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### Extracellular chaperones

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## Extracellular chaperones

### Abstract

The maintenance of the levels and correct folding state of proteins (proteostasis) is a fundamental prerequisite for life. Life has evolved complex mechanisms to maintain proteostasis and many of these that operate inside cells are now well understood. The same cannot yet be said of corresponding processes in extracellular fluids of the human body, where inappropriate protein aggregation is known to underpin many serious diseases such as Alzheimer's disease, type II diabetes and prion diseases. Recent research has uncovered a growing family of abundant extracellular chaperones in body fluids which appear to selectively bind to exposed regions of hydrophobicity on misfolded proteins to inhibit their toxicity and prevent them from aggregating to form insoluble deposits. These extracellular chaperones are also implicated in clearing the soluble, stabilized misfolded proteins from body fluids via receptor-mediated endocytosis for subsequent lysosomal degradation. Recent work also raises the possibility that extracellular chaperones may play roles in modulating the immune response. Future work will better define the *in vivo* functions of extracellular chaperones in proteostasis and immunology and pave the way for the development of new treatments for serious diseases.

### Keywords

extracellular, chaperones, CMMB

### Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

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## 1 Introduction

It has been estimated that about 400 grams of protein are synthesized and degraded each day in the human body. Individual proteins are degraded at extremely varied rates, with half-lives ranging from several minutes to many hours. Intracellularly, this variation in half-life has been attributed to differences in the intrinsic stability of proteins and the recognition of non-native structures by highly selective and precisely regulated protein quality control systems. Molecular chaperones have been identified as key players in orchestrating the control of protein folding, but almost all previous studies have been restricted to a focus on intracellular events. The average 70 kg human contains 15 liters of extracellular fluids, including five liters of blood. Although the concentration of proteins is lower in extracellular than intracellular fluids (6% in plasma and 2% in interstitial fluid, 30% in cytosol), extracellular conditions are more oxidizing [1]. In addition, uniquely, extracellular fluids are continuously subjected to shear stress (e.g. the pumping of fluids around the body) which is known to induce protein unfolding and aggregation [2]. The relatively harsh extracellular conditions suggest that mechanisms to sense and control the folding state of extracellular proteins are likely to be essential for the maintenance of human (and other large animal) life.

Uncontrolled protein unfolding or misfolding and the consequent accumulation of protein aggregates are implicated in the pathology of many diseases collectively known as Protein Deposition Diseases (PDD). PDDs are typically late-onset [3], suggesting that the underlying cause of the disease may be disruption or overwhelming of protein folding quality control mechanisms that were once able to maintain existing proteins in their native conformation. Although the reasons for the progressive impairment of fundamental physiological processes in aging is not fully understood, it is likely that the combination of declining protein folding quality control and exposure to thermal, ionic, heavy metal or oxidative stress may be responsible for late-onset PDDs. All PDDs involve protein misfolding leading to the deposition in tissues of insoluble protein aggregates; however, the type of aggregate formed varies between the individual diseases. In many PDDs including Alzheimer's disease, type II diabetes, systemic amyloidosis and transmissible spongiform encephalitis, proteins deposit as highly ordered,  $\beta$ -sheet-rich fibrillar aggregates known as amyloid. In other PDDs the nature of the protein deposits is fibrillar, but not amyloid – for example, Lewy bodies, which are found in Parkinson's and Alzheimer's disease. In still other PDDs, amorphous (unstructured), non-filamentous extracellular aggregates are formed. For example, such aggregates are formed by IgG light chain and/or IgG heavy chain in non-amyloidotic monoclonal IgG deposition disease (NAMIDD) [4]. In addition, drusen are amorphous extracellular deposits that accumulate in patients with age-related macular degeneration. In healthy eyes drusen are not found in the macula,

however they may exist in the retinal periphery and their size and number are considered a risk factor for developing age-related macular degeneration later in life [5].

It is notable that many PDDs are associated with *extracellular* protein deposits. Thus the previous near-exclusive focus of studies on intracellular processes to control protein folding may not provide the knowledge needed to treat these diseases. Intracellular chaperones (e.g. Hsp70 & Hsp90) may be released from necrotic [6] or apoptotic [7] cells, during viral cell lysis, secreted in exosomes [8, 9], or via other specific mechanisms [10-12]; they have been discovered in human plasma and associated with cell surfaces, in particular cancer cells. Numerous extracellular roles have been postulated for these chaperones, such as cancer cell invasiveness [13] and immune presentation [14-21]. These “moonlighting” functions for normally intracellular chaperones may be very important. However, the low abundance of this class of chaperone in extracellular fluids makes it unlikely that they can play a major role in controlling the folding state of abundant extracellular proteins in body fluids.

It has only recently become apparent that abundant extracellular counterparts to the intracellular molecular chaperones exist. Clusterin was the first abundant extracellular chaperone (EC) to be identified [22, 23] but the number of known ECs continues to grow and now includes at least 7 members. This Chapter outlines properties of each of the proteins that may function as mammalian ECs, and proposes a model for how they act as key elements in a system to monitor and control the folding state of extracellular proteins. The model presented will also propose how the ECs may play important complementary roles in the immune system.

## **2 Abundant Extracellular Chaperones**

There are seven currently known abundant extracellular proteins likely to function as chaperones (outlined in table 1). The strength of the available evidence for this varies with each protein. For more detailed information see the corresponding sections below.

Table 1: Overview of the currently known extracellular chaperones

Chaperone	Abundance	Chaperone function	Disease association	References
Clusterin	35-105 $\mu\text{g/ml}$ (blood plasma) 1.2-3.6 $\mu\text{g/ml}$ (CSF) 2-15 $\text{mg/ml}$ (seminal plasma)	Holdase-type chaperone activity similar to the small heat shock proteins	* Associated with extracellular deposits tested including age related macular degeneration, Creutzfeldt-Jakob disease, atherosclerosis, Alzheimer's disease * Upregulated in experimental models of stress * Genetic association with Alzheimer's disease	[22-54]
$\alpha_2$ -macroglobulin	1.5-2 $\text{mg/ml}$ (blood plasma) 1-3.6 $\mu\text{g/ml}$ (CSF)	Holdase-type chaperone activity similar to the small heat shock proteins	* Promotes phagocytosis of pathogen <i>Trypanosoma cruzi</i> * Associated with extracellular deposits in Alzheimer's disease, dialysis related amyloidosis and Creutzfeldt-Jakob disease * Able to stimulate a cytotoxic T lymphocyte response against chaperoned peptides	[55-65]
Haptoglobin	0.3-2 $\text{mg/ml}$ (blood plasma) 0.5-2 $\mu\text{g/ml}$ (CSF)	Holdase-type chaperone activity similar to the small heat shock proteins	* Upregulated during infection, neoplasia, trauma, and other inflammatory conditions * Co-deposits with amyloid in senile plaques, drusen with age-related macular degeneration and in protein deposits associated with chronic glomerulonephritis	[66-74]
Apolipoprotein E	4-6.4 $\mu\text{g/ml}$ (blood plasma) 1.8-5.7 $\mu\text{g/ml}$ (CSF)	Stabilizes proteins in solution	* Strong genetic association with Alzheimer's disease * Co-localizes with Alzheimer's and Creutzfeldt-Jakob plaques	[75-81]
Serum Amyloid P Component (SAP)	40 $\mu\text{g/ml}$ (blood plasma) 8.5 $\text{ng/ml}$ (CSF)	ATP-independent re-folding activity	* Binds with high specificity to amyloid and is universally found in amyloid deposits * Elevated SAP levels in CSF of Alzheimer's patients	[82-90]
Caseins	$\sim 80\%$ of milk protein	$\alpha_{\text{S1}}$ - and $\beta$ -casein have a holdase-type chaperone activity similar to the small heat shock proteins	* Associated with amyloid-like deposits in mammary tissue	[26, 91-103]
$\alpha_{\text{E}}$ C-Fibrinogen	2-4.5 $\text{mg/ml}$ (blood plasma)	Stabilizes proteins in	* Plasma levels are elevated under periods of stress	[104-106]

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## 2.1 Clusterin

Clusterin (also known as apolipoprotein J, sulfated glycoprotein 2 and SP-40, 40) was the first normally secreted protein identified as an abundant extracellular chaperone [22]. This heat-stable glycoprotein has an extremely broad biological distribution and exhibits high sequence homology (70-80%) across a wide range of mammalian species, suggesting that it performs a fundamentally important function *in vivo* [107]. Clusterin has been detected in all extracellular fluids that have been tested. In humans, clusterin is present in the range of 35-105  $\mu\text{g/ml}$  in blood plasma [24], 1.2-3.6  $\mu\text{g/ml}$  in cerebral spinal fluid (CSF) [25], and 2-15  $\text{mg/ml}$  in seminal plasma [25]. Determining the biological importance of clusterin has been complicated by the propensity of the protein to interact with a large number of structurally diverse molecules. It is likely that many of these interactions result from a single underlying property of clusterin, which is relevant to its primary function. Regardless, many alternative biological functions for clusterin have been proposed including roles in lipid transport [108], sperm maturation [109], complement regulation [107], membrane recycling [110] and apoptosis [111].

Clusterin is encoded by a single gene and the translated product is internally cleaved to produce two subunits,  $\alpha$  and  $\beta$ , prior to secretion from the cell. Matrix-assisted laser desorption ionization mass spectrometry has identified two primary forms of human plasma clusterin at about 58 kDa and 63.5 kDa, which are likely to be different glycoforms [112]. Approximately 17-27% of the mass of clusterin is comprised of branched, sialic acid-rich, N-linked carbohydrates [112]. This high carbohydrate content in addition to a high level of disorder and a tendency to form oligomers has impeded structural analysis of clusterin, however, sequence analysis has allowed for the prediction of several structural elements. These include three predicted amphipathic  $\alpha$ -helices (residues 173–184, 234–250 and 424–441) [108] and two predicted coiled-coil helices (residues 40-99 and 318-350) [113]. The five predicted  $\alpha$ -helical regions are thought to be significant in the chaperone activity of clusterin. It has been proposed that the  $\alpha$ -helical regions form a molten globule-like binding pocket that is the site of interaction for a variety of ligands [114].

Many reports have suggested that clusterin may have intracellular importance, for example in DNA repair [115], transcription [116], microtubule organization [117], or apoptosis [115, 118, 119]. Various mechanisms have been proposed to explain the presence of clusterin in intracellular compartments. This includes the reuptake of secreted clusterin back into the cytosol [117], retrotranslation of clusterin from the golgi to the cytosol [120], and the generation of nuclear isoforms via alternative initiation of transcription to yield a 43 kDa isoform [121] or via alternative splicing to yield a 49 kDa isoform [122]. Given that none of these latter studies sequenced the intracellular clusterin, it is unknown whether the putative “isoforms” are indeed the result of alternative transcription initiation or

splicing or whether they simply represent clusterin at different stages of maturation (e.g., cleaved or uncleaved, at different stages of glycosylation). Unambiguous structural identification of these intracellular isoforms of clusterin is required before their existence can be firmly accepted and their function(s) meaningfully assigned.

### 2.1.1 In Vitro Chaperone Activity

The hypothesis that clusterin may function as a molecular chaperone was first proposed over 10 years ago [22]. Since that time many studies have shown that clusterin has chaperone activity similar to that of the small heat-shock proteins (sHsps) [22, 23, 26-29, 33]. At substoichiometric concentrations, clusterin inhibits the stress-induced amorphous aggregation of a large number of unrelated client proteins by binding, in an ATP-independent manner, to areas of exposed hydrophobicity on partially unfolded intermediates [22, 26-29, 31]. While clusterin alone has no refolding activity, it can preserve heat-stressed enzymes in a state competent for subsequent ATP-dependent refolding by Hsc70 [112]. The chaperone activity of clusterin involves the sequestration of client proteins into soluble high molecular weight (HMW) complexes; when generated *in vitro*, these complexes have diameters of 50 - 100 nm and are  $\geq 4 \times 10^7$  Da [31]. The maximum "loading" of clusterin appears to correspond to a mass ratio of 1:2 (clusterin:client) regardless of the client protein [31]. Immunoaffinity depletion of clusterin renders proteins in human plasma more susceptible to aggregation and precipitation [27]. Fibrinogen, ceruloplasmin and albumin are major endogenous clients for clusterin when human plasma is subjected to physiologically relevant stress [30]. However, the method used to detect endogenous clients is biased towards those proteins that are relatively less stable and more abundant; it is likely that clusterin acts globally to stabilize a very broad range of clients *in vivo*.

The chaperone activity of clusterin is not limited to those proteins that form amorphous aggregates. Clusterin also inhibits the fibrillar aggregation of a large number of amyloid forming clients including amyloid  $\beta$  (A $\beta$ ) peptide [34, 35], PrP106–126 [36], apolipoprotein C-II [37], disease-associated variants of lysozyme [33],  $\alpha$ -synuclein, calcitonin,  $\kappa$ -casein, SH3 and CC $\beta$ w [32]. While clusterin appears to prevent amyloid formation in a dose dependent manner, in some cases very low levels of clusterin (relative to client protein) significantly increased amyloid formation [32]. It was proposed that when present at very low concentrations, clusterin may stabilize prefibrillar oligomers that "seed" fibril growth and are believed to be primarily responsible for amyloid-associated cytotoxicity. Thus, the clusterin:client protein ratio is an important determinant of the effects of clusterin on amyloid formation and toxicity. It is unknown exactly how clusterin is able to interfere with amyloid formation although the existing evidence suggests that it interacts predominantly with prefibrillar oligomeric species formed during the early stages of amyloid aggregation [32, 33]. These early aggregating species possess

surface-exposed hydrophobicity [123], thus the interaction of clusterin with amyloid-forming proteins may, as in the case of amorphously aggregating proteins, arise from hydrophobic interactions.

A number of investigations have focused on identifying possible interactions between members of the LDL receptor superfamily and clusterin [124-131]. Cellular internalization of clusterin via the LDL receptor megalin was the first reported clusterin-LDL receptor superfamily interaction [124]. Subsequent reports described the internalization of free clusterin and clusterin-A $\beta$  peptide complexes by the same receptor [125, 126]. Recently, two other human members of the LDL receptor superfamily, ApoE receptor 2 and very low density lipoprotein receptor, were reported to bind and internalize free clusterin and leptin-clusterin complexes using transfected cell models [131]. Interactions of clusterin with chicken oocyte-specific LDL receptors have also been described [128]. A recent study has suggested that megalin and LRP are capable of mediating the clusterin-dependent clearance of cellular debris into non-professional phagocytes [129]. However, the previous report of Kounnas *et al.* (1995) indicated that megalin, but not LRP, binds clusterin. Additional unidentified mechanisms of clusterin-dependent internalization were also suggested by Bartl *et al.* (2001). The affinity of clusterin binding to megalin is increased by the association of clusterin with lipids [127]. It is currently unknown how binding interactions with other molecules, such as stressed chaperone client proteins, affect the binding affinity of clusterin for megalin or other members of the LDL receptor superfamily. However, it has been shown that clusterin has independent binding sites for megalin, stressed proteins and unstressed ligands [130].

### 2.1.2 Evidence for In Vivo Chaperone Action/Disease Involvement

Clusterin is found associated with extracellular protein deposits in numerous diseases including in normal peripheral drusen and drusen in age-related macular degeneration patients [38], with membrane attack complex in renal immunoglobulin deposits [39], in prion deposits in Creutzfeldt-Jakob disease [40, 41], with PEX material in pseudoexfoliation (PEX) syndrome [42], in atherosclerotic plaques [43] and in amyloid plaques or with soluble A $\beta$  peptide in Alzheimer's disease [44, 45]. Two genome wide studies have recently implicated certain single nucleotide polymorphisms in the clusterin gene as risk factors for Alzheimer's disease [53, 54]. The overexpression of clusterin has been reported in a diverse range of renal and neurodegenerative diseases in addition to cancers, atherosclerosis and diabetes [46]. Additionally, clusterin is upregulated in experimental models of pathological stress including oxidative stress [47], shear stress [48], proteotoxic stress (generated by inhibition of the proteasome) [49], heat stress [50], ionizing radiation [51] and exposure to heavy metals [52].

In clusterin knock-out mice, damage to testicular cells is increased after heat-shock and the removal of damaged cells is impaired [132]. After myosin-induced auto-immune myocarditis, cell damage is also more severe in clusterin-deficient mice [133] and post-ischemic brain injury is more severe [134]. Together this data suggests that stress-induced increase in clusterin expression is a cytoprotective response. In an Alzheimer's disease model, compared to control mice, mice in which the clusterin and ApoE genes were knocked out showed early disease onset and a marked increase in A $\beta$  peptide levels and amyloid formation. The researchers concluded that apoE and clusterin work synergistically to inhibit the deposition of fibrillar A $\beta$  [135]. A more recent study has demonstrated that clusterin knock-out mice develop progressive glomerulopathy which is characterized by the accumulation of insoluble protein deposits in the kidneys [136]. This directly implicates clusterin in the clearance of potentially pathological aggregating proteins, although the precise mechanism underlying this has yet to be described.

## 2.2 $\alpha_2$ -Macroglobulin ( $\alpha_2$ M)

$\alpha_2$ M is a large secreted glycoprotein, assembled from four identical 180 kDa subunits into a 720 kDa tetramer; disulphide linked dimers of the individual 180 kDa subunits interact non-covalently to form the final tetrameric quaternary structure [137]. The secreted molecule is comprised of ~ 10% carbohydrate by mass. It is synthesized mainly in the liver, but is secreted from a range of different cell types (such as astrocytes) and can be found in human plasma and cerebrospinal fluid at 1500-2000 [56] and 1-3.6  $\mu$ g/ml [57], respectively. It is best known for its ability to inhibit a broad spectrum of proteases, which it accomplishes using a unique trapping method.  $\alpha_2$ M contains a 'bait region' which undergoes limited proteolysis upon encountering a protease, resulting in a large conformational change and exposure of a thiol ester bond. The protease forms a covalent linkage with  $\alpha_2$ M by reacting with the intramolecular thiol ester bond, which leads to further conformational changes exposing a receptor recognition site for low density lipoprotein receptor related protein (LRP). Overall, these structural changes produce a more compact molecule (known as "activated" or "fast"  $\alpha_2$ M) and inhibits the protease by physically trapping it within a steric "cage" [56]. By directly interacting with the thiol ester bond, small nucleophiles such as methylamine can also activate  $\alpha_2$ M [138]. Although human  $\alpha_2$ M is best known for its protease inhibitor function, it has also been shown to bind to and promote clearance of other endogenous and exogenous molecules, consistent with a broader protective function.  $\alpha_2$ M is known to bind to cytokines and growth factors (without converting to activated  $\alpha_2$ M), including transforming growth factor- $\beta$  (TGF- $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ), interleukin 8 (IL-8), platelet derived growth factor-BB (PDGF-BB), nerve growth factor- $\beta$  (NGF- $\beta$ ), and vascular endothelial growth factor (VEGF) (reviewed in refs [139, 140]). The affinity of  $\alpha_2$ M for most cytokines is higher in the activated state, and while in this state  $\alpha_2$ M can deliver them via receptor mediated endocytosis to lysosomes for degradation

[141]. In addition,  $\alpha_2\text{M}$  has been shown to bind to the pathogen *Trypanosoma cruzi* and promote its phagocytosis [142].  $\alpha_2\text{M}$  has also been found to bind to endogenous proteins found in proteinaceous deposits associated with disease.  $\alpha_2\text{M}$  is known to bind to the A $\beta$  peptide associated with Alzheimer's disease [58, 59],  $\beta_2$ -microglobulin which forms insoluble deposits in dialysis related amyloidosis [60], and prion protein associated with plaques in Creutzfeldt-Jakob disease [61].

### 2.2.1 In Vitro Chaperone Activity

$\alpha_2\text{M}$  forms stable complexes with misfolded proteins to inhibit their stress-induced aggregation and precipitation but is unable to independently promote their refolding [62]. In addition, depletion of  $\alpha_2\text{M}$  from whole human plasma renders proteins in this fluid more susceptible to aggregation and precipitation, even at 37 °C [62]. The formation of complexes between  $\alpha_2\text{M}$  and misfolded proteins is thought to be, at least in part, due to hydrophobic interactions [62]. The binding of a misfolded substrate protein does not activate  $\alpha_2\text{M}$  and as a result the complex formed is not bound by LRP. However, while complexed with misfolded client proteins,  $\alpha_2\text{M}$  remains able to interact with proteases and subsequently adopt its activated conformation and then interact with LRP [62]. Although LRP is the only known receptor for  $\alpha_2\text{M}$ , it remains possible that non-activated  $\alpha_2\text{M}$ /misfolded client protein complexes are taken up via other cell surface receptors. As an example, scavenger receptors have been shown to bind to methylamine activated forms of  $\alpha_2\text{M}$  [143]. In addition to inhibiting amorphous aggregation,  $\alpha_2\text{M}$  has been shown to inhibit amyloid fibril formation. This effect can be seen even at sub-stoichiometric levels of  $\alpha_2\text{M}$  (as low as a 1:100 molar ratio of  $\alpha_2\text{M}$ :substrate) [144, 145]. It is thought that  $\alpha_2\text{M}$  interacts with lowly populated oligomeric species affecting the formation of stable nuclei from which amyloid formation proceeds [58, 145].

$\alpha_2\text{M}$ -client protein complexes are thought to be removed from the extracellular space by receptor mediated endocytosis.  $\alpha_2\text{M}$ -A $\beta$  complexes are internalized via LRP expressed on U87 cells and are subsequently degraded [58]. In addition, complexes formed from  $\alpha_2\text{M}$  and heat-stressed citrate synthase (or glutathione-S-transferase, GST) that have been incubated with trypsin also bind to LRP on the surface of JEG-3 cells [62]. This uptake of complexes may protect cells from the toxicity of aggregating species. However, under certain conditions,  $\alpha_2\text{M}$  was shown to promote the neurotoxicity of A $\beta$  [63]. In stark contrast, using primary rat mixed neuronal cultures, others have demonstrated that  $\alpha_2\text{M}$  can protect cells from A $\beta$  toxicity [146]. The different effects observed may be explained by differences between systems in the extent of receptor mediated removal of complexes from the extracellular environment. This is illustrated by the demonstration that in the presence of  $\alpha_2\text{M}$  (but not otherwise), SH-SY5Y cells expressing the  $\alpha_2\text{M}$  receptor (LRP) are more resistant to A $\beta$  toxicity than cells that do not [63]. The protective effect of  $\alpha_2\text{M}$  could be inhibited by RAP (a pan-specific inhibitor of LRP

ligand binding). Furthermore,  $\alpha_2\text{M}$  promoted A $\beta$  toxicity against LRP-negative LAN5 cells but had the opposite effect with LRP-expressing LAN5 transfectants [63]. Importantly, this function has been demonstrated *in vivo*: the normally rapid removal of radiolabelled A $\beta$  from mouse brain is significantly inhibited by the LDL family inhibitor RAP and antibodies against LRP-1 and  $\alpha_2\text{M}$ .

### 2.2.2 Evidence for In Vivo Chaperone Action/Disease Involvement

$\alpha_2\text{M}$  has been found co-localized with A $\beta$  and prion plaques in Alzheimer's disease and CJD respectively [61, 63]. In addition, levels of circulating complexes formed between  $\alpha_2\text{M}$  and  $\beta_2\text{m}$  in plasma of haemodialysis patients are correlated with the severity of dialysis related amyloidosis [60]. In addition,  $\alpha_2\text{M}$  has been found in complex with prion protein in human plasma [64]. Lastly, the ability of  $\alpha_2\text{M}$  to promote the removal of A $\beta$  from the extracellular space has been shown *in vivo*; the normally rapid removal of radiolabelled A $\beta$  from mouse brain is significantly inhibited by the LDL family inhibitor RAP and antibodies against LRP-1 and  $\alpha_2\text{M}$  [147].

### 2.2.3 Potential Application of $\alpha_2\text{M}$ in Anti-Cancer Treatments

$\alpha_2\text{M}$  shares a common receptor ( $\alpha_2\text{M}$  receptor/ LRP1) with a variety of intracellular chaperones [148-150] which have been implicated in the re-presentation of chaperoned peptides to stimulate an immune response [8, 14, 16, 18-21, 148, 150-154]. This has led to the ability of  $\alpha_2\text{M}$  to perform a similar immunological function being examined. It was shown that  $\alpha_2\text{M}$ -peptide complexes are able to induce the re-presentation of the chaperoned peptides on MHC class I molecules *in vitro* and subsequently prime a cytotoxic T lymphocyte response in  $\alpha_2\text{M}$ -peptide immunized mice [55]. As both intra- and extracellular chaperones have now both been shown to elicit such a response, it has been proposed that  $\alpha_2\text{M}$  samples the extracellular space and Hsps the intracellular milieu. In this model, LRP1 facilitates the sampling of the entire antigenic milieu of an organism [55]. Unlike Hsps which are ubiquitously expressed, many tumors do not express  $\alpha_2\text{M}$ . Thus, in order to explore the use of  $\alpha_2\text{M}$ -peptide complexes as an anti-cancer treatment, exogenous  $\alpha_2\text{M}$  has been added to tumor cell lysates to generate the complexes [65].  $\alpha_2\text{M}$ -peptide complexes made *in vitro* induced anti-tumor responses and protection against tumor challenge similar to that of GP96 [155]. Thus,  $\alpha_2\text{M}$  and perhaps the other extracellular chaperones all offer potential vehicles for peptide-specific control of the immune response and immune modulatory therapies.

### 2.3 Haptoglobin

Haptoglobin is a secreted glycoprotein with many known biological functions, however, it is best known as a hemoglobin binding protein. The non-covalent interaction between haptoglobin and hemoglobin is particularly strong with a reported  $K_d \sim 10^{-15}$  M [66]. This interaction prevents the loss of hemoglobin and iron via glomerular filtration by redirecting the haptoglobin-hemoglobin complex to the liver [156]. The interaction of haptoglobin with hemoglobin also reduces the amount of free hemoglobin and iron available to catalyse oxidation reactions [157], and has an inhibitory effect on nitric oxide [158] and prostaglandin synthesis [159]. Haptoglobin also has a bacteriostatic effect on organisms unable to obtain heme from the hemoglobin-haptoglobin complex [160] and appears to play an important role in angiogenesis [161]. Finally, haptoglobin has been implicated in the regulation of lymphocyte transformation [162]. Haptoglobin is found in most body fluids. Its plasma concentration is between 0.3-2 mg/ml [67] and it is found in CSF between 0.5-2  $\mu$ g/ml [66]. Sequence analysis has identified haptoglobin as a chymotrypsinogen-like serine protease homolog, although it has a distinct biological function [163]. Humans express one of three different haptoglobin phenotypes (Hp 1-1, Hp 1-2 or Hp 2-2) depending on the presence of two principal alleles (Hp1 and Hp2) coding for the  $\alpha$  and  $\beta$  chains which associate covalently via disulphide linkage. The  $\alpha^1$ ,  $\alpha^2$  and  $\beta$  chain peptides are 9.2 kDa, 15.9 kDa and 27.2 kDa, respectively [68]. Similar to clusterin, haptoglobin is heavily glycosylated.

#### 2.3.1 In Vitro Chaperone activity

Human haptoglobin specifically inhibits the precipitation of a wide variety of proteins induced by heat or oxidative stress [68, 69]. All three human haptoglobin phenotypes exert this chaperone action, although Hp1-1 appears to be the most efficient. Like clusterin, haptoglobin forms stable, soluble high molecular weight complexes with partially unfolded clients in an ATP-independent manner, but has no independent refolding activity. The possibility that haptoglobin holds misfolded proteins in a state competent for refolding by other chaperones is currently untested. Immunoaffinity depletion of haptoglobin from human serum significantly increases the amount of protein that precipitated in response to stresses [164]. At substoichiometric levels, haptoglobin has been shown to dose-dependently inhibit amyloid formation by A $\beta$ , cc $\beta_w$ , calcitonin, and the lysozyme variant I59T [164].

Haptoglobin is a known ligand of the CD11b/CD18 receptor on natural killer cells [145]. With much lower affinity, haptoglobin also binds to CD4 and CD8 receptors on T lymphocytes [165]. Neutrophils and monocytes also possess binding sites for haptoglobin and are responsible for haptoglobin uptake in peripheral

blood [165]. Additionally, the acute-phase macrophage protein CD163 has been identified as a scavenger receptor for hemoglobin-haptoglobin complexes [166]. This high affinity receptor ligand interaction is  $\text{Ca}^{2+}$ -dependent and mediates endocytosis of the hemoglobin-haptoglobin complex [70]. It is possible that haptoglobin may facilitate the clearance of misfolded proteins via a similar mechanism to the clearance of hemoglobin-haptoglobin complexes, although this is yet to be investigated.

### 2.3.2 Evidence for In Vivo Chaperone Action/Disease Involvement

Haptoglobin is upregulated during a variety of conditions including infection, neoplasia, pregnancy, trauma, acute myocardial infarction and other inflammatory conditions [70]. Its possible chaperone role *in vivo* is supported by co-deposition with amyloid in senile plaques [71], with drusen in age-related macular degeneration [72], and in protein deposits resulting from chronic glomerulonephritis [73]. Surprisingly, haptoglobin gene knockout does not impair the clearance of free plasma hemoglobin, however haptoglobin-null mice display reduced postnatal viability and greater oxidative damage after induced hemolysis [74].

## 2.4 ApoE

Apolipoprotein E (ApoE), is a 35 kDa secreted glycoprotein, synthesized primarily by the liver, but can be found expressed by astrocytes, microglia and oligodendrocytes in the brain. It exists in three isoforms, E2, E3, and E4, which differ only by single amino acid variations. The prevalence of the alleles coding for these isoforms, E2, E3 and E4, is approximately 7–8%, 75–80% and 14–15%, respectively [167, 168]. ApoE is an amphipathic protein that is known for its ability to mediate transport and clearance of cholesterol, triglycerides and other lipids [169]. It mediates lipid transport through binding to the low density lipoprotein (LDL) receptor. ApoE is best known for its association with Alzheimer's disease; APOE  $\epsilon 4/\epsilon 4$  homozygotic individuals have a significantly greater risk of developing Alzheimer's disease [170].

### 2.4.1 In Vitro Chaperone activity

ApoE has been shown to have the ability to bind to aggregation prone polypeptides, such as tau and A $\beta$  [77, 78]. Interestingly, binding of ApoE to A $\beta$  is isoform-dependent with the binding of ApoE4 being of lowest affinity (E2>E3>E4) [78, 171]. The stoichiometry of the interaction between A $\beta$  and ApoE has been estimated at 5 A $\beta$  peptide molecules per ApoE molecule [172]. This interaction is likely to be the driving force behind the ability of ApoE to affect the aggregation of A $\beta$ . It has been shown to both promote and inhibit A $\beta$  aggregation depending on the conditions and specific variant of A $\beta$  peptide used. The formation of complexes between ApoE and A $\beta_{1-40}$  has been shown to inhibit the formation of amy-

loid, at a 100:1 molar ratio of A $\beta$ :ApoE [173]. The complexes made were added to monomeric A $\beta$  and were unable to act as “seeds” for amyloid formation. However, the complexes formed still reacted with thioflavin T [173]. In addition, it has been shown that ApoE can prolong the lag phase of A $\beta$  aggregation without affecting the amount of fibrillar material finally formed [174]. Similarly, ApoE lengthens the lag phase of amyloid formation from A $\beta$ <sub>29-40</sub> and A $\beta$ <sub>29-42</sub> by forming complexes with the respective peptides [172]. Interestingly, the E4 isotype had no effect on the lag phase. In contrast, there are many reports that suggest that ApoE can promote the formation of A $\beta$  fibrils. ApoE was shown to enhance the formation of thioflavin T positive material from A $\beta$ <sub>1-40</sub> [175], and promoted fibril formation by A $\beta$ <sub>1-42</sub> (as judged by thioflavin T and transmission electron microscopy) [176].

#### 2.4.2 Evidence for In Vivo Chaperone Action/Disease Involvement

The major focus on ApoE work has been its role in chaperoning A $\beta$  due to its strong genetic association with Alzheimer’s disease [79]. In humans ApoE has been found co-localized with Alzheimer’s and CJD plaques [80]. To further complicate understanding of the role of ApoE in amyloid formation (see above), mouse studies have been similarly conflicting. In initial studies, both A $\beta$  immunoreactivity and amyloid formation were reduced in ApoE knockout mice [177, 178]. In contrast, expression of human ApoE in transgenic mice suppressed A $\beta$  deposition [179]. Regardless of its effect on amyloid formation, just as observed for other extracellular chaperones such as clusterin and  $\alpha_2$ M, complexes formed between ApoE and A $\beta$  are efficiently taken up by receptor mediated endocytosis and promote subsequent degradation of A $\beta$ . It has been shown that A $\beta$ -ApoE complexes bind to the cell surface receptor megalin while free A $\beta$  does not [180]. In addition, LRP1 binds to A $\beta$ -ApoE complexes and internalizes them for subsequent degradation in lysosomes (or transport into plasma) [147]. Furthermore, it has been suggested that ApoE facilitates internalization and degradation of A $\beta$  by astrocytes [81]. As A $\beta$  is known to activate glial cells, its incorporation into complexes and its ApoE-dependant receptor mediated uptake may play a role in modulating the immune response. Consistent with this idea, it has been shown that the formation of ApoE- A $\beta$  complexes inhibits the activation of astrocytes by A $\beta$  [181].

#### 2.5 Serum Amyloid P Component

Serum Amyloid P Component (SAP) is a member of the highly conserved pentraxin family and consists of five identical 25 kDa subunits arranged in a ring [182]. As for other pentraxins, SAP displays calcium-dependent ligand binding and tertiary structure similar to legume lectins [183]. It is estimated that over 8% of the mass of the molecule is N-linked oligosaccharide [183]. It has been proposed that SAP circulates as a decamer with two pentameric rings noncovalently

bound [183, 184]. However, other reports claim that SAP exists as a single pentamer in the body and that the decameric form is obtained only upon purification [185, 186]. Human SAP shares approximately 51% amino acid homology with C-reactive protein, a classical human acute phase protein. In contrast, SAP does not behave as an acute phase protein in humans [82]; it is generally present in human plasma at around 40  $\mu\text{g/ml}$  [83] and in CSF at around 8.5  $\text{ng/ml}$  [84]. Although to date no clear biological function has been ascribed to SAP, it is known to interact with a diverse range of molecules *in vitro*. For example, SAP binds to glycosaminoglycans [187], DNA and chromatin [188-190], complement components [191, 192], fibronectin [193], C-reactive protein [194], aggregated IgG [195], phosphatidylethanolamine [196] and endotoxin [183, 197]. Of particular interest in the current context, SAP binds highly specifically to amyloid and is universally found in amyloid deposits [85-88, 191].

### 2.5.1 In Vitro Chaperone Activity

There is currently little evidence for the existence of efficient refolding chaperones in the extracellular milieu, however, it has been reported that SAP has ATP-independent refolding activity [89]. When present at a 10-fold molar excess, SAP was able to recover 25% of the initial enzyme activity of denatured lactate dehydrogenase. Whether this activity would be enhanced by the presence of ATP or “helper” chaperones is currently unknown. Further studies are needed before the potential physiological significance of this refolding activity becomes clear. SAP binds to synthetic A $\beta$  at physiological concentrations of  $\text{Ca}^{2+}$  [198] and binds to all types of amyloid fibrils tested *in vitro* [199]. SAP has a protease-resistant  $\beta$ -pleated sheet structure that in the presence of  $\text{Ca}^{2+}$  is resistant to proteolysis [200]. Consequently, SAP binding to amyloid fibrils is thought to inhibit their proteolytic digestion.

### 2.5.2 Evidence for In Vivo Chaperone Action/Disease Involvement

SAP constitutes up to 15% of the mass of amyloid deposits *in vivo*, which is remarkable considering it is only present in plasma at trace concentrations. Also, strongly supporting a role for SAP in amyloid pathogenesis is the frequency with which it is found localized in amyloid deposits *in vivo* [85-88, 90]. SAP knockout mice are viable and fertile with no obvious abnormalities, however, they display delayed amyloid deposition in models of systemic amyloidosis [89]. These results support that SAP plays a role in amyloid pathogenesis and that inhibition of SAP binding to amyloid is a potential therapeutic target. Given that SAP does not appear to be expressed in the brain, localization of SAP with cerebral amyloid deposits suggest that either specific active transport mechanisms exist to transport it from one side of the blood-brain barrier to the other or that damage to the blood-brain barrier is sufficient to allow the protein to leak into the brain during disease.

Regardless of the mechanism by which it gets there, the CSF concentration of SAP is higher in patients with Alzheimer's disease [85].

## 2.6 Caseins

Casein is the main constituent of milk (~ 80% of protein in bovine milk) and is made up of a heterogeneous mixture of phosphoproteins that includes four unrelated gene products,  $\alpha_{S1}$ -,  $\alpha_{S2}$ -,  $\beta$ - and  $\kappa$ -casein. In their monomeric forms, the caseins themselves are small, ranging in molecular mass between 19 and 25 kDa. However, the casein proteins exhibit a strong tendency to associate with each other, through hydrophobic and ionic interactions, which, in the presence of calcium and other ions, leads to the formation of casein micelles [91]. The micelles range in mass between  $10^3$  and  $3 \times 10^6$  kDa and represent the primary nutritional source of calcium (in the form of calcium phosphate) to the neonate [91]. The caseins have been classified as intrinsically disordered proteins, as they are extremely flexible, essentially unfolded and have relatively little secondary or tertiary structure under physiological conditions [201]. Their open, dynamic and malleable conformations suggest that they exist in a molten globule-like state, with extensive regions of solvent-exposed and clustered hydrophobicity [202]. As a result, it is unlikely that detailed X-ray crystal structures of full-length casein protein will be achieved; however, three-dimensional energy-minimized molecular models are available [203, 204]. Two of the casein proteins,  $\alpha_{S1}$ - and  $\beta$ -casein, have been found to have molecular chaperone-like activity, similar to the small heat shock proteins (sHsps) [26, 92]. The open, flexible nature of  $\alpha_{S1}$ -casein and  $\beta$ -casein results from the high percentage of proline residues in their amino acid sequence (9% of the amino acid sequence of  $\alpha_{S1}$ -casein and 18% of  $\beta$ -casein) and lack of disulfide bonds. Both  $\alpha_{S1}$ -casein and  $\beta$ -casein also possess a high degree of overall hydrophobicity, with well separated hydrophilic and hydrophobic domains. Such properties, which they share with other molecular chaperones such as the sHsps and clusterin [26], likely accounts for their ability to bind to a wide-range of destabilized, partially unfolded target proteins to prevent their aggregation [92].

### 2.6.1 In Vitro Chaperone Activity

To date, studies on the chaperone-like activity of casein proteins have been performed with bovine whole casein (comprising all four casein proteins) or with  $\alpha_S$ -casein (comprising both  $\alpha_{S1}$ - and  $\alpha_{S2}$ -casein) or  $\beta$ -casein. Thus,  $\alpha_S$ -casein and  $\beta$ -casein have been shown to inhibit the amorphous aggregation of a range of unrelated target proteins induced by heating [93-97], reduction [93, 95, 96] and UV-light [93]. They do so by forming high molecular weight complexes with the target protein, and stabilizing them in order to prevent their aggregation and potential precipitation. They have no intrinsic re-folding ability [95, 96] and thus their mechanism of action is akin to the sHsps and clusterin [26]. The chaperone-like activity of  $\alpha_S$ -casein and  $\beta$ -casein against amorphously aggregating target proteins

is phosphorylation-dependent, dephosphorylation decreasing their chaperone efficacy [94, 205]. The caseins are heavily phosphorylated (typically 8 phosphate residues per mole for  $\alpha_{S1}$ -casein and 5 for  $\beta$ -casein) which, apart from its role in calcium-binding and stabilization of the casein micelle, appears to play a significant role (via their negative charge) in maintaining the solubility of the complexes formed between the caseins and target proteins [205]. It has recently been suggested that the chaperone-like activity of these caseins may be exploited in order to control protein aggregation during food production [92].  $\alpha_S$ -casein and  $\beta$ -casein also appear to possess a generic ability to prevent protein aggregation associated with fibril formation. For example, whole and  $\beta$ -casein inhibit heat-induced fibril formation by ovalbumin [206],  $\alpha_S$ -casein and  $\beta$ -casein inhibit  $\kappa$ -casein fibril formation [205, 207], and  $\alpha_{S1}$ -casein inhibits  $\alpha_{S2}$ -fibril formation [208].

### 2.6.2 Evidence for In Vivo Chaperone Action/Disease Involvement

Caseins are uniquely synthesized in the mammary gland and immediately associate to form casein micelles, which are secreted into the alveolar lumen [209]. There is no direct evidence that a failure in the chaperone action of  $\alpha_S$ -casein and  $\beta$ -casein is involved in disease, however, amyloid-like deposits (known as *corpora amylacea*) have been identified in mammary tissue from a variety of species [98-100], and bundles of fibrils have been reported in the cytoplasm of cells that surround these calcified deposits [101]. The proteins present in these deposits and fibrils include the caseins [102, 103]. When isolated from the other caseins,  $\alpha_{S2}$ - and  $\kappa$ -casein readily form fibrils when incubated under conditions of physiological pH and temperature (i.e. pH 7.0-7.4, 37 °C) [207, 208, 210, 211] which suggests that these proteins may form fibrils *in vivo*. However, fibril formation by  $\alpha_{S2}$ - and  $\kappa$ -casein is inhibited by physiological concentrations of  $\alpha_{S1}$ - and  $\beta$ -casein *in vitro* [207, 208] and thus, the tendency of caseins to associate together acts as a protective mechanism to prevent this form of aggregation. Indeed, the fact that amyloid deposits in mammary tissue are not more prevalent is most likely attributable to the chaperone-like ability of  $\alpha_{S1}$ - and  $\beta$ -casein, which act to prevent the release of the amyloidogenic  $\alpha_{S2}$ - and  $\kappa$ -casein precursors by binding them into casein micelles.

## 2.7 Fibrinogen

Fibrinogen is synthesized by the liver and circulates in human plasma at a concentration of 2-4.5 mg/ml [212]. It is the 340 kDa glycoprotein precursor to fibrin, which forms clots in the wound response. Fibrinogen is an “acute phase protein” and its levels in plasma are elevated in response to a variety of stresses including stroke, atherosclerotic diseases, age and acute myocardial infarction [212]. Fibrinogen molecules are comprised of two sets of disulfide-linked A $\alpha$ -, B $\beta$ -, and  $\gamma$ -chains. Fibrin is formed after cleavage of fibrinopeptide A (FPA) from fibrinogen

A $\alpha$ -chains, which initiates fibrin polymerization. In addition to its well known role in providing a scaffold for clots, fibrinogen also has other biological functions involving a range of binding sites, some of which are only exposed as a consequence of fibrin formation. These other functions include recruiting platelets into clots, down-regulation of circulating levels of thrombin, and plasminogen activation [212].

### 2.7.1 In Vitro Chaperone Activity

Two publications only have appeared so far describing the chaperone activity of fibrinogen. The first of these presented results suggesting that human plasma fibrinogen (i) specifically, and independently of ATP, inhibited the thermally induced aggregation of citrate synthase and firefly luciferase, (ii) held the heat-stressed forms of these proteins in a state competent for refolding by a rabbit reticulocyte lysate, (iii) inhibited amyloid formation by yeast prion protein Sup35, and (iv) inhibited heat-induced aggregation of proteins in undiluted mouse plasma [105]. These studies were described as having been done using fibrinogen sourced from a commercial supplier (which would be expected to be overwhelmingly comprised of the usual 340 kDa form). However, in a subsequent study by the same group, similar chaperone properties were attributed specifically to the  $\alpha_E$ C C-terminal extension of fibrinogen, present only in a much less abundant 420 kDa isoform of the protein (fibrinogen-420) [106]. Fibrinogen-420 is normally present in human plasma at 20-150 mg/ml (i.e. making up about 0.4-7.5% of the circulating fibrinogen pool). Our own experiments failed to show any chaperone activity for the 340 kDa form of fibrinogen (A. Wyatt, unpublished). The reason(s) for the apparent discrepancy between these two publications is unclear. However, the balance of evidence suggests that the abundant 340 kDa form of fibrinogen is not a chaperone, but that the  $\alpha_E$ C moiety in fibrinogen-420 is a chaperone-active species.

### 2.7.2 Evidence for In Vivo Chaperone Action/Disease Involvement

The level of  $\alpha_E$ C can be regulated by proteases (such as matrix metalloproteases and plasmin) which can rapidly release it from fibrinogen-420. It has been suggested that fibrinogen-420 acts as a delivery vehicle for  $\alpha_E$ C [106]. Evidence for an *in vivo* chaperone role for fibrinogen-420/ $\alpha_E$ C is currently limited to the demonstration that (i) proteins in plasma of fibrinogen knock-out mice aggregate to a greater extent when incubated for 48 h at 43 °C than those in the plasma of wild-type mice [106], and (ii) exogenously added  $\alpha_E$ C formed complexes with a variety of proteins in human plasma heated for 30 min at 50 °C [106]. Future work will hopefully further define the *in vivo* role(s) of the fibrinogen-420/ $\alpha_E$ C chaperone activity and its potential involvement in diseases.

## 2.9 In Vivo Functions of Extracellular Chaperones

It is clear from the growing number of abundant ECs identified that they are likely to play very important roles in the maintenance of normal physiological functions. The precise details of these roles are currently under investigation but are likely to include (i) selective binding to exposed regions of hydrophobicity on extracellular proteins induced to misfold by (for example) various physical or chemical stresses, leading to (ii) inhibition of their toxicity towards cells and (iii) stabilization of their structure so that they are prevented from aggregating to form insoluble deposits. Soluble complexes formed between ECs and misfolded proteins are probably internalized via receptor-mediated endocytosis and subsequently degraded by (for example) lysosomal proteolysis. However, it is also feasible that within antigen-presenting cells ECs can directly bound protein antigens to other intracellular proteolytic systems such as the proteasome, and that peptide fragments of the degraded chaperone client proteins are later presented at the cell surface in association with Class I and/or II major histocompatibility antigens. In this way, ECs may play multiple critical roles *in vivo*, protecting the body from the dangers of inappropriate aggregation of extracellular proteins but also playing a pivotal role in the processing of extracellular protein antigens necessary for eliciting protective immune responses (Figure 1).

## 3 Conclusions

It is barely over a decade since the first abundant mammalian extracellular chaperone (clusterin) was identified. Since that time there have been a series of discoveries of other extracellular chaperones such that we now know there are at least 7 present at (in some cases) substantial concentrations in human blood. Collectively, by mass, these chaperones account for possibly in excess of 7% of all blood proteins. The caseins are also abundant in another important extracellular fluid, milk. It will be unsurprising if further extracellular chaperones are identified in coming years. The sheer abundance of these chaperones in body fluids strongly suggests that they perform vital biological functions. These functions may include roles in stabilizing misfolded proteins aggregating via either the amorphous or amyloid-forming pathways, mediating the clearance of these aggregation-prone (and often toxic) proteins from the body, and modulating the response of the immune system to extracellular antigens. The processes governing the development of the many serious human diseases linked to inappropriate aggregation of extracellular proteins are poorly understood. Clearly, advances in knowledge of extracellular chaperones will impact upon our ability to prevent and treat these diseases, and may allow us to better harness the power of the immune system to fight conditions such as cancers. Furthermore, extracellular chaperones may exert powerful but currently poorly characterized effects on the delivery and efficacy of systemically administered hydrophobic drugs. All of these considerations point to the

importance of future work to identify the *in vivo* roles of the growing family of extracellular chaperones.

### References

1. Sitia, R. and I. Braakman (2003) Quality control in the endoplasmic reticulum protein factory. *Nature* 426:891-894
2. Ker, Y.C. and R.H. Chen (1998) Stress-induced conformational changes and gelation of soy protein isolate suspensions. *Lebenson Wiss Technol* 31:107-113
3. Bucciantini, M., et al. (2002) Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* 416:507-510
4. Buxbaum, J. and G. Gallo (1999) Nonamyloidotic monoclonal immunoglobulin deposition disease. Light-chain, heavy-chain, and light- and heavy-chain deposition diseases. *Hematol Oncol Clin North Am* 13:1235-1248
5. Mullins, R.F., et al. (2000) Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. *FASEB J* 14:835-846
6. Saito, K., Y. Dai, and K. Ohtsuka (2005) Enhanced expression of heat shock proteins in gradually dying cells and their release from necrotically dead cells. *Exp Cell Res* 310:229-236
7. Feng, H., et al. (2001) Stressed Apoptotic Tumor Cells Express Heat Shock Proteins and Elicit Tumor-Specific Immunity. *Blood* 97:3503-3512
8. Gastpar, R., et al. (2005) Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. *Cancer Res* 65:5238-5247
9. Lancaster, G.I. and M.A. Febbraio (2005) Exosome-dependent trafficking of HSP70: a novel secretory pathway for cellular stress proteins. *J Biol Chem* 280:23349-23355
10. Mambula, S.S. and S.K. Calderwood (2006) Heat shock protein 70 is secreted from tumor cells by a nonclassical pathway involving lysosomal endosomes. *J Immunol* 177:7849-7857
11. Mambula, S.S., et al. (2007) Mechanisms for Hsp70 secretion: Crossing Membrane without a leader. *Methods* 43:168-175
12. Merendino, A.M., et al. (2010) Hsp60 is actively secreted by human tumor cells. *PLoS One* 5:e9247
13. Eustace, B.K., et al. (2004) Functional proteomic screens reveal an essential extracellular role for hsp90 alpha in cancer cell invasiveness. *Nat Cell Biol* 6:507-514

14. Srivastava, P.K., et al. (1998) Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. *Immunity* 8:657-665
15. Srivastava, P. (2002) Roles of heat-shock proteins in innate and adaptive immunity. *Nat Rev Immunol* 2:185-194
16. Basu, S. and P.K. Srivastava (1999) Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. *J Exp Med* 189:797-802
17. Maki, R.G., et al. (2007) A phase I pilot study of autologous heat shock protein vaccine HSPPC-96 in patients with resected pancreatic adenocarcinoma. *Dig Dis Sci* 52:1964-1972
18. Rivoltini, L., et al. (2003) Human tumor-derived heat shock protein 96 mediates in vitro activation and in vivo expansion of melanoma- and colon carcinoma-specific T cells. *J Immunol* 171:3467-3474
19. Srivastava, P.K., A.B. DeLeo, and L.J. Old (1986) Tumour Rejection Antigens of Chemically Induced Sarcomas of Inbred Mice. *Proc Natl Acad Sci U S A* 83:3407-3411
20. Suto, R. and P.K. Srivastava (1995) A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* 269:1585-1588
21. Udono, H. and P.K. Srivastava (1993) Heat shock protein 70-associated peptides elicit specific cancer immunity. *J Exp Med* 178:1391-1396
22. Humphreys, D.T., et al. (1999) Clusterin has chaperone-like activity similar to that of small heat shock proteins. *J Biol Chem* 274:6875-6881
23. Wilson, M.R. and S.B. Easterbrook-Smith (2000) Clusterin is a secreted mammalian chaperone. *Trends Biochem Sci* 25:95-98
24. Murphy, B.F., et al. (1988) SP-40,40, a newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. *J Clin Invest* 81:1858-1864
25. Choi, N.H., et al. (1990) Sandwich ELISA for quantitative measurement of SP-40,40 in seminal plasma and serum. *J Immunol Methods* 131:159-163
26. Carver, J.A., et al. (2003) Small heat-shock proteins and clusterin: intra- and extracellular molecular chaperones with a common mechanism of action and function. *IUBMB Life* 55:661-668
27. Poon, S., et al. (2002) Mildly acidic pH activates the extracellular molecular chaperone clusterin. *J Biol Chem* 277:39532-39540
28. Poon, S., et al. (2000) Clusterin is an ATP-independent chaperone with a very broad substrate specificity that stabilizes stressed proteins in a folding-competent state. *Biochemistry* 39:15953-15960
29. Poon, S., et al. (2002) Clusterin is an extracellular chaperone that specifically interacts with slowly aggregating proteins on their off-folding pathway. *FEBS Letters* 513:259-266

30. Wyatt, A.R. and M.R. Wilson (2010) Identification of human plasma proteins as major clients for the extracellular chaperone clusterin. *J Biol Chem* 285:3532-3539
31. Wyatt, A.R., J.J. Yerbury, and M.R. Wilson (2009) Structural characterization of clusterin-client protein complexes. *J Biol Chem* 284:21920-21927
32. Yerbury, J.J., et al. (2007) The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with pre-fibrillar structures. *FASEB J* 21:2312-2322
33. Kumita, J.R., et al. (2007) The extracellular chaperone clusterin potentially inhibits amyloid formation by interacting with prefibrillar species. *J Mol Biol* 369:157-167
34. Matsubara, E., B. Frangione, and J. Ghiso (1995) Characterization of apolipoprotein J-Alzheimer's a-beta interaction. *J Biol Chem* 270:7563-7567
35. Oda, T., et al. (1995) Clusterin (apoJ) alters the aggregation of amyloid beta peptide 1-42 and forms slowly sedimenting A-beta complexes that cause oxidative stress. *Exp Neurol* 136:22-31
36. McHattie, S. and N. Edington (1999) Clusterin prevents aggregation of neuropeptide 106-126 in vitro. *Biochem Biophys Res Commun* 259:336-340
37. Hatters, D.M., et al. (2002) Suppression of apolipoprotein C-II amyloid formation by the extracellular chaperone, clusterin. *Eur J Biochem* 269:2789-2794
38. Crabb, J.W., et al. (2002) Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. *Proc Natl Acad Sci U S A* 99:14682-14687
39. French, L.E., J. Tschopp, and J.A. Schifferli (1992) Clusterin in renal tissue: preferential localization with the terminal complement complex and immunoglobulin deposits in glomeruli. *Clin Exp Immunol* 88:389-393
40. Sasaki, K., et al. (2002) Clusterin/apolipoprotein J is associated with cortical Lewy bodies: immunohistochemical study in cases with alpha-synucleinopathies. *Acta Neuropathol* 104:225-230
41. Freixes, M., et al. (2004) Clusterin solubility and aggregation in Creutzfeldt-Jakob disease. *Acta Neuropathol* 108:295-301
42. Zenkel, M., et al. (2006) Clusterin deficiency in eyes with pseudoexfoliation syndrome may be implicated in the aggregation and deposition of pseudoexfoliative material. *Invest Ophthalmol Vis Sci* 47:1982-1990
43. Mackness, B., et al. (1997) Increased immunolocalization of paraoxonase, clusterin and apolipoprotein A-I in the human artery wall with the progression of atherosclerosis. *Arterioscler Thromb Vasc Biol* 17:1233-1238

44. Witte, D.P., et al. (1993) Platelet activation releases megakaryocyte-synthesized apolipoprotein J, a highly abundant protein in a atheromatous lesions. *Am J Pathol* 143:763-773
45. Ghiso, J., et al. (1993) The cerebrospinal-fluid soluble form of Alzheimer's amyloid beta is complexed to SP-40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex. *Biochem J* 293:27-30
46. Calero, M., et al. (2000) Apolipoprotein J (clusterin) and Alzheimer's disease. *Microsc Res Tech* 50:305-315
47. Rosenberg, M.E. and J. Silksen (1995) Clusterin: Physiologic and pathophysiologic considerations. *Int J Biochem Cell Biol* 27:633-645
48. Strocchi, P., et al. (2006) Clusterin up-regulation following sub-lethal oxidative stress and lipid peroxidation in human neuroblastoma cells. *Neurobiol Aging* 27:1588-1594
49. Ubrich, C., et al. (2000) Laminar shear stress upregulates the complement-inhibitory protein clusterin. *Circulation* 101:352-355
50. Loison, F., et al. (2006) Up-regulation of the clusterin gene after proteotoxic stress: implications of HSF1-HSF2 heterocomplexes. *Biochem J* 395:223-231
51. Michel, D., et al. (1997) Stress-induced transcription of the clusterin/apoJ gene. *Biochem J* 328:45-50
52. Criswell, T., et al. (2005) Delayed activation of insulin-like growth factor-1 receptor/Src/MAPK/Egr-1 signaling regulates clusterin expression, a pro-survival factor. *J Biol Chem* 280:14212-14221
53. Harold, D., et al. (2009) Genome-wide association study identifies variants at *CLU* and *PICALM* associated with Alzheimer's disease. *Nat Genet* 41:1088-1093
54. Lambert, J.C., et al. (2009) Genome-wide association study identifies variants at *CLU* and *CR1* associated with Alzheimer's disease. *Nat Genet* 41:1094-1099
55. Binder, R.J., D. Karimeddini, and P.K. Srivastava (2001) Adjuvanticity of alpha2-Macroglobulin, an Independent Ligand for the Heat Shock Protein Receptor CD91. *J Immunol* 166:4968-4972
56. Sottrup-Jensen, L. (1989) Alpha-macroglobulins: structure shape and mechanism of proteinase complex formation. *J Biol Chem* 264:11539-11542
57. Biringer, R.G., et al. (2006) Enhanced sequence coverage of proteins in human cerebrospinal fluid using multiple enzymatic digestion and linear ion trap LC-MS/MS. *Brief Funct Genomic Proteomic* 5:144-153
58. Narita, M., et al. (1997) Alpha2-macroglobulin complexes with and mediates the endocytosis of beta-amyloid peptide via cell surface low-density lipoprotein receptor-related protein. *J Neurochem* 69:1904-1911
59. Mettenburg, J.M., D.J. Webb, and S.L. Gonias (2002) Distinct binding sites in the structure of alpha 2-macroglobulin mediate the interaction

- with beta-amyloid peptide and growth factors. *J Biol Chem* 277:13338-13345
60. Motomiya, Y., et al. (2003) Circulating levels of alpha2-macroglobulin-beta2-microglobulin complex in hemodialysis patients. *Kidney Int* 64:2244-2252
  61. Adler, V. and V. Kryukov (2007) Serum macroglobulin induces prion protein transition. *Neurochem J* 1:43-52
  62. French, K., J.J. Yerbury, and M.R. Wilson (2008) Protease activation of alpha2-macroglobulin modulates a chaperone-like action with broad specificity. *Biochemistry* 47:1176-1185
  63. Fabrizi, C., et al. (2001) Role of alpha2-macroglobulin in regulating amyloid -protein neurotoxicity: protective or detrimental factor? *J Neurochem* 78:406-412
  64. Adler, V., et al. (2007) Alpha2-macroglobulin is a potential facilitator of prion protein transformation. *Amyloid* 14:1-10
  65. Binder, R.J. (2004) Purification of Alpha2-Macroglobulin and the Construction of Immunogenic Alpha2-Macroglobulin-Peptide Complexes for Use as Cancer Vaccines. *Methods* 32:29-31
  66. Bowman, B.H. and A. Kurosky (1982) Haptoglobin: The evolutionary product of duplication, unequal crossing over, and point mutation. *Adv Hum Genet* 12:189-261
  67. Baskies, A.M., et al. (1980) Serum glycoproteins in cancer patients: first reports of correlations with in vitro and in vivo parameters of cellular immunity. *Cancer* 45:3050-3060
  68. Kurosky, A., et al. (1980) Covalent structure of human haptoglobin: a serine protease homolog. *Proc Natl Acad Sci U S A* 77:3388-3392
  69. Pavlicek, Z. and R. Etrich (1999) Chaperone-like activity of human haptoglobin: Similarity with a-crystallin. *Collect Czech Chem Comm* 64:717-725
  70. Kristiansen, M., et al. (2001) Identification of the haemoglobin scavenger receptor. *Nature* 409:198-201
  71. Langlois, M.R. and J.R. Delanghe (1996) Biological and clinical significance of haptoglobin polymorphisms in humans. *Clin Chem* 42:1589-1600
  72. Powers, J.M., et al. (1981) An immunoperoxidase study of senile cerebral amyloidosis with pathogenetic considerations. *J Neuropathol Exp Neurol* 40:592-612
  73. Kliffen, M., P.T. de Jong, and T.M. Luiders (1995) Protein analysis of human maculae in relation to age-related maculopathy. *Lab Invest* 72:267-272
  74. Tomino, Y., et al. (1981) Immunofluorescent studies on acute phase reactants in patients with various types of chronic glomerulonephritis. *Tokai J Exp Clin Med* 6:435-441

75. Phillips, N.R., R.J. Havel, and J.P. Kane (1983) Sex-related differences in the concentrations of apolipoprotein E in human blood plasma and plasma lipoproteins. *J Lipid Res* 24:1525-1531
76. Landén, M., et al. (1996) Apolipoprotein E in cerebrospinal fluid from patients with Alzheimer's disease and other forms of dementia is reduced but without any correlation to the apoE4 isoform. *Dementia* 7:273-278
77. Strittmatter, W.J., et al. (1994) Isoform-specific interactions of apolipoprotein E with microtubule-associated tau: Implications for Alzheimer disease. *Proc Natl Acad Sci U S A* 91:11183-11186
78. Strittmatter, W.J., et al. (1993) Binding of human apolipoprotein E to synthetic amyloid b peptide: Isoform specific-effects and implications for late-onset Alzheimer disease. *Proc Natl Acad Sci U S A* 90:8098-8102
79. Corder, E.H., et al. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 261:921-923
80. Namba, Y., et al. (1991) Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Res* 541:163-166
81. Koistinaho, M., et al. (2004) Apolipoprotein E promotes astrocyte colocalization and degradation of deposited amyloid-beta peptides. *Nat Med* 10:719-726
82. Aquilina, J.A. and C.V. Robinson (2003) Investigating interactions of the pentraxins serum amyloid P component and C-reactive protein by mass spectrometry. *Biochem J* 375:323-328
83. Pepys, M.B., et al. (1978) Comparative clinical study of protein SAP (amyloid P component) and C-reactive protein in serum. *Clin Exp Immunol* 32:119-124
84. Hutchinson, W.L., et al. (1994) The pentraxins, C-reactive protein and serum amyloid P component, are cleared and catabolized by hepatocytes in vivo. *J Clin Invest* 94:1390-1396
85. Botto, M., et al. (1997) Amyloid deposition is delayed in mice with targeted deletion of the serum amyloid P component gene. *Nat Med* 3:885-889
86. Coria, F., et al. (1988) Isolation and characterization of amyloid P component from Alzheimer's disease and other types of cerebral amyloidosis. *Lab Invest* 58:454-458
87. Breathnach, S.M., et al. (1981) Amyloid P component is located on elastic fibre microfibrils in normal human tissue. *Nature* 293:652-654
88. Kalaria, R.N., et al. (1991) Serum amyloid P in Alzheimer's disease. Implications for dysfunction of the blood-brain barrier. *Ann NY Acad Sci* 640:145-148
89. Yang, G.C., et al. (1992) Ultrastructural immunohistochemical localization of polyclonal IgG, C3, and amyloid P component on the congo red-

- negative amyloid-like fibrils of fibrillary glomerulopathy. *Am J Pathol* 141:409-410
90. Tennent, G.A., L.B. Lovat, and M.B. Pepys (1995) Serum amyloid P component prevents proteolysis of the amyloid fibrils of Alzheimer's disease and systemic amyloidosis. *Proc Natl Acad Sci U S A* 92:4299-4303
  91. Swaisgood, H.E (2003) Chemistry of the caseins. In: Fox PF and McSweeney PLH (ed) *Advanced Dairy Chemistry*, Kluwer Academic/Plenum Publishers: New York
  92. Thorn, D.C., H. Ecroyd, and J.A. Carver (2009) The two-faced nature of milk casein proteins: amyloid fibril formation and chaperone-like activity. *Aust J Dairy Technol* 64:36-40
  93. Bhattacharyya, J. and K.P. Das (1999) Molecular chaperone-like properties of an unfolded protein, alpha(s)-casein. *J Biol Chem* 274:15505-15509
  94. Matsudomi, N., et al. (2004) Ability of alphas-Casein to suppress the heat aggregation of ovotransferrin. *J Agric Food Chem* 52:4882-4886
  95. Morgan, P.E., et al. (2005) Casein proteins as molecular chaperones. *J Agric Food Chem* 53:2670-2683
  96. Zhang, X., et al. (2005) Chaperone-like activity of beta-casein. *Int J Biochem Cell Biol* 37:1232-1240
  97. Hassanisadi, M., et al. (2008) Chemometric study of the aggregation of alcohol dehydrogenase and its suppression by beta-caseins: a mechanistic perspective. *Anal Chim Acta* 613:40-47
  98. Reid, I.M. (1972) Corpora amylacea of the bovine mammary gland. Histochemical and electron microscopic evidence for their amyloid nature. *J Comp Pathol* 82:409-413
  99. Taniyama, H., et al. (2000) Localized amyloidosis in canine mammary tumors. *Vet Pathol* 37:104-107
  100. Gruys, E. (2004) Protein folding pathology in domestic animals. *J Zhejiang Univ Sci* 5:1226-1238
  101. Nickerson, S.C. (1987) Amyloid fibril formation in the bovine mammary gland: an ultrastructural study. *Cytobios* 51:81-92
  102. Claudon, C., et al. (1998) Proteic composition of corpora amylacea in the bovine mammary gland. *Tissue Cell* 30:589-595
  103. Niewold, T.A., et al. (1999) Casein related amyloid, characterization of a new and unique amyloid protein isolated from bovine corpora amylacea. *Amyloid* 6:244-249
  104. Mosesson, M.W. (2005) Fibrinogen and fibrin structure and functions. *J Thromb Haemost* 3:1894-1904
  105. Tang, H., et al. (2009) Fibrinogen has chaperone-like activity. *Biochem Biophys Res Commun* 378:662-667
  106. Tang, H., et al. (2009) Alpha(E)C, the C-terminal extension of fibrinogen, has chaperone-like activity. *Biochemistry* 48:3967-3976

107. Jenne, D.E. and J. Tschopp (1992) Clusterin: the intriguing guises of a widely expressed glycoprotein. *Trends Biochem Sci* 17:154-159
108. de Silva, H.V., et al. (1990) Apolipoprotein J: Structure and tissue distribution. *Biochemistry* 29:5380-5389
109. Hermo, L., K. Barin, and R. Oko (1994) Developmental expression of sulfated glycoprotein-2 in the epididymis of the rat. *Anat Rec* 240:327-344
110. Jordan-Starck, T.C., et al. (1992) Apolipoprotein J: a membrane policeman? *Curr Opin Lipidol* 3:75-85
111. Buttyan, R., et al. (1989) Induction of the Trpm-2 gene in cells undergoing programmed death. *Mol Cell Biol* 9:3473-3481
112. Kapron, J.T., et al. (1997) Identification and characterization of glycosylation sites in human serum clusterin. *Protein Sci* 6:2120-2123
113. Lupas, A. (1991) Predicting coiled-coils from protein sequences. *Science* 252:1162-1164
114. Bailey, R.W., et al. (2001) Clusterin, a Binding Protein with a Molten Globule-like Region. *Biochemistry* 40:11828-11840
115. Yang, C.R., et al. (2000) Nuclear clusterin/XIP8, an x-ray induced Ku70-binding protein that signals cell death. *Proc Natl Acad Sci U S A* 97:5907-5912
116. Santilli, G., B.J. Aronow, and A. Sala (2003) Essential requirement of apolipoprotein J (clusterin) signaling for Ikappa B expression and regulation of NF-kappaB activity. *J Biol Chem* 278:38214-38219
117. Kang, S.W., et al. (2005) Clusterin interacts with SCLIP (SCG10-like protein) and promotes neurite outgrowth of PC12. *Exp Cell Res* 309:305-315
118. Debure, L., et al. (2003) Intracellular clusterin causes juxtannuclear aggregate formation and mitochondrial alteration. *J Cell Sci* 116:3109-3121
119. Zhang, H.L., et al. (2005) Clusterin inhibits apoptosis by interacting with activated Bax. *Nat Cell Biol* 7:909-915
120. Nizard, P., et al. (2007) Stress-induced retrotranslocation of clusterin/ApoJ into the cytosol. *Traffic* 8:554-565
121. Reddy, K.B., et al. (1996) Transforming growthfactor b (TGFb)-induced nuclear localization of apolipoprotein J/clusterin in epithelial cells. *Biochemistry* 35:6157-6163
122. Leskov, K.S., et al. (2003) Synthesis and functional analyses of nuclear clusterin, a cell death protein. *J Biol Chem* 278:11590-11600
123. Bucciantini, M., et al. (2004) Pre-fibrillar amyloid protein aggregates share common features of cytotoxicity. *J Biol Chem* 279:31374-31382
124. Kounnas, M.Z., et al. (1995) Identification of Glycoprotein 330 as an endocytic receptor for apolipoprotein J/Clusterin. *Biochemistry* 270:13070-13075
125. Zlokovic, B.V., et al. (1996) Glycoprotein 330 megalin: Probable role in receptor-mediated transport of apolipoprotein J alone and in a complex

- with Alzheimer disease amyloid  $\beta$  at the blood-brain and blood-cerebrospinal fluid barriers. *Proc Natl Acad Sci U S A* 93:4229-4234
126. Hammad, S.M., et al. (1997) Interaction of Apolipoprotein J-Amyloid B-peptide Complex with Low Density Lipoprotein Receptor-Related Protein-2/Megalin. *J Biol Chem* 272:18644-18649
  127. Calero, M., et al. (1999) Functional and structural properties of lipid-associated apolipoprotein J (clusterin). *Biochem J* 344:375-383
  128. Mahon, M.G., et al. (1999) Multiple involvement of clusterin in chicken ovarian follicle development. *J Biol Chem* 274:4036-4044
  129. Bartl, M.M., et al. (2001) Multiple receptors mediate apoJ-dependent clearance of cellular debris into nonprofessional phagocytes. *Exp Cell Res* 271:130-141
  130. Lakins, J.N., et al. (2002) Evidence that clusterin has discrete chaperone and ligand binding sites. *Biochemistry* 41:282-291
  131. Bajari, T.M., et al. (2003) A model for modulation of leptin activity by association with clusterin. *FASEB J* 17:1505-1507
  132. Trougakos, I.P., et al. (2006) Clusterin/apolipoprotein J up-regulation after zinc exposure, replicative senescence or differentiation of human haematopoietic cells. *Biogerontology* 7:375-382
  133. Bailey, R.W., et al. (2002) Heat shock-initiated apoptosis is accelerated and removal of damaged cells is delayed in the testis of clusterin/apoJ knock-out mice. *Biol Reprod* 66:1042
  134. McLaughlin, L., et al. (2000) Apolipoprotein J/clusterin limits the severity of murine autoimmune myocarditis. *J Clin Invest* 106:1105-1113
  135. Wehrli, P., et al. (2001) Inhibition of post-ischemic brain injury by clusterin overexpression. *Nat Med* 7:977-978
  136. DeMattos, R.B., et al. (2004) ApoE and clusterin cooperatively suppress Abeta levels and deposition: Evidence that ApoE regulates extracellular Abeta metabolism in vivo. *Neuron* 41:193-202
  137. Jensen, P.E. and L. Sottrup-Jensen (1986) Primary structure of human alpha-2 macroglobulin. Complete disulfide bridge assignment and localization of two interchain bridges in the dimeric and proteinase binding unit. *J Biol Chem* 261:15863-15869
  138. Imber, M.J. and S.V. Pizzo (1981) Clearance and binding of two electrophoretic "fast" forms of human alpha 2-macroglobulin. *J Biol Chem* 256:8134-9
  139. LaMarre, J., et al. (1991) Cytokine binding and clearance properties of proteinase-activated alpha 2-macroglobulins. *Lab Invest* 65:3-14
  140. Feige, J.J., et al. (1996) Alpha 2-macroglobulin: a binding protein for transforming growth factor-beta and various cytokines. *Horm Res* 45:227-232
  141. Crookston, K.P., et al. (1994) Classification of alpha 2-macroglobulin-cytokine interactions based on affinity of noncovalent association in solution under apparent equilibrium conditions. *J Biol Chem* 269:1533-1540

142. Araujo-Jorge, T.C., N. de Meirelles Mde, and L. Isaac (1990) Trypanosoma cruzi: killing and enhanced uptake by resident peritoneal macrophages treated with alpha-2-macroglobulin. *Parasitol Res* 76:545-552
143. van Dijk, M.C., et al. (1992) Role of the scavenger receptor in the uptake of methylamine-activated alpha 2-macroglobulin by rat liver. *Biochem J* 287 ( Pt 2):447-455
144. Hughes, S.R., et al. (1998) Alpha2-macroglobulin associates with beta-amyloid and prevents fibril formation. *Proc Natl Acad Sci U S A* 95:3275-3280
145. Yerbury, J.J., et al. (2009) Alpha 2 macroglobulin and haptoglobin suppress amyloid formation by interacting with prefibrillar protein species. *J Biol Chem* 284:4246-4254
146. Du, Y., et al. (1997) Alpha2-macroglobulin as a beta-amyloid peptide-binding plasma protein. *J Neurochem* 69:299-305
147. Shibata, M., et al. (2000) Clearance of Alzheimer's amyloid-ss(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. *J Clin Invest* 106:1489-1499
148. Basu, S., et al. (2001) CD91, a common receptor for heat shock proteins gp96, Hsp90, hsp70 and calreticulin. *Immunity* 14:303-313
149. Binder, R.J., D.K. Han, and P.K. Srivastava (2000) CD91: A Receptor for Heat Shock Protein Gp96. *Nat Immunol* 1:151-155
150. Binder, R.J. and P.K. Srivastava (2004) Essential Role of Cd91 in Representation of Gp96-Chaperoned Peptides. *Proc Natl Acad Sci U S A* 101:6128-6133
151. Arnold-Schild, D., et al. (1999) Receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. *J Immunol* 162:3757-3760
152. Henderson, B., et al. (2010) Caught with their PAMPs down? The extracellular signaling actions of molecular chaperones are not due to microbial contaminants. *Cell Stress Chaperones* 15:123-141
153. Pockley, A.G., M. Muthana, and S.K. Calderwood (2008) The dual immunoregulatory roles of stress proteins. *Trends Biochem Sci* 33:71-79
154. Quintana, F.J., et al. (2004) Inhibition of adjuvant-induced arthritis by DNA vaccination with the 70-kd or the 90-kd human heat-shock protein: immune cross-regulation with the 60-kd heat-shock protein. *Arthritis Rheum* 50:3712-3720
155. Binder, R.J., S.K. Kumar, and P.K. Srivastava (2002) Naturally Formed or Artificially Reconstituted Non-Covalent Alpha2-Macroglobulin-Peptide Complexes Elicit Cd91-Dependent Cellular Immunity. *Cancer Immun* 2:16
156. Dobryszczyka, W. (1997) Biological functions of haptoglobin - new pieces to an old puzzle. *Eur J Clin Chem Clin Biochem* 35:647-654
157. Giblett, E.R. (1968) The haptoglobin system. *Ser Haematol* 1:3-20

158. Gutteridge, J.M. (1987) The antioxidant activity of haptoglobin towards haemoglobin-stimulated lipid peroxidation. *Biochim Biophys Acta* 917:219-223
159. Edwards, D.H., et al. (1986) Haptoglobin-haemoglobin complex in human plasma inhibits endothelium dependent relaxation: evidence that endothelium derived relaxing factor acts as a local autocooid. *Cardiovasc Res* 20:549-556
160. Lange, V. (1992) Haptoglobin polymorphisms - not only a genetic marker. *Anthropol Anz* 50:281-302
161. Barclay, R. (1985) The role of iron in infection. *Med Lab Sci* 42:166-177
162. Cid, M.C., et al. (1993) Identification of haptoglobin as an angiogenic factor in sera from patients with systemic vasculitis. *J Clin Invest* 91:977-985
163. Sobek, O. and P. Adam (2003) On S. Seyfert, V. Kunzmann, N. Schwetfeger, H. C. Koch, A. Faulstich: Determinants of lumbar CSF protein concentration. *J Neurol* 250:371-372
164. Yerbury, J.J., et al. (2005) The acute phase protein haptoglobin is a mammalian extracellular chaperone with an action similar to clusterin. *Biochemistry* 44:10914-10925
165. El Ghmati, S.M., et al. (1996) Identification of haptoglobin as an alternative ligand for CD11b/CD18. *J Immunol* 156:2542-2552
166. Wagner, L., et al. (1996) Haptoglobin phenotyping by newly developed monoclonal antibodies: Demonstration of haptoglobin uptake into peripheral blood neutrophils and monocytes. *J Immunol* 156:1989-1996
167. Cedazo-Minguez, A. and R.F. Cowburn (2001) Apolipoprotein E: a major piece in the Alzheimer's disease puzzle. *J Cell Mol Med* 5:254-266
168. Zannis, V.I., D. Kardassis, and E.E. Zanni (1993) Genetic mutations affecting human lipoproteins, their receptors, and their enzymes. *Adv Hum Genet* 21:145-319
169. Li, W.H., et al. (1988) The apolipoprotein multigene family: biosynthesis, structure, structure-function relationships, and evolution. *J Lipid Res* 29:245-271
170. Strittmatter, W.J., et al. (1993) Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci U S A* 90:1977-81
171. LaDu, M.J., et al. (1994) Isoform-specific binding of apolipoprotein E to beta-amyloid. *J Biol Chem* 269:23403-23406
172. Pillot, T., et al. (1997) Specific modulation of the fusogenic properties of the Alzheimer beta-amyloid peptide by apolipoprotein E isoforms. *Eur J Biochem* 243:650-659
173. Wood, S.J., W. Chan, and R. Wetzel (1996) An ApoE-Abeta inhibition complex in Abeta fibril extension. *Chem Biol* 3:949-956
174. Evans, K.C., et al. (1995) Apolipoprotein E is a kinetic but not a thermodynamic inhibitor of amyloid formation: implications for the pathogene-

- sis and treatment of Alzheimer disease. *Proc Natl Acad Sci U S A* 92:763-767
175. Castano, E.M., et al. (1995) Fibrillogenesis in Alzheimer's disease of amyloid beta peptides and apolipoprotein E. *Biochem J* 306 (Pt 2):599-604
176. Ma, J., et al. (1994) Amyloid-associated proteins alpha 1-antichymotrypsin and apolipoprotein E promote assembly of Alzheimer beta-protein into filaments. *Nature* 372:92-94
177. Bales, K.R., et al. (1997) Lack of apolipoprotein E dramatically reduces amyloid beta-peptide deposition. *Nat Genet* 17:263-264
178. Bales, K.R., et al. (1999) Apolipoprotein E is essential for amyloid deposition in the APP(V717F) transgenic mouse model of Alzheimer's disease. *Proc Natl Acad Sci U S A* 96:15233-15238
179. Holtzman, D.M., et al. (1999) Expression of human apolipoprotein E reduces amyloid-beta deposition in a mouse model of Alzheimer's disease. *J Clin Invest* 103:R15-R21
180. Mackic, J.B., et al. (1998) Human blood-brain barrier receptors for Alzheimer's amyloid-beta 1-40. Asymmetrical binding, endocytosis, and transcytosis at the apical side of brain microvascular endothelial cell monolayer. *J Clin Invest* 102:734-743
181. Hu, J., M.J. LaDu, and L.J. Van Eldik (1998) Apolipoprotein E attenuates beta-amyloid-induced astrocyte activation. *J Neurochem* 71:1626-1634
182. Lim, S.K., et al. (1998) Increased susceptibility in Hp knockout mice during acute hemolysis. *Blood* 92:1870-1877
183. Emsley, J., et al. (1994) Structure of Pentameric Human Serum Amyloid-P Component. *Nature* 367:338-345
184. Pepys, M.B., et al. (1994) Human serum amyloid P component is an invariant constituent of amyloid deposits and has a uniquely homogeneous structure. *Proc Natl Acad Sci U S A* 91:5602-5606
185. Wood, S.P., et al. (1988) A pentameric form of human serum amyloid P component. Crystallization, X-ray diffraction and neutron scattering studies. *J Mol Biol* 202:169-173
186. Sorensen, I.J., et al. (1995) Native human serum amyloid P component is a single pentamer. *Scand J Immunol* 41:263-267
187. Hawkins, P.N., et al. (1994) Concentration of serum amyloid P component in the CSF as a possible marker of cerebral amyloid deposits in Alzheimer's disease. *Biochem Biophys Res Commun* 201:722-726
188. Bickerstaff, M.C.M., et al. (1999) Serum amyloid P component controls chromatin degradation and prevents antinuclear autoimmunity. *Nat Med* 5:694-697
189. Breathnach, S.M., et al. (1989) Serum amyloid P component binds to cell nuclei in vitro and to in vivo deposits of extracellular chromatin in systemic lupus erythematosus. *J Exp Med* 170:1433-1438

190. Sorensen, I.J., et al. (2000) Complexes of serum amyloid P component and DNA in serum from healthy individuals and systemic lupus erythematosus patients. *J Clin Immunol* 20:408-415
191. de Haas, C.J.C. (1999) New insights into the role of serum amyloid P component, a novel lipopolysaccharide-binding protein. *FEMS Immunol Med Microbiol* 26:197-202
192. Sorensen, I.J., et al. (1996) Binding of complement proteins C1q and C4bp to serum amyloid P component (SAP) in solid contra liquid phase. *Scand J Immunol* 44:401-407
193. Barbashov, S.F., C. Wang, and A. Nicholson-Weller (1997) Serum amyloid P component forms a stable complex with human C5b6. *J Immunol*. 158:3830-3858
194. de Beer, F.C., et al. (1981) Fibronectin and C4-binding protein are selectively bound by aggregated amyloid P component. *J Exp Med* 154:1134-1139
195. Swanson, S.J., R.B. Christner, and R.F. Mortensen (1992) Human serum amyloid P-component (SAP) selectively binds to immobilized or bound forms of C-reactive protein (CRP). *Biochim Biophys Acta* 1160:309-316
196. Brown, M.R. and B.E. Anderson (1993) Receptor-ligand interactions between serum amyloid P component and model soluble immune complexes. *J Immunol* 151:2087-2095
197. de Haas, C.J.C., et al. (1998) A synthetic lipopolysaccharide (LPS)-binding peptide based on amino acids 27- 39 of serum amyloid P component inhibits LPS-induced responses in human blood. *J Immunol* 161:3607-3615
198. Coker, A.R., et al. (2000) Molecular chaperone properties of serum amyloid P component. *FEBS Letters* 473:199-202
199. Hamazaki, H. (1995) Ca(2+)-dependent binding of human serum amyloid P component to Alzheimer's beta-amyloid peptide. *J Biol Chem* 270:10392-10394
200. Pepys, M.B., et al. (1979) Binding of serum amyloid P component (SAP) by amyloid fibrils. *Clin Exp Immunol* 38:284-293
201. Uversky, V.N. (2002) What does it mean to be natively unfolded? *Eur J Biochem* 269:2-12
202. Farrell, H.M., Jr., et al. (2002) Molten globule structures in milk proteins: implications for potential new structure-function relationships. *J Dairy Sci* 85:459-471
203. Kumosinski, T.F., E.M. Brown, and H.M. Farrell, Jr. (1993) Three-dimensional molecular modeling of bovine caseins: a refined, energy-minimized kappa-casein structure. *J Dairy Sci* 76:2507-2520
204. Farrell, H.M., Jr., et al. (2009) Review of the chemistry of alphaS2-casein and the generation of a homologous molecular model to explain its properties. *J Dairy Sci* 92:1338-1353

205. Koudelka, T., P. Hoffmann, and J.A. Carver (2009) Dephosphorylation of alpha(s)- and beta-caseins and its effect on chaperone activity: a structural and functional investigation. *J Agric Food Chem* 57:5956-5964
206. Khodarahmi, R., M. Beyrami, and H. Soori (2008) Appraisal of casein's inhibitory effects on aggregation accompanying carbonic anhydrase refolding and heat-induced ovalbumin fibrillogenesis. *Arch Biochem Biophys* 477:67-76
207. Thorn, D.C., et al. (2005) Amyloid fibril formation by bovine milk kappa-casein and its inhibition by the molecular chaperones alphaS- and beta-casein. *Biochemistry* 44:17027-17036
208. Thorn, D.C., et al. (2008) Amyloid fibril formation by bovine milk alpha s2-casein occurs under physiological conditions yet is prevented by its natural counterpart, alpha s1-casein. *Biochemistry* 47:3926-3936
209. Farrell, H.M., Jr., et al. (2006) Casein micelle structure: What can be learned from milk synthesis and structural biology. *Curr Opin Colloid In* 11:135-147
210. Farrell, H.M., Jr., et al. (2003) Environmental influences on bovine kappa-casein: reduction and conversion to fibrillar (amyloid) structures. *J Protein Chem* 22:259-273
211. Ecroyd, H., et al. (2008) Dissociation from the oligomeric state is the rate-limiting step in fibril formation by kappa-casein. *J Biol Chem* 283:9012-9022
212. Moesson, M.W. (2005) Fibrinogen and fibrin structure. *J Thromb Haemost* 3:1894-1904

### Figure legend

Figure 1 Theoretical model for the involvement of ECs in extracellular proteostasis and the immune response. Under normal physiological conditions misfolded extracellular proteins are bound by (a) scavenger-like receptors directly, or (b) circulating ECs, keeping them soluble and facilitating their subsequent transport to cell surface scavenger-like receptors. EC-client protein complexes may be internalized and subsequently degraded by lysosomal proteolysis. Alternatively, on antigen presenting cells (c), EC (or IC)-client protein complexes may be (i) bound and internalized by a variety of receptors, (ii) subsequently processed intracellularly by yet to be established mechanisms, and then (iii) re-presented as peptides on the cell surface associated with major histocompatibility (MHC) antigen class I or II molecules to trigger the release of cytokines and an immune response.

