

## Review Article

# Dysfunction of the ubiquitin-proteasome system in atherosclerotic cardiovascular disease

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**Abstract:** The ubiquitin-proteasome system (UPS) is an integral part of the protein metabolism and protein quality control in eukaryotic cells. It is involved in a number of biological processes of significance for vascular biology and pathology such as oxidative stress, inflammation, foam cell formation, and apoptosis. This review summarizes both indirect and direct lines of evidence for a role of the UPS in atherosclerosis from the initiation to the progression and complication stage and concludes with a future perspective.

**Keywords:** Atherosclerosis, inflammation, oxidative stress, proteasome, ubiquitin

The discovery of the ubiquitin-proteasome system (UPS) as the central protein degradation system in living cells was a milestone that was awarded the Nobel Prize in Chemistry in 2004. The central proteolytic element of this system is the proteasome, a high molecular, multi-catalytic cylinder composed of four stacked rings, two outer alpha and two inner beta rings [1, 2]. The connection with the ubiquitin system is made via a so-called 19S cap unit to either side of the cylinder. This unit serves as the recognition site for chains of at least four ubiquitin molecules attached to target proteins. Tagging target proteins in this particular manner is mediated by the ubiquitin system in three successive steps (activation, transfer and linking of ubiquitin molecules). Substrate specificity is determined by the last step, which is catalyzed by ubiquitin ligases [1].

Given the broad range of substrates, the UPS is involved in numerous biological processes including proliferation, apoptosis, and inflammation, which sparked interest in this system with regards to atherosclerosis (**Figure 1** and **Table 1**). Our initial studies were pursued on autopsy cases of patients who died from acute myocardial infarction [3]. This first study on the expression of ubiquitin showed increased ubiquitin levels in culprit plaques. Subsequent studies indicated that this was the consequence of

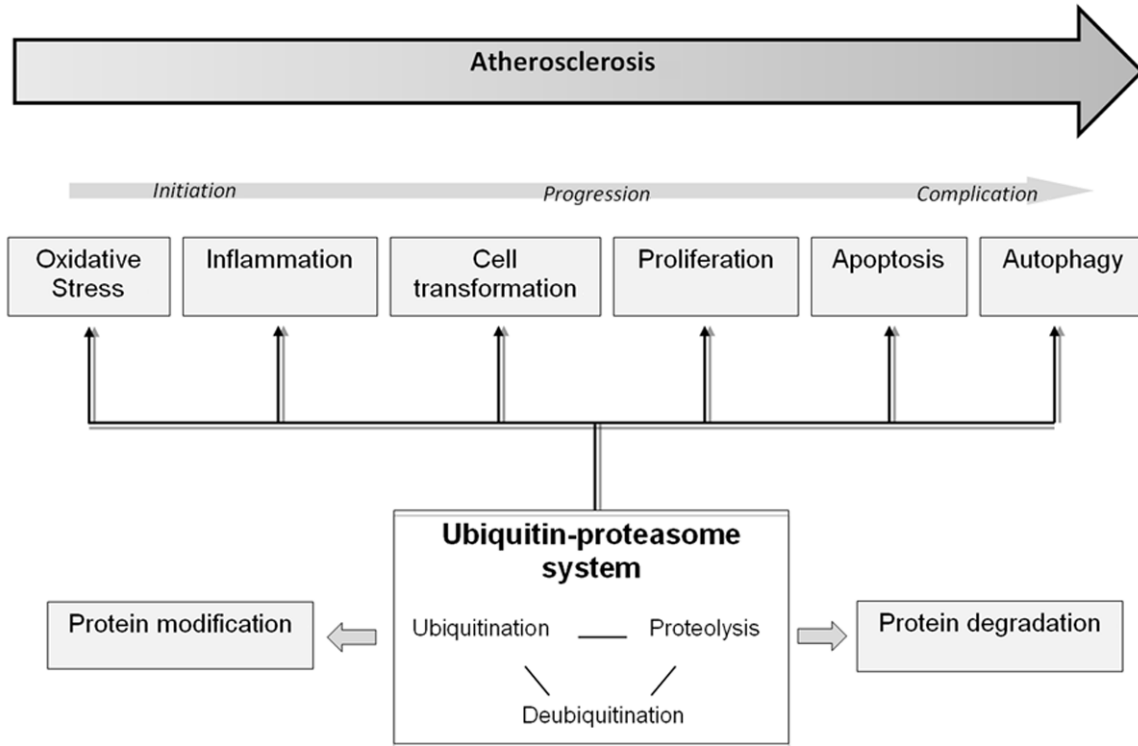
increased ubiquitin-tagged protein substrates with a decrease in proteasome activity as a contributing factor [4]. Since then more attention has been directed to the effect of proteasome inhibition.

In this article we review established and new evidence for the involvement of the UPS in atherosclerosis and its pathological elements.

### UPS - elements and substrates of significance for atherosclerosis

The proteasome is responsible for the degradation of 90% of all proteins in eukaryotic cells. Intriguingly, the assembly and disassembly of the proteasome itself is a fairly dynamic process and its final composition is determined by the biological milieu of the cellular environment [5]. The so-called constitutive proteasome is the classical proteasome subtype, the immunoproteasome is the next most familiar, and the thymoproteasome the least common subtype [6]. As the name implies, the thymoproteasome is expressed in cells of the thymus and the immunoproteasome in immune cells, especially antigen-presenting cells. However, the constitutive proteasome can be switched to the immunoproteasome in non-immune cells under conditions of inflammation, exposure to interferon (IFN)- $\gamma$  or tumor necrosis factor (TNF)- $\alpha$ , oxidative stress, and hypoxia [7]. Under these cir-

# UPS function and dysfunction in atherosclerosis

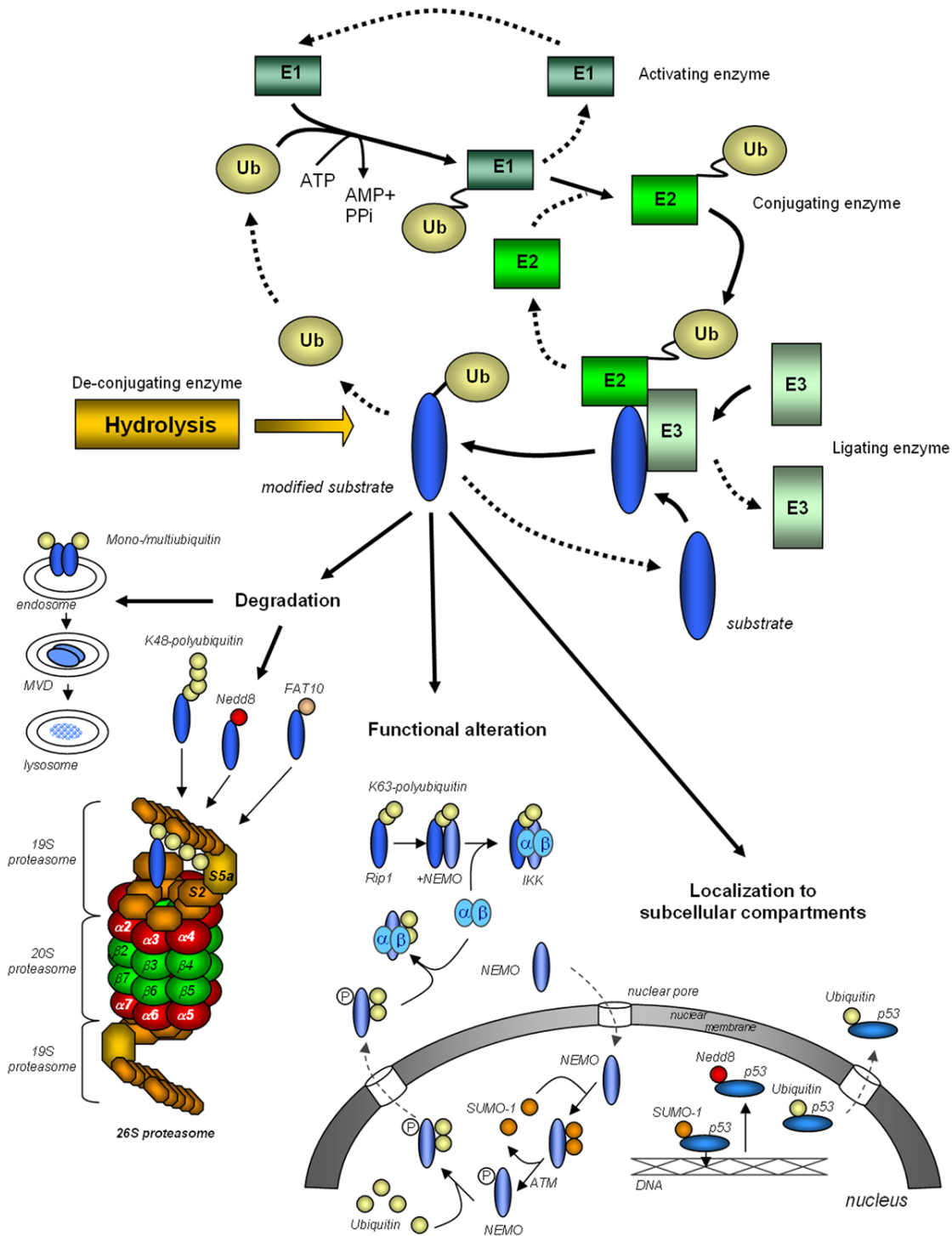


**Figure 1.** Overview of the involvement of the ubiquitin-proteasome system in atherosclerosis.

**Table 1.** Substrates of the UPS of interest for atherosclerosis

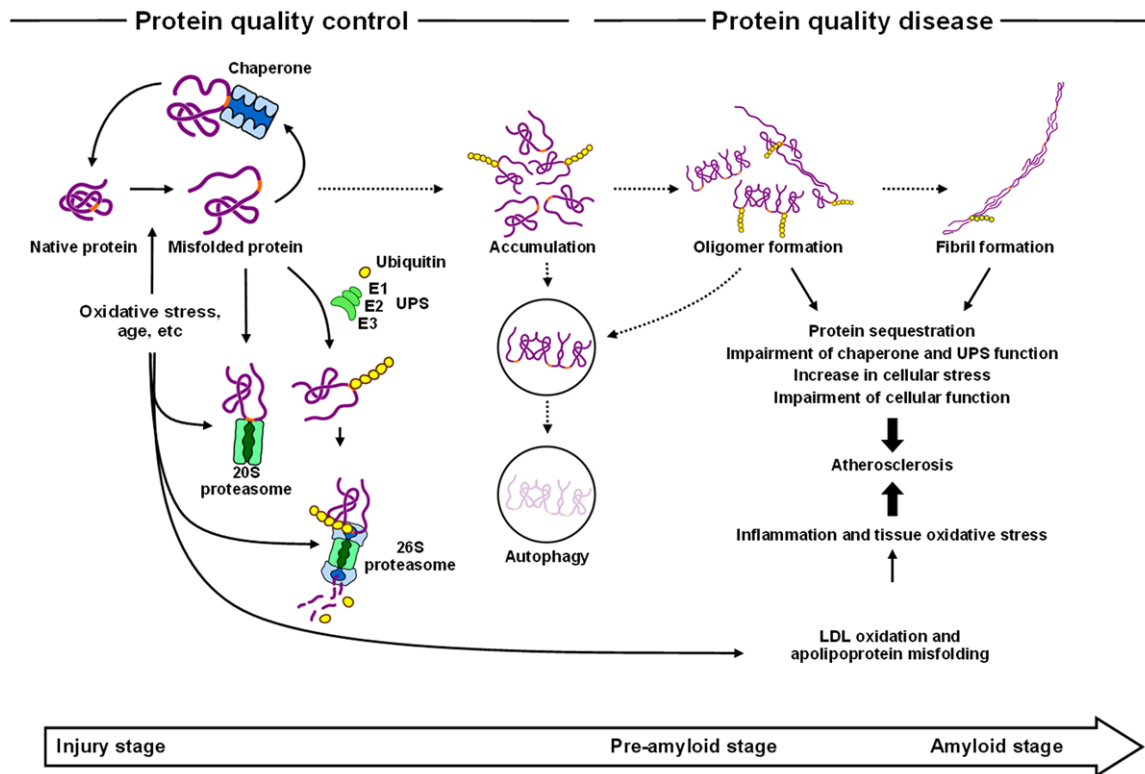
Functional class	Protein substrates
Positive cell cycle regulators	Cyclins, e.g. Cyclin A Cyclin-dependent kinase activators, e.g. Cdc25 A DNA replication initiation factors, e.g. Cdc6
Negative cell cycle regulators	Cyclin-dependent kinase inhibitors, e.g. p19INK4d, p21WAF1/Cip1, p27Kip1, p57Kip2 Retinoblastoma family of proteins, e.g. pRb, p107, p130
Pro-apoptotic molecules	p53 Bax Bid
Anti-apoptotic molecules	Caspase 3, caspase 7 c-IAP-1, XIAP Mdm2 Bcl-2
Transcription factors	NFκB1/NFκB2 precursor proteins p105/p100 HIF-1α c-myc c-fos/c-jun Nrf-2
Transcription regulators (signal transduction)	IκBα, IκBβ, IκBe c-Cbl, Cbl-b, Cbl-c Intracellular receptors, e.g. inositol 1,4,5-trisphosphate receptors, estrogen-receptor α Cell surface G-protein-coupled receptors, e.g. platelet-activating factor receptor Cell surface receptor tyrosine kinases, e.g. ErbB1, ErbB2, ErbB3, PDGF α and β receptors VEGF receptor type 2 (KDR/Flk-1), insulin-like growth factor-1 receptor Macrophage-stimulating factor receptor RON Non-receptor tyrosine kinases, e.g. src, abl, Lck, syk, ZAP-70

## UPS function and dysfunction in atherosclerosis



**Figure 2.** Illustration of protein modification by the ubiquitin proteasome system. Ubiquitin is activated, transferred and conjugated to a target protein by the action of E1, E2, and E3 enzymes. This process is balanced by the action of de-conjugating enzymes. Modified proteins can undergo degradation, either via the endosome/multi-vesicular-body (MVB)/lysosome pathway or via the 26S proteasome with and without adaptor proteins. Alternatively, they can undergo functional activation (e.g. I $\kappa$ B kinase (IKK) via ubiquitination of its regulatory subunit NEMO or associates) or translocation (e.g. polyubiquitination of p53). Proteins, modified by ubiquitin or ubiquitin-like modifiers such as NEDD8 or FAT10, are escorted to and recognized by the 26S proteasome by ubiquitin-domain proteins. Besides this recognition and substrate binding, the 19S complex facilitates deubiquitination and unfolding of the target protein by virtue of its ATPase activity as well as opening of the proteolytic tunnel of the 20S proteasome, which is composed of two flanking alpha and two central beta rings. The poly-amino acid-fragments of the target proteins are subsequently degraded further by proteases. Modified from [15].

## UPS function and dysfunction in atherosclerosis



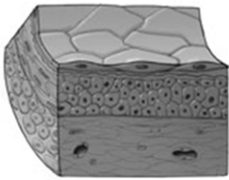
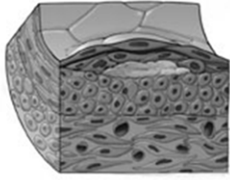
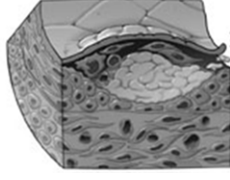
**Figure 3.** Compensatory up-regulation of the expression and activity of chaperones and the ubiquitin-proteasome system (UPS) prevents the accumulation of damaged and dysfunctional proteins in vascular cells subjected to the stress of cardiovascular risk factor exposure. The UPS activity also contributes to the classical activation pathway of nuclear factor kappa-B and thereby to inflammation and cell proliferation. With the formation and growth of a metabolically active atherosclerotic plaque, there is further production of misfolded and damaged proteins in the progression phase. Once the classical protein quality mechanisms are overwhelmed and fail, these dysfunctional proteins accumulate (and aggregate) and autophagy remains the final clearance pathway. The accumulating proteins can undergo further oxidation, ubiquitination, and cross-linking. As yet another unique characteristic, beta-pleated sheets can be formed and hence amyloid fibrils via the intermediate steps of pre-amyloid oligomers and protofibrils. In addition to intracellular proteins, proteins in the extracellular matrix can undergo conformational changes. For instance, oxidation and phospholipid hydrolysis of low-density lipoprotein (LDL) produces oxidatively modified and electronegative particles with unfolding of the apolipoprotein components. Recognition of amyloid-like fibrils by CD36 (and conceivably the receptor for advanced glycation end-products) leads to the production of reactive oxygen species, chemokines, and cytokines, which contributes further to the atherosclerotic disease process, including its complication phase. Modified from [98].

cumstances, the three proteolytic subunits of the constitutive proteasome are replaced with inducible subunits of the immunoproteasome. Furthermore, two homologous subunits, PA28 $\alpha$  and PA28 $\beta$ , can be added as unique caps (i.e. 11S proteasome). These changes allow for better protein processing for antigen presentation [8]. However, functions beyond this traditional role of the immunoproteasome have been discovered including more efficient degradation of oxidized/damaged proteins and modification of cytokine production and T-cell survival and function [7]. To further complicate the matter, an intermediate- or hybrid-type of the proteasome containing both constitutive and induc-

ible subunits has also been identified [9] and even extracellular forms of the proteasome (so-called circulating proteasome) have been recognized [10].

While some proteins can be degraded directly by the proteasome complex, a substantial number of proteins require “escorting” to the proteasome by modifier molecules (Figure 2) [1]. Ubiquitin was the first modifier molecule to be recognized, and the entire protein modification-degradation sequence has been referred to as the “ubiquitin-proteasome system” (UPS). Ubiquitin-tagging of protein substrates (“ubiquitination”) involves three successive steps for the

## UPS function and dysfunction in atherosclerosis

	Cell type focus	Mechanism focus	Proteasome activity	UPS involvement
<b>Initiation stage</b> 	Endothelial cell	Oxidative stress	Increased	Protective
	Macrophage	Inflammation		
<b>Progression stage</b> 	Foam cells	Survival/apoptosis	Preserved	Permissive
	Smooth muscle cell	Proliferation		
	Endothelial cells	Angiogenesis		
<b>Complication stage</b> 	Inflammatory cells	Inflammation	Decreased	Contributory
	All of the above	Apoptosis/autophagy		

**Figure 4.** Conceptual outline and summary of the potential role of the ubiquitin proteasome system in atherosclerosis.

individual ubiquitin moiety: 1) activation, 2) transfer, and 3) ligation to the protein substrate and previously attached ubiquitin molecules [1]. This linking is mediated by epsilon-NH2 lysine residue of the substrate and Lys48 residues of previously conjugated ubiquitin. In addition, ubiquitin molecules can undergo K63 linking, which does not lead to the degradation but the modification of proteins [1].

The following paragraphs will reflect on the current level of evidence for a role of the UPS in atherosclerosis, stratified by stage of the disease process. Conceptualized as such in our previous reviews, recent experimental studies have lent support for this view of a differential role of the UPS in atherosclerosis. A comprehensive “omics” approach to atherosclerosis including mRNA, miRNA, and DNA analysis identified the UPS as a major system of alteration besides the biological processes of inflammation, proliferation, and blood vessel formation [11]. Similarly, a genetic approach to the differences between atherosclerosis-susceptible and atherosclerosis-resistant animals found one of the most striking divergences in proteasome activity [12]. Exclusive to the resistant

strain was the expression of the proteasome maturation protein, which is central to the assembly of the protein, thus suggesting that a functional proteasome complex may avert atherosclerosis.

### The UPS in the initial stage of atherosclerosis

#### *Mechanistic considerations*

The prevailing theory views atherosclerosis as a response-to-injury process whereby dysfunction and activation of the endothelium take central stage [13, 14]. On molecular level, decreased bioavailability of nitric oxide (NO) is the central pathological element with numerous consequences all in favor of atherosclerosis development [2, 15]. The reduction in vascular NO bioavailability is the consequence of both reduced NO production and increased consumption. In endothelial cells, NO is produced by the enzyme endothelial NO synthase (eNOS), which remains in an inactive state in interaction with caveolin-1 in flask-shaped invaginations of the plasma membrane called caveolae. Activation of eNOS occurs when its inhibitory conformation with caveolin-1 is

## UPS function and dysfunction in atherosclerosis

reversed by excess  $\text{Ca}^{2+}$ /calmodulin and Akt-induced phosphorylation of eNOS [16]. This is reversed by protein phosphatase 2A, which upon ubiquitination translocates from the cytosol to the membrane where it associates with and dephosphorylates eNOS [17]. Dimerization of two eNOS molecules is furthermore necessary for enzymatic activity as is the essential co-factor tetrahydro-L-biopterin (BH4). In a BH4-reduced state, as mediated by cardiovascular risk factors, eNOS produces superoxide instead of NO (referred to as “eNOS uncoupling”). Superoxide reacts with NO avidly, generating various products such as peroxynitrite, which reduces NO and BH4 levels further. Intriguingly, it has been demonstrated that hyperglycemia stimulates degradation of guanosine 5'-triphosphate cyclohydrolase I (GTPCH) via the UPS, leading to BH4 deficiency and eNOS uncoupling in endothelial cells [18]. This sequence seems to be operational even in vivo, and proteasome inhibition does reverse the reduction of GTPCH, BH4 and endothelial dysfunction in streptozotocin-induced diabetes mellitus. Oxidative stress products such as 4-hydroxynonenal (4-HNE) are also capable of decreasing GTPCH levels and activity in endothelial cells via the proteasomal pathway with the aforementioned consequences [19]. Thus, this pathway complements the BH4 depletion due to superoxide production by NADPH oxidase, as noted with systemic hypertension and hyperlipidemia [20]. On the other hand, it has been demonstrated that proteasomal degradation of intracellular proteins contributes to the substrate supply of eNOS, and combined proteasome and lysosome impairment results in a profound decrease in eNOS activity [21]. Finally, vasorelaxation, the cardinal reflection of endothelial NO production, is linked to cyclic GMP production in vascular SMC as a consequence of NO-mediated activation of soluble guanylyl cyclase (sGC). Importantly, sGC levels are controlled by the co-chaperone/ubiquitin ligase carboxyl terminus of Hsc70 interacting protein (CHIP), which mediates ubiquitination and proteasomal degradation of sGC, thereby attenuating NO donor-induced relaxation of rat aortic rings [22].

With regards to oxidative stress, the UPS has an important role in regulating protective efforts via the transcription factor nuclear erythroid 2-related factor 2 (Nrf2). Nrf2 undergoes constitutive ubiquitination and degradation via

the proteasome as long as the level of oxidative stress is low and Keap-1 serves as a substrate adaptor for a Cul3-dependent E3 ubiquitin ligase complex [15]. Under circumstances of increased cellular oxidative stress, however, Keap-1 itself becomes a substrate for UPS-mediated degradation, and consequently, Nrf2 is stabilized and binds to genome sequences with an anti-oxidant response element (ARE). This leads to expression of genes encoding for proteins which have been linked to the amelioration of oxidative stress such as NQO1, HO-1, SOD-1, and GCLC [23]. Of note, Nrf2 also leads to an upregulation of proteasome subunits, and their overexpression via the Nrf2 pathway has been found to increase cellular resistance against toxic misfolded proteins [24]. Of further note, proteasome inhibitors can “shield” against oxidative injury in an Nrf2-dependent manner at low dose but may have the opposite effect at higher doses [25-27]. Vice versa, oxidative stress and its products can increase proteasome activity at low levels but decrease proteasome activity at high levels [28, 29].

Increased oxidative stress also stimulates the activation of another important transcription factor and regulator of oxygen homeostasis: hypoxia-inducible factor (HIF)-1 $\alpha$  [30]. HIF-1 $\alpha$  undergoes hydroxylation under normoxic conditions, allowing for its recognition by the ubiquitin ligase von-Hippel-Lindau (VHL) and its ubiquitination and degradation by the proteasome. This constitutive degradation ceases under conditions of hypoxia, which prevents hydroxylation of HIF-1 $\alpha$  and allows for its transcriptional activity on sequences with a hypoxia response element. Among the genes controlled by HIF-1 $\alpha$  is the one encoding for vascular endothelial growth factor (VEGF). This angiogenic growth factor is highly significant for vasa vasorum neovascularization, which starts already in the initiation stage of atherosclerosis and later influences progression and complications of atherosclerotic plaques [31]. Moreover, HIF-1 $\alpha$  promotes smooth muscle cell proliferation and foam cell formation [32, 33].

Interestingly, nuclear factor kappa B (NF $\kappa$ B), the central transcription factor responsible for endothelial cell activation, is activated by oxidative stress and an interplay with HIF-1 $\alpha$  has been noted as well. The UPS has a central role in the classical activation pathway of NF $\kappa$ B based on proteolysis of its endogenous inhibi-

## UPS function and dysfunction in atherosclerosis

tors (I $\kappa$ Bs) and proteolytic processing of precursor molecules [34]. A20, a member of the dual deubiquitinase and ubiquitin ligase family of enzymes highlights the significance of UPS for NF $\kappa$ B and NF $\kappa$ B for atherosclerosis. A20 exerts an important negatively modulating function on NF $\kappa$ B and maps to an important atherosclerosis susceptibility locus in mice [35]. High glucose and hyperglycemia reduce A20 levels in resident vascular cells by post-translational O-Gluco-samine-N-Acetylation, which leads to its ubiquitination and targeting for proteasomal degradation favoring NF $\kappa$ B activation [36]. On the other hand, the UPS is also involved in the termination of NF $\kappa$ B activity by degradation of the promoter-bound dimeric transcription factor complexes [37]. This applies to both the classical (UPS-dependent) as well as the non-classical (UPS-independent) activation pathway. The premise that the latter takes a more important role under conditions of oxidative stress in the vasculature would imply that inhibition of the proteasome under such conditions would lead to persistent activation of NF $\kappa$ B rather than its inhibition [38]. One of the gene sequences controlled by NF $\kappa$ B encodes for the pro-molecule of endothelin-1 (ET-1) as well as adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and E-selectin and chemoattractants such as monocyte chemoattractant protein (MCP)-1. Related to the interference with the NF $\kappa$ B pathway, proteasome inhibition has been shown to decrease vascular ET-1 content and adhesion molecule expression [39]. Most recently, not related to potent NF $\kappa$ B inhibition but reduction of oxidative stress, low-dose proteasome inhibitor treatment reduced adhesion molecule expression in the aorta of hypertensive rats [40]. In this context it is also pertinent to consider that S-glutathionylation of the redox-sensitive MAPK phosphatase (MKP)-1 upon LDL and high glucose stress leads to their proteasomal degradation. This step primes monocytes to endothelial adhesion, migration, and conversion [41].

### *Experimental evidence*

Studies on early disease stages of atherosclerosis have yielded a spectrum of results. Ubiquitin staining is not very prominent in coronary arteries and aortas of normal animals but can become evident under conditions of hypercholesterolemia and most notably in the smooth muscle cells of the media likely because of the

larger cellular volume [42, 43]. This is more apparent with immunoblotting, which also provides an instructing reflection of the molecular spectrum of ubiquitinated proteins. The amount of proteins modified in such a manner is potentially reduced by antioxidant treatment, which may in part be related to a stimulating effect on proteasome activity. Higher vascular proteasome activities are noted under conditions of chronic hypercholesterolemia and renal insufficiency, presumably indicting a compensatory up-regulation to meet increased demand [42-45]. Studies on human coronary and cerebral arteries echo these results indicating low background ubiquitin staining in non-diseased vessels but increasing levels with developing disease [3, 46]. Thus, there is not only rationale but also evidence for the activation of the UPS in the early disease stages of atherosclerosis.

### **The UPS in the progression stage of atherosclerosis**

#### *Mechanistic consideration*

The reduced barrier function of the dysfunctional endothelium allows for circulating molecules such as low-density lipoprotein (LDL) particles to enter the subendothelial space, where they get entrapped and undergo (oxidative) modifications. The concomitant activation of the endothelium allows for the attraction and invasion of monocytes into the intimal space where they turn into macrophages. An imbalance of cholesterol uptake and efflux along with enhanced esterification of cholesterol and its storage in cytoplasmic droplets leads to the conversion of these macrophages to foam cells, the traditional signature cell of atherosclerosis [47]. The other cell transformation taking place at this stage of the disease process is the transition of vascular SMCs from a contractile to a metabolic phenotype. Importantly, the UPS is involved in all of these processes.

Similar to SR-A, the scavenger receptor CD36 is subject to UPS-mediated degradation [48]. It is regulated by protein kinase C alpha (PKC $\alpha$ ), whose activity is downregulated by ubiquitination, leading to increased expression of CD36, intracellular cholesterol accumulation and the development of atherosclerosis [49]. Esterification of intracellular cholesterol is key for their entrapment and also to reduce cytotoxicity.

## UPS function and dysfunction in atherosclerosis

This key step is catalyzed by Acyl-coenzyme A: cholesterol acyltransferase-1 (ACAT-1), whose levels are also regulated by the UPS [50]. Free cholesterol, on the other hand, suppresses the ubiquitination of the ATP-binding cassette transporter molecules ABCA1 and ABCG1 and thereby their proteasomal degradation, which facilitates cholesterol efflux [51, 52].

Adipose differentiation-related protein is of further significance as it is associated with lipid droplets in various cell types including foam cells, and a functional UPS is required for the regression of these cells [53]. Finally, the very first link between the UPS and foam cells was made by the observation that aggregated LDL particles stimulate the expression of a ubiquitin-conjugating enzyme that mediates the ubiquitination and degradation of p53, thereby facilitating the suppression of apoptosis of lipid-bearing macrophages and contributing to foam cell formation [54].

The initial disease process matures as smooth muscle cells (SMCs) invade from the media into areas of foam cell, intracellular and extracellular cholesterol accumulation and lipid pool formation. They transform into a proliferating and metabolically active cell population that produces collagen and a fibrous cap over the lipid pool. Importantly, the conversion of smooth muscle cells from a contractile to a metabolic phenotype is prevented by proteasome inhibition [55, 56]. This is at least in part related to myocardin, a transcriptional co-activator that favors the expression of genes for a contractile phenotype but is targeted for proteasomal degradation by the ubiquitin ligase CHIP [57]. Besides migration, the UPS also influences cell cycle progression and viability of vascular SMCs [58, 59]. This has been attributed to an upregulation of the p21 cyclin-dependent kinase inhibitor and a shift in the balance in favor of pro-apoptotic molecules such as Bad [60]. Moreover, modulation of the pro-proliferation and pro-survival effects of NF $\kappa$ B seems of significance, and in fact, overexpression of the deubiquitinating enzyme, ubiquitin C-terminal hydroxylase L1 (UCHL1) inhibited NF $\kappa$ B activation as potently as proteasome inhibitors, likely related to reduced I $\kappa$ B $\alpha$  ubiquitination [61]. Similar results were obtained for cylindromatosis (CYLD), another deubiquitinating enzyme targeting TRAF2, a central molecule in the intra-

cellular TNF $\alpha$ -receptor signaling cascade upstream from I $\kappa$ Bs [62].

Regarding extracellular remodeling in atherosclerosis, one important mediator of matrix formation is transforming growth factor (TGF)- $\beta$ . Intriguingly, the diminishing NO bioavailability in the diseased arterial wall removes potent inhibition on TGF- $\beta$  signaling as NO directs Smad2 to proteasomal degradation [63]. Other downstream mediators in the TGF- $\beta$  signaling, including its receptor, have been shown to undergo proteolysis by the proteasome [64]. However, other studies show that proteasome inhibition can actually reduce matrix metalloproteinase and matrix production [65]. These find their echo in recent experiments indicating that proteasome activity contributes to angiotensin-II-induced hypertension and hypertensive aortic vascular remodeling [66].

### *Experimental evidence*

There is evidence of both accumulation of ubiquitinated proteins and relatively preserved proteasome activity with early and developing stages of atherosclerosis. In human arteries ubiquitin immunoreactivity can be noted in endothelial and smooth muscle cells in the early disease stages and in almost all cells thereafter [3]. As such, it is a reflection of cellularity as well as UPS substrates generation and turn over. Furthermore, prominent accumulation can also be noted around and within the lipid core, reflecting remnants of necrotic foam cells [3]. A recent study confirms the aforementioned observations made in the initiation stage in animal models in early human atherosclerotic lesions [11]. These have an increased spectrum of ubiquitinated proteins and increased proteasome proteolytic activity.

Taken together, the activity of the UPS is upregulated over baseline levels in early developing and progressing atherosclerotic lesions. The increase in ubiquitinated proteins is not the consequence of decreasing proteasome activity in this stage but rather increasing substrate availability. The UPS is inherently involved in cholesterol metabolism, foam cell formation and viability as well as SMC transformation, proliferation, and migration and therefore in the central elements of atherosclerosis progression.



### The UPS in the complication stage of atherosclerosis

#### *Mechanistic considerations*

With expansion of the necrotic lipid core and thinning of the overlying fibrous cap, the atherosclerotic plaque reaches its critical limit in sustaining structural integrity. This is further weakened by the release of proteolytic enzymes, formation and leakage of plaque neovessels in the shoulder area, and apoptosis of various protective cells under circumstances of high levels of inflammation. The consequences are endothelial cell shearing off the basement membrane leading to erosion or tearing of the fibrous cap leading to plaque rupture, thrombus formation, and complete or near complete vascular occlusion as the substrate of acute clinical presentations [47].

T cells have an important role in amplifying or decreasing the extent of tissue inflammation and apoptosis [67]. In these cells the strength and duration of the pro-inflammatory IKK/NF $\kappa$ B signaling pathway is controlled by the balance of K63-linked ubiquitination of Malt-1 upon T cell receptor (TCR)/CD 28 costimulation and the Malt-1-deubiquitinating activity of A20 [68]. Bcl10 promotes the activation of the IKK complex but undergoes phosphorylation as a consequence and thereby becomes a target for UPS-mediated degradation in negative feedback loop manner [69]. However, as T cells in atherosclerotic plaques often lack the costimulatory receptor CD28 [67], the significance of this activation mode for atherosclerotic cardiovascular disease remains questionable.

AlphaCD3/CD28-costimulated T-cells from healthy volunteers and patients with rheumatoid arthritis do show a marked reduction of the release of several NF $\kappa$ B-inducible cytokines (including tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6 and IL-10) within the first 24 hours of treatment with the proteasome inhibitor bortezomib. Beyond this time frame, proteasome inhibition leads to a reduction of T cell activation and induction of T-cell apoptosis [70]. These findings likely relate to the proteasome-mediated activation and nuclear translocation of nuclear factor of activated T cells [15]. This transcription factor controls the expression of the activation-associated cell surface receptors CD25, CD28, CD120b and CD134 as

well as production of interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-4 and IL-5. Furthermore, T cells can be arrested in the G (1) phase by accumulation of cyclin-dependent kinase inhibitors p21 (WAF1/CIP1) and p27 (KIP1) and the disappearance of cyclin A, cyclin D2 and proliferating cell nuclear antigen. Finally, with prolonged inhibition of proteasome function, T cells undergo apoptosis via the mitochondrial p53 pathway. Intriguingly, oxLDL induces changes outlined above, and most notably apoptosis of CD4+/CD25+ regulatory T cells in a time- and concentration-dependent manner [71]. This is important as apoptosis of regulatory T cells removes a cell population so vital for the stabilization of atherosclerotic plaque as these cells down-regulate T cell responses to foreign and self-antigens. On the other hand, a very recent study just pointed out that the aforementioned unique population of CD4+CD28+ T cells is relatively apoptosis-resistant relating to an alteration in the balance of pro- and anti-apoptotic molecules, and that inhibition of the proteasome renders these cells susceptible to apoptosis again [72].

Dendritic cells (DCs) are the main antigen-presenting cells and stimulators for T cells and are present in atherosclerotic plaques, primarily in the shoulder regions and particularly in complicated and symptomatic plaques [47, 73]. They remain functionally competent under these conditions as suggested by the observation that they maintain antigen-presenting properties and their ability to prime CD4(+) T cells even under hypercholesterolemic conditions [74]. However, proteasome inhibition leads to the impairment of the maturation and function of DCs. As a consequence, DCs fail to stimulate allogeneic CD4(+) and CD8(+) T cells and autologous CD4(+) T cells sufficiently and they lose their ability to regulate innate and adaptive anti-tumor immunity [75-77]. Proteasome inhibition also induces apoptosis of DCs via the mitochondrial pathway, and the sensitivity to this effects is related to the activity of the NF $\kappa$ B pro-survival pathway [78, 79]. NF $\kappa$ B also leads to changes of the composition of the proteasome in these cells, namely upregulation of the expression of the PA28 $\beta$  unit of the immunoproteasome, which is the limiting factor for proper PA28 $\alpha\beta$  complex formation [80]. Contrary to most other cells, expression of the 11S proteasome units in DCs is differentially regulated and not solely dependent on IFN- $\gamma$  [81].

## UPS function and dysfunction in atherosclerosis

Intriguingly, the immunoproteasome was recently identified as a potential link between inflammation and apoptosis of plaque cells [82]. In the presence of IFN- $\gamma$ , vascular SMCs in the fibrous cap are sensitized to apoptosis via the Fas/Fas ligand pathway, related to the induction of the inducible  $\beta 5$  subunit of the immunoproteasome. This switch in proteasome subtype allowed for proteolytic processing of myeloid cell leukemia (Mcl)-1, thereby removing a potent inhibitor (sequester) of pro-apoptotic molecules (59) [83]. This mode of regulation is in addition to the possible degradation of Mcl-1 via the conventional UPS [84, 85].

With regards to apoptosis, the UPS targets a variety of pro- and anti-apoptotic molecules whereby the interaction with Bcl-2 needs to be highlighted as Bcl-2 can negatively influence proteasome activity and vice versa [86]. Furthermore, one of the most important factors degraded by proteasomes during apoptosis may be the inhibitors of apoptosis (IAPs) [86]. These bind and target caspases for degradation. However, in response to an apoptotic stimulus, they undergo auto-ubiquitination and degradation. Their expression is regulated by NF $\kappa$ B, which relates to the significance of this transcription factor as a pro-survival factor, and the importance of the UPS for the activity of this transcription factor was outlined above. Furthermore, the UPS impacts cell apoptosis via control of cellular levels of p53. This transcription factor not only regulates the expression of various proteins but also their post-transcriptional level by “triaging” them to the UPS [86]. A prime example is FLIP, which relates to Mcl-1 in its regulation by the UPS in TNF-mediated apoptosis. Thereby p53 seems to take an important role in rendering cells sensitive to apoptosis, including those, which had become resistant, for instance, senescent cells [87]. Finally, the UPS is responsible for degradation of the pro-proliferative transcription factor c-Myc, and the accumulation of this transcription factor due to impairment of UPS function promotes cellular apoptosis via the Fas/FasLigand pathway [86]. All of these factors are of significance in atherosclerosis, underscored by studies demonstrating that transfer of p53 to atherosclerotic lesions generates experimental models of vulnerable, complicated plaques [88-95].

In addition to apoptosis, autophagic cell death has been recognized as a separate entity in

atherosclerotic plaques [96]. Autophagy is activated under circumstances of impaired proteasome function likely as a consequence of endoplasmic reticulum stress caused by misfolded proteins and as a compensatory effort to remove polyubiquitinated protein aggregates [97]. Accordingly, atherosclerosis bears characteristics of other protein quality diseases (**Figure 3**) [98]. A number of mechanisms, however, can induce autophagy in the atherosclerotic plaque independent of the impairment of proteasome function [96]. Moreover, autophagy can also have cytoprotective effects and the detrimental effects prevail only with excessive stimulation of autophagic activity [96].

### *Experimental evidence*

Only one study investigated UPS activity in advanced atherosclerotic plaques in an animal model [99]. Contrary to the experimental studies on animals exposed to cardiovascular risk factors, there was no evidence of increased ubiquitin levels even though these animals were subjected to dietary changes consistent with cardiovascular risk. There was no report on proteasome activities, however, challenging the interpretation and perspective of these results.

More information has been obtained from studies on human samples. Studies on coronary, carotid, and cerebral arteries consistently outlined an increase in ubiquitin conjugates [3, 4, 46]. Furthermore, one recent study also noted increased expression of the proteasome in atherosclerotic plaques, which did not decline with further progression of disease [11]. Yet, advanced plaques from patients who died of an acute myocardial infarction have a notable decrease in proteasome activity, even below baseline level of non-diseased vessels [11]. A reduction in proteasome activity was also found in symptomatic carotid plaques and correlated with parameters of oxidative stress and apoptosis [4]. Other studies, however, noted an increase in proteasome activity in high risk plaques and even in macrophages extracted from these plaques [100, 101]. These differences have been explained by differences in the study population, as, for instance, proteasome activity declines with age and is lower in atherosclerotic plaques of patients > 60 years of age [102, 103]. Collectively, these data raise the question if it is, in fact, a decrease in proteasome function that contributes to the senes-

## UPS function and dysfunction in atherosclerosis

**Table 2.** Listing of experimental studies investigating the effect of proteasome inhibition on atherosclerosis

<i>Author</i>	<i>Year</i>	<i>Model</i>	<i>Disease stage</i>	<i>Proteasome inhibitor</i>	<i>Dose</i>	<i>Duration</i>	<i>Degree of Inhibition</i>	<i>Major findings</i>
Herrmann et al.	2007	N and HC pigs	Initiation stage	MLN-274	0.08 mg/kg SQ twice weekly	12 weeks	68-72%	Induction of oxidative stress, Impairment in endothelial function, Increase in intima-media ratio
Van Herck et al.	2009	Apo E <sup>-/-</sup> mice on Western diet and with carotid artery collar	Progression stage (start of treatment 2 weeks after cuff placement)	Bortezomib	Low dose: 0.1 mcg/g body weight IP Every 3 days High dose: 0.5 mcg/g body weight IP every 3 days	4 weeks	37% (aorta) 49% (liver) 57% (aorta) 77% (liver)	No change in plaque area or composition
Van Herck et al.	2009	Apo E <sup>-/-</sup> mice on Western diet and with carotid artery collar	Complication stage (start of treatment 6 weeks after cuff placement)	Bortezomib	Low dose: 0.1 mcg/g body weight IP Every 3 days High dose: 0.5 mcg/g body weight IP every 3 days	4 weeks	37% (aorta) 49% (liver) 57% (aorta) 77% (liver)	No change in plaque area or macrophage content but decrease in $\alpha$ -SMC-actin+ cells and collagen content, increase in apoptosis and necrotic core
Feng et al.	2010	New Zealand white rabbits undergoing sham operation or 5/6 nephrectomy	Initiation stage	MG-132	20 mcg/kg IM daily	4, 8, and 12 weeks	Max. 40% in normal and 61% in uremic animals	Reduction of NF $\kappa$ B activation, TNF $\alpha$ production, and intimal thickening
Wilck et al.	2012	LDLR <sup>-/-</sup> mice on Western diet	Initiation to progression stage	Bortezomib	50 mcg/kg body IP twice weekly	6 weeks	33% (liver)	Reduction in oxidative stress, macrophage accumulation, and plaque formation No impairment in vasoreactivity

cence of the vascular system, which includes atherosclerotic changes. Then again the question is how a decrease in proteasome activity could contribute to plaque destabilization if unimpaired UPS activity relates to inflammation. It may be speculated that chronic impairment in proteasome function results in apoptosis of plaque cells and thereby contributes to plaque destabilization. In fact, the detrimental effect on cell viability over time may well be the prevailing one.

Taken together, along with the evolution of the atherosclerotic disease process from progression to complication, there is a notable decrease in proteasome activity. While this decrease in proteasome activity may be assumed to have beneficial effects in reducing inflammation, it may rather lead to increased oxidative stress and accumulation of its products with subsequent cell decay. This diminishes the pool of endothelial cells and matrix-producing smooth muscle cells and thereby the viable lining of plaque neovessels and plaque surface, contributing to complications such as plaque erosion, plaque rupture and plaque hemorrhage.

### **Proteasome inhibition - for better or worse in atherosclerosis**

With regards to the modulation of the activity of the UPS, the focus has been on the proteasome for very pragmatic reasons. Such an approach, however, is broad in its consequences and still not all encompassing. Only a few studies have been pursued in atherosclerosis models over the past years as summarized in **Table 2**. At first glance, the results vary greatly based on the disease model and the type and administration of the proteasome inhibitor. At second view, a correlation with the degree of proteasome inhibition and possibly the underlying disease burden becomes evident. Higher degrees of proteasome inhibition (60-80%) seemingly contribute to atherosclerosis, inducing as well as advancing it, even to the point of a more complication-prone phenotype. Lower degrees of proteasome inhibition (40%), on the other hand, seem to have the opposite effect. Another study related at least some of the anti-inflammatory effects of aspirin to an inhibition of the activation of nuclear factor kappa B via proteasome inhibition [43]. Interestingly, the

20S proteasome was significantly more sensitive to both the impact of hypercholesterolemia and aspirin in this animal model. Similarly, two different polyphenols of green tea were found not only to decrease intimal thickening in an early atherosclerosis model but also to decrease otherwise upregulated arterial proteasome activities (mainly chymotrypsin-like) [45]. No further experiments were performed to establish that inhibition of the proteasome is a causal element to the anti-atherosclerotic effects of these substances. Intriguingly, even in these studies the effect of these substances differed by vascular segments, and in fact, an increase in chymotrypsin-like activity was noted in the venous system. Indeed, adaptive responses to proteasome inhibition need to be considered which add to the complexity of the field [104].

### **Future directions**

An extraordinary number of studies have been pursued on the role of the UPS in atherosclerosis over the past years. In keeping with the primary research direction the focus has been on the intracellular UPS. However, studies have reported the presence of the extracellular UPS and it has been suggested that it functions as an endogenous immune modulator [10, 105]. Most recently, and also related to the CXCR4 receptor pathway, extracellular ubiquitin has been shown to exert pro-angiogenic effects on cardiac microvascular endothelial cells [106]. This is therefore an area that is highly relevant and interesting for atherosclerosis and future studies are to examine the role of the extracellular UPS in atherosclerosis further. This includes the extracellular proteasome and the circulating proteasome, the levels of which are elevated under conditions of inflammation such as rheumatoid arthritis [107]. Another subtype of the proteasome recognized to exert a putatively important role in rheumatoid arthritis but not studied much up to this point in atherosclerosis is the immunoproteasome [108]. Studies on this subtype are supported by the availability of specific immunoproteasome inhibitors [108]. Such specific targeting may circumvent some of the hazards associated with general proteasome inhibitors. Indeed, the FDA has issued a warning on the proteasome inhibitor carfilzomib because of reports of cardiovascular events [109].

## Summary

The UPS is a vital part of protein metabolism and protein quality control mechanisms, and a reduction in the function of the vascular proteasome could lead to a degenerative disease process that shares similarities with neurodegenerative disorders and bears characteristics of protein quality diseases. Upon cardiovascular risk factor exposure and in the initial disease stages, the activity of the UPS is increased but with uncertainty whether this generates pathophysiological momentum or constitutes a compensatory mechanism. The fact that proteasome function is lower in complicated than in non-complicated advanced plaques implies that a decrease in proteasome activity is a manifestation of a rather late disease stages (**Figure 4**). Proteasome inhibition is therefore likely not to accomplish much in the complicated disease stage. In prior stages of the disease, depending on the environment, dose and duration of proteasome inhibition, the effects can be rather diverse and broad limiting its therapeutic utility. While second generation proteasome inhibitors have become available, these have been associated with cardiovascular events. Thus, more specific targeting of specific subtypes of the proteasome such as the immunoproteasome might be a more promising approach. In addition, specific inhibitors of the ubiquitin system might allow a more differential assessment of the pathophysiological role of the individual components of the UPS in atherosclerotic cardiovascular disease and possibly even of the extracellular UPS. These are the next chapters to be written in the book on UPS and atherosclerosis.

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## Disclosure of conflict of interest

There is no conflict of interest for any author of this manuscript.

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## UPS function and dysfunction in atherosclerosis

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