Chapter II

Review of Literature
Human Neutrophil Elastase

Introduction

Human neutrophil elastase, also known as Human leukocyte elastase (HLE) is a serine protease found in the azurophilic granules of the neutrophils (18). Human Polymorphonuclear Neutrophils (PMNs) represent 35-75% of the circulating leukocytes and are the abundant type of white blood cell in mammals (Fig 1). They are classified as granulocytes because of their intracytoplasmic granule content and are characterized by a multilobular nucleus. Neutrophils develop from pluripotent stem cells in the bone marrow and are released into the bloodstream where they reach a concentration of 1.5 to 5x10^9 cells/liter. Neutrophils play an important role in innate immune defense against invading pathogens and are among the primary mediators of inflammatory response (6).

![Fig 1: Structure of human polymorphonuclear neutrophil](image)

Neutrophils are the first white blood cells to be recruited to areas of inflammation and recruitment of neutrophils to the site of inflammation is one of the first lines of host defense against infection (15, 36). Activated PMN destroy microorganisms using an array of weapons that include ROS, antimicrobial peptides, proteases and other compounds interfering with bacterial growth and metabolism (15, 37).
Proteases (also Proteinases, Peptidases) are hydrolases (EC 3.4.21-24 and 99) and are degradative enzymes which cleave proteins into smaller peptides and amino acids (38). They represent a class of enzymes which occupy a central position with regard to their biological functions and commercial applications. Proteases are classified into six different types viz; serine proteases, aspartic proteases, cysteine proteases, threonine proteases, glutamate proteases and metalloproteases. This classification is based on the key amino acid residues present at their active sites which involve in catalytic processes. There are at least 500 to 600 different proteases in humans; most of them are serine, cysteine, and metalloproteases (39).

The serine proteases (EC 3.4.21.-) are characterized by the presence of a highly reactive serine residue as part of the catalytic center. The catalytic triad consists of serine, histidine and aspartic acid residues. They are widely distributed in prokaryotes and eukaryotes and include exopeptidases, endopeptidases and oligopeptidases (15, 40, 41). They comprise of nearly one third of all proteases known and are implicated in many important biological processes such as digestion, blood clotting and immune response. They include digestive enzymes of exocrine glands, clotting factors and leukocyte granule associated proteases such as neutrophil elastase, proteinase 3 and cathepsin G (6).

The term elastase defines a group of serine proteases that possess the ability to cleave the important connective tissue protein elastin, which is widely distributed in vertebrate tissue and is particularly abundant in the lung, arteries, skin and ligaments (42, 16). Discovered by Balo et al., elastase was extracted and purified from the pancreas as an enzyme by Banga in 1949 (43). They are heterogeneous with differing substrate specificities and catalytic mechanisms. Despite the differences in the catalytic mechanisms, all of these elastases share a common specificity for cleaving peptide bonds associated with hydrophobic or aromatic amino acids (44). These elastases include: the neutrophil elastase (NE), the pancreatic elastase (PE), the macrophage elastase (MMP-12)
and the fibroblast elastase (16). Amongst these the most widely studied elastases are HNE and PE (44). Although the activity of leukocyte proteases had been described early in the 20th century, HNE was identified only in 1968 by Janoff and Scherer (6).

**Biosynthesis and Processing**

The genes encoding HNE consists of five exons and four introns. The gene for HNE, ELA2 (formerly called) or ELANE (newly dubbed) is located within a 50-kilobase segment in the terminal region of the short arm of chromosome 19 (45). The synthesis of HNE is regulated first at the transcriptional level during granulocyte development and second at the post-translational level before they are stored in their proteolytically active mature form within neutrophil azurophilic granules (6). HNE is synthesized as an inactive prepro-protein containing a signal peptide and an amino-terminal prodipeptide that require two separate amino-terminal processing steps to become active. They also contain a C-terminal propeptide, the removal of which is not necessary for HNE activity. Shortly after synthesis, the signal peptide is removed by a signal peptidase, leaving a N-terminal pro-sequence of two amino-acid residues. Removal of this prodipeptide occurs before or during transport to the granules and requires the activity of dipeptidyl peptidase I (DPPI; also known as cathepsin C) (46, 47). Although the prodipeptide is not required for the sorting of an enzyme to a granule, its removal is crucial for activation of enzymatic activity (48). In addition, N-terminal processing is essential for the optimal storage of neutrophil serine proteases in azurophil granules. In the absence of DPPI, the pro-forms of serine proteases might either be constitutively secreted or more easily degraded (49). Biosynthesis and processing of HNE is depicted in Fig.2.
Fig 2: Newly synthesized pre-pro-enzymes are processed by a signal peptidase and shuttled from the rough endoplasmic reticulum (ER) through the Golgi complex (a). The pro-enzyme probably encounters dipeptidyl peptidase I (DPPI), which removes the pro-dipeptide, as it leaves the Golgi complex and before being packaged in granules (b). Carboxy-terminal truncation by an unknown protease (c) allows the enzyme to interact with the adaptor protein AP3 and be packaged in granules where it awaits the proper signal for regulated secretion. In the absence of DPPI, the pro-enzyme is either degraded (d) or constitutively secreted (e). If the C terminus remains intact, the interaction with AP3 is blocked and the enzyme is alternatively routed to the cell surface (f) where it might be in a transmembrane conformation (adopted from Pham CT. Neutrophil serine proteases: specific regulators of inflammation. Nat Rev Immunol. 2006).

**Localization in neutrophils**

There are four types of granules in neutrophils: primary (also known as azurophil), secondary (also known as specific), tertiary (also known as gelatinase) and secretory granules (13). Fully processed mature HNE is primarily stored in azurophil granules of neutrophils in their active form at high concentration (5mM) (42, 29). In addition to being stored in the azurophilic granules, immunostaining and electron microscopy have shown that NE is also localized in the nuclear envelope (50). A list of the granules and the protein constituents is shown in the Fig. 3.
Degranulation of neutrophils or in vivo release of HNE

Once neutrophils are activated at inflammatory sites, these granules translocate to phagosomes and the plasma membrane where they release their contents. The translocation process has two steps. The first step depends on cytoskeleton remodeling and microtubule assembly. The second step involves interaction between soluble-N-ethylmaleimide-sensitive-factor accessory-protein receptors (SNAREs) present on the plasma membrane and the granule which facilitates fusion (51). Multiple different SNAREs have been identified in neutrophils and it is likely that different granule SNAREs interact differently with the plasma membrane SNAREs thereby dictating the rate of exocytosis (51, 52). After they are released, the neutrophil serine proteases are fully active. The pro-inflammatory cytokines including TNF-α, IL-6, IL-8 and lipopolysaccharides (LPS) produced during inflammatory response are shown to be involved in the migration, activation and degranulation of neutrophils resulting in release of its contents.
**The in vitro release of HNE**

Recognition of stimuli by neutrophils triggers series of processes, including phagocytosis and release of its granular contents. NE is the major secreted product of activated neutrophils. Various factors are shown to stimulate elastase release by neutrophils. These include cytokines, endotoxins, platelet aggregation factor (PAF), and formyl-methionyl-leucyl-phenylalanine (fMLP) (53).

Since in inflammatory disease conditions, activated neutrophils release elastase in abundance, recent experimental studies have assessed the effects of various disease specific molecules on the elastase release from neutrophils in in vitro conditions. Neutrophils exposed to high glucose, homocysteine (HCys) and IL-6 have been shown to form NETs in increased quantity and neutrophil elastase, a major associated component of NETs was also elevated significantly. Studies have observed increased circulating markers of NETs (i.e. elastase) released from neutrophils exposed to high glucose concentration compared to low glucose concentration (54). Joshi et al found increased elastase activity in neutrophils isolated from T2 diabetic subjects with hyperhomocysteinemia (55). HCys is considered as an independent risk factor for development of cardiovascular diseases (56, 57). Isolated smooth muscle cells from sublingual arteries when incubated with varying concentrations of HCys were shown to induce synthesis of elastase. Through elastase activation, HCys may increase the degradation of Extracellular matrix (ECM) and subsequent release of elastin peptides which induce vascular smooth muscle cell proliferation and migration into the subendothelium, leading to neointima formation and progressive vascular occlusion (58).

In a culture of human neutrophils, phorbol myristate acetate (PMA) and calcium ionophore A23187 increased elastase activity in the supernatants (24). PMA and A23187 are powerful stimulator of protein kinase C which in turn activates neutrophil membrane–
bound NADPH-oxidase resulting in enhanced ROS production which triggers the release of elastase from neutrophil granules (59, 60).

It is well known that tobacco components play a pivotal role in lung damage. Nicotine is shown to have direct effect on neutrophil functions with elevated elastase activity observed in the culture supernatants. A direct correlation between elastase activity release and nicotine concentration was also observed. The results indicate that nicotine plays an active role in the lung damage inducing neutrophils to release elastase activity (61, 62).

The role of serine proteases from PMNs in ischemic conditions was tested by Dang QB et al. When PMNs were co-incubated with cerebral endothelial cells and submitted to oxygen-glucose deprivation conditions for 4 hours, they were able to induce blood brain barrier (BBB) disruption. In particular, elastase was shown to be the major determinant of BBB breakdown. When culture media was supplemented with purified HDL-C, BBB breakdown was limited suggesting protective role of HDL in acute stroke patients (63).

The increasing knowledge of the role of HNE in several disease conditions has considerably increased the interest in discovering and/or developing useful potent therapeutic elastase inhibitors. Many natural compounds have been demonstrated to inhibit the release of elastase from neutrophils (16). Various phenolic compounds have been reported to inhibit HNE release. The flavanol quercetin was the first natural compound which was shown to inhibit the degranulation of neutrophils (64). Resvertol, a natural phenol from red wine and heparin were also reported to inhibit elastase release from neutrophils (65, 66).
Physical Properties, Structural Characteristics and Mechanism of Action

HNE is a basic glycoprotein whose primary structure shows considerable homology with proteinase 3 (54%) and cathepsin G (37%). It has been assigned a unique number by the Enzyme Commission of the International Union of Biochemistry based on its activity (E.C. 3.4.21.37) (18). Main characteristic features of HNE are represented in Table 1.

Table 1: Main characteristics of HNE

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HNE</th>
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<tbody>
<tr>
<td>EC number</td>
<td>3.4.21.37</td>
</tr>
<tr>
<td>Amino acid residues</td>
<td>218</td>
</tr>
<tr>
<td>Molecular mass</td>
<td>29-33kDa</td>
</tr>
<tr>
<td>Pi</td>
<td>( \approx 10.5 )</td>
</tr>
<tr>
<td>Number of glycosylation sites</td>
<td>2</td>
</tr>
<tr>
<td>Number of disulfide bridges</td>
<td>4</td>
</tr>
<tr>
<td>Optimal pH for activity</td>
<td>8.0-8.5</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td>Small hydrophobic residues: Val, Cys, Ala, Met, Ile, Leu, Ser</td>
</tr>
<tr>
<td>Natural substrates</td>
<td>Elastin, cartilage proteoglycans, collagen types I,II and IV, Fibronectin</td>
</tr>
<tr>
<td>Endogenous inhibitors</td>
<td>Alpha1-antitrypsin, alpha2-macroglobulin, elafin, pre-elafin</td>
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The sequence analysis of HNE shows that it is a single chain polypeptide of 218 amino acids with four intramolecular disulfide bonds linking eight half-cystine residues; these residues have been established by x-ray crystallography to be linked as follows: Cys-26 to Cys-42, Cys-122 to Cys-179, Cys-132 to Cys-158, Cys-169 to Cys-194. There are two positions at which the protein is N-glycosylated (Asn-95 and Asn-144). A
detailed analysis of both the type and structure of such side chains indicate that the composition reflects that of a complex carbohydrate of variable composition which accounts for its three major isoforms (67). Analysis of a minor form of elastase (E-1) indicates that the carbohydrate structures at each glycosylation site are complex-type bi-antennary chains usually associated with secretory glycoproteins. In contrast, the isoform E-3, the major form of elastase, contain exclusively truncated, oligomannose-type chains at the same positions in the sequence of each protein. These data suggest the possibility that certain elastase (E-1) might be destined for secretory, others (E-3) for lysosomal functions (68).

HNE display a three dimensional structure consisting of two homologous β-barrels and a C-terminal α-helix (Fig.4). Each barrel contains six antiparallel β-sheets connected through a linker segment. Residues of the catalytic triad i.e. Ser 195, Asp 102 and His 57 are located at the junction of the two β-barrels, whereas the active site cleft runs perpendicular to this junction. This arrangement of amino acids in the active site presumably allows neutrophil attack by Ser 195 on the carbonyl carbon (C=O) of the substrate scissile bond, thus setting off the catalytic process (6).

Fig.4: Three – dimensional structure of HNE. Ribbon plot of neutrophil elastase showing the two asymmetric β-barrels and the C-terminal α-helix, front view on the left, back view after a rotation of 1800 around a vertical y-axis on the right. N- and C-terminal β-barrels are represented in red and yellow respectively. The three flexible loops of the C-terminal β-barrels forming the activation domain are colored in pink and indicated by arrows. Disulfide bonds are depicted in green (adopted from Korkmaz B et al. Neutrophil Elastase, Proteinase 3, and Cathepsin G as therapeutic targets in human diseases. Pharmacol Rev 2010).
The catalytic triad of nucleophilic elastase is shown in figure 5.

![Figure 5: Catalytic triad of HNE](image)

The catalytic site of elastase is flanked on one or both sides by specificity subsites, each able to accommodate the side chain of a single amino acid residue from the substrate. These sites are numbered as shown in Fig.6 (69). Catalysis by HNE involves three steps, namely, substrate binding, acylation of Ser195, and deacylation. Briefly, covalently linked enzyme-peptide intermediate is formed with the loss of the amino acid or peptide fragment. This acylation step is followed by a deacylation process which occurs by a nucleophilic attack on the intermediate by water, resulting in hydrolysis of the peptide. Elastase preferentially cleaves the substrate after small hydrophobic residues (2, 69).

![Figure 6: Active sites of proteases](image)

Fig.6: Active sites of proteases. The catalytic site of proteases is indicated by * and the scissile bond is indicated by -; S1 through Sn and S1’ through Sn’ are the specificity subsites on the enzyme, while the residues they accommodate from the substrate are numbered P1 through Pn and P1’ through Pn’ respectively (adopted from Rao MB, et al. Molecular and Biotechnological aspects of Microbial Proteases. Microbiol Mol Biol Rev 1998).
Biological functions of HNE

HNE is currently viewed as a multifunctional enzyme involved in destroying pathogenic organisms and in inflammatory process regulation (6).

1) Intracellular effects: The primary role of the intracellular HNE is the proteolysis of foreign proteins (from bacteria) during phagocytosis by neutrophils. This antibacterial role of HNE is directed towards Gram-negative but not gram-positive bacteria (16).

Belaouaj et al. showed that NE deficient (NE-/−) mice were more susceptible to sepsis and death following intraperitoneal infection with gram negative, but not gram positive, bacteria than wild-type mice. They demonstrated in vitro that HNE mediates the killing of Gram-negative bacteria by degrading purified outer membrane protein A (OmpA) found on the surface of *Escherichia Coli* (E. coli) (70). Furthermore, incubation of neutrophil elastase with E. coli leads to a loss of bacterial integrity and lysis of bacteria in vitro.

A study by Weinrauch et al. demonstrated a role for NE in controlling enterobacteria such as *Salmonella enterica, Yersinia enterocolitica* and *Shigella flexneri*. They showed that NE targets and cleaves virulence factors of these bacteria at a lower concentration than required to degrade other proteins and in the absence of neutrophil elastase, these bacteria escape from the phagolysosome leading to their increased survival in the cytoplasm of infected neutrophils (71). In addition, a study by Reeves et al. suggested that activated serine proteases, not ROS, are primarily responsible for the destruction of bacteria. They found that mice made deficient in neutrophil granule proteases but normal with respect to superoxide production and iodinating capacity were unable to resist staphylococcal and candida infections. They also suggested that, myeloperoxidase (MPO) protects serine proteases from inactivation by oxidation by breaking down H₂O₂ to hypochloric acid (HOCl) (72). In contrast, Hirche et al. showed that MPO mediates oxidative inactivation of neutrophil elastase. They observed that MPO-deficient mice were more susceptible to infection with Klebsiella pneumonia (a
Gram-negative bacteria), indicating a more direct role for MPO in bacterial killing (73). These observations indicate that both MPO and neutrophil serine proteases are required for optimal intracellular killing of microorganisms in the mouse system.

2) Extracellular effects: In addition to the intracellular activities, HNE also exhibits extracellular antibacterial effects. Brinkmann et al have shown that serine proteases released from neutrophils form NETs with chromatin and that NETs bind Gram-positive and Gram-negative bacteria. These NETs allow neutrophils to deliver high concentrations of serine proteases that degrade virulence factors and kill bacteria extracellularly (11). Extracellular neutrophil elastase can also cleave the bacterial virulence factor flagellin that has pro-inflammatory effect on epithelial cells, thereby abrogating the ability of flagellin to induce a pro-inflammatory host response (74).

   In summary, as was discussed above, NE have both intra- and extracellular effects mediating host defense against infection.

3) Role in inflammation: In addition to having a role in host defense, NE has been associated with non-infectious, inflammatory processes. NE secreted into the extracellular environment from activated neutrophils in response to inflammation is inhibited by multiple proteinase inhibitors. In order for NE to regulate inflammation, it must escape the effects of these endogenous inhibitors present in the extracellular environment. The neutrophil has an elaborate repertoire of methods capable of circumventing these defenses (18). First, the tight adhesion of neutrophils to the ECM leads to the compartmentalization of the released proteases between the neutrophil and ECM where the proteases are released and this microenvironment excludes the large, circulating protease inhibitors (75). Second, antiproteases are sensitive to inactivation by oxidants released from activated neutrophils, which oxidize a critical methionine residue in the active site. Third, a large proportion of the serine proteases released from neutrophils bind to the plasma membrane with their catalytic activity preserved and that this tight binding
makes them inaccessible to large endogenous inhibitors (76). Hence, despite the presence of potent inhibitors, HNE is able to act locally in the pericellular and subjacent regions of neutrophils (18).

Several studies in animal models of inflammation have shown that inhibition of NE reduces neutrophil infiltration and neutrophil-mediated injury, suggesting it having a role in inflammation. For example, in a hamster model of endotoxin-induced acute lung injury Kawabata et al. showed an increase in inflammatory cell count in bronchoalveolar lavage fluid that peaked at 24 hours and correlated with NE activity in the fluid. When hamsters were treated with an NE-inhibitor, there was a change in the inflammatory cell count and histopathologic analysis of the lung tissue showed a decrease in hemorrhage and inflammation (77). Similarly, a role for NE in inflammation has also been shown in models of ischemia-reperfusion and collagen-induced arthritis (78, 79). In contrast, a study by Zhi Liu et al in NE-null mutant mice, showed that the absence of neutrophil elastase protected mice from bullous pemphigoid, which is an inflammatory skin disease characterized by blister formation (80). Taken together, these observations suggest that elastase have an important regulatory role in the local inflammatory response.

HNE is shown to have both anti- and pro-inflammatory activities (6, 18). It is capable of degrading mature TNF-α and IL-1β (81). In vitro studies have also demonstrated that HNE can degrade both IL-2 and IL-6 (82). Inactivation of these pro-inflammatory cytokines might limit the activation of neutrophils and dampen the inflammatory process suggesting that HNE may be involved in the down-regulation of inflammation. Conversely, it can enhance influx of neutrophils to the site of inflammation by inducing the secretion of GM-CSF, IL-6, and IL-8 from epithelial cells (16, 18). HNE cleaves α1-PI, generating a fragment that is chemotactic for neutrophils. In addition, HNE can degrade interendothelial (VE-cadherin) and interepithelial (E-cadherin) junctional
protein potentially promoting endothelial and epithelial permeability and pulmonary edema (18).

**Regulation of HNE activity or Role of Physiological Inhibitors**

The regulation of elastase in tissues is a prerequisite for the maintenance of homeostasis. During phagocytosis and neutrophil turnover, HNE is released into the extracellular space as active proteases. They may be potentially damaging when present at high concentrations. So they are tightly regulated in the extracellular and pericellular space to avoid degradation of connective tissue proteins such as elastin, collagen and proteoglycans (6). The basic level of control is normally achieved by regulated expression/secretion, by activation of inactive precursors or zymogens of proteases, and by degradation of the mature enzymes (83). A second level of regulation is by inhibition of their proteolytic activity by endogenous protein inhibitors which ultimately control the activity of HNE (6, 84). They belong to three main families: serpins (serine protease inhibitors), the canonical inhibitors, and the macroglobulins (6).

1) **Serine Protease Inhibitors (Serpins)**

Serpins are the largest and most diverse family of protease inhibitors with 350-500 amino acids (85) that regulate diverse physiological processes such as coagulation, fibrinolysis, complement activation, angiogenesis, apoptosis, inflammation, neoplasia and viral pathogenesis. However, the primary function of most members of the serpin family is to neutralize overexpressed serine proteinase activity (86). More than 1500 types have been identified in human and other organisms. The majority of serpins inhibit serine proteases, but serpins that inhibit caspases and papain-like cysteine proteases have also been identified (87). Serpins employ a unique, irreversible suicide substrate- like inhibitory mechanism to inhibit proteases (85). The X-ray crystal structures of serpins have shown
that they are globular proteins with nine α-helices and three β-sheets. The reactive center loop is localized in an approximately 20 amino acid long peptide segment on the molecular surface. It is linked C-terminally to strand 1 of β-sheet C and N-terminally to strand 5 of β-sheet A (86). Huntington et al with the crystallographic structure of a typical serpin-protease complex explained the mechanism of inhibition. In the inhibitory pathway, the protease initially recognizes the inhibitor as a potential substrate by reaction of the active serine of the protease with the reactive center of the serpin that acts as a 'bait' for a serine or cysteine proteinase. This cleaves the reactive center between P1 and P1' (Fig 6). This cleavage allows insertion of the cleaved reactive center loop into the β-sheet of the serpin, dragging the protease with it and moving it over 71Å to the opposite pole of the serpin to form a 1:1 stoichiometric covalent inhibitory complex. The tight linkage of the two molecules and resulting overlap of their structures does not affect the hyperstable serpin, but causes a 37% loss of structure in the protease. This is induced by the displacement of the serine from its active site, together with breakage of interactions formed during zymogen activation. The distortion of the catalytic site structure prevents the release of the protease from the complex, and the structural disorder allows its proteolytic destruction (88).

The principal serpins are α1-Protease Inhibitor (α1-Antitrypsin), α1-Antichymotrypsin and Monocyte/Neutrophil elastase inhibitor (MNEI, also called Serpin B1) of which α1-antitrypsin is the principal inhibitor of HNE.

**Alpha1-Protease Inhibitor (α1-PI)/ α1-Antitrypsin (α1-AT):** α1-PI was originally named α1-antitrypsin (α1-AT) because of its ability to irreversibly bind trypsin. It is now recognized that α1-AT is a potent inhibitor of multiple serine proteases with particularly high activity toward the neutrophil serine proteases, neutrophil elastase and proteinase-3 (28). It was first isolated in 1955 by Shultze and is one of the most abundant serine protease inhibitors in the circulation (86, 89).
α1-AT is a highly polymorphic, acute-phase glycoprotein synthesized mainly in hepatocytes and subsequently secreted into the plasma. Besides liver, it is also synthesized in extrahepatic tissues and cells, including neutrophils, monocytes and macrophages, alveolar macrophages, intestinal epithelial cells, carcinoma cells and the cornea. Extra hepatic synthesis of α1-AT is important in preventing tissue damage in the site of inflammation or injury (90). α1-AT is an acute-phase reactant whose plasma concentration can rise by 3-4-fold above normal (1.34 mg/ml) during inflammation, infection and malignant diseases. Although the mechanisms responsible for the increase of α1-AT are poorly understood, it has been shown that human neutrophils, monocytes and alveolar macrophages can increase expression of α1-AT in response to inflammatory mediators, such as IL-6, bacterial LPS and in response to α1-AT itself when complexed with neutrophil elastase (6, 86). Its normal circulating concentration functions to maintain the elasticity of the lung by preventing the hydrolytic destruction of elastin fibers. Severely diminished circulating concentrations of α1-AT, resulting from the impaired secretion of genetic variants can function as an etiologic agent for the development of COPD (91).

**Genetics of α1-AT**

The α1-AT is encoded by SERPINA1 gene located in proteinase inhibitor (Pi) locus on the long arm of chromosome 14 (14q32.1). The Pi locus is 12.2 kb long and consists of 4 coding exons, 3 non-coding exons and 6 introns. At the 5′ region of the SERPINA1 gene there are three non-protein coding exons (IA, IB, IC) which control gene transcription. Exons referred as exons II–V are coding and containing the sequence information that defines the protein itself. The region coding for the reactive loop with the active inhibitory center Met358 is within exon V (90).
**Polymorphism of α₁-AT**

The α₁-AT coding gene SERPINA1 is a highly polymorphic gene, with more than 125 single nucleotide polymorphisms (SNPs) reported in public SNP databases (Entrez SNP). Variants of α₁-AT are classified by the protease inhibitor system and each variant is identified by migration on agarose gel electrophoresis. All α₁-AT variants are categorized according to the serum level and functional activity as normal, deficient, null and dysfunctional (6, 90).

**Normal variants of α₁-AT**

More than 100 different naturally occurring genetic variants of α₁-AT have been identified (6, 91). Normal α₁-AT variants have normal serum level and functional activity to inhibit NE. More than 95% of normal variants are the “common” M1 (Ala213), M1 (Val213), M2 and M3 (90).

**Deficient variants of α₁-AT (α₁-ATD)**

Several mutations associated with α₁-ATD have been identified, and the most common are Z and S types (6, 90). The classical form of α₁-ATD is characterized by a point mutation (Glu342 → Lys) that leads to misfolding of mutant α₁-ATZ. α₁-ATZ accounts for almost 95% of all cases of α₁-ATD. Z mutation slows the folding of the α₁-AT molecule with subsequent increase in the concentration of intermediate which then polymerizes and accumulates in the endoplasmic reticulum of cells in which it is synthesized with reduced secretion resulting in lower or undetectable serum level of α₁-AT than normal variants. Since liver is the predominant site of α₁-AT synthesis, accumulation of mutant α₁-ATZ within the ER of liver cells leads to toxic consequences, including hepatic fibrosis/cirrhosis and carcinogenesis (85, 92).
Structure of $\alpha_1$-AT and Mechanism of Inhibition

$\alpha_1$-AT is a single-chain, globular glycoprotein consisting of 394 amino acids and a total molecular weight of 52kDa in the mature form. The main characteristics of the protein are: Met358 residue at the active site, isoelectric point ranging from 4.4 to 4.6, and crystallographic analysis of the mature protein reveals that it exhibits a number of glycoforms. There are three N-linked glycosylation sites on the external surface of the one end of the molecule. The side chains are composed of N-acetyl glucosamine, mannose, galactose and sialic acid and they are linked to amino acids Asn46, Asn83 and Asn247 (6, 90). The internal structure of $\alpha_1$-AT is highly ordered with 30 percent $\alpha$-helices and 40 percent $\beta$-pleated sheets. $\alpha_1$-AT obeys the general structure of serpins having nine $\alpha$-helices and three $\beta$-sheets with an exposed reactive center loop (RCL).

Structure as depicted in Figure 7.

Fig. 7: Schematic picture of the native $\alpha_1$-antitrypsin. The reactive center (yellow), $\beta$-sheet A (red) and C-terminal peptide (dark blue) are shown in relation to the rest of the structure (light blue) (adopted from Janciauskiene S. Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles. Biochimica et Biophysica Acta 2001).

Similar to other serpins, $\alpha_1$-AT is “suicide” or “single use” inhibitor that employs a unique and extensive conformational change in the process of inhibition of target
proteases (Fig. 8). The exposed RCL of α₁-AT is highly stressed external loop protruding from the molecule with Met358-Ser359 in the active center. Inhibitory process begins by docking of the α₁-AT and the neutrophil elastase and formation of Michaelis complex. Like the other inhibitory serpins, the structure of the RCL is crucial for the ability of the inhibitor to undergo a “stressed to relaxed” (S→R) conformational change. The active α₁-AT is in metastable or “stressed form”, which is essential for inhibition of proteases. During the process of inhibition, α₁-AT is like mousetrap with spring-like shift from a metastable to a hyperstable state. After the formation of Michaelis complex there are two possible different ending of the reaction. One is inactivation of protease, where serpin has undergone the S → R transition, and the protease hangs distorted at the base of the molecule. The other possibility is α₁-AT substrate-like behavior, where RCL forms the fourth β-sheet, providing the opportunity for the protease to escape the conformational trap, leaving active protease and inactive cleaved serpin (87, 88, 90).

Fig. 8: The inhibitory mechanism of serpin (adopted from Ruby HP Law et al. An overview of the serpin superfamily. Genome Biology 2006).
**Biological functions of α₁-AT**

In addition to protease inhibition, recent studies have demonstrated that α₁-AT also has anti-inflammatory, immunomodulatory, antiapoptotic and antimicrobial roles (93). α₁-AT is an irreversible inhibitor for kallikreins 7 and 14 (94). Anti-inflammatory action of α₁-AT is facilitated by blockade of pro-inflammatory cytokine release from human peripheral blood mononuclear cells (95). Specifically, α₁-AT decreases the production of two typical upstream mediators of inflammation TNF-α and IL-1β. α₁-AT is also shown to increase the levels of IL-10, an anti-inflammatory cytokine in various experimental conditions (95). The activity of pro-inflammatory cytokines appears to consistently diminish in the presence of elevated α₁-AT, while the release of anti-inflammatory mediators increases (96). α₁-AT also lowers the levels of the IL-8 and monocyte chemotactic protein (MCP)-1, two major chemokines involved in the chemotaxis of inflammatory cells. α₁-AT has also been reported to inhibit neutrophil superoxide production (97) and to enhance insulin-mediated mitogenesis in various cell lines (98). Findings have also revealed a role of α₁-AT in iron metabolism that it enhances the synthesis of both transferrin receptor and ferritin (99). It has recently been demonstrated that a specific 20-residue fragment of α₁-AT binds to the gp41 fusion peptide of HIV-1 and prevents the virus from entering target cells, thereby inhibiting HIV-1 infection. These findings suggest that α₁-AT may play a protective role in HIV-1-infected individuals (100).

Several studies have shown that α₁-AT inhibits the activity of caspase-3, an intracellular cysteine protease which plays an essential role in cell apoptosis (101, 102). Recent studies show that α₁-AT stimulates insulin secretion and protects β-cells against
cytokine-induced apoptosis. Animal studies provide further evidence that α1-AT therapy prolongs islet graft survival in transplanted allogeneic diabetic mice (103, 104).

2) Canonical inhibitors

The largest groups of protein inhibitors are canonical inhibitors that act according to the standard mechanism of inhibition. A huge number of canonical inhibitors have been isolated from various cells, tissues, and organisms. Canonical inhibitors are widely distributed in essentially all groups of organisms. SLPI and elafin are the examples for this group of inhibitors (6, 105).

SLPI, also known as secretory leukoprotease or leukocyte protease inhibitor or antileukoproteinase is an 11.7-kDa cationic, nonglycosylated, highly basic, acid-stable, cysteine-rich, 107-amino acid, single-chain polypeptide (106). SLPI is constitutively expressed at many mucosal surfaces and is produced by a number of cell types including neutrophils, macrophages and epithelial cells lining the respiratory and alimentary tracts. The evidence to date suggests that the function of SLPI in vivo is to protect local tissue against the detrimental consequences of inflammation not only as a result of its anti-inflammatory activities but also via its antiprotease and antimicrobial properties (107). SLPI protects the tissues by inhibiting the proteases such as elastase, cathepsin G, trypsin, chymotrypsin, chymase, and tryptase. However, its physiological target is neutrophil elastase, because SLPI accounts for approximately 90% of all elastase inhibitors in human bronchial secretions, and it has high affinity for neutrophil elastase (106, 108). Because of its lower molecular weight compared with α1-AT, SLPI can access the space between adherent neutrophil and ECM, thus protecting ECM proteins from proteolysis (75).

Elafin also known as Peptidase inhibitor 3 or skin-derived anti leukoprotease (SKALP), is a basic, nonglycosylated 6-kDa inhibitor. The elafin precursor protein trappin-2 (pre-elafin) is cleaved to form the mature protein elafin (109). Elafin shares
40% of sequence identity with SLPI, and the active site residues are also familiar. Elafin interacts with HNE through its active site centered on the Ala24-Met25 peptide bond (6).

3) Alpha-macroglobulins

The alpha-macroglobulin family includes protease inhibitor, human \( \alpha_2 \)-macroglobulins (\( \alpha_2 \)-MG) which is a tetrameric, polyvalent inhibitor of 720kDa. \( \alpha_2 \)-MG constitutes a large part of the plasma proteins and in early childhood serum concentration is up to 4-5 g/l. Nearly all \( \alpha_2 \)-MG in the blood is native, since \( \alpha_2 \)-MG -proteinase complex is subject to rapid receptor-mediated clearance. The serum concentration in adults is about 2 g/l. The level is slightly higher in women than in men (32). The \( \alpha_2 \)-MG molecule is synthesized mainly in liver, but also locally by macrophages, fibroblasts and adrenocortical cells. Because its high molecular mass impairs diffusion to inflammatory sites during neutrophil extravasation, its major role is probably restricted to controlling protease activity within the circulation (6). It inhibits all classes of proteases such as serine, cysteine, aspartic and metalloproteases. Proteases bind and cleave the bait region of \( \alpha_2 \)-MG that is a segment particularly susceptible to proteolytic cleavage which initiates a conformational change so that the \( \alpha_2 \)-MG collapses about the protease and forms a \( \alpha_2 \)-MG – protease complex. This complex is recognized by macrophage receptors and cleared from the system. \( \alpha_2 \)-MG also functions as an inhibitor of fibrinolysis by inhibiting plasmin and as an inhibitor of coagulation by inhibiting thrombin. It also acts as carrier protein as it binds to numerous hormones, growth factors and cytokines (110).

**HNE and its endogenous inhibitors in Infectious diseases**

It has become clear that serine protease HNE is capable of modulating many biological functions. Dysregulation of its activity resulting in its accumulation have been associated
with the pathogenesis of several disorders. Infectious diseases are characterized by the invasion of host’s body tissues by disease-causing agents, their multiplication and the reaction of host tissues to these pathogens and the toxins they produce. As stated earlier, host innate immune response is one of the factors involved in the setting of infection, which involves a coordinated expression of inflammatory cytokines with chemoattractant activity and subsequent recruitment of various cell types particularly immune cells aimed at clearing the pathogenic agent (6, 7). Neutrophils play a crucial role in protecting the host against microbial pathogens but they produce proteolytic enzymes such as NE, the uncontrolled activity of which can destroy tissue and lead to organ failure (6, 7, 15). Counteracting NE activity through the use of clinically tested protease inhibitors has proven to be beneficial in a number of human inflammatory conditions and mouse models (6, 16).

Dengue is one of the most important viral infections of humans. Dengue viruses (DENVs) are transmitted by mosquitoes of the genus *Aedes* in the form of four distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). DENVs are members of the Flaviviridae family, which are single stranded RNA viruses (111). Estimation of the global incidence of dengue infections per year is close to 400 million (112). DENV infection may result in an asymptomatic or mild self-limiting acute febrile illness, dengue fever (DF) or life-threatening severe illnesses, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (113). The pathogenesis of DENV infection has been linked to the ability of the dengue virus to infect monocytes. Dengue virus infected monocytes in turn are shown to produce various inflammatory mediators such as neutrophils, plasma cascade systems and cytokines (112, 114). Pro-inflammatory cytokines including IL-6 and IL-8 are shown to activate neutrophil functions and thus are involved in the migration and activation of neutrophils (115). Neutropenia is one of the salient clinical findings in dengue patients, suggesting that neutrophils have a crucial role in the pathogenesis of DENV infection (116). In a study by Juffrie et al, significant
elevation in elastase was observed in patients with DENV infection. They also found significant correlation between elastase and levels of IL-8 suggesting the involvement of IL-8 in neutrophil activation in DENV infection. A significantly higher elastase in patients with shock than in normotensive patients suggests the role of elastase in pathogenesis of shock (115). This action of elastase may be facilitated by activation of complement that contribute to vasodilatation and increased vascular permeability (117).

Many proteomic studies have identified a larger number of differentially-expressed serum proteins in dengue fever of which most were acute phase proteins followed by serine protease inhibitors which were regular indicators of infection and inflammation. Elevated serum levels of α₁-AT were observed in DF & DHF. Upregulation of this protease inhibitor might be a consequence of the host response to prevent the inflammation and vascular damage triggered by dengue virus infection (118, 119). Brasier et al identified α₂-MG as most informative protein biomarker in the DENV infection associated complications. In the Mass Spectrometry analysis they found increased α₂-MG in Dengue Fever Complicated (DFC) and levels were decreased in DHF cases (120). Albuquerque LM et al also found decreased expression of the inhibitor in severe dengue cases (121). These results suggest the consumption of plasma anti-protease which may play a role in the evolution to DHF. Kumar Y et al observed elevated levels of α₂-MG in dengue patients (122).

Pneumonia is inflammation of the lungs that can be caused by bacteria, viruses, fungi or parasites but most commonly it is bacterial. Pneumonia can be categorized as: community-acquired, hospital-acquired and pneumonia occurring in immunocompromised individuals. In normal conditions, alveolar macrophages (AMs) are the main cells that respond to bacteria that reach lower airways. However, if the microbial inoculum is too high or too virulent to be stopped by AM alone, these cells recruit PMNs
into the alveoli from the vascular compartment. Cytokines and other pro-inflammatory mediators secreted by the AM attract PMN to destroy the invading pathogens (123). After activation, neutrophils exit the vasculature and migrate through the interstitium into the alveolar space. The degree of neutrophil activation, generation of ROS and the release of antimicrobial molecules play a key role in pathogen clearance. However, prolonged neutrophil activation may contribute to lung injury and poor outcomes in pneumonia. Studies have attributed this to protease-antiprotease imbalance. NE besides its important antimicrobial functions holds an evident role in the pathogenesis of lung injury. Adequate antiprotease activity is required in the lung tissues to counteract the damaging effects of NE. Clinical studies have found significantly elevated NE and altered levels of its endogenous inhibitors in pneumonia patients. The elevated levels correlated with the severity of the lung injury. Boutten A et al estimated total NE and α1-AT concentrations in BAL fluid from both pneumonia-infected and non-involved lung. While they found significantly elevated total NE in BAL fluid from involved lung, elastase-inhibitory capacity of α1-AT in the involved lung was reduced (124). Braun J et al found approximately 40 times higher concentration of elastase-α1-AT complex in pneumonia group than in the control group (125). In a study by Umeki S et al., the plasma levels of α2-MG were slightly increased in patients with pneumonia (126). In vitro studies on animal models have provided evidence for the protective effect of elastase inhibition. Pott GB et al demonstrated human α1-AT as a suppressor of bacterial pneumonia and pneumonia-related pathogenesis. In α1-AT+/+ transgenic mice that express human α1-AT in lungs, mortality due to Pseudomonas aeruginosa pneumonia was reduced 90% compared to non-transgenic control animals. Exogenous human α1-AT given to non-transgenic mice also significantly reduced pneumonia mortality (95).

Recent studies have demonstrated the role of NE and its inhibitors in other infectious diseases. Pukrittayakamee et al. found that elastase levels on admission were
elevated in all patients with severe malaria and it was elevated only in 86.6% and 65% of the moderately severe and mild patients respectively. They also observed decrease in the levels of elastase as the patients became afebrile and aparasitaemic (127). In vitro studies have demonstrated that α1-AT has potent anti-HIV activity. It was found to inhibit HIV-1 replication (128, 129).

**HNE and its endogenous inhibitors in Non-Infectious diseases**

Non infectious disease is a medical condition or disease which is non-infectious. They include diabetes mellitus, heart disease, stroke, cancer, preeclampsia, asthma, chronic kidney disease, osteoporosis, cataracts and more. Risk factors such as a person’s background, lifestyle and environment are known to increase the likelihood of certain non-infectious diseases (130).

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs leading to complications of micro-vascular and macro-vascular systems. Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes (131, 132). According to the latest 2016 World Health Organization (WHO) data globally, estimated 422 million adults are living with diabetes mellitus (133). Diabetes currently affects more than 62 million Indians, which is more
than 7.1% of the adult population. The average age of onset is 42.5 years and nearly 1 million Indians die due to diabetes every year (134).

Several pathogenic processes are involved in the development of diabetes. Hyperglycemia at the onset induces the stimulation of various pro-inflammatory mediators such as IL-1β, TNF-α and IL-6 that could cause tissue specific inflammation (135). Chronic, low-grade inflammation is causally linked to systemic insulin resistance and low tissue insulin sensitivity, both hallmarks of T2DM. Several studies have indicated Type 2 diabetes as an inflammatory disease (135, 136, 137, 138). Typical elements of inflammatory processes are seen in the vasculature in diabetes which include altered levels of specific chemokines and cytokines, changes in the number and activation state of various leukocyte populations, increased expression of vascular adhesion molecules leading to recruitment of leukocytes to the vascular wall (135, 139). High concentrations of glucose in T2DM may activate neutrophils chronically to release elastase. A direct association between elastase and diabetes has also been studied (140, 141). Concentration of elastin peptides (biological markers of ECM destruction) (140) and elastase activity (141) were significantly increased in diabetic patients and serum glucose was correlated to serum elastase activity (141). Agnieszka P et al found significantly higher leukocyte elastase/ α1-proteinase inhibitor complexes in plasma and neutrophil extracts in type 2 diabetic subjects compared to controls. They also observed a significantly elevated elastase in patients with both micro- and macro-angiopathy in comparison to the group with only micro- or macro-angiopathy (142). A recent study by Talukdar et al implicates neutrophils in the genesis of insulin resistance, via the action of neutrophil elastase. From their experimental study on lean and obese mouse as well as human liver and adipose tissue cell lines, they concluded that NE appears to a) cause Insulin Receptor Substrate 1 (IRS1) degradation, b) reduce insulin signaling c) enhance
glucose production and d) derange lipid metabolism, all of which contributes to increased cellular insulin resistance (137).

Recent studies have raised the possibility of an association between α₁-AT and diabetes. In support of this, a few clinical studies demonstrated that plasma α₁-AT levels and activity were lower in diabetic patients than in non-diabetic controls (143). Invitro studies have demonstrated that over-expression of α₁-AT significantly reduces insulitis and prevents the development of overt hyperglycemia in non-obese mice (103). It has also been shown that administration of clinical grade human α₁-AT prolongs pancreatic islet allograft survival and exhibits cytoprotective effects (102). α₁-AT is under evaluation for treatment of Type1 diabetes in multiple clinical trials. Initial results suggest that α₁-AT therapy could potentially improve insulin production without adverse effects. Up to 50% of individuals displayed improved islet function (26). In a study by Rachmiel M et al evaluated safety and tolerability of α₁-AT in a pediatric population with recent onset T1DM and they have observed that treatment with α₁-AT was safe and well tolerated (144).

Human α₂-MG, a plasma glycoprotein, traps and inhibits proteolytic enzymes which participate in inflammation and homeostasis. Significant role of α₂-MG in immunoregulatory processes has been established. Previous studies have investigated the levels of α₂-MG in patients with diabetes mellitus and found an association between their levels and complications of diabetes (33). A recent study explored the association among salivary α₂-MG, plasma α₂-MG and blood glucose variants in type 2 diabetic patients. They found that salivary α₂-MG statistically correlated with plasma α₂-MG (145).

Preeclampsia (PE) is a pregnancy specific hypertensive disease with multisystem involvement. PE complicates 3%–8% of pregnancies in Western countries and constitutes a major source of morbidity and mortality worldwide. In India, the incidence of PE is reported to be 8-10%. Overall, 10%–15% of maternal deaths are directly associated with
PE and eclampsia. PE was formerly defined by new onset of hypertension i.e. systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg as measured twice, 4-6 hours apart and less than 7 days apart and proteinuria (> 300 mg/24 h) arising after 20 weeks of gestation in a previously normotensive woman and disappearance of all these abnormalities before the end of the 6th week postpartum (146, 147). According to the new guidelines by the American College of Obstetricians and Gynecologists proteinuria is no longer required for the diagnosis of PE (146). New development of decreased platelets, changes in the kidney or liver function, fluids in the lungs, or signs of brain disorder such as seizures and/or visual disturbances along with persistent high blood pressure is now used to diagnose PE.

PE has a complex pathophysiology, the primary cause being abnormal placentation. Abnormalities in the development of placental vasculature early in pregnancy may result in relative placental under perfusion/hypoxia/ischemia, which then leads to release of factors into the maternal circulation, culminating in the clinical signs, symptoms and complications of PE. All of the clinical manifestations of PE can be attributed to glomerular endotheliosis, increased vascular permeability, and a systemic inflammatory response (146, 148).

Women with PE exhibit chronic inflammation characterized by oxidative stress, pro-inflammatory cytokines and autoantibodies (149). Both observational and experimental studies have demonstrated an association between inflammation and endothelial dysfunction in PE (150). An inflammatory response is usually accompanied by increased concentrations of pro-inflammatory cytokines leading to neutrophil activation (151). TNF-α and IL-6 are some of the pro-inflammatory cytokines that are known to play a role in neutrophil activation in PE (152). During neutrophil activation, there is a metabolic activation and release of contents of their granules contributing to increased
inflammatory response and/or oxidative stress. Elastase is frequently measured in plasma as a cell free marker to quantify such activation (153) and studies have reported elevated elastase levels in preeclampsia establishing its role in complications of PE (151, 154).

The circulating levels of $\alpha_1$-AT rises during normal pregnancy. $\alpha_1$-AT deficiency is associated with several pregnancy and placental disorders. Since unopposed inflammation contributes to PE, this disease is might be associated with lower than normal levels and activity of $\alpha_1$-AT. Guy T et al reported significantly lowered $\alpha_1$-AT levels in severe PE women (155). Feng et al in a proteomic study using 2D PAGE identified that the normal full-term pregnant women expressed the most $\alpha_1$-AT and in late-onset PE women $\alpha_1$-AT was down regulated. This differential expression was also consistent with the peripheral circulating levels of $\alpha_1$-AT as the concentration was highest in full-term pregnancy group, moderate in the early-onset and lowest in the late-onset PE group (156). A recent study by same authors on PE animal model demonstrated that $\alpha_1$-AT injection significantly relieved the high blood pressure, increased fetal weight and reduced urine protein levels in a dose-dependent manner and thus improve PE. The authors also showed that exogenous $\alpha_1$-AT injection increased the antioxidants levels and suppressed oxidative stress in pregnant PE mice model. Thus they concluded that $\alpha_1$-AT would become a potential strategy for PE therapy. In this study, they also found a significantly decreased levels of $\alpha_1$-AT in placental tissues from women with PE compared to that of healthy women (157).

The levels of $\alpha_2$-MG is marginally increased during pregnancy, possibly due to estrogen. Decreased $\alpha_2$-MG concentration typically results from increased clearance of $\alpha_2$-MG- proteinase complex and usually occurs in conditions associated with increased proteolytic activity. Increased serum $\alpha_2$-MG is frequently seen in nephrotic conditions
and reflect a change in plasma volume as well as in its metabolism (32). Horne et al found high α₂-MG levels in PE with proteinuria as compared to normal pregnant women (158).

Stroke is a serious neurological disease, and constitutes a major cause of death and disability throughout the world (159). It is one of the most devastating manifestations of two common diseases, atherosclerosis and hypertension. Besides age, hypertension is the most important cardiovascular risk factor for developing both ischemic and hemorrhagic stroke (160). It is estimated that 25% or more of strokes may be attributable to hypertension. Lowering BP reduces the risk of stroke. Epidemiological studies have shown that for each 10 mm Hg lower SBP, there is a decrease in risk of stroke of approximately one third in persons aged 60 to 79 years.

The pathophysiology of stroke is complex and involves excitotoxicity mechanisms, inflammatory pathways, oxidative damage, ionic imbalances, apoptosis, angiogenesis and neuroprotection (159). Inflammatory response after ischemic brain injury is initiated by the rapid production of many different inflammatory mediators including a number of cytokines and damage associated molecular patterns (DAMPs). These promote neutrophil recruitment and activation. Recent evidences suggests that neutrophils play an important role in the pathogenesis of stroke. Neutrophil recruitment to the cerebral ischemic regions occurs as early as thirty minutes after ischemia and reperfusion and the degree of accumulation is correlated with the extent of brain infarct and clinical outcome. Activated neutrophils adhere to activated endothelium through adhesion molecules that promote neutrophil–endothelial interactions and neutrophil migration. Proteolytic enzymes such as elastase and free oxygen radicals and cytokines released from neutrophils contributes to pathogenesis of acute stroke with resulting effects on the BBB and brain parenchyma (161) (Fig. 9).
Neutrophils and their contents have emerged as treatment targets for ischemic stroke on account of their important role in pathogenesis of the disease. In a rat model of middle cerebral artery occlusion, neutrophil depletion was shown to limit edema and infarct size area (162) and also to reduce BBB breakdown and inflammation following intracerebral hemorrhage and improve stroke outcomes (163). Experimental studies have shown elastase to have major role in breakdown of BBB (63). In a mice model of transient middle cerebral occlusion, authors have found increased levels and activity of tissue elastase. Furthermore, pharmacological inhibition of NE and also genetic deletion of NE, significantly reduced infarct volume, BBB disruption, vasogenic edema, and leukocyte-endothelial adherence. The study findings confirm the involvement of NE in neurovascular stroke pathology (164). In an earlier study by Akira et al on rat model of middle cerebral artery occlusion, neutropenia by anti-neutrophil antibody injection significantly reduced the size of cerebral damage. Administration of elastase- specific inhibitor, ONO-5046 also reduced the size of damage indicating the role of elastase in the...
development of cerebral damage (165). Clinical studies in stroke patients have also shown higher levels of elastase as compared to healthy individuals. Grau et al. reported higher levels of elastase-inhibitor complex in subjects with risk factors compared to healthy individuals without risk factors (166).

As post-ischemic inflammation contributes significantly to the ultimate pathology, studies have targeted this aspects of ischemia to improve the outcome of stroke. α₁-AT with potent anti-inflammatory, antiapoptotic and cytoprotective along with its anti-elastase properties is tested by various researchers for its beneficial role in stroke. Konrad C et al reported a case report of a 45 year old male patient with homozygous α₁-AT deficiency in whom spontaneous internal carotid artery dissection occurred in the absence of any other known risk factors (167). In a study by Moldthan HL et al, intravenous/intracranial delivery of human α₁-AT into rats with induced middle artery occlusion significantly reduced the infarct volume at 72 hours compared with control rats, concluding that human α₁-AT could be a potential novel therapeutic drug for the protection against neurodegeneration following ischemic stroke (168).

α₂-MG is a protease inhibitor that enhances procoagulant properties via the neutralization of plasmin, plasminogen activators and metallopeptinases. Recent studies have reported increased levels of α₂-MG in stroke patients and also associated the levels with high-grade white matter lesions suggesting the involvement of this inhibitor in the pathophysiology of acute ischemic stroke (34). Beheiri et al showed elevated α₂-MG levels independently increase the odds of stroke and deep vein thrombosis in white children through its procoagulant properties (169).

NE and its endogenous inhibitors; α₁-AT and α₂-MG have been implicated in the initiation and progression of inflammatory chronic lung diseases such as COPD, acute lung injury (ALI), ARDS and cystic fibrosis (CF) (6, 170, 171). Leukocyte elastase has been implicated in the process of atherosclerosis by inducing degradation of endothelial
membrane and sub endothelial matrix proteins (172, 173). α2-MG is known to be involved in the development of foam cells in atherosclerotic lesions (174). The presence of elastinolytic activities and role of NE in proliferation and tumorigenesis in human breast cancer tissue has been demonstrated (175, 176, 177). The role of NE as a putative diagnostic marker and therapeutic target for patients with colorectal cancer was also studied (178). In an experimental study, a specific NE inhibitor completely suppressed growth of cancer cells transplanted into severe combined immunodeficiency mice (179). Various experimental studies and clinical findings have indicated that a deficiency or decreased α1-AT is associated with increased risk of various cancers such as liver cancer, bladder cancer, gall bladder cancer, malignant lymphoma and lung cancer (180). α2-MG is shown to be valuable for the prognosis of pancreatic cancer (181). Studies have shown elevated plasma levels of elastase in rheumatoid arthritis patients indicating activation of polymorphonuclear leukocytes in response to inflammation. Very high concentrations of elastase-α1-PI complex have also been reported in rheumatoid arthritis patients (182). The presence of proteolytically active NE in diseased epidermis, which is known to contain specific inhibitors of this enzyme, suggests a pathophysiologic role of this enzyme in psoriasis, contact dermatitis, and atopic dermatitis (183, 184). Elastase is shown to play a role in in vivo destruction of epidermal-dermal junction (184).

It is evident from the above stated review of literature that the normal circulating concentrations of neutrophil elastase and elastase inhibitors are important to maintain the integrity of elastic tissues. Its dysregulation can have devastating effects by playing a significant role in the development and progression of both infectious and non-infectious disease. The worldwide increased morbidity of COPD, severity of AATD and new findings on HNE during inflammation has prompted extensive experimental and clinical research to develop efficient elastase inhibitors.
Lacunae of Knowledge
There are several studies implicating direct role of elastase and its inhibitors in the development and progression of tissue specific inflammatory processes in various disease conditions. All these studies have been conducted in isolated and specific disease conditions and there are not many comparative studies on the behavior of the levels of elastase and its inhibitors among various types of diseases. Therefore it was considered worthwhile evaluating the levels of free elastase, elastase in complex form as well as the levels of its endogenous inhibitors in various infectious and non-infectious diseases. The outcome of the study is expected to provide insight to the behavior of these molecules and their roles in diagnosis, management/treatment and prognosis in various diseases. In addition, a systematic approach to investigate the factors triggering elastase release from neutrophils was also intended in the study to corroborate its role in the disease development and progression.
Aims and Objectives
• To compare the levels of elastase and its endogenous inhibitors in infectious and non-infectious diseases to evaluate if there existed any difference in the levels of these molecules among these disease groups.

• To correlate diagnostic parameters and risk factors of infectious and non-infectious diseases with the levels of elastase and its inhibitors to propose add on biomarkers for various disease conditions.

• To assess by in vitro methods the effects of disease specific molecules on the release of elastase from neutrophils to associate with the diagnostic parameters.

• To ascertain the usefulness of measuring the levels of these molecules as differential inflammatory markers or as add on markers for diagnosis and prognosis of infectious and non-infectious diseases.
General Materials and Methods
The study was carried out in the Department of Biochemistry of Sri Devaraj Urs Medical College, constituent college of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka. The study involved randomly selected subjects attending or admitted in the respective departments of R. L. Jalappa Hospital and Research Centre, the teaching hospital of the medical college. Every enrolled patient or their relatives gave informed written consent to participate in the study. Ethical approval for the study was obtained from Institutional Ethical Committee and the study complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration.

Chemicals and Reagents

All reagents used for the study were of analytical grade unless otherwise specified. Succinyl tri- L-alanyl-p-nitroaniline (STANA, Cat.No. S4760) was purchased from Sigma, USA. α1-AT and α2-MG Enzyme Linked Immunosorbent Assay (ELISA) kits were purchased from Immunology Consultants laboratory, Inc, USA and Neutrophil elastase- α1-antitrypsin complex (NE–α1-AT complex) ELISA kit was from Calbiochem.

Assay Description

1) Determination of Elastase activity:

Plasma elastase was estimated using STANA as substrate at 410nm as per the procedure described by Beith.J, et al (185). Briefly, the assay system comprised of 2mM of STANA in 0.2M of Tris Hcl buffer pH 7.6. The reaction was initiated by the addition of plasma. After a 15 minute incubation at 37°C, the reaction was stopped by the addition of 1.0 ml of 30% Acetic acid. The optical density of p-nitroaniline liberated was measured at 410 nm. The measurement is based on the following reaction:
\[
\text{SucAla}_3\text{-pNA} + \text{H}_2\text{O} \rightarrow \text{SucAla}_3\text{+pNA}
\]

where:

\[
\begin{align*}
\text{SucAla}_3\text{-pNA} &= \text{STANA} \\
\text{SucAla}_3 &= \text{N-Succinyl-trialanine} \\
\text{pNA} &= \text{p- nitroaniline}
\end{align*}
\]

Unit definition:

One unit of elastase activity was defined as the amount of enzyme required to release 1.0 µmole of p-nitroaniline per unit time under standard assay conditions.

\[
\text{Units/ml enzyme} = \frac{\text{Absorbance} \times \text{Total volume of Assay} \times \text{DF}}{8.8 \times \text{volume of plasma used}}
\]

where:

DF= dilution factor

8.8=millimolar extinction coefficient of p-nitroaniline at 410nm.

2) Plasma \(\alpha_1\)-AT assay:

Plasma levels were quantified using two-site ELISA as per manufacturer’s instructions. Briefly, appropriately diluted samples were added to wells coated with anti- \(\alpha_1\)-AT antibodies. After incubation and washing, the solid-phase bound complexes were further incubated with anti-\(\alpha_1\)-AT antibodies conjugated with horseradish peroxidase. Following another washing step, a chromogenic substrate, 3, 3’, 5, 5’-tetramethylbenzidine was added to the samples. The quantity of solid-phase bound complexes which varies directly with the concentration of \(\alpha_1\)-AT in the tested samples were measured at 450nm. The concentration of \(\alpha_1\)-AT in each tested sample was calculated from the standard curve constructed from the standards after considering dilution factor.
3) Plasma α2-MG assay:

The α2-MG concentration was determined using two-site ELISA kit as per manufacturer’s specifications. Briefly, diluted samples were added to wells coated with anti- α2-MG antibodies. After incubation and washing, anti-α2-MG antibodies conjugated with horseradish peroxidase were added each well. These enzyme-labelled antibodies form complexes with the previously bound α2-MG. After further washing steps, the bound enzyme was assayed by the addition of a chromogenic substrate, 3, 3’, 5, 5’-tetramethylbenzidine. The quantity of bound enzyme directly varies with the concentration of α2-MG in the tested samples. Absorbance was read at 450nm in an ELISA reader. The quantity of α2-MG in the tested sample was calculated from the standard curve constructed from the standards and corrected for sample dilution.

4) Plasma NE–α1-AT complex assay:

The complex concentration was measured in the plasma using ELISA method according to the manufacturer’s instructions. Briefly, diluted samples were added to wells coated with antibody specific for human PMN elastase. After an hour of incubation, unbound proteins were removed by washing. This step was followed by the addition of anti-α1-PI polyclonal antibodies conjugated with horseradish peroxidase to all the wells. These enzyme-labelled antibodies form complexes with the previously bound PMN elastase. Following another incubation and washing steps, the bound complex was assayed by the addition of a chromogenic substrate, 3, 3’, 5, 5’-tetramethylbenzidine. Absorbance was read at 450nm using an ELISA reader. The concentration of complex for each sample was determined from the standard curve constructed from the standards after considering dilution factor.