Chapter 1

Introduction and Literature Review.

1.1 Introduction:

Although primarily an anti-asthma agent, the cysteinyl leukotriene receptor antagonist, montelukast, has been reported to be useful in the treatment of other acute and chronic inflammatory diseases in which the neutrophil is believed to be the primary offender. As neutrophils do not synthesize cysteinyl leukotrienes, it is possible that montelukast possesses neutrophil-directed anti-inflammatory properties that are unrelated to its conventional cysteinyl leukotriene receptor ($CysLT_1R$)-targeted activities. These putative alternative neutrophil-directed anti-inflammatory activities of montelukast represent the focus of the current study.

1.2. Literature Review:

This literature review will focus on neutrophils, including activation of neutrophils and their role in the pathogenesis of disease, with emphasis on asthma and new targets for pharmacotherapy in controlling neutrophilic inflammation, especially in asthma.

1.2.1 Resting Neutrophils:

Neutrophils play a key role in the innate immune system of host defence (Kobayashi *et al*, 2005). Host defence systems consist of a number of cellular and protein components that interact to protect the host environment from outside attack.

Neutrophils are formed in the bone marrow from a pluripotent progenitor cell. Differentiation leads to the formation of monopotent progenitor cells, myeloblasts, promyeloblasts, metamyelocytes to band forms and maturing neutrophils. This maturation process takes 10-15 days to complete. Mature neutrophils are incapable of cell division (Adkinson *et al*, 2003). Mature neutrophils contain a variety of granules containing pre-formed products necessary to participate in the eradication of microbial pathogens, and also have the capacity to synthesise potent antimicrobial agents, once activated (Borregaard, Sørensen & Theilgaard-Mönch, 2007). Among these products are different proteases, oxygen radicals, defensins and certain lipid mediators. Neutrophils are also equipped to express genes to form products such as Fc-receptors, complement components, NADPH oxidase proteins and a number of cytokines and chemokines which play an important role in inflammatory and immune responses (Scapini *et al*, 2000).

1.2.2 Migration of neutrophils:

1.2.2.1 Margination:

Mature neutrophils are released from the bone marrow and migrate through the circulation, reaching tissue sites within 6-8 hours. This process is known as margination and ensures that large numbers of these cells are available when needed in the defence against harmful microbes.

Neutrophil migration from the circulation to the site of infection or inflammation is controlled by vascular endothelial interactions. Loose tethering of the neutrophils on the endothelium is mediated by L-selectin on the neutrophil surface and ligands on the surface of endothelial cells, including E- and P-selectin, and P-selectin glycoprotein ligand-1 (PSGL-1) (Wright *et al*, 2010). Tight adhesion of the neutrophil to the endothelium is mediated though interactions between ligands expressed on the surface of leucocytes with ligands on the endothelium such as intercellular adhesion molecule-1(ICAM-1), ICAM-2 and vascular cell adhesion molecule-1 (VCAM-1), as well as mucosal addressin cell adhesion molecule-1(MADCAM-1) (Wright *et al*, 2010).

A large number of marginated neutrophils are found in the pulmonary capillaries. At any given time, there are 60-100 times more neutrophils in the pulmonary capillaries than in the systemic circulation (Hogg & Doerschuk, 1995).

Migration of the neutrophils through the pulmonary capillaries is slow due to the fact that they have to deform to be able to pass through the narrow spaces in the capillary bed (Doerschuk *et al*, 1993). The slow movement of the neutrophils contributes to the high number in the pulmonary circulation.

1.2.2.2 Transmigration:

Following margination of the neutrophil to the wall of the vessel, tethering, rolling and adhesion to the vascular endothelium, the process of neutrophil transendothelial migration (TEM), across the vessel takes place. Diapedesis

across the vessel structure occurs by movement either through endothelial cell (EC) junctions (paracellular route) or through the body of the EC itself (transcellular route) (Gane & Stockley, 2011).

Paracellular transmigration occurs typically at the intersection of three or more ECs known as the tricellular corner (Burns *et al*, 1997). Neutrophil migration through the EC is enhanced by activation of the endothelial cell, or the neutrophil, or both, and the presence of a chemotactic gradient across the endothelium (Yang *et al*, 2005). Activation of these these cells can be induced by cytokines such as tumor necrosis factor (TNF), interleukin-1β (IL-1β) (Kumar *et al*, 2011) or endothelial-bound chemoattractants, including leukotriene B_4 (LTB_4), C5a and interleukin 8 (IL-8), or bacterial endotoxin (Gane & Stockley, 2011). Neutrophils can also change shape and migrate through an EC junction. This process can be mediated by adhesion molecules that relocate to allow the passage of the neutrophil, or it could be mediated by neutrophil proteases as was suggested by some studies (Su, Chen & Jen, 2002).

Diapedesis through the basement membrane (BM) has to be achieved without causing damage to the barrier function of the vessel (Hallman *et al,* 2005). Low expression regions (LERs) within the BM express less key matrix proteins and collagen IV and these regions have been shown to align with the EC junctions (Voisin, Woodfin & Nourshargh, 2009). Neutrophils adhere mostly to the endothelium close to these LERs. Neutrophils have the ability to increase the size of the LER, a process possibly mediated by elastase (Wang *et al,* 2006).

1.2.2.3 Transendothelial migration in the lung:

Integrins are transmembrane-spanning CAMs on the surface of leukocytes. Complement receptor 3 (CR3) was the first integrin identified and has several synonyms: Mac-1, Mo-1, αMβ2, CD11b/CD18. It is a member of the β2 integrins, which share the common CD18 (β2) subunit and are expressed exclusively by leukocytes; the other members are LFA-1 (CD11a/CD18), CR4 (CD11c/CD18), and the enigmatic αDβ2 (CD11d/CD18) (Ehlers, 2000).

Neutrophil migration to the lung tissue occurs via two different pathways, either via a β2-integrin-dependent, or an -independent mechanism (Doerschuk, Tasaka & Wang, 2000). The specific stimulus will determine the pathway chosen, as well as the inflammatory stimulus, whether acute or chronic. Mackarel *et al,* have shown in a study done in COPD, cystic fibrosis or bronchiectasis patients, compared to healthy controls, that a difference could be determined. Neutrophils in healthy adults and the stable patients, transmigrated via a CD18-independent way in response to IL-8 and LTB_4 stimulation, whereas the neutrophils from patients with acute exacerbations migrated via a CD18-independent way. Neutrophils of all the groups migrated via a CD18-dependent mechanism on N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) stimulation, confirming the stimulus specific nature of the process (Mackarel *et al,* 2001). Once neutrophils have passed through the endothelium, migration follows a chemotactic gradient.

1.2.3 Chemotactic Mediators:

Several chemokines, bacterial products, complement split products and lipid mediators act as chemoattractants for neutrophils. Important chemoattractants with respect to the lung include IL-8 and LTB_4.

1.2.3.1 Chemokines:

More than 40 chemokines have been identified in humans and are classified into two major groups: cysteine-X-cysteine (CXC) and the C-C (double-cysteine molecules) subfamilies. Chemokines bind to trans-membrane-spanning receptors signalling through G-protein interactions (Thelen & Didichenko, 1997).

Interleukin-8 (IL-8) is a potent chemoattractant for neutrophils in the lung. IL-8 can act as an activator of neutrophils through coupling of the chemokine to receptors (CXCR1 or CXCR2) on the neutrophils (Murphy, 1997). Sources of IL-8 include macrophages, neutrophils, endothelial cells, epithelial cells, fibroblasts and smooth muscle cells. Neutrophils are thus both a source of IL-8 and a target for the chemokine. This was demonstrated in a study done by Jatakanon *et al.* in severe persistent asthma, which demonstrated high concentrations of both IL-8 and neutrophils in the sputum of these patients (Jatakanon *et al,* 1999).

1.2.3.2 Lipid mediators:

Leukotriene B_4 (LTB_4) is a lipid mediator with potent chemoattractant properties that is rapidly generated from activated innate immune cells such as neutrophils,

macrophages, and mast cells (Ohnishi, Miyahara & Gelfand, 2008). The production of leukotrienes from membrane-derived arachidonic acid will be discussed in detail in a later section.

Three receptors have been identified to bind LTB_4. BLT1 and BLT2 are both G protein-coupled seven transmembrane domain receptors on the cell surface of specific cells. BLT1 is a high affinity receptor expressed predominantly on granulocytes, monocytes/ macrophages, mast cells, dendritic cells, and effector T cells (Tager & Luster, 2003). BLT2 also plays a role in chemotaxis of neutrophils (Yokomizo *et al,* 2001). The third receptor, PPARα, is a nuclear receptor that binds eicosanoids including LTB_4. Interaction with PPARα promotes degradation of the lipid mediators (Devchand *et al,* 1996).

Leukotriene B_4 is not only a chemoattractant for neutrophils, but also an activator of these cells. Complement 5a also acts as a chemotactic factor for neutrophils (Czermak *et al,* 1998).

1.2.4 Neutrophil Priming and Activation:

1.2.4.1 Priming:

Neutrophil priming refers to a process whereby exposure of these cells to various inflammatory mediators such as lipopolysaccharide (LPS), TNF, or granulocyte macrophage colony stimulating factor (GM-CSF) greatly enhances subsequent agonist-induced respiratory burst activity and degranulation responses

(Cadwallader *et al,* 2002). Priming of neutrophils has an effect on neutrophil survival and integrin/selectin expression (Condliffe *et al,* 1998). This process leads to enhanced killing of microbes, but will lead to tissue damage if uncontrolled.

Neutrophils are primed by binding of agonists to a number of different membrane receptors. These receptors include: (1) G-protein-linked seven-transmembrane-domain receptors which are single-transmembrane-domain receptors that require crosslinking for activation such as Fc-receptors; and (2) single-transmembrane-domain receptors for growth-regulating cytokines including tumor necrosis factor (TNF) and GM-CSF. The G-protein-linked seven-transmembrane-domain receptors bind platelet-activating factor (PAF), complement component 5a (C5a), substance P and IL-8. Activation of any of these receptors will prime the oxidase of neutrophils for subsequent activation. Activation of the seven-transmembrane-domain receptors, excluding IL-8, only leads to priming (Hallet & Lloyds, 1995), while cross-linking of the receptors will prime the neutrophil oxidase at low concentrations and will activate it at high concentrations. The growth-regulating cytokine receptors will only lead to priming (Guichard *et al,* 2007).

Intracellular signalling pathways involved in the regulation of the neutrophil oxidase include those driven by phosphoinositide 3-kinase (PI3K), phospholipase C (PLC)/Ca^{2+}-dependent protein kinase C (PKC), phospholipase D (PLD), phospholipase A2 (PLA2), and p38/Erk (Condliffe *et al,* 2005).

Activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase results from an increase in the cytosolic free Ca^{2+} in neutrophils (Guichard *et al,* 2007). Once the membrane receptors of the neutrophils interact with the chemoattractants and adhesion molecules, cytosolic Ca^{2+} is abruptly and transiently increased, which is a prerequisite for the initiation of the crucial pro-inflammatory actions of neutrophils, including generation of superoxide by the membrane-associated electron transporting NADPH oxidase, adhesion to vascular endothelium, degranulation, activation of cytosolic phospholipase A2 and 5-lipoxygenase, as well as synthesis of IL-8 (Tintinger *et al,* 2008).

1.2.4.2 Calcium Homeostasis:

Uncontrolled release of the mediators of activated neutrophils can lead to tissue damage and needs strict control. This is neatly done by the control mechanisms of Ca^{2+} homeostasis in neutrophils.

Mobilisation and restoration of Ca^{2+} homeostasis will be explained according to the diagram in Figure 1.1 (page 11). (Reprinted from Drug Design, development and Therapy. 2008;2:95-104) (Tintinger *et al,* 2008).

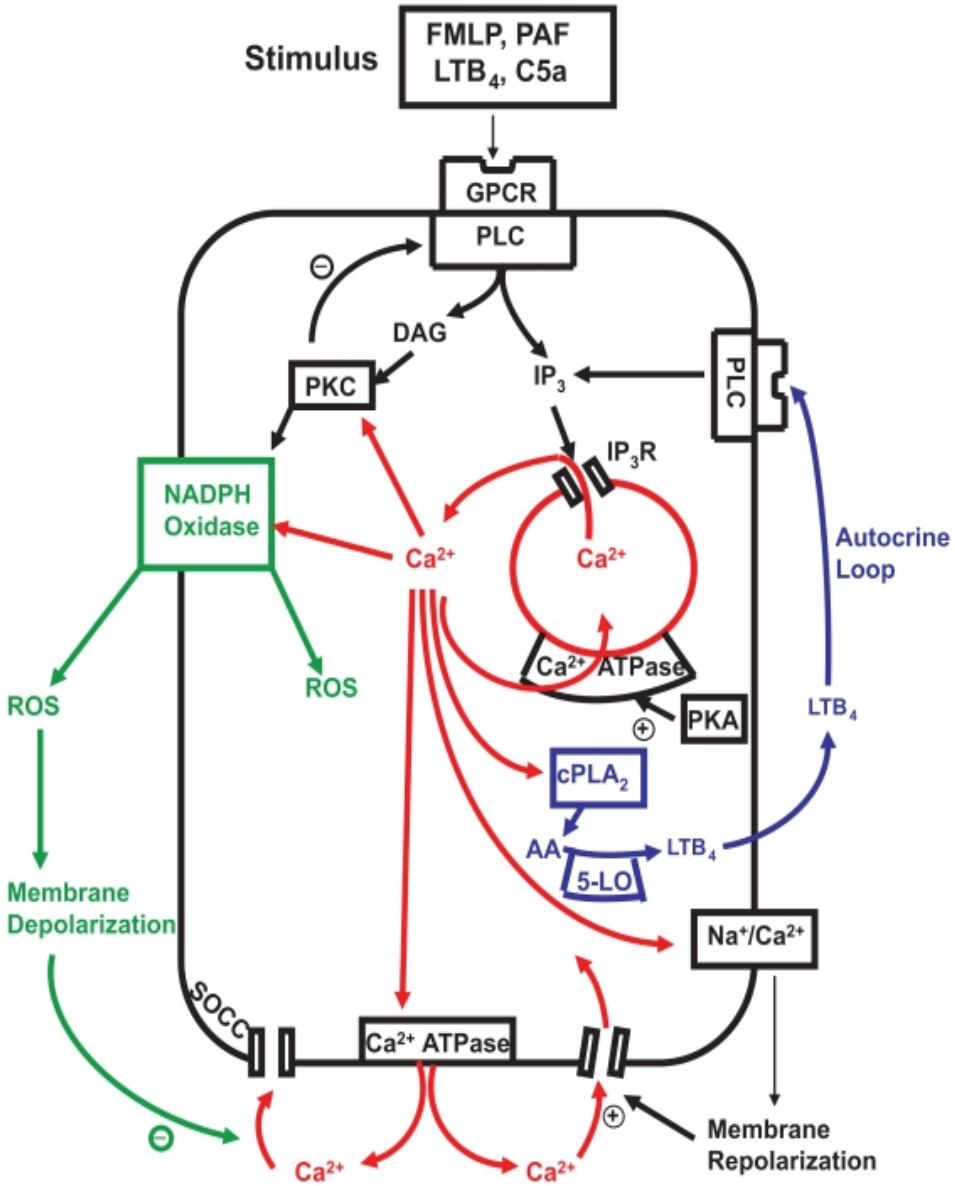

Figure 1.1: Calcium-mobilizing stimuli interact with membrane G-protein coupled receptors (GPCR) to activate phospholipase C (PLC) generating inositol triphosphate (IP3) which interacts with IP3 receptors (IP3R) releasing Ca^{2+} from storage vesicles. Cytosolic phospholipase A2 (cPLA2) mobilizes arachidonic acid (AA) for the 5-lipoxygenase (5-LO) pathway. The AA metabolite leukotriene B_4 (LTB_4) is actively transported to the cell exterior where it binds to its receptor to activate PLC, completing a positive feedback autocrine loop. Ca^{2+} released into the cytosol is rapidly extruded from the cell by the plasma membrane Ca^{2+} ATPase and re-sequestered into storage vesicles by the protein kinase A (PKA)-sensitive endomembrane Ca^{2+} ATPase. Protein kinase C (PKC) activated by Ca^{2+} and diacylglycerol (DAG) facilitates assembly and activation of NADPH oxidase on the outer membrane which generates reactive oxygen species (ROS) with concomitant membrane depolarization. The depolarized membrane potential delays Ca^{2+} entry through store operated channels (SOCCs) until the Ca^{2+} -activatible Na+/Ca^{2+} exchanger, operating in reverse mode, mediates recovery of the membrane potential promoting Ca^{2+} reuptake via SOCCs. PKC down-regulates PLC as part of a negative feedback loop to terminate IP3 production (Pharmacological control of neutrophil-mediated inflammation: Strategies targeting calcium handling by activated polymorphonuclear leucocytes. Drug design, Develop and Therapy. 2008;2:95-104)

Following receptor-mediated activation by chemoattractants including C5a, IL-8, FMLP, PAF and LTB_4, Ca^{2+} is released from intracellular stores of the neutrophil to reach a concentration of free cytosolic Ca^{2+}, 5-10 fold higher than basal value. Binding to the 7-transmembrane, G-protein-coupled receptors mentioned earlier, is controlled by various Gα and Bβγ subunits and leads to activation of β isomers of phospholipase C (PLC). PLC mediates production of inositol-1,4,5-triphosphate (IP_3) by hydrolysis of phosphatidylinositol-4,5,-biphosphate. Interaction between IP_3 and Ca^{2+}-mobilising receptors on intracellular storage vesicles results in discharge of stored Ca^{2+} into the cytosol (Tintinger *et al,* 2008). The presence of two distinct intracellular Ca^{2+} stores in human neutrophils has been demonstrated, one immediately below the plasma membrane, and the other at the centre of the cell, near the nuclear lobes (Pettit & Hallett, 1998).

The duration of the peak increase in the cytosolic Ca^{2+} is usually brief and is followed by a progressive decline, returning to basal levels. The duration of the peak cytosolic concentration of Ca^{2+} and the rate of return to basal level is determined by several different, albeit coordinated, mechanisms. At least four mechanisms have been identified: (1) shuttling of Ca^{2+} between the stores and the cytosol (Anderson, Steel & Tintinger, 2005); (2) activation of a secondary wave of Ca^{2+} influx stimulated by endogenously-generated LTB_4 by activated neutrophils (Steel *et al,* 2007); (3) the efficiency of mechanisms promoting clearance of Ca^{2+} from the cytosol (Steel & Anderson, 2002); and (4) the regulatory mechanisms controlling the time of onset, rate and magnitude of influx of extracellular Ca^{2+} (Tintinger *et al,* 2008).

The removal of Ca^{2+} from the cytosol of activated neutrophils is essential to avoid hyperactivity of these cells that can cause tissue damage. This is achieved by two separate adenosine triphosphate (ATP)-driven pumps. These are the plasma membrane Ca^{2+}-ATPase, mediating Ca^{2+} efflux, and the endomembrane ATPase, which is responsible for resequestration. The contributions of these pumps in removing the cation from the cytosol are equal (Pettit & Hallett, 1998).

Activation of neutrophils by chemoattractants like FMLP leads to activation of PLC and release of stored Ca^{2+}, but also transient activation of adenylate cyclase, resulting from the interaction of adenosine with G-protein/adenylyl cyclase-coupled adenosine receptors (AR) of the $A2_A$ subtype on the neutrophil membrane (Iannone, Wolberg & Zimmerman 1989; Theron *et al,* 2002), which leads to activation of adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase A (PKA). Phospholamban on the endomembrane Ca^{2+}-ATPase undergoes PKA-mediated phosphorylation which leads to up-regulation of the Ca^{2+} sequestrating/ resequestrating activity of the pump (Chu *et al,* 2000).

The membrane depolarisation action of NADPH oxidase facilitates the effective Ca^{2+}clearance actions of the two Ca^{2+-}ATPase pumps. Depolarisation restricts the influx of extracellular Ca^{2+} (Tintinger *et al,* 2008). The electrogenic properties of the oxidase lead to an abrupt and steep decrease in membrane potential (Steel & Anderson, 2002). The driving force for Ca^{2+} entry is abolished when depolarisation of the cells occurs, due to the reduction of the electrical component of the

electrochemical gradient that promotes Ca^{2+} entry. "Consequently, NADPH oxidase-mediated membrane depolarization enables the plasma membrane and endomembrane Ca^{2+}-ATPases to mediate clearance of Ca^{2+} from the cytosol of activated neutrophils, unhindered by influx of extracellular Ca^{2+}" (Tintinger *et al,* 2001).

Following the depletion of the intracellular Ca^{2+} stores, refilling occurs through a process known as store-operated Ca^{2+} influx. The duration of NADPH oxidase activity, as well as the intensity of the activity will determine the onset and rate of store-operated Ca^{2+} influx in neutrophils. Inefficient activators of the oxidase, such as PAF, cause rapid influx of Ca^{2+} which overwhelms the Ca^{2+}-ATPases, resulting in prolonged elevations in peak cytosolic Ca^{2+} concentrations (Tintinger *et al,* 2001). On the other hand, extracellular Ca^{2+} uptake into neutrophils activated by FMLP is delayed due to the activities of the oxidase, with the influx only being detectable at ± 1 min after activation by the chemoattractant. When NADPH oxidase activity declines, influx proceeds gradually over 5 min. This rate of Ca^{2+} influx is superimposable on that of membrane repolarisation (Tintinger, Steel & Anderson, 2005).

From the above, it is clear that calcium signalling in neutrophils is triggered by the activity of PLC, leading to the opening of IP3-gated channels in the membranes of intracellular calcium stores. During receptor stimulated Ca^{2+} release, the stores are depleted of Ca^{2+}, leading to store-operated Ca^{2+} entry (SOCE) (Schaff *et al,* 2010).

The main components of the SOCE pathway are the sensor of calcium store depletion, STIM 1 (Collins & Meyer, 2011), on the endoplasmic reticulum, and the plasma membrane component Orial 1 (Feske *et al,* 2006). Orial 1 mediates Ca^{2+} entry in neutrophils, in cooperation with transient receptor potential channels (TRPCs). TRPC 6 is the SOCE channel, regulating Ca^{2+} influx in neutrophils activated by E-selectin and G-protein coupled receptors (Bréchard, 2008).

SOCE play a role in neutrophil recruitment. A rise in Ca^{2+} causes an increase in the affinity of the β_2-integrin, MAC-1, which was mentioned before to play a role in the migration of neutrophils.

1.2.5 Activated Neutrophils:

Activated neutrophils can respond via two separate mechanisms. Either with rapid release of pre-formed serine proteinases stored in primary (azurophilic) granules and release of oxygen radicals, or by activating transcription factors that trigger *de novo* expression of molecules (e.g. receptors and cytokines) (Kasama *et al,* 2005).

1.2.5.1 Proteases:

Neutrophils take part in antibacterial activities via three routes: phagocytosis, degranulation and formation of neutrophil extracellular traps (NETs) (Urban *et al,* 2009). Neutrophil granules are formed during the maturation process and are released into the extracellular space from activated neutrophils by degranulation. Three types of granules have been identified: Azurophil granules (primary),

specific granules (secondary) and small storage granules (tertiary) (Wright *et al,* 2010). The contents of these granules are shown in Table 1.1.

Table 1.1: Neutrophil cytoplasmic granules and their major contents.

Azurophil Granules	*Specific Granules*	*Small Storage Granules*
a) Antimicrobial Substances • Myeloperoxidase • Defensins • Lysozyme • BPI (bacterial/permeability-increasing protein)	*a) Specific Granules* • Lactoferrin • Lysozyme	*a) Proteinases* • Gelatinase B (MMP-9)
b) Serine Proteases • Elastase • Cathepsin G • Proteinase 3	*b) Proteinases* • Collagenase (MMP-8) • Gelatinase (MMP-9) • Complement activator	*b) Membrane Receptors* • FMLP receptors • MAC-1 receptor
c) Acid Hydrolases • Cathepsin B • Cathepsin D	*c) Membrane receptors* • FMLP-receptor • MAC-1 • Laminin receptor	*c) Acid Hydrolases* • Cathepsin D • DAG lipase
d) Phospholipases • Secretory phospholipase A_2	*d) Other* • Histaminase • Cytochrome b_{558}	*d) Other* • B2-microglobulin

Neutrophil proteases include matrix metalloproteinases (MMPs) and neutrophil serine proteases (NSP).

1.2.5.1.1 Serine Proteases

Neutrophil serine proteases (NSP) contain the amino acid, serine, as the key residue of their enzymatic centre, which initiates the cleavage of the protein substrates (Kessenbrock, Dau & Jenne, 2011).

The serine proteases include, neutrophil cathepsin G (CG), neutrophil elastase (NE) and proteinase 3 (PR3), which are synthesized as inactive zymogens. The inactive molecules require two N-terminal proteolytic modifications to become active. After the signal peptide removal, the proenzyme is further processed by the lysosomal cysteine protease dipeptidyl peptidase I (DPPI, also known as cathepsin C) en route to the granules where they are stored as active enzymes (Pham, 2008).

They play an important role in bacterial defence, but can also alter the immune response by altering, either enhancing or abolishing the function of cytokines and chemokines by cleaving these proteins (Shapiro, 2002).

N-terminal proteolytic modification of IL-8 by the NSPs can alter cell function in different ways. PR3 converts it to a more potent neutrophil activating stimulant, whereas NE and CG will inactivate IL-8 (Padrines *et al,* 1994).

Progranulin (PGRN) also known as granulin-epithelin precursor, has been described to suppress the adhesion-dependent oxidative burst and protease

release by neutrophils in the presence of TNF. However, PRN loses its anti-inflammatory potential once cleaved by NE (Kessenbrock *et al,* 2008).

Once the NSPs are released into the extracellular space, they bind to surface receptors. The presence of CG on the surface of cells triggers integrin clustering on neutrophils favouring interaction with immobilised immune complexes, leading to cytoskeletal rearrangements, increased reactive oxygen species (ROS) production and the secretion of chemokines (Raptis *et al,* 2005).

Integrins may also provide binding sites for NSP on the cell surface. Neutrophil elastase binds directly to the integrin CD11b/CD18 (CR3, Mac-1), regulating integrin-mediated cellular attachment and detachment (Cai & Wright, 1996). Release of IL-8, cathepsin B and matrix metalloproteinase- 2 (MMP-2), can be induced by NE through a MyD88/IRAK/TNF-receptor-associated factor 6 (TRAF-6)-dependent pathway which also involves Toll-like receptor 4 (TLR4) (Geraghty *et al,* 2007). The Toll-like receptors are intra- and extracellular membrane-anchored molecules. These receptors sense inflammatory stimuli such as bacterial DNA or lipopolysaccharide (LPS) and, via stimulation of the nuclear κB pathway, mediate cellular activation (Xu *et al,* 2010).

As mentioned before, it was shown that NE plays a role in neutrophil transmigration though the vascular wall into the tissue (Wang *et al,* 2006). The mechanisms involved include: (1) cooperation with platelet/endothelial-cell adhesion molecule 1 (PECAM-1) and alpha 6 integrins (Wang *et al,* 2005); (2)

cleavage of adhesion molecules by NE and CG. The process of cleavage, especially at EC cell junctions, might cause gaps through which the neutrophils transmigrate (Robledo *et al,* 2003). These adhesion molecules include vascular endothelial cadherins (E-cadherins) (Mayerle *et al,* 2005), ICAM-1 (Robledo *et al,* 2003) and VCAM-1 ((Xu *et al,* 2005).

Uncontrolled proteolysis would be harmful for the host and tight control is necessary. Serine protease inhibitors are produced by the liver to neutralise the NSPs after their release (Kessenbrock, Dau & Jenne, 2011). Imbalance between proteases and their inhibitors can lead to certain autoimmune diseases (Heutinck *et al,* 2010), while emphysema can develop in patients with a genetic deficiency of α1-antitrypsin (Fregonese & Stolk, 2008).

There are ways that the NSPs maintain their activity and protect themselves: (1) NSPs are present in high concentrations which overwhelm the inhibitors (Owen & Campbell, 1995); (2) NSPs localise close to the vascular cell surface to shield themselves from their inhibitors (Campbell, Campbell & Owen, 2000); and (3) Neutrophil-released proteases such as MMP-9, can degrade inhibitors of NSPs (Liu *et al,* 2000).

1.2.5.1.2 Matrix Metalloproteinase:

Matrix metalloproteinases (MMPs) are a family of enzymes responsible for digestion of structural components of the extracellular matrix, including membranes collagens, elastin, laminin and fibronectin (Kessenbrock, Plaks & Werb, 2010). The

MMPs can also cleave other proteins such as receptors, growth factors, cytokines, chemokines and other proteases (McCawley & Matrisian, 2001).

In humans, more than 27 different MMPs have been described. They are grouped into five categories: Collagenases, gelatinases, stromelysins, matrilysins and membrane-associated types, and others. Activities of the MMPs are regulated by tissue inhibitors of metalloproteinases (TIMP) (Tandon & Sinha (2011).

Neutrophils produce high levels of MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B). These MMPs remodel the extracellular matrix, facilitating leucocyte trafficking through the endothelial barriers into solid organs. In a study conducted by Jung *et al* (2009), it was demonstrated that neutrophils are key mediators of recruitment of both Th1 and Th2 cells to the airways. The ratio between MMP-8, MMP-9 and TIMP-1 is crucial in the recruitment process. The study suggested that both neutrophils and MMPs could be targets for new anti-inflammatory asthma treatment (Jung *et al,* 2009).

Uncontrolled activity of the MMPs, has been implicated in chronic lung disorders, including asthma (Cauwe & Opdenakker, 2010) cystic fibrosis (Flifiel *et al,* 2006), and chronic obstructive pulmonary disease (Köhrmann *et al,* 2009). MMP-2,-8 and -9 were also found in acute-onset pulmonary diseases such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (Kong *et al,* 2009).

Increased expression of MMP-8 in bronchial biopsies correlated well with disease severity and was a good predictor of COPD development in smokers (Ilumets *et al,* 2007). In a study by Prikk *et al,* a correlation was shown between the level of MMP-8 and -9, neutrophils and markers of neutrophil activation such as myeloperoxidase (MPO), a marker of oxidant generation (Prikk *et al,* 2002).

1.2.5.2 Oxygen Radicals:

Already in 1933, it was observed that neutrophils demonstrated an increase in oxygen consumption during phagocytosis, a process known as the respiratory burst (Baldridge & Gerard, 1933). The respiratory burst is essential for effective bacterial killing as is clearly evident from chronic granulomatous disease (CGD) in which the absence of this process leads to overwhelming infection (Holmes, Page & Good, 1967). This oxygen-dependent form of bacterial killing leads to consumption of molecular oxygen due to the formation of superoxide and other reactive oxygen species (ROS).

Once activated, neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multicomponent enzyme, transfers electrons from cytoplasmic NADPH to molecular oxygen to generate superoxide:

$$NADPH + O_2 \rightarrow NADPH^+ + O_2^- + H^+$$

O_2^- is converted to H_2O_2, either spontaneously or by superoxide dismutase. H_2O_2 has antimicrobial potential in its own right, but is converted to the more potent

ROS, HOCl (reaction 1), by the primary granule enzyme myeloperoxidase (MPO). The reaction of H_2O_2 + HOCl leads to formation of singlet oxygen (reaction 2, $^1O_2$) (Babior, Lambeth & Nauseef, 2002).

1. $H_2O_2 + Cl^- \rightarrow HOCl + H_2O$
2. $H_2O_2 + OCl^- \rightarrow {}^1O_2 + H_2O + Cl^-$

The components of NADPH oxidase are the membrane-bound cytochrome b_{558}, comprising gp91^{phox} (NOX2) and p22^{phox} (phox stands for phagocyte oxidase), and four cytosolic components, p47^{phox}, p67^{phox}, p40^{phox} and guanosine diphospate (GDP)-bound GTP-binding protein Rac1/2 (Chessa *et al,* 2010). NOX2 acts as the catalytic subunit of the enzyme.

Cytochrome b_{558} is a membrane-bound flavohemoprotein which contains NOX2 and p22^{phox}, but also a flavin adenine dinucleotide (FAD), which serves as a NADPH-binding site, and two hemo-prosthetic groups, one of which binds gp91^{phox} only, and the other binds both gp91^{phox} and p22^{phox}. In resting neutrophils, 15% of these subunits of the cytochrome b_{558} are plasma membrane bound and 85% within membranes of specific granules and secretory vesicles which translocate to the plasma membrane on oxidase activation (Sheppard *et al,* 2005).

The different units of NADPH oxidase are influenced by neutrophil priming agents. However, priming does not cause activation of the enzyme. Priming of the NADPH oxidase is defined operationally as augmentation of superoxide generation in

response to a second, activating stimulus (Sheppard *et al,* 2005). The time it takes to reach maximal augmentation in this priming process, differs for different priming agents binding to the G-protein-coupled receptor of the neutrophil and can be a few minutes or up to 2h.

When primed by a long-acting agent such as lysophosphatidylcholine (LPC) or GM-CSF, p47^{phox} translocates to the plasma membrane (Mansfield *et al,* 2002). Phosphorylation is a prerequisite for translocation and the degree of phosphorylation correlates with the potency of the priming agent. For instance, in the case of TNF, only partial phosphorylation of p47^{phox} takes place and the molecule is not translocated (Dewas *et al,* 2003). However, the rapid primer PAF, causes phosphorylation of p67^{phox}, p40^{phox}, and Rac2, but not p47^{phox} (Gay, 1990).

Lipid mediators such as LPC, arachidonic acid and LTB_4 can also increase the release of O_2^- in response to a subsequent stimulus (Palmblad *et al,* 1984). LTB_4 participates in translocation of Rac2 (Abdel-Latif *et al,* 2004). Lipid mediators may activate PKC and phosphoinositide-3 kinase (PI-3K) (Brown *et al,* 2003). A key component of neutrophil priming is Ca^{2+}, many of the aforementioned priming activities being Ca^{2+} dependent, such as activation of PKC (Heyworth & Badwey, 1990). Many signalling cascades essential for priming and assembly of the NADPH oxidase components, are initiated by a rise in the cytosolic Ca^{2+} concentration (Silliman *et al,* 2003). Priming of the neutrophil will lead to fusion of the cytoplasmic granules with the plasma membrane which will allow gp91^{phox} and p22^{phox} to interact with the membrane (Borregaard, 1988).

In the activated neutrophil, additional phosphorylation of p47^{phox}, p67^{phox} and p40^{phox} and translocation to the plasma membrane is necessary to complete assembly of the oxidase and is the final step in activation of the enzyme.

These final steps include: "guanine-5'-triphosphate loading of Rac, allowing its translocation to the membrane and its interaction with the tetratricopeptide repeat domain of p67^{phox} (Lapouge *et al,* 2000); the interaction of p67^{phox} with gp91^{phox}; and the phosphorylation of p47^{phox} (Chessa *et al,* 2010)." Phosphorylation of p47^{phox} leads to the exposure of the SH3 domain allowing interaction with p22^{phox}. The C-terminus of p47^{phox} binds to the C-terminal SH3 domain of p67^{phox}. The *phox* homology (PX) domain of p47^{phox} interacts with phosphatidylinositol 3,4-biphosphate (PtdIns(3,4)P_2) and phosphatidic acid. This binding may play a role in the efficiency of the assembly and activation of the enzyme (Karathanassis *et al,* 2002). The subunit p40^{phox} binds to p67^{phox}. It also contains a SH3 domain as well as a threonine (T) 154 -, and serine (S) 315 conserved phosphorylation site (Bouin *et al,* 1998). Phosphorylation of both these sites is triggered by PKC, an enzyme shown to be important in activation of the oxidase. PKC isoforms also cause phosphorylation of p22^{phox}, enhancing the activity of NADPH and regulating the p22^{phox}-p47^{phox} interaction in the membrane (Lewis *et al,* 2010). A schematic diagram of NOX/NADPH and NOX/DUOX reproduced from an article in the *Journal of Pharmacological Science*, 2010, by M Katsuyama is shown in Figure 1.2 (page 25) (used with permission)

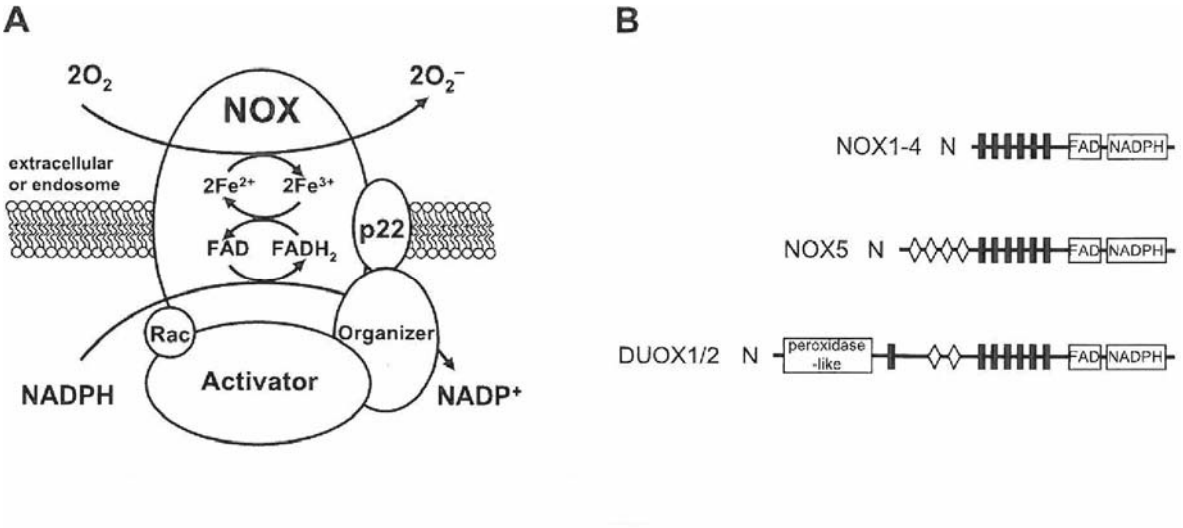

Figure 1.2: Schematic diagrams of NOX/NADPH oxidases. A membrane association of NOX and other components. Requirement of other components depends on the NOX isoforms. B. Schematic diagrams of NOX/DUOX. Closed boxes, transmembrane regions. Open diamonds, EF-hands.

Production of O_2^- in an NADPH-dependent manner has been reported in non-phagocytic cells including vascular smooth muscle cells (VSMC) (Katsuyama, 2010) and endothelial cells (Frey, Ushio-Fukai & Malik, 2009). NOX2 is not formed in these cells, but non-phagocyte NOX2 homologs have been identified. Five NOX isoforms (NOX1-5) and two related enzymes (DUOX1/2) have been reported (Katsuyama, 2010).

1.2.5.3. Leukotriene B_4:

The lipid mediator, Leukotriene B_4 (LTB_4), is rapidly generated by activated neutrophils and acts as a potent chemoattractant. LTB_4 is also produced by macrophages and mast cells (Monteiro *et al,* 2011). The cysteinyl leukotrienes together with LTB_4 form the family of leukotrienes.

The leukotrienes are formed from arachidonic acid, which is released from cellular membranes by cytosolic phospholipase A2 (cPLA2), and further converted by the 5-lipoxygenase (5-LO) pathway (Peters-Golden & Henderson, 2007). The pathway will be explained via a diagram (Figure 1.3, page 27) from an article by Paul Rubin and Karl W. Mollison which was published in "Prostaglandins and other Lipid Mediators" in 2007 (Rubin & Mollison, 2007).

In activated cells, arachidonic acid is released from the nuclear membrane and is metabolised by 5-LO in conjunction with the 5-lipoxygenase-activating protein (FLAP) to form 5-hydroperoxyeicosatetraenoic acid (5-HETE) and subsequently, Leukotriene A (LTA_4). LTA_4 can be conjugated with glutathione by LTC_4 synthase,

leading to the production of the cysteinyl leukotrienes, or it can be metabolised by LTA_4 hydrolase to form LTB_4 (Singh *et al,* 2010). Studies have identified two new branches of the 5-LO pathway, producing 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) which shares the OXE receptor with 5-HETE (Brink *et al,* 2004), and can be transformed to 5-oxo-7-glutathionyl-8,11,14-eicosatetraenoic acid (FOG7) which has a distinct receptor (Bowers *et al,* 2000).

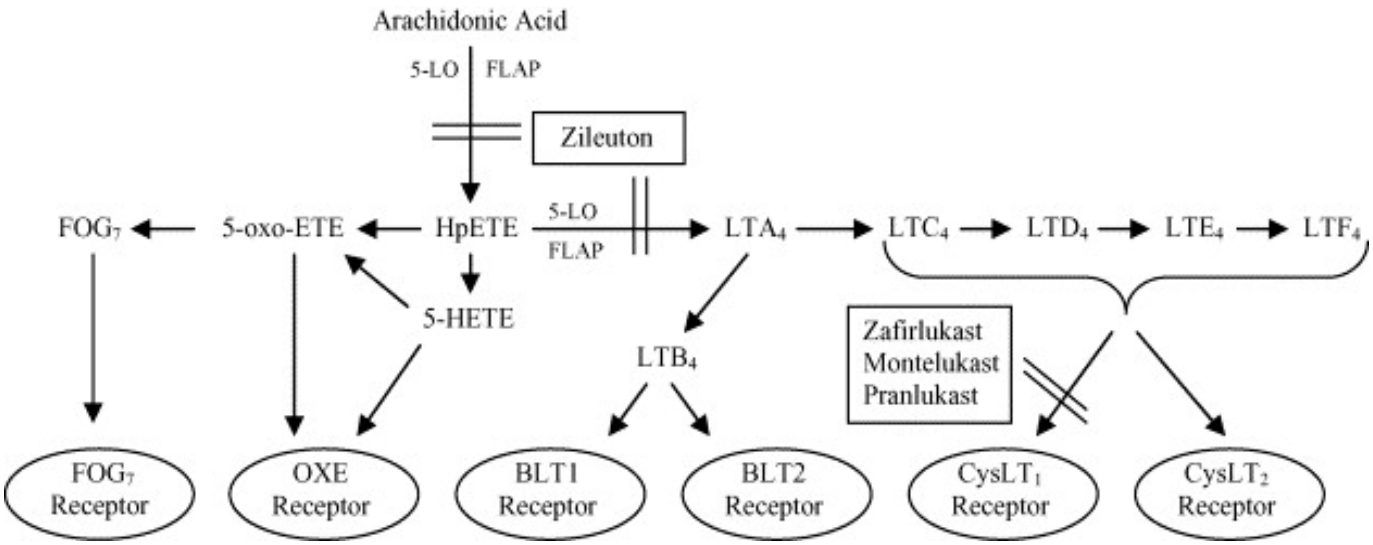

Figure 1.3: The 5-LO pathway eicosanoids and inhibitors. Arachidonic acid metabolism initiated by 5-lipoxygenase leads to the bioactive eicosanoids shown here along with their receptors. Points of intervention are indicated for currently approved therapeutic agents. (Paul Rubin P, Mollison KW. Pharmacotherapy of diseases mediated by 5-lipoxygenase pathway eicosanoids. Prostaglandins Other Lipid Mediat. 2007 May;83(3):188-97)

Two LTB_4 receptors have been identified, BLT_1 and BLT_2 (Tager & Luster, 2003). Chemotaxis of neutrophils can be mediated by OXE (Brink *et al*, 2004), FOG7 (Bowers *et al,* 2000) or BLT1/2.

The majority of evidence suggests that the chemotactic action of LTB_4 is mediated through binding to the high affinity BLT1 receptor on target cells (Yokomizo *et al,* 2001). Neutrophil influx is a known feature in severe asthma and high levels of LTB_4 (Jatakanon *et al,* 1999) are found in the lungs of these patients, which may be due to the involvement of more than one receptor (Rubin & Mollison, 2007). Both BLT_1 and BLT_2 are G protein-coupled seven transmembrane domain receptors. Binding of LTB_4 to either of these receptors activates a range of intracellular activities including intracellular Ca^{2+} mobilisation (Salmon & Ahluwalia, 2010), activation of extracellular signal-regulated kinase 1 / 2, phosphoinositide-3 kinase, as well as degranulation (Lundeen *et al,* 2006).

Another receptor that plays a role in the metabolism of LTB_4 is the PPARα that binds eicosanoids, promoting degradation of the lipid mediators. They play an important role in clearance of LTB_4, controlling the inflammatory process (Narala *et al,* 2010).

Levels of LTB_4 are elevated in the sputum and plasma in asthmatic patients during an acute asthma attack and not in normal individuals (Sampson *et al,* 1995). LTB_4 was also shown to be elevated during nocturnal asthma and correlates with nocturnal fall in forced expiratory volume in one second (FEV_1) (Wenzel *et al,*

1994). In patients with aspirin-sensitive asthma (ASA), a condition associated with severe asthma, aspirin sensitivity and severe rhinosinusitis with recurrent nasal polyposis, high levels of LTB_4, as well as CysLTs in the airways, have been reported (Sousa *et al,* 2002).

LTB_4 has been detected in higher than normal levels in other allergic diseases such as allergic rhinitis (Ohnishi, Miyahara & Gelfand, 2008), atopic dermatitis (Reilly *et al,* 2000) and allergic conjunctivitis (Akman, Irkeç & Orhan, 1998).

A significant correlation was found between the annual fall in post-bronchodilator FEV_1 and numbers of peri-bronchial $CD8^+$ T cells, a correlation that was not evident with eosinophils, $CD4^+$ T cells, or mast cells (Van Rensen *et al,* 2005). In a study by Gelfand *et al*, $CD8^+$/$BLT1^+$/IL-13^+ T cells were found in broncho-alveolar lavage (BAL) fluid of asthmatics, but not normal subjects (Gelfand & Dakhama, 2006). BLT1-expressing effector memory $CD8^+$ T cells are more resistant to corticosteroids than $CD4^+$ T cells, and corticosteroids can even enhance the activation and effector function of these $CD8^+$ T cells due to up-regulation of BLT1 expression. Corticosteroid-mediated up-regulation of BLT1 on effector memory $CD8^+$ T cells may contribute to the ability of these agents to enhance the development of allergic airway inflammation (Ohnishi *et al,* 2008).

1.2.6 Neutrophil Clearance

The resolution of the inflammatory process relies on the effective 'switching off' of the neutrophil, the promotion of apoptosis and the successful clearance of these cells (Fox *et al,* 2010).

Apoptosis is a physiological process of programmed cell death, and, in the case of inflammatory cells, is necessary to control tissue damage. Many neutrophils will undergo apoptosis even before leaving the bone marrow to maintain cell numbers. Certain mediators play a role in delaying apoptosis for instance, GM-CSF and granulocyte colony-stimulating factor (G-CSF), are associated with suppression of neutrophil apoptosis (Fox *et al,* 2010). Activation of nuclear factor-κB (NF-κB) has been shown to be of importance in the regulation of human granulocyte apoptosis, possibly via the regulation of the production proteins, which protect the neutrophil from cytotoxic effects of cytokines such as TNF (Ward *et al,* 1999).

Two pathways of apoptosis have been identified. The key protein in the intrinsic pathway, regulating constitutive neutrophil apoptosis, is the anti-apoptotic protein, myeloid cell leukaemia-1 (Mcl-1). This protein is rapidly expressed and has a short half-life of 2-3h. Once the survival signal is lost, it is degraded and the cellular level of the protein correlates with apoptosis (Leuenroth *et al,* 2000). Mcl-1 is a member of the anti-apoptotic members of the Bcl-2 family; the others being Bcl-X_L and A1. The pro-apoptotic members include Bax, Bad, Bcl-Xs, Bak and Bid (Andina *et al,* 2009). Death receptors, including Fas, TNF-related apoptosis-inducing ligand

(TRAIL) receptors-1 and -2, and TNF receptors-1 and -2 regulate the extrinsic pathway of apoptosis. Binding of the death receptors with their ligands induces apoptosis via caspase-8 activation. Mcl-1 is also a target of caspase-8 (Akgul & Edwards, 2003).

In a study reported by Petrin *et al* (2006), it was illustrated that LTB_4 inhibition of neutrophil apoptosis involves upregulation of Mcl-1 and a decrease in Bax proteins. LTB_4 activates phosphatidylinositol 3-kinase (PI3-K) via its BLT1 receptor. Neutrophil exposure to GM-CSF or IL-8 delays apoptosis by activating PI3-K and ERK-dependent pathways (Pétrin *et al,* 2006).

Glucocorticosteroids also prolong neutrophil survival due to anti-apoptotic effects. These include high levels of the glucocorticoid receptor β (GRβ) in comparison to the GRα (Marwick, Adcock & Chung, 2010). Strickland *et al*., have shown that neutrophil survival was prolonged if exposed to dexamethasone and that the ratio of GRβ to GRα was increased. This effect was enhanced if the neutrophils were pre-incubated with IL-8 (Strickland *et al,* 2001). The fact that neutrophil apoptosis is inhibited by glucocorticoids, is compatible with a role for this mechanism in steroid-resistant asthma.

1.2.7 Asthma

Asthma is a complex clinical syndrome characterised by variable symptoms of airway obstruction and bronchial hyperresponsiveness due to chronic airway

inflammation (National Heart, Lung and Blood Institute, 2007). Although it is a chronic disease, acute exacerbations and symptoms can be induced by a number of triggers, including amongst others, respiratory viral infections, allergen exposure and exercise (Gravett *et al,* 2010). Inflammatory cell infiltration in the airways during acute episodes may involve eosinophils, which are traditionally associated with asthmatic inflammation, as well as neutrophils and lymphocytes (Cosmi *et al,* 2011). The chronic inflammation leads to hypertrophy of the airway smooth muscle, thickening of the basement membrane, and mucus production. This process can lead to airway remodeling and fixed airway obstruction if poorly treated (Durrani, Viswanathan & Busse, 2011).

Over the years, asthma has proven to be a complex disease that includes different clinical presentations, inflammatory processes and diverse genetic profiles. These groups respond differently to available treatment regimens, and present in a range of age groups with varying grades of severity irrespective of the length of time that the individual has been diagnosed with asthma. In an attempt to better classify the groups and optimum therapies more accurately; different phenotypes have been described in the literature. According to The Encarta World Dictionary, the definition of “Phenotype” is: “the visible characteristics of an organism resulting from the interaction between its genetic makeup and the environment”. In a review in the Lancet of August/September 2006, Wenzel proposes the following potential phenotype categories: “Some patients will have asthma that can be classified under more than one phenotype and this division still will not give a clear indication of the underlying disease process or response to treatment” (Wenzel, 2006).

Clinical or physiological phenotypes

Severity-defined

Exacerbation-prone

Defined by chronic restriction

Treatment-resistant

Defined by age at onset

Phenotypes related to the following triggers

Aspirin or non-steroidal anti-inflammatory drugs

Environmental allergens

Occupational allergens or irritants

Menses

Exercise

Inflammatory phenotypes

Eosinophilic

Neutrophilic

Pauci-granulocytic

Lötvall *et al*, therefore suggest a classification of "endotypes" where endotypes are defined as: "a subtype of a condition, which is defined by a distinct functional or pathophysiological mechanism" (Lötvall *et al,* 2011). The possible relationship between asthma phenotypes and endotypes is shown below in Table 1.2 (page 34).

Table 1.2: Proposed relationship between asthma phenotypes and endotypes: asthma phenotypes can be present in more than 1 endotype, and endotypes can contain more than 1 phenotype**.**

Phenotype:	Eosinophilic asthma
	Endotypes: allergic asthma (adult), aspirin-sensitive asthma, severe late-onset hypereosinophilic asthma, ABPM (allergic broncho-pulmonary mycosis)
Phenotype:	Exacerbation-prone asthma
	Endotypes: allergic asthma (adult),aspirin-sensitive asthma, late-onset hypereosinophilic asthma, API-positive (asthma-predictive indices) preschool wheezer, ABPM, viral-exacerbated asthma, premenstrual asthma
Phenotype:	Obesity-related asthma
	Endotypes: airflow obstruction caused by obesity, severe steroid-dependent asthma, severe late-onset hypereosinophilic asthma
Phenotype:	Exercise-induced asthma
	Endotypes: cross-country skiers' asthma, other forms of elite-athlete asthma, allergic asthma, API-positive preschool wheezer
Phenotype:	Adult-onset asthma
	Endotypes: aspirin-sensitive asthma, infection-induced asthma, severe late-onset hypereosinophilic asthma
Phenotype:	Fixed airflow limitation
	Endotypes: noneosinophilic (neutrophilic) asthma
Phenotype:	Poorly steroid-responsive asthma
	Endotypes: noneosinophilic (neutrophilic) asthma, steroid-insensitive eosinophilic asthma, airflow obstruction caused by obesity

This table is reproduced from: Journal of Allergy and Clinical Immunology Volume 127, Issue 2, February 2011, Pages 355-360

From the above discussion, it is clear that more questions than answers exist with respect to the understanding of asthma and predicting outcomes with treatment.

Treatment aims in chronic inflammatory diseases such as rheumatoid arthritis and ulcerative colitis, are usually to induce remission, but in all the guidelines of asthma treatment, control is the target. In a recent article by Upham & James on remission in asthma, it is again acknowledged that the heterogeneity of asthma and the natural history of the disease have to be taken into consideration when defining remission (Upham & James, 2011). Early treatment of asthma in children did not prevent disease progression. Eosinophils are generally considered to be the key inflammatory cell in asthmatic inflammation, but blocking Interleukin-5 (IL-5), a known growth factor for eosinophils, did not lead to asthma control. This may again be due to the fact that other inflammatory cells including neutrophils may play an important role in the chronic inflammation and that reducing the eosinophil count is not enough (Upham & James, 2011).

1.2.7.1 Neutrophils in Asthma:

Neutrophils are present in the airways of some asthmatic patients, especially those with acute exacerbations (Ito *et al,* 2008), and fatal asthma that may occur within hours of the exacerbation (Lamblin *et al,* 1998), has been described in nocturnal asthma (Nadif *et al,* 2009) and severe-steroid resistant asthma (Wenzel *et al,* 1997).

Neutrophils are present in bronchoalveolar lavage fluid in some patients following allergen challenge in both the acute phase, as well as the late phase of asthma, which might be due to the fact that neutrophils express high-affinity receptors for IgE (FcεRI). Activation of these receptors leads to IL-8 release (Gounni *et al,* 2001). Interleukin-9 (IL-9) can also activate neutrophils through surface receptor stimulation causing an increase in IL-8 release. IL-9 receptor expression is increased in asthma, being induced by the Th2 cytokine, IL-4, as well as GM-CSF (Abdelilah *et al,* 2001).

In acute asthma, neutrophils will release oxygen radicals and proteases that contribute to tissue damage and increased mucus gland hyperplasia, mucus secretion, and epithelial damage. Elastase exposure increases fibroblast migration that may lead to airway remodeling (Vignola, Kips & Bousquet, 2000).

In sudden-onset fatal asthma, neutrophils have been found to be the predominant inflammatory cells involved (Carroll *et al,* 1996).

Chronic severe asthma is another type of asthma in which neutrophils are found in high concentrations in sputum, correlating with increased levels of IL-8 and myeloperoxidase (MPO). This group of severe asthmatics is poorly responsive to corticosteroid treatment. It is debatable whether the neutrophil infiltration in these severe cases is a consequence or cause of treatment with high doses of corticosteroids (Jatakanon *et al,* 1999).

Asthmatics that smoke also represent a group with high levels of neutrophils in the airways (Cowan *et al,* 2009).

1.2.7.2 Cysteinyl Leukotrienes (CysLTs):

The synthesis of leukotrienes was discussed earlier in this chapter, with emphasis on LTB_4. However, the CysLTs will be discussed in more detail because of the important role they play in the pathogenesis of asthma, and the fact that they are the primary target of treatment with montelukast. Of necessity this section will repeat some of the detail mentioned in the preceding section on leukotriene synthesis.

Feldberg and Kellaway identified a material they named "slow reaction smooth muscle-stimulating substance (SRS)" 60 years ago (Feldberg & Kellaway, 1938). This substance was observed to be released by antigen challenge in human lungs and was renamed, "slow-reacting substance of anaphylaxis (SRS-A) (Brocklehurst, 1960). The discovery of the precise biochemical nature of SRS-A was anticipated by the isolation of 5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HETE) in polymorphonuclear leucocytes (Borgeat & Samuelsson, 1979a; Borgeat & Samuelsson 1979b; Borgeat & Sameulsson, 1979c) and finally identified as a cysteine-containing derivative of 5-hydroxy-7,9,14-eicosatetraenoic acid (Murphy, Hammaström & Sameulsson, 1979. These compounds were named leukotrienes as they were found in leucocytes and were characterised by the presence of three conjugated double-bonds (Samuelsson, 1983).

The synthesis of leukotrienes from phospholipase A_2 was depicted earlier in this literature review (Figure 1.3, page 27). The cysteinyl leukotrienes are synthesised from LTA_4 (Peters-Golden & Henderson, 2007). This intermediate is converted by either of the two enzymes, LTA_4 hydrolase or LTC_4 synthase, to form LTB_4 or LTC_4, respectively. In order for 5-lipoxygenase to function effectively in cells, 5-lipoxygenase-activating protein (FLAP) needs to be present. FLAP does not have any enzymatic activity, but rather enhances the activity of 5-lipoxygenase (Peters-Golden & Brock, 2003). The tripepetide side chain of LTC_4 may be cleaved in two successive steps to generate LTD_4 and LTE_4, which together with the parent compound, comprise the cysteinyl-leukotrienes (cys-LTs) (Duroudier, Tulah & Sayers, 2009).

The capacity to generate large amounts of leukotrienes from arachidonate is largely confined to leukocytes; however, the amounts of LTB_4 and cys-LTs that various types of leukocytes produce depend on the distal enzymes LTA_4 hydrolase and LTC_4 synthase, respectively. Although non-leukocyte cells generally do not have sufficient 5-lipoxygenase and FLAP to synthesize appreciable amounts of leukotrienes from arachidonate, such cells expressing distal LTA_4 –metabolizing enzymes can take up leukocyte-derived LTA_4 and metabolize it into bioactive leukotrienes, a process that is termed “transcellular bio-synthesis (Folko & Murphy, 2006).

The output of the leukotriene synthetic pathway is regulated by: (1) the amount of free arachidonate that phospholipase A_2 releases from cell-membrane phospholipids (Uozumi *et al,* 1997; Henderson *et al,* 2007); (2) the level of each of the proteins in the 5-lipoxygenase pathway; (3) the catalytic activity per enzyme molecule (e.g., modulated by protein kinase-directed phosphorylation); and (4) the availability of small molecules (e.g., ATP, nitric oxide (Coffey, Phare & Peters-Golden, 2000), and reactive oxygen intermediates) that modulate 5-lipoxygenase activity.

Leukotrienes act by binding to specific heptahelical receptors of the rhodosin class that are located on the outer plasma membrane of structural and inflammatory cells (Kanaoka & Boyce, 2004; Salmon & Ahluwalia, 2010). Once ligated by the leukotriene, the receptor interacts with G-proteins in the cytoplasm, thereby eliciting increases in intracellular calcium and reductions in intracellular 3'-5'-cyclic adenosine monophosphate (cAMP).

CysLTs in humans appear to function through at least two receptors ($CysLT_1$ and $CysLT_2$) (Capra, 2004). $CysLT_1$Rs have a relatively restricted occurrence, being expressed on the plasma membrane of epithelial cells, fibroblasts/myoblasts, smooth muscle cells and endothelial cells in the structural group of cells, while the inflammatory group includes neutrophils, monocytes/macrophages, basophils, mast cells, dendritic cells, B lymphoctes and $CD4^+$ T cells (Yoshisue *et al,* 2007). Interaction of the CysLTs with the $CysLT_1$ receptor on the structural cells, leads to mucus production and collagen synthesis and release with implications for airway

remodelling, contractility, proliferation of smooth muscle, vascular permeability, and oedema (Hui & Funk, 2002).

Although receptors are found on the aforementioned inflammatory cells, only mast cells, basophils, eosinophils and, to a lesser extent, monocytes/macrophages, possess the necessary enzymes to convert LTA_4 to CysLTs (Peters-Golden & Henderson, 2007). Interaction of the CysLTs with $CysLT_1Rs$ on the inflammatory cells: (1) recruits and activates T_H2 cells and eosinophils; (2) prolongs eosinophil survival; (3) and may be able to act as co-factor for the enhanced production of eosinophils from the bone marrow in combination with GM-CSF; (4) increases the production of ROS by neutrophils, eosinophils and monocytes/macrophages, these oxidants being mediators of vascular permeability and bronchial hyper-reactivity; and (5) induce the release of proteolytic enzymes such as elastase and matrix metalloproteinases from phagocytic cells, which promote airway re-modelling (Holgate *et al,* 2003).

Certain reported actions of Cys-LTs are not readily explained by their interactions with either $CysLT_1$ or $CysLT_2$, raising the possibility of the presence of $CysLT_1$-$CysLT_2$ heterodimers or additional receptors (Daniele *et al,* 2011). One candidate is G protein-coupled receptor 17 (GPR17), a dual-uracil nucleotide-cysteinyl leukotriene receptor (Ciana *et al,* 2006).

1.2.7.3 Treatment of Asthma:

The aim of treatment of chronic asthma is to gain control (Bateman *et al,* 2008). In patients with intermittent asthma, short acting β2 stimulants are used as reliever medication. When this is needed more than three times per week, the asthma is classified as persistent asthma, and according to the GINA guidelines, persistent asthma should be treated with a controller (anti-inflammatory) medication with short-acting β2-stimulants only being used as reliever treatment if symptoms are experienced (Bateman *et al,* 2008).

Inhaled corticosteroids are the first line anti-inflammatory agents with the addition of a long-acting β2-stimulant (LABA) if the patient remains symptomatic. A leukotriene antagonist can be used as an additional controller agent in adults being added either to an inhaled steroid or to the combination of an inhaled steroid and LABA (Sears, 2011). Outcomes with combination therapy with inhaled steroids and long-acting beta 2-receptor agonists (LABAs), either in separate devices, or combined in single inhalers, are well documented, and show improved control (Maneechotesuwan *et al,* 2005). The guidelines in children provide for options to use leukotriene antagonists as first-line treatment (Bacharier *et al,* 2008). Montelukast is the only drug in this group that has registration for use in children below the age of twelve.

Phenotypes of asthma with predominantly neutrophilic inflammation will generally respond poorly to inhaled steroids due to mechanisms mentioned previously (Strickland *et al,* 2001; Marwick, Adcock & Chung, 2010). Clinical studies in

asthmatics that smoke, show reduced efficacy of corticosteroids (Lazarus *et al,* 2007) and emphasise the need for new targets for controlling inflammation in asthma. A few trials with combination anti-inflammatory drugs, have underscored the need to target neutrophils as well as eosinophils (Hanania, 2008).

There is evidence that salmeterol reduces the number of neutrophils in the airway mucosa, as well as in broncho-alveolar lavage fluid of patients with mild asthma, whereas the inhaled corticosteroid, fluticasone, was ineffective. Treatment with salmeterol resulted in more symptom-free days than treatment with fluticasone or placebo (Jeffery *et al,* 2002). Formoterol inhalation reduced sputum neutrophils after four weeks of treatment, without any effect on eosinophil counts, while budesonide had the reverse effect with a reduction in eosinophils and no effect on neutrophils (Maneechotesuwan *et al,* 2005). In fact, corticosteroids prolong neutrophil survival by inhibiting their apoptosis, as mentioned previously (Strickland *et al,* 2001; Marwick, Adcock & Chung, 2010). These findings may explain why the combination of inhaled steroids and LABA's is more effective in the treatment of asthma than inhaled steroids alone. Treatment with a LABA without concurrent use of an inhaled steroid is contraindicated due to the potential increase in severe and life-threatening asthma exacerbations (Salpeter *et al,* 2006).

Acute exacerbations of asthma are associated with an increase of both eosinophils and neutrophils. The combination product of formoterol and budesonide, known as Symbicord, has efficacy as reliever therapy in acute asthma. This may be due to the complementary action of the agents and the fact that formoterol has a quick

onset of action (Bousquet *et al,* 2007). The combination of salmeterol and fluticasone is not suitable as reliever therapy because salmeterol has a slow onset of action.

Addition of either LABAs or montelukast to inhaled steroid treatment has also been studied, and montelukast was proven to be as effective as addition of salmeterol to fluticasone (Ilowite *et al,* 2004).

A pilot study conducted by Dupont *et al*, suggested that the addition of montelukast to a fixed-association inhaled steroid and LABA, may result in significant improvement in asthma control (Dupont *et al,* 2005). Beneficial additive effects of salmeterol and montelukast in asthma control were also reported in two other studies (Dempsey *et al,* 2000; Deykin *et al,* 2007).

Theophylline has been used in the treatment of asthma and COPD for many years. It is known that theophylline inhibits all phosphodiesterase (PDE) isozymes non-selectively. This might contribute to the therapeutic effects in asthma but the non-selective inhibition of all PDEs, leads to side effects that limit its usefulness as does its antagonist actions on type $A2_A$ adenosine receptors (Fukuda *et al,* 2011).

1.2.7.4 New Targets for Treatment:

Total control of asthma in all the different groups, remains a challenge and new targets for pharmacotherapy are constantly being investigated. In a review by Walsh published in *Discovery Medicine* in April 2011, the limitations of new

cytokine-directed therapies of asthma are discussed. Omalizumab, a humanised monoclonal antibody directed at the FcεRI binding domain of human IgE, is the only biologic drug that has proven efficacy in the treatment of asthma. It is mainly used in severe allergic asthmatics. Additionally, anti-IL-13 monoclonal antibodies have been reported to be therapeutically useful in a subset of patients with bronchial asthma, specifically those patients with high circulating levels of periostin (Corren *et al*., 2011). Revisiting the potential of existing drugs is also a target for investigation (Walsh 2011).

1.2.7.4.1 Phosphodiesterase Inhibitors:

Cyclic AMP (3'-5'-cyclic adenosine monophosphate cAMP), the "original second messenger," is generated when a first messenger, including chemokines, lipid mediators, hormones or drugs, bind to a seven-transmembrane-spanning G protein-coupled receptor (GPCR), which in turn is coupled to a stimulatory G protein α subunit (Gαs) (Serezani *et al,* 2008). This leads to the exchange of GDP for GTP on the Gαs protein and dissociation of the βγ subunit complex. The enzyme adenylyl cyclase (AC) is then activated to catalyse the cyclisation of ATP to generate cAMP and pyrophosphate. Ligands which activate Gαi subunits on the other hand inhibit AC and the production of cAMP, and include LTB_4, LTC_4 and LTD_4. Intracellular levels of cAMP are controlled by AC and the enzyme cAMP phosphodiesterase (PDE). To date, eleven distinct cyclic nucleotide PDE gene families are recognised (Torphy, 1998).

Inactivation of cyclic nucleotides by PDEs is mediated via hydrolytic cleavage of the 3'-phosphoester bond to an inactive 5'-nucleotide monophosphate. The affinities of the various PDEs differ for cAMP and cGMP. PDE4 and PDE7 are selective for cAMP. PDE4 is also the predominant PDE expressed in neutrophils (Torphy, 1998).

Roflumilast, an orally administered selective PDE4 inhibitor for the treatment of COPD, was first identified in 1993 (Rabe, 2011). *In vitro*, inhibition of PDE4 will lead to a reduction in apoptosis and release of inflammatory mediators from neutrophils (Hatzelmann *et al,* 2010). *In vivo*, this reduction will lead to inhibition of cell movement and cytokine and chemokine release from inflammatory cells such as neutrophils, eosinophils, macrophages and T-cells (Sanz, Cortijo & Morcillo, 2005). Roflumilast has been approved for use in COPD in the USA and Europe (Page & Spina, 2011). The gastro-intestinal side-effects such as nausea and vomiting might, however, limit its use in practise (Field, 2008). The molecule has been studied in asthmatics with effects comparable to those of inhaled corticosteroids (Rabe, 2011).

In addition, GlaxoSmithKline plc is developing 256066, an inhaled formulation of a PDE4 inhibitor that has demonstrated efficacy in trials in asthma (Higgs, 2010).

Although the effects of montelukast on PDEs are unknown, it is noteworthy that the early, first generation experimental $CysLT_1R$ antagonists were found to possess PDE inhibitory activity (Fleish, Rinkema & Marshall, 1984).

1.2.7.4.2 Montelukast:

Montelukast sodium, 2-[1[[1-[3-[2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-2[1-hydroxy-1-methyl-ethyl)phenyl]-propyl]sulphanyl-methyl]-propyl]cyclopropyl] ethanoic acid, is a selective, pharmacological antagonist of type 1 cysteinyl leukotriene receptors ($CysLT_1Rs$) (Al Omari *et al,* 2007).

The primary mode of action of montelukast is via antagonism of the $CysLT_1Rs$, blocking the pro-asthmatic/ pro-inflammatory effects of the CysTLs. This mode of action forms the basis of the use of this agent in the treatment of the inflammation in asthma (Bateman *et al,* 2008). Montelukast is marketed by Merck (Merck Research Laboratories, Rahway, NJ, USA) as Singulair.

The varied clinical responses in different individuals after oral intake of montelukast might be due to genetic variations of the efflux and uptake transport proteins (Lima, 2007). It is rapidly absorbed from the intestine after intake of the different oral formulations. The mean peak plasma concentration is achieved at 3-4 hours after intake of the 10mg tablet in fasted adults and at 2-2.5 hours after intake of the 5 mg chewable tablet in fasted adults. In fasted 2 to 5 year old children, the mean peak plasma concentration after intake of the 4 mg chewable tablet was reached at 2 hours while the 4 mg granule formulation is bioequivalent to the 4 mg chewable tablet after oral intake by fasted adults.

The mean oral bioavailability in those taking the 10 mg tablet was 64% while those of the 4 mg and 5 mg chewable tablets were 63% and 73% respectively (Merck

Research Laboratories). Maximum plasma concentrations (Cmax) after oral administration of a 10 mg tablet ranged between 350-385 ng/ml in healthy adults (Cheng *et al,* 1996). In 2-5 year old children, the Cmax value after intake of 4 mg chewable tablets was 471 ng/ml, while in 6-14 month old children the Cmax was 514 ng/ml after administration of 4 mg oral granules (Knorr *et al,* 2001).

Montelukast is considered to be safe in children from 6 months of age and is the only CysLT antagonist that has registration in children under the age of 12 years as mentioned above (Gravett *et al,* 2010).

Evidence of successful treatment of patients with several different phenotypes of asthma with montelukast is well documented, including, atopic asthma (Riccioni *et al,* 2007), aspirin-sensitive asthma (Currie & McLaughlin, 2006), acute exacerbations due to viral infections (Bisgaard, 2003), asthma in children (Bacharier *et al,* 2008), exercise-induced asthma (Currie & McLaughlin, 2006), and asthma in patients that smoke (Rabinovich et al, 2008). The efficacy of montelukast in these patient groups is primarily due to the blockade of CysTLRs, but may also involve other mechanisms. In this context montelukast has also been shown to play a role in reducing airway remodelling via reducing fibronectin-induced migration of human lung fibroblasts that stimulate smooth muscle proliferation (Tokuriki *et al,* 2007), while studies have shown secondary anti-inflammatory effects of montelukast, unrelated to the antagonism of the $CysLT_1Rs$, such as:

- Inhibition of 5-lipoxygenase in activated neutrophils as well as monocytes/ macrophages, which leads to a decrease in the synthesis of CysLTs as well as in LTB_4. The mechanisms have not been fully described, but are not via $CysLT_1R$ blockade. The concentrations of montelukast needed for this action, are slightly higher than those needed for antagonism of the $CysLT_1Rs$ (Ramires *et al,* 2004). The blocking of LTB_4 production is potentially an added benefit in treating neutrophil-mediated inflammation in asthma, which was mentioned to be corticosteroid-resistant.
- Inhibition of adherence of eosinophils to vascular endothelium by interfering with the interaction of the eosinophil adherence molecule, $\alpha_4\beta1$, with vascular cell adhesion molecule-1, its counter receptor (Robinson *et al,* 2008). This inhibition was unaffected by the inclusion of the 5-lipoxygenase-activating protein inhibitor, MK886, confirming the $CysLT_1R$-independent mechanism. Montelukast also decreased eosinophil migration activated by of the chemoattractant, 5-oxo-6,8,11,14-eicosatetraenoic acid, which was also associated with a decreased expression of the urokinase plasminogen receptor and a decrease in secretion of MMP-9, which in turn protects against tissue extracellular matrix digestion (Lanlois *et al,* 2006).
- Montelukast was reported, together with pranlukast and zafirlukast, to antagonise the effects of nucleotides acting at P2Y receptors on both a monocyte/macrophage cell line and primary human monocytes, effects that were characterised by inhibition of phospholipase C. This inhibition leads to the

failure to produce inositol triphosphate and Ca^{2+} mobilisation from intracellular stores with decreased production of IL-8 (Mamedova *et al,* 2005).

- In studies on some of the earlier $CysLT_1R$ antagonists such as FLP55712 (Fleish, Rinkema & Marshall, 1984) and LY171883 (Hay *et al,* 1987) and the novel agent CR3465 (Ferrari *et al,* 2004), secondary, non-specific PDE inhibition was described. CR3465 like montelukast possesses a quinoline moiety which may underpin the inhibition of the PDEs.

Another agent, ibudilast, also known as KC-404, AV-411 and MN-166, was developed in Japan and has been marketed there for the treatment of asthma and cerebrovascular disorders. This molecule combines $CysLT_1R$ antagonism and PDE inhibitory properties (Barkhof *et al,* 2010).

The successful use of montelukast in the treatment of diseases other than asthma is well documented. In patients with moderate-to-severe COPD, montelukast therapy leads to clinical improvement (Drakatos *et al,* 2009), while in another study, montelukast added to existing treatment regimes, resulted in decreases in serum levels of LTB_4, IL-8 and TNF (Gueli *et al,* 2011). Several studies done in patients with cystic fibrosis have shown efficacy of montelukast treatment (Stelmach *et al,* 2005), while in children with reactive airway disease following respiratory syncytial virus infection, montelukast led to reduction of symptoms and a delay in acute exacerbations (Bisgaard, 2003). In a recent study done on patients with bronchiolitis obliterans syndrome after lung transplantation, montelukast

added to azithromycin resulted in a decrease in FEV_1 decline (Verleden *et al,* 2011).

Besides lung diseases, montelukast was also reported to be of benefit in sepsis-induced hepatic and ileal injury in a rat model (Sener *et al,* 2005), and in another study also in rats, it led to improved wound healing in burn injury (Turtay *et al,* 2010). In a model of gouty arthritis, montelukast led to a decrease in the total inflammatory cell count, with a predominant effect on polymorphonuclear cells (Ponce *et al,* 2011). Data from a study in atherosclerotic rabbits showed that montelukast inhibited neointimal hyperplasia which was associated with decreased expression of MMP-2 and MMP-9 independent of plasma lipid levels (Liu *et al,* 2009). In a recent study by Wang *et al* in experimented autoimmune encephalomyelitis, montelukast and zafirlukast, both targeting CysTLR1, effectively blocked central nervous system infiltration by inflammatory cells, indicative of potential use in the treatment of multiple sclerosis (Wang *et al,* 2011).

In conclusion, this literature review suggests that neutrophilic inflammation in asthma should be targeted in treatment regimens and that new properties of existing drugs should be explored. It also emphasises the potential of montelukast in controlling inflammation.

1.3 Hypothesis

The hypothesis which forms the basis of this study is that the anti-asthma agent, montelukast, possesses secondary neutrophil-directed anti-inflammatory properties which are distinct from conventional $CysLT_1R$ antagonism. Alternatively, the pro-inflammatory function of these cells is not affected by montelukast.

Objectives:

The primary objectives of the laboratory research described in this thesis were to investigate the effects of:

1) The cysteinyl leukotrienes (CysLTs) C_4 and D_4 on isolated human neutrophils, which may result in hyperreactivity of the cells on subsequent exposure to chemoattractants.
2) Montelukast, at therapeutically relevant concentrations, on the mobilization of stored and extracellular Ca^{2+}, as well as on several Ca^{2+}-dependent, pro-inflammatory activities of neutrophils, in relation to alterations in intracellular cAMP levels, and activities of cAMP and cGMP PDEs following exposure of isolated human neutrophils to chemoattractants.
3) Formoterol and montelukast, individually and in combination, on the generation of ROS and LTB_4, as well as release of the matrix metalloproteinases, MMP-8 and MMP-9, and expression of the neutrophil adhesion molecule CR3 in

relation to alterations in the intracellular concentrations of cAMP and cytosolic Ca^{2+}.