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## Acute Phase Proteins: Structure and Function Relationship

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### 1. Introduction

The acute-phase response is critical to the body's ability to successfully respond to injury. It normally lasts only few days; however, if continued unchecked, the acute phase response may contribute to the development of chronic inflammatory states, tissue damage and disease. The acute phase response is typically characterized by fever and changes in vascular permeability, along with profound changes in the biosynthetic profile of various acute phase proteins (APPs) (Hack et al., 1997, Gabay & Kushner, 1999). APPs are an evolutionarily conserved family of proteins produced mainly in the liver in response to infection and inflammation. In all mammalian species, the synthesis of the APPs is mainly regulated by inflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor (TNF). For instance, haptoglobin (Hp), C-reactive protein (CRP), serum amyloid A (SAA), alpha-1 acid glycoprotein (AGP) and hemopexin are regulated mainly by IL-1 or combinations of IL-1 and IL-6, whereas fibrinogen, alpha-1 antichymotrypsin (ACT) and alpha-1 antitrypsin (AAT) are regulated by IL-6 (Koj, 1985; Kushner & Mackiewicz, 1993). Exogenous glucocorticoids can also influence APPs by their effect on cytokines. The decreased synthesis of albumin during the inflammatory reaction has also been shown to be the result of monocyte/macrophage-derived products, such as IL-1 (Moshage et al., 1987). The concentration of specific blood APPs varies during inflammatory states; increasing or decreasing by at least 25 percent (Kushner et al., 1982). Indeed, ceruloplasmin concentrations can increase by 50 percent and CRP and serum amyloid A by a 1000-fold (Kushner et al., 1981; Dinarello, 1983; Blackburn, 1994; Gruys et al., 1994; Ingenbleek & Bernstein, 1999).

The rise in the plasma concentration of APPs can assist host defense by aiding recognition of invading microbes, mobilization of leukocytes into the circulation, and increasing blood flow to injured or infected sites. These actions favor the accumulation of effector molecules and leukocytes at locally inflamed sites; in essence, they enhance local inflammation and antimicrobial defense. Concurrently, the APPs also prevent inflammation in uninvolved tissues by neutralizing inflammation-induced molecules (such as cytokines, proteases, and oxidants) that enter the bloodstream, by diminishing the proinflammatory responses of circulating leukocytes, and by forestalling endothelial activation.

A particularly important role for APPs in the establishment of host defense is also suggested by the magnitude and rapidity of changes in concentrations of APPs, together with their short half-life. This is also supported by the known functional capabilities of the APPs, and hence theories as to how they might serve useful purposes in inflammation, healing, or adaptation to a noxious stimulus. The functional activities of APPs as well as the relationship between protein structure and function are discussed in this chapter.

## **2. Diverse functional activities of acute phase proteins (APPs)**

APPs are regarded as both mediators and inhibitors of inflammation operating at multiple possible sites. The classic complement components, many of which are APPs, have central proinflammatory roles in immunity. Complement activation leads to chemotaxis, plasma protein exudation at inflammatory sites, and opsonization of infectious agents and damaged cells. Other APPs such as fibrinogen, plasminogen, tissue plasminogen activator (tPA), urokinase and plasminogen activator inhibitor-I (PAI-1) play an active role in tissue repair and tissue remodelling (Gabay & Kushner, 1999). APPs also have antiinflammatory actions. For example, the antioxidants haptoglobin and hemopexin protect against reactive oxygen species, and AAT and ACT both antagonize the activity of proteolytic enzymes (Janciauskiene, 2001). Some metal chelating proteins, such as ceruloplasmin, that binds copper, and hemopexin, that binds heme, act more directly against pathogens. Other proteins are directly involved in the innate immunity against pathogens. LPS-binding protein (LPS-BP), for example, interacts with bacterial lipopolysaccharide (LPS) transferring it to CD14, a receptor on the surface of macrophages and B-cells. Following the presentation of LPS by LBP, a lipopolysaccharide recognition complex is formed on the membrane via the recruitment of a second receptor, Toll Like Receptor 4. These events drive the TLR signaling pathway that induces the activation of several inflammatory and immune-response genes, including pro-inflammatory cytokines (Gutsmann et al., 2001). Some APPs might act as protectors against cell death by apoptosis. For example, alpha 1-acid glycoprotein (AGP) and AAT have been shown to inhibit the major mediators of apoptosis, namely caspase-3 and caspase-7 (Van Molle et al., 1999). There are many diseases where induction of specific APPs parallels the degree and evolution of the inflammatory processes, hence, elevated APPs can be of diagnostic and prognostic value. The pathogenic role of fibrin in thrombosis is well known. CRP has been demonstrated to enhance ischemia/reperfusion injury by activating the complement system (Lu et al., 2009). Elevated serum values of CRP are known to be associated with an increased risk of human atherosclerosis. Ferritin, another APP, is a primary iron-storage protein and often measured to assess a patient's iron status. Procalcitonin (PCT), was discovered recently as a marker of bacterial infection (Assicot et al., 1993). On the other hand, APPs can be considered as putative drugs for the treatment of various inflammatory diseases. Different experimental studies have demonstrated how the administration of a specific APP prior to or after the initiation of an acute-phase response can switch the pro-inflammatory to the anti-inflammatory pathway necessary for the resolution of inflammation. In this regard, purified plasma AAT is used for the treatment of emphysema and other diseases in patients with inherited AAT deficiency and shows anti-inflammatory and immune modulatory effects.

### 3. Multifunctional activities of single APP

Despite vast pro- and anti-inflammatory properties ascribed to individual APPs, their role during infections remains incompletely defined as to the functional advantages acquiring from changes in plasma concentrations of the APPs. So far, existing data provide evidence that APPs act on a variety of cells involved in the early and late stages of inflammation and that their effects are time, concentration and molecular conformation-dependent (Figure 1). Many APPs have a dual function; amplifying inflammatory responses when the inciting pathogen is present within the host and down-regulating the response when the pathogen has been eradicated.

## Pleiotropism of Acute Phase Proteins (APPs)

A single protein has different functional activities

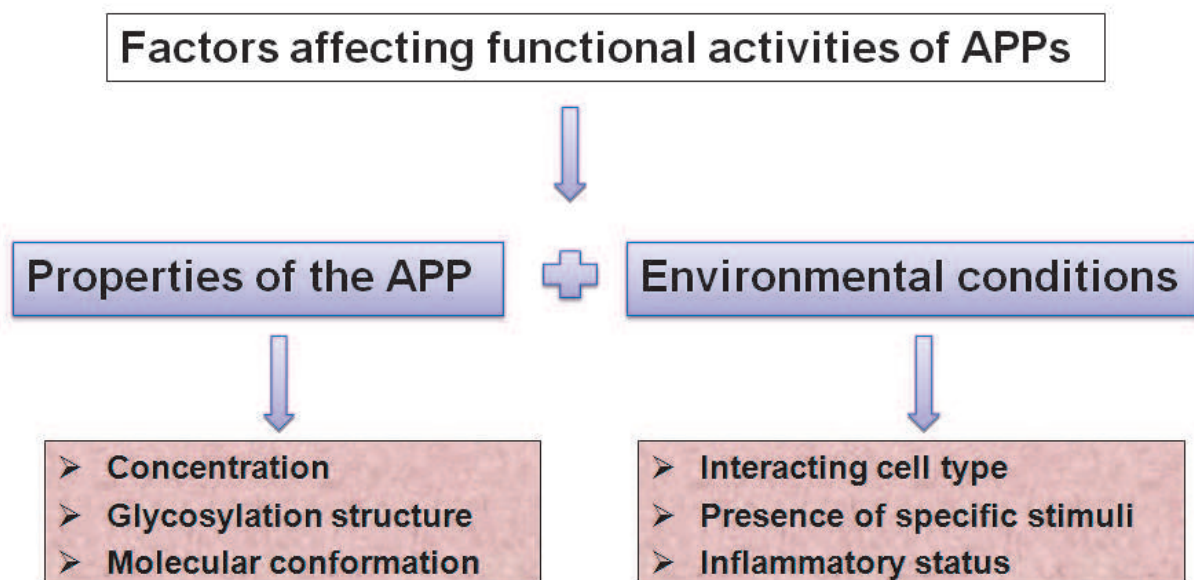


Fig. 1. Schematic presentation of factors that may affect activities of APPs.

#### 3.1 C-reactive protein (CRP)

C-reactive protein (CRP) is a member of the pentraxin family of proteins, which are serum opsonins, which bind to damaged membranes and nuclear autoantigens. CRP has an ability to recognize pathogens and to mediate their elimination by recruiting the complement system and phagocytic cells, thus making it an important member of the first line of innate host defense. The normal concentration in healthy human serum is usually lower than 10 mg/L, increasing slightly with age. Current research suggests that subjects with elevated basal levels of CRP are at an increased risk of diabetes (Pradhan et al., 2001, Dehghan et al., 2007), hypertension and cardiovascular disease (Koenig et al., 2006). CRP is an ancient protein whose biological role appears quite complex. Although, originally CRP was suggested to be purely a biomarker, recent studies have pointed that it may in fact be a direct mediator of patho-physiological processes. It is likely that the activity of CRP in

humans, either pro- or anti-inflammatory is dependent on the context in which it is acting, and thus CRP may be more versatile than previously thought.

### 3.1.1 Pro-inflammatory effects of CRP

CRP displays pro-inflammatory effects by activating the complement system and inducing inflammatory cytokines and tissue factor production in monocytes. The binding of phosphocholine and the complement pathway component (C1q) by CRP is part of innate immunity that activates the classical complement pathway (Gabay & Kushner, 1999; Du Clos, 2000). Data on the consumption of complement components and cell lysis have indicated that CRP-initiated complement activation is restricted to the formation of the C3 convertase (Berman et al., 1986). Formation of the alternative pathway amplification convertase and of C5 convertases is inhibited by factor H (Mold et al., 1984), which binds directly to CRP (Mold et al., 1999). The interactions between CRP and its diverse ligands, such as phosphocholine or Fc $\gamma$  receptors, has the potential to influence a variety of cells and pathways with the potential to affect: apoptotic cells (Gershov et al., 2000), damaged cell membranes (Volanakis & Wirtz, 1979), phagocytic cells (Ballou & Lozanski, 1992), smooth muscle cells (Hattori et al., 2003), and endothelial cells (Pasceri et al., 2000). Experimental evidence for the binding of CRP to apoptotic cells was provided recently (Gershov et al., 2000). The distribution of CRP on the surface of such cells is similar to that of the complement membrane attack complex. In addition to the membrane of intact injured cells, CRP also binds to membranes and nuclear constituents of necrotic cells. Several nuclear constituents, including histones (Du Clos et al., 1988), small nuclear ribonucleoproteins (Du Clos, 1989) and ribonucleoprotein particles (Pepys et al., 1994) have been shown to bind CRP in a calcium-dependent fashion, and CRP deposition to the nuclei of necrotic cells at sites of inflammation has been observed (Gitlin et al., 1977).

To date, experiments with monocytes have shown that CRP induces the production of inflammatory cytokines (IL-1, IL-6, TNF $\alpha$ , IL-8) (Ballou & Lozanski, 1992; Xie et al., 2005), the generation of reactive oxygen species (Zeller & Sullivan, 1992), leads to increased expression of tissue factor (Cermak et al., 1993), and affects cell chemotaxis (Whisler et al., 1986; Kew et al., 1990). Recently, Hanriot et al. (2008), investigating human monocytes exposed to CRP have confirmed the results of earlier studies on CRP-mediated induction of expression of numerous proinflammatory cytokine genes (with the exception of TNF $\alpha$ ) and further evidenced increased expression of PAI-2, MCP-1, GRO- $\alpha$ , GRO- $\beta$ , and the chemokine receptors CCR8 and CXCR4. It has also been demonstrated that isolated from serum and recombinant CRP can stimulate expression of the monocytic surface integrin CD11b and downregulate that of CD31 antigen (Woollard et al., 2002). Numerous reports in the literature document the role of CRP in atherogenesis. Epidemiological evidence reveals an association between elevated plasma CRP and atherosclerosis (Haverkate et al., 1997; Ridker et al., 1997; Koenig et al., 1999). Infusion of recombinant CRP in healthy men results in the activation of inflammation and coagulation (Bisoendial *et al.*, 2005). *In vitro*, CRP has been shown to exert a direct proinflammatory and proatherosclerotic effect on vascular cells, as exemplified by: (1) induction of an increased expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (Pasceri et al., 2000); (2) stimulation of secretion of monocyte chemoattractant protein-1 (MCP-1) (Pasceri et al., 2001); and (3) facilitation of macrophage low-density lipoprotein (LDL) uptake (Verma et al., 2002a). Transcription of genes encoding the cell



adhesion molecules (VCAM-1, ICAM-1, E-selectin) and chemokines is tightly regulated by the transcription factor NF- $\kappa$ B, which has been implicated as a key mediator of atherosclerosis (Brand et al., 1996; Marumo et al., 1997; Thurberg & Collins, 1998; De Martin et al., 2000). However, with regard to the effects of CRP on endothelial cells, published studies are somewhat controversial. CRP has been shown to promote production of pro-angiogenic molecules such as endothelin-1 and IL-6 in human saphenous vein endothelial cells (Verma et al., 2002), and activate NF- $\kappa$ B signalling through the CD32 receptor (Liang et al., 2006). However, other studies suggested that endothelial cell activation by CRP is due to contamination of the commercially obtained protein with LPS and/or sodium azide (Liu et al., 2005, Taylor et al., 2005).

### 3.1.2 Anti-inflammatory effects of CRP

#### 3.1.2.1 *In vitro*

CRP appears to express anti-inflammatory properties. For example, in monocytic cells CRP has been shown to increase the synthesis of interleukin-1-receptor antagonist (IL-1ra), to up-regulate vascular endothelial growth factor A (VEGF-A) expression (Tilg et al, 1993), to increase the release of the anti-inflammatory cytokine IL-10 (Mold et al, 2002; Szalai et al, 2002) and to repress the synthesis of IFN- $\gamma$  (Szalai et al, 2002). Furthermore, CRP has been reported to display anti-inflammatory effects in monocytes through down-regulation of alpha2-macroglobulin expression and up-regulation of liver X receptor  $\alpha$  expression (Hanriot et al., 2008). CRP was found to bind to a ligand on *Leishmania donovani* and phosphorylcholine-expressing *Neisseria meningitidis*, and hence increase their uptake by human phagocytes (Culley et al., 1996, Casey et al., 2008). Thomas-Rudolph et al (2007) have also reported that innate recognition by CRP enhances effective uptake and presentation of bacterial antigens through Fc-gamma receptors on dendritic cells and stimulates protective adaptive immunity. CRP has been shown to express multiple anti-inflammatory effects on neutrophils. CRP down-regulates the generation of superoxide by activated neutrophils which leads to a significant reduction of intracellular protein phosphorylation. IL-8-, formyl-methionyl-leucyl-phenylalanine (fMLP)-, and the complement component C5a-induced chemotactic responses of neutrophils are also found to be inhibited by CRP (Zhong et al., 1998). Inhibition of neutrophil chemotactic responses by CRP correlates with a reduction of chemotactic peptide-induced p38 kinase activity (Heuertz et al., 1999). In addition, CRP has been reported to induce cleavage and shedding of L-selectin from the surface of neutrophils, and markedly attenuate the attachment of human neutrophils to endothelial cells (Zouki et al., 1997). Finally, CRP has been shown to mediate shedding of the membrane-bound IL-6 receptor from neutrophils (Jones et al., 1999). The combined data on the effects of CRP on neutrophils indicate that this protein can limit the inflammatory response.

#### 3.1.2.2 *In vivo*

A variety of studies utilizing exogenous or transgenic CRP have probed the effects of CRP *in vivo*. The ability of CRP to protect mice against bacterial infection by various species has been well established. These species include *S. pneumoniae* (Mold et al., 1981; Szalai et al., 1995) and *Haemophilus influenza* (Weiser et al., 1998; Lysenko et al., 2000), which have phosphocholine-rich surfaces, and *Salmonella enterica* serovar Typhimurium, which has no known surface phosphocholine, although its cell membrane is known to be rich in another

CRP ligand, phosphoethanolamine (Szalai et al., 2000). Protection is presumably mediated through CRP binding to phosphocholine or phosphoethanolamine, followed by activation of the classical complement pathway. CRP protection of mice infected with *S. pneumoniae* has been shown to require an intact complement system, but does not require interaction with FcγRs (Mold et al., 2002; Szalai et al., 2002). CRP's protective effects are not limited to bacteria. It has been shown to play a protective role in a variety of inflammatory conditions, including endotoxin-mediated shock (Mold et al., 2002; Xia et al., 1997). Transgenic mice expressing human CRP are resistant to experimental allergic encephalomyelitis (Szalai et al., 2002). The study by Heuertz and collaborators has demonstrated that pretreatment of rabbits with purified human CRP intravenously significantly reduces neutrophil infiltration and C5a-induced alveolitis (Heuertz et al., 1993). One of the most commonly studied animal models of human systemic lupus erythematosus (SLE) is the female NZB x NZW F mouse model (B/W). These mice, as with human SLE patients, develop high levels of autoantibodies to nuclear antigens, leading to circulating immune complexes, the renal deposition of immune complexes and renal pathology. In this experimental model a single injection of human CRP not only markedly delayed the development of proteinuria, but also reversed ongoing active nephritis. The half-life of CRP in mice is only 4 hours (Kushner et al., 1978), however, despite the rapid clearance of injected CRP, the downregulation of the inflammatory process in the kidney persisted for more than 2 months. This suggests that CRP is most likely to be a direct regulator of the inflammation induced by immune complex deposition. In support of this B/W mice transgenic for human CRP also show a delayed onset of proteinuria and enhanced survival compared with non-transgenic B/W mice (Rodriguez et al., 2005). A single injection of CRP both prevents and reverses accelerated nephrotoxic nephritis (NTN) in C57BL/6 mice (Rodriguez et al., 2005) indicating that CRP-induced suppression of immune complex-mediated inflammation is not limited to autoimmune nephritis. Protection from NTN by CRP was associated with a decrease in inflammatory and pathologic changes in glomeruli and a marked reduction in renal expression of IL-1β and other macrophage chemoattractants (Rodriguez et al., 2007).

### 3.2 Alpha1-acid glycoprotein (AGP)

AGP is another example of an APP with multiple biological effects. AGP belongs to the lipocalin family, a group of proteins sharing a similar three-dimensional structure capable of binding and carrying hydrophobic molecules. The normal range of AGP concentration in the serum is 0.5-1.0 g/L, which can be increased several-fold in response to inflammation, infection, and systemic tissue injury (Engstrom et al., 2004; Lind et al., 2004). AGP function is still unknown; however, this protein is suggested to have a complex role by differentially regulating inflammatory responses (Logdberg & Wester, 2000; Fournier et al., 2000). So far, the most important function of AGP is linked to its ability to inhibit platelet aggregation (Snyder & Coodley, 1976; Costello et al., 1979).

#### 3.2.1 Pro-inflammatory effects of AGP

Earlier studies have shown that AGP can activate monocytes, induce T cell proliferation (Singh & Fudenberg, 1986) and enhance TNFα, IL-1, and IL-6 secretion (Boutten et al., 1992; Drenth et al., 1996; Su & Yeh, 1996). More recently it has been demonstrated that AGP activates human monocytes to secrete TNFα through a tyrosine kinase dependent pathway and this can be enhanced in the presence of serum AGP-binding proteins. Since TNFα can

also trigger the synthesis and secretion of AGP, the increase in TNF $\alpha$  secretion by AGP-stimulated monocytes may represent a positive feedback of APPs to amplify the inflammatory signal (Su et al., 1999). A current study evaluated the effects of bovine AGP on neutrophil pro-inflammatory responses, including respiratory burst activity and cytokine production, and found that bovine AGP enhanced neutrophil production of IL-8 in a dose-dependent manner (Rinaldi et al., 2008)

### 3.2.2 Anti-inflammatory effects of AGP

#### 3.2.2.1 *In vitro*

AGP has been shown to induce macrophage expression of IL-1ra and soluble TNF receptor, which antagonize the activity of IL-1 $\beta$  and TNF $\alpha$ , respectively (Tilg et al., 1993, Hocheppied et al., 2003). Sorensson and colleagues (1999) reported that endothelial barrier functions are dependent on the presence of AGP. A number of studies have investigated the effects of AGP on neutrophil function. At physiological concentrations, human AGP has inhibitory effects on neutrophil chemotactic responses after stimulation with fMLP and the complement component C5a (Laine et al., 1990, Vasson et al., 1994). Moreover, it has been shown that low doses of AGP promote neutrophil aggregation, while higher doses inhibit this response (Laine et al., 1990). Neutrophil respiratory burst activity is also reported to be modulated by AGP, and several studies have demonstrated that human AGP can inhibit the extracellular release of superoxide anion after activation with opsonized zymosan or phorbol 12-myristate,13-acetate (PMA) (Costello et al., 1984; Vasson et al., 1994). Bovine AGP inhibited zymosan-induced neutrophil extracellular release of superoxide anion and hydrogen peroxide without affecting the capacity of neutrophils to engulf and kill *Staphylococcus aureus*. Interestingly, AGP exerted its effect on free radical production regardless of whether neutrophils were exposed to AGP prior to or after activation (Rinaldi et al., 2008).

#### 3.2.2.2 *In vivo*

Several studies have shown that AGP may function as an immune modulator displaying a protective effect in different models of shock. In a model of bacterial septic shock, using the gram-negative *Klebsiella pneumoniae*, AGP showed clear protection when given prior to the lethal challenge (Fournier et al., 2000). Furthermore, AGP was found to inhibit apoptosis and inflammation in murine models, and to induce cAMP-dependent signaling in the endothelial cells (Libert et al., 1994; Costello et al., 1979). Using *Escherichia coli* LPS, an initiator of the acute inflammatory response associated with septic shock, Morre DF and coworkers (1997) demonstrated that AGP-LPS complexes can activate mouse macrophages *in vitro* and that AGP protects against sepsis. It has also reported that AGP protects mice from lethal shock induced by TNF $\alpha$  or endotoxin. The protection was observed in both normal and in galactosamine-sensitized mice; with optimal desensitization requiring at least 3 mg of AGP administered 2 hours before the lethal challenge. Under these conditions, complete inhibition of all TNF-induced metabolic changes was observed (Libert et al., 1994). In another model, AGP has been found to significantly increase survival rate (48 hours) in rats with septic peritonitis. This effect was seen when AGP (200 mg/kg i.v.) was given 15 min prior to and 24 hours after cecal puncture. In a hemorrhagic/hypovolemic shock model (including a defined trauma) in rats treated with 200 mg/kg AGP resulted in significantly higher values of mean arterial blood pressure, cardiac output and stroke volume when



compared to corresponding values obtained after resuscitation with Ringer's solution or intravenous albumin (Muchitsch et al., 1998). In addition, AGP has been found to be protective against ischemia reperfusion in kidneys (Daemen et al., 2000). According to the results of this study AGP and AAT administered at reperfusion prevented apoptosis at 2 hours and 24 hours and exerted anti-inflammatory effects, as indicated by reduced renal TNF- $\alpha$  expression and neutrophil influx after 24 hours leading to improved renal function. Administration of AGP and AAT 2 hours after reperfusion resulted in a similar trend but without functional improvement. Moreover ischemia reperfusion elicited an acute phase response, as reflected by elevated serum AGP and serum amyloid P (SAP) levels after 24 hours, and increased hepatic acute phase protein mRNA levels after 18 hours of renal reperfusion. Other useful physiological effects of AGP include protection against brain edema formation after experimental stroke (Pichler et al., 1999), and injuries after intestinal ischemia (Williams et al., 1997).

### 3.3 Alpha1-antitrypsin (AAT)

Alpha1-Antitrypsin (AAT), also referred to as alpha<sub>1</sub>-proteinase inhibitor or SERPINA1, is the prototypical member of the SERPIN (an acronym for serine proteinase inhibitor) family of protease inhibitors (Carrell, 1986). The normal plasma concentration of AAT ranges from 0.9 to 1.75 g/L. AAT is present in all tissues and biological fluids including cerebrospinal fluid, saliva, tears, breast milk, semen, urine and bile. Over 100 alleles of AAT have been identified to date, of which at least 20 affect either the amount or the function of the AAT molecule *in vivo* (Gooptu & Lomas, 2009). The genes are inherited as co-dominant alleles (products of both genes can be found in the circulation). Individuals with plasma AAT values below 0.7 g/L are considered to be AAT deficient. In very rare circumstances individuals may inherit AAT null alleles which are characterized by very low levels of serum AAT. AAT deficiency typically results from point mutations causing a perturbation in protein structure and resulting in increased intracellular polymerization and retention in the cell of synthesis. Retained AAT polymers in the endoplasmic reticulum of hepatocytes can promote liver damage with a variable clinical presentation, from neonatal hepatitis to liver cirrhosis and hepatocellular carcinoma in adults. The lack of circulating protein predisposes to the development of early-onset COPD (Carrell & Lomas, 1997). AAT deficiency has also been associated with a number of other inflammatory diseases, although the association is only moderate or weak. These include bronchial asthma, bronchiectasis, systemic vasculitis, rheumatoid arthritis, inflammatory bowel diseases, intracranial and abdominal aneurysms, arterial dissections, psoriasis, chronic urticaria, mesangiocapillary glomerulonephritis, pancreatitis and pancreatic tumors, multiple sclerosis, and other occasionally reported conditions (Janciauskiene et al., 2011).

#### 3.3.1 Anti-inflammatory effects of AAT

##### 3.3.1.1 *In vitro*

It was previously thought that the primary function of AAT was to inhibit neutrophil elastase and proteinase 3 (Gettings, 2002). However, current studies demonstrate that AAT is an irreversible inhibitor for kallikreins 7 and 14 (Schapira et al., 1982; Luo et al., 2006), and that AAT also inhibits intracellular and cell-surface proteases. Matriptase, a cell surface serine protease involved in the activation of epithelial sodium channels, is one such protease (Tseng et al., 2008; Janciauskiene et al., 2008). AAT also inhibits the activity of caspase-3, an

intracellular cysteine protease which plays an essential role in cell apoptosis (Petrache et al., 2006). A recent study provides new evidence that AAT inhibits ADAM-17 activity, also called TACE (tumor necrosis factor- $\alpha$ -converting enzyme) (Bergin et al., 2010). We recently found that AAT inhibits calpain I which is implicated in numerous pathological conditions including Alzheimer's disease, demyelination events of multiple sclerosis, neuronal damage after spinal cord injury and hypoxic/ischaemic injury to brain, kidney and heart organs, and tumour development and invasion (Al-Omari et al., 2011). The ability of AAT to inhibit neutrophil calpain I was related to intracellular entry of AAT via lipid rafts (Subramaniam et al., 2010), a transient rise in intracellular calcium, increase in intracellular cholesterol esters, activation of the Rho GTPases, Rac1 and Cdc42, and extracellular signal-regulated kinase (ERK1/2). Furthermore, AAT caused a significant inhibition of non-stimulated as well as formyl-met-leu-phe (fMLP)-stimulated neutrophil adhesion to fibronectin, inhibited lipopolysaccharide (LPS)-induced IL-8 release and slightly delayed neutrophil apoptosis (Al-Omari M et al 2011). Recently, AAT was found to bind to IL-8 and to inhibit IL-8 interaction with its receptor CXCR1 (Bergin et al., 2010). AAT plays an immunoregulatory role, to inhibit neutrophil superoxide production, to enhance insulin-induced mitogenesis in various cell lines, and to induce IL-1ra expression (Bucurenci et al., 1992; She et al., 2000; Tilg et al., 1993). Findings that AAT enhances the synthesis of both transferrin receptor and ferritin revealed a role of AAT in iron metabolism (Graziadei et al., 1997). Interestingly, AAT has been shown to regulate heme oxygenase-1 activity in Alzheimer's disease patients (Maes et al., 2006). AAT has also been found to bind to the secreted enteropathogenic *Escherichia coli* proteins (EspB, EspD), thereby reducing their hemolysis of red blood cells (Knappstein et al., 2004). An interaction between AAT and *Cryptosporidium parvum* (Forney et al., 1996), a protozoan parasite, has been shown to inhibit *Cryptosporidium parvum* infection, suggesting a potential role for AAT in cryptosporidiosis. AAT inhibits endotoxin-stimulated TNF $\alpha$ , IL-6, IL-1 $\beta$  and enhances IL-10 expression in human monocytes, neutrophils, endothelial cells (Janciauskiene et al., 2007) AAT also expresses a broad anti-inflammatory profile in gene expression studies on primary human lung microvascular endothelial cells, including the suppression of self-induced TNF $\alpha$  expression (Subramaniam et al., 2008). Current studies provide further evidence that AAT therapy prolongs islet graft survival in transplanted allogeneic diabetic mice (Lewis et al., 2008) and show that AAT stimulates insulin secretion and protects  $\beta$ -cells against cytokine-induced apoptosis, and these effects of AAT also seem to be mediated through the cAMP pathway (Kalis et al., 2010). In view of these novel findings, it is suggested that AAT may act as an anti-inflammatory compound to protect  $\beta$ -cells under immunological attack in type 1 diabetes, and also raise the possibility of a new therapeutic strategy to potentiate insulin secretion in type 2 diabetes (Koulmanda et al., 2008).

AAT has also been found to express dual, time-dependent effects. Both *in vitro* and *in vivo* studies have shown that within a short time (2 to 4 hours) AAT amplifies endotoxin (LPS)-induced pro-inflammatory responses whereas after 18-48 hours AAT significantly inhibits LPS-induced TNF $\alpha$ , IL-1 $\beta$  and IL-8 expression and release, and enhances IL-10 synthesis (Subramaniam et al., 2010). This finding point to hypothesis that AAT can regulate the progression and resolution of the acute-phase reaction in a time-dependent manner. The overall view that arises from the current data is that short-term enhancement of LPS-induced cell activation may be the key mechanism by which the function of AAT is accomplished. In keeping with this, several *in vitro* and *in vivo* studies have been published in which prior initiation of an acute-phase response or administration of a specific APP has

been shown to switch the pro-inflammatory to the anti-inflammatory pathways necessary for the resolution of inflammation.

### 3.3.1.2 *In vivo*

AAT has been found to significantly protect against the lethality induced by TNF $\alpha$  or endotoxin in mice (Libert et al., 1996). The protection is optimal with a single dose of at least 300  $\mu$ g i.p. or 100  $\mu$ g i.v. given 2 hours before a lethal challenge, either with a low dose of TNF $\alpha$  in the presence of galactosamine or a higher dose of murine TNF alone. Under optimal conditions, the drop in body temperature, the release of liver transaminases, and the increase in clotting time are also inhibited. Similarly, Jie and co-workers (2003) have shown that pretreatment with AAT (120 mg/kg) can attenuate acute lung injury in rabbits induced with endotoxin. The pretreatment of AAT attenuated the deterioration of oxygenation, the reduction of compliance and the deterioration of other physiological and biochemical parameters mentioned above. In agreement, we currently found that pre-treatment with AAT protects mice against LPS-induced lung injury, inhibits LPS-induced pro-inflammatory genes and enhances the expression of genes associated with tissue repair and regeneration (unpublished data). In another model, Churg et al, (2001) have demonstrated that at 2 hours after dust administration, AAT completely suppressed silica-induced neutrophil influx into the lung and macrophage inflammatory protein-2 (MIP-2)/monocyte chemoattractant protein-1 (MCP-1) (neutrophil/macrophage chemoattractant) gene expression, partially suppressed nuclear transcription factor - $\kappa$ B (NF- $\kappa$ B) translocation, and increased inhibitor of NF- $\kappa$ B (I $\kappa$ B) levels. By 24 hours, PMN influx and connective tissue breakdown measured as lavage desmosine or hydroxyproline were still at, or close to, control levels after AAT treatment. In the recent study by Lewis et al., (2005), diabetic mice were grafted with allogeneic islets and treated with AAT monotherapy. After 14 days of treatment, mice remained normoglycemic and islet allografts were functional for up to 120 treatment-free days. After graft removal and retransplantation, mice accepted same-strain islets but rejected third-strain islets, thus confirming that specific immune tolerance had been induced. Explanted grafts exhibited a population of T regulatory cells in transplant sites. Grafts also contained high levels of mRNA for foxp3, cytotoxic T lymphocyte antigen-4, TGF- $\beta$ , IL-10, and IL-1 receptor antagonist, but expression of pro-inflammatory mediators was low or absent. After implantation of skin allografts, AAT-treated mice had greater numbers of foxp3-positive cells in draining lymph nodes compared with control treatment mice. Moreover, dendritic cells exhibited an immature phenotype with a decrease in the activation marker CD86. Although the number of CD3 transcripts decreased in the DLNs, AAT did not affect IL-2 activity *in vitro*. AAT monotherapy provides allografts with anti-inflammatory conditions that favor development of antigen-specific T regulatory cells. AAT treatment in humans is known to be safe, hence its use during human transplantation may be considered.

### 3.3.1.3 Augmentation therapy with AAT in patients

Based on the protease-antiprotease hypothesis, augmentation therapy was introduced for COPD patients with severe (ZZ) AAT deficiency during the 1980s. The major concept behind augmentation therapy was that raising the levels of blood and tissue AAT would protect the lungs from continuous destruction by proteases, particularly neutrophil elastase. Whether this biochemical normalization of AAT levels influences the pathogenic processes of COPD is still under debate. However, recent results do suggest that augmentation therapy may have beneficial effects including reducing the frequency of lung infections and

reducing the rate of decline of lung function. Several non-randomized observational studies and one meta analysis on the clinical effectiveness of AAT augmentation treatment showed a favorable result regarding lung function (FEV<sub>1</sub>) in AAT-deficient COPD patients with moderate disease undergoing augmentation therapy (Chapman et al, 2009; Stockley et al, 2009).

Clinical studies provide evidence that augmentation therapy with AAT reduces the incidence of lung infections in patients with AAT deficiency-related emphysema and reduces levels of the chemoattractant leukotriene B<sub>4</sub>. A study by Lieberman and co-workers (2000) showed that augmentation therapy with AAT is associated with a marked reduction in the frequency of lung infections in the majority of patients. Most patients reported a frequency of three to five infections per year before starting AAT therapy, which dropped to zero to one infection per year while receiving AAT. In two patients with a prior history of continuous lung infections, AAT therapy was associated with the complete absence of infection in one patient and with one to two infections per year in the second. It was also reported that aerosolized AAT suppresses bacterial proliferation in a rat model of chronic *Pseudomonas aeruginosa* lung infection (Kueppers et al., 2011)

Several case reports support the beneficial effects of AAT augmentation therapy in other clinical conditions. Two ZZ AAT Spanish sisters with fibromyalgia experienced a rapid, progressive, and constant control of their fibromyalgia symptoms during AAT augmentation therapy (Blanco et al, 2006). Another report described a 21yr old a ZZ AAT female with septal panniculitis which was poorly responsive to dapsone and doxycycline treatment, who was successfully treated with intravenous infusion of AAT (Gross et al., 2008). Recently Chowdhury and collaborators have described a 33-year-old ZZ AAT woman with rapidly progressing panniculitis and extensive skin necrosis. Augmentation therapy with AAT proved to be life saving (Chowdhury et al., 2002). Cutaneous vasculitis in a 49-year-old man with AAT deficiency persisted despite treatment with colchicine, prednisone, and antibiotics, but has been effectively controlled with the administration of AAT (Dowd et al., 1995). In addition, Griese et al, 2007 examined the effect of 4 weeks of AAT inhalation on lung function, protease-antiprotease balance and airway inflammation in Cystic Fibrosis (CF) patients. In a prospective, randomised study, 52 CF patients received a daily inhalation of 25 mg AAT for 4 weeks targeting their peripheral or bronchial compartment. Inhalation of AAT increased AAT levels and decreased the levels of elastase activity, neutrophils, pro-inflammatory cytokines and the numbers of *P. aeruginosa*. However, it had no effect on lung function. No difference was found between the peripheral and bronchial mode of administration. In conclusion, although no effect on lung function was observed, the clear reduction of airway inflammation after AAT treatment may precede pulmonary structural changes.

### **3.4 Other examples of multifunctional APPs**

#### **3.4.1 Haptoglobin (Hp)**

Haptoglobin (Hp) is homologous to the serine proteases of the chymotrypsinogen family but has no serine protease activity (Kurosky et al, 1980). Hp exists in two allelic forms in the human population, so-called Hp1 and Hp2, the latter one having arisen due to the partial duplication of the Hp1 gene. Plasma haptoglobin levels change during life, with Hp levels in healthy infants being lower than in healthy adults. In healthy adults, the haptoglobin concentration in plasma is 0.38-2.08 g/L (Javid, 1978). Hp, by binding hemoglobin and



removing it from the circulation, prevents iron-stimulated formation of oxygen radicals and has an important role as an antioxidant (Sadrzadek & Bozorgmehr, 2004). Both *in vitro* and *in vivo* studies have established that subjects with the Hp1-1 phenotype are more likely to resist cellular oxidative stress than those with the Hp2-2 phenotype, with Hp2-1 being intermediate (Tseng et al., 2004). Hp has been shown to play an antioxidant/anti-inflammatory role, to contribute to neutrophil activation, to maintain reverse cholesterol transport, to modulate the inhibition of cyclooxygenase and lipoxygenase, and to inhibit monocyte and macrophage functions amongst other activities. For instance, Hp inhibited respiratory burst activity in neutrophils stimulated with fMLP, arachidonic acid, and opsonized zymosan (Oh et al., 1990), inhibited phagocytosis and reduced intracellular bactericidal activities of granulocytes (Rossbacher et al., 1999). Moreover, Hp has been found to stimulate the formation of prostaglandin E2 in osteoblast-like cells, and to potentiate the stimulatory effect of bradykinin and thrombin on PGE2 formation (Frohlander et al., 1991, Lerner & Frohlander, 1992). Hp has also been shown to support angiogenesis (Cid et al., 1993). It has been suggested that the increased levels of Hp found in chronic inflammatory conditions may play an important role in tissue repair. In systemic vasculitis, Hp might also compensate for ischemia by promoting the development of collateral vessels. By enhancing the Th1 cellular response, Hp establishes Th1-Th2 balance *in vitro* (Arredouani et al., 2003). Hp also inhibits epidermal Langerhans cells in the skin and might have a role in preventing T cell-dependent skin disorders (Pagano et al., 1982). In addition, Hp inhibits cathepsin B and L and decreases neutrophil metabolism and antibody production in response to inflammation (Oh et al., 1990, Pagano et al., 1982). Iron is one of the essential elements for bacterial growth. However, once bound to Hp, hemoglobin and iron are no longer available to bacteria that require iron, such as *Escherichia coli*. Indeed, Eaton and collaborators showed that a fatal consequence of intra-peritoneally injected *Escherichia coli* and hemoglobin in rats can be prevented by the administration of Hp (Eaton et al., 1982). In the lungs, Hp is synthesized locally and is a major source of antimicrobial activity in the mucous layer and alveolar fluid and also has an important role in protecting against infection (Yang et al., 1995). Hp-hemoglobin complexes in human plasma inhibit endothelium dependent relaxation (Edwards et al., 1986).

### 3.4.2 Serum amyloid A (SAA)

SAA structurally resembles an apolipoprotein, and is mainly transported in association with lipoprotein particles, particularly high-density lipoprotein (HDL) (Eriksen & Benditt, 1980). The SAA concentration of serum/plasma samples ranges from 1-5 µg/ml. During an acute phase response, SAA becomes the main apolipoprotein on HDL, and the displaced Apo-AI then becomes available to extract cellular free cholesterol upon interacting with cell-surface (Tam et al., 2008). For this reason, and because SAA itself may also extract cholesterol from cells (Stonik et al., 2004), it is thought that SAA plays a role in cholesterol metabolism and atherosclerosis (Jahangiri et al., 2009). Whether SAA is pro- or anti-atherogenic is not yet clear, since putative beneficial effects on cholesterol metabolism may be mitigated by effects on inflammation- a known risk factor for atherosclerosis (Libby et al., 2002). Some of the effects described for SAA seem to be minimized or abolished by its association with HDL (Barter et al., 2004). The very high expression of SAA gives rise to a completely different pathological problem: the continuous high expression of SAA is the prerequisite for the development of secondary amyloidosis, caused by the conformational change of SAA in an

insoluble proteolytic peptide, AA, that deposits as insoluble plaque in major organs (Malle & De Beer, 2003). In attempt to understand the biological role of SAA and of its association with HDL, it was demonstrated that SAA is able, for instance, to induce leukocyte migration (Connolly et al, 2010) and collagenase (Brinckerhoff et al., 1989), and to inhibit the TNF $\alpha$  and IL-1 $\beta$ - induced hypothalamic PGE2 synthesis (Tilg et al., 1993). Several other SAA activities have been described including increasing cleavage of triacylglycerols into glycerol and fatty acids on HDL<sub>3</sub> by enhancing the activity of secretory phospholipase (Sullivan et al., 2009). SAA also directly acts on the cholesterol molecule by decreasing its esterification, and increases its uptake by hepatocytes (Steinmetz et al., 1989). Recent studies reveal that serum SAA also has a dual role in modulating neutrophil function. SAA induces the differentiation of interleukin 10 (IL-10)-secreting neutrophils via signaling dependent on the G protein-coupled protein FPR2 (formyl peptide receptor 2), but also promotes the interaction of neutrophils with invariant natural killer T cells (iNKT cells), restoring T cell proliferation by abolishing IL-10 secretion. The final process is dependent on the antigen-presenting molecule CD1d and co-stimulatory molecule CD40 and results in less production of IL-10 and enhanced production of IL-12, thus limiting the suppressive activity of neutrophils (De Santo et al., 2010). SAA may affect inflammatory responses by activating its putative receptor on neutrophils (FPRL1), leading to increased production of IL-8 (He et al., 2003). SAA is also thought to be able to activate TLR2- and TLR4- dependent signaling (Cheng et al., 2008). Recent reports suggest that SAA may also play a role in host defense, notably in the clearance of Gram-negative bacteria. Shah et al. demonstrated that SAA binds to the outer membrane protein A of *Escherichia coli*, which facilitates bacterial clearance by phagocytes (Shah et al., 2006). Such a bactericidal effect of SAA is intriguing in light of the reported expression of SAA in intestinal epithelia of rodents and humans, since these cells are exposed to many gram-negative bacteria (Berg, 1996). Intestinal epithelial expression of SAA protects from colitis by reducing bacterial load (Eckhardt et al., 2010).

### 3.5 Importance of the co-ordinated expression and biological activity of APPs

APP expression represents one of the most important and highly effective mechanisms of innate immunity. The wide range of defensive and repair functions fulfilled by APPs not only reduces pathologic damage, but also acts as a homeostatic mechanism. On the other hand APPs may also play pro-inflammatory roles and produce detrimental effects. Importantly, changes in different APPs occur at different rates and to different degrees. Ceruloplasmin and the complement components C3 and C4 exhibit relatively modest acute-phase behaviour (typically about a 50% increases). Concentrations of Hp, AGP, AAT, ACT, and fibrinogen ordinarily increase about 2–5-fold. CRP and SAA are normally present in only trace amounts, but may exhibit a dramatic increase (1000-fold or more) in individuals with severe infections. In contrast, plasma concentrations of negative APPs such as albumin, transferrin, transthyretin, alpha-fetoprotein, typically decrease during the acute-phase response. These orchestrated alterations in specific APP production during inflammatory states are not completely understood. However, the known functional capabilities of many of the APPs leads to the logical speculation that specific changes in APP expression serve useful purposes in inflammation, healing or adaptation to infection or injury. Moreover, current knowledge clearly indicates that during the acute phase reaction a single APP can play multiply roles, and that diverse APPs can possess very similar biological activities (Figure 3). The combined action of two or more APPs may produce effects that no single protein would be able to achieve.

For example, diverse APPs like AAT, AGP, Hp, and CRP can have similar anti-inflammatory and immuno-modulatory roles in experimental models *in vitro* and *in vivo*. AAT which is an archetypal member of the SERPIN superfamily, a main inhibitor of neutrophil elastase, and AGP a member of the lipocalin family, a group of proteins sharing a similar three-dimensional structure capable of binding and carrying hydrophobic molecules, both inhibit cell apoptosis, inhibit neutrophil chemotaxis and adhesion, inhibit neutrophil activation and induce macrophage-derived interleukin-1 receptor antagonist release, and protects mice from endotoxin-induced septic shock. Similarly, CRP (opsonin) and Hp (hemoglobin binder) inhibit neutrophil activation, including chemotaxis and superoxide production and degranulation. Thus, it seems that all these APPs, specifically in neutrophil models, show very similar effects. It cannot be excluded that these proteins may have more common characteristics and biological effects however the lack of high quality purified endotoxin or contaminant-free proteins limits expanding our current understanding. A more detailed knowledge of the separate and combined APP functional pathways is essential in order to prevent or control development of various pathological conditions as well as to develop safe and effective anti-inflammatory therapies.

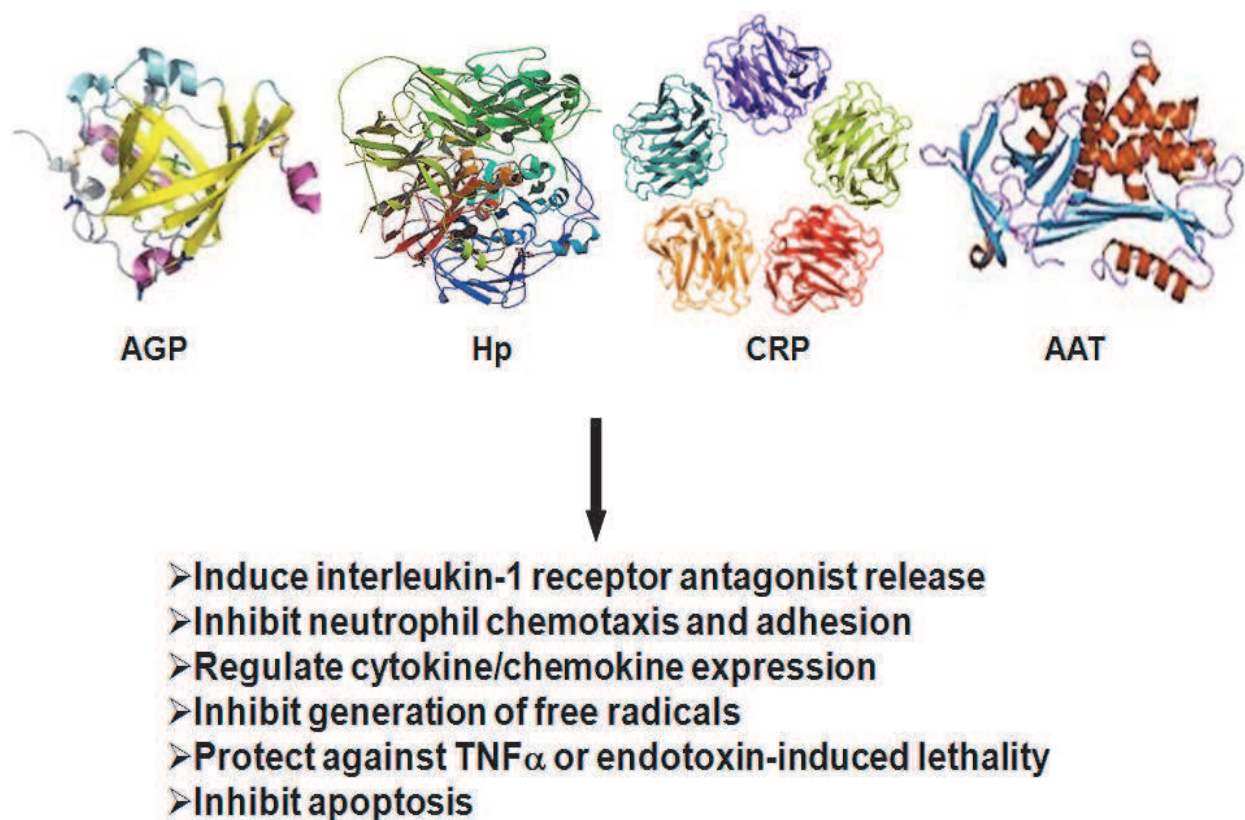


Fig. 3. Diverse APPs express similar anti-inflammatory activities.

#### 4. APPs structure-function relationship

Post-translational modifications of proteins can regulate their function by causing changes in protein activity, their cellular location and dynamic interaction with other proteins. Virtually all proteins function by interacting with other molecules and these interactions can have numerous effects on the physical, structural, biochemical and functional properties of

proteins. There are also different types of interactions on a protein-protein and protein-environment level which lead to complex formation, protein degradation, self-assembly or other modifications in protein structures, such as oxidation. The ability to undergo post-translational conformational changes is crucial for the physiological function of many proteins, including APPs. Similarly, such changes could alter both physicochemical and functional properties of the proteins with potential unforeseen physiological or pathological consequences. Conformational modification may lead to an acquired deficiency of specific APP, but also to the generation of new molecular forms with potent biological activities. The altered forms of APPs are detected in tissues and fluids recovered from inflammatory sites, but the important questions of how they are generated, what their biological activities are, and which of them are directly linked to pathological processes and/or may be useful markers to characterise disease states, remain to be answered. Glycosylation is one of the most important post-translational modifications of APPs, and has been widely acknowledged as one of the most important ways to modulate both protein function and lifespan. Glycosylation of APPs which is partially regulated by cytokines may be distinct in disease and provide useful disease markers.

#### 4.1 Glycosylation of APPs

The N-glycan chains of APP glycoproteins differ in their branching, showing bi-, tri-, and tetra-antennary structures. Inflammatory states are usually associated with changes in the glycosylation profile of APPs. It has been demonstrated that there is an increased concentration of the conA-reactive microheterogeneous forms of APPs in patients with acute inflammation, *e.g.* acute bacterial infections and burns. Conversely, a shift in the population of APPs towards those with a higher content of conA-nonreactive tri- and tetra-antennary carbohydrates has been shown in the sera of patients with chronic inflammatory diseases (*e.g.* chronic bacterial infections, rheumatoid arthritis, ankylosing spondylitis) (Hrycaj *et al.*, 1996; Stibler *et al.*, 1998). An intriguing question is whether the changes in the glycosylation profile of APPs might affect their biological activity and/or function.

##### 4.1.1 AGP

AGP purified from human plasma consists of a mixture of AGP with different degrees of sialylation and glycosylation. It has been demonstrated that microheterogeneity variants of AGP differ with regard to their immunomodulatory properties: the conA-nonreactive variant of AGP is more effective in modulating of lymphocyte proliferation than conA-reactive AGP variants. It has also been shown that AGP has an affinity for E-selectin and that this affinity can be changed by *in vitro* fucosylation of AGP (Mackiewicz *et al.* 1987). The highly branched and sialylated form of AGP which is the ligand for cell adhesion molecules such as E-selectin and P-selectin, inhibits migration of neutrophils, monocytes and T-cells, and modifies complement activity (Hrycaj *et al.*, 1993).

Marked changes in AGP glycoforms are observed during acute-phase reactions. The changes comprise alterations in branching pattern as revealed by reactivity with concanavalin A and the fucose-binding lectin (Elliott *et al.*, 1997). Thus analysis of sialo- and asialo-oligosaccharides of AGP as well as its glycoforms is important for understanding the biological roles of AGP (Kakehi *et al.*, 2002; Sei *et al.*, 2002). For example, the asialylated carbohydrate-deficient variant of AGP appears mainly in sera of patients after acute inflammation, infection, burns or other severe tissue damage (Fournier *et al.*, 2000).



The expression of a sialyl Lewis x{sLe<sup>x</sup>, NeuAcα2-3Galβ1-4(Fuca1-3)GlcNAc-} portion in the carbohydrate chains of human AGP molecules has been shown to be of importance in inflammation. De Graaf et al. (1993) found a direct relationship between the reactivity of human AGP to the fucose-specific binding lectin (*Aleuria aurantia*) and staining of human AGP by anti-sLe<sup>x</sup> monoclonal antibody under healthy and disease conditions.

In a recent study a recombinant form of sialyl Lewis<sup>x</sup>(sLe<sup>x</sup>)-bearing (sAGP) was administered intravenously to rats after 50 min of intestinal ischemia just before 4 h of reperfusion. A non-sLe<sup>x</sup>-bearing form of AGP (nsAGP) was used as control. sAGP-treated animals had a 62% reduction in remote lung injury, assessed by <sup>125</sup>I-albumin permeability, compared with those treated with nsAGP. There was a reduction in pulmonary myeloperoxidase levels in sAGP-treated rats compared with nsAGP-treated rats. Complement-dependent intestinal injury, assessed by <sup>125</sup>I-albumin permeability was reduced by 28% in animals treated with sAGP compared with those treated with nsAGP leading the authors to conclude that sAGP ameliorates both complement- and neutrophil-mediated injury (Williams et al., 1997).

The changes in the glycosylation pattern of AGP has been found in patients with ulcerative colitis (Ryden et al., 1997), and in patients with various liver diseases (alcoholic liver disease, hepatitis B, hepatitis C cirrhosis). For example, hyperfucosylation occurred in all cases of liver disease, although the hepatitis B and C samples showed a more significant increase in comparison with the others. Additionally N-acetylgalactosamine (GalNAc) was detected in the majority of the hepatitis C samples, which was unexpected since this monosaccharide is not a usual component of the N-linked oligosaccharide chains (Anderson et al., 2002).

In the group of Type I diabetic patients with increased urinary albumin excretion, a significant increase in alpha3-fucosylation of AGP could be detected. Therefore, the increased alpha3-fucosylation of AGP can be used as a putative marker for the development of vascular complications in Type I diabetic patients (Poland et al., 2001).

AGP and its derivatives, prepared by sequential enzymatic cleavage of the carbohydrate units, were tested for their nerve-growth-promoting activities with explants of whole dorsal root ganglia from chick embryos. The results showed that the AGP derivatives with terminal galactose, N-acetylglucosamine, or mannose have marked neurite-promoting activities (Liu et al., 1988).

#### 4.1.2 Other APPs

AAT has a molecular weight of  $M_r$  52 000, and is ~12% carbohydrate by weight. The AAT molecule carries a high negative charge because of sialic acid residues on the three complex glycans attached to asparagine residues 46, 83, and 247. Isoelectric focusing of plasma AAT leads to the detection of eight bands, which are numbered M1 to M8 (anodal-low pH to cathodal-high pH). The bands M4 and M6 are the most abundant of the isoforms, making up 40% and 34% of the total plasma AAT, respectively, whereas M3 and M5 are present in only trace amounts (Mills et al., 2001). Fucosylated AAT was analyzed individually and in combination with the currently used marker, alpha-fetoprotein, for the ability to distinguish between a diagnosis of cirrhosis and hepatocellular carcinoma (HCC). The levels of fucosylated AAT were significantly higher in patients with HCC compared to those with cirrhosis (Wang et al., 2009). Remarkably, non-glycosylated AAT showed shorter half life and no ability to interact with IL-8 than compared to glycosylated form of AAT (Bergin et al., 2010). This suggests an importance of AAT glycosylation for its biological activities.

ACT (alpha 1-antichymotrypsin), a serine anti-protease with specificity against neutrophil cathepsin G, is homologous with AAT, plasminogen activator inhibitor and angiotensinogen. ACT is an APP with carbohydrate content 24% of molecular weight. As for other glycoproteins, micro-heterogeneity of ACT may be ascribed to differences in carbohydrate structure, and indeed different patterns of ACT micro-heterogeneity has been shown in different diseases including cancer, heart failure and rheumatoid arthritis (Saldova et al., 2008; Kazmierczak et al., 1995; Havenaar et al., 1998). A low content of terminal GlcNAc glycans and sialic acid in peripheral ACT has been suggested as a marker of progression in Alzheimer's disease (Ianni et al., 2010). The changes in patterns of glycosylation of transferrin (Tf) towards highly branched glycans have been observed in iron deficiency anaemia, rheumatoid arthritis, liver cirrhosis or in physiological state such as pregnancy. Differences in glycosylation of Tf seems to alter the metabolism of iron (Yang et al., 2005; van Pelt J et al., 1996; Dupre et al., 2001). Changes in the glycosylation pattern of major serum APPs such as Hp, AGP, AAT, Tf and alpha-fetoprotein have been recently shown in patients with pancreatic cancer and chronic pancreatitis (Sarrats et al., 2010)

#### 4.2 Cleaved forms of AAPs

The cleaved modifications of APPs may lead to a functional deficiency of the protein, but the cleaved forms of APPs may themselves express new biological activities. For example, antithrombin which functions as an inhibitor of thrombin and other enzymes, has potent antiangiogenic and antitumour activity in its cleaved conformation.

##### 4.2.1 CRP

CRP comprises five identical, non-covalently bound subunits of 206 amino acids (23,017 daltons) arranged in cyclic symmetry (Oliveira et al., 1979). One side of the pentamer participates in binding ligands such as phosphorylcholine, and the other side binds effector molecules such as C1q. When CRP is exposed to denaturing conditions in the presence of a chelating agent, the CRP pentamer is altered to form both individual subunits and aggregates (Gotschlich & Edelman, 1967) designated as modified-CRP (mCRP) (Potempa et al., 1983). The half-life of mCRP in the circulation is <5 min in mice (Motie et al., 1998). These findings indicate that the transport of mCRP from circulation to various sites in the body most likely to be faster than pentameric CRP. mCRP displays antigenic, electrophoretic, and ligand binding reactivities distinct from pentameric CRP (Potempa et al., 1987). Currently has been reported that mCRP is much more potent than pentameric CRP in binding to modified LDL (Singh & Fudenberg, 2009).

Since extravasation and activation of neutrophil granulocytes are essential in the inflammatory response, the effects of CRP on these cells are of particular importance. Stimulation of neutrophils activates a membrane-associated serine protease which leads to the cleavage of biologically active peptides from CRP. CRP peptides 77-82 and 201-206 have been found to inhibit neutrophil chemotaxis to fMLP *in vitro* and to diminish neutrophil influx and protein leakage into alveoli after fMLP induced inflammation in mice (Zouki et al., 1997).

Neutrophil extravasation into inflamed or injured areas involves a complex interaction of leukocytes with endothelial cells *via* regulated expression of surface adhesion molecules. The initial attachment of neutrophils to endothelium is mediated by L-selectin (CD62L) (Díaz-González et al., 1995; Walcheck et al., 1996). L-Selectin is constitutively expressed by

neutrophils and is released from neutrophils by proteolytic cleavage within minutes after activation with a concomitant upregulation of Mac-1 (CD11b/CD18). The monomeric CRP (but not nCRP) has been found to up-regulate CD11b/CD18 expression and extracellular signal regulated kinase (ERK) activity, suggesting that mCRP may participate in the promotion of neutrophil adhesion to endothelial cells (Zouki et al., 2001). In contrast to CRP, mCRP induces IL-8 secretion in neutrophils (Khreiss et al., 2005) and human coronary artery endothelial cells (Devaraj S et al., 2004), promotes neutrophil-endothelial cell adhesion (Zouki et al., 2001), and delays apoptosis of human neutrophils (Schwedler et al., 2006).

mCRP binds to a number of different ligands to CRP and also exhibits a different set of biologic activities. More recently, it has been shown that mCRP has profound inhibitory effects on tumor growth and metastatic ability of an adenocarcinoma in mice (Kresl et al., 1999). Cross-reactive epitopes of mCRP have also been detected in the fibrous elements of blood vessels and lymphatic organs suggesting that mCRP may be present in extracellular spaces (Samberg et al., 1988). Since mCRP can self-associate into a matrix-like structure (Motie et al., 1996), the naturally occurring antigen may be self-associated aggregates of mCRP, and thus represent a tissue-based as opposed to a blood based form of CRP. mCRP, but not CRP, binds immune complexes (Khreiss et al., 2004), potentiates the activities of activated leukocytes and platelets (Zouki et al., 2001) and stimulates megakaryocyte differentiation in mice (Potempa et al., 1996).

Additionally, both CRP conformations interact differently with components of the complement cascade. M Mihlan et al., 2009, identified for the first time that mCRP, but not pCRP, has a complement-modulating effect. mCRP recruits the complement inhibitor Factor H to the surface of damaged cells or particles, and enhances local complement inhibition both in the fluid phase and on the cellular surface. Thus, by recruiting C1q to the surface of damaged cells, mCRP triggers complement activation resulting in the formation of C3 convertases and C3b surface deposition. However, by binding inhibitor Factor H and enhancing the inhibitory activity, further complement activation, amplification, cytokine release, C9 deposition and terminal membrane attack assembly are inhibited. Furthermore, the phagocytosis of apoptotic particles is increased. This shows how CRP can contribute to an anti-inflammatory scenario and explains how mCRP contributes to the safe removal of damaged apoptotic particles and necrotic cells which may be relevant for diseases such as atherosclerosis (Zipfel & Skerka, 2009).

Using immunofluorescence microscopy (Eisenhardt et al., 2009) have shown the generation of mCRP from CRP on adherent activated platelets, together with the immunohistological colocalization of mCRP with the CD41 antigen in atherosclerotic plaques. These findings suggest that in atherosclerosis mCRP is generated from circulating CRP, and mCRP is then deposited at the atherosclerotic plaque, exerting strong proinflammatory effects (Agrawal et al., 2010).

Despite the growing interest in mCRP, it remains unclear how mCRP is generated and whether it contributes to inflammatory processes such as atherosclerosis. Thus, it appears that both CRP and altered forms of CRP, including mCRP, may each serve important distinct functions in the acute phase and the host defense response to trauma and infectious agents. Therefore, it is important to ascertain the extent of conversion and reversion of CRP to mCRP and possible intermediate forms to help define and understand the biological function(s) of the CRP molecule.

#### 4.2.2 AAT

AAT is another example of AAP which can be found *in vivo* in cleaved forms. Cleaved forms of AAT are known to occur when AAT forms an inhibitor complex with serine protease which subsequently dissociates or is degraded, or when it is cleaved by non-target proteases, usually at sites in its reactive loop, without the formation of stable inhibitor complexes. Such cleavage generates a 4 kDa carboxyl-terminal fragment of 36 residues, which remains non-covalently bound to the cleaved AAT. Human cathepsin L, collagenase and stromelysin, and bacterial proteinases from *Staphylococcus aureus*, *Serratia marcescens* metalloproteinase and *Pseudomonas aeruginosa* elastase (Rapala-Kozik et al., 1999) all fall into the latter class and exhibit efficient AAT degrading activity. Recent studies established AAT as a key substrate for gelatinase B (MMP-9) *in vivo* (Liu et al., 2000). It has long been hypothesized that neutrophil elastase-mediated tissue destruction in certain inflammatory diseases such as emphysema, is caused by an imbalance in the ratio of elastase to AAT (Weiss, 1989). The studies of Liu and collaborators provide *in vivo* evidence that this mechanism, mediated by the proteolytic inactivation of AAT by gelatinase B, underlies the pathology of the inflammatory skin disorder called bullous pemphigoid which is initiated by deposit formation at the basement membrane (Jordon et al., 1985). Generated cleaved forms of AAT may contribute to the later phase of polymorphonuclear leukocyte infiltration. Indeed, cleaved AAT was shown to form fibrillar structures and to be a potent chemoattractant for monocytes (Janciauskiene et al., 1995; Banda et al., 1988).

Fragments of AAT have been found in human bile, atherosclerotic plaque, urine and plasma, and have been shown to regulate lipid metabolism, inflammatory cell activation and even to inhibit human HIV-1 expression. It has recently been demonstrated that a specific 20-residue fragment of AAT (C-terminal peptide, residues 377–396, referred to as VIRIP) binds to the gp41 fusion peptide of HIV-1 and prevents the virus from entering target cells, thereby inhibiting HIV-1 infection (Münch et al., 2007). These findings suggest that AAT may play a protective role in HIV-1-infected individuals (Forssmann et al., 2010). We found that the C-terminal fragment of AAT, C-36 peptide, corresponding to residues 359–394 suppresses bile acid synthesis *in vitro* and *in vivo*. The DNA element involved in the C-36-mediated regulation of 7 $\alpha$ - and 12 $\alpha$ -hydroxylase promoters mapped to the alpha1-fetoprotein transcription factor site in both promoters. The C-36 peptide prevented binding of FTF to its target DNA recognition site by direct interaction with FTF (Gerbod-Giannone et al., 2002). Hence, the effects of AAT peptides as potential drugs for systemic lupus erythematosus are being studied (Shapira et al., 2011).

#### 4.3 Oxidized and nitrosylated forms of APPs

Under conditions of compromised oxygen supply, such as occurs in injury, infection or malignancy, oxygen species with free unpaired electrons are generated during mitochondrial electron transport. Referred to as highly reactive oxygen species (ROS), their production causes damage to cell membranes and macromolecules (lipids, proteins and DNA) (Valko et al., 2007). Oxidative changes of protein structure can have a wide range of downstream functional consequences, such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity. It is now recognized that oxidation of proteins plays an essential role in the pathogenesis of an important number of degenerative diseases. Compared to controls more oxidized proteins are found in tissues from animals and patients



suffering from Alzheimer's disease, rheumatoid arthritis, atherosclerosis or amyotrophic lateral sclerosis (Banfi et al., 2008). For example, in plasma from patients with Alzheimer's disease the most obviously oxidized proteins were identified as isoforms of AAT and fibrinogen  $\gamma$ -chain precursor proteins. Both these proteins have been suggested to be involved in inflammation processes in Alzheimer's disease (Choi et al., 2002).

S-nitrosylation is another important post-transcriptional modification of the APPs and their peptides, which may be involved in NO-dependent signal transductions. For example, a recent study indicates that S-nitrosylation can be effectively catalyzed by the copper ion of ceruloplasmin, a major multicopper-containing plasma protein, under physiological conditions (Mani et al., 2004). It is now also conceivable that nitrosylation of thiols is involved in modulation of various biological events, such as functional regulation of receptors, ion channels and synaptic vesicle fusion (Miyamoto et al., 2000).

#### 4.3.1 AAT

Oxidized AAT has been found in inflammatory exudates at levels of ~5–10% that of total AAT (Wong & Travis, 1988), and AAT recovered from BAL fluid in smokers is 40% less active compared with non-smokers due to oxidation of the P1 methionine (methionine 358 at the active site) to methionine sulfoxide (Carp et al., 1982). The oxidation of AAT by cigarette smoke or free radicals in vivo has been proposed as a mechanism by which elastin and thus alveolar destruction occurs in COPD (Gadek et al., 1979; Beatty et al., 1984). Oxidation of the P1 methionine (methionine 358) or methionine 351 to methionine sulfoxide significantly reduces the ability of AAT to inhibit neutrophil elastase (Taggart et al., 2000). Hydrogen peroxide in cigarette smoke and N-chloroamines and hypochlorous acid in neutrophils can oxidize and inactivate AAT (Ossanna et al., 1986; Scott et al., 1999). Thus the oxidation of AAT by cigarette smoke or free radicals in vivo could lead to a relative deficiency of elastase inhibitors and has been suggested as a mechanism contributing to the development of emphysema and other diseases such as cystic fibrosis, adult respiratory distress syndrome, and bronchiectasis (McGuire et al., 1982; Roum et al., 1993; Izumi-Yoneda et al., 2009). In addition, oxidative inactivation can enhance the susceptibility of AAT to proteolytic attack, particularly by neutrophil elastase and certain bacterial proteases, including thermolysin, aureolysin, serralysin, pseudolysin, *Staphylococcus aureus* serine proteinase, streptopain and periodontain. Thus, oxidation and proteolytic processes in some cases may work synergistically. Moreover, oxidized AAT by itself amplifies and perpetuates the inflammatory processes by directly affecting the functional activities of structural and inflammatory cells or by interacting with other molecules such as IgA and low-density lipoproteins. It has been shown that oxidized AAT significantly induces the production of IL-8 and MCP-1 from a lung epithelial cell line (A549 cells) and in a time- and dose-dependent manner and attracts macrophages (Li et al., 2009). Release of oxidants by these inflammatory cells could oxidize newly synthesized AAT, which has diffused into the airways and would perpetuate the cycle. This process may be amplified by oxidized AAT induction of MCP-1 synthesis from monocytes (Moraga & Janciauskiene, 2000). These pathways may be one explanation as to why inflammation persists after smoking cessation in chronic obstructive pulmonary disease (Retamales et al., 2001). A complex of oxidized AAT and LDL was isolated from human plasma and was detected in human atherosclerotic lesions of the coronary artery (Donners et al., 2005). The product of AAT nitrosylation, S-NO-AAT, has been shown to have multiple biological functions, including potent anti-microbial activity

and inhibition of cysteine protease. In a study by Ikebe and co-workers (Ikebe et al., 2000) it was suggested that S-NO-AAT exerted a potent cytoprotective effect in liver ischemia-reperfusion injury by maintaining the tissue blood flow, inducing hemeoxygenase 1, and suppressing neutrophil-induced liver damage and apoptosis. Interestingly, it was verified that S-NO-AAT expressed similar serine protease inhibitory activity towards pancreatic trypsin and pancreatic and neutrophil elastase as native AAT. Thus, S-NO-AAT may function not only as a simple NO (nitroso) donor but also as a protease inhibitor with a broad inhibitory spectrum.

#### 4.4 Complexed and polymerized forms of APPs

Function of some APPs is dependent on their complex formation with other molecules and/or on their polymerization. For example, CRP binds to phosphocholine, as well as phosphoethanolamine, microbial surface proteins, chromatin and other ligands (Thompson et al., 1999; Agrawal et al., 2002, Okemefuna et al, 2010). CRP activates the classical pathway of complement by binding to C1q, but its binding to CFH in the alternative pathway has turned out to be more controversial. CRP-ligand interactions lead to the recognition of damaged or apoptotic cells and bacterial pathogens.  $Ca^{2+}$  and phosphocholine bind to the B (binding) face of the pentameric ring, whereas the other A ( $\alpha$ -helix) face binds to macromolecular ligands such as C1q. The complex between Hp and Hemoglobin has been studied for decades and represents one of the strongest non-covalent interactions reported in plasma (Nielsen & Moestrup, 2009). Hp also binds apolipoprotein A-I (ApoA-I), and impairs its stimulation of lecithin: cholesterol acyltransferase (LCAT) which plays a major role in reverse cholesterol transport (Cigliano et al., 2009). Hp binds and protects ApoE from oxidative damage (Salvatore et al., 2009). Some APPs are regulated by co-factors which are needed to expose or maintain the functional conformation of the APP. The best example of this phenomenon is antithrombin, which is activated by heparin through induced and transmitted conformational changes that stabilise the proteinase-sensitive active site (Jodan et al., 1987). Another APP, PAI-1, which normally exists in a latent form, can be maintained in its functional form in the presence of plasma vitronectin (Wiman et al., 1988). As well as stabilising PAI-1 in the active conformation, vitronectin also alters the specificity of PAI-1, making it an efficient inhibitor of thrombin. The finding that active PAI-1 specifically inhibits integrin attachment to vitronectin (Stefansson & Lawrence, 1996) further illustrates the unique functional interdependence that exists between PAI-1 and vitronectin. Complexes between APPs and other proteins are also found to be associated with specific diseases. For example, in sera from patients with myeloma and Bence-Jones proteinuria, complexes between AAT and the kappa light chain of immunoglobulins were detected (Laurell et al., 1974). In plasma from diabetic subjects, complexes between AAT and factor Xia, and AAT and heat shock protein-70 (HSP70), as well as glycosylated forms of AAT were detected (Austin et al., 1987; Scott et al., 1998; Finotti et al., 2004). Moreover, complexes between immunoglobulin A and AAT have been detected in the sera and synovial fluid of patients with rheumatoid arthritis, systemic lupus erythematosus and ankylosing spondylitis (Scott et al., 1998). Human tissue kallikrein 3, a serine proteinase commonly known as a prostate-specific antigen (PSA) which correlates with prostate hypertrophy and malignancy, is also known to bind to AAT in sera of subjects with high PSA concentrations (Zhang et al., 2000). Localization of AAT-low-density-lipoprotein (LDL) complexes in atherosclerotic lesions and enhanced degradation of AAT-LDL by macrophages suggested

the involvement of the complex in atherogenesis (Donners et al., 2005). Alpha1-antichymotrypsin/Alzheimer's peptide Abeta (1-42) complexes and ACT polymers have been associated with Alzheimer's disease (Licastro et al., 1997; Sun et al., 2002). Studies of the functional and conformational polymorphism of inhibitory APPs clearly show that some proteins can undergo polymerization due to an inherited mutation, or chemical modification, and obtain new biological activities or reflect undergoing pathological process. A well-characterised example of a mutant APP associated with a disease state is AAT. The most widely studied deficiency variants of AAT are Z and S, which have E342K and E264V mutations respectively (Carrell et al., 1996; Carrell et al., 1892). Polymerisation of these mutants of AAT is known to be involved in AAT deficiency-related diseases such as emphysema, liver cirrhosis, neonatal hepatitis, hepatocellular carcinoma and lung emphysema (Eriksson, 1990) The liver pathology is characterised by the formation of intracellular inclusions of polymerised AAT. Recent studies now indicate that extra-hepatic AAT polymerization may also occur. For example, AAT polymers have been identified in the lungs and circulation of Z AAT deficiency subjects. Importantly, like other modified forms, AAT polymers lack protease inhibitor activity which will exaggerate the severe deficiency, but also exhibit additional biological functions that may be relevant in pathological processes (Mahadeva et al., 2005; Mulgrew et al., 2004) (Figure 5). The susceptibility of Z AAT individuals who smoke to develop chronic obstructive pulmonary disease is in part related to the combination of the severe anti-elastase deficiency arising from an absolute and functional reduction in neutrophil elastase inhibitory capacity (Stoller & Aboussouan, 2005), and the independent multiple effects of cigarette smoke on inflammatory cells and molecules (Barnes et al., 2003). Our current data clearly demonstrate that cigarette smoke promotes polymerization of Z mutant AAT, but not of the normal, M variant of AAT. Thus cigarette smoke directly accelerates polymerization of Z AAT *via* oxidation of the protein leading to further depletion of the neutrophil protease protection in the lung and enhanced neutrophil influx (Alam et al., 2010). The data uniquely suggests that rather than the major risk factors for chronic obstructive pulmonary disease namely cigarette smoke and Z AAT deficiency having independent additive effects, they directly interact to create an effect greater than the sum of the individual risks.

## 5. General conclusions

Inflammation is a complex, highly orchestrated process involving many cell types and molecules, some of which initiate, amplify, or sustain the process, some of which attenuate it, and some of which aid resolution. A number of the participating AAPs are multifunctional and contribute to both the enhancement and the inhibition of inflammation at different points in its evolution. The outcome of the acute inflammatory response is most likely to be determined by the orchestrated generation of a specific profile of APPs, their concentrations and molecular forms in the microenvironment. Diseases associated with chronic inflammation may be due to an inadequate acute-phase response driven by APPs, their concentration and molecular form and/or an inability to eliminate invading pathogens and to rapidly to resolve the inflammatory processes. Often, a single APP is regarded as a marker for inflammation to aid in diagnosis and assesses response to treatment, however we believe one needs to see the profile of several APPs in action to understand function in relation to disease which may help in turn to determine prognosis.

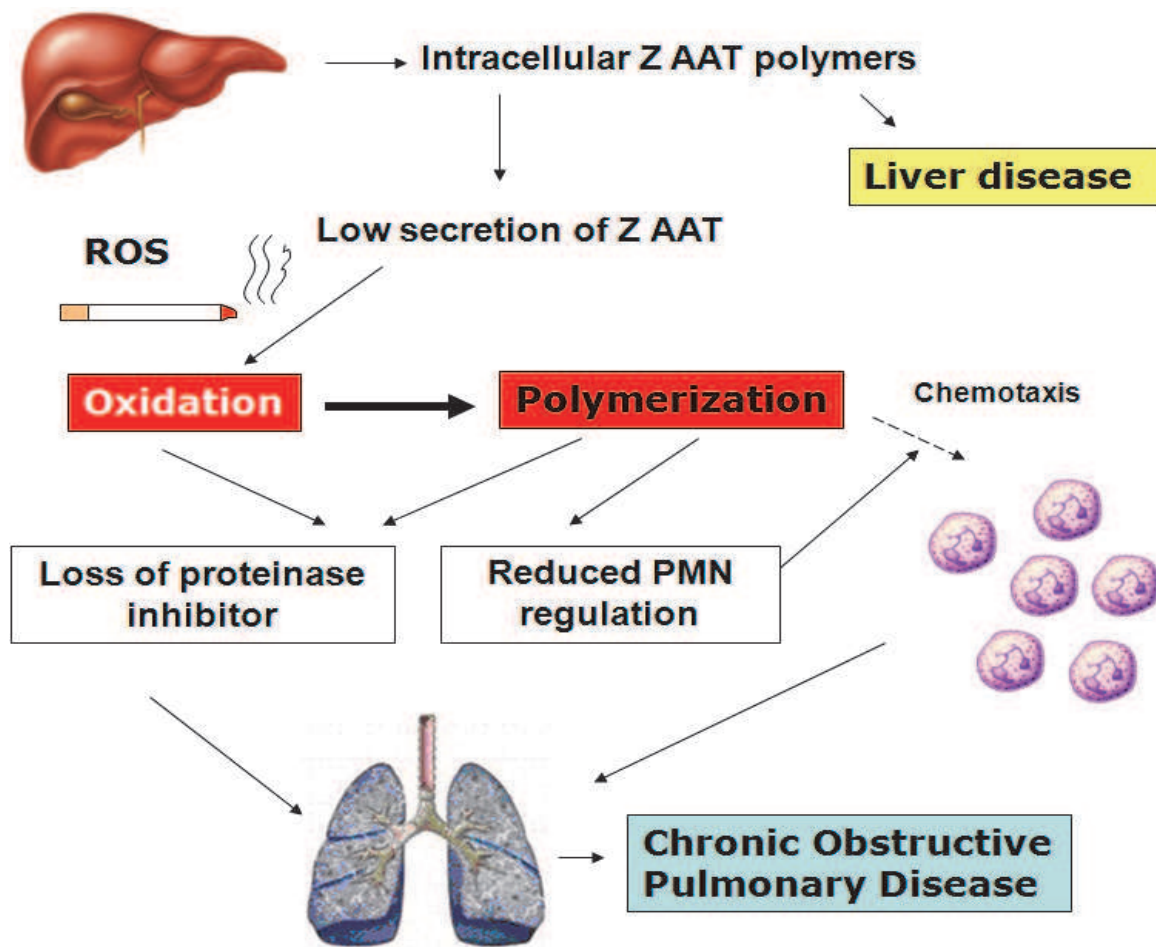


Fig. 4. Schematic diagram showing proposed model for the pathogenesis of emphysema in patients with inherited Z AAT deficiency. Cigarette smoke induces significant oxidation of Z AAT, which accelerates AAT protein polymerization. The plasma deficiency and reduced inhibitory activity of Z AAT would be exacerbated by the oxidation and polymerization of AAT within the lungs, thereby further reducing the antiproteinase screen. This together with the loss of the normal inhibitory effect of AAT on neutrophil chemotaxis and the chemoattractant ability of AAT polymers thereby increase tissue damage and accelerate emphysema progression.

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## 7. References

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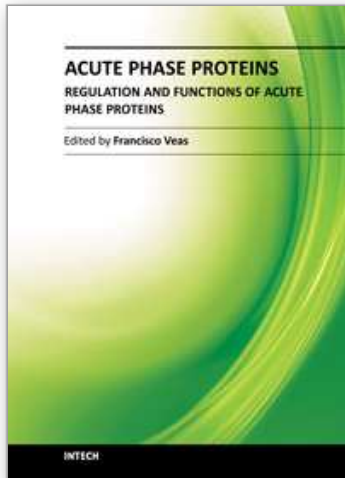
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The two volumes of Acute Phase Proteins book consist of chapters that give a large panel of fundamental and applied knowledge on one of the major elements of the inflammatory process during the acute phase response, i.e., the acute phase proteins expression and functions that regulate homeostasis. We have organized this book in two volumes - the first volume, mainly containing chapters on structure, biology and functions of APP, the second volume discussing different uses of APP as diagnostic tools in human and veterinary medicine.

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