Heat Shock Protein-60 and Risk for Cardiovascular Disease

Manfredi Rizzo1,2, Alberto J.L. Macario1,2,3, Everly Conway de Macario3, Ioanna Gouni-Berthold4, Heiner K. Berthold5, Giovam Battista Rini1, Giovanni Zumbo6 and Francesco Cappello2,6,*

1Department of Internal Medicine and Medical Specialties, University of Palermo, Palermo, Italy; 2Euro-Mediterranean Institute of Science and Technology, Italy; 3Department of Microbiology and Immunology, School of Medicine, and IMET, University of Maryland, Baltimore (MD), USA; 4Center for Endocrinology, Diabetes and Preventive Medicine, University of Cologne, Cologne, Germany; 5Lipid Clinic at the Interdisciplinary Metabolism Center, Virchow Clinic Campus, and Evangelical Geriatrics Center Berlin, Charité University Medicine Berlin, Berlin, Germany; 6Department of Experimental Biomedicine and Clinical Neurosciences, University of Palermo, Palermo, Italy

Abstract: Cardiovascular disease (CVD) is a leading cause of morbidity and mortality worldwide. There is growing evidence that molecular chaperones, many of which are heat shock proteins HSPs, are involved in CVD pathogenesis. In this review we focus on HSP60, the human mitochondrial chaperone that also displays extramitochondrial and extracellular functions. HSP60 is typically cytoprotective but a number of stress conditions determine its conversion to a potentially toxic molecule for cells and tissues. We present illustrative examples of specific subtypes of CVD where HSP60 is implicated in the initiation and/or progression of disease. The data not only indicate a pathogenic role for HSP60 but also its potential as a biomarker with applications for diagnosis, assessing prognosis and response to treatment, as well as for preventing and treating CVD.

Keywords: Chaperonin, heat shock protein 60, cardiomyocytes, heart failure, cardiovascular disease, atherosclerosis.

1. INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality worldwide, for both men and women [1,2]. An efficacious prevention strategy includes the treatment of various CVD risk factors, such as smoking, hypertension, hypercholesterolemia and diabetes. However, the absence of “traditional” risk factors does not completely exclude the possibility of CVD. For example, epidemiological data have suggested that inflammation can also be a risk factor for the development of atherosclerosis [2]. As a consequence, the National Cholesterol Education Program, through the Adult Treatment Panel III guidelines, identified inflammation as one of the “emerging risk factors,” and suggested that its detection and measurement will improve the estimation of absolute risk that would be obtained by using only the traditional CVD risk factors [2].

In the past two decades growing evidence has accumulated indicating that a group of molecules highly conserved during evolution and assembled under the name of molecular chaperones/heat shock proteins (HSPs) can participate in the pathogenesis of atherosclerosis and CVD. HSPs-chaperones constitute a physiological system, now called chaperoning system, that plays a crucial role in maintaining cell and tissue homeostasis [3,4]. For this reason, qualitative and quantitative modifications of HSPs due to genetic or acquired defects cause a variety of pathological conditions now referred to as chaperonopathies [5,6].

The levels and functional quality of intracellular HSPs, as a rule, decline with age but their overexpression often occurs in response to a variety of stressors, resulting in their intracellular accumulation. This intracellular accumulation and post-translational modification of HSPs is followed by their release from intact, not damaged cells into the circulation [4]. HSPs interact with the immune system in a manner that contributes to generating/maintaining the inflammatory status, especially in elderly people [4]. In this regard, it is relevant that human and bacterial HSPs share a high degree of homology and can immunologically cross react. Therefore, antibodies elicited by a bacterial HSP can also react against the human counterpart present in the infected individual and, thereby, initiate various pathogenic cascades. [8].

HSP60 is the human mitochondrial chaperonin that works in conjunction with its co-chaperonin HSP10 to facilitate protein folding. Due to its key role in supporting mitochondrial functionality, HSP60 has been regarded as one of the most important proteins for cell survival. Additionally, the interest toward this chaperonin has been growing since it was also found in extramitochondrial sites (e.g. cytosol, cell membrane and extracellular space), where it displays roles not directly pertinent to protein folding, such as regulation of apoptosis, induction of inflammatory phenomena and cell signaling [10].

We review the main experimental results about the involvement of HSP60 in CVD. We discuss epidemiologic and mechanistic data regarding the role of HSP60 in the pathogenesis of CVD as well as its potential therapeutic implications. We also consider the most recent attempts to use HSP60 for chaperonotherapy of CVD. In order to do so, we have followed the following search strategy, evaluating the existing literature about HSPs, molecular chaperones, chaperonins, HSP60 and risk for cardiovascular disease, heart failure, atherosclerosis, ischaemia/reperfusion, hypertension, atrial fibrillation and metabolic diseases. The search strategy involved PubMed®, Scopus, ISI-Knowledge, Google Scholar and other minor databases.

2. HSP60 CAN PROTECT CARDIOMYOCYTES FROM ISCHAEMIA/REPERFUSION AND HEAT SHOCK-INDUCED DAMAGE

The levels of HSP60 protein and mRNA were found doubled in human subjects with ischaemic and dilated cardiomyopathy compared with normal controls [11,12]. This observation encouraged research on protein and mRNA levels of HSP60 in ischaemia/reperfusion (I/R) animal models. No changes in HSP60 mRNA levels were observed after 20 min of ischaemia followed by 30 min of reperfusion in rat heart [13]. Myocardial protection against I/R insult, induced by mild exercise, was independent of HSP60 [13,14]. In rabbits, HSP60 increased after 30 min of ischaemia preceded by a 5 min ischaemic pre-treatment and the increase in the chaperonin correlated with myocardial protection [15]. Likewise,
HSP60 increased during ischaemia and decreased during reperfusion in rat heart, as shown by a proteomic approach [16]. Doxorubicin treatment after I/R induced HSP60 up-regulation [17,18]. All together, these data show that HSP60 expression is finely regulated in myocardium, and that the chaperonin is part of the molecular network contributing to myocardial protection during I/R processes.

Along these lines, other data indicate participation of HSP60 in the cardiovascular reaction to stress. Coronary artery ligation induced an increase of HSP60 levels and this was related to a decrease of mitochondrial oxygen consumption rate in rats [19]. Myocardial infarction induced early release of HSP60 into the circulation, both in rats [20] and humans [21]. Elevated levels of circulating HSP60 were predictive of post-infarct adverse events [21], and thus this protein was proposed as a prognostic tool in post-infarct follow-up. HSP60 levels did not change after a single episode of hypothermic ischaemia, cardioplegic arrest, and reperfusion in subjects undergoing cardiac surgery, and this may reflect the effective cardioprotection of extracorporeal circulation [22]. In contrast, myocardial levels of HSP60 protein increased following acute rejection, probably reflecting the acute stress of this condition [23].

A number of studies compared the effects of single or combined hypoxia/ischaemia and heat stress on HSP60 levels in cardiomyocytes (CMC). These studies were conducted mostly on embryonic or neonatal CMC since these cells are more likely to replicate in culture than their adult counterparts. HSP60 mRNA levels increased in neonatal rat CMC after ischaemia but not after a sub-lethal heat stress [24]. In contrast, embryonic rat-derived CMC subjected to a sub-lethal heat stress showed elevated levels of HSP60 protein that was associated with an increased survival against a subsequent lethal heat stress [25]. The same cells transfected with HSP60-bearing plasmids showed no increased survival against either heat stress or hypoxia [25]. This was possibly due to the lack of HSP10, the HSP60 mitochondrial co-chaperonin. Indeed, only simultaneous overexpression of HSP60 and HSP10 by adenoviral constructs in neonatal rat CMC protected against simulated ischaemia, while single overexpression of any of these 2 chaperonins was ineffective [26]. Conversely, in another study on neonatal rat CMC, overexpression of HSP60 and HSP10 either together or separately protected myocytes against apoptosis, maintaining mitochondrial integrity and capacity for ATP generation after simulated ischaemia and reperfusion [27]. The discrepant results may be attributed to the use of neonatal rather than embryonic CMC. Finally, a very recent study on transgenic mice showed that myocardial ischemia activates an innate immune signaling via HSP60 and toll-like receptor (TLR)4 that in turn plays an important role in mediating apoptosis and inflammation during I/R [28]. All together, these data show that the involvement of HSP60 in CMC apoptosis is a controversial topic that we will address in the following paragraph.

3. HSP60 HAS ANTI-APOPTOTIC EFFECTS IN CARDIOMICYOCYTES

As already mentioned, HSP60 in normal cells is present mainly in mitochondria but also in extramitochondrial sites, such as cytosol and cell membranes [10]. Cytosolic HSP60 (cHSP60) can be found in 2 forms, namely, with or without the mitochondrial signal sequence, a sort of “tail” that the molecule, translated in the cytosol, loses when it enters into this organelle [29]. It is not clear if cHSP60 forms the same heptameric structure, i.e. the “barrel” that it loses when it enters into this organelle. cHSP60 has been shown to display functions distinct from those of the intramitochondrial counterpart (that is dedicated to assisting protein folding). For example, cHSP60 forms complexes with other proteins involved in apoptosis, as Bak and Bax. HSP60 decrease in CMC induced by antisense treatment resulted in the dissociation of the complexes between the chaperonin and Bak and Bax, which in turn precipitates apoptosis, as documented by cytochrome C release, caspase 3 activation, and DNA fragmentation [30,31]. Also, hypoxia induced dissociation of the HSP60-Bax complex in adult rat CMC with translocation of cHSP60 to plasma membrane and Bak to mitochondria, the latter, in turn, triggering apoptosis [31,32]. In contrast, in the same model levels of mitochondrial HSP60 were unchanged [32]. Hence, redistribution of cHSP60 to the plasma membrane during hypoxia appeared to contribute to the initiation of the apoptotic cascade. HSP60 presence in plasma membrane is per se a pathogenic mechanism of heart failure (HF), as we will discuss later.

The study of the effects of microRNAs (miRs) on cells and tissues is a relatively young and growing research field. miRs are small, non-coding single-stranded RNA molecules that downregulate gene expression. It has been shown that by using miRs it is possible to modify CMC proliferation, differentiation, and apoptosis. For example, miR-1 has been found to be pro-apoptotic [33]. miR-1 has a single target site in the 3′-untranslated region of HSP60 mRNA, thus determining post-transcriptional repression of HSP60 synthesis in rat ventricular CMC [34]. Similar effects have also been demonstrated for miR-206 [34]. This miR-induced reduction of HSP60 can trigger CMC apoptosis. However, even though studies on the participation of HSP60 in apoptosis of CMC are numerous, information on the role of this chaperonin in other forms of cell death in the myocardium is, to our knowledge, still lacking. Studies are needed to examine the mechanisms of myocardial cell death and the role played by HSP60 in this process.

4. HSP60 AND THE PATHOGENESIS OF HEART FAILURE (HF) AND HYPERTENSION

Since HSP60 can influence cardiac apoptosis, it has been postulated that this chaperonin participates in the pathogenesis of HF. This condition is essentially a state of chronic inflammation and progressive fatal injury of cardiac muscle. It arises as complication of a number of genetic or acquired disorders that result in cell death and the subsequent reduction of the heart’s pumping function. Among the disease entities causing HF are dilated cardiomyopathy and myocardial infarction. In these conditions, the levels of HSP60 protein and mRNA were found increased (from 2 to 5-fold) [11,12]. These data were confirmed in another study that showed a cHSP60 decrease, while mitochondrial HSP60 increased in CMC from both dilated cardiomyopathy and ischemic hearts [34]. In patients with dilated cardiomyopathy HSP60 was found not only in CMC but also in connective tissue [12]. The latter observation is in agreement with other data demonstrating that cHSP60 reduction in CMC during hypoxia is accompanied by HSP60 localisation in the cell membrane [31,32] and, in turn, its secretion via the exosomal pathway [36]. HSP60 localisation in the cell membrane correlated with increased myocardial apoptosis (as mentioned before), while its release into the extracellular space and, in turn, into the bloodstream and lymphatic system, can activate the innate immune system and promote a pro-inflammatory state (including an increase in TNF-alpha) [37]. Thus, the “abnormal” trafficking of HSP60 from cytosol to cell membrane may cause CMC death and HF progression. However, it is not known what determines HSP60 localization in the cell membrane. It could be postulated that post-translational modifications and/or single nucleotide polymorphisms may be involved. Additionally, elevated HSP60 levels in plasma may stimulate activation of TLR4, which has been shown in adult rats to be responsible for CMC death [38].

Increase of myocardial HSP60 was also found in a model of HF induced by coronary artery ligation in rats [39] as well as in a model of HF induced in ventricular-tachypaced dogs [40]. Subsequent work with a rat model showed that treatment with trendapril reversed the HSP60 increases [41]. Elevated levels of circulating HSP60 have also been described in human subjects with HF due to non-atherosclerotic cardiac disease [42]. Patients with more se-
Atrial fibrillation (AF) is a common condition especially in elderly people [45, 46]. It represents a stress for cardiomyocytes (they are not stimulated in a coordinated fashion thus do not achieve efficient blood flow). There is convincing evidence that HSP60 is increased in the myocardium during AF, and current efforts focus on understanding the physiological relevance of this HSP60 accumulation, i.e. whether it is the cause or effect of AF.

HSP60 protein was found to be increased in atrial myocardial samples of patients with chronic AF compared with patients in sinus rhythm [47,48]. HSP10 was also found to be increased [48]. Interestingly, HSP60 levels in atrial myocardium of moderate, severe, and profound myolysis groups of patients with AF were significantly lower than in specimens from patients with light myolysis, while no differences were found for other HSPs [49]. Although this HSP60 reduction has yet to be explained, one can speculate that myolysis is accompanied by leakage of HSP60 and other proteins from damaged cells which then enter the circulation. This is in agreement with recent observations by our group in blood samples of patients with chronic AF compared with controls receiving a high-cholesterol diet alone [72]. A significant enhancement in the size of fatty-streak lesions was recorded in immunized animals receiving a high-cholesterol diet as well as of anti-HSP60 antibodies [65-69]. Both chaperonin and anti-chaperonin antibodies have been shown to elicit production of pro-inflammatory cytokines [59,70,71]. An indirect confirmation of the pro-atherogenic role of HSP60 and anti-HSP60 antibodies came from studies done with mice immunized against human HSP60. A significant enhancement in the size of fatty-streak lesions was recorded in immunized animals receiving a high-cholesterol diet compared with controls receiving a high-cholesterol diet alone [72].

Studies examining potential human and bacterial HSP60 structural homologies found amino acid sequence similarities representing the basis for immunologic cross-reaction between the two molecules (molecular mimicry). This cross-reaction may explain why antibodies generated against a bacterial HSP60 become also “autoantibodies” recognizing the human autologous chaperonin [73]. These autoantibodies are able to recognize HSP60 anywhere, including when the chaperonin becomes localized on the endothelial-cell membrane as a consequence of stress caused by a variety of stressors [74]. Interaction of membrane HSP60 with the autoneuroimmune system most likely cause cell death. These autoantibodies can recognize HSP60 localized on the endothelial cell membrane, potentially causing cell death and thus initiating the atherosclerotic process.

The probable sources of these autoantibodies can be infections by distinct pathogens in various locations, such as lung (Chlamydia pneumonia [67,75] or Mycobacterium tuberculosis [75-77]), genital tract (Chlamydia trachomatis [78,79]), oral cavity (Porphyromonas gingivalis [70,80]), and stomach (Helicobacter pylori [81,82]). Also, antibodies that can destroy endothelial cells overexpressing HSP60 have been observed in viral (e.g. cytomegalovirus) infections [83,84]. A significant reduction of anti-HSP60 antibody levels was found in a group of 275 subjects aged 40-60 after statin therapy [85]. This effect was attributed to direct immunomodulatory proper-

5. HSP60 INCREASE IN ATRIAL FIBRILLATION: CAUSE OR CONSEQUENCE?

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Another recent study showed that some HSPs can play important roles in the stabilization of restored sinus rhythm after mitral valve surgery in AF patients [50]. The authors suggested also that low circulating levels of HSP60 pre-operatively predict a stable outcome [51]. However, it was not clear whether the anti-HSP60 auto-antibodies were detected because of the possible role of bacterial HSP60 (i.e. GroEL) in triggering autoimmunity, as we will discuss in a following section.

A recent study showed that increase of HSP60 – both at the mRNA and protein levels – during HF is driven mostly by NFkB activation [43]. The promoter region of HSP60 contains a p65-binding element and in a cardiac cell line pre-treatment with siRNA inhibiting p65 prevented HSP60 overexpression subsequent to treatment with TNF-alpha (which was used to test the role of NFkB activation in HSP60 expression) [43]. This study opened a new frontier for the investigation of reasons how HSP60 overexpression occurs in CVD and also in other pathologies, such as cancer and aging-related disorders.

Finally, elevated levels of anti-HSP70 and anti-HSP65 have been found by enzyme immunoassay in subjects with hypertension independently of age, smoking habits and blood lipids, while HSP60, HSP70 and anti-HSP60 antibody levels were similar to those in normotensive controls [44]. However, it was not clear how elevated levels of anti-HSP70 and anti-HSP65 influence the subjects with hypertension. Hence, further studies are still required to better evaluate the factors inducing, and the clinical significance of, circulating HSPs in this group of patients.

6. HSP60 AND METABOLIC DISORDERS: A NEGLECTED TOPIC

HSP60 has been considered for a long time an intramitochondrial molecule involved in protein folding. However, it is now clear that this chaperonin and other HSPs are involved also in various other intracellular functions. For example, a study performed in yeasts whose HSP60 has a high grade of structural and functional homology with the human counterpart [51], showed that the chaperonin concentrations influenced the expression of 6-phosphofructokinase, suggesting a regulatory effect of HSP60 on glycolysis [52]. These results perhaps suggest that HSP60 may play a role in controlling metabolic pathways; it can then be inferred that if HSP60 is quanti- or qualitatively deficient (chaperonopathy) some metabolic disturbance may arise, which in turn could have a deleterious impact on the cardiovascular system.

Circulating levels of HSP60 have been found increased in diabetic patients [53]. This suggest a role of this mitochondrial cell stress protein in the CVD associated with diabetes (e.g. aortic stiffness), as already postulated for HSP70 [54]. This clinical observation has been supported by other experimental studies. Chaperonins HSP10 and HSP60 have been implicated in the development of diabetic cardiomyopathy [55]. Moreover, HSP60 and HSP10 overexpression in neonatal CMC amplified insulin-like growth factor-1 receptor (IGF-1R) activity through post-translational modifications [55]. In agreement with these data, diabetic myocardial lesions due to insulin deficiency were associated with reduction of HSP60 levels and, in turn, this reduction resulted in a decline of IGF-1R signalling functions [55,56]. The above reports represent the initial contributions to the relatively new field of molecular chaperones and chaperonopathies and metabolic diseases, which is bound to expand considerably in the near future.

7. HSP60 AND ITS BACTERIAL COUNTERPART ARE PRO-ATHEROGENIC MOLECULES

Since atherosclerosis (ATS) was initially proposed to be “an autoimmune disease due to an immune reaction against heat-shock protein 60” [references in 57,58], there has been a number of publications addressing this topic [for comprehensive reviews, see 57-61]. Many population-based studies showed a strong correlation between ATS and high levels of soluble, circulating HSP60 [62-66] as well as of anti-HSP60 antibodies [65-69]. Both chaperonin and anti-chaperonin antibodies have been shown to elicit production of pro-inflammatory cytokines [59,70,71]. An indirect confirmation of the pro-atherogenic role of HSP60 and anti-HSP60 antibodies came from studies done with mice immunized against human HSP60. A significant enhancement in the size of fatty-streak lesions was recorded in immunized animals receiving a high-cholesterol diet compared with controls receiving a high-cholesterol diet alone [72].

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ties of statins through their known effects on lymphocyte function. If confirmed, these data may provide another mechanism through which statins exert their antiatherosclerotic effects.

Several studies directed attention to the proatherogenic role of *Chlamydia pneumoniae* HSP60. This chaperonin activates T-cells isolated from atheromatous plaques, stimulating a pro-inflammatory mechanism which could contribute to the pathogenesis of ATS [86]. At the same time, HSP60 induced cellular oxidation of low-density lipoproteins, a crucial step that alters the lipoprotein into a highly atherogenic form [87]. Analogously, it has been proposed that HSP60 is the molecular link between ATS and periodontitis [88]. In a recent study, we found that increased levels of HSP60 in blood of patients with mild periodontitis correlated inversely with high-density lipoproteins and positively with triglycerides and small, dense low-density lipoproteins (Rizzo and Cappello, unpublished observations), one of the most atherogenic forms of lipoproteins [89,90]. What molecular mechanisms are responsible for these correlations remains to be clarified.

In addition to destruction of endothelial cells as described above, human HSP60 may act by stimulating vascular smooth muscle cell (VSMC) proliferation by autocrine/paracrine mechanisms [91], while it has been shown that HSP70 improves viability of stressed vascular smooth muscle cells, possibly via its chaperone functions [92]. This proliferative mechanism may be enhanced by *Chlamydia pneumonia* infection, which induces endogenous HSP60 overexpression in VSMC [93]. These observations suggest that a timely treatment of infectious diseases may reduce the progression of ATS and related complications. In addition, efforts are under way to develop an HSP60-targetted chaperonotherapy for ATS (see below). However, in a recent study in which the correlations among anti-hHSP60 antibodies, *Chlamydia pneumonia* infection, and CVD in a high-risk population, such as patients undergoing hemodialysis have been evaluated, they found that anti-hHSP60 did not correlate to global absolute risk for cardiovascular disease, C-reactive protein serum levels, and incidence of CVD [94]. The authors concluded suggesting that anti-hHSP60 autoimmune response is not related to *Chlamydia pneumonia* infection and *Chlamydia pneumonia* -related CVD risk, at least in patients undergoing hemodialysis.

Regulatory T-cells (Treg) have a crucial role in controlling immune responses against self and non-self antigens [95]. Their potent immunosuppressive properties suggest that they might be useful for treating diseases with an immune component [96]. Treg can infiltrate atherosclerotic plaques as shown by adoptive transfer of an *in vitro* expanded HSP60-specific Treg population into mice that resulted in the inhibition of plaque formation [97]. This effect was dependent on HSP60-specific activation, as adoptive transfer of equal numbers of ovalbumin-specific Treg did not produce the same results.

Similarly, another research group induced Treg activation by oral administration of either HSP60 or an HSP60-derived peptide (amino acids 253 to 268) in mice [98]. This oral tolerance induction produced a significant reduction of plaque size. Another team of investigators obtained similar results by subcutaneous immunization with *Helicobacter pylori*-HSP60 in mice that showed reduction of ATS and related complications. In addition, efforts are under way to develop an HSP60-targetted chaperonotherapy for ATS (see below).

**Fig. (1).** HSP60 (both human and bacterial) may be an active player during heart failure and atherosclerosis pathogenesis

HSP60 in normal cells is confined mainly inside mitochondria (1). However, after stress (2), its levels increase and it can accumulate in the cytosol, having protective roles in the cells (e.g. preventing apoptosis in cardiomyocytes). If stress is accompanied by other, mostly unknown factors such as single nucleotide polymorphisms (SNP) or post-translational modifications (PTM), HSP60 localizes in the cell membrane (mHSP60) and this is a pre-requisite for its secretion (3a). At the same time, GroEL (the bacterial HSP60 homologue) may be released by cells infected by any or a number of pathogens (3b). HSP60 and GroEL reach blood and lymphatic vessels, in turn stimulating both adaptive and innate immunities either in the circulation or in lymph nodes (4). As a consequence, mHSP60 becomes a target for the immune system that causes lysis of the stressed cells (5) with release of cytosolic HSP60 (6), initiating a vicious circle of chronic inflammation (7).
of atherosclerotic plaque formation and decline of Th1 differentiation [99]. Finally, very recently 2 independent groups showed that immunization with a combination of human HSP60 and apolipoprotein B (apoB) peptides caused a significant reduction of atherosclerotic lesions compared with mice immunized with either human HSP60 or apoB alone [100,101].

In conclusion, although HSP60 has to be considered as one of many pathogenic factors of ATS and CVD, it seems to play a significant role in the development of the inflammatory state that characterizes ATS. Therefore, HSP60 should be considered a target for agents aiming at slowing atherosclerotic plaque formation and the development of CVD.

8. CONCLUSIONS

There is increasing evidence supporting participation of HSP60 in the development of various CVD. In particular, both human and bacterial HSP60 may be an active player in the pathogenesis of HF and ATS (Fig. 1). This chaperonin plays variable roles, most likely depending on the existing conditions at the cell or tissue level. HSP60 may play an antiatherogenic or a proatherogenic role, depending on currently unknown mechanisms. In addition, the roles of HSP60 may be influenced by its interactions with other intracellular proteins and other processes, including secretion into the extracellular space with the chaperonin becoming an extracellular chaperone, signal molecule, autoantigen, or cytokine-like or endocrine-like molecule.

However, it is yet unclear where the HSP60 molecules that are found in lesions come from, namely from local stressed cells, or also from other cell types via intercellular space and/or blood stream. In other words, is atherosclerotic plaque formation a local phenomenon (in what regards participation of HSP60 in its pathogenesis), or is it a local manifestation of a systemic dysregulation of the chaperoning system? Answering these questions may provide invaluable clues regarding the pathogenesis, treatment, and, above all, prevention of ATS and ATS-related CVD, as myocardial infarction and HF. In the meantime, measurement of HSPs, HSP60 in particular, should be considered a potentially useful tool for diagnostic and prognostic purposes, and to guide therapeutic efforts. CVD may be, at least in part, chaperonopathies with all the potential therapeutic options that such a association implies.

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