn from the diet, percentage of sputum neutrophils increases and asthma symptoms worsen [447, 591]. Epidemiological studies provide further support for an association between intake of antioxidant-rich foods such as fruits and vegetables and respiratory outcomes, in particular lung function [613, 614]. It has also been reported that total intake of vegetables and fruits has a negative relationship with the prevalence of asthma [552], but does not have any association with FEV₁ [615], or airway obstruction [616]. There are other evidences for an association between intake of antioxidant-rich foods, such as fruits and vegetables and respiratory outcomes, in particular lung function [613, 614]. These foods may have a modulatory effect on bronchial reactivity because of their antioxidant content [617]. Among the antioxidants, some carotenoids have been extensively studied [618]. It has been shown that increased oxidative stress occurs in asthma [571, 619] due to white blood cells and macrophages producing more reactive oxygen species (ROS) compared to healthy controls [620, 621]. Thus, antioxidants may play a vital role in asthma therapy [589].

Carotenoids are natural fat-soluble pigments found mainly in plants [135] and act as antioxidants in the human body [156-160]. Among these lycopene found mainly in tomato and tomato products [188], is the most powerful antioxidant [44, 173]. Studies show that dietary supplementation with lycopene could be an effective way to reduce
the level of oxidative stress and improve the inflammatory status [583]. It has been shown that despite similar dietary intake, circulating carotenoids (including lycopene) are low in asthmatics compared to healthy people [241, 622, 623]. Other studies reported that blood carotenoid levels can be raised after oral supplementation with carotenoids and drinking tomato juice [160, 583, 624]. A limited number of supplementation studies have also shown that lycopene and β-carotene can reduce broncho-constriction following exercise in exercise induced asthma (EIA) [589, 590]. It is also shown that tomato based food protects against asthma onset [548]. The study by wood et al, also demonstrated that carotenoids, in particular lycopene, can reduce neutrophilic airway inflammation [591]. It has also been shown that carotenoids have a negative correlation with some inflammatory biomarkers such as interleukin-6 (IL-6) [449], tumour necrosis factor-α (TNF-α) [388, 450] and C-reactive protein (CRP) [444, 450, 453].

There is limited information about the effects of lycopene supplementation or tomato juice consumption on inflammation biomarkers among asthmatics. The aim of this study was to extend the observations of Wood et al [591] and examine the therapeutic effects of tomato juice and lycopene capsules on systemic inflammatory biomarkers in asthmatics. The effects of dietary lycopene supplementation in the form of tomato juice or tomato extract on some systemic inflammation biomarkers in asthmatic patients were examined.

3. 2. Materials and Methods:

Study plan and recruiting the patients:
Thirty two asthma patients recruited from the Respiratory Clinic at the John Hunter Hospital, Newcastle, NSW, Australia consumed a low antioxidant diet for 10 days. Following collection of baseline data, subjects were instructed to consume a diet low in antioxidants. This diet included no more than one piece of fruit and two serves of vegetables per day and avoidance of tea, coffee, red wine, fruit juices, nuts, seeds, vitamin or mineral supplements and aspirin. Twenty-two of these then consumed a randomised controlled cross-over trials involving 7 clinic visits over a 51-day period. Seventeen subjects completed the trial. Subjects received 3 different treatments (7 days each), in random order. The treatments included lycopene capsules (3/day), tomato juice
(840mL/day) and placebo (3 capsules/day). The capsules of lycopene supplements used were Lyco-mato [LycoRed Natural Products Industries Limited, Bersheva, Israel] and they provided 15mg of lycopene per capsule. Patients were asked to take 3 capsules a day, providing 45mg of lycopene daily. Patients consumed 840mL of provided tomato juice daily, providing an equivalent dose of 45mg lycopene per day. The placebo contained soybean oil. Prior to commencing the study and between each treatment, there was a 10-day washout period. For the duration of the study patients consumed a diet low in antioxidants. Patients were given advice and a list of foods they needed to avoid for the duration of the study. Compliance with the diet was confirmed using 24 dietary recall records. Compliance with capsules was measured using diary cards and the pill count back method.

**Study design:**

<table>
<thead>
<tr>
<th>Washout (10 days)</th>
<th>Lycopene capsules (3 capsules/day) (7 days)</th>
<th>Washout (10 days)</th>
<th>Tomato juice (840mL/day) (7 days)</th>
<th>Washout (10 days)</th>
<th>Placebo (3 capsules /day) (7 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit 1 (Day 0)</td>
<td>Visit 2 (Day 10, randomisation) (Day 17)</td>
<td>Visit 3 (Day 27)</td>
<td>Visit 4 (Day 34)</td>
<td>Visit 5 (Day 44)</td>
<td>Visit 6 (Day 51)</td>
</tr>
</tbody>
</table>

**Clinical assessments:**

At every visit blood samples were taken from the participants (method described in chapter 2). At the first visit anthropometry data (method described in chapter 2), information regarding lung function and asthma diagnosis (method described in chapter 2) were collected.

**Biochemical analysis:**

Using ELISA kits the concentration of plasma IL-6, IL-8, TNF-α and CRP were measured (method described in chapter 2). Carotenoid levels of plasma also measured using high performance liquid chromatography (HPLC) (method described in chapter 2).

**Statistic analysis:**
The data was tested whether they are normally distributed. The differences between the medians [Q1-Q3] of different cytokines after each treatment were analysed by Friedman test and the change during the initial washout period was analysed by Wilcoxon rank test. P<0.05 was considered to be significant. Statistical analysis was undertaken using Graph-pad software (Graphpad Prism 4, CA, USA) compatible with Microsoft Windows XP.

**Ethics approval:**
The project that involves in human participants was approved by University of Newcastle Ethics Committee (Approval Reference No: H -783-0304) and the Hunter Area Research Ethics Committee (Approval Reference No: 03/09/10/3.13). All the participants gave written informed consent.

### 3.3. Results

**Demographic and some clinical information of participants enrolled in the study:**
Table 3.3.1 shows some general information including demographic and clinical characteristic of participants. The participants were mostly women with the average age of 54.5 (for all participants). Their FEV$_1$ and FVC was 84 and 95 percent of predicted for their age and sex. They use 1000µg beclamethasone equivalent as inhaled corticosteroid. Study started with 32 people droping down to 22 in the last day of intervention, mostly due to decreasing their interest to participating in the study as they are asked to consume 840mL tomato juice daily.

<table>
<thead>
<tr>
<th>Table 3.3.1 Demographic and clinical characteristic of participants enrolled in the study:</th>
</tr>
</thead>
<tbody>
<tr>
<td>N 22</td>
</tr>
<tr>
<td>Sex (%27.6 Male), (%72.4 female)</td>
</tr>
<tr>
<td>Age (years) 54.5 [35-61]</td>
</tr>
<tr>
<td>% predicted FEV$_1$ (SD) 84.24 (16.7)</td>
</tr>
<tr>
<td>% predicted FVC$^b$ (SD) 95 (14.6)</td>
</tr>
<tr>
<td>ICS$^c$ 1000 [475-1525]$^d$</td>
</tr>
</tbody>
</table>
Comparison of plasma lycopene, IL-6, IL-8, CRP and TNF-α concentrations after each treatment (tomato juice, lycopene capsules and placebo):

Table 3.3.2 shows the comparison between the concentration of plasma lycopene, IL-6, IL-8, CRP and TNF-α after each treatment (tomato juice, lycopene capsules and placebo). Plasma concentrations of IL-6, IL-8, CRP and TNF-α were not significantly changed following dietary supplementation with tomato juice, lycopene or placebo capsules. The lycopene levels increased significantly in the treatment group.

Table 3.3.2 Comparison between mean (SEM) of concentration of plasma lycopene, IL-6, IL-8, CRP and TNF-α after each treatment (tomato juice, lycopene capsules and placebo):

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>After Placebo</th>
<th>After tomato juice</th>
<th>After lycopene capsules</th>
<th>P-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma lycopene</td>
<td>16</td>
<td>0.24 (0.07-0.47)</td>
<td>0.6 (0.41-1.07)</td>
<td>0.7 (0.46-1.26)</td>
<td>P=0.0001</td>
<td>Significant</td>
</tr>
<tr>
<td>Plasma IL-6</td>
<td>12</td>
<td>3.971 (0.748)</td>
<td>3.909 (0.888)</td>
<td>3.346 (0.543)</td>
<td>P=0.75</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma IL-8</td>
<td>17</td>
<td>0.766 (0.94)</td>
<td>0.868 (0.173)</td>
<td>0.949 (0.163)</td>
<td>P=0.056</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma CRP</td>
<td>16</td>
<td>4.134 (0.73)</td>
<td>3.64 (0.83)</td>
<td>3.68 (0.65)</td>
<td>P=0.662</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma TNF-α</td>
<td>6</td>
<td>1.385 (0.12)</td>
<td>1.41 (0.12)</td>
<td>1.44 (0.144)</td>
<td>P=0.187</td>
<td>NS</td>
</tr>
</tbody>
</table>

SEM = Standard Error of Mean

a: data presented as median [Q1, Q3]

b: data presented as mean (SEM)
The concentration [Mean (SEM)] of plasma IL-6, IL-8, CRP and TNF-α before and after washout period:

Table 3.3.3 shows comparison between the concentration of the IL-6, IL-8, CRP and TNF-α before and after washout period. Results show that during 10-day washout period in which participants consumed a low-antioxidant diet, there is no statistically significant change in the concentration of plasma IL-6, IL-8, CRP and TNF-α.

Table 3.3.3 Concentration [Mean (SE)] of the plasma IL-6, IL-8, CRP and TNF-α before and after washout period:

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Before washout</th>
<th>After washout</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/mL)</td>
<td>3.25 (0.4)</td>
<td>3.74 (0.44)</td>
<td>P= 0.4</td>
</tr>
<tr>
<td>IL-8 (pg/mL)</td>
<td>0.80 (0.079)</td>
<td>0.94 (0.1)</td>
<td>P=0.82</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>4.74 (0.89)</td>
<td>5.07 (1.09)</td>
<td>P=0.96</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>1.42 (0.1)</td>
<td>1.33 (0.09)</td>
<td>P=0.56</td>
</tr>
</tbody>
</table>

Median [Q1-Q3] of the different inflammatory biomarkers after placebo, tomato juice and tomato extract:

Figure 3.3.4 show the median [Q1-Q3] of different inflammatory biomarkers after placebo, tomato juice and tomato extract. Results show that the plasma concentrations of IL-6, IL-8, CRP and TNF-α are not changed after treatments.

Figure 3.3.4 Median [Q1-Q3] of different inflammatory biomarkers after placebo, tomato juice and tomato extract:
Correlation between some of the inflammatory biomarkers versus plasma lycopene levels, FVC predicted% and sputum neutrophil count following 10 days on low antioxidant diet.

Figures 3.3.5, 3.3.6, and 3.3.7 show the correlation between some of the inflammatory biomarkers versus plasma lycopene levels, FVC predicted% and sputum neutrophil count following 10 days on low antioxidant diet.

Correlation between plasma CRP versus lycopene levels following 10 days on low antioxidant diet:

Figure 3.3.5 shows that people with higher plasma lycopene significantly have lower plasma CRP levels following 10 days on low antioxidant diet.

Figure 3.3.5 Correlation between plasma CRP versus lycopene levels following 10 days on low antioxidant diet:
Correlation between plasma CRP versus FVC predicted% following 10 days on low antioxidant diet:

Figure 3.3.6 shows that people with higher plasma CRP levels significantly have lower FVC predicted% following 10 days on low antioxidant diet.
3.3.7 Correlation between changes in plasma IL-6 concentrations versus change of sputum neutrophil count:

Figure 3.3.7 shows that participants with increased plasma IL-6 levels have increased sputum neutrophil count following 10 days on low antioxidant diet.
3.4. Discussion:
Changes in airway inflammation were observed as a result of supplementation, thus we were interested in how systemic inflammation was affected. However, due to the large variability in the data obtained from ELISA assays, it is difficult to observe a definite effect of supplementation on inflammatory biomarkers levels.

The effect of lycopene supplementation on asthma has not been widely studied. The only study which investigated the effect of the lycopene supplementation on exercise-induced asthma is conducted by Neuman et al. In this study using similar supplementation dose and period as we followed in this experiment (7 days supplementation with 30mg/day lycopene) it was demonstrated that exercise-induced bronchoconstriction among asthmatics was significantly reduced [589]. Riso et al supplemented 26 healthy subjects with 30mg lycopene/day for 26 days and found decreased whole blood TNF-α levels that confirmed beneficial effect of lycopene on systemic inflammation biomarkers [625]. However, Watzl et al did not find any decrease in blood TNF-α and other cytokines concentration after supplementation with higher dose of lycopene via tomato juice (37mg lycopene per day) in healthy subjects.
Hughes (2001) postulated that moderate carotenoid supplementation may not have any beneficial effect on many diseases in well-nourished subjects, but it might show some beneficial effects in undernourished people [626]. Thus, as asthmatics have lower concentrations of circulatory carotenoids [241, 622, 623], a beneficial response after carotenoid supplementation in asthmatics may be predicted. Recently it has been reported that lycopene supplementation does not decrease plasma CRP concentration [627]. Our data confirms this.

Our results show that 7 days lycopene supplementation results in increased plasma lycopene concentrations that confirms results of other study with the same supplementation period [628]. Results also showed a trend towards decreased plasma IL-6 and CRP after both the treatments (tomato juice and lycopene capsules) and plasma IL-8 (after lycopene extract). However, these trends were not statistically significant. Due to an unexpected variability in the ELISA data, particularly for the tomato juice the study was not adequately powered to detect these changes. For example, following tomato juice supplementation, the study had only 5% power to detect a change in IL-6 compared to placebo and 6% power to detect a change in TNF-α. A probable explanation for higher variability of results could be the high inter-subject variability in the uptake of lycopene, which has contributed to the high variability in the inflammatory markers. Our finding regarding the plasma inflammatory biomarkers levels as well as their probable high variability is consistent with some of the other studies [629, 630].

Our data showed a significantly negative correlation between plasma CRP and lycopene levels. This confirms the results published by Rovley et al which reported a negative relationship of plasma CRP and lycopene [631]. It was also shown that there is a significant correlation between CRP versus FVC predicted% that confirms recently published data by Melbye et al [632] and Fogarty et al [633]. It was also found that participants with increased IL-6 levels have increased sputum neutrophil count following 10 days on low antioxidant diet. This finding confirms the data published by Jagielo et al [634].

It is possible that if we enrolled more participants, we could show a beneficial effect of lycopene supplementation on systemic inflammation. Thus we conclude that lycopene
supplementation in asthma does have an anti-inflammatory effect, but in a study of this size, changes in systemic inflammation could not be detected.
Lycopene enrichment of cultured epithelial airway cells
4.1. Introduction

Carotenoids are natural pigments in plants, and initially were noticed as the probable precursor of vitamin A. Recently it has been found that carotenoids may have a protective effect against diseases such as different types of cancer [635-637], cardiovascular disease [638, 639], degenerative eye disorders [640, 641] and asthma [583, 589, 590, 642]. It has been shown that asthmatics are relatively deficient in carotenoids [241, 622, 623] and that low plasma carotenoid levels are associated with more severe asthma [583]. It has also been shown that carotenoid supplementation could have a beneficial effect on asthma [583, 589, 590]. The role of carotenoids as antioxidants and immune regulators has been explored [626].

Lycopene is the most powerful antioxidant among the carotenoids [44, 173] and can be found in high concentrations in tomato and tomato based products [188]. Lycopene is sensitive to oxygen, extreme low and high pH and temperature [643]. It protects against singlet oxygen induced damage [644]. Dietary supplementation studies show that supplementation of lycopene could be an effective way to improve the inflammatory status of colitis in a rat model [591], reduce broncho-constriction in exercise induced asthma (EIA) [589] and reduce neutrophilic airway inflammation in asthma [591].

As a fat soluble compound, lycopene is poorly soluble in aqueous systems. In the human small intestine, lycopene molecules are solubilised by bile salts and absorbed like other lipids where they are placed in the low density lipoprotein (LDL) and high density lipoprotein (HDL) fractions by the liver to be circulated via the bloodstream [148]. But in cell culture experiments, lycopene has to be solubilised in a water based media to be taken up by cultured cells [645]. For this purpose, lycopene can be dissolved in solvents or bound to allow entry into cultured cells. These solvents or carriers must be safe and non-toxic to the cultured cells and deliver lycopene into the cells efficiently [597]. There are a limited numbers of studies using lycopene enrichment of cultured cells and in vitro studies using lycopene in cell culture models are limited by the lack of an adequate method of lycopene solubilisation [645].

Cyclodextrin is a common carrier of lycopene that is used for incorporation of dietary lycopene into plasma and isolated membranes [646, 647], however, it is not a common carrier of lycopene into intact cells [645]. Dimethylsulfoxide (DMSO) has been used as
a co-solvent of lycopene, although DMSO can only dissolve a small amount of lycopene (less than 0.01mg of lycopene per mL comparing to 4mg of lycopene dissolved in a mL of tetrahydrofuran) [597]. Ethanol and n-hexane have also been tested as lycopene solvents, however, their toxicity and the low stability of lycopene dissolved in them are some disadvantages of using these solvents to incorporate lycopene into cultured cells [648].

Recently, tetrahydrofuran (THF) as a lycopene co-solvent [452, 649] and lycopene liposomes (or micelles) as a lycopene carrier [597] have been considered to be the most useful methods for lycopene enrichment of cultured cells. THF which is a low viscosity liquid is used as a solvent in chemical reactions and as mentioned above can dissolve large amounts of lycopene (more than 4mg lycopene per mL) [597]. On the other hand, liposomes are spherical particles containing hydrophilic compounds on their surface and hydrophobic compounds in their core. They are vesicular delivery systems which are made from assembly of phospholipids consisting of surfactant bilayers [650].

Lycopene enrichment of cultured prostate tumour and lung cells has been compared using two methods of THF and liposome and the solubility and stability of the lycopene have been determined. In this experiment, it was found that the stability of lycopene was higher using lycopene liposomes compared to lycopene dissolved in THF [597]. Another study successfully reported using liposomes for lycopene enrichment of human umbilical vein endothelial cells [648]. It was shown that liposomes can carry more lycopene into the cultured cells compared to THF and the half life of lycopene in cell culture media is longer using liposomes [597]. However, other researchers have criticized the use of liposomes for lycopene enrichment due to carotenoid instability [651, 652].

Solubilisation of lycopene using THF was presented first by Cooney et al in 1993, where it was found that THF could successfully dissolve all of the carotenoids safely into the cells in cell culture experiments [649]. Martin et al used pre-enrichment of cells with lycopene/THF [452], which involves enriching the cells with lycopene and removing the supplementation medium before undertaking any additional treatment on cells. However, Xu et al believed that adding large amounts of THF to cell medium could be toxic to some of the cell types and it was recommended that as low
concentration of THF as possible be used in the medium (0.7% (v/v) or less). It has also been recommended to use THF for the most hydrophobic carotenoid (lycopene) rather than other carotenoids [597]. Another method to solubilise and stabilise β-carotene was developed, where carotenoids were localized in human lipoproteins in vivo [652]. This method is a safe method for carotenoid enrichment of cultured human liver cells [652]. However, this method is time consuming, expensive and dependent on human lipoprotein availability. In summary, the available literature is inconsistent and recommends several different methods for enrichment of airway epithelial cells with lycopene. Therefore, we undertook several experiments to determine, which carrier in what concentration and what supplementation period would be the most suitable for lycopene enrichment of cultured airway epithelial cells (Calu-3 cells). Calu-3 cell line [653] is derived from a lung adenocarcinoma [654]. This cell line is widely used as a model for human sub-mucosal airway gland serous cells [654-657]. The aim of this study is to compare lycopene enrichment of cultured airway epithelial cells (Calu-3 cells) using liposomes (as a carrier) and THF (as a solvent).
4.2. Material and methods:

Study design:

In this study, confluent Calu-3 cells were incubated (for 24 hours) with (1) different concentrations of lycopene dissolved in THF and (2) different concentrations of lycopene carried by liposomes. Cells were visually inspected after lycopene supplementation with lycopene and media and cells were taken to analyse interleukin-6 (as an inflammatory biomarker) and lactate dehydrogenase (as cell death biomarker) released by cells as well as extracellular and intracellular lycopene concentrations.

**Enriching the airway epithelial cells (Calu-3) with lycopene using liposomes:**

As described in chapter 2, lycopene liposomes were prepared by combination of 100μM monoolein, 33.3μM oleic acid, 2μM sodium taurocholate and 16.7μM L-α-phosphatidylcholine in a glass container, followed by 15 minutes sonication in a water bath (Sanophon, Ultrasonic Industries, USA) at room temperature. As described in chapter 2, passages 25-31 of airway epithelial cells (Calu-3, ATCC, USA) were cultured in 12 well plates in minimum essential medium (MEM) containing 10% foetal calf serum/minimum essential medium (10%FCS/MEM), 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine (all from Invitrogen Corporation, Carlsbad, USA) and 2.2g/L NaHCO₃ (Sigma) at 37°C in the presence of 5% CO₂. When cells were confluent, the medium was replaced by fresh medium.
containing different concentrations of lycopene liposomes in 1% foetal calf serum/minimum essential medium (1% FCS/MEM). The final concentrations of lycopene were 0, 5, and 10µg lycopene/mL of media (i.e. 0, 9.31 and 18.63µM). Cells were incubated for 24 hours at 37°C in presence of 5% CO₂. Cell culture experiment was performed three times.

*Enriching the airway cells with lycopene solubilised in tetrahydrofuran:*

As described in chapter 2, passages 25-31 of airway epithelial cells (Calu-3, ATCC, USA) were cultured in 12 well plates in minimum essential medium (MEM) containing 10% foetal calf serum (10%FCS/MEM), 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine (all from Invitrogen Corporation, Carlsbad, USA) and 2.2g/L NaHCO₃ (Sigma) at 37°C in the presence of 5% CO₂. When cells were confluent, the medium was replaced by fresh medium containing different concentrations of lycopene/THF in 1% foetal calf serum/minimum essential medium (1% FCS/MEM). The final concentrations of lycopene in the media were 0, 2.5, 5, 10 and 25µg lycopene/mL of media (i.e. 0, 4.65, 9.31, 18.63 and 46.5µM). The medium containing THF without lycopene was used as control. Cells were incubated for 24 hours at 37°C in presence of 5% CO₂. Cell culture experiment was performed three times.

*Visual inspection of Calu-3 cells:*

Cellular viability of cultured Calu-3 cells was checked after lycopene supplementation (Olympus microscope, TL4).

*Analysing inflammatory biomarkers:*

The media was removed from the cells and stored at -80°C (method described in chapter 2). The cells were also detached using trypsin and stored -80°C freezer (method described in chapter 2). Interleukin-6 levels in the media were analysed using R&D Systems ELISA kits (Minneapolis, MN, USA) according to the kit instructions (method described in chapter 2). ELISA assay were performed for each of three cell culture samples in duplicate. The mean (SEM) of IL-6 released by cells co-cultured with and without lycopene was compared using paired t-test.
Analysing cell death biomarkers:
Lactate dehydrogenase (LDH) concentration in the media was measured by the Hunter Area Pathology Service (HAPS), John Hunter Hospital, NSW, Australia. Lactate dehydrogenase is assayed using enzymatic method on the Dade Behring RXL Dimension platform (Dade Behring Inc. USA). The assay had a coefficient of variation of 4.9% at 350U/L. Lactate dehydrogenase assay were performed for each of three cell culture samples in duplicate. The mean (SEM) of LDH released by cells co-cultured with and without lycopene was compared using paired t-test.

Analysing extracellular and intracellular lycopene:
In order to measure the lycopene concentration of media as well as cells, Calu-3 cells were grown in a large flask (T175). After enriching the cells with different concentrations of lycopene (final concentrations of 0, 1.25, 2.5 and 5µg/mL lycopene) for 24 hours, media and cells were taken into butylated hydroxytoluene (BHT) coated tubes and the concentration of lycopene in cells and media were measured by HPLC (method described in chapter 2). Lycopene concentrations of culture media were described as µg/mL and concentrations in Calu-3 cells were described as µg/cell pellets of 20 million. This assay was performed three times.

4.3. Results
Figures 4.3.1 and 4.3.2 show Calu-3 cells considered as 50% and 100% confluent under the microscope. Calu-3 cells usually get confluent (100%) in a T175 flask around 8 days.

Figure 4.3.1 Calu-3 50% confluent in the bottom of flask:
Figure 4.3.2 Calu-3 100% confluent in the bottom of flask:

Enriching the airway cells with lycopene using lycopene liposomes:
Visual inspection of the cells and medium indicated that 24 hours of incubation with lycopene liposomes (all concentrations) killed all the Calu-3 cells (Figure 4.3.3). The bodies of cells were detached, erupted and floated in the medium. Thus, the other biomarkers in medium were not analysed. All Calu-3 cells which were not supplemented by lycopene liposome were viable.

Figure 4.3.3 Cells incubated with lycopene liposomes:

Enriching airway epithelial cells with lycopene using THF/lycopene:
Visual inspection of Calu-3 cells by microscope indicated that 24 hours incubation of cells with medium containing 0.5% and 1% THF/lycopene (v/v) did not kill the cells. However, media containing 2% THF and THF/lycopene (v/v) killed large amounts of cells and media containing 5% THF and THF/lycopene killed all of Calu-3 cells.
Following figures show the Calu-3 cells after 24 hours incubation with 1% FCS/MEM containing 0%, 0.5%, 2% and 5% of THF/lycopene.

*Figure 4.3.4* Calu-3 cells after 24 hours incubation with no THF

*Figure 4.3.5* Calu-3 cells after 24 hours incubation with 0.5% THF and/or THF/lycopene:

*Figure 4.3.6* Calu-3 cells after incubation with 2% THF and/or THF/lycopene:
Figure 4.3.7 Calu-3 cells after incubation with 5% THF and/or THF/lycopene:

Figure 4.3.8 shows the concentrations of IL-6 released by Calu-3 cells after 24 hours incubation with different concentrations of lycopene dissolved in THF. Both 5% THF and 5% THF/lycopene resulted in cell death and IL-6 was undetectable. The use of lycopene significantly decreased the IL-6 concentration produced by cells, in 0.5%, 1%, and 2% group.
Figure 4.3.8 IL-6 released by Calu-3 cells after 24 hours incubation with different concentrations of THF and THF/lycopene

LDH released by Calu-3 cells after 24 hours incubation with different concentrations of THF/lycopene:

Figure 4.3.9 shows the concentration of LDH released by Calu-3 cells after 24 hours incubation with different concentrations of lycopene dissolved in THF. It shows that LDH levels increased as the concentration of THF increased in the media. However, the release of LDH by cells incubated with 0.25% and 0.5% THF was minimal.
Figure 4.3.9 LDH released by Calu-3 cells after 24 hours incubation with 1% FCS/MMEM containing different concentrations of THF/lycopene (n=3):

Lycopene uptake of Calu-3 cells after 24 hours incubation with different concentration of lycopene/THF:

Figure 4.3.10 shows (a) extracellular and (b) intracellular lycopene concentrations after 24 hours incubation of Calu-3 cells with different concentrations of lycopene. It shows lycopene concentration of media as well as lycopene levels of cell pellets of Calu-3 cells (µg/20 million cells) after 24 hours incubation with different concentration of lycopene/THF. Results show a dose dependant uptake of lycopene by cells from media.
Figure 4.3.10 The (a) extracellular and (b) intracellular lycopene concentrations after 24 hours incubation of Calu-3 cells with different concentrations of lycopene (n=3):
4.4. Discussion

This study demonstrated that dissolution of lycopene in THF is the most suitable method of enriching airway epithelial cells with lycopene. Although, it has been reported that lycopene enrichment of human prostate tumour cells, lung tumour cells and umbilical vein endothelial cells using liposomes is not toxic to cells [597, 648], our results showed that adding liposomes prepared in the same way as reported was toxic to cultured airway epithelial cells (Calu-3).

Visual inspection of cells after 24 hours incubation with different concentrations of lycopene carried by liposomes confirmed that Calu-3 cells were detached from the flask, erupted and floated in the medium and there were no viable Calu-3 cells found. However, cells with no liposomes were all viable. The presence of erupted cells in the medium may be due to the presence of sodium taurocholate salt crystals (one of the liposome components) in the medium (figure 4.3.3) which would increase the hypertonicity of the medium. A toxic effect of sodium taurocholate in cell culture experiments has previously been reported [658]. However, Xu et al did not report any cytotoxic effect of sodium taurocholate in their experiment [597]. In this experiment all of the media containing liposomes were sterile filtered, thus infection cannot explain detachment of the cells from the flask. It is prudent to conclude that co-culture of Calu-3 cells with lycopene liposomes containing 1%FCS/MEM medium is not a satisfactory method of enriching airway epithelial cells (Calu-3 cells) with lycopene.

After 24 hours incubation of Calu-3 cells with different concentrations of lycopene/THF, it was found that cells enriched with low levels of lycopene/THF (0.5% and 1% \(v/v\)) were viable and cells enriched with 2% and 5%, lycopene/THF \(v/v\) were partially and completely dead respectively. This is in line with other studies suggesting use of THF concentration of no more than 0.7% of medium for human prostate tumour and human lung tumour cells [597]. Our study confirms this finding, where visual inspection of cells confirmed that higher concentrations of THF in the medium resulted in cell death.

Studies report that lycopene delivered by THF into human aortic endothelial cells, decreased inflammation in these cells [452]. Therefore lycopene is expected to decrease IL-6 released by cells as IL-6 is an acute inflammatory response agent that cells release
in inflammatory situation [373]. Our data demonstrated a reduction in IL-6 release in the presence of lycopene, with IL-6 decreased by between 7 and 24%. Interestingly, our data also demonstrated a mild pro-inflammatory effect of THF. IL-6 levels of the medium treated with 0.5%, 1% and 2% THF showed a dose dependant increase in the IL-6 levels. This pro-inflammatory effect of THF is confirmed by some animal studies [659]. As described in figure 4.3.8, cells incubated with 5% THF and 5% THF/lycopene (25µg lycopene) produced undetectable amount of IL-6, confirming that Calu-3 cells were destroyed after addition of 5% THF. Thus there were no viable cells to produce cytokines.

Lactate dehydrogenase (LDH) is an intracellular enzyme that catalyses the conversion of lactate to pyruvate and its extracellular concentration increases with cell death or cell damage [660]. LDH has been commonly used as a cell death biomarker after lycopene enrichment of cells [648]. LDH levels of medium after 24 hours incubation with different concentrations of THF/lycopene showed an increased LDH levels after incubation with THF/lycopene. It shows that the highest LDH levels related to the highest concentration of THF/lycopene and lowest LDH levels related to the control group (with no THF/lycopene). Visual inspection of the cells confirms both the IL-6 and LDH results in which indicated cell death occurred on treatment with 5% lycopene/THF. This confirms a cyto-toxic effect of THF.

Our data showed a dose dependant lycopene uptake by Calu-3 cells from the media. It was demonstrated that increased extracellular lycopene resulted in significantly increased intracellular lycopene. Comparing the concentration of lycopene before (the amount of added lycopene to the fresh media) and after 24 hours of incubation (as shown in figure 4.3.10) confirms that lycopene levels in the media decreased after 24 hours. This was probably due to (1) cellular uptake of lycopene during 24 hours incubation period (2) degradation and oxidization of lycopene in cell culture condition (3) cis-trans isomerisation of lycopene (which makes lycopene undetectable at its usual retention time in the HPLC analysis) (4) de-stabilizing of lycopene due to use of polystyrene flasks and plates for cell incubation (compared to glass containers) [597].

It has been reported that lycopene uptake after 24 hours incubation is almost twice of its uptake after 12 hours, but it does not show any further increase after 48 hours [661]. It
has also been reported that after 48 hours incubation, more than 30% of lycopene in the medium is reduced and cells can obtain only 1% of lycopene [648]. Therefore, it seems that 24 hours incubation of cells with lycopene is the best supplementation period. One study showed that lycopene enrichment of cells at concentrations more than 5μM has a growth inhibitory effect on some of the cell lines [661]. Therefore in this experiment, 24 hours enrichment of cells with 0.5% THF/lycopene (containing 2.5μg lycopene/mL of medium) is recommended. This amount of lycopene in the medium is almost two times higher than circulating plasma lycopene levels in healthy people, which is reported to be 0.8-1.34μg/mL [242, 243]. Therefore, the intracellular dose we achieved after our recommended method is probably biologically relevant to human physiologic systems. In conclusion it was found that THF is more suitable carrier of lycopene for enriching airway epithelial cells than liposomes. By using THF/lycopene, cellular uptake of lycopene occurs in a dose dependant manner. Enrichment of airway epithelial cells with 2.5μg/mL (4.65μM) lycopene dissolved in 0.5% THF v/v is the most suitable and safe method of lycopene enrichment of airway epithelial cells (Calu-3 cell).
Anti-inflammatory effects of lycopene enrichment on infected cultured airway epithelial cells
5.1. Introduction:
The results of supplementation trials discussed in chapter 3 [354, 363, 662, 663], indicated that lycopene may act to prevent asthma by affecting the innate immune response. Some key components of the innate immune system are described in figure 5.1.1.

*Figure 5.1.1* Innate immunity pathway leading to hyper-responsiveness and exacerbation in asthmatics:

As shown in the pathway above, the innate immune response may be activated by triggers such as viruses and endotoxin. IL-8 mediated neutrophil influx, has a role leading to airway hyper-responsiveness in asthmatics [663].

Rhinovirus (RV) is the most common cause of the common cold [363] and the major cause of exacerbation in asthma among adults [364] and children [364, 365]. RV infection can also lead to airway obstruction in asthmatics [386]. RV is the cause of more than 50% of upper respiratory tract infections [664]. The mechanism of asthma worsening by RV is not completely understood [376]. RVs are divided into two subclasses; major and minor. Major group RV (e. g. RV-43) bind to intercellular
adhesion molecule-1 (ICAM-1) receptors and minor group (e.g. RV-1B) bind to the LDL receptor of the cells [366, 367]. This binding results in internalisation of virus particles into the cytoplasm of the cells [368], where RV replicates [369].

RVs are transmitted via a direct contact [370] and target epithelial cells, in which they replicate, uncoat [371] and initiate innate immune responses [372, 373] locally and systemically [372]. The presence of viral ribonucleic acid (vRNA) (due to replication of single stranded RNA) results in transcription of interferons (IFN) which may induce apoptosis [374]. After RV infection, the integrity and viability of epithelial cells are not changed dramatically [371, 665], confirming that there are no toxic materials released due to the infection [376]. After RV attack, large numbers of neutrophils and lymphocytes are recruited to the airways [375]. As a result, epithelial cells produce various cytokines, chemokines and other inflammatory mediators that might contribute to the host defence and inflammatory responses [376]. TLRs play an important role in innate immunity by recognising the invaders and their activation initiate the activation of the downstream signalling cascades leading to the activation of NF-κB [666, 667]. NF-κB mediates the transcription of many inflammatory biomarkers such as cytokines and chemokines [124]. Some of the inflammatory biomarkers released after RV infection include interleukin-1 (IL-1) [377], IL-6 [125, 371, 378], IL-8 [126, 371, 378-381], IL-11 [382], granulocyte-monocyte-colony stimulation factor (GM-CSF) [371, 381], RANTES [380, 383], intercellular adhesion molecule-1 (ICAM-1) [377, 384-386] and interferon-gamma induced protein-10 (IP-10) [376]. RV infection also increases oxidative stress in airway epithelial cells most probably via increased production of reactive oxidative species (ROS) [337, 387]. Increased production of ROS activates NF-κB [337], that mediates the transcription of inflammatory biomarkers such as interleukin-6 (IL-6) and IL-8 after RV infection [124-126]. There is an strong evidence regarding increased activation of NF-κB after rhinovirus infection [125, 126, 337].

Elevated levels of IL-6 during viral infection reflect the role of IL-6 as a cytokine and an acute inflammatory response agent [125, 373]. IL-6 has various functions as a central factor of growth and differentiation of immune cells [390-392]. IL-8 may also be elevated during viral infection and is a chemotactic agent for neutrophils and activated eosinophils [126, 373, 668]. Another mediator important in virus infection is IFN-γ induced protein-10 (IP-10) which is produced by epithelial [417] as well as
inflammatory cells [421], increases T-lymphocyte number and their chemotaxis properties [376]. Increased IP-10 secretion by airway cells contributes to airway hyper-responsiveness and inflammation [669]. IP-10 levels are believed to be associated with rhinovirus replication [376], however, it has been also reported that IP-10 levels could be independent of viral replication [421].

Lipopolysaccharide (LPS) is a bacterial endotoxin that is one of the components of gram-negative bacterial cell walls, and causes inflammation in epithelial cells and in experimental animals and this acts as a positive control for viral infection experiments [432, 460, 670]. LPS-exposed cells release large amounts cytokines such as TNF-α, IL-1β, IL-6, IL-8 and IL-12 [432, 460, 671, 672], probably due to activation of macrophages through the binding of LPS-binding protein to CD14 that is a membrane glycoprophosphatidylinositol anchored protein [673]. LPS causes inflammation in human neutrophils as well [670, 674], that may be due to increased expression of IL-6 gene [674].

Neutrophils are important in viral infection and they are known as one of the major inflammatory cells present in the sputum of asthmatics [521] and have been found to be elevated specifically during acute exacerbations [505]. Neutrophils are recruited and activated by inflammatory biomarkers and their activation result in airway obstruction [522], most probably via IL-8 activity [523]. Neutrophils also generate free radicals during the respiratory burst, thus they attempt to destroy the invading pathogen [604].

It has been reported that various antioxidants have anti-viral activities [675-679]. Among them, carotenoids are natural fat-soluble compounds [135], which act as antioxidant in the human body [156-160]. The antioxidant properties of carotenoids are mainly associated with their singlet oxygen quenching [173, 174] and their radical scavenging abilities [166, 175, 176]. Among carotenoids, lycopene which can be found in high concentrations in tomato and tomato products [188] is the most powerful antioxidant [44, 173]. It has been shown that circulating levels of lycopene is lower in asthmatics compared to healthy people [241, 622, 623]. Lycopene has been shown to improve inflammatory status in animal models [432, 448, 626] and reduce exercise-induced bronchoconstriction in asthmatics [589]. Studies show that lycopene supplementation of cultured cells decreases the production of cytokines and
inflammatory biomarkers including, ICAM-1 expression, released IL-6, IL-8, IL-12 and nitric oxide, and also the activation of NF-κB after LPS exposure of various types of cells [452, 460, 680]. It has also been reported that lycopene down-regulates the expression of co-stimulatory molecules (CD80 and CD86) on antigen presenting cells [432]. Studies showed that dietary supplementation of lycopene may reduce oxidative stress and improve inflammatory status in colitis [448]. It has also been shown that lycopene reduces inflammatory signals in lateral prostate lobe [461] and inhibits the expression of inflammatory agents in hyperhomocysteinemic rats [462]. While, there are limited studies on the effect of lycopene on viral infections [681, 682], there is no evidence regarding the effect of lycopene on the inflammatory response of airway cells when exposed to common triggers, such as rhinovirus infection and LPS exposure.

We hypothesised that lycopene can decrease inflammation in airway epithelial cells infected with rhinovirus. The mechanism may be via suppression of viral replication, which can be mediated by IP-10 levels [376]. Alternatively, through its effect as an antioxidant [173], lycopene may decrease the oxidative stress induced by rhinovirus infection [387] and it would influence redox-based NF-κB activation [432].
Figure 5.1.2 Mechanisms by which lycopene may affect the inflammatory response to viruses [374].

The aim of this study is to determine whether lycopene supplementation affects inflammatory response of airway epithelial cells infected by RV (major and minor subclass) or exposed to LPS.
5.2. Materials and Methods:

Study design:

Airway epithelial cell culture:
As described in chapter 2, airway epithelial cells (Calu-3, Passage 30-33, ATCC, USA) were cultured in minimum essential medium (MEM) containing 10% foetal calf serum, 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine (all from Invitrogen Corporation, Carlsbad, USA) and 2.2g/L NaHCO₃ at 37°C in the presence of 5% CO₂. Cell culture experiments were performed three times.

Lycopene enrichment of cultured epithelial airway cells:
As described in chapter 2, confluent Calu-3 cells were incubated (for 24 hours) with lycopene (0 and 2.5μg/mL) dissolved in tetrahydrofuran (THF) (0 and 0.5% THF in cell culture medium).

Rhinovirus and LPS challenge of Calu-3 cells:
As described in chapter 2, after removing the supplementation media (as mentioned above) from Calu-3 cells, they were either infected with (a) RV-43 at multiplicity of infection (MOI) of 0.32 and ultraviolet inactivated RV-43 (b) RV-1B at multiplicity of infection (MOI) of 0.56 and ultraviolet inactivated RV-1B and (c) lipopolycaccharide 1µg/mL (Sigma). Plates were incubated for 48 hours at 37°C in the presence of 5% CO₂. This assay was undertaken three times.

**Visual inspection of Calu-3 cells:**
Cellular viability of cultured Calu-3 cells was checked after each step (Olympus microscope, TL4).

**Analysing the inflammatory biomarkers concentration of media:**
As described in chapter 2, interleukin-6 (IL-6), IL-8 and interferon-gamma induced protein-10 (IP-10) concentrations of medium were measured by ELISA (R&D Systems Minneapolis, MN, USA) according to manufacturer’s instructions. These ELISA assays were undertaken in duplicate for each cell culture assay. The concentration of biomarkers released by cells enriched with and without lycopene was compared.

**Relative expression of inflammatory biomarkers gene by real-time quantitative PCR:**
As described in chapter 2, gene expression of cytokines was measured by reverse transcription polymerase chain reaction (RT-PCR) after RNA extraction (RNeasy kit, Qiagen, Carlsbad, USA) and reverse transcription to cDNA (Qiagen, Carlsbad, USA) according to manufacturer’s instructions. This assay was undertaken in duplicated. The gene expression of cytokines in cells enriched with and without lycopene was compared.

**Lactate dehydrogenase assay:**
Lactate dehydrogenase (LDH) released by cells was measured by the Hunter Area Pathology Service (HAPS), John Hunter Hospital, NSW, Australia. Lactate dehydrogenase is assayed using enzymatic method on the Dade Behring RXL Dimension platform (Dade Behring Inc. USA). The assay had a co-efficient of variation of 4.9% at 350 U/L.

**Analysing extracellular and intracellular lycopene concentrations:**
As described in chapter 2, in order to measure the concentration of extracellular and intracellular lycopene of cells enriched with different concentrations of lycopene, Barua’s method was employed [141]. Briefly, extracellular (500µL of media) and intracellular (cell pellets of cell count of 20 million) lycopene was extracted from the samples by ethyl acetate and hexane and incorporated in dichloromethane: methanol; 1:2 v/v). Samples were analysed by high performance liquid chromatography (HPLC) 1200 Series (Agilent Technologies, USA) by injecting (5μL, with flow rate: 0.3mL/min) into Hypersil ODS C18 column (Thermo Electron Corporation, USA) and detecting by the diode array detector (DAD) (Agilent Technologies, USA) at a preset 450nm wavelength. This assay was undertaken three times.

**Viral titration assay:**

As described in chapter 2, TCID$_{50}$ experiments were performed using near confluent RD-ICAM-1 cells seeded in 96-well tissue culture plates (NUNC, Roskilde, Denmark). Cells were infected by either media alone or virus containing media at varying dilutions. Serial ten-fold dilutions of the samples were prepared and 4 individual wells were infected with each dilution. For titration of samples 6 dilutions were prepared. Additionally, for every dilution 2 controls wells were prepared with media alone. After four days the plates were read and TCID$_{50}$ calculated. Infected wells were scored based on the cytopathic effect (CPE) seen, >50% CPE was demonstrated by light microscopy was considered a positive result. Viral titers of the samples determined by cell titration assay using RD-ICAM-1 cells and the viral titer was calculated and expressed as tissue culture infectious dose at 50% in log value (TCID$_{50}$ log$_{10}$) [595] and using the Karber formula for the tissue culture infective dose 50% (TCID$_{50}$) [596].

**Statistical analysis:**

Differences between lycopene supplemented and un-supplemented groups were analysed using paired t-test and difference between different concentrations of LDH and lycopene uptake were analysed using analysis of variances (ANOVA) by Graphpad software (Graphpad Prism 4, CA, USA). P-values less than 0.05 were considered as statistically significant.
5.3. Results

*Visual inspection of Calu-3 cells:*

Visual inspections of the cultured Calu-3 cells after each step confirmed that cells were viable.

*IL-6 released by Calu-3 cells infected with rhinoviruses and LPS:*

Figure 5.3.1 shows IL-6 released by Calu-3 cells after 24 and 48 hours incubation with RV and LPS. Data shows a time dependent increase in IL-6 levels after 24 and 48 hours infection with RV and LPS.

*Figure 5.3.1 IL-6 released by Calu-3 cells incubated with and without RV-1B, RV-43, UV inactivated RV-1B and LPS for 24 and 48 hours:*

*IL-6 released by Calu-3 cells enriched with and without lycopene before and after infection induced by Rhinovirus-43 (RV-43), Rhinovirus-1B (RV-1B) and Lipopolysaccharide (LPS) exposure:*

Figure 5.3.2 shows that the Calu-3 cells released more IL-6 after RV-1B infection and LPS exposure. This effect was significantly reduced by lycopene enrichment, for both RV-1B and LPS.
Figure 5.3.2 IL-6 (mean ± SEM) released by Calu-3 cells enriched with and without lycopene before and after infection induced by (a) Rhinovirus-43 (RV-43), (b) Rhinovirus-1B (RV-1B) and (c) Lipopolysaccharide (LPS) exposure:

(a)

(b)

(c)
IL-8 released by Calu-3 cells enriched with and without lycopene before and after infection induced by Rhinovirus-43 (RV-43), Rhinovirus-1B (RV-1B) and Lipopolysaccharide (LPS) exposure:

Figure 5.3.3 shows that the concentration of IL-8 after different rhinovirus serotypes and LPS was not changed. Lycopene supplementation reduced IL-8 concentration only in cells exposed to LPS.
Figure 5.3.3 IL-8 (mean ± SEM) released by Calu-3 cells enriched with and without lycopene before and after infection induced by (a) Rhinovirus-43 (RV-43), (b) Rhinovirus-1B (RV-1B) and (c) Lipopolysaccharide (LPS) exposure:
IP-10 released by Calu-3 cells enriched with and without lycopene before and after infection induced by Rhinovirus-43 (RV-43), Rhinovirus-1B (RV-1B) and Lipopolysaccharide (LPS) exposure:

Figure 5.3.4 shows that IP-10 released by Calu-3 cells increased after RV-43 and RV-1B infection as well as LPS exposure. Lycopene supplementation led to a significant decrease in IP-10 after RV-43 infection and LPS exposure and a non-significant decrease after RV-1B infection.
Figure 5.3.4 IP-10 (mean ± SEM) released by Calu-3 cells enriched with and without lycopene before and after infection induced by (a) Rhinovirus-43 (RV-43), (b) Rhinovirus-1B (RV-1B) and (c) Lipopolysaccharide (LPS) exposure:

(a)

(b)

(c)
IL-6 mRNA levels (Relative Expression Ratio %) in cells enriched with and without lycopene before and after infection:

Figures 5.3.5 shows that the level of IL-6 mRNA increased due to RV-43 and RV-1B infection, as well as LPS exposure. Lycopene enrichment led to a significant reduction in IL-6 expression.
Figure 5.3.5 IL-6 mRNA levels (Relative Expression Ratio %) in cells enriched with and without lycopene before and after infection induced by (a) rhinovirus-43 (RV-43), (b) rhinovirus-1B (RV-1B) and (c) Lipopolysaccharide (LPS) exposure:

(a)

(b)

(c)
**IL-8 mRNA levels (Relative Expression Ratio %) in cells enriched with and without lycopene before and after infection:**

Figure 5.3.6 shows that LPS exposure resulted in increased IL-8 mRNA levels in Calu-3 cells, followed by significant decrease due to lycopene enrichment. IL-8 mRNA level was not changed after rhinovirus infection or lycopene enrichment in cells infected with rhinoviruses.
Figure 5.3.6 IL-8 mRNA levels (Relative Expression Ratio %) in cells enriched with and
without lycopene before and after infection induced by (a) rhinovirus-43 (RV-43), (b)
rhinovirus-1B (RV-1B) and (c) Lipopolysaccharide (LPS) exposure:

(a)

(b)

(c)
IP-10 mRNA levels (Relative Expression Ratio %) in cells enriched with and without lycopene before and after infection:

Figure 5.3.7 shows that RV-1B resulted in increased IP-10 mRNA level in Calu-3 cells, followed by significant decrease due to lycopene enrichment. IP-10 mRNA levels decreased after lycopene enrichment of Calu-3 cells infected with RV-43.
Figure 5.3.7 IP-10 mRNA levels (Relative Expression Ratio %) in cells enriched with and without lycopene before and after infection induced by (a) rhinovirus-43 (RV-43), (b) rhinovirus-1B (RV-1B) and (c) Lipopolysaccharide (LPS) exposure:
Virus titre of RV-43 and RV-1B in Calu-3 cells enriched with and without lycopene: 
TCID\textsubscript{50} results showed that lycopene supplementation decreased viral replication of RV-1B by 85% significantly (p=0.025) and also RV-43 by 53% non-significantly (p=0.18).

Figure 5.3.8 TCID\textsubscript{50} (mean ± SEM) of RV-43 and RV-1B in the Calu-3 cells enriched with and without lycopene (50% Tissue Culture Infective Dose):

5.4. Discussion
As an antioxidant lycopene can reduce cellular oxidative stress [44] and acts as one of the most probable inhibitors of cytokine production via suppression of NF-κB activity due to its antioxidant action [337, 432, 460]. Our results showed that lycopene significantly decreased IL-6 release by Calu-3 cells following RV-1B infection (p=0.026), IL-8 release after LPS exposure (p=0.004) and IP-10 release after RV-43 infection (p=0.001) and LPS exposure (p=0.02). These decreases in cytokine release were consistent with the results of cytokine mRNA expression in which lycopene decreased IL-6 mRNA expression after RV-1B infection (p=0.004), IL-8 mRNA expression after LPS exposure (p=0.01) and IP-10 mRNA expression after RV-43 infection (p=0.006). Lycopene also decreased viral replication of RV-1B by 85% (p<0.025).
The expression of IL-6 and IL-8 mRNA is mediated by nuclear factor-κB (NF-κB) [125]. NF-κB has a central role in expression of many inflammatory biomarkers (reviewed in [124]) including IL-6 and IL-8 in inflammatory situation such as RV infection [125, 126]. NF-κB starts transcription of these genes by being attached to DNA in the promoter regions of the target genes [405]. Thus, inhibition of NF-κB activity can down regulate protein expression and protein levels of these cytokines [125, 126, 432]. Bai et al found that β-carotene can inhibit NF-κB activity in LPS exposed cells. This effect of β-carotene on the redox-based NF-κB activation is attributed to the antioxidant effect of β-carotene [436]. Lycopene has also been shown to decrease the nuclear translocation of NF-κB p65 subunit in LPS-stimulated dendritic cells [432]. Lycopene is expected to have greater effects on the redox-based NF-κB activation, because based on the various carotenoid’s inhibition of thiobarbituric acid-reactive substances formation, lycopene is the most effective antioxidant with β-carotene ranked fifth [44].

Our results show that RV infection and LPS exposure result in increased IL-6 concentrations by cultured airway epithelial cells. This increase was evident at 24 hours and nearly doubled by 48 hours incubation. Zalman et al also infected airway epithelial cells with rhinovirus and reported the same pattern of increased IL-6 and IL-8 following 24 and 48 hours infection [683]. Therefore, in order to observe a larger inflammatory response by Calu-3 cells, it was decided to use 48 hours infection time in our experiment. Pre-supplementation of cultured cells with lycopene for 24 hours [452] (where lycopene is added to the medium of cells and then removed from cells before induction of infection to the cells) is a common method of lycopene enrichment of cultured cells [432, 452]. In previous chapter, it was found that the best lycopene concentration for pre-supplementation is 2.5µM (dissolved in 0.5% THF). That concentration has also been used in other in vitro studies [452, 680]. Therefore, in the current experiment it was decided to pre-supplement Calu-3 cell monolayer with 2.5µM/mL lycopene for 24 hours and infect the cells with RVs and LPS for 48 hours.

Our data showed that IL-6 and IP-10 released by Calu-3 cells and their mRNA levels increased significantly after 48 hours infection with RV-43 and RV-1B as well as LPS exposure (p<0.05). This confirms the results of other studies in which RV infection resulted in increased inflammation in airway cells [125, 371, 376]. These data also
confirm a study reporting the key role of IL-6 expression in LPS induced inflammation [674]. As expected, ultraviolet-in-activated RV (non-infectious virus) did not increase inflammation in Calu-3 cells significantly, confirming that there was no viable virus after UV-inactivation of RV stock.

IL-8 released by Calu-3 cells was not significantly changed due to infection as well as lycopene supplementation. In cell culture experiments IL-8 production is induced at two time points. The first time point is in the second hour of virus infection (early event) [684] and the second peak is at 48-72 hours (late event) of incubation [126]. Therefore, it is likely that the 48 hours infection that was used in this experiment is not the appropriate time to detect IL-8 released by the cells. Low MOI of RV used in the experiments also is a probable explanation for unexpected results of IL-8 [685] and higher doses of RVs may be needed to elicit release of mediators of neutrophil chemotaxis in confluent Calu 3 cells.

We hypothesised that lycopene may reduce RV replication in the cultured airway cells, which may explain the reduced cytokine production we showed following RV infection. It has been reported that some nutrients such as flavone [106], vitamin A [686] and ascorbic acid [687, 688] decrease the replication of some viruses. This occurs with the interference with the viral replication between viral un-coating and the initiation of viral RNA (vRNA) synthesis [106]. Our TCID$_{50}$ results showed that lycopene significantly decreased viral replication of RV-1B by 85% (p<0.025). We also showed that lycopene may decrease viral replication of RV-43; however, due to the variability in the data, this was not statistically significant. By decreasing viral replication, lycopene could decrease the inflammatory mediators released by cells and reduce infection of neighbouring cells. Decreased IP-10 production by cells, is reportedly associated with low rhinovirus replication in the cells [376], that is consistent with our finding of decreased IP-10 levels due to lycopene.

In summary, we found that RV infection and LPS exposure for 48 hours can increase the inflammatory biomarkers in airway epithelial cells. Cells pre-supplemented with 2.5µg/mL lycopene for 24 hours (before the infection period), reduced IL-6 and IP-10 levels, possibly due to the inhibitory effect of lycopene on rhinovirus replication. In addition, the reduction in inflammatory cytokines levels may occur as a result of
lycopene scavenging the reactive oxygen species that are produced following infection and thereby suppressing the activation of NF-κB. This would be an interesting area for future research.
Anti-inflammatory effects of long chain n-3 polyunsaturated fatty acids on rhinovirus-infected cultured airway epithelial cells
6.1. Introduction
Kromann and Green (1980) compared the prevalence of inflammatory disease among Eskimos in Greenland with age/gender-matched people from Denmark. They found a lower incidence of inflammatory disorders including coronary heart disease, asthma, multiple sclerosis and psoriasis among people from Greenland [463]. Later this was attributed to the anti-inflammatory effect of a high intake of long chain n-3 polyunsaturated fatty acids (LCn-3PUFA) obtained from seafood dominated diets [464]. In the 1980s many studies confirmed the anti-inflammatory properties of LCn-3PUFA [277]. LCn-3PUFA mostly refer to fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) [246]. In the human body EPA and DHA are synthesized from the essential fatty acid, α-linolenic acid [275]. The key source of LCn-3PUFA is seafood [282].

In addition to being a source of energy in the human body, LCn-3PUFA can also be converted to eicosanoids which play an important regulatory role [244], in regulating inflammation [283, 284]. Eicosanoids affect inflammation via the cyclooxygenase (COX) and lipoxygenase (LOX). Arachidonic acid (AA) is converted to prostaglandin-2 (PG₂), thromboxane-2 (TX₂) and leukotrienes-4 (LT₄) which are more potent inflammatory mediators than the eicosanoids produced from EPA, including PG₃, TX₃ and LT₅ [245, 278, 689]. For example, while both of PG₂ and PG₃ are pro-inflammatory agents [275], LTB₄ (produced from AA) is 10-30 times more potent as a chemo-attractant of leukocytes than LT₅ (produced from EPA) and is also a potent inducer of ROS production [277, 278, 689]. As a chemoattractant, LTB₄ increases the number of neutrophils at the site of inflammation which induce more inflammation [503, 690, 691]. TX₂ (produced from AA) is also a stronger vasoconstrictor and platelet aggregator compared to TX₃ produced from LCn-3PUFA [277, 278].

It has been shown that supplementation with EPA and DHA decreases LTB₄ production by leukocytes [284]. It has also been shown that daily oral supplementation of EPA and DHA decreases neutrophil chemotaxis due to LTB₄ by up to 70% [284]. It has been demonstrated that EPA and to a lesser extent DHA [275] compete with AA leading to a reduction in active eicosanoids [276, 277]. It has also been shown that DHA decreases the release of AA from the phospholipids of cellular membranes via decreasing the
enzymatic activity of phospholipase A\textsubscript{2} [692]. Moreover, LCn-3PUFA decrease the incorporation of AA into the cellular membrane [693].

It has also been shown that AA converts to isoprostanes (8-iso-PGF\textsubscript{2α}) [694] via cyclooxygenase-independent [694, 695] and dependent [696] pathways, which are catalysed by free radicals [694, 695]. Isoprostanes (F\textsubscript{2}-isoprostanes) are considered markers of lipid peroxidation in vivo [697]. They are biologically active, as they have vasoconstrictive activity [695, 698] and may worsen asthma [699]. It has also been shown that production of PGF\textsubscript{2α} [700] is decreased in vivo after high intake of DHA and EPA [309-311]. Therefore the net effect of increased LCn-3PUFA content of the cellular membrane is reduced inflammation via substitution for AA, thereby removing the substrate for the lipoxygenase (LOX) and cyclooxygenase (COX) enzymes and leading to production of less potent eicosanoids [275, 277, 465].

There is evidence that increased levels of LCn-3PUFA in the cellular membrane decreases cytokine production and also minimizes the response to pro-inflammatory cytokines [288, 289]. It has been reported that oral supplementation of LCn-3PUFA decreases systemic inflammation via reducing levels of acute inflammatory biomarkers such as C-reactive protein (CRP) and interleukin-6 (IL-6) [290]. This anti-inflammatory effect has been confirmed by an in vitro study in human mononuclear cells in which LCn-3PUFA supplementation reduced the production of IL-6 [291]. In vivo studies in mice have also shown a decrease in circulating levels of IL-6, IL-10, TNF-α [292-294], IL-12, IL-1β and IFN-γ [293] due to LCn-3PUFA supplementation. It has also been found that n-6 fatty acids, such as AA, have a pro-inflammatory effect that is opposite to the effect of LCn-3PUFA such as EPA and DHA [296-298]. Anti-inflammatory effects of LCn-3PUFA are attributed to their potent effect on suppression of nuclear factor-κB (NF-κB) [286, 299-301, 468] most probably via inactivation of toll-like receptors (TLRs) [285], which have a key role in activation of NF-κB [667]. NF-κB has a proven effect on the production of various pro-inflammatory cytokines (reviewed in [124]) including IL-6 and IL-8 production in rhinovirus infection [124-126]. It has been reported that LCn-3PUFA suppresses NF-κB via blocking degradation of I-κB (the inhibitor of NF-κB) and also blocking the mitogen-activated protein kinase (MAPK) [124, 299]. It has been also shown that while AA activates TLRs (TLR2 and TLR4),
LCn-3PUFA inactivate TLR2 [285, 287]. Recently, it has been found that DHA is more potent than EPA in suppression of NF-κB [300].

It has also been reported that supplementation of high levels of fish oil in vivo (in which fish oil provides more than 20% of total fatty acids in the diet) decreases (a) lymphocytes proliferation, (b) natural killer cells and monocyte activities and (c) neutrophil and monocyte chemotaxis [278, 288, 289, 291, 302, 303]. However, it has also been shown that lower levels of fish oil supplementation (in which EPA and DHA provided 4.4% of total fatty acids or 1.7% of dietary daily energy) is sufficient to demonstrate some of the above mentioned effects [304]. It has been found that the supplementation of lower levels of EPA and DHA, equivalent to levels consumed in a normal balanced human diet [244] inhibits lymphocyte proliferation and dietary EPA (but not DHA) inhibits natural killer cell activity [305]. Investigating the relationship between LCn-3PUFA, cytokines and eicosanoids is an interesting area for future research [701]. Figure 6.1.1 schematically shows the probable mechanism by which LCn-3PUFA decrease AA inflammatory pathway.
Rhinovirus (RV) is the most common cause of the common cold [363] and the major cause of exacerbation in asthma among adults [364] and children [364, 365]. RV infection can lead to airway obstruction in asthmatics [386]. The binding of RV to the cellular receptors results in internalisation of virus particles into the cytoplasm of the cells [368], where RV replicates [369]. RVs target epithelial cells, in which they replicate and un-coat [371]. After RV infection, large numbers of neutrophils and lymphocytes are recruited to the airways [375]. As a result, epithelial cells produce various cytokines, chemokines and other inflammatory mediators that might contribute to the host defence and inflammatory responses [376]. Induction of the immune response starts with activation of TLRs which activates NF-κB [285, 667]. NF-κB mediates the transcription of many inflammatory biomarkers (reviewed in [124]) including cytokines and chemokines [125, 126]. Some of these inflammatory molecules include interleukin-1 (IL-1) [377], IL-6 [125, 371, 378], IL-8 [126, 371, 378-381], IL-11 [382], granulocyte-monocyte-colony stimulation factor (GM-CSF) [371, 381], intercellular adhesion molecule-1 (ICAM-1) [377, 384-386] and interferon-gamma induced protein-10 (IP-10) [376]. Rhinovirus infection increases oxidative stress in
airway epithelial cells most probably via increased production of reactive oxidative species (ROS) [337, 387]. Increased production of ROS activates NF-κB [337].

Asthma is considered as a chronic inflammatory disease [312-314]. It has been shown that dietary components such as fatty acids and antioxidants most strongly implicated in asthma aetiology [279, 544]. LCn-3PUFA have been demonstrated as useful intervention for a range of inflammatory diseases, including asthma [544]. However, clinical [263] and meta-analysis studies have failed to show any beneficial effect of LCn-3PUFA on asthma situation in vivo [321].

There is no evidence regarding whether LCn-3PUFA can improve inflammation in airway epithelial cells infected with RV. However, some studies have shown an anti-inflammatory effect of EPA and DHA on different cell types induced by LPS in vitro [299-301]. All of these studies found that EPA and DHA suppress NF-κB activation in the cells [299, 300]. We hypothesised that LCn-3PUFA may decrease inflammation in rhinovirus-infected airway epithelial cells. The aim of this study was to determine the effect of EPA and DHA supplementation on the inflammatory response of airway epithelial cells to RV infection.
6.2. Materials and Methods:

Study design:

Airway epithelial cell culture:
As described in chapter 2, airway epithelial cells (Calu-3, Passage 40-43, ATCC, USA) were cultured in minimum essential medium containing 10% foetal calf serum (10%FCS/MEM), containing 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, and 2.2g/L NaHCO₃ (all from Invitrogen Corporation, Carlsbad, USA) at 37°C in the presence of 5% CO₂. All experiments were carried out in cells with >80% confluence.

LCn-3PUFA enrichment of cultured epithelial airway cells:
As method described in chapter 2, in order to prepare the medium containing DHA, EPA AA, the method of Nair et al was employed [598]. Final concentration of 0, 10, 200 and 400µM of each fatty acid (all were purchased from Sigma) was dissolved in 0.04% ethanol (400µL ethanol/L of medium) and added to 10% FCS/MEM.

Visual inspection of Calu-3 cells:
Cellular viability of cultured Calu-3 cells was checked after each step of supplementation and infection (Olympus microscope, TL4).
Rhinovirus infection of Calu-3 cells:
As method described in chapter 2, after removing fatty acid contained media, cells were infected with rhinovirus-43 (RV-43; multiplicity of infection: MOI=7.2) and rhinovirus-1B (RV-1B; multiplicity of infection: MOI=7.2). Plates were incubated for 48 hours at 37°C in the presence of 5% CO₂. This assay was undertaken three times.

Analysing the inflammatory biomarkers concentration of media:
As method described in chapter 2, interleukin-6 (IL-6), IL-8 and interferon-gamma induced protein-10 (IP-10) concentrations of media were measured by ELISA (R&D Systems Minneapolis, MN, USA) according to manufacturer’s instructions. This assay was undertaken in duplicated for each (three passages of) cell culture assay.

Lactate dehydrogenase assay:
Lactate dehydrogenase (LDH) concentration in the media was measured by the Hunter Area Pathology Service (HAPS), the John Hunter Hospital, NSW, Australia. Lactate dehydrogenase is assayed using enzymatic method on the Dade Behring RXL Dimension platform (Dade Behring Inc. USA). The assay has a co-efficient of variation of 4.9% at 350 U/L. This assay was undertaken three times.

Fatty acids incorporation:
The incorporation of fatty acids (including EPA, DHA and AA) into Calu-3 cells was analysed using gas chromatography (GC) [594]. The cell pellet was suspended in 2mL of a methanol/toluene mixture (4:1 v/v), containing C19:0 (0.02mg/mL) and BHT (0.12g/L) and vortexed vigorously. The samples were methylated by adding 200μL acetyl chloride drop-wise while vortexing, followed by heating to 100°C for 1 hr. After cooling, the reaction was stopped by adding 5mL of 6% K₂CO₃ followed by vigorous mixing by vortex. The sample was centrifuged at 3000 x G at 4°C for 10 min to facilitate separation of layers. The upper toluene layer containing the fatty acid methyl esters was transferred to a 2mL glass vial and crimp sealed with a teflon lined cap for analysis by gas chromatography (GC). GC analysis was conducted using a 30m x 0.25mm (DB-225) fused carbon-silica column, coated with cyanopropylphenyl (J & W Scientific, Folsom, CA). Both injector and detector port temperatures were set at 250°C. The oven temperature was 170°C for 2 min, increased 10°C/min to 190°C, held for 1 min, then increased 3°C/min up to 220°C and maintained to give a total run time of 30
min. A split ratio of 10:1 and an injection volume of 3μL were used. The chromatograph was equipped with a flame ionization detector, auto-sampler and auto-detector. Sample fatty acid methyl ester peaks were identified by comparing their retention times with those of a standard mixture of fatty acid methyl esters and quantified using a Hewlett Packard 6890 Series Gas Chromatograph with Chemstations Version A.04.02.

**Viral titration assay:**
As method described in chapter 2, TCID$_{50}$ experiments were performed using confluent RD-ICAM-1 cells seeded in 96-well tissue culture plates (NUNC, Roskilde, Denmark). Cells were infected by either media alone or virus containing media at varying dilutions. Serial ten-fold dilutions of the samples were prepared and 4 individual wells were infected with each dilution. For titration of samples 6 dilutions were prepared. Additionally, for every dilution 2 control wells were prepared with media alone. After four days the plates were read and TCID$_{50}$ calculated. Infected wells were scored based on the cytopathic effect (CPE) seen, >50% CPE was demonstrated by light microscopy was considered a positive result. Viral titers of the samples determined by cell titration assay using RD-ICAM-1 cells and the viral titer was calculated and expressed as tissue culture infectious dose at 50% in log value (TCID$_{50}$ log$_{10}$) [595] and using the Karber formula for the tissue culture infective dose 50% (TCID$_{50}$) [596].

**Statistics:**
Paired t-test, analysis of variances (ANOVA) and correlations were performed by Graphpad software (Graphpad Prism 4, CA, USA). P-values less than 0.05 were considered as statistically significant.
6.3. Results

*Visual inspection of the cells:*

Visual inspection of the cultured Calu-3 cells after each step confirmed that cells were viable.

*Cellular uptake of EPA, DHA and AA by Calu-3 cells:*

Figure 6.3.1 shows the incorporation% of different concentrations of EPA, DHA and AA into Calu-3 cells. Figure shows that fatty acids incorporated into the cultured airway epithelial cells in dose dependant manner. Incorporation of EPA (P=0.0001) and DHA (P=0.0046) into cellular membrane increased significantly as the concentration of the fatty acids are increased in the media. Incorporation of AA into the cells is increased as AA is increased in the media; however, its trend is not statistically significant (P=0.23). Results show at the highest concentration (400µM) of EPA, DHA and AA incorporated 16.3%, 26% and 6% into the cellular membrane of the Calu-3 cells respectively. Therefore, DHA has the highest incorporation into the airway epithelial cells.
Figure 6.3.1 Incorporation% of different concentrations of (a) EPA, (b) DHA and (c) AA into Calu-3 cells:

![Graphs showing incorporation% of different concentrations of EPA, DHA, and AA into Calu-3 cells.]

Incorporation of AA in control cells compared to the cells supplemented with EPA and DHA:

Figure 6.3.2 shows the incorporation of AA in control cells compared to the cells supplemented with EPA and DHA. Results show that supplementation with DHA (400µM) significantly decreased the incorporation of AA in cellular membrane of Calu-3 cells.
Figure 6.3.2 Incorporation of AA in control cells compared to the cells supplemented with EPA and DHA (n=3):

Lactate dehydrogenase (LDH) released by Calu-3 cells incubated with different concentrations of EPA, DHA and AA:

Figure 6.3.3 shows the LDH released by Calu-3 cells supplemented with different concentrations (0, 10, 200 and 400µM) of EPA, DHA and AA. Results do not show any significant change in LDH released by cells due to different concentrations of fatty acids.
Figure 6.3.3 Lactate dehydrogenase (LDH) released by Calu-3 cells incubated with different concentrations of (a) EPA, (b) DHA and (c) AA:

Inflammatory biomarkers released by Calu-3 cells supplemented with and without different concentrations of EPA, DHA and AA following infection induced by RV-43 and RV-1B:

Figures 6.3.4, 6.3.5, 6.3.6, 6.3.7, 6.3.8 and 6.3.9 show IL-6, IL-8 and IP-10 released by Calu-3 cells supplemented with different concentrations of EPA, DHA and AA following infection with RV-43 and RV-1B. Results show that both of RV-43 and RV-1B infection resulted in increased release of IL-6, IL-8 and IP-10. DHA (400µM) resulted in a significant 16% reduction in IL-6 after RV-43 (6.3.4b), 29% reduction in
interleukin-6 after rhinovirus-1B infection (6.3.5b), 28% reduction in IP-10 after rhinovirus-43 infection (6.3.8b) and 23% reduction in IP-10 after rhinovirus-1B infection (6.3.9b). The lower concentration of DHA (200µM) also decreased IL-6 released by Calu-3 cells infected with RV-1B (6.3.5b). Decreased release of IL-6 by Calu-3 cells infected with RV-1B due to DHA is dose dependent (6.3.5b).
Figure 6.3.4 IL-6 released by Calu-3 cells enriched with and without different concentrations of (a) EPA, (b) DHA and (c) AA following infection induced by Rhinovirus-43:
Figure 6.3.5 IL-6 released by Calu-3 cells enriched with and without different concentrations of (a) EPA, (b) DHA and (c) AA following infection induced by Rhinovirus-1B:
Figure 6.3.6 IL-8 released by Calu-3 cells enriched with and without different concentrations of (a) EPA, (b) DHA and (c) AA following infection induced by Rhinovirus-43:

(a)

(b)

(c)
Figure 6.3.7 IL-8 released by Calu-3 cells enriched with and without different concentrations of (a) EPA, (b) DHA and (c) AA following infection induced by Rhinovirus-1B:
Figure 6.3.8 IP-10 released by Calu-3 cells enriched with and without different concentrations of (a) EPA, (b) DHA and (c) AA following infection induced by Rhinovirus-43:
Figure 6.3.9 IP-10 released by Calu-3 cells enriched with and without different concentrations of (a) EPA, (b) DHA and (c) AA following infection induced by Rhinovirus-1B:

(a) $P=0.002$

(b) $P=0.002$, $P=0.01$

(c) $P=0.002$
Comparison of the effect of supplementation of each fatty acid (400µM) on (a) IL-6, (b) IL-8 and (c) IP-10 released by Calu-3 cells infected with rhinovirus:

Figure 6.3.10 and 6.3.11 show the comparison between the levels of IL-6, IL-8 and IP-10 released by Calu-3 cells supplemented with highest concentration of fatty acids (400µM). Figures show that DHA (400µM) decreased IL-6 and IP-10 more than the highest concentration of other fatty acids. Figures also show that there is a decreasing trend in concentration of IL-6 and IP-10 supplemented with EPA (400µM); however, this trend is not statistically significant. Results also show that there is an increasing trend in the concentrations of IP-10 supplemented with AA (400µM); however, this trend is not statistically significant.
Figure 6.3.10 Comparison of the effect of supplementation of each fatty acid (400µM) on (a) IL-6, (b) IL-8 and (c) IP-10 released by Calu-3 cells infected by RV-43:

(a) IL-6 (pg/mL)

(b) IL-8 (pg/mL)

(c) IP-10 (pg/mL)
Figure 6.3.11 Comparison of the effect of supplementation of each fatty acid (400µM) on (a) IL-6, (b) IL-8 and (c) IP-10 released by Calu-3 cells infected by RV-1B:

Viral replication of RV-43 and RV-1B in Calu-3 cells supplemented with and without the highest concentration (400µM) of EPA, DHA and AA:

Figure 6.3.12 shows viral replication of RV-43 and RV-1B in Calu-3 cells supplemented with and without the highest concentration (400µM) of EPA, DHA and AA. Results show that none of the fatty acids has changed the viral replication of rhinoviruses significantly.
Figure 6.3.12 Viral replication of (a) RV-43 and (b) RV-1B in Calu-3 cells supplemented with and without the highest concentration (400µM) of EPA, DHA and AA:

(a)

(b)
Correlations between DHA content of the cells and pro-inflammatory cytokine released by cells after RV-1B infection:

Figure 6.3.13 shows the correlations between DHA content of the cells with (a) IL-6 and (b) IP-10 released by cells after RV-1B infection. Results show that DHA content of the cells negatively correlated with IL-6 (Spearman’s rho = -0.775, P = 0.003) and IP-10 (Spearman’s rho = -0.697, P = 0.012) released by the cells after infection with RV-1B.

Figure 6.3.13 Correlations between DHA content of the cells with (a) IL-6 and (b) IP-10 released by cells after RV-1B infection:
6.4. Discussion

This is the first study to investigate the effect of EPA and DHA on rhinovirus infected airway epithelial cells (Calu-3 cells). We showed that DHA (400µM) decreased the release of IL-6 and IP-10 by airway epithelial cells infected with RV-43 and RV-1B. It was also shown that EPA and AA have no effect on reducing the release of measured inflammatory biomarkers. It was also shown that DHA, EPA and AA have no effect on virus replication of RV-43 and RV-1B.

Our results showed that EPA, DHA and AA are incorporated into the cultured airway epithelial cells in dose dependent manner (Figure 6.3.1). Our results showed that at the highest concentration (400µM), DHA incorporation is highest followed by incorporation of EPA and AA. DHA (400µM) incorporation into the cells (26%) was nearly two times of incorporation of EPA (16.3%) and four times of incorporation of AA (6%). Other studies reported a dose dependent incorporation of LCn-3PUFA into human breast cancer cells after 24 hours [702]. It has been shown that higher incorporation of DHA into cellular membrane compared to EPA is attributed to more efficient acylation of DHA than EPA into membrane phospholipids [703].

Our results also showed that AA incorporation% in cells supplemented with DHA decreased significantly compared to control cells with no supplementation. This is in agreement with other studies showing LCn-3PUFA decrease the incorporation of AA in cellular membrane of airway epithelial cells [693]. Our results also show that DHA decreased AA incorporation more than EPA. The probable explanation for this is higher incorporation of DHA into the cellular membrane compared to EPA that is shown in Figure 6.3.1. This substitution of DHA/EPA for AA is important, as the anti-inflammatory effect of LCn-3PUFA is dependent, not only on increasing the level of LCn-3PUFA, but reducing the level of AA, thus ensuring a reduction in production of pro-inflammatory eicosanoids.

Supplementation of different concentrations (10, 200 and 400µM) of EPA, DHA and AA did not cause any cytotoxic effect on the cultured cells. Cellular viability was confirmed by visual inspection of the cells as well as lactate dehydrogenase (LDH) released by the cells after supplementation with LCn-3PUFA (Figure 6.3.3). LDH is a commonly used cell death biomarker [648]. In vitro studies have been shown that
incorporation of high levels of DHA and EPA (more than 500µM) increase cell cytotoxicity and apoptosis in different cell types [272, 704, 705]. However, in low concentration (as has been used in this experiment), there is no cell toxicity has been reported [299, 598].

Our results showed that the concentration of IL-6, IL-8 and IP-10 increased significantly after infection with RV-43 and RV-1B. This is in agreement with the other studies which have found rhinovirus infection increases the production of IL-6, IL-8 [125, 126, 683] and IP-10 [376] in different types of cultured cells. Our results also showed that DHA (400µM) significantly decreased the release of IL-6 and IP-10 by Calu-3 cells infected with RV-43 and RV-1B. Our results also showed that DHA in 200µM concentration decreased IL-6 release by Calu-3 cells infected with RV-1B. The results also showed strong negative correlation between DHA content of cells and IL-6 and IP-10 released by cells. Other in vitro [291], human [290] and animal studies [292] reported a decrease in IL-6 release after supplementation with LCn-3PUFA. Weldon et al reported that DHA is more potent than EPA in decreasing the production of inflammatory biomarkers [300]. In their study, the incorporation of DHA and EPA was not compared. Our study showed that increased incorporation of DHA compared to EPA is probably the explanation of higher anti-inflammatory effect of DHA, because it may be accompanied with inhibited inflammatory pathway of AA (due to low AA content of cellular membrane) [275, 277, 465] and decreased levels of different inflammatory biomarkers (due to increased DHA content of cellular membrane) [290-294]. The potential effect of LCn-3PUFA on decreasing cytokine production via suppression of NF-κB has been well described [286, 299-301]. NF-κB has a proven effect on production of vast variety of pro-inflammatory cytokines (reviewed in [124]) including IL-6 and IL-8 after RV infection [125, 126]. It has been reported that LCn-3PUFA suppress NF-κB via (a) inactivation of toll-like receptor-2 (TLR2) [285, 287, 301], (b) blocking I-κB (NF-κB inhibitor) degradation and also (c) blocking the mitogen-activated protein kinase (MAPK) [299]. LCn-3PUFA also inhibit AA inflammatory pathway via competing with AA to produce less inflammatory active eicosanoids [275-277], decreasing the release of AA from the phospholipids of cellular membrane via decreasing the enzymatic activity of phospholipase A2 [692], decreasing AA incorporation into the cellular membrane [693] and suppressing the activation of cyclooxygenase-2 (COX-2) which converts AA to PG2 and TX2 [285, 286].
probable mechanism for this suppression is also related to inactivation of toll-like receptor-2 (TLR2) [285, 287]. Therefore the most probable mechanism by which DHA decreased the production of inflammatory biomarkers in our study is its inhibitory effect on (a) NF-κB activation and (b) AA inflammatory pathway, both via decreasing TLRs activity.

Our results show that viral replication of RV-43 and RV-1B was not changed due to supplementation of fatty acids. Therefore, RV replication is independent of fatty acid composition of cellular membrane. There is no evidence regarding the effect of EPA, DHA and AA on the viral replication of rhinoviruses in the literature.

Our data also showed that DHA decreased IP-10 production of Calu-3 cells infected with rhinoviruses. However, there is no evidence regarding the effect of LCn-3PUFA on the release of IP-10 from the airway epithelial or other type of cells. It has been reported that response elements of NF-κB in the promoter region of the IP-10 gene are involved in transcriptional activation of IP-10 [706-709]. Therefore, decreased NF-κB activation may affect the release of IP-10. It has been suggested that IP-10 concentration is mostly related to viral replication in the cells [376]. However, according to our unchanged viral replication data in cells supplemented with different fatty acids, decreased concentration of IP-10 due to DHA observed in our study is probably independent of viral replication. The viral-replication-independent release of IP-10 has been reported previously [421].

In summary we found that DHA decreases inflammation in the airway epithelial cells infected with RV-43 and RV-1B via decreasing IL-6 and IP-10 released by cells. It was found that after supplementation of equal amounts of EPA and DHA, DHA incorporation into the airway epithelial cells is higher than EPA. DHA decreased AA incorporation in the cellular membrane. These two findings might be a probable explanation for the higher anti-inflammatory effect of DHA. It has been also found that enrichment of airway epithelial cells with DHA, EPA and AA did not change the replication of RV-43 and RV-1B. Therefore, DHA decreases the inflammatory response of airway epithelial cells to RV infection.
The synergistic anti-inflammatory effect of lycopene and long chain n-3 polyunsaturated fatty acids on rhinovirus infected airway epithelial cells
7.1. Introduction

Lipids are oxidized by reactive oxygen species (ROS) produced as by-products of normal metabolism in the body [244]. Reactive oxygen species include compounds such as superoxide (\( \cdot O_2^- \)), the hydroxyl radical (\( \cdot OH \)), hydrogen peroxide (\( \cdot H_2O_2 \)), singlet oxygen (\( \cdot O_2 \)) and hypochlorous acid (\( \cdot HOCl^- \)) [561, 710, 711]. It is generally suggested that lipid oxidation proceeds via a free radical mechanism called autoxidation, which includes initiation, propagation and termination stages and predominantly occurs with polyunsaturated fatty acids (PUFA) [712, 713]. Lipid oxidation occurs via both non-enzymatic and enzymatic pathways in the cellular membrane [712]. A peroxidation sequence in PUFA is initiated by the attack of free radicals with sufficient reactivity to abstract a hydrogen atom from an allelic methylene group of a polyunsaturated fatty acid [244]. Different stages of autoxidation of lipids are followed [244].

1. **Initiation:** \( X' + LH \rightarrow L' + XH \)
2. **Propagation:** \( L' + O_2 \rightarrow LOO' \)
3. \( LOO' + LH \rightarrow L' + LOOH \)
4. **Termination:** \( LO' + LO' \rightarrow \) non-radical polymers
5. \( LOO' + LOO' \rightarrow \)
6. \( L' + L' \rightarrow \)
7. \( LOO' + L' \rightarrow \)

It has been suggested that increased levels of LCN-3PUFA in cellular membranes results in increased susceptibility to lipid peroxidation [306, 714-716], due to increased oxidizability of the polyunsaturated fatty acid content of the membrane [307]. It has been suggested that polyunsaturation of fatty acids is the most important target of lipid peroxidation [715]. An animal study showed that increased LCN-3PUFA content of red blood cells via consumption of fish oil increases lipid peroxidation until a critical low level of intact lipids is reached in the membrane of cells [717]. Human studies also confirmed an increased susceptibility of LDL to lipid peroxidation due to increased LCN-3PUFA content of LDL particles [718].

It has been confirmed by high performance liquid chromatography (HPLC) analysis that lipids are converted mainly to hydroxyl and hydrogen peroxyl radicals as products and markers of lipid peroxidation and oxidative stress [719]. Other markers of lipid peroxidation also include isoprostanes [720-724], malondialdehyde (MDA) [725-727],
total hydroxy-octadecadienoic [728, 729], exhaled ethane [730, 731] and blood glutathione [732]. To avoid increased susceptibility of cells to oxidative stress during LCn-3PUFA supplementation, cosupplementation with antioxidants is recommended, to minimize fatty acid peroxidation [716, 733].

Antioxidants can scavenge free radicals; thereby preventing LCn-3PUFA from oxidative degradation [44]. Antioxidants are substances that inhibit oxidation of other molecules in the body [1, 2]. They can prevent cellular damage due to oxidative stress [3] via: decreasing the concentration of O₂ [4], catalytic metal ions [5-7] and H₂O₂ [8-10], scavenging the free radicals that initiate oxidative stress [11, 12], breaking the initiated sequence chain of oxidation [13-15], quenching the singlet oxygen [16-18] and repairing oxidative damage caused by free radicals [19-22]. Carotenoids are an important class of antioxidants, found mostly in fruit and vegetables. They generally scavenge peroxyl radicals via electron transfer, hydrogen abstraction and addition [135, 166, 177-180]. The following equations show the peroxyl radical scavenging property of carotenoids (CAR):

\[
\begin{align*}
\text{CAR} + \text{ROO}^- & \rightarrow \text{CAR}^{\cdot} + \text{ROO}^- \\
\text{CAR} + \text{ROO}^- & \rightarrow \text{CAR}^{\cdot} + \text{ROOH} \\
\text{CAR} + \text{ROO}^- & \rightarrow \text{ROOCAR}^{\cdot}
\end{align*}
\]

There is evidence that carotenoid supplementation can inhibit the lipid peroxidation caused by increasing LCn-3PUFA content of cell membranes. This is demonstrated by an increased lag time before oxidative deterioration [734]. Co-supplementation of LCn-3PUFA with antioxidants such as natural vitamin E [719, 735-738] and its synthetic forms [739] has also been shown to be effective in preventing oxidative stress. Antioxidants such as vitamin E [740] and lycopene [179, 180] can terminate lipid peroxidation via hydrogen abstraction and result in oxidative damage prevention [741]. It has also been reported that some other antioxidants such as dimethylthiourea (DMTU) [742], lutein, zeaxanthin [743] and genistein [744] have synergistic effects with different functions of LCn-3PUFA. The effect of vitamin C on peroxidation of fatty acid has also been studied, with inconsistent results [745, 746]. However, the beneficial effect of a high intake of antioxidants on lipid peroxidation is generally well accepted [747].
We have found that lycopene decreases inflammation via decreased cytokine release from airway epithelial cells infected with rhinovirus (chapter 5). We have also demonstrated that DHA has a similar anti-inflammatory effect (chapter 6). It has been proposed that the probable anti-inflammatory mechanism of both nutrients may involve suppression of nuclear factor-κB (NF-κB). Lycopene is a potent antioxidant [44, 173] and most probably exerts its anti-inflammatory effect via suppression of NF-κB by its redox based activity [432]. On the other hand, LCn-3PUFA which do not have antioxidant property [275, 300], might exert their effect on inflammation via non-redox based suppression of NF-κB [285, 299, 301]. The synergistic effect of lycopene and LCn-3PUFA on inflammation is unknown and even in a most recent review article regarding the synergistic effects of antioxidants and fatty acids in inflammation, lycopene seems to be ignored [701]. We hypothesised that lycopene may increase the anti-inflammatory effect of LCn-3PUFA via preventing oxidation. Therefore, we aimed to examine the probable synergistic anti-inflammatory effect of lycopene and LCn-3PUFA on airway epithelial cells infected with rhinovirus.
7.2. Materials and methods

Study design:

Airway epithelial cell culture:
As described in chapter 2, airway epithelial cells (Calu-3, Passage 45-48, ATCC, USA) were cultured in minimum essential medium (MEM) containing 10% foetal calf serum, 2% penicillin-streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% L-glutamine, and 2.2g/L NaHCO₃ (Invitrogen Corporation, Carlsbad, USA) at 37°C in the presence of 5% CO₂. Cell culture experiments were repeated three times.

Supplementation of Calu-3 cells with combination of lycopene, EPA and DHA:
As described in chapter 5 (for lycopene supplementation) and 6 (for LCn-3PUFA supplementation), Calu-3 cells were incubated with 1%FCS/MEM containing final concentrations of the following supplements; lycopene (2.5µg lycopene dissolved in
Visual inspection of Calu-3 cells:
Cellular viability of cultured Calu-3 cells was checked after each step (Olympus microscope, TL4).

Intracellular lycopene analysis:
As described in chapter 2, in order to measure the concentration of intracellular lycopene, Barua’s method was employed [141]. Briefly, intracellular (20 million cell pellets) lycopene was extracted from the samples by ethyl acetate and hexane, and incorporated in dichloromethane: methanol; 1:2 v/v. Samples were analysed by high performance liquid chromatography (HPLC) 1200 Series (Agilent Technologies, USA) by injecting (5µL, with flow rate: 0.3mL/min) into Hypersil ODS C18 column (Thermo Electron Corporation, USA) and detecting by diode array detector (DAD) (Agilent Technologies, USA) at a preset 450nm wavelength. This assay was undertaken three times.

Fatty acids incorporation:
The incorporation of fatty acids (including EPA, DHA and AA) into Calu-3 cells was analysed using gas chromatography (GC) [594]. The cell pellet was suspended in 2mL of a methanol/toluene mixture (4:1 v/v), containing C19:0 (0.02mg/mL) and BHT (0.12g/L) and vortexed vigorously. The samples were methylated by adding 200µL acetyl chloride drop-wise while vortexing, followed by heating to 100ºC for 1 hr. After cooling, the reaction was stopped by adding 5mL of 6% K$_2$CO$_3$ followed by vigorous mixing by vortex. The sample was centrifuged at 3000 x G at 4ºC for 10 min to facilitate separation of layers. The upper toluene layer containing the fatty acid methyl esters was transferred to a 2mL glass vial and crimp sealed with a teflon lined cap for analysis by gas chromatography (GC). GC analysis was conducted using a 30m x 0.25mm (DB-225) fused carbon-silica column, coated with cyanopropylphenyl (J & W Scientific, Folsom, CA). Both injector and detector port temperatures were set at 250ºC. The oven temperature was 170ºC for 2 min, increased 10ºC/min to 190ºC, held for 1 min, then increased 3ºC/min up to 220ºC and maintained to give a total run time of 30
min. A split ratio of 10:1 and an injection volume of 3μL were used. The chromatograph was equipped with a flame ionization detector, auto-sampler and auto-detector. Sample fatty acid methyl ester peaks were identified by comparing their retention times with those of a standard mixture of fatty acid methyl esters and quantified using a Hewlett Packard 6890 Series Gas Chromatograph with Chemstations Version A.04.02.

**Rhinovirus infection of Calu-3 cells:**
As described in chapter 2, after removing the supplemented media, cells were infected by rhinovirus-43 (RV-43) (multiplicity of infection: MOI = 7.2) and rhinovirus-1B (RV-1B) (multiplicity of infection: MOI = 7.2). Plates were incubated for 48 hours at 37°C in the presence of 5% CO₂.

**Analysing the inflammatory biomarkers concentration of media:**
As described in chapter 2, IL-6, IL-8, and IP-10 concentrations in the media were measured by ELISA (R&D Systems Minneapolis, MN, USA) according to manufacturer’s instructions. This assay was undertaken in duplicate for each cell culture assay. The concentration of biomarkers released by cells enriched with and without different supplements was compared.

**Lactate dehydrogenase assay:**
Lactate dehydrogenase (LDH) concentration in the media was measured by the Hunter Area Pathology Service (HAPS), the John Hunter Hospital, NSW, Australia. Lactate dehydrogenase is assayed using an enzymatic method on the Dade Behring RXL Dimension platform (Dade Behring Inc. USA). The assay has a co-efficient of variation of 4.9% at 350 U/L.

**Viral titration assay:**
As described in chapter 2, TCID₅₀ experiments were performed using confluent RD-ICAM-1 cells seeded in 96-well tissue culture plates (NUNC, Roskilde, Denmark). Cells were infected by either media alone or virus containing media at varying dilutions. Serial ten-fold dilutions of the samples were prepared and 4 individual wells were infected with each dilution. For titration of samples, 6 dilutions were prepared. Additionally, for every dilution, 2 control wells were prepared with media alone. After
four days the plates were read and TCID$_{50}$ calculated. Infected wells were scored based on the cytopathic effect (CPE) seen, >50% CPE demonstrated by light microscopy was considered a positive result. Viral titers of the samples determined by cell titration assay using RD-ICAM-1 cells and the viral titer was calculated and expressed as tissue culture infectious dose at 50% in log value (TCID$_{50}$ log$_{10}$) [595] and using the Karber formula for the tissue culture infective dose 50% (TCID$_{50}$) [596]. This assay was undertaken three times.

Statistics:
Differences between supplemented and unsupplemented groups were analysed using paired t-tests and analysis of variance (ANOVA) by Graphpad software (Graphpad Prism 4, CA, USA). P-values less than 0.05 were considered as statistically significant.
7.3. Results

*Visual inspection of Calu-3 cells:*

Visual inspection of the cultured Calu-3 cells after each step confirmed that cells were viable.

*Lactate dehydrogenase (LDH) released by Calu-3 cells incubated with EPA (400µM), DHA (400µM), lycopene (2.5µg/mL) and their combinations:*

Figure 7.3.1 shows lactate dehydrogenase (LDH) released by Calu-3 cells incubated with EPA (400µM), DHA (400µM), lycopene (2.5µg/mL) and their combinations. Results show that there is no difference between LDH released by Calu-3 cells supplemented with EPA (400µM), DHA (400µM), lycopene (2.5µg/mL) and their combinations.

*Figure 7.3.1 Lactate dehydrogenase (LDH) released by Calu-3 cells incubated with EPA (400µM), DHA (400µM), lycopene (2.5µg/mL) and their combinations:*

![Graph showing LDH released by Calu-3 cells](image)

*Incorporation% of (a) EPA and (b) DHA into Calu-3 cells supplemented with EPA (400µM), DHA (400µM) and their combination with lycopene (2.5µg/mL):*

Figure 7.3.2 shows the incorporation% of (a) EPA and (b) DHA into Calu-3 cells supplemented with EPA (400µM), DHA (400µM) and their combination with lycopene (2.5µg/mL). The results show that incorporation of both of fatty acids increased after supplementation of fatty acids combined with and without lycopene. The results also
show that there is no significant change in the incorporation of EPA and DHA due to concomitant lycopene supplementation. The results also show that incorporation of DHA (19.5%) is higher than EPA (11.5%).

Figure 7.3.2 Incorporation% of (a) EPA and (b) DHA into Calu-3 cells supplemented with EPA (400µM), DHA (400µM) and their combination with lycopene (2.5µg/mL):

(a)

Incorporation% of EPA

(b)

Incorporation% of DHA

Intracellular lycopene concentration of Calu-3 cells supplemented with lycopene (2.5µg/mL) and its combination with EPA (400µM) and DHA (400µM): Figure 7.3.3 shows the concentration of lycopene in Calu-3 cells (20 million cells) supplemented with lycopene (2.5µg/mL) and its combination with EPA (400µM) and DHA (400µM). The results show that lycopene concentrations of Calu-3 cells supplemented with lycopene-EPA and lycopene-DHA are less than cells supplemented
with lycopene. The results also show that lycopene concentration of Calu-3 cells supplemented with lycopene-DHA is less than lycopene concentration of cells supplemented with lycopene-EPA.

Figure 7.3.3 Intracellular lycopene concentration of Calu-3 cells supplemented with lycopene (2.5µg/mL) and its combination with DHA (400µM) and EPA (400µM):

Inflammatory biomarkers released by Calu-3 cells supplemented with EPA, DHA, lycopene and their combinations following rhinovirus infection:

Figures 7.3.4, 7.3.5, 7.3.6 show IL-6, IL-8 and IP-10 released by Calu-3 cells supplemented with lycopene, EPA, DHA and their combinations followed by RV-43 and RV-1B infection. The results show that supplementation with lycopene-DHA resulted in decreased IL-6 and IP-10 released by Calu-3 cells infected with RV-1B. Changes due to supplementation of EPA, DHA, lycopene and their combinations before and after infection with RV-43 are not statistically significant. The results also show that supplementation of lycopene-EPA and lycopene-DHA did not change IL-8 released from Calu-3 cells infected with both of RVs.
Figure 7.3.4 IL-6 released by RV infected Calu-3 cells supplemented with EPA, DHA, lycopene and their combinations:

Figure 7.3.5 IL-8 released by RV infected Calu-3 cells supplemented with EPA, DHA, lycopene and their combinations:
Figure 7.3.6 IP-10 released by RV infected Calu-3 cells supplemented with EPA, DHA, lycopene and their combinations:

Viral replication of (a) RV-43 and (b) RV-1B in Calu-3 cells supplemented with EPA, DHA, lycopene and their combinations:

Figure 7.3.7 shows viral replication of (a) RV-43 and (b) RV-1B in Calu-3 cells supplemented with lycopene-EPA and lycopene-DHA. The results show that lycopene-DHA significantly decreased the replication of RV-1B. Trends toward decreased viral replication of RV-43 and RV-1B in the cells co-cultured with lycopene-EPA and lycopene-DHA are not statistically significant.
Figure 7.3.7 Viral replication of (a) RV-43 and (b) RV-1B in Calu-3 cells supplemented with lycopene-EPA and lycopene-DHA:

Comparison of the effect of lycopene, DHA and lycopene-DHA on the inflammation of Calu-3 cells infected with RVs:

Table 7.3.1 shows the comparison of changes due to lycopene, DHA and lycopene-DHA on inflammation in Calu-3 cells infected with RVs. The changes due to lycopene-DHA are smaller than the changes due to lycopene or DHA alone. Comparing the
magnitude of the changes shows that lycopene-DHA does not further decrease the inflammatory biomarkers and RV replication compared to lycopene or DHA.

Table 7.3.1 Comparison of the effect of lycopene, DHA and lycopene-DHA on the inflammation of Calu-3 cells infected with RVs:

<table>
<thead>
<tr>
<th>Supplements</th>
<th>Changes due to lycopene</th>
<th>Changes due to DHA</th>
<th>Changes due to lycopene-DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>RV-43</td>
<td>No significant change</td>
<td>16% Decrease</td>
</tr>
<tr>
<td></td>
<td>RV-1B</td>
<td>19% Decrease</td>
<td>29% Decrease</td>
</tr>
<tr>
<td>IL-8</td>
<td>RV-43</td>
<td>No significant change</td>
<td>No significant change</td>
</tr>
<tr>
<td></td>
<td>RV-1B</td>
<td>No significant change</td>
<td>No significant change</td>
</tr>
<tr>
<td>IP-10</td>
<td>RV-43</td>
<td>23% Decrease</td>
<td>28% Decrease</td>
</tr>
<tr>
<td></td>
<td>RV-1B</td>
<td>No significant change</td>
<td>23% Decrease</td>
</tr>
<tr>
<td>RV replication</td>
<td>RV-43</td>
<td>No significant change</td>
<td>No significant change</td>
</tr>
<tr>
<td></td>
<td>RV-1B</td>
<td>85% Decrease</td>
<td>No significant change</td>
</tr>
</tbody>
</table>

7.4. Discussion

This is the first study investigating the synergistic anti-inflammatory effect of lycopene and LCn-3PUFA in RV infected airway epithelial cells. We found that the combination of lycopene and DHA did not further decrease the production of inflammatory biomarkers and rhinovirus replication. We also found that DHA incorporation into Calu-3 cells was higher than EPA when incubated in equimolar concentrations and its increased incorporation resulted in decreased intracellular lycopene levels most probably due to increased antioxidant usage. Therefore, lycopene does not have synergistic effect with DHA in decreasing the inflammatory response of airway epithelial cells to RV infection.

Visual inspection of cultured Calu-3 cells and also lactate dehydrogenase concentrations confirmed that supplementation of airway epithelial cells with lycopene-EPA and lycopene-DHA did not cause any cytotoxic effect on Calu-3 cells. Cells were viable after each step of supplementation and infection and the results of the lactate dehydrogenase assay showed that there is no difference between LDH released by Calu-3 cells supplemented with DHA, EPA, lycopene and their combinations (Figure 7.3.1). Previously it was shown that supplementation with lycopene (Chapter 5), EPA and
DHA (Chapter 6) individually did not have any cytotoxic effect on Calu-3 cells. This study now demonstrates that the combinations of these nutrients also have no cytotoxic effect on Calu-3 cells.

The results show that there was no significant change in incorporation of EPA or DHA when combined with lycopene (Figure 7.3.2). As reported in chapter 6, the incorporation of DHA is greater than EPA. Thus supplementation of similar doses of EPA and DHA (400µM) resulted in higher incorporation of DHA (19.5%) compared to EPA (11.5%). However, lycopene cosupplementation did not enhance LCn-3PUFA uptake.

Our data showed that lycopene concentrations of Calu-3 cells supplemented with lycopene-EPA and lycopene-DHA were lower than cells supplemented with lycopene alone (Figure 7.3.3). Increased intracellular content of LCn-3PUFA results in increased susceptibility to lipid peroxidation that is associated with increased usage of antioxidants [736]. Thus the probable explanation for lower concentrations of lycopene in cells with increased EPA and DHA content is increased usage of lycopene as it protects LCn-3PUFA from oxidation. Decreased levels of plasma vitamin E, another lipid soluble antioxidant, have also been found by Meydani et al following oral administration of EPA and DHA [736]. Our results show that the lycopene content of Calu-3 cells supplemented with lycopene-DHA is even lower than the lycopene content of cells supplemented with lycopene-EPA. This is probably due to the higher incorporation of DHA compared to EPA which we have observed in current and previous chapter (Chapter 6).

Our results showed that supplementation with lycopene-EPA and lycopene-DHA resulted in decreased IL-6 and IP-10 release by Calu-3 cells infected with RV-1B. Comparing the magnitude of the changes due to lycopene, DHA, EPA and their combinations indicates that the lycopene-DHA did not further decrease the production of inflammatory biomarkers, compared to lycopene or DHA alone. Some studies have reported a probable synergistic effect of antioxidants with LCn-3PUFA, with decreased levels of inflammatory biomarkers such as TNF-α being reported [748]. We found that lycopene and DHA do not have a significant synergistic anti-inflammatory effect on airway epithelial cells infected with RV. Considering that lycopene and DHA alone
decrease the release of IL-6 and IP-10 (Chapters 5 and 6), decreased intracellular lycopene levels in cells supplemented with EPA and DHA could explain the observed weak anti-inflammatory effect of the combination of lycopene and DHA.

These experiments show that supplementation of lycopene-DHA significantly decreased the replication of RV-1B but not RV-43. Supplementation of lycopene-EPA did not change replication of RV-43 or RV-1B, however there was a trend towards decreased replication. This is probably due to the effect of lycopene, rather than EPA and DHA, as we have demonstrated (Chapter 5) that lycopene decreases the viral replication of RV-1B (85%), while DHA did not affect viral replication (Chapter 6). This chapter shows that lycopene-DHA decreased the viral replication of RV-1B up to 40%, however the effect was not as strong as for lycopene alone (85%). Assuming that lycopene is responsible for the decrease in viral replication, decreased intracellular lycopene levels in the cells supplemented with lycopene-DHA (Figure 7.3.3) could explain the weaker effect of lycopene-DHA on decreasing viral replication of RV-1B. There are no previous reports regarding the synergistic effect of lycopene and LCn-3PUFA on RV replication in literature. However, this experiment does not show any benefit of cosupplementation of lycopene and DHA on viral replication.

In summary we found that lycopene and DHA do not have a significant synergistic anti-inflammatory effect on airway epithelial cells infected with RVs. It was also found that supplementation with DHA increased the utilisation of lycopene, most probably due to increased unsaturated fatty acid content of the cells. The decreased intracellular levels of lycopene that were achieved when delivered in combination with DHA supplementation may explain the weaker anti-inflammatory effect of the combination.
Chapter 8

General discussion and future directions
The burden of asthma is high in Australia, as it affects 1 in 10 adults and 1 in 6 children [474, 600]. Asthma is a disease linked to inflammation [312-314] and oxidative stress [313, 562-564]. There is considerable evidence that antioxidant nutrients may have a significant beneficial effect on asthma [749]. Furthermore, many studies show the beneficial effect of antioxidant-rich foods such as fruit and vegetable on asthma prevalence. For example, in adults there is a negative association between the intake of fresh fruits [750] and vegetables [751] and asthma prevalence. It has also been found that introduction of fresh fruits and vegetables in childhood may decrease the risk of asthma in the later life [752]. Lycopene is a potent antioxidant found in fruits and vegetables [44, 173, 187] that can reduce oxidative stress and improve inflammatory status [448, 459, 461, 462, 753]. Lycopene has been shown to decrease the inflammation via a redox-based effect on inactivation of nuclear factor-κB (NF-κB) [432, 434, 435], a transcription factor that mediates the production of vast varieties of inflammatory biomarkers [124-126]. Redox-based effect of antioxidants on NF-κB suppression highlights an important link between antioxidants and inflammation. Lycopene is of particular interest in asthma, as its circulating level is low in asthmatics compared to healthy people [241, 622, 623]

Several experiments exploring the role of lycopene in reducing inflammation in asthma were undertaken. Firstly, the effect of oral supplementation with lycopene on systemic inflammation in patients with stable asthma (Chapter 3) was examined. The results presented show that there is a trend towards decreased plasma CRP and IL-6 after intake of lycopene-rich treatments. Decreased CRP [753] and IL-6 [449] levels following daily lycopene supplementation has been reported recently. A negative correlation was observed between plasma lycopene and CRP levels, suggesting that low lycopene levels drive systemic inflammation. A negative correlation between plasma CRP and lung function (%FVC) was also evident, suggesting that systemic inflammation may contribute to the worsening of clinical outcomes. Furthermore, the correlation observed between plasma IL-6 and sputum neutrophil count, suggests that there is an association between airway and systemic inflammation. This data confirm previous observations that lycopene protects against inflammation in asthma [631-634].

A series of \textit{in vitro} experiments were conducted to examine the effects of lycopene supplementation on inflammation in airway epithelial cells. This required establishment
of a suitable method for enriching cultured airway epithelial cells with lycopene, which has poor water solubility. Tetrahydrofuran (THF), as a lycopene co-solvent [452, 649] and liposomes as lycopene carriers [597, 650] have been used for lycopene transport into different types of cultured cells. The effectiveness of THF and liposomes in delivering lycopene into airway epithelial cells was compared and it was found that pre-supplementing with lycopene at the final concentration of 2.5µg/mL dissolved in 0.5% THF (in cell culture media) for 24 hours delivers lycopene into Calu-3 cells efficiently without serious cytotoxic effects compared to liposomes. This method which has been used by other researchers for incorporation of lycopene and other carotenoids in vitro studies to decrease inflammation [452], was followed for the experiments involving lycopene enrichment of Calu-3 cells.

Many researchers continue to optimise methods for lycopene delivery into cultured cells. Recently, Lin et al have reported a new method of lycopene delivery into cultured human prostate cancer cells. They compared fetal bovine serum (FBS) and THF, and found that fetal bovine serum can carry larger amounts of lycopene into the cells [754]. This method can be used in case of mega-dose supplementation of lycopene. This method was not available when our cell culture experiments were conducted, however, it can be investigated further in future studies.

Chapter 5 describes the anti-inflammatory effects of lycopene in Calu-3 cells infected with two different serotypes (major and minor) of rhinovirus (RV) and lipopolysaccharide (LPS). It was found that pre-supplementation of Calu-3 cells with lycopene reduces the release of IL-6 and IP-10 following RV infection and is associated with a significant reduction in RV replication. The results suggest that lycopene is likely to be working in two ways; firstly it may have a direct inhibitory effect on the formation of reactive oxygen species (ROS) that occur following infection, thereby reduce the activation of NF-κB via lycopene’s redox based activity. Secondly, it is also likely that it causes a reduction in viral replication directly and reduces the release of IP-10. The probable mechanism by which lycopene decreases the production of inflammatory biomarkers is NF-κB suppression, in which lycopene inhibits nuclear translocation of p65 subunit of NF-κB [432, 434, 435]. NF-κB is a transcription factor that mediates the production of many inflammatory cytokines [124-126]. It has been found that the activity of NF-κB changes with the cellular redox state and increased production of
ROS results in activated NF-κB [425, 432, 434, 437, 439, 440, 755, 756]. Therefore, increased antioxidant capacity of cells due to lycopene [189], may decrease the activation of NF-κB [425, 432, 434, 437, 439, 440, 755, 756] and the production of inflammatory biomarkers [425, 432, 434, 437, 439, 440, 755-757]. The probable mechanism by which lycopene decreases RV replication is its interference with the viral replication between viral uncoating and the initiation of viral RNA (vRNA) synthesis [106].

Other antioxidant nutrients such as β-carotene [436] quercetin [434], vitamin C [437, 438] and vitamin E [437, 439, 440] are shown to suppress NF-κB activity and decrease inflammation via the same mechanism as lycopene. An interesting direction for future work would be the investigation of the anti-inflammatory effects of other dietary antioxidants such as carotenoids other than lycopene, vitamin E, flavonoids and vitamin C on airway inflammation. This would provide a broader picture of the beneficial effect of fruit and vegetable intake on managing inflammation, specifically in relation to asthma and respiratory health [750-752].

In chapter 6, the anti-inflammatory effects of LCn-3PUFA have been reported. It was hypothesised that supplementation of long chain n-3 polyunsaturated fatty acids (LCn-3PUFA) would decrease inflammation in airway epithelial cells infected with RV. Many studies have reported an anti-inflammatory property of LCn-3PUFA in terms of decreasing inflammatory biomarkers [290-294]. LCn-3PUFAs inhibit production of potent eicosanoids and play an important role in the regulation of inflammation in the human body [244, 283]. The net effect of increased LCn-3PUFA content of cellular membrane is inhibition of the inflammatory pathway of arachidonic acid (AA) [275, 277, 465] and decreased levels of inflammatory cytokines [290-294]. Anti-inflammatory effects of EPA and DHA are attributed to their potent effect on suppression of NF-κB [286, 299-301] due to (a) decreased action of toll-like receptors (TLR2, 3, 4, 5, or 9) [285, 287, 301], (b) blocking I-κB degradation (inhibitor of NF-κB activation) and also (c) blocking the mitogen-activated protein kinase (MAPK) [299]. It has also been shown that DHA and to a less extent EPA decrease the activation of NF-κB via IL-1 and TNF-α pathways [300], which are redox insensitive pathways [756, 758, 759]. In order to test the hypothesis, airway epithelial cells were supplemented with different concentrations of EPA, DHA and AA followed by rhinovirus infection
(major and minor serotypes). The results showed that DHA, but not EPA decreased inflammation in the airway epithelial cells infected by RV-43 and RV-1B by decreasing IL-6 and IP-10 released by cells. A higher proportion of DHA was incorporated into cells than EPA, and this corresponded with reduced AA content. Increased DHA content and decreased AA content of cell membranes might explain the anti-inflammatory effect of DHA compared to EPA, as substitution of DHA for AA inhibits the inflammatory pathway of arachidonic acid (AA) [275, 277, 465].

The anti-inflammatory effects of LCn-3PUFA that was found in our in vitro experiments is in agreement with several epidemiological studies that have also shown that asthma risk is reduced with increased intake of LCn-3PUFA [275, 277, 279, 760]. Therefore, consuming adequate amounts of seafood or DHA fortified food is probably a useful way to manage inflammation in asthma. Findings regarding the beneficial effects of DHA supplementation on inflammation in airway epithelial cells, suggest that the anti-inflammatory effect of DHA in asthma in vivo should be closely examined. In particular, the effect of DHA on the frequency and severity of virus-induced exacerbations should be investigated.

Results presented in chapter 7 on the anti-inflammatory effects of lycopene and LCn-3PUFA confirmed that both lycopene and DHA decrease the production of inflammatory biomarkers. It is also known that supplementation of LCn-3PUFA results in increased susceptibility of cells to lipid peroxidation [306, 714-716], due to increased oxidizability of polyunsaturated fatty acids of cellular membrane [307]. Therefore, it was hypothesised that co-supplementation with an antioxidant (lycopene) and LCn-3PUFA may prevent LCn-3PUFA from lipid peroxidation and have a greater anti-inflammatory effect compared to lycopene or LCn-3PUFA alone. Meydani et al found that vitamin E protected against lipid peroxidation due to supplementation of EPA and DHA [736]. It has also been reported that adding vitamin C and E to EPA and DHA supplements results in decreased IFN-γ released from mononuclear cells [761]. Therefore, it was hypothesised that there would be a synergistic anti-inflammatory effect of lycopene and LCn-3PUFA on airway epithelial cells infected with rhinovirus, when applied in combination. It was found that DHA uptake by Calu-3 cells was higher than EPA uptake. Furthermore, uptake of DHA was associated with decreased intracellular lycopene levels. This finding probably confirms increased usage of
lycopene as an antioxidant in the cells with higher levels of LCn-3PUFA, similar to decreased circulating vitamin E levels of elderly people following EPA and DHA supplementation [736]. It was found that lycopene-DHA (but not lycopene-EPA) decreased the production of IL-6 and IP-10 following RV-1B infection and the replication of RV-1B. However, the magnitude of these changes is smaller than the changes due to lycopene and DHA individually. It is concluded that lycopene and DHA do not have any significant synergistic anti-inflammatory effect on airway epithelial cells infected with RVs. Decreased intracellular levels of lycopene due to DHA supplementation is probably the explanation of the weak anti-inflammatory effect of lycopene-DHA compared to lycopene.

The results of these experiments show that lycopene, DHA and lycopene-DHA consistently caused a decrease in IP-10 and IL-6 released by Calu-3 cells infected with RV-1B. IL-6 is an acute inflammatory response cytokine [125, 373] and IP-10 which is produced by epithelial [417] and inflammatory cells [421], is a chemokine [376], levels of which may be dependent [376], or independent of viral replication [421]. Therefore, experiments show that lycopene, DHA and lycopene-DHA, decrease acute inflammation induced by a minor serotype of RV (RV-1B). Thus, it is proposed that increased dietary consumption of nutrients such as lycopene and DHA by asthmatics could be an effective way to prevent exacerbation induced by RV infection. As discussed in chapter 1, 5, and 6, decreased release of cytokines (such as IL-6) and chemokines (such as IP-10), by airway epithelial cells and also decreased replication of RV (RV-1B) in airway epithelial cells, result in decreased leukocyte recruitment and their ROS production which further inhibits inflammation in the airways. This would be an interesting area for further research.

This PhD project has used an experimental model to investigate the effect of nutrient supplementation on airway inflammation. In the human body, which is more complex than cell culture models, the interactions between inflammatory pathways are more complicated and may be different to what we observed in our in vitro model. Therefore, clinical studies are needed to confirm our observations in an in vivo setting.
In summary, the following conclusions were made from our *in vivo* and *in vitro* studies on the effect of lycopene and LCn-3PUFA on airway inflammation:

1. Oral intake of lycopene is associated with lower levels of systemic inflammatory biomarkers in asthma (Chapter 3).

2. Enriching airway epithelial cells with lycopene (2.5µg/mL) dissolved in THF (0.5%) for 24 hours is a safe method of lycopene supplementation (Chapter 4).

3. Lycopene decreases the production of inflammatory biomarkers (IL-6 and IP-10) as well as RV-1B replication in cultured airway epithelial cells infected by RVs. This is most probably due to the redox-based effect of lycopene (Chapter 5).

4. DHA (but not EPA) decreases the production of inflammatory biomarkers (IL-6 and IP-10) in cultured airway epithelial cells infected by RVs. This is most probably due to increased incorporation of DHA and decreased incorporation of AA into the membranes of airway epithelial cells (Chapter 6).

5. The combination of lycopene and DHA decreased inflammatory biomarkers released from airway epithelial cells infected with RV. However, no synergistic anti-inflammatory effect of their combination was observed, most probably due to decreased intracellular lycopene concentrations resulting from increased susceptibility of DHA-enriched membranes to oxidation (Chapter 7).

Conclusion:
The results presented in this PhD thesis have demonstrated an anti-inflammatory role for lycopene and DHA in asthma. Further studies examining the role of these nutrients in reducing the frequency and severity of asthma exacerbations are warranted.
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