# Studies on Elastase and Elastase-Inhibitors in Infectious and Non- Infectious Diseases

Thesis submitted to Sri Devaraj Urs Academy of Higher Education & Research for the award of the degree of

**Doctor of Philosophy (Ph.D)**

In

**Biochemistry**

**Under the Faculty of Medicine**

By


**Mamatha Kunder**

Department of Biochemistry

Under the guidance of

**Dr. A.V.M.Kutty**, Professor

Sri Devaraj Urs Medical College, Tamaka, Kolar

(constituent Institute of SDUAHER, Tamaka, Kolar, Karnataka)



June 2017

# CERTIFICATE OF DECLARATION BY THE CANDIDATE

I, **Mamatha Kunder** do hereby declare that the research work presented in the thesis titled **"Studies on Elastase and Elastase-Inhibitors in Infectious and Non-Infectious diseases"** is an original work carried out under the guidance of Dr. A.V.M. Kutty, Ph.D, Professor, Department of Biochemistry and Co–guidance of Dr. V. Lakshmaiah, MD, Professor, Department of Medicine, Sri Devaraj Urs Medical College for the award of the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associate ship, fellowship or any other similar title or recognition.

Date: Signature of the candidate

Place: Kolar

Mamatha

Mrs. Mamatha Kunder

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Guide

Dr. A.V.M. Kutty, Ph.D
Professor
Department of Biochemistry
Sri Devaraj Urs Medical College
Tamaka, Kolar, Karnataka

Date: 23rd June 2017

Co – Guide

Dr. V. Lakshmaiah, MD
Professor
Department of Medicine
Sri Devaraj Urs Medical College
Tamaka, Kolar, Karnataka

Date:

Dr. V. LAKSHMAIAH
KMC No. 20861
DATE 23/6/17 KOLAR

# ENDORSEMENT BY THE HOD/ PRINCIPAL/HEAD OF THE INSTITUTION

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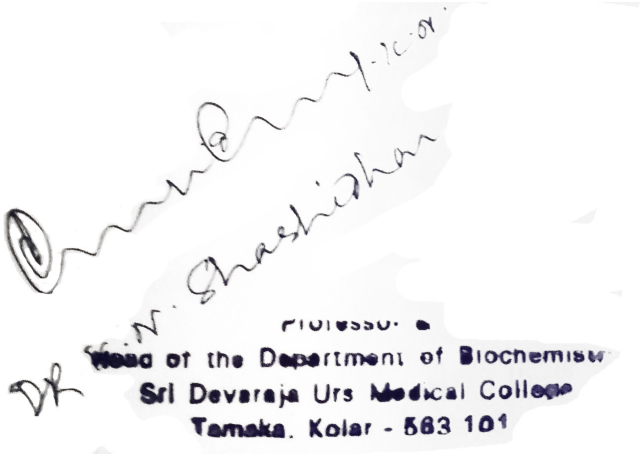


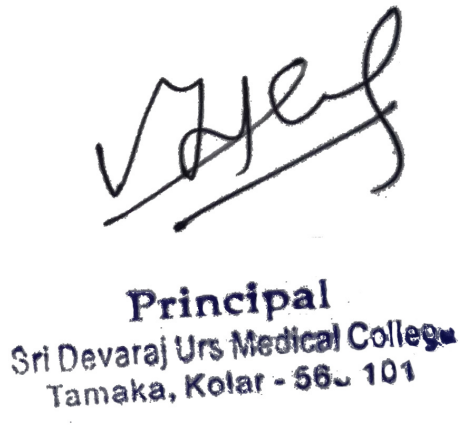


Signature of the HOD

Signature of the Principal

Place: Kolar

Date: 23.6.17

Place: Kolar

Date: 23/06/2017

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Mamatha Kunder

# ABBREVIATIONS

$\alpha_1$-AT – $\alpha_1$-Antitrypsin

$\alpha_2$-MG – $\alpha_2$-Macroglobulin

$\alpha_1$-PI - $\alpha_1$-Proteinase Inhibitor

AATD- $\alpha_1$-Antitrypsin Deficiency

ARDS-Acute respiratory distress syndrome

AMs- Alveolar macrophages

BBB- Blood brain barrier

COPD-Chronic Obstructive Pulmonary Disease

CRP-C-Reactive Protein

DPPI- Dipeptidyl peptidase I

DENV- Dengue virus

DF- Dengue fever

DHF- Dengue hemorrhagic fever

DSS- Dengue shock syndrome

DBP- Diastolic blood pressure

DAMPs- Damage associated molecular patterns

DN- Diabetic Nephropathy

DR- Diabetic Retinopathy

ELISA- Enzyme Linked Immunosorbent Assay

ECM – Extracellular Matrix

FBS-Fasting Blood Sugar

2% FBS- 2% Fetal Bovine Serum

GM-CSF- Granulocyte Macrophage-Colony Stimulating Factor

HbA1c- Glycosylated hemoglobin

HNE-Human Neutrophil Elastase

Hcys- Homocysteine

HBSS-Hank's Balanced Salt Solution

IL- Interleukins

ICAM-1-Intracellular Adhesion Molecule-1

LPS- Lipopolysaccharides

MPO- Myeloperoxidase

NCDs- Non-Communicable diseases

NE-Neutrophil Elastase

NETs- Neutrophil Extracellular traps

NE–$\alpha_1$-AT complex – Neutrophil elastase- $\alpha_1$-antitrypsin complex

PMNs- Polymorphonuclear Neutrophils

PE-Pancreatic elastase

PMA- Phorbol myristate acetate

PE- Preeclampsia

PBS-Phosphate Buffer Saline

ROS- Reactive Oxygen Species

RCL- Reactive center loop

RPMI-1640 medium - Roswell Park Memorial Institute-1640 medium

SLPI – Secretory Leukocyte Protease Inhibitor

Serpins – Serine Protease Inhibitors

STANA – Succinyl tri- L-alanyl-p-nitroaniline

SNPs- Single nucleotide polymorphisms

SBP- systolic blood pressure

SNAREs- Soluble-N-ethylmalemide-sensitive-factor accessory-protein receptors

TNF-α- Tumor Necrosis Factor- α T2DM-Type2 diabetes mellitus

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# Chapter I

Introduction

Infectious diseases, also known as contagious or communicable diseases, are diseases that are caused by infectious agents such as bacteria, viruses, fungi or parasites those are passed directly or indirectly, from one person to another. Infectious diseases remain a leading cause of morbidity and mortality worldwide with HIV, tuberculosis and malaria estimated to cause 10% of all deaths each year and account for nearly half of India's disease burden (1). Despite the commendable successes in control afforded by improved sanitation, immunization, and antimicrobial therapy, the infectious diseases continue to be a common and significant problem of modern medicine. There is a continuous emergence of new infectious diseases as demonstrated by the SARS epidemic in 2003, the swine flu pandemic in 2009, MERS CoV in 2013 and Zika in 2016. In the recent past, India has witnessed many outbreaks of emerging infections. Human pathogens emerge and re-emerge due to interaction of multiple complex factors between the host and the pathogen, each driven by the need to secure the success of the species in changing environments (2).

Infectious diseases comprise clinically evident illness resulting from the infection, presence and growth of pathogenic biological agents in host tissue. In all of these cases the pathogen must have the ability to recognize, become associated with, exploit the nutrient reserve and combat the defense responses of its specific hosts. To accomplish these tasks, pathogens use an extensive battery of virulence and related factors. The infectious diseases are usually characterized by the major organ system involved. Thus infections can be classified as respiratory infections, gastrointestinal infections, genitourinary infections, nervous system infections, skin and soft tissue infections, bone and joint infections, cardiovascular infections and generalized infections (3).

In contrast to infectious diseases, non-infectious diseases or non-communicable diseases (NCDs) are those that are not caused by a pathogen and are not contagious. They are of long duration and generally progress slowly. These diseases are primarily caused by four major modifiable risk factors that include unhealthy diets, physical inactivity, the harmful use of alcohol and tobacco use. For example, unhealthy diets may show up in individuals as raised blood pressure, increased blood glucose, elevated blood lipids and obesity which can lead to cardiovascular diseases and other microvascular diseases. The main types of NCDs are cardiovascular diseases (like heart attacks and stroke), cancers, chronic respiratory diseases (such as chronic obstructive pulmonary disease and asthma) and diabetes mellitus which are responsible for almost 70% of all deaths worldwide.

Inflammation is a physiological, protective immune response of the host to any injury or infection. It is one of the most important host defense mechanisms to resolve the initial injury, remove debris, initiate tissue repair and regeneration, suppress and prevent spread of an infection. It comprises a series of changes in the terminal vascular bed, in blood and in connective tissues to eliminate the offending irritant and repair the damaged tissue. However, inflammatory response sometimes results in further injury and organ dysfunction. The process of inflammation is characterized by endothelial cell activation, leukocyte recruitment and activation, vasodilation and increased vascular permeability (4, 5, 6). It involves cellular / tissue, humoral and chemical (cytokines) participants.

Neutrophils are the first white blood cells to migrate towards the infected or damaged site. They participate in the innate immune response at the early stage of inflammation and migrate to areas of inflammation within minutes. Neutrophil recruitment is initiated by changes on the surface of endothelium that result from stimulation by inflammatory mediators including histamine, cysteinyl-leukotrienes and cytokines [tumor necrosis factor- α (TNF-α), IL-1β and IL-6]. These inflammatory

mediators are released from tissue resident sentinel leukocytes when they come into contact with pathogens. Activation of endothelial cells leads to increased expression of adhesion molecules, such as selectins and integrin ligands, and presentation of chemokines upon their surfaces, which participate in the neutrophil adhesion to endothelial cells. This step is followed by transmigration and extravasation of neutrophils from circulation to sites of inflammation or infection (7, 8). Although neutrophils are major effectors of acute inflammation, several recent evidence indicate that they also contribute to chronic inflammatory conditions and adaptive immune responses (7).

Once at the site of infection, neutrophils encounter microorganisms and phagocytose them using both oxidative and non-oxidative mechanisms. After they are encapsulated in phagosomes, neutrophils mediate direct killing of the pathogens by using reactive oxygen species (ROS) generated by membrane-bound NADPH oxidase system (oxidative mechanism) or antimicrobial proteases and peptides (non-oxidative mechanism). These antimicrobial proteins are released from the neutrophil granules either into phagosomes or into the extracellular environment, thus acting on either intra- or extracellular pathogens, respectively (9, 10). Neutrophils can also eliminate extracellular microorganisms by releasing neutrophil extracellular traps (NETs). NETs are composed of decondensed chromatin to which histones, proteins (for example lactoferrin and cathepsins) and enzymes (for example myeloperoxidase and neutrophil elastase) released from neutrophil granules are attached. NETs immobilize pathogens, thus preventing them from spreading and they are also thought to directly kill pathogens by means of antimicrobial histones and proteases such as elastase (11, 12).

The non-oxidative arm of antimicrobial action is mediated by the release of several potent cytotoxic proteins and proteases. These molecules are stored in the cytoplasmic granules of neutrophils. Neutrophils carry four different kinds of granules

and each are able to release up to forty varieties of molecules (13, 14). These granules undergo differential exocytosis following activation of neutrophils upon exposure to various inflammatory mediators such as cytokines and chemokines and lead to release of variety of proteins (15, 16). Proteases secreted by the azurophil granules and particularly the human neutrophil elastase (HNE) are the most deleterious molecules, if not properly controlled they could cause severe damage to healthy tissue. The term elastase refers to a group of enzymes capable of release of soluble peptides from insoluble elastin by proteolysis (17).

HNE, a serine protease exhibits number of biological effects. In addition to its intra-and extracellular effects mediating host defense against infection, NE has been associated with non-infectious, inflammatory process regulation. It functions as a negative regulator of inflammation by degrading various pro-inflammatory cytokines such as IL-6, IL-1 and TNF-α (16, 18). This inactivation might limit further activation of neutrophils and dampen the inflammatory process (15). In vitro studies have suggested that HNE may also play a role in neutrophil adhesion. It can cleave cell-bound intercellular adhesion molecule-1 (ICAM-1), required for the adhesion of neutrophils to the endothelial surface and decreasing neutrophil adhesion and migration (19). Even though elastase has these positive attributes, this enzyme also poses a significant challenge to the body because it has been shown to have potentially pro-inflammatory effects (16, 18, 20). For example, it can enhance neutrophil migration by inducing the secretion of pro-inflammatory cytokines granulocyte macrophage-colony stimulating factor (GM-CSF), IL-6, and IL-8 from epithelial cells enhancing the inflammatory responses (18). HNE cleaves $\alpha_1$-antiprotease inhibitor, generating a fragment that is chemotactic for neutrophils (21). HNE is a proteolytic enzyme that digests virtually every type of tissue matrix proteins such as insoluble elastin, collagen, fibronectin, proteoglycan

and cadherins (16, 22). Elastase is the only protease capable of degrading the mature elastin that imparts elastic recoil to tissues. Uncontrolled elastase activity results in excessive degradation of the elastin network and thus is implicated in various infectious as well as non-infectious diseases. High concentrations of neutrophil elastase, component of NETs, can cause degradation of the wound matrix and delay healing (23).

As mentioned above, elastase could be potentially damaging when over expressed or when present in high concentrations as uncontrolled secretion can trigger off destructive processes associated with various chronic diseases. Hence, in vivo the proteolytic activity of NE, released to the extra cellular region is well regulated and controlled by a number of potent endogenous macromolecular antiproteases, such as alpha$_1$-proteinase inhibitor or alpha$_1$-antitrypsin ($\alpha_1$-PI/$\alpha_1$-AT), $\alpha_2$-macroglobulin ($\alpha_2$-MG) and secretory leukoproteinase inhibitor (SLPI). The inhibition of elastase activity by these endogenous inhibitors represents a major mechanism limiting host tissue destruction. These inhibitors rapidly and irreversibly interact with the free enzyme, resulting in formation of a stable protease inhibitor complex that is incapable of showing enzymatic activity (24, 25). The ability to evade these antiproteinases is key to the success of NE in the extracellular environment. In inflammatory states where large numbers of polymorphonuclear neutrophils are infiltered and activated, enormous amount of elastase is released. It is known that during infection neutrophils release ROS such as superoxides, $H_2O_2$ and hydroxyl radicals (25). These have been shown to inactivate proteins including the endogenous protease inhibitors. Thus, during infection or inflammation the possibility of enhanced NE activity is very high resulting in excessive proteolysis of extracellular matrix proteins causing crucial tissue damage and progress of disease. Physiological balance between elastase-antielastase factors is essential to maintain normal integrity of tissues and an imbalance has been implicated in the

pathogenesis of several acute and chronic inflammatory diseases. Thus, study of the role of elastase and its endogenous inhibitors is still an important area of investigation as both these factors play important roles in the disease progression or its prevention.

HNE was first recognized as protein-degrading enzyme but have now proven to be multifunctional protease participating in a variety of pathophysiological processes. Thus, there has been a significant interest in investigating the properties of elastase and its inhibitors in the recent years. Neutrophil proteases, particularly NE have long been therapeutic targets. Designing a specific inhibitor capable of targeting and inhibiting elastase would probably help reduce progression of inflammation. The available data from experimental and clinical studies suggest that inhibition of NE using therapeutic inhibitors would suppress or attenuate deleterious effects of inflammation including lung diseases (6). At present, a number of natural and synthetic inhibitors of elastase have been used in the treatment of HNE - related diseases (16).

$\alpha_1$-AT is the major circulating serine protease inhibitor which exhibits diverse roles in living cells. It is a potent inhibitor of multiple serine proteases with high activity against neutrophil serine proteases, neutrophil elastase and proteinase 3. In addition to its anti-protease activity, $\alpha_1$-AT expresses anti-inflammatory, antiapoptotic, immunomodulatory and antimicrobial effects (26, 27, 28). These multiple functions highlight the importance of this inhibitor in health and diseases. $\alpha_1$-AT deficiency (AATD), an inherited disease, is associated with excessive proteolytic activity and is well established in the development of pulmonary emphysema, Chronic Obstructive Pulmonary Disease (COPD), acute lung injury, cystic fibrosis and acute respiratory distress syndrome (ARDS) (29). The intravenous or intratracheal aerosol administration of recombinant or purified, plasma derived $\alpha_1$-AT is a standard therapy used for individuals with AATD and pulmonary emphysema (30). Recent studies suggest that, $\alpha_1$-

AT therapy could be beneficial in patients with type 1 and type 2 diabetes, acute myocardial infarction, stroke, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and transplant rejection (27) and is currently under evaluation in multiple clinical trials to assess its efficacy in maintaining pancreatic beta cell reserve and glycemic control in type 1 diabetes patients. Various research groups working on experimental diabetes in animal models and retinal pericyte cell cultures have demonstrated potential, protective role of $\alpha_1$-AT on retinal vasculature and it was also suggested that early use of $\alpha_1$-AT therapy may be an effective strategy to prevent or hinder the progression of diabetic retinopathy (31). $\alpha_2$-MG, a major antiprotease within the circulation is able to inactivate variety of proteinases and its increased or decreased levels are implicated in the pathogenesis of various inflammatory disease conditions such as nephrotic syndrome, type 2 diabetes, stroke, preeclampsia, atherosclerosis and cancer (32, 33, 34, 35).

In view of the profound and cardinal roles that NE and its endogenous inhibitors could play in various infectious and non-infectious diseases, they might be of use as add on biomarkers in various disease conditions which could support diagnosis, prognosis and treatment. Research in this area is likely to yield insights that will contribute to our understanding on the role of these multifunctional molecules in health and disease.

# 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).

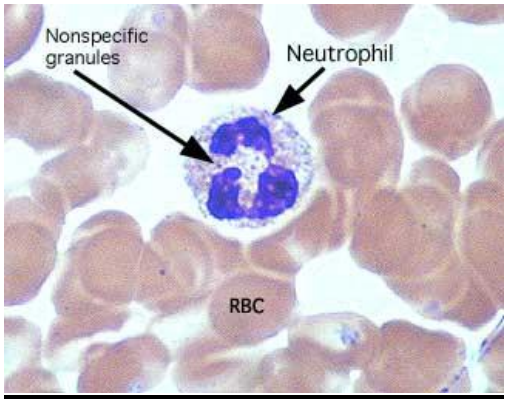


Fig 1: Structure of human polymorphonuclear neutrophil

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 in to 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 20$^{th}$ 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.

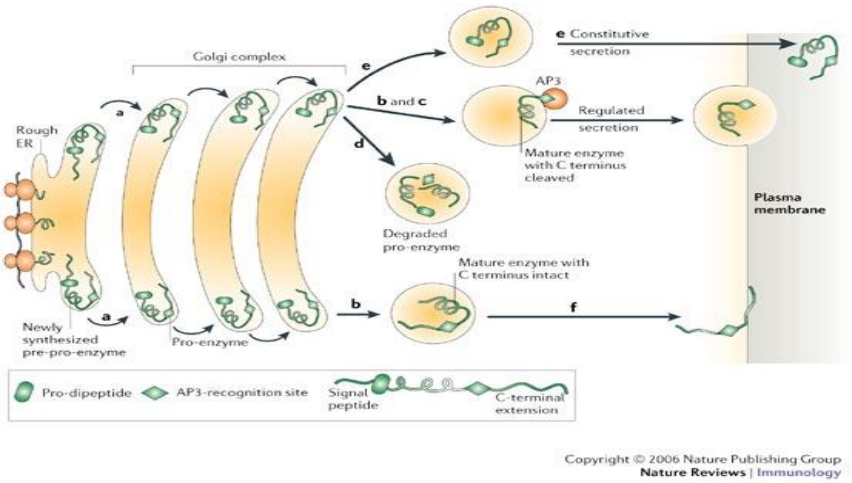


Fig 2: Newly synthesized pre-pro-enzymes are processed by a signal peptidase and shuttled from the rough endoplasmic reticulum (ER) through the Golgi complex (a). The pro-enzyme probably encounters dipeptidyl peptidase I (DPPI), which removes the pro-dipeptide, as it leaves the Golgi complex and before 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.

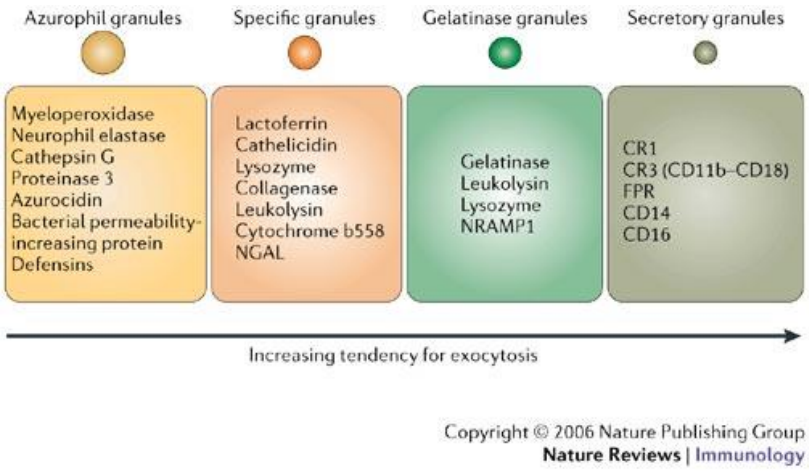


Fig.3: Neutrophil granule content. CR, Complement Receptor; FPR, Formyl Peptide Receptor; NGAL, Neutrophil gelatinase associated lipocalin; NRAMP 1, natural-resistance associated macrophage protein 1 (adopted from Pham CT. Neutrophil serine proteases: specific regulators of inflammation. Nat Rev Immunol. 2006).

## 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-ethylmalemide-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 sub-lingual 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

| **Characteristics** | **HNE** |
|---|---|
| EC number | 3.4.21.37 |
| Amino acid residues | 218 |
| Molecular mass | 29-33kDa |
| Pi | ≈ 10.5 |
| Number of glycosylation sites | 2 |
| Number of disulfide bridges | 4 |
| Optimal pH for activity | 8.0-8.5 |
| Substrate specificity | Small hydrophobic residues: Val, Cys, Ala, Met, Ile, Leu, Ser |
| Natural substrates | Elastin, cartilage proteoglycans, collagen types I,II and IV, Fibronectin |
| Endogenous inhibitors | Alpha1-antitrypsin, alpha2-macroglobulin, elafin, pre-elafin |

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).

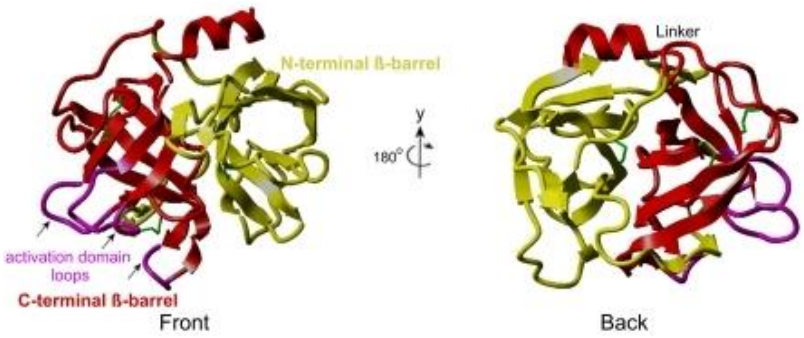


Fig.4: Three – dimensional structure of HNE. Ribbon plot of neutrophil elastase showing the two asymmetric β-barrels and the C-terminal α-helix, front view on the left, back view after a rotation of 1800 around a vertical y-axis on the right. N- and C-terminal β-barrels are represented in red and yellow 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.

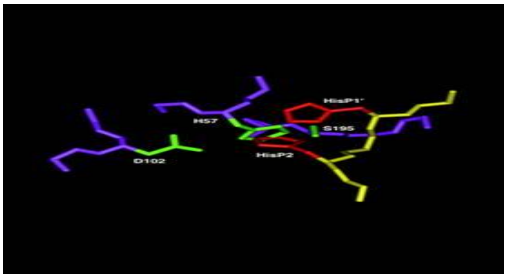


Fig. 5: Catalytic triad of HNE

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).

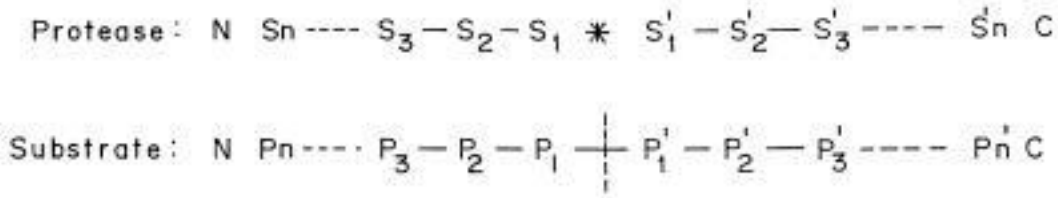


Fig.6: Active sites of proteases. The catalytic site of proteases is indicated by ∗ and the scissile bond is indicated by ┼; S1 through Sn and S1′ through Sn′ are the specificity subsites on the enzyme, while the residues they accommodate from the substrate are numbered P1 through Pn and P1′ through 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). Belaaouaj 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 $H_2O_2$ 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 $\alpha_1$-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) **Ser**ine **P**rotease **In**hibitor**s** (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 $71A^0$ 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 $\alpha_1$-Protease Inhibitor ($\alpha_1$-Antitrypsin), $\alpha_1$-Antichymotrypsin and Monocyte/Neutrophil elastase inhibitor (MNEI, also called Serpin B1) of which $\alpha_1$-antitrypsin is the principal inhibitor of HNE.

**Alpha$_1$-Protease Inhibitor ($\alpha_1$-PI)/ $\alpha_1$-Antitrypsin ($\alpha_1$-AT)**: $\alpha_1$-PI was originally named $\alpha_1$-antitrypsin ($\alpha_1$-AT) because of its ability to irreversibly bind trypsin. It is now recognized that $\alpha_1$-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).

$\alpha_1$-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 $\alpha_1$-AT is important in preventing tissue damage in the site of inflammation or injury (90). $\alpha_1$-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 $\alpha_1$-AT are poorly understood, it has been shown that human neutrophils, monocytes and alveolar macrophages can increase expression of $\alpha_1$-AT in response to inflammatory mediators, such as IL-6, bacterial LPS and in response to $\alpha_1$-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 $\alpha_1$-AT, resulting from the impaired secretion of genetic variants can function as an etiologic agent for the development of COPD (91).

## Genetics of $\alpha_1$-AT

The $\alpha_1$-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 $\alpha_1$-AT

The $\alpha_1$-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 $\alpha_1$-AT are classified by the protease inhibitor system and each variant is identified by migration on agarose gel electrophoresis. All $\alpha_1$-AT variants are categorized according to the serum level and functional activity as normal, deficient, null and dysfunctional (6, 90).

## Normal variants of $\alpha_1$-AT

More than 100 different naturally occurring genetic variants of $\alpha_1$-AT have been identified (6, 91). Normal $\alpha_1$-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 $\alpha_1$-AT ($\alpha_1$-ATD)

Several mutations associated with $\alpha_1$-ATD have been identified, and the most common are Z and S types (6, 90). The classical form of $\alpha_1$-ATD is characterized by a point mutation (Glu342  Lys) that leads to misfolding of mutant $\alpha_1$-ATZ. $\alpha_1$-ATZ accounts for almost 95% of all cases of $\alpha_1$-ATD. Z mutation slows the folding of the $\alpha_1$-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 $\alpha_1$-AT than normal variants. Since liver is the predominant site of $\alpha_1$-AT synthesis, accumulation of mutant $\alpha_1$-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 α-helices and 40 percent β-pleated sheets. $\alpha_1$-AT obeys the general structure of serpins having nine α-helices and three β-sheets with an exposed reactive center loop (RCL). Structure as depicted in Figure 7.

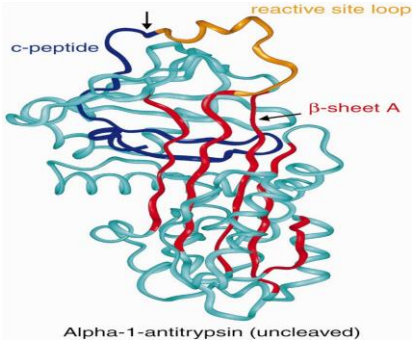


Fig. 7: Schematic picture of the native α $_1$-antitrypsin. The reactive center (yellow), β-sheet A (red) and C-terminal peptide (dark blue) are shown in relation to the rest of the structure (light blue) (adopted from Janciauskiene S. Conformational properties of serine proteinase inhibitors (serpins) 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 $\alpha_1$-AT is highly stressed external loop protruding from the molecule with Met358-Ser359 in the active center. Inhibitory process begins by docking of the $\alpha_1$-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 $\alpha_1$-AT is in metastable or "stressed form", which is essential for inhibition of proteases. During the process of inhibition, $\alpha_1$-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 $\alpha_1$-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).

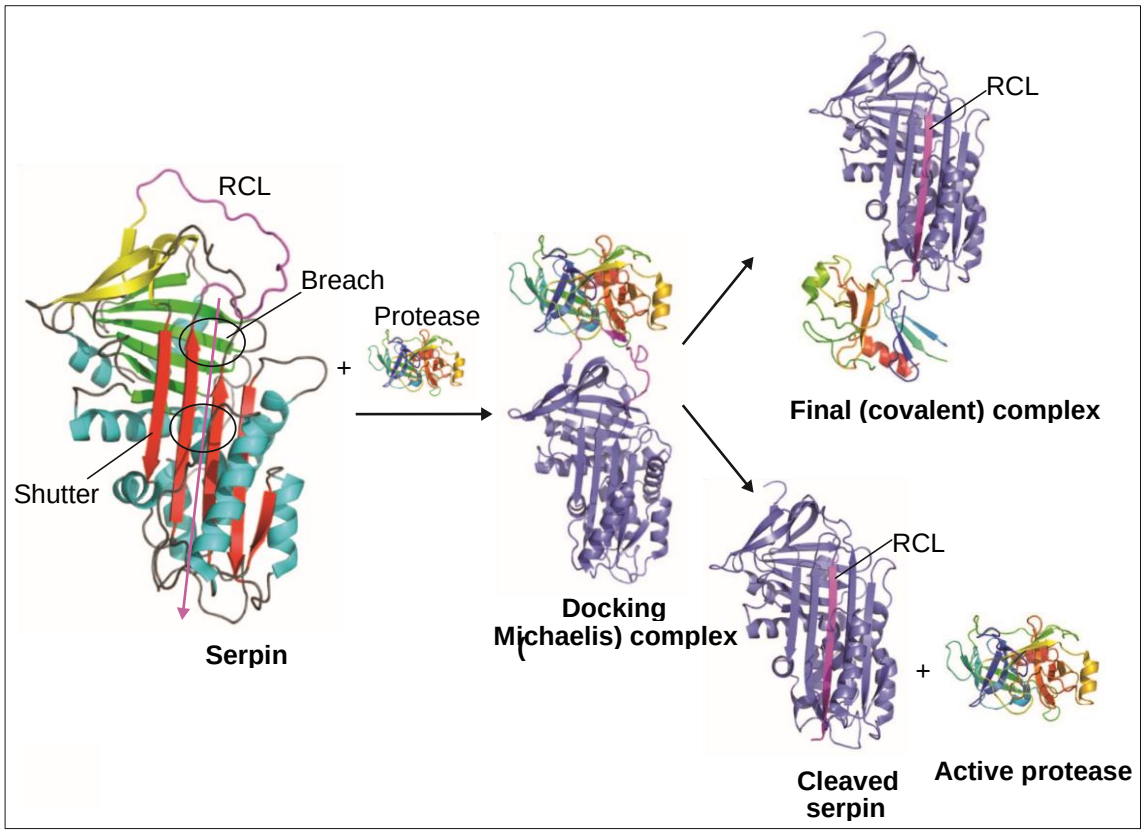


Fig. 8: The inhibitory mechanism of serpin (adopted from Ruby HP Law et al. An overview of the serpin superfamily. Genome Biology 2006).

## Biological functions of $\alpha_1$-AT

In addition to protease inhibition, recent studies have demonstrated that $\alpha_1$-AT also has anti-inflammatory, immunomodulatory, antiapoptotic and antimicrobial roles (93). $\alpha_1$-AT is an irreversible inhibitor for kallikriens 7 and 14 (94). Anti-inflammatory action of $\alpha_1$-AT is facilitated by blockade of pro-inflammatory cytokine release from human peripheral blood mononuclear cells (95). Specifically, $\alpha_1$-AT decreases the production of two typical upstream mediators of inflammation TNF-α and IL-1β. $\alpha_1$-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 $\alpha_1$-AT, while the release of anti-inflammatory mediators increases (96). $\alpha_1$-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. $\alpha_1$-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 $\alpha_1$-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 $\alpha_1$-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 $\alpha_1$-AT may play a protective role in HIV-1-infected individuals (100).

Several studies have shown that $\alpha_1$-AT inhibits the activity of caspase-3, an intra-cellular cysteine protease which plays an essential role in cell apoptosis (101, 102). Recent studies show that $\alpha_1$-AT stimulates insulin secretion and protects β-cells against

cytokine - induced apoptosis. Animal studies provide further evidence that $\alpha_1$-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 $\alpha_1$-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 $\alpha_1$-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 $\alpha_2$-MG as most informative protein biomarker in the DENV infection associated complications. In the Mass Spectrometry analysis they found increased $\alpha_2$-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 $\alpha_2$-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 $\alpha_1$-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 $\alpha_1$-AT in the involved lung was reduced (124). Braun J et al found approximately 40 times higher concentration of elastase- $\alpha_1$-AT complex in pneumonia group than in the control group (125). In a study by Umeki S et al., the plasma levels of $\alpha_2$-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 $\alpha_1$-AT as a suppressor of bacterial pneumonia and pneumonia-related pathogenesis. In $\alpha_1$-AT$^{+/+}$ transgenic mice that express human $\alpha_1$-AT in lungs, mortality due to *Pseudomonas aeruginosa* pneumonia was reduced 90% compared to non-transgenic control animals. Exogenous human $\alpha_1$-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 $\alpha_1$-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/ $\alpha_1$-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 $\alpha_1$-AT and diabetes. In support of this, a few clinical studies demonstrated that plasma $\alpha_1$-AT levels and activity were lower in diabetic patients than in non-diabetic controls (143). Invitro studies have demonstrated that over-expression of $\alpha_1$-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 $\alpha_1$-AT prolongs pancreatic islet allograft survival and exhibits cytoprotective effects (102). $\alpha_1$-AT is under evaluation for treatment of Type1 diabetes in multiple clinical trials. Initial results suggest that $\alpha_1$-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 $\alpha_1$-AT in a pediatric population with recent onset T1DM and they have observed that treatment with $\alpha_1$-AT was safe and well tolerated (144).

Human $\alpha_2$-MG, a plasma glycoprotein, traps and inhibits proteolytic enzymes which participate in inflammation and homeostasis. Significant role of $\alpha_2$-MG in immunoregulatory processes has been established. Previous studies have investigated the levels of $\alpha_2$-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 $\alpha_2$-MG, plasma $\alpha_2$-MG and blood glucose variants in type 2 diabetic patients. They found that salivary $\alpha_2$-MG statistically correlated with plasma $\alpha_2$-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 $\alpha_2$-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).

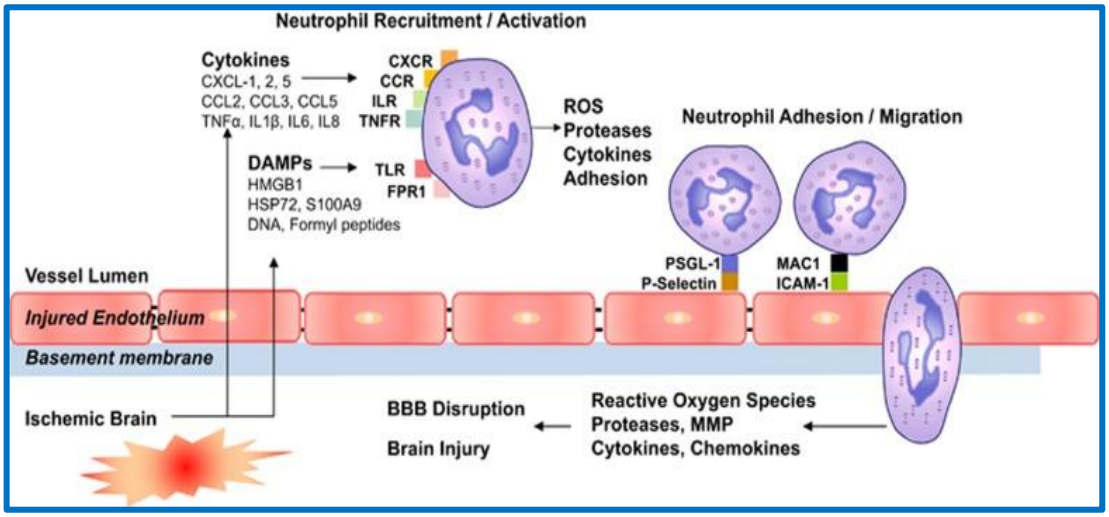


Fig 9: Neutrophils activation and adhesion in acute ischemic stroke. CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; CCR, CC-chemokine receptor; CXCR, CXC chemokine receptor; DAMPs, damage-associated molecular patterns; IL, interleukin; ILR, interleukin receptor; FPR, formyl peptide receptor; HMGB1, high mobility group box 1; HSP72, heat-shock protein 72; ICAM-1, intracellular adhesion molecule-1; MAC-1, macrophage 1 antigen; MMP-9, matrix metalloproteinase 9; PSGL-1, P-selectin glycoprotein ligand-1; TLR, toll-like receptor; TNF$\alpha$, tumor necrosis factor alpha (adopted from Jickling GC et al. Targeting neutrophils in ischemic stroke: translational insights from experimental studies. J Cereb Blood Flow Metab 2015).

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. $\alpha_1$-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 $\alpha_1$-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 $\alpha_1$-AT into rats with induced middle artery occlusion significantly reduced the infarct volume at 72 hours compared with control rats, concluding that human $\alpha_1$-AT could be a potential novel therapeutic drug for the protection against neurodegeneration following ischemic stroke (168).

$\alpha_2$-MG is a protease inhibitor that enhances procoagulant properties via the neutralization of plasmin, plasminogen activators and metalloproteinases. Recent studies have reported increased levels of $\alpha_2$-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 $\alpha_2$-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; $\alpha_1$-AT and $\alpha_2$-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). $\alpha_2$-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 $\alpha_1$-AT is associated with increased risk of various cancers such as liver cancer, bladder cancer, gall bladder cancer, malignant lymphoma and lung cancer (180). $\alpha_2$-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-$\alpha_1$-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. $\alpha_1$-AT and $\alpha_2$-MG Enzyme Linked Immunosorbent Assay (ELISA) kits were purchased from Immunology Consultants laboratory, Inc, USA and Neutrophil elastase- $\alpha_1$-antitrypsin complex (NE–$\alpha_1$-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$^{o}$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:

$$SucAla_3\text{-}pNA + H2O \xrightarrow{\text{Elastase}} SucAla_3 + pNA$$

where:

$SucAla_3$-pNA = STANA

$SucAla_3$ = N-Succinyl-trialanine

pNA = p- nitroaniline

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.

$$\text{Units/ml enzyme} = \frac{\text{Absorbance x Total volume of Assay x DF}}{\text{8.8 x volume of plasma used}}$$

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 $\alpha_2$-MG assay:

The $\alpha_2$-MG concentration was determined using two-site ELISA kit as per manufacturer's specifications. Briefly, diluted samples were added to wells coated with anti- $\alpha_2$-MG antibodies. After incubation and washing, anti-$\alpha_2$-MG antibodies conjugated with horseradish peroxidase were added each well. These enzyme-labelled antibodies form complexes with the previously bound $\alpha_2$-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 $\alpha_2$-MG in the tested samples. Absorbance was read at 450nm in an ELISA reader. The quantity of $\alpha_2$-MG in the tested sample was calculated from the standard curve constructed from the standards and corrected for sample dilution.

### 4) Plasma NE–$\alpha_1$-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-$\alpha_1$-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.

# Chapter III

## Comparative analysis of levels of elastase and its endogenous inhibitors in infectious and non-infectious diseases

## Introduction

It is evident from the foregoing review of literature that pathophysiologic differences exist in the way tissue destruction takes place in infectious and non-infectious diseases. Infectious diseases are caused by microbial pathogens while many pathogenic risk factors are involved in the pathogenesis of non-infectious diseases. Oxidative stress which develop and progress in these diseases result in the production and release of pro-inflammatory mediators such as cytokines and chemokines (113, 115, 152, 162, 186). These pro-inflammatory mediators once activated facilitate inflammatory process by recruiting neutrophils and macrophages. NE, released from neutrophils play a pivotal role in protecting the host but the uncontrolled activity of it can cause tissue damage and progress of disease (187). Several studies have been conducted to study the levels of elastase and its endogenous inhibitors in different diseases. However, most of these studies are discrete. Since a comparative evaluation of neutrophil enzyme, elastase and its endogenous inhibitors has not been done in infectious and non-infectious disease conditions, the focus of the study detailed in this chapter is to evaluate the levels of these molecules among patients diagnosed with infectious and non-infectious diseases of larger prevalence and relevance such as dengue, pneumonia, diabetes, preeclampsia and stroke to ascertain if they could be of use as differential inflammatory markers. Further, a comparative evaluation of these molecules has been done in patients diagnosed with dengue (viral infection) and pneumonia (bacterial infection) to gain insights into whether or not the levels of these molecules are differentially affected among bacterial and viral infection.

## Materials and Methods

The number of subjects in each disease condition, inclusion and exclusion criteria considered while collecting blood samples in infectious and non-infectious diseases are depicted in Table 2 & Table 3 respectively.

**Table 2:** Inclusion & exclusion criteria of infectious disease groups

| Sl.No. | Study Groups | No. of Subjects & Inclusion Criteria | Exclusion Criteria |
|---|---|---|---|
| 1 | Control | **n=52**: clinically proven healthy individuals in the age group of 30-70years. | Patients with acute and chronic infections, T2DM, chronic diseases such as cardiac diseases, malignancy, stroke, chronic obstructive lung disease, liver disorders, acute renal failures, history of smoking, pregnancy. |
| 2 | Dengue | **n=50 (DF=39; DHF=11):** Patients were recruited upon confirmation of their dengue status. Dengue was confirmed by IgM and/or IgG positive. Samples from dengue confirmed patients were obtained on day 5 to day 7 after onset of fever.<br>The age of the study group ranged from 40-55years.<br>DHF was clinically confirmed by petechiae, purpura, ecchymosis and hematoma; reduced PL count, prolonged bleeding time, PT and APTT | |
| 3 | Pneumonia | **n=50:** Clinically and radiologically confirmed cases of both community and hospital acquired pneumonia. | |

**Table 3:** Inclusion & exclusion criteria of non-infectious disease groups

| Sl.No. | Study Groups | No. of Subjects & Inclusion Criteria | Exclusion Criteria |
|---|---|---|---|
| 1 | Control | **n=60**: clinically proven healthy individuals in the age group of 30-70years whose fasting blood glucose was in the range of 90-105mg/dl. | Patients with acute and chronic infections, non-diabetic cases with retinopathy/ nephropathy, diabetic patients with chronic diseases such as cardiac diseases, malignancy, stroke, chronic obstructive lung disease, liver disorders, acute renal failures, gestational diabetes mellitus and history of smoking. |
| 2 | T2DM | **1) DM (n=60)**: clinically proven & confirmed diabetes mellitus patients without complications whose fasting blood glucose was ≥126mg/dl and 2 hours PPBS was ≥200mg/dl.<br>**2) Diabetic Nephropathy (DN) (n=60)**: Clinically proven cases of diabetic nephropathy diagnosed on the basis of persistent proteinuria (Urinary Albumin excretion Rate >300mg/24hr; confirmed by dip-stick method) by the consulting physician and abnormal renal function as represented by an abnormality in serum Creatinine.<br>3) **Diabetic Retinopathy (DR) (n=60):** Clinically proven cases of diabetic retinopathy confirmed by fundoscopy.<br>All the patients were in the age group of 30-70 years. | |
| 3 | Preeclampsia | **1) Normotensive Pregnant women (n=50):** on the basis of clinical and ultrasound evaluation and with normal course and | Patients with any infection, twins, history of pregestational diabetes, gestational diabetes |

| | | | |
|---|---|---|---|
| | | outcome of pregnancy.<br>**2) Mild Preeclamptic women (n=27)**<br>diagnosed by the presence of<br>i) hypertension ≥140mmHg SBP and ≥90mmHg DBP) on two occasions with 4-6hours apart<br>ii. proteinuria (≥1+ by urine dipstick method)<br>iii. with or without pathological edema.<br>**3) Severe Preeclamptic Pregnant women (n=23)**<br>PE was considered as severe, if the subjects had at least two of the following: ≥160mmHg systolic BP; ≥110mmHg diastolic BP; dipstick proteinuria of 3+ or more.<br>All the women were in the age group of 19-36 years and were over 20 weeks of gestation. | mellitus, renal disease, liver disease, cardiovascular disease and hypertension. |
| 4 | Stroke | **n=40**: First ever stroke patients; within 48 hours of hospital admission | Patients with recent history of myocardial infarction, diabetes mellitus, malignancy, pregnancy, renal or hepatic failure. |

## Sample collection

6ml of blood (fasting sample for FBS and in other cases non-fasting samples) was collected under complete aseptic precautions from an antecubital vein into tubes containing sodium fluoride (for FBS), EDTA (for HbA1c, hematologic studies); Sodium

Heparin (for NE, $\alpha_1$-AT, $\alpha_2$-MG and NE–$\alpha_1$-AT complex estimation). For investigations like C- reactive protein (CRP), renal function and liver function tests blood was collected in tubes without anticoagulant. The blood samples were centrifuged for 15 minutes at 3000rpm within 2hours of collection. After centrifugation, serum and plasma were separated and aliquots were stored at -70$^{o}$C until assayed. Samples were thawed at room temperature, vortexed and centrifuged before analysis.

## Assays

Basic biochemical investigations were done by standard methods using Vitros 250 Dry chemistry analyzer (Johnson & Johnson). Glycosylated hemoglobin (HbA1c) was analyzed by HPLC method using BIORAD D-10. Complete Blood Count was performed by Beckman- Coulter, an automatic blood cell counter. CRP estimation was done by rapid latex slide tests. Assay procedures for plasma elastase activity, $\alpha_1$-AT, $\alpha_2$-MG and NE–$\alpha_1$-AT complex levels are described under general materials and methods section.

## Statistical Analyses

The data were statistically analyzed by SPSS software version 22 (licensed version). The results are expressed as Mean$\pm$SD. All variables were checked for normal distribution by Shapiro-Wilk test. For statistical differences in means between groups ANOVA (Analysis of variance) with post-hoc test (for normally distributed parameters) or Kruskal-Wallis test (for not-normally distributed parameters) were used as appropriate. P value $\leq$0.05was considered statistically significant and <0.001as highly significant.

## Results

Baseline characteristics and plasma levels of elastase activity, $\alpha_1$-AT, $\alpha_2$-MG and NE- $\alpha_1$-AT complex in the infectious disease groups are presented in the Table 4 and Table 5 respectively.

**Table 4:** Basic characteristics of infectious disease groups

| Study Groups / Variables | Control (n=52) | Dengue (n=50) | Pneumonia (n=50) |
|---|---|---|---|
| Age (years) | 48.48±6.39 | 48.50±4.34 | 57.5±16 |
| Gender(male/female) | 32/20 (61.5%/38.5%) | 30/20 (60%/40%) | 22/28 (44%/56%) |
| HCT (%)* | 42.27±3.63 | 43.01± 5.22 | 35.13±5.35 |
| PL count ($10^3$/ L) | 272.23±61.24 | 71.94±39.17 | 267.64±40.04 |
| Total WBCs($10^3$/ L) | 7.04±1.02 | 4.38±1.81 | 14.25±2.75 |
| Neutrophils (%) | 51.27±7.37 | 40.68±12.37 | 82.42±6.86 |
| Lymphocytes (%) | 37.86±6.62 | 33.99±9.20 | 7.21±2.68 |
| Monocytes (%) | 5.01±1 | 14.24±6.09 | 6.64±1.73 |
| CRP (ug/ml)* | 0 | 22.92±7.6 | 28.68+5.54 |

Values are expressed as Mean±SD. *p value highly significant ($p<0.001$) for all the parameters except for HCT and CRP.

The data indicate that baseline variables reflected the clinical features.

**Table 5:** Plasma elastase activity, $\alpha_1$-AT, $\alpha_2$-MG and NE- $\alpha_1$-AT complex in the infectious disease groups

| Study Groups / Variables | Control (n=52) | Dengue (n=50) | Pneumonia (n=50) |
|---|---|---|---|
| Plasma Elastase Activity (U/ml) | 0.35±0.21 | 0.78±0.20 | 0.70±.09 |
| Plasma $\alpha_1$-AT (mg/dl)* | 123.35±25.66 | 66.25±7.75 | 116.06±63.91 |
| Plasma $\alpha_2$-MG (mg/dl) | 209.42±31.15 | 162.75±24.16 | 262.73±46.99 |
| Plasma NE- $\alpha_1$-AT complex(ng/ml) | 214.89±13.05 | 238.38±62.47 | 356.30±31.81 |

Values are expressed as Mean±SD. *p value highly significant ($p<0.001$) for all the parameters except for $\alpha_1$-AT ($p=0.394$).

The mean plasma elastase activity were significantly higher in both DENV infection (0.78±0.20) and pneumonia (0.70±0.09) patients than in healthy subjects (0.35±0.21) with p value <0.001. On the other hand, the mean $\alpha_1$-AT levels were decreased significantly in patients with dengue (66.25±7.75) compared to controls (123.35±25.66) and in patients with pneumonia (116.06±63.91). However, the comparison of levels of $\alpha_1$-AT between controls and pneumonia did not reveal any significance. The mean $\alpha_2$-MG levels were significantly decreased in patients with dengue (162.75±24.16) and increased in patients with pneumonia (262.73±46.99) when compared to controls (209.42±31.15).When plasma NE- $\alpha_1$-AT complex was compared, the pneumonia cases exhibited significant increase reflective of the increased levels of NE and $\alpha_1$-AT, while the dengue cases presented marginal increase which was not statistically significant.

When elastase activity was compared between DF and DHF patients, DHF patients had higher activity of 1.044±0.17 U/ml compared to DF group with elastase activity of 0.70±0.15 U/ml (Fig 1). However there was no significant difference in the levels of $\alpha_1$-AT, $\alpha_2$-MG and NE- $\alpha_1$-AT complex between DF and DHF groups.

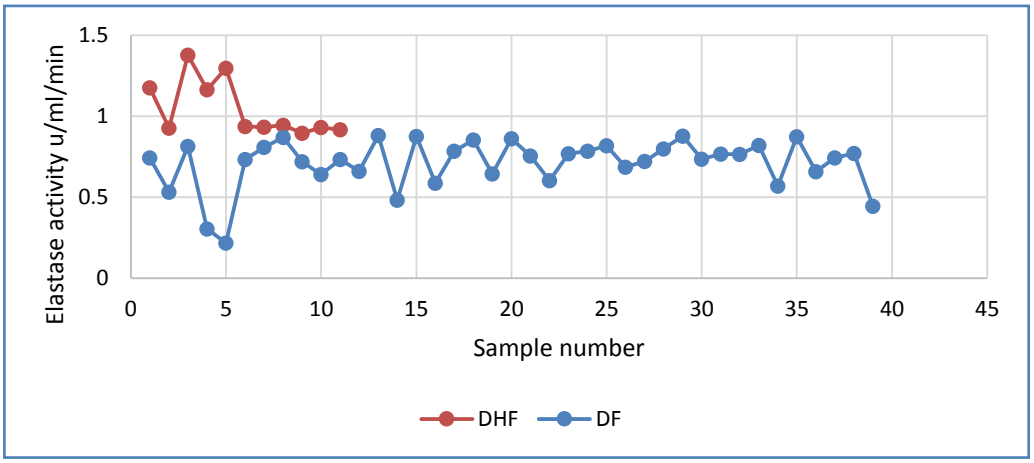


**Fig 10:** Elastase activity in DF and DHF patients

The general characteristics and baseline biochemical parameters of non-infectious disease groups are depicted in Table 6 and 7 respectively.

**Table 6:** General characteristics of non-infectious disease groups

| VARIABLES | DISEASE GROUPS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | T2DM | | | PE | | | |
| | Control (n=60) | DM (n=60) | DN (n=60) | DR (n=60) | Normotensive Pregnant (n=50) | Mild PE (n=27) | Severe PE (n=23) | **STROKE** (n=40) |
| Age (years) | 50.47±7.99 | 54.22±9.51 | 58.82±6.98 | 56.40±9.00 | - | - | - | 65.60±9.36 |
| Gender (male/ female) | 35/25 | 38/22 | 39/20 | 36/24 | - | - | - | 32/8 |
| Duration of diabetes (years) | - | 5.10±2.14 | 13.10±4.40 | 9.95±3.83 | - | - | - | - |
| Maternal age (years) | - | - | - | - | 23.62±2.98 | 25.11±4.20 | 25.70±4.35 | - |
| Gestational age (weeks) | - | - | - | - | 37.18±3.05 | 34.70±3.37 | 34.96±3.11 | - |
| Blood Pressure (mmHg)<br>Systolic<br>Diastolic | - | - | - | - | 120.28±8.70<br>80.20±5.88 | 147.11±9.35<br>100.22±8.84 | 170.87±14.11<br>106.52±11.12 | 200.5±36.08<br>114.0±14.82 |
| Cases with proteinuria (n (%)<br>• Traces<br>• 1+<br>• 2+<br>• 3+ | - | - | - | - | - | 4 (14.8)<br>16 (59.3)<br>7 (25.9)<br>- | 1(4.3)<br>3(13.0)<br>5(21.7)<br>14(60.9) | - |

Values are expressed as Mean±SD; DM=Diabetes Mellitus; DN=Diabetes Nephropathy; DR=Diabetes Retinopathy; PE=Preeclampsia.

**Table 7:** Baseline biochemical characteristics of non-infectious disease groups

| VARIABLES | DISEASE GROUPS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control (n=60) | T2DM | | | PE | | | STROKE (n=40) |
| | | DM (n=60) | DN (n=60) | DR (n=60) | Normotensive Pregnant (n=50) | Mild PE (n=27) | Severe PE (n=23) | |
| FBS(mg/dl) | 91.33 ± 10.47 | 165.18 ± 69.72 | 191.80 ± 71.92 | 186.43 ± 71.82 | - | - | - | - |
| PPBS(mg/dl) | - | 224.48 ± 84.81 | 272.40 ± 83.99 | 285.17 ± 86.91 | - | - | - | - |
| HbA1c (%) | - | 8.78 ± 2.20 | 9.80 ± 2.92 | 10.02 ± 2.31 | - | - | - | - |
| Blood Urea(mg/dl) | 22.02±7.80 | 26.57±7.56 | 59.62±16.46 | 30.97±14.13 | - | - | - | - |
| Serum Creatinine (mg/dl) | 0.93±0.25 | 1.02±0.12 | 2.25±0.50 | 1.22±0.85 | - | - | - | - |
| Serum uric acid (mg/dl) | - | - | - | - | 4.53±1.30 | 7.53±1.35 | 8.16±1.57 | - |
| Serum CRP (ug/ml) | 0 | 17.90±6.68 | 43.40±8.72 | 23.80±4.68 | 0 | 12.22±2.2 | 14.35±2.92 | 25.35±5.62 |

Values are expressed as Mean±SD; DM=Diabetes Mellitus; DN=Diabetes Nephropathy; DR=Diabetes Retinopathy; PE=Preeclampsia.

Plasma levels of elastase activity, $\alpha_1$-AT, $\alpha_2$-MG and NE- $\alpha_1$-AT complex in non-infectious disease groups are depicted in Table 8.

**Table 8:** Plasma levels of elastase activity, $\alpha_1$-AT, $\alpha_2$-MG and NE- $\alpha_1$-AT complex in non-infectious disease groups

| VARIABLES | DISEASE GROUPS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control (n=60) | T2DM | | | PE | | | STROKE (n=40) |
| | | DM (n=60) | DN (n=60) | DR (n=60) | Normotensive Pregnant (n=50) | Mild PE (n=27) | Severe PE (n=23) | |
| Plasma Elastase Activity (U/ml) | 0.35±0.20 | 0.73±0.31 | 0.87±0.35 | 0.76±0.41 | 0.35±0.10 | 0.37±0.03 | 0.62±0.08 | 0.51±0.1 |
| Plasma $\alpha_1$-AT (mg/dl) | 122.95±25.71 | 41.13±14.06 | 26.26±6.16 | 8.77±2.85 | 110.26±42.39 | 83.94±25.08 | 68.58±26.39 | 87.63±27.69 |
| Plasma $\alpha_2$-MG (mg/dl) | 208.87±31.16 | 167.29±30.45 | 144.66±13.72 | 104.67±11.47 | 265.37±66.91 | 201.06±38.23 | 298.79±32.52 | 247.33±63.41 |
| Plasma NE- $\alpha_1$-AT complex(ng/ml) | 215.83±13.61 | 98.85±23.85 | 129.26±20.40 | 153.25±17.11 | 171.08±23.81 | 176.19±9.27 | 164.31±11.63 | 346.2±57.89 |

Values are expressed as Mean±SD; p value highly significant (p=0.001) across all the parameters for all the groups; DM=Diabetes Mellitus; DN=Diabetes Nephropathy; DR=Diabetes Retinopathy; PE=Preeclampsia.

The analysis of the values in non-infectious disease groups indicated that there was a significant increase in the levels of elastase activity in all the three disease groups compared to controls (0.35±0.20). The mean values were as follows for diabetic group with or without complications: DM (0.73±0.31), DN (0.87±0.35) and DR (0.76±0.41). The activity was increased two fold in severe preeclampsia (0.62±0.08) in comparison to normal (0.35±0.10) and mild preeclamptic subjects (0.37±0.03). Stroke patients also showed increased elastase activity (0.51±0.1).

The results on the levels of $\alpha_1$-AT showed decrease in all the disease conditions compared to controls (122.95±25.71). The level was lowered the most in DR (8.77±2.85) than in the DN group (26.26±6.16) in comparison with diabetics without complication (41.13±14.06). The reduction in $\alpha_1$-AT was 60% in severe while it was 40% in mild PE. Significantly reduced $\alpha_1$-AT levels with mean value of 87.63±27.69 was observed in stroke patients.

The values for $\alpha_2$-MG presented significant decrease in diabetes groups with (DN 144.66±13.72; DR 104.67±11.47) or without complications (DM 167.29±30.45) compared to normal (208.87±31.16). While the levels were significantly elevated in severe preeclamptic women (298.79±32.52) and stroke patients (247.33±63.41).

Plasma NE-$\alpha_1$-AT complex concentration was significantly lower in the diabetic group with complications (DN 129.26±20.40; DR 153.25±17.11) and without complications (DM 98.85±23.85) as compared to control group (215.83±13.61) as reflective of lowered $\alpha_1$-AT. However, the complex estimation did not evince any significant changes in PE group. Levels were significantly increased in stroke patients (346.2±57.89).

Overall, the analysis of the results of these molecules between infectious and non-infectious disease groups demonstrated significant increase in the levels of elastase activity in both the disease groups. While the levels of $\alpha_1$-AT were significantly

decreased in both these distinct disease groups. However, the values for $\alpha_2$-MG and complex showed differential picture.

## Discussion

There are many studies assessing the levels of neutrophil elastase, $\alpha_1$-AT and $\alpha_2$-MG in infectious diseases such as dengue (115), pneumonia (124, 186), malaria (127), HIV infection (128) as well as in non-infectious diseases like diabetes Mellitus (142), preeclampsia (152), stroke (167, 188), cancer (176), atherosclerosis (173) etc. These studies have shown altered levels of these molecules as part of pathophysiological processes either in the development or progression of diseases. All these studies have been focusing on one disease at a time and therefore the alterations observed were interpreted for the possible roles of elastase, $\alpha_1$-AT and $\alpha_2$-MG in specific disease condition. Several studies have shown that inflammatory processes are the primary response to altered homeostasis or infection (7, 8, 10). Therefore, in the current study meticulous comparative analyses have been carried out in order to evaluate the usefulness of the measurement of these molecules as specific markers or add on markers in conjunction with the primary causative agent.

Since neutrophils are the first cells to respond to inflammation, it releases several proteases, particularly NE which has the ability to degrade extracellular matrix proteins (15). In biological systems they are well regulated and controlled by a number of endogenous protease inhibitors such as $\alpha_1$-AT and $\alpha_2$-MG and adequate levels of these are shown to have some effects in mitigating the severity of inflammatory processes. Many of the protease inhibitors interact with the target proteases by contact with the active site, resulting in formation of a stable protease inhibitor complex that is incapable of showing enzymatic activity (24, 25).

One of the most important observations in this comparative study was elevated levels of elastase activity in all the infectious and non-infectious diseases as compared to controls. This could be attributed to enhanced neutrophil activation and degranulation contributing to progressive inflammation. This observation is in consensus with the available data (115, 124, 141, 142, 152, 167, 187, 188).

Since all disease conditions exhibited elevated levels of NE, attempt was made to compare the levels of this enzyme with severity of the disease conditions and it was observed that the levels of NE was elevated in DHF compared to DF. This observation is of relevance with regard to hemorrhagic manifestation in DHF as elastase is known to disrupt the vascular tissues. There are reports on increased levels of NE in patients of DM, PE and stroke (141, 142, 152, 167, 188). In the current study, the elevated levels of NE activity observed in patients suffering from severe PE was significantly higher in comparison to mild PE or normotensive pregnant women. Thus, the measurement of NE in these disease conditions makes it relevant to assess the severity and potential damage that could happen during the progression of dengue fever and pregnancy associated with preeclampsia.

$\alpha_1$-Antitrypsin is the major circulating serine protease inhibitor (serpin) and a potent inhibitor of multiple serine proteases with high activity against neutrophil serine proteases, neutrophil elastase and proteinase 3 (189). An adequate activity of this inhibitor is critical for the maintenance of protease –antiprotease homeostasis and prevention of proteolytic tissue damage (24). Contrary to expectation of an increased level of $\alpha_1$-AT in the patient groups in response to elevated elastase activity as natural defense mechanism, the levels of $\alpha_1$-AT were decreased significantly in all the diseases studied.

Though there was generalized decrease in the levels of $\alpha_1$-AT in disease conditions, there was significant decrease in the levels of $\alpha_1$-AT in diabetic patients compared to preeclamptic women and stroke patients. On comparison of the levels of $\alpha_1$-AT among diabetic patients, DR group patients exhibited significant decrease in the levels of $\alpha_1$-AT compared to DN or diabetes without complications. The decrease in the level was to the extent of 15 fold and 5 fold in retinopathy and nephropathy patients respectively compared to normal subjects. However, in diabetic patients without complications the fold decrease in the levels of $\alpha_1$-AT was to the extent of only 3 fold in comparison to normal. Thus the results of this study strongly indicate a significant attenuation of $\alpha_1$-AT levels in the patients suffering from diabetic retinopathy.

It is well known that the patients suffering from infections, diabetes, PE or stroke exhibit an increase in oxidative stress and generate free radicals (190, 191, 192, 193). These free radicals have been shown to destroy $\alpha_1$-AT. Accordingly the decrease in the level of this inhibitor observed in this study could be attributed to destruction of this molecule by the free radicals. Though in the current study an analysis of ROS had not been done, the data available in the literature is reflective of explanation cited above. However, the marked decrease in $\alpha_1$-AT levels in diabetic retinopathy patients than other diabetic patients raised doubts on the possibility of destruction of $\alpha_1$-AT by free radicals alone as hyperglycemia and increased oxidative stress are common features in diabetes. This observation paved way to consider other molecular mechanisms for the decreased levels of $\alpha_1$-AT in retinopathy. The possibilities could include decreased expression or mutation in the $\alpha_1$-AT gene resulting in decreased synthesis or defective/truncated $\alpha_1$-AT formation in diabetes retinopathy patients. The data indicate that $\alpha_1$-AT could be a most critical molecule and might serve as a molecule of interest to explore in order to get insight into the molecular mechanism of the vascular changes observed in disease groups particularly DR. Moreover, the role of $\alpha_1$-AT in development of DR has been observed

recently in experimental and clinical studies (31) which substantiates that $\alpha_1$-AT levels are important in DR.

Alpha$_1$-proteinase inhibitor binds with elastase at a 1:1 molar ratio and forms an 80KD NE- $\alpha_1$-AT complex. The formation of complex reflects a rapid response of the organism to infection/inflammation (194). The complex formation between NE and $\alpha_1$-AT was increased in pneumonia which was reflective of the increased NE and near normal levels of $\alpha_1$-AT. There was no significant increase in the levels of NE- $\alpha_1$-AT complex in dengue patients as expected on account of the decreased levels of $\alpha_1$-AT. Thus it is evident that the decreased $\alpha_1$-AT observed in dengue is distinct and reflective. However, this difference observed is attributable and characteristic of a viral infection needed to be studied by extrapolating the study in other viral diseases and levels of $\alpha_1$-AT. Similarly increased levels of NE- $\alpha_1$-AT complex in pneumonia could be a characteristic feature in bacterial infections which has to be explored by extending the studies in bacterial infections.

There was no increase in the levels of NE-$\alpha_1$-AT complex in diabetic and preeclamptic women consequent to the increased elastase activity. The data was of no relevance, suggesting that the measurement of complex is of not much significance in the assessment of complications of diabetes or PE. Differing from this, stroke patients had a significantly increased complex levels. This observation is in agreement with a study conducted by Grau et al who reported higher levels of elastase inhibitor complex in subjects with risk factors compared to healthy controls without risk factors (166). These findings further support the actual concept that plasma elastase reflects the chronic activation of neutrophils by vascular risk factors such as hyperglycemia and dyslipidemia (141, 142).

With regard to the levels of $\alpha_2$-MG, the patients suffering from pneumonia had higher levels compared to patients suffering from dengue and controls. Similarly, severe

PE and stroke patients had higher levels of $\alpha_2$-MG in comparison to diabetes group and controls. These findings observed in pneumonia, severe PE and stroke are in accordance with the previous studies conducted (34, 126, 158, 169). $\alpha_2$-MG is a broad spectrum protease inhibitor that is known to neutralize α-thrombin, plasmin, and activated protein C, which suggests that it has anticoagulant as well as procoagulant properties (34, 169). The procoagulant properties of elevated $\alpha_2$-MG levels independently contribute to the intravascular coagulation adding to further severe complications of pneumonia, severe PE and stroke (34, 126, 158, 169). $\alpha_2$-MG forms a complex with elastase and is cleared rapidly from the plasma by macrophages (32). Therefore, the decrease observed in this study could be attributed to the binding of $\alpha_2$-MG to elevated elastase and getting rapidly cleared from the plasma by macrophages.

## Conclusion

This study further reiterates elevated neutrophil elastase activities in both infectious and non-infectious disease conditions in conformity with characteristics of inflammatory processes. However, the study establishes that the levels of this enzyme are associated with severity of the diseases particularly in cases of DF and PE. These findings suggest the destructive role of NE in diseases and their complications thus making NE a potential target molecule that could be regulated through therapeutic interventions to prevent the disease development and complications.

Decreased levels of $\alpha_1$-AT observed in all the disease groups clearly indicate possible role of this molecule in the development of diseases and their complications. Reduction in the levels of $\alpha_1$-AT in viral infection in comparison to bacterial infection is an observation of relevance and studies have to be extrapolated to other viral infections to confirm the finding whether it is a unique feature for viral infections or otherwise. The other significant observation is the increased $\alpha_2$-MG in bacterial infection which needed

to be validated further with other bacterial infections. Thus a decreased $\alpha_1$-AT in viral infection and increased $\alpha_2$-MG in bacterial infection could be of differential diagnostic significance.

The observation of significant decrease in $\alpha_1$-AT levels in diabetic retinopathy patients opens up a plethora of questions on the underlying reason for the decrease. It is known that chronic diabetes leads to microvascular diseases. However, there are no predictive diagnostic means on the nature of the complications that diabetic patients are predisposed to. The results of this study are promising and implicate that $\alpha_1$-AT could have a predictive role in indicating the possibility of developing diabetic retinopathy. The study also suggests the possible involvement of $\alpha_2$-MG in the development of complications of preeclampsia and stroke for its implied role in coagulation.

# Chapter IV

## Correlation of diagnostic parameters and risk factors of infectious and non-infectious diseases with the levels of elastase and its inhibitors

## Introduction

Efficient and accurate diagnosis of diseases is of primary importance for clinical care, surveillance activities and outbreak control. Early laboratory confirmation and clinical diagnosis are important because some patients progress in a short period from mild disease to severe and at times to death. Therefore early diagnosis and early intervention can be sometimes life-saving. Diagnosis of a disease based only on clinical symptoms could be at times unreliable, as many diseases share common clinical symptoms (195, 196).

Clinical manifestations such as fever, respiratory symptoms, and chest x-rays are usually recommended for the diagnosis of pneumonia (197). On the other hand, viral culture, nucleic acid amplification, and serological assays have been used for the diagnosis of dengue (198). These physical and routine laboratory examinations are generally sufficient to identify diseases. However, there are no means for early prediction on the severity of these diseases as these diseases are known to turn severe without any warning signs leading to complications.

The diagnosis of diabetes mellitus depends solely on the demonstration of hyperglycemia. The recommended diagnostic criteria for diabetes mellitus are: classic symptoms of diabetes and random plasma glucose concentration $\geq$200mg/dl or FBS $\geq$126mg/dl and 2-h post load plasma glucose concentration $\geq$200mg/dl during the OGCT or HbA1C $\geq$6.5% (199). Symptoms of hyperglycemia may be relatively late development in the course of T2 diabetes delaying the diagnosis and thus approximately 30% of patients are presented with complications of diabetes such as retinopathy, nephropathy and neuromuscular disease at the clinical diagnosis of type 2 diabetes (200). Prolonged hyperglycemia has been shown to result in chronic inflammatory state ultimately leading

to multi organ dysfunction. Two predominant complications of diabetes are retinopathy and nephropathy as consequences of uncontrolled hyperglycemia. Studies have indicated that not all patients develop multiple complications but invariably develop one of the complications on account of prolonged hyperglycemic state. The assessment of renal function is used in the diagnosis of nephropathy and its severity. However there are no specific biomarkers for diabetic retinopathy which is diagnosed exclusively by fundoscopy (131).

Despite considerable research, identification of patients with severe form of PE continues to challenge clinicians. Progression from mild to severe on the disease spectrum may be gradual or rapid. Severity of PE is classified based on substantially increased BP and proteinuria (201). However, recent research have shown that waiting for proteinuria to present can result in delayed intervention or missed diagnosis, as not all women with PE will develop proteinuria. Hence, an early measurable indicator of the severity of PE is desirable. PE, characterized by enhanced inflammatory response is accompanied by increasing concentrations of pro-inflammatory cytokines, acute phase proteins and leukocyte activation (151). Studies have indicated elevated elastase levels in preeclamptic women (154).

Current diagnosis of stroke relies on clinical examination and is supplemented further with various neuroimaging techniques. However, interpretation of brain imaging appearances can be difficult, as computerized tomography (CT) is often normal after the onset of ischemia and may remain normal in patients with mild ischemic strokes. Achieving an accurate diagnosis quickly in patients with acute stroke is extremely important for prompt initiation of treatment. Thus, disease specific molecules to support clinical diagnosis, to identify patients at risk of disease and guide treatment and prognosis would be valuable to improve care of patients.

It is well known that simple laboratory investigations such as platelet, WBC, neutrophil counts, FBS, HbA1c, renal function parameters, BP, among others would aid in diagnosis of above mentioned diseases. However, a specific biological marker for the diagnosis of disease and assess treatment outcome is therefore vital and it might be appropriate to have some adjunct parameters reflective of diagnosis, progression, and severity of diseases for differential diagnosis. Since disease conditions considered in this study mimic inflammatory pattern, a systematic measurements of elastase and its endogenous inhibitors were relevant. Therefore, a correlative study was planned on the association of these molecules with basic diagnostic parameters of diseases under study to ascertain any association and to utilize the results for diagnosis, treatment and prognosis.

## Statistical Analysis

Pearson's correlation coefficient was used to analyze the correlation between continuous variables. P value $\leq$0.05was considered statistically significant and <0.001as highly significant.

## Results

Correlation studies were carried out with parameters which were significantly increased or decreased in comparison to the normal values.

In pneumonia, the base parameter neutrophil counts correlation with NE and $\alpha_2$-MG presented positive associations (Fig 11).

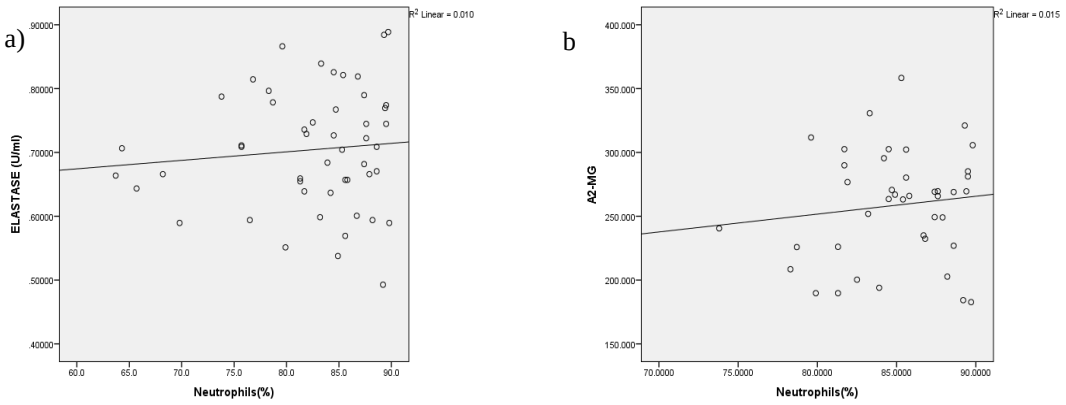


Fig 11: Linear correlation plots of neutrophil count with NE and $\alpha_2$-MG in pneumonia. Pearson's coefficient and p values are as follows: a) r = 0.098, p=0.500; b) r= 0.121, p=0.449.

Correlation of platelet counts as base parameter with elastase and $\alpha_1$-AT in dengue patients revealed that elastase associated negatively and $\alpha_1$-AT correlated positively. While neutrophil counts with elastase correlated negatively (Fig 12).

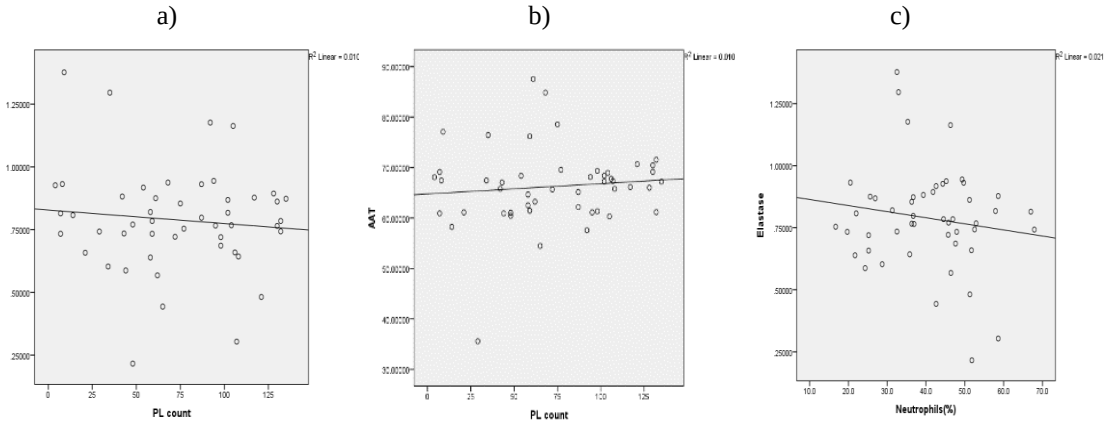


Fig 12: Linear correlation plots of a) platelet count with NE: r = -0.100, p=0.493; b) platelet count with $\alpha_1$-AT (AAT): r=0.102, p=0.486. c) neutrophil count with NE: r=-0.146, p=0.315 in dengue.

Among non-infectious diseases, association studies of FBS with $\alpha_1$-AT in DN and DR indicated negative association. However the association was strongly negative with $\alpha_1$-AT in patients' suffering from retinopathy (Fig 13).

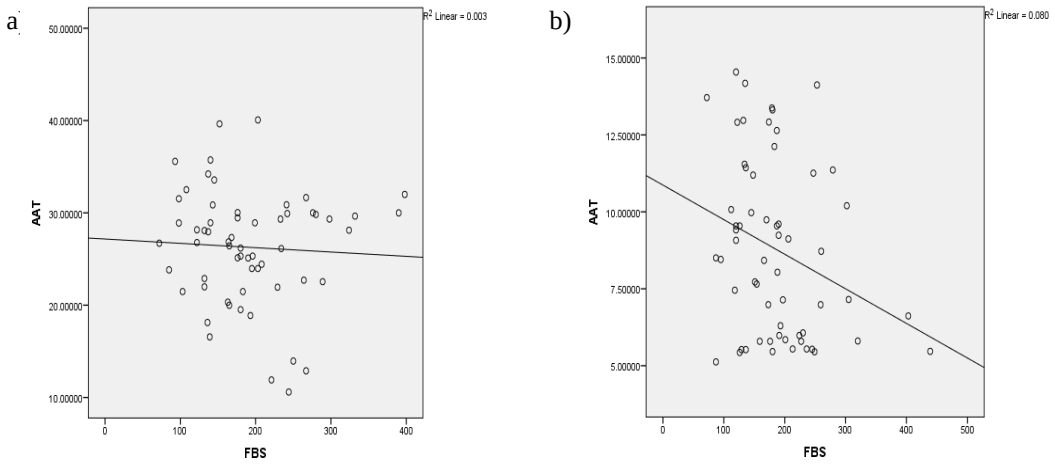


Fig 13: Linear correlation plots of FBS with $\alpha_1$-AT: a) in DN r = - 0.055, p=0.678; b) in DR r= -0.282*, p=0.029 (*correlation significant at p<0.05).

When the levels of NE and its endogenous inhibitors were correlated with the severity parameter proteinuria of PE, a strong positive correlation was obtained with NE and $\alpha_2$-MG. On the other hand, a strong negative association was indicated with $\alpha_1$-AT (Fig 14).

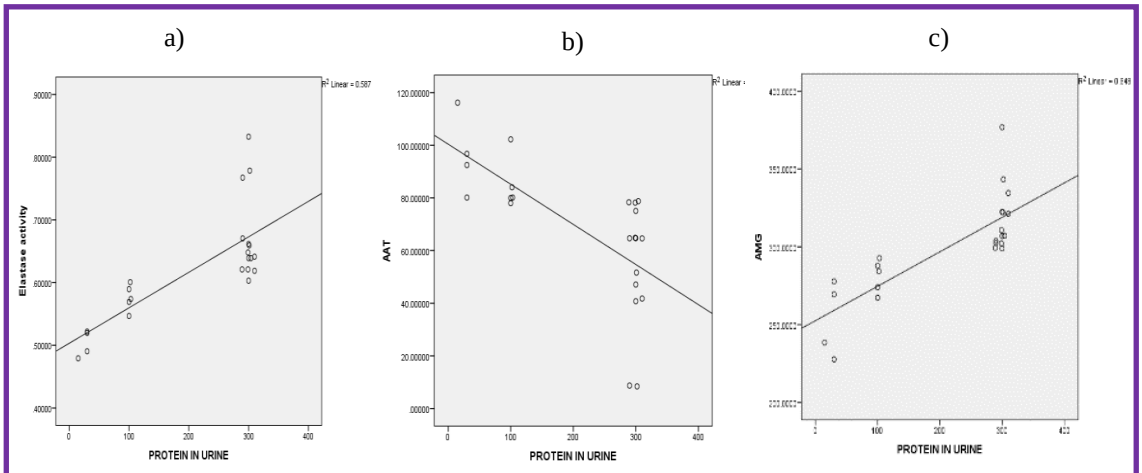


Fig 14: Linear correlation plots of proteinuria with elastase, $\alpha_1$-AT, $\alpha_2$-MG in severe PE patients: a) r = 0.766**, p=0.000; b) r= - 0.682**, p=0.000; c) r=0.805**, p=0.000 (**correlation highly significant at p<0.01).

Correlation analysis of SBP in stroke patients indicated a positive correlation with elastase and $\alpha_2$-MG and negative association with $\alpha_1$-AT (Fig 15).

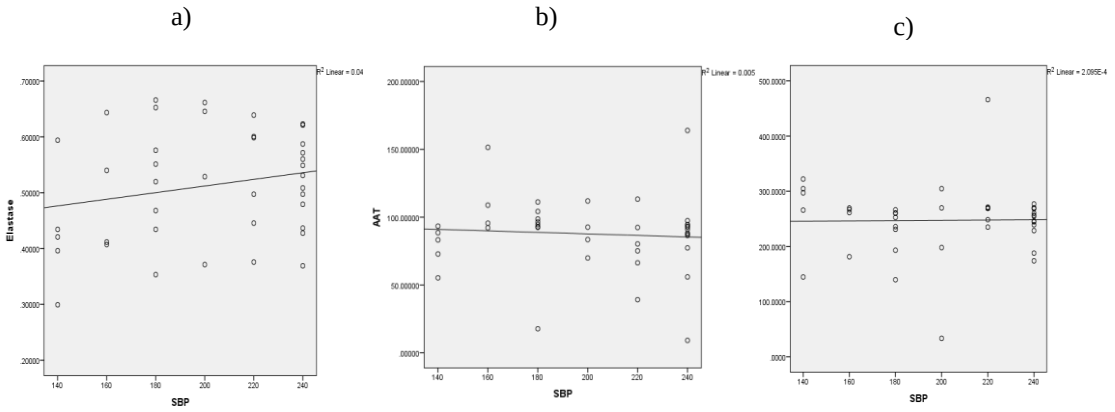


Fig 15: Linear correlation plots of SBP with elastase, $\alpha_1$-AT, $\alpha_2$-MG in stroke patients: a) r = 0.215, p=0.183; b) r= -0.073, p=0.656; c) r=0.014, p=0.929.

## Discussion

Correlation or association studies are conducted in many disease conditions to evolve newer diagnostic and therapeutic strategies. Since all the disease conditions studied had significantly altered levels of NE and its endogenous inhibitors, attempt was made to correlate levels of these molecules with the basic diagnostic and risk parameters of diseases under study to propose add on biomarkers which would aid in diagnosis, treatment and prognosis.

Pneumonia is infection of the lungs that can cause mild to severe illness. The difference of clinical course is associated with the virulence of etiologic agents and/or the host immune status. The circulating immune cells including neutrophils, lymphocytes, and monocytes could be involved in the pathogenesis of pneumonia. The pathogenesis of pneumonia in each etiologic agent may be different; in general, patients with typical bacterial pneumonia manifest more toxic clinical symptoms with leukocytosis,

neutrophilia and bacteremia (202). In pneumonia lesions, mainly activated neutrophils and mononuclear phagocytes are predominantly observed, and mediators such as proteolytic enzymes, oxygen radicals, and cytokines from these cells could be associated with host lung injury (203). Significant increases in plasma NE, IL-6 and IL-8 have been confirmed at infectious sites of pneumonia and IL-8 positively correlated with the number of neutrophils or NE (124). In a study on association of plasma NE with other inflammatory mediators and clinical features it has been shown that NE appears to play a critical role in severe pneumonia and determination of its concentration in blood could be a useful indicator of severity (187). In this study a significant elevation in elastase and $\alpha_2$-MG were observed and these parameters were correlated with neutrophil count. A positive correlation of neutrophil counts with elastase activity and $\alpha_2$-MG was observed. These observations could be suggestive of some significance as adjunct parameters for diagnosis of pneumonia.

Dengue virus infection symptoms vary from mild to severe; severe forms include DHF and DSS. Severe forms are characterized by hemodynamic disturbances, increased vascular permeability, hypovolemia, hypotension, and shock. Thrombocytopenia and platelet dysfunction are common features and are related to the clinical outcome (111, 204). Physiologically, platelets are involved in hemostasis, wound healing, and inflammation (111). Activated platelets release many cytokines and chemokines which in turn are involved in induction of immune cells such as neutrophils and monocytes to the site of injury (205). DENV infection activates platelets and induce their consumption due to ongoing disseminated intravascular coagulation, platelet destruction due to increased apoptosis and suppression of bone marrow causing platelets dysfunction and thrombocytopenia (116, 206, 207). Neutropenia observed in dengue patients here could be attributed to activated platelet-neutrophil aggregation followed by its destruction which is reflected in the elevated elastase levels observed in the study. There was also a

significant decrease in the levels of $\alpha_1$-AT in dengue patients. Correlation studies indicated negative correlation of platelets and neutrophils with elastase signifying the protective role of platelets and neutrophils against dengue pathogen and destructive effect of elastase leading to further complications. Juffrie et al also found negative correlation between platelet count and elastase (115). Incidentally, a significant decrease in the neutrophil counts was observed in DHF patients compared to DF patients. Neutropenia though not a characteristic feature in DHF patients needed to be evaluated with a larger sample size study as well as multicentric study. Thus, significantly elevated elastase activity in DHF patients with reduced neutrophil count implies that NE can be a relevant marker for severity in DENV infection. A positive association between platelet counts and $\alpha_1$-AT also indicates the destruction of $\alpha_1$-AT in DENV infection. Monitoring the plasma levels of these molecules thus could be of use for evaluation of severity of dengue infection.

One unique finding in this study is the differential levels of $\alpha_1$-AT and $\alpha_2$-MG in pneumonia and dengue. The levels of $\alpha_2$-MG increased in pneumonia with near normal $\alpha_1$-AT levels while the picture was reverse with $\alpha_1$-AT decrease with almost normal $\alpha_2$-MG in dengue. Are these observations presented in this study characteristic to bacterial infection (pneumonia) and viral infection (dengue) needed to be explored. If such consistencies are observed in different bacterial and viral infections, it would help in differential diagnosis of bacterial and viral diseases.

Diabetes is a chronic disease and is associated with multiple complications. The overall health condition of the diabetic patient largely depend upon the status of hyperglycemia, environmental factors and in some cases due to genetic factors. Results of first chapter showed that, there was a noticeable reduction in levels of $\alpha_1$-AT in diabetic complications, particularly in retinopathy patients and so correlation of this parameter

with FBS was made in DR and DN patients. A negative correlation was observed in both the groups. However, a significant negative correlation was observed in DR patients as compared to DN patients suggesting that $\alpha_1$-AT levels in diabetes could be a predictive marker molecule for retinopathy. Recent experimental and clinical studies have shown protective effect of $\alpha_1$-AT in diabetic retinopathy (31). The possible explanation for decrease in $\alpha_1$-AT in retinopathy have been discussed in chapter III.

Preeclampsia is a progressive inflammatory disease characterized by elevation in levels of inflammatory mediators in maternal circulation (151). There are studies indicating significant increase in the activity of elastase in PE (154). However, a correlation of severity parameters with inflammatory parameters have not been reported. Though correlation of these molecules with both proteinuria and BP was carried out in this study, the most meaningful and relevant correlation was observed only with proteinuria and not with BP. The observation of significant increase in the levels of NE and $\alpha_2$-MG in severe PE and their significant positive correlation with severity marker proteinuria makes the measurement of NE and $\alpha_2$-MG as dependable parameters in the determination of severity of PE. The correlation was converse in the case of $\alpha_1$-AT. Reduced levels of $\alpha_1$-AT in PE group could be due to renal loss of this protein. Urinary estimation of $\alpha_1$-AT would have explained this. The correlation analyses suggests that measurement of levels of NE and $\alpha_2$-MG along with $\alpha_1$-AT could strengthen the assessment of severity of PE.

Stroke is a leading cause of death and severe, long-term disability. Most of the first - time stroke patients have high BP. Hypertension acts as a major determinant of endothelial dysfunction and vascular damage, promoting inflammatory activation of endothelial cells and recruitment of inflammatory cells including neutrophils, into the ischemic brain tissue (208, 209). Results presented in chapter III showed significant

increase in the levels of NE and $\alpha_2$-MG in stroke patients and so correlation of these parameters with SBP was made. The correlation study indicated positive association of elastase and $\alpha_2$-MG with SBP. The positive association between SBP and elastase indeed indicates that stroke outcome is mediated by an inflammatory response. This observation also indicates possible role of these molecules in the development of complications of stroke.

## Conclusion

The outcomes of correlation studies suggest that the measurement of NE, $\alpha_1$-AT and $\alpha_2$-MG could be of relevance in diagnosis of diseases in combination with other tests and clinical signs preferentially for differential diagnosis. These molecules are also relevant in determining the severity of dengue fever on account of increased NE in DHF with associated neutropenia. This study opens up the notion that in bacterial infection there is an increase in $\alpha_2$-MG and in viral infection a decrease $\alpha_1$-AT. Though it is premature to emphasis on this, the question whether these are characteristics of host response in bacterial and viral diseases or otherwise needed to be studied.

Similarly, measurement of NE, $\alpha_1$-AT and $\alpha_2$-MG in non-infectious diseases also make sense, as there were some relevant and significant correlations with complications of DM and severity of the disease. Measurement of $\alpha_1$-AT levels in diabetic patients could be of relevance as a marker for retinopathy complications of diabetes. The characteristic feature in two disease conditions of PE and stroke, both attributed to hypertension are the increased levels of NE and $\alpha_2$-MG but with a significant decrease in $\alpha_1$-AT which is seen in severe PE and can be attributed to renal loss in PE associated with proteinuria. As, this is the first of its kind of study, more investigations are needed to take the results of this study to a logical end and in to clinical practice.

# Chapter V

## Invitro culture of neutrophils to study the effects of triggering factors on elastase release from neutrophils

## Introduction

Non-infectious diseases included in this study are diabetes mellitus, preeclampsia and stroke. A common observation noted among them were increased levels of NE and a positive correlation of NE with the causative and/or risk parameters of all disease conditions. It is known that hyperglycemia leads to release of NE through inflammatory mediators, leading to complications of diabetes (135). Further, it is established that increased glucose levels could release elastase in Invitro culture system (54, 210). On the other hand, hyperhomocysteinemia is considered either as a co-morbid condition or a modifiable, independent risk factor in the development of cardiovascular diseases such as stroke, coronary artery disease, preeclampsia and diabetes (55, 56, 57).

Homocysteine (Hcys) is a thiol- group containing amino acid intermediate in methionine synthesis and is utilized in the body for transmethylation reaction. Hcys above the physiological levels exerts diverse deleterious effects on vascular and immune cells through synthesis of ROS leading to endothelial injury, platelet activation, smooth muscle cell proliferation, oxidation of LDL and induction of endothelial-monocyte interactions (211, 212). Invitro study by Bryushkova et al. demonstrated expression of N-methyl-D-aspartate (NMDA) receptors by neutrophils on their membranes and subsequent degranulation upon incubation with Hcys (213). Alvarez-Maqueda M et al also showed Hcys increased intracellular $H_2O_2$ via NADPH oxidase leading to activation and phosphorylation of mitogen-activated protein kinases (MAPKs) resulting in chemotaxis and migration of human peripheral neutrophils (214). In a comprehensive study conducted by Joshi et al it was shown for the first time that Hcys acts as a potential inducer of NET formation and associated release of elastase (55). As briefed above, Hcys has been implicated as a stimulating factor for neutrophils leading to pathophysiological

changes in the body. These reports prompted for designing a simple experimental setup to look for the effect of Hcys on neutrophils and elastase activity by using varying physiological and pathological concentrations of Hcys. To ensure that the system designed is dependable, a validation was carried out by using varying concentrations of glucose as a reference molecule.

## Materials and methods

## Chemicals and Reagents

All reagents used were of analytical grade unless otherwise specified. 2% Fetal Bovine Serum (2% FBS) (Cat. No. 10270106) and Roswell Park Memorial Institute -1640 media (RPMI-1640) (Cat. No. 31800022) were purchased from Invitrogen. Ficoll-paque Plus was purchased from GE Healthcare (Cat.No. 17-1440-02), 20% dextran was from Himedia (Cat.No. RM 4187) and 96-well tissue culture plates from Genetix. DL-Homocysteine was procured from Sigma, USA (Cat.No. 44925).

## Isolation and Invitro culture of neutrophils

Ficoll- dextran density gradient method was employed for the isolation of human PMNs as described by Joshi et al (210). PMNs were isolated from blood of healthy volunteers with informed consent. Briefly, 5ml of blood was collected in sodium heparinized tubes and was transferred to a 15ml falcon tube and diluted it with 2ml of phosphate buffer saline (PBS). Diluted blood was gently layered on top of 4ml of Ficoll-paque Plus gradient solution in a fresh falcon tube and centrifuged the tube at 2000rpm for 20 minutes at room temperature. Top two layers were discarded carefully without disturbing the bottom layer. Cell pellets were suspended in 9ml of Hank's balanced salt solution (HBSS) and 0.5ml of 20% dextran followed by gentle mixing. After incubation for 1 hour within laminar air flow hood, the supernatant was carefully transferred into a new falcon

tube and centrifuged at 1800rpm for 10 minutes at room temperature. Supernatant was discarded and 2ml of RBC lysis buffer (1X) was added to the pellet. Pellet was mixed carefully and incubated for 3-4 minutes at room temperature. This was followed by addition of 9 ml of HBSS buffer and centrifugation at 1800 rpm for 10 minutes at room temperature. Supernatant was discarded and the white pellet obtained consisted of PMNs. Pellets were then suspended in appropriate volume of HBSS to adjust the concentration of neutrophils to $2.5x10^6$ cells per ml. An aliquot was used for counting neutrophils by Trypan- blue dye exclusion method using hemocytometer and for cell purity assessment by Giemsa staining.

Cell suspension was centrifuged at 1800 rpm for 10 minute at room temperature, supernatant was discarded and the pellet was resuspended in 1 ml of RPMI with 2% FBS. Cells ($2.5x10^6$) were then seeded on 96-well tissue culture plates and were incubated without or with increasing concentrations of glucose (5.5mM, 11mM, 22M/L) and homocysteine (5, 11, 25, 50, 200µM/L) in a final volume of 100µl per well at 37ºC in $CO_2$ incubator for 2hours at an interval of minutes. Three independent experiments were carried out for each concentrations and incubation time. Supernatants were collected after each incubation period and elastase activity quantified immediately using STANA as substrate.

## Results

**Influence of glucose on elastase release:** To assess the impact of high glucose conditions on the release of elastase from neutrophils, isolated neutrophils from healthy individuals were cultured in the presence and absence of varying concentrations of glucose. The result obtained is represented in Fig 16.

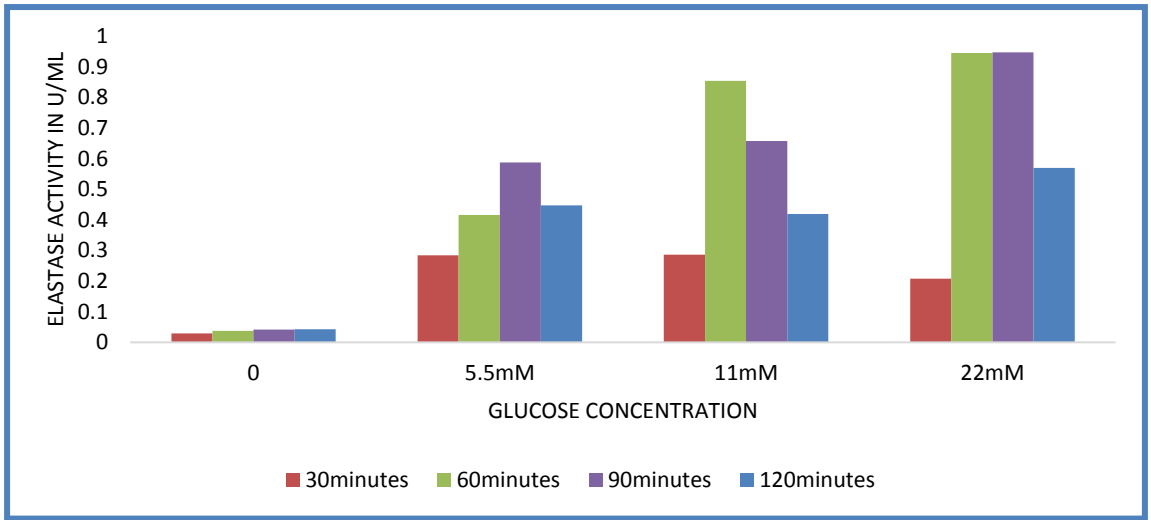


**Fig 16:** Influence of Glucose on elastase release

The results showed that, the increase in glucose concentrations had direct effect on elastase release which is reflected in terms of increased elastase activity. On exposure to 5.5mM glucose, maximum elastase activity of 0.588±0.02 U/ml/min was recorded after 90 minutes of incubation, while 11mM glucose yielded maximum activity of 0.855±0.06 U/ml/min measured after 60 minutes of incubation followed by a decreased elastase activity (0.658±0.00 U/ml/min) after 90 minutes of incubation. On incubation with 22mM glucose concentration maximum activity was recorded at both 60 (0.946±0.04 U/ml/min) and 90 (0.948±0.04 U/ml/min) minutes of incubation. At all the provided conditions, up to 90 minutes of incubation there was an increase in elastase activity but after 120 minutes the activity decreased considerably (0.448±0.04 with 5.5mM; 0.42±0.00 with 11mM and 0.57±0.01 with 22mM of glucose).

**Influence of Hcys on elastase release:** Freshly isolated peripheral neutrophils were exposed to varying concentrations of Hcys and the culture supernatants were assayed for elastase activity at different time intervals. The results obtained are represented in Fig.17.

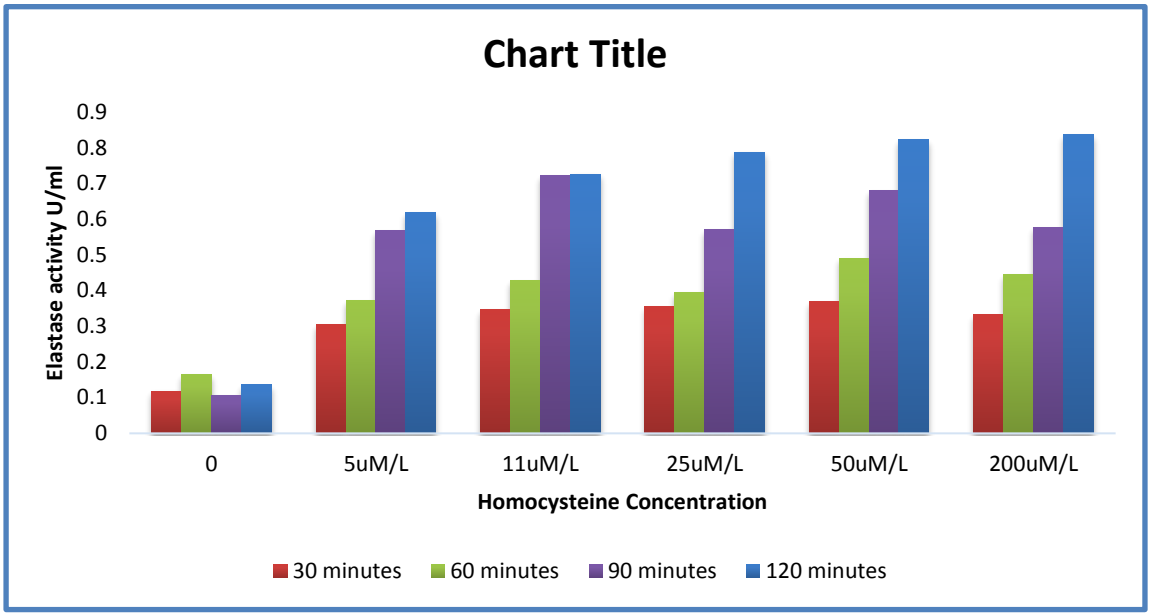


**Fig 17:** Influence of Homocysteine on elastase release

As indicated, elastase activity gradually increased with time at every concentration and maximum activity was recorded at 200uM/L Hcys concentration with 120 minutes of incubation. Further increase in Hcys concentration led to inhibition of elastase release. The elastase activity recorded are as follows: for 5µM/L Hcys concentration - 0.306±0.01; 0.373±0.07; 0.568±0.11 and 0.618±0.01U/ml/min; for 11µM/L- 0.347±0.07; 0.428±0.02; 0.722±0.02 and 0.726±0.05U/ml/min; for 25µM/L- 0.356±0.05; 0.394±0.09; 0.572±0.48 and 0.788±0.09U/ml/min; for 50µM/L- 0.37±0.23; 0.491±0.10; 0.681±0.23 and 0.822±0.04U/ml/min and for 200µM/L- 0.333±0.09; 0.446±0.02; 0.578±0.003 and 0.838±0.01U/ml/min after 30, 60, 90 and 120minutes of incubation respectively.

## Discussion

Deregulation of metabolic and immune response pathways in T2DM has been studied well. Neutrophils play an important role in innate immune response (7). Clinical studies and data from experimental models suggest hyperglycemia induced neutrophil chemotaxis, phagocytosis and bactericidal properties (215). Lowering of blood glucose

levels using anti-diabetic drugs has been shown to improve and re-sensitize neutrophil activity (216). Joshi et al demonstrated that a concentration dependent increase in glucose levels (15mM and 20mM) significantly increased NETs formation (210). Menegazzo L also observed increased release of NETs and circulating markers of NETs (i.e. elastase) with high glucose (25mM) compared to low glucose (5mM) concentrations (54). The results of the present study are similar to the observations made by Joshi et al and Menegazzo L. In this study concentrations of glucose used were in the range of 5.5mM to 22mM with incubation time ranging from 30minutes to 120 minutes. Results showed that there was concentration and time dependent increase in the elastase activity. The finding of increased elastase activity with increasing glucose concentrations support for the validation and the hypothesis that hyperglycemia activates neutrophils resulting in excessive release of elastase. The decrease in elastase activity observed after 120minutes of incubation at all concentrations of glucose could be attributed to autolysis of elastase in the medium.

Neutrophil extracellular traps (NETs) are networks of extracellular fibril matrix of histones and granular proteins bound chromatin expelled from neutrophils. The process of formation of NETs is called as NETosis. The protein component of NETs include elastase, myeloperoxidase, cathepsin G, bactericidal permeability increasing protein and others bound to DNA backbone. Elastase is one of the main components of NETs. NETs can either fight disease or cause disease. Neutrophils, in addition to various mechanisms such as phagocytosis and release of antimicrobial factors, are known to eliminate pathogens by producing NETs. NETs have been also shown to induce host tissue damage associated with autoinflammatory diseases, preeclampsia, acute pancreatitis, Alzheimer's disease and cancer metastasis (11, 55, 217).

High plasma homocysteine concentration is considered as an independent risk factor for developing cardiovascular diseases (56, 57). Since elastase also plays a key role in progression of inflammatory processes in cardiovascular diseases, this study was done to establish a relationship between elevated Hcys levels and elastase activity. The results showed that the activity of elastase released from freshly isolated neutrophils gradually increased with time and increasing concentrations of Hcys. After reaching a maximum activity at 120 minutes of incubation further increase of Hcys concentrations led to the inhibition of elastase release. These findings are in agreement with a study conducted by Joshi et al which showed increased release of NETs and associated elastase activity from neutrophils isolated from peripheral blood of healthy donors on increasing concentrations of Hcys. The authors also observed that in presence of Hcys, glucose primed neutrophils respond additively to produce NETs suggesting an association between hyperhomocysteinemia and diabetes (55). The present study results are in agreement with the earlier studies and supports that both hyperglycemia and hyperhomocysteinemia are inducers of release of elastase from neutrophils and could bring in detrimental effects leading to progressive disease conditions.

## Conclusion

Higher concentrations of glucose and homocysteine increase release of elastase from neutrophils in vitro. The findings demonstrate that risk factors of diabetes and stroke activates neutrophils and the release of elastase. Thus elevated levels of neutrophil elastase could be an important determinant in the vascular complications of diabetes and stroke.

# Chapter VI

Summary and Conclusion

The current study was aimed at evaluating the circulating levels of elastase, its inhibitors – $\alpha_1$-AT, $\alpha_2$-MG and NE in complex with $\alpha_1$-AT in infectious and non-infectious diseases. The data obtained indicated that all the diseases studied had significantly higher elastase activity which was almost two fold in comparison to controls. In dengue and preeclampsia, the levels of NE was associated with severity of the diseases. The findings highlights its importance in the progression of inflammation. This study also support the protective role of $\alpha_1$-AT as decreased levels were associated with complications of disease conditions. One of the salient features of this study was significantly decreased levels of $\alpha_1$-AT in diabetic retinopathy patients. This observation implicates that $\alpha_1$-AT could be a predictive marker of diabetic retinopathy. The other noteworthy observation is $\alpha_1$-AT reduction in viral infection in comparison to bacterial infection. The study has to be extrapolated to other viral infections to confirm the finding whether it is a feature in viral infections or otherwise.

The plasma concentrations of $\alpha_2$-MG showed different picture in different conditions with significantly high values observed in severe preeclampsia, stroke and pneumonia. In diabetes and dengue, levels were considerably decreased. Thus an associated decreased $\alpha_1$-AT in viral infection and increased $\alpha_2$-MG in bacterial infection could be add on parameters of differential diagnostic significance. The significantly elevated $\alpha_2$-MG levels in severe preeclampsia and stroke, both hypertension-associated diseases, suggest the possible involvement of this inhibitor in the complications associated these disorders for its implied role in coagulation. The data on NE - $\alpha_1$-AT complex was also diverse; diabetes with and without complications patients had significantly reduced levels and pneumonia and stroke patients had significantly elevated plasma concentration. Hence, data on the NE - $\alpha_1$-AT complex did not contribute much for drawing any conclusion or association.

The correlation studies indicated that the measurement of NE and $\alpha_2$-MG in pneumonia and NE and $\alpha_1$-AT in dengue could be of some relevance. Similarly the measurements of the levels of NE and $\alpha_1$-AT in diabetic patients particularly in retinopathy patients and NE and $\alpha_2$-MG in PE and stroke would be of relevance in diagnosis and prognosis in combination with other tests and clinical signs.

The first chapter provided background for extension of this study to assess the effect of glucose and homocysteine on elastase release from neutrophils in in vitro conditions. The results obtained clearly indicated that high glucose and homocysteine increased the release of elastase. The findings demonstrate that risk factors of diabetes and stroke activates neutrophils to release elastase and could bring in damaging effects leading to progressive disease conditions.

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# Publications

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ORIGINAL ARTICLE

# Plasma Neutrophil Elastase, $\alpha_1$-Antitrypsin, $\alpha_2$-Macroglobulin and Neutrophil Elastase–$\alpha_1$-Antitrypsin Complex Levels in patients with Dengue Fever

**Mamatha Kunder[1] · V. Lakshmaiah[2] · A. V. Moideen Kutty[1]**

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**Abstract** Dengue fever (DF) is characterized by systemic inflammatory response including neutrophil activation leading to uncontrolled elastase activity. This study was aimed to measure the activity of plasma neutrophil elastase (NE), its endogenous inhibitors $\alpha_1$-antitrypsin ($\alpha_1$-AT) and $\alpha_2$-macroglobulin ($\alpha_2$-MG) and elastase in complex with $\alpha_1$-AT (NE–$\alpha_1$-AT complex) in DF. 50 dengue patients [39 DF and 11 dengue hemorrhagic fever (DHF)] and 52 healthy subjects were included in the study. NE was measured using *N*-succinyl-tri-alanine-*p*-nitroanilide as substrate. $\alpha_1$-AT, $\alpha_2$-MG and NE–$\alpha_1$-AT complex were estimated by ELISA. The result analysis indicated that the dengue patients had significantly higher elastase activity with significantly reduced inhibitor levels compared to controls. Between DF and DHF patients, DHF group had significantly higher elastase activity. In conclusion, significantly elevated NE and reduced inhibitors level in dengue fever indicate these parameters could be of significance in DF particularly for the assessment of progression of inflammatory processes.

**Keywords** Dengue fever · Neutrophil elastase · $\alpha_1$-Antitrypsin · $\alpha_2$-Macroglobulin · NE–$\alpha_1$-AT complex

✉ A. V. Moideen Kutty
kuttyavm@gmail.com

[1] Department of Biochemistry, Sri Devaraj Urs Medical College, A Constituent College of Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka 563103, India

[2] Department of Medicine, Sri Devaraj Urs Medical College, A Constituent College of Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka, India

## Introduction

Dengue fever (DF) is an acute infectious disease of viral etiology with an estimated global incidence of close to 400 million per year [1, 2]. In response to any infection or tissue injury, the body initiates a series of cellular events including recruitment of neutrophils to the site of injury. The outcome of these events is an induction of a localized inflammatory response that allows cells to move out of the vessels to the site of infection/injury to repair cellular damage and restrict the replication of a pathogen. During dengue virus (DENV) infection, this normal process is affected. Studies demonstrate that DENV can be considered as an infectious trigger of an acute vascular disorder associated with inflammatory response [3].

The pathogenesis of DENV infection has been linked to the ability of the DENV to produce various inflammatory mediators such as neutrophils, plasma cascade systems and cytokines [1]. Neutrophils are known to have a crucial role in the pathogenesis of DENV infection as neutropenia is one of the most salient clinical features of dengue infection [4]. Elevated production of IL-8, a chemoattractant cytokine with potential pro-inflammatory effects in DENV infection [1, 3, 5] is known to activate and degranulate neutrophils [1, 6]. It is expected during DENV infection degranulation of neutrophils could release NE and consequent endothelial damage [7, 8]. Therefore it was considered worthwhile to study the levels of elastase and its endogenous inhibitors as in inflammatory states like DENV infection where large numbers of polymorphonuclear leukocytes are infiltered and activated.

Thus this study was aimed to determine the levels of plasma elastase activity, its two inhibitors (antiproteases):

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alpha$_1$-antitrypsin ($\alpha_1$-AT) and alpha$_2$-macroglobulin ($\alpha_2$-MG) and elastase in complex with $\alpha_1$-AT (NE–$\alpha_1$-AT complex) in DENV infection to associate with inflammatory processes.

## Materials and Methods

### Study Population

The present study is a case control study carried out in the Department of Biochemistry, Sri Devaraj Urs Medical College, the constituent college of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka. A total of 50 dengue patients admitted in the Department of Medicine of R. L. Jalappa Hospital and Research Centre, the teaching hospital of the medical college were included in the study; of them 39 were dengue fever (DF) and 11 were dengue hemorrhagic fever (DHF) patients. Dengue was confirmed by non-structural 1 antigen (NS1 antigen). Samples from dengue confirmed patients were obtained on day 5 to day 7 after onset of fever. 52 age and gender matched normal healthy individuals without fever were selected as controls. Every enrolled individual gave their informed written consent to participate in the study. The study protocol was approved by Institutional Ethical Committee.

Patients with acute and chronic infections, T2DM, cardiac diseases, malignancy, stroke, chronic obstructive lung disease, liver disorders, acute renal failures, history of smoking were excluded from the study.

### Sample Collection

6 ml of fasting venous blood was collected from all the subjects in tubes containing sodium fluoride (for FBS estimation), EDTA (for hematologic studies), Sodium Heparin (for elastase, $\alpha_1$-AT, $\alpha_2$-MG and NE–$\alpha_1$-AT complex estimation). For investigations like CRP, renal function and liver function tests blood was collected in tubes without anticoagulant. Blood samples were centrifuged within 2 h of collection. After centrifugation, serum and plasma were separated and aliquots were stored at −70 °C until assayed.

### Methods

Basic blood chemistry measurements were done by standard methods using Vitros 250 Dry chemistry analyzer (Johnson & Johnson). Complete Blood Count was performed by Beckman-Coulter, automatic blood cell counter. Serum C-reactive protein (CRP) estimation was done by rapid latex slide tests. Plasma elastase was estimated using succinyl tri-L-alanyl-*p*-nitroanilide (STANA, SIGMA) as substrate at 410 nm as per the procedure described by Beith et al. [9]. Plasma $\alpha_1$-AT and $\alpha_2$-MG were analyzed using Enzyme Linked Immunosorbent Assay, Immunology Consultants Laboratory, Inc, USA. NE–$\alpha_1$-AT complex was quantified by ELISA (Calbiochem).

### Statistical Analysis

The results were analyzed by SPSS software version 22 (licensed version) for statistical significance. The results are expressed as mean ± SD. All variables were checked for normal distribution by Shapiro–Wilk test. Differences between groups were analyzed using Student's unpaired *t* test for normally distributed parameters and Mann–Whitney *U* test for not-normally distributed parameters. *p* value <0.05 was considered statistically significant and <0.001 as highly significant.

## Results

*Baseline characteristics of study groups* are shown in Table 1. The mean age was 48 years. Majority were males, 61.5% and females were 38.5% in control group and in dengue group 60% were males and 40% were females. There were significant changes in hemodynamic parameters between control and patient group. Significant decrease in platelet count (272.23 ± 61.24–71.94 ± 39.17 × $10^3$/l, $p < 0.001$), total WBC count (7.04 ± 1.02–4.38 ± 1.81 × $10^3$/l, $p < 0.001$), neutrophils (51.27 ± 7.37–40.68 ± 12.37%, $p < 0.001$) and lymphocytes (37.86 ± 6.62–33.99 ± 9.20, $p < 0.05$) observed in dengue patients whereas we found significant increase in monocytes in dengue patients as compared to controls (5.01 ± 1–14.24 ± 6.09, $p < 0.001$).

*Plasma levels of elastase activity, $\alpha_1$-AT, $\alpha_2$-MG and NE–$\alpha_1$-AT complex in the study groups* are presented in the Table 2. Plasma elastase activity in patients with DENV infection were significantly higher than in healthy subjects ($p < 0.001$) whereas plasma levels of inhibitors $\alpha_1$-AT and $\alpha_2$-MG were significantly lower in dengue patients as compared to healthy control group ($p < 0.001$). When plasma NE–$\alpha_1$-AT complex was estimated in the two group, dengue patients had higher concentration but the results were not statistically significant ($p = 0.068$).

When elastase activity was compared between DF and DHF patients, DHF patients had higher activity of 1.044 ± 0.17 U/ml/min compared to DF group with elastase activity of 0.70 ± 0.15 U/ml/min (Fig. 1). However there was no significant difference in the levels of $\alpha_1$-AT, $\alpha_2$-MG and NE–$\alpha_1$-AT complex.

**Table 1** Basic characteristics of study groups

| Variables | Controls (n = 52) | Cases (n = 50) | *p* value |
|---|---|---|---|
| Age (years) | 48.48 ± 6.39[a] | 48.50 ± 4.34[a] | 0.986 |
| Gender (male/female) | 32/20 (61.5%/38.5%) | 30/20 (60%/40%) | – |
| HCT (%) | 42.27 ± 3.63[a] | 43.01 ± 5.22[a] | 0.407 |
| PL count ($10^3$/l) | 272.23 ± 61.24[b] | 71.94 ± 39.17[b] | <0.001** |
| Total WBCs ($10^3$/l) | 7.04 ± 1.02[b] | 4.38 ± 1.81[b] | <0.001** |
| Neutrophils (%) | 51.27 ± 7.37[a] | 40.68 ± 12.37[a] | <0.001** |
| Lymphocytes (%) | 37.86 ± 6.62[a] | 33.99 ± 9.20[a] | <0.05* |
| Monocytes (%) | 5.01 ± 1[b] | 14.24 ± 6.09[b] | <0.001** |

[a] Student's unpaired *t* test and [b] Mann–Whitney *U* test were used. Values are expressed as mean ± SD

* Statistically significant; ** Statistically highly significant

**Table 2** Comparison of plasma NE activity, $\alpha_1$-AT, $\alpha_2$-MG and NE–$\alpha_1$-AT complex between cases and controls

| Parameters | Controls (n = 52) | Cases (n = 50) | *p* value |
|---|---|---|---|
| Elastase activity (U/ml/min) | 0.35 ± 0.21[b] | 0.78 ± 0.20[b] | <0.001* |
| $\alpha_1$-AT (mg/dl) | 123.35 ± 25.66[b] | 66.25 ± 7.75[b] | <0.001* |
| $\alpha_2$-MG (mg/dl) | 209.42 ± 31.15[b] | 162.75 ± 24.16[b] | <0.001* |
| NE–$\alpha_1$-AT complex (ng/ml) | 214.89 ± 13.05[a] | 238.38 ± 62.47[a] | 0.068 |

[a] Student's unpaired *t* test and [b] Mann–Whitney *U* test were used. Values are expressed as mean ± SD

* Statistically highly significant

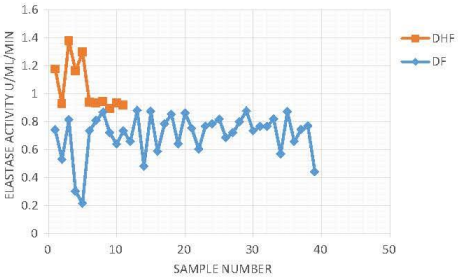


**Fig. 1** Elastase activity in DF and DHF patients

## Discussion

Pathogenesis of dengue, a systemic viral infection condition, is multifactorial and complex. Host immune response is one of the factors involved in the pathogenesis of dengue infection. Clinical and in vitro observations showed that monocytes and splenic macrophages are the principal direct targets of DENV [10, 11]. Infected monocytes in turn produce high levels of several cytokines with chemoattractant activity which are known to activate and degranulate neutrophils [1, 6]. Neutropenia, an established clinical feature of DENV infection could be attributed to degranulation of neutrophils and this in turn would reflect an elevated elastase activity in dengue patients than in normal. To obtain an insight into the levels of neutrophil elastase with the severity of dengue infection, the dengue patients were divided into DF and DHF groups. The data showed significantly higher plasma elastase activity in DHF patients suggestive of more destructive sequela in DHF patients (Fig. 1). However, a larger DHF patient group would be necessary to statistically associate the same. The finding of elevated elastase activity in dengue patients is in accordance with the study conducted by Juffrie et al. [1].

Though an increased levels of antiproteases ($\alpha_1$-AT and $\alpha_2$-MG) were expected in the patient group to counteract the elevated elastase activity as natural defense mechanism, the levels of the inhibitors were significantly decreased in dengue cases. It is known that the patients suffering from dengue exhibit an increase in oxidative stress and generate free radicals [12, 13]. Free radicals have been shown to destroy $\alpha_1$-AT and $\alpha_2$-MG and thus a possible explanation for observation of decreased levels of these inhibitors in this study may be due to the destruction of these molecules by free radicals.

Alpha$_1$-proteinase inhibitor binds with elastase at a 1:1 molar ratio and forms an 80KD elastase–$\alpha_1$-AT complex. The formation of complex reflects a rapid response of the organism to infection [14]. We have observed no increase in the levels of complex in dengue group consequent to the increased elastase activity. This observation indicates normal receptor-mediated clearance of complex from the plasma in DENV infection.

## Conclusion

An increased elastase activity and decreased levels of elastase inhibitors in dengue patients suggest their role in the inflammatory processes and its progression in dengue infection. The present study also indicates an elevated elastase activity in dengue hemorrhagic fever in comparison to dengue fever suggesting that it could be a possible marker for assessment of severity of dengue fever.

**Compliance with Ethical Standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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Original Article

Biochemistry Section

# Correlation of Plasma Neutrophil Elastase Activity and Endogenous Protease Inhibitor Levels with the Severity of Pre-eclampsia

MAMATHA KUNDER[1], AV MOIDEEN KUTTY[2], V LAKSHMAIAH[3], SR SHEELA[4]

## ABSTRACT

**Introduction:** Pre-eclampsia (PE) is a common maternal syndrome characterized by severe systemic inflammatory response including neutrophil activation leading to uncontrolled activity of elastase. The excessive activity of elastase would lead to destroyal of the integrity of endothelial cells and could exacerbate the pathophysiological symptoms in PE. Thus, assessment of NE activity and its control mechanisms would be of relevance in the determination of severity of PE.

**Aim:** To correlate the activity of plasma NE and its endogenous inhibitors $\alpha_1$-antitrypsin ($\alpha_1$-AT) and $\alpha_2$-macroglobulin ($\alpha_2$-MG) with severity of PE.

**Materials and Methods:** A comparative study was conducted between normotensive pregnant (n=50) and pre-eclamptic (n=50) women. Serum C-Reactive Protein (CRP) was estimated by rapid latex slide and uric acid by uricase method. Plasma elastase was estimated using succinyl tri- L-alanyl-p-nitroanilide as substrate. Plasma $\alpha_1$-AT, $\alpha_2$-MG and NE- $\alpha_1$-AT complex were quantified by ELISA. ANOVA and Pearson's correlation tests were used to analyze the data. The results were expressed as mean±SD and p-value <0.001 was considered statistically highly significant.

**Results:** The activity of elastase was increased significantly in severe PE (0.62±0.08) in comparison to normal (0.35±0.10) and mild pre-eclamptic subjects (0.37±0.03). The values of $\alpha_1$-AT were significantly less in mild (83.94±25.08) and severe PE (68.58+26.39) in comparison to normal (110.26±42.39). There was a significant rise in the levels of $\alpha_2$-MG in severe PE. However, the complex estimation did not evince any significant changes.

**Conclusion:** The results of the present study indicate a significantly elevated elastase activity, $\alpha_2$-MG levels and decreased $\alpha_1$-AT in severe PE patients. The correlation analyses of PE severity parameters with NE, $\alpha_1$-AT and $\alpha_2$-MG further support the roles of these molecules in the assessment of severity of PE.

**Keywords:** $\alpha_1$-antitrypsin, $\alpha_2$-macroglobulin, NE- $\alpha_1$-AT complex

## INTRODUCTION

PE is a major cause of maternal and neonatal morbidity and mortality. It is a multisystem disorder which is characterized by vasoconstriction [1], leukocyte activation [2], enhanced inflammatory response [3] and oxidative stress [4]. The causes for the development of PE are still unclear and are a topic of active investigation. The pathological lesions of decidual vessels in PE have similarity to atherotic lesions of arteries [5]. Neutrophils have been implicated in the pathogenesis of atherotic changes and endothelial dysfunction through release of variety of substances. Elastase is one of such molecules released from neutrophils and is an established marker for neutrophil activation [6-8].

Neutrophil Elastase (NE), a serine protease stored in the primary granules of neutrophils, is capable of degrading various extracellular matrix proteins such as elastin, collagen, fibrinogen and proteoglycans [9]. Therefore, it can cause vascular basement membrane damage and can facilitate tissue infiltration of neutrophils. Activation of neutrophils is implicated in PE and consequently contributes to vascular basement membrane damage leading to oedema and proteinuria [10], a usual observation in PE. A positive correlation have been demonstrated between von Willebrand Factor (a marker of endothelial damage) and NE by Greer IA et al., indicating that neutrophil activation could contribute to endothelial damage and dysfunction in PE [11].

Thus, uncontrolled neutrophil activation can lead to destroyal of the integrity of endothelial cells and could exacerbate the pathophysiological symptoms in PE. It is well established that PE is manifested as mild, moderate and severe forms in pregnant women but it is unclear what exaggerates the symptoms and the severity. This study is an attempt in this direction to correlate the activity of neutrophil elastase and its endogenous inhibitors α1-antitrypsin ($\alpha_1$-AT) and $\alpha_2$-macroglobulin ($\alpha_2$-MG) with severity of PE.

## MATERIALS AND METHODS

The present study is a comparative study conducted during the period of October 2015 to April 2016. The subjects of this study were the pregnant women attending or admitted in the Department of Obstetrics and Gynecology, RL Jalappa Hospital and Research Center, the teaching hospital of Sri Devaraj Urs Medical College (SDUMC) and the biochemical evaluation was carried out in the Department of Biochemistry of SDUMC, a constituent college of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India. Every enrolled pregnant woman gave their informed written consent to participate in the study. This study was performed after obtaining Institutional Ethical Committee approval and the study complied with the Helsinki Declaration.

A total of 50 pregnant normotensive women and 50 pre-eclamptic pregnant women (27 mild and 23 severe cases), were included in the study. All the women were in the age group of 19-36 years and were over 20 weeks of gestation. Normal pregnancy was diagnosed on the basis of clinical and ultrasound evaluation and all of them presented a normal course and outcome of pregnancy. The pre-eclamptic patients were diagnosed by the presence of hypertension (≥140 mmHg systolic BP and ≥90 mmHg diastolic BP) on two occasions

with 4-6 hours apart, proteinuria (≥1+ by urine dipstick method) with or without pathological oedema. PE was considered as severe, if the subjects had at least two of the following: ≥160 mmHg systolic BP; ≥110 mmHg diastolic BP; dipstick proteinuria of 3+ or more. All the other cases were considered as mild PE. All patients with any infection, twins, history of pregestational diabetes, gestational diabetes mellitus, renal disease, liver disease, cardiovascular disease and hypertension were excluded from the study.

Almost 6 ml of blood was collected from an antecubital vein from all the subjects in tubes containing EDTA (for haematologic studies); Sodium heparin (for NE, $\alpha_1$-AT, $\alpha_2$-MG and NE- $\alpha_1$-ATcomplex estimation) and in tubes without anticoagulant (for CRP estimation). Blood samples were centrifuged within two hours of collection. After centrifugation, serum and plasma were separated and aliquots were stored at -70°C until assayed. Samples were thawed at room temperature, vortexed and centrifuged before analysis.

Complete blood count was performed by Beckman- Coulter, an automatic blood cell counter. Serum C - Reactive Protein (CRP) estimation was done by rapid latex slide tests. Serum uric acid was estimated by uricase method [12] using Dry Chemistry Vitros 250 Johnson and Johnson analyzer. Estimation of plasma elastase was done using succinyl tri- L-alanyl-p-nitroanilide (STANA, from SIGMA) as substrate at 410 nm as per the procedure described by Beith J et al., [13]. Plasma $\alpha_1$-AT and $\alpha_2$-MG were analyzed using ELISA kit purchased from Immunology Consultants laboratory, Inc, USA. NE-$\alpha_1$-AT complex was quantified by ELISA (Calbiochem).

## STATISTICAL ANALYSIS

The data were statistically analyzed by SPSS software version 22.0. The results were expressed as mean±SD. For statistical differences in means between the groups ANOVA (Analysis of variance) was used. Pearson's correlation coefficient test was used to analyze the correlation of severity parameters (BP and proteinuria) with elastase and its inhibitors. A p-value <0.001 was considered highly significant.

## RESULTS

The baseline physical and biochemical characteristics of the normal, mild and severe pre-eclamptic subjects are depicted in [Table/Fig-1]. The gestational age was in the range of 34 to 37 weeks for normal, and 31 to 36 weeks for mild to severe PE subjects. The blood pressure was elevated significantly in the case of mild and severe cases of PE in comparison to normal. The blood pressure was also significantly higher in severe PE compared to mild PE.

| Variables | Normal pregnancy (n=50) | Mild pre-eclampsia (n=27) | Severe pre-eclampsia (n=23) | p-value |
|---|---|---|---|---|
| Maternal age (years) | 23.62±2.98 | 25.11±4.20 | 25.70±4.35 | |
| Gestational age (weeks) | 37.18±3.05 | 34.70±3.37 | 34.96±3.11 | |
| Blood Pressure (mmHg) | | | | |
| Systolic | 120.28±8.70 | 147.11±9.35 | 170.87±14.11 | *p<0.001 |
| Diastolic | 80.20±5.88 | 100.22±8.84 | 106.52±11.12 | |
| Cases with proteinuria n (%) | - | | | |
| • Traces | | 4 (14.8) | 1(4.3) | |
| • 1+ | | 16 (59.3) | 3(13.0) | |
| • 2+ | | 7 (25.9) | 5(21.7) | |
| • 3+ | | - | 14(60.9) | |
| Serum uric acid (mg/dl) | 4.53±1.30 | 7.53±1.35 | 8.16±1.57 | *p<0.001 |
| Serum CRP (ug/ml) | 0 | 12.44±11.40 | 14.35±13.98 | *p<0.001 |

**[Table/Fig-1]:** Baseline characteristics of study groups.
ANOVA with Bonferroni test was used. Results represented as mean±SD. *p<0.001 statistically highly significant.

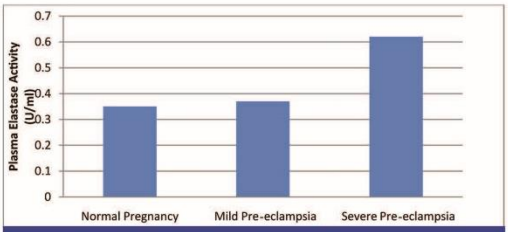
The data on proteinuria was suggestive of PE as per the criteria defined. Serum uric acid showed significant rise in PE group (mild 7.53±1.35; severe 8.16±1.57) compared to controls (4.53±1.30). When serum CRP was compared, mild (12.44±11.40) and severe (14.35±13.98) pre-eclamptic women presented significantly higher CRP levels as compared to normotensive pregnant women.

The data on NE, $\alpha_1$-AT, $\alpha_2$-MG and NE- $\alpha_1$-AT complex are presented in [Table/Fig-2]. The activity of neutrophil elastase was increased two fold in severe PE (0.62±0.08) in comparison to normal (0.35+0.10) and mild preeclamptic subjects (0.37±0.03) and was statistically highly significant. The values of $\alpha_1$-AT have been on the decline and were significantly less in mild and severe PE in comparison to normal; a 60% reduction in severe and 40% reduction in mild. There was a significant rise in the levels of $\alpha_2$-MG in severe pre-eclamptic women. However, the complex estimation did not evince any significant changes indicating normal balance and did not contribute to analytic value. Significant association between elastase activity and disease severity is depicted in [Table/Fig-3].

Correlation studies of the severity parameter; proteinuria with the levels of NE and $\alpha_1$-AT indicated a positive and negative picture respectively in severe form of PE [Table/Fig-4]. On the other hand,

| Parameters | Normal pregnancy (n=50) | Mild pre-eclampsia (n=27) | Severe pre-eclampsia (n=23) | p-value |
|---|---|---|---|---|
| Plasma Elastase Activity (U/ml) | 0.35±0.10 | 0.37±0.03 | 0.62±0.08 | *p<0.001 |
| Plasma $\alpha_1$-AT (mg/dl) | 110.26±42.39 | 83.94±25.08 | 68.58±26.39 | *p<0.001 |
| Plasma $\alpha_2$-MG (mg/dl) | 265.37±66.91 | 201.06±38.23 | 298.79±32.52 | *p<0.001 |
| Plasma NE-$\alpha_1$-ATcomplex (ng/ml) | 171.08±23.81 | 176.19±9.27 | 164.31±11.63 | p=0.285 |

**[Table/Fig-2]:** Plasma levels of elastase activity, $\alpha_1$-AT, $\alpha_2$-MG and NE-$\alpha_1$-AT complex in the study groups.
ANOVA with Bonferroni test was used. Results represented as mean±SD. *p<0.001 statistically highly significant.




**[Table/Fig-3]:** Elastase activity expressed in U/ml of plasma in study groups.

the correlation analysis of $\alpha_2$-MG with proteinuria presented a negative correlation in mild and a positive correlation in severe group. However, complex correlation with the severity parameters did not present any definite correlations.

## DISCUSSION

PE exhibits characteristics of an inflammatory disease including neutrophil activation [2,14,15]. The activation of neutrophils in PE may be due to some pro-inflammatory cytokines and chemoattractants released during an inflammatory response (i.e., TNF-$\alpha$, IL-6 and IL-8) [16]. Elastase activity is measured as marker of neutrophil activation in several inflammatory conditions including PE [7,8,14,15]. The complications induced by PE state are detrimental to both the mother and the foetus and have been a serious subject of investigation. Research often focuses on the changes in the biochemical parameters with no data on its onset and progress.

| Parameters | Plasma Elastase Activity (U/ml) | | Plasma $\alpha_1$-AT (mg/dl) | | Plasma $\alpha_2$-MG (mg/dl) | | Plasma NE-$\alpha_1$-ATcomplex (ng/ml) | |
|---|---|---|---|---|---|---|---|---|
| | r | P | r | P | r | P | r | P |
| **Mild PE** | | | | | | | | |
| Systolic BP (mmHg) | -0.178 | 0.373 | 0.361 | 0.064 | 0.131 | 0.156 | -0.130 | 0.673 |
| Diastolic BP (mmHg) | -0.004 | 0.983 | -0.201 | 0.314 | 0.005 | 0.978 | 0.280 | 0.354 |
| Proteinuria –traces | 0.899 | 0.101 | -0.690 | 0.310 | -0.856 | 0.144 | 1.000 | - |
| Proteinuria -1+ | 0.014 | 0.960 | 0.266 | 0.338 | -0.436 | 0.104 | -0.338 | 0.412 |
| Proteinuria -2+ | 0.090 | 0.832 | 0.046 | 0.914 | -0.076 | 0.857 | -0.987 | 0.104 |
| **Severe PE** | | | | | | | | |
| Systolic BP (mmHg) | -0.015 | 0.945 | -0.075 | 0.733 | -0.263 | 0.224 | -0.258 | 0.419 |
| Diastolic BP (mmHg) | -0.376 | 0.077 | 0.153 | 0.486 | 0.054 | 0.808 | 0.182 | 0.572 |
| Proteinuria -1+ | 0.064 | 0.959 | -0.247 | 0.841 | 0.933 | 0.234 | 0.920 | 0.256 |
| Proteinuria -2+ | 0.366 | 0.544 | -0.282 | 0.645 | 0.692 | 0.195 | -0.331 | 0.586 |
| Proteinuria -3+ | 0.169 | 0.565 | -0.171 | 0.558 | 0.290 | 0.315 | 0.329 | 0.589 |

**[Table/Fig-4]:** Correlation of plasma levels of elastase activity, $\alpha_1$-AT, $\alpha_2$-MG and NE-$\alpha_1$- AT complex with severity markers in mild and severe groups. Pearson's correlation coefficient test was used.

In the present study we have measured and compared the plasma levels of NE, $\alpha_1$-AT, $\alpha_2$-MG and NE- $\alpha_1$-AT complex in patients suffering from mild and severe PE with normal pregnant women [Table/Fig-2]. The analysis of NE activity indicated a significant increase in the severe PE which is in agreement with previous studies [7,8,11,14] and it could contribute to progressive inflammation.

α1-antitrypsin inhibits several serine proteases (mainly NE), and adequate activity of this inhibitor is critical for the maintenance of protease–antiprotease homeostasis and the prevention against proteolytic tissue damage [17]. Determination of the plasma $\alpha_1$-AT level demonstrates the available level of the inhibitor capable of inhibiting intravascular proteases. Contrary to expectation of an increased level of $\alpha_1$-AT in inflammation, a decreased level was observed in the study groups compared to the normal and it was highly significant paving ways to overpowering role of elastase in the complications of PE.

It is also pertinent to note that, there was no increase in the levels of NE-$\alpha_1$-AT complex in PE group consequent to the increased elastase activity. This observation could be an indication of decreased synthesis of $\alpha_1$-AT rather than its involvement in complex formation to control elastase activity. The reason for decreased $\alpha_1$-AT is an area of concern and is suggestive that supplementation of $\alpha_1$-AT would be able to minimize the destructive effects of NE on vascular tissues.

We have observed a significantly higher $\alpha_2$-MG level in severe PE patients compared with normal or mild PE patients against an expected reduced $\alpha_2$-MG concentration in severe PE as it is supposed to bind to elastase and get rapidly cleared from the plasma through macrophage receptors [18]. Raised levels observed in this study could be attributed to renal insufficiency, a common feature in PE patients. Moreover, increased levels of this inhibitor in severe PE possibly contribute to the intravascular coagulation as $\alpha_2$-MG has antiplasmin activity [19] adding to further severe complications. Horne CHW et al., also found high $\alpha_2$-MG levels in PE with proteinuria as compared to normal pregnant women [19].

In order to understand the relation of PE severity parameters with the levels of NE, $\alpha_1$-AT, $\alpha_2$-MG and NE- $\alpha_1$-AT complex correlation analyses were carried out. Though we have correlated these molecules with both proteinuria and BP, the most meaningful and relevant correlation was observed only with proteinuria and not with BP. The observation of increase in the activity of NE in severe PE and its positive correlation with severity marker proteinuria makes the measurement of NE a dependable parameter in the determination of severity of PE. Similar picture but in a negative direction was observed in case of $\alpha_1$-AT suggesting that measurement of $\alpha_1$-AT along with the activity of NE could strengthen the assessment of severity of PE.

As reported by earlier studies [20-22], a significant increase in the levels of serum uric acid and CRP in PE compared to controls was observed and points to generalised inflammation in these patients. However, the levels did not yield any information on the severity of the PE.

## LIMITATION

Since PE is a progressive disease, a follow up of mild preeclamptic women to assess the progression of these women to severe form would have been the better study design to emphatically conclude the role of elastase enzyme and its inhibitors in severity of PE. Moreover, urinary estimation of $\alpha_1$-AT would have explained the reduced levels of $\alpha_1$-AT in PE group. We do agree a larger sample size definitely would have supported the results obtained.

## CONCLUSION

The present study clearly indicates an association between increased levels of NE and decreased $\alpha_1$-AT with the severity of PE suggesting that both would be relevant markers for assessment of severity. Monitoring the plasma levels of these molecules thus could be of use for evaluation of the status of PE.

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**PARTICULARS OF CONTRIBUTORS:**
1. Lecturer, Department of Biochemistry, Sri Devaraj Urs Medical College, Kolar, Karnataka, India.
2. Professor, Department of Biochemistry, Sri Devaraj Urs Medical College, Kolar, Karnataka, India.
3. Professor, Department of Medicine, Sri Devaraj Urs Medical College, Kolar, Karnataka, India.
4. Professor, Department of Obstetrics and Gynaecology, Sri Devaraj Urs Medical College, Kolar, Karnataka, India.

**NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:**
Dr. AV Moideen Kutty,
Registrar, Professor, Sri Devaraj Urs Academy of Higher Education and Research,
Tamaka, Kolar-563103, Karnataka, India.
E-mail: kuttyavm@gmail.com

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