The Diagnostic and Prognostic Application of Heat Shock Proteins and their Post-Translational Modifications from Liquid Biopsies

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Abstract

Liquid biopsies contain numerous proteins coming from extracellular vesicles (EVs), be it microvesicles or exosomes, released by both normal and tumour cells, as well as the presence of any circulating tumour cells (CTCs). Such proteins can be used as biomarkers for early diagnosis, prognostic assessment, disease progression monitoring, therapy selection and treatment response, particularly in oncology. EVs have been identified as mediators of cell-to-cell communication in both normal and pathological conditions and suggested to play a role in promoting and maintaining cancer dissemination and progression by altering the tumour microenvironment through immune suppression, angiogenesis and metastasis. One class of proteins garnering particular interest are extracellular heat shock proteins (HSPs) (secreted despite no consensus secretory sequence), and their post-translational modifications (PTMs), which are thought to act as key players in intercellular crosstalk and activation of signalling pathways during stress conditions. This review will focus on how characterising and quantifying these proteins can indicate the condition of the physiological system in a variety of pathological contexts.

Keywords: extracellular vesicles, biomarkers, heat shock proteins (HSPs), post-translational modifications (PTMs), intercellular crosstalk

1. Introduction

Liquid biopsies may contain a wide variety of biomolecules including DNA, RNA, proteins and metabolites. When considering the presentation of the numerous proteins within a liquid

biopsy, these can be free in the plasma, encapsulated within or on the surface of extracellular vesicles (EVs) or still inside cells within the biopsy (such as in the case of circulating tumour cells (CTCs)).

One class of proteins garnering particular interest as part of liquid biopsies are extracellular heat shock proteins (HSPs), and their post-translational modifications (PTMs), mainly because they should not be present in body fluids at the concentrations observed due to their lack of an export sequence and also as a result of the growing evidence supporting the notion that these proteins can mediate intercellular crosstalk and act as messengers that activate signalling pathways during stress conditions.

1.1. Heat shock proteins (HSPs)

HSPs are a class of chaperone proteins ubiquitously expressed in the cells of both prokaryotic and eukaryotic organisms. HSPs have been traditionally named and subdivided into six groups or families based on their molecular weight, namely, the small HSPs (which include HSP27), HSP40, HSP60, HSP70, HSP90 and HSP100 family. However, more recently, a new nomenclature and classification system based on the naming issued by the Human Genome Organisation (HUGO) Gene Nomenclature Committee (HGNC) has been proposed for classifying human HSPs into the following groups: HSPA (HSP70), HSPB (small HSPs including HSP27), HSPC (HSP90), HSPD/HSPE (HSP60/HSP10), HSPH (HSP110) and DnaJ (HSP40) [1]. Each of these families has members that are constitutively expressed and others that are inducible upon stress.

Under normal physiological conditions, constitutive HSPs fulfil important regulatory roles in a wide range of cellular processes including the synthesis, folding, translocation, assembly and in some cases activation of the proteins they interact with. On the other hand, after an episode of cellular stress, inducible HSPs help to refold and prevent aggregation of misfolded proteins, as well as assist in the proteasomal degradation of misfolded proteins which cannot be recovered. Moreover, HSPs can block apoptotic signalling and increase tolerance to subsequent insults [2].

However, it is now starting to emerge that during stress, the role of HSPs goes beyond what is expected to be their intracellular chaperoning functions for recovery from multiple stress conditions. Despite HSPs acting predominantly intracellularly, they have also been found expressed in the cell plasma membrane and in the extracellular space. Numerous HSPs have been reported to be present in the extracellular space and general circulation, activating a range of signalling pathways depending on the effector cell type or target organ. The role of such extracellular HSPs appears to be that of a systemic warning system of stressful events or chronic conditions, acting by priming the body, of which the immune system is a major effector, in order to prepare for and counteract the spread of the stress insult. Extracellular HSPs thus seem to act as a form of intercellular communication system during stress conditions, particularly those responses linked to oxidative stress, immunity or inflammation [3].

1.2. The presence of HSPs outside cells

When HSPs are present outside cells, they can be found as free proteins in solution or forming part of EVs. EVs can be of various types, with distinct structural and biochemical properties

as well as intracellular site of origin. These include large microvesicles (up to 1500 nm) that are heterogeneous in shape and produced from the plasma membrane, small (50–100 nm) and more uniformly shaped exosomes released from endosomes via the endocytic pathway and apoptotic vesicles produced upon cell death [4, 5].

EVs are released by almost all cell types, both healthy and diseased (including tumour cells). Such vesicles carry a wide range of biologically active molecules including growth factors, cytokines, mRNAs and microRNAs, extracellular matrix constituents and also proteins [6]. The protein fraction consists of cytosolic or plasma membrane components, either inside or on the surface. Their molecular contents have been shown to mediate intercellular communication in a variety of cellular processes, in both normal and pathological conditions, with the transfer of such biomolecules altering the function of the target cells. In the context of cancer, for example, EVs can modulate both the tumour microenvironment and cells and tissues which are located at a distance, affecting the immunity in the area, promoting angiogenesis and bringing about metastasis [7, 8].

EVs are also released by cells in response to being exposed to a stressor or as a result of chronic cellular stress. Such EVs contain particular molecules, including HSPs, whose expression level is directly linked to or induced by the stress insult. Upon reaching their effector cells, and especially when interacting with cells of the immune system, some EV components act as signalling molecules, activating a response in the effector cells which pre-empts the stress insult prior to its spread [3].

Proteomic studies have shown that EVs from serum, saliva, milk or plural effusions contain HSP27, HSP60, HSP70 and HSP90 [9–16] at high concentrations, with the ability to synergise with other encapsulated factors [3]. The delivery of HSPs in EVs provides a much stronger signal to effector cells as exemplified by EVs containing HSP70 producing a 250-fold higher activation of macrophages than an equal concentration of HSP70 in solution [17].

The HSPs encapsulated within or presented on the surface of such EVs, together with changing levels in free HSPs, can thus be valuable disease biomarkers for early detection, diagnosis and therapy selection. However, in order to access them, these proteins need to be purified from the body fluids of patients, characterised, quantified and compared to what is known in the healthy condition.

2. Secretion and uptake of extracellular heat shock proteins

When cells are exposed to a stressor, which includes but is not limited to heat shock, osmotic stress, exposure to heavy metals, hypoxia, ischemia or pathogens, these release signalling molecules in order to alert the rest of the system that a stressful condition is being experienced in some part of the organism and which might potentially lead to a situation of systemic damage. Among the stress signals which can be released by cells in response to an incidence of cellular stress are HSPs and other components of the chaperone (**Figure 1**). It is worth noting that most HSPs lack the consensus signal required for secretion via the classical endoplasmic reticulum (ER)-Golgi pathway [3, 18]. So far, it appears that the secretion of HSPs is achieved via a number of alternative pathways; however, these are still not well defined. Presently, the HSP release mechanisms identified are (but might not be limited to) processes via:

i. Cell lysis—where the process can be the result of a physiologically regulated release of cytokines or necrosis resulting from a pathological condition. Extracellular HSP70 has been suggested to be released into circulation under a variety of pathological conditions which cause widespread cell death as well as the following necrosis of tumour cells [19].

ii. Endolysosomal pathway—where the HSP is translocated into lysosomes and instead of being degraded is translocated out of the cell via endocytosis. HSP27 (dephosphorylated at S15 and S82) [20] and HSP70 [21] have been shown to enter endolysosomes, which are then secreted extracellularly in an ATP-dependent manner, from both tumuor cells and macrophages possibly via some pathway analogous to the ATP-binding cassette (ABC) transport system [21].

iii. Exosomal pathway—where the HSP is contained in secretory vesicles (exosome lumen) which rupture or are lysed once present in the extracellular space. A number of HSPs have been detected within extracellular vesicles including HSP27, HSP70, HSC70, GRP75, GRP78 and HSP90 [22–25].

iv. Inclusion in the exosomal membrane—where the HSP is inserted into the membrane of the secretory vesicles rather than being in the lumen. The isolation of HSP70-containing vesicles, derived from the plasma membrane, indicates that the surface of the vesicle can be used as an export system [17, 26, 27].

v. Secretory-like granules—where the vesicles used to transport the HSP are neither lipid bodies, nor endosomes, or lysosomes. Tumour cells were found to release HSP70 in structures that were only positive for chromogranin A, which is a marker of secretory granules [28].

Once in the extracellular space or general circulation, these HSPs can stimulate a wide range of cell types. However, similar to the secretion mechanisms, the recognition and uptake of HSPs by cells, as well as the role that extracellular HSPs play in cell activation, are poorly understood. HSPs have been reported to bind to a wide variety of receptors on target cells, among which are:

i. Low-density lipoprotein (LDL) receptor-related protein 1 (LRP1; CD91)—a receptor involved in receptor-mediated endocytosis, which is found on numerous cell types including antigen-presenting cells (APCs), known to bind to HSP70, HSP90 and calreticulin [29, 30].

ii. CD40—a member of the tumour necrosis factor (TNF) receptor family that is essential in mediating a broad variety of immune and inflammatory responses and can bind to HSP70 [31, 32].

iii. C-C chemokine receptor type 5 (CCR5; CD195)—a receptor on white blood cells involved in the process by which T cells are attracted to target areas via cytokines, which has also been shown to bind to mycobacterial HSP70 [33].

iv. Toll-like receptors (TLRs)—of the ten TLR receptors found in humans, only TLR2 and TLR4 are so far known to act as HSP receptors. They are known to bind to HSP60, HSP70 and HSP90 [34–37]. It has been suggested that TLR activation by HSP is most likely not the result of a direct binding of HSP70 to these receptors but rather either a low affinity interaction or a secondary activation involving the prior binding of HSP to another receptor [38].

v. CD14—a co-receptor for TLR4 activation, which was found to be also required for HSP70 induction of cytokines [35].

vi. Scavenger receptors (SR)—a family of receptors currently classified into ten subclasses (A–J) based on structure and biological function [39]. At least three SRs bind to and internalise HSPs, namely, lectin-like oxidised LDL receptor 1 (LOX-1), scavenger receptor expressed by endothelial cell 1 (SREC-1) and fasciclin and EGF-like, laminin-type EGF-like and link domain-containing scavenger receptor 1(FEEL-1)/common lymphatic endothelial and vascular endothelial receptor 1 (CLEVER-1), with HSP70 binding to all three, HSP60 binding to LOX-1, HSP90 binding to LOX-1 and SREC-1 and calreticulin binding SREC-1 but not LOX-1 [38, 40–43]. Furthermore, scavenger receptor-A (SR-A) can bind to and internalise HSP90 and calreticulin as well as HSP110 and GRP170 [42, 44]. The sialic acid-binding immunoglobulin-type lectin (Siglec) receptors Siglec-5 and Siglec-14 have also been found to bind to HSP70 [45, 46].
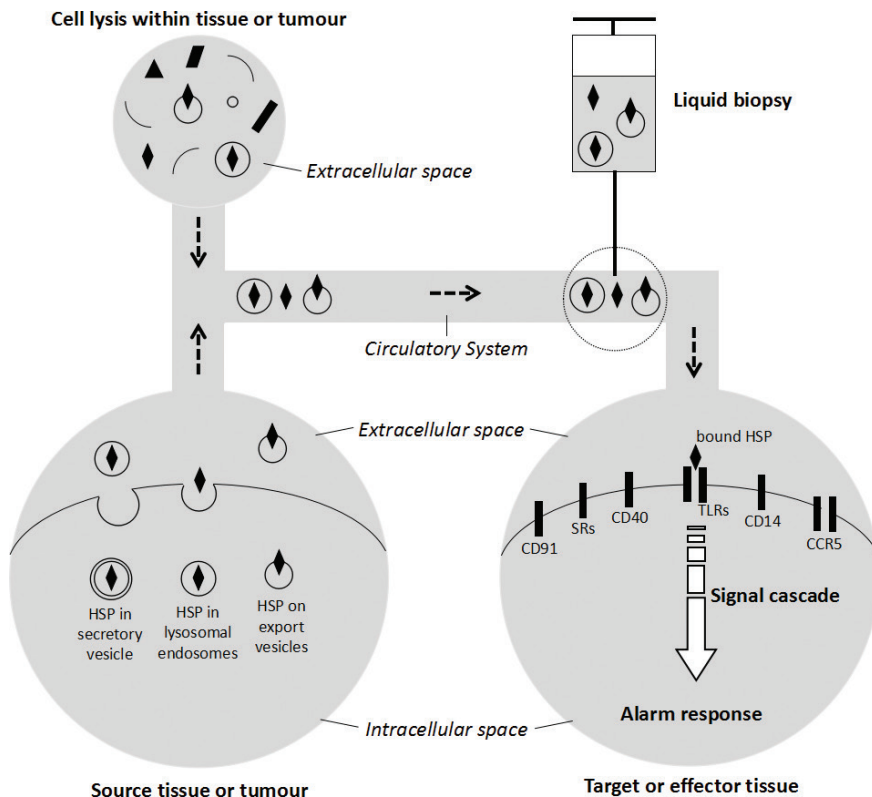

Figure 1. Heat shock proteins (HSPs) are exported into the extracellular space and general circulation via a number of different processes including cell lysis, secretory vesicles, lysosomal endosomes or export vesicles. Once these extracellular HSPs reach the target tissues, they bind to a variety of receptors, which initiate an alarm response. When these extracellular HSPs are collected from patients with chronic diseases and quantified, they can have diagnostic or prognostic value.

3. Biomarker potential of extracellular heat shock proteins

Changes in extracellular HSPs have been detected and implied to be actively involved in many chronic pathological conditions including arthritis, cardiovascular disease, cancer, type 2 diabetes mellitus (T2DM), chronic obstructive pulmonary disease (COPD) and neurodegenerative diseases. However, in order for these extracellular proteins to be used as biomarkers for early diagnosis, prognostic assessment, disease progression monitoring, therapy selection or treatment response, it is essential to characterise their functions and quantify their levels in the selected body fluids for liquid biopsies under both normal physiological conditions and the various pathological contexts.

For example, with respect to cancer, a wide range of studies have linked changes in extracellular HSPs to key mechanisms involved in either the process of malignant transformation or the progression of a tumour via evasion of apoptosis, increased cell proliferation and immortality, invasiveness and metastasis. On the other hand, when it comes to T2DM, because the biochemical mechanisms are not well understood, it is more difficult to link extracellular HSPs to the aetiology of the condition. However, T2DM patients present a two- to fourfold higher risk of developing macrovascular diseases, including coronary artery disease, stroke and peripheral vascular disease, making episodes of cardiovascular complications the major fatality in such patients [47]. Moreover, the sustained hyperglycaemia brings about cellular dysfunction via systematic biochemical changes due to oxidative stress, accumulation of advanced glycated end products (AGEs) and chronic inflammation [48], which are processes highly associated with HSPs.

3.1. HSP27

Extracellular HSP27 has been so far linked to three major functions, immune response modulation, angiogenesis and atheroprotection through a number of mechanisms, which in the contexts of cancer and T2DM can have a significant contribution to the aetiology or progression of the disease.

Immune signalling is activated by extracellular HSP27 via interaction with receptors on the surface of immune or endothelial cells, leading to the differential production and release of cytokines and growth factors, in order to modulate the immune response, cellular migration and proliferation. Extracellular HSP27 interacts with TLR2, TLR3 and TLR4, bringing about NF-κB transcriptional activation and the upregulation of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1), leading to the secretion of TNF-α, IL-6, IL-8, IL-10, IL-1β, IL-12p35 and IL-12p40, colony-stimulating factor 2 (CSF2) and vascular endothelial growth factor (VEGF) [49–52]. The release of IL-10 induced by extracellular HSP27 was found to involve the phosphorylation of p38 and MAPKAPK-2, whilst the upregulation of TNF-α was attributed to the activation of both p38 and ERK1/ERK2 signalling pathways [53]. HSP27 was also found to interact with oestrogen receptor-β (ER-β) [54, 55].

In cancer, extracellular Hsp27 has been reported to exert pro-angiogenic effects via the stimulation of the transcription of the vascular endothelial growth factor (VEGF) gene [50]. Increased

VEGF expression promoted HSP27 phosphorylation through the stress-activated protein kinase 2 (SAPK-2)/p38 pathway, resulting in cytoskeletal rearrangements and endothelial cell migration [56]. Furthermore, HSP27 phosphorylation not only reduced the release of HSP27 in the extracellular space, where the released HSP27 binds to and blocks VEGF [20], but also enhanced intracellular VEGF expression by interacting with the TLR3 on endothelial cells [50].

In the context of diabetes, T2DM patients with cardiovascular disease presented no significant change in serum HSP27 than non-diabetic controls [57]. However, extracellular HSP27 levels were found to be inversely correlated to progression, complexity and instability of plaques found in atherosclerotic human coronary arteries [54, 58], with HSP27 secretion being greatly reduced in atherosclerotic lesions and almost absent in complicated plaques [59]. Lower levels of serum HSP27 were described as being predictive of subsequent heart attacks, strokes or cardiovascular death within the following 5 years [60]. Atheroprotection is thought to be mediated through oestrogen (for the extracellular release of HSP27) as well as via modulation of various processes involved in atherosclerosis, such as cholesterol homeostasis and trafficking, regional inflammation (including mobility of immune cells in plaques and macrophage activation into foam cells) and plaque remodelling by extracellular HSP27 [61]. Extracellular HSP27 seems to be involved in reduced lipid engulfment by macrophages and foam cell formation through the blocking and downregulation of macrophage scavenger receptor A [62, 63], as well as the promotion of cholesterol efflux by enhancing ATP-binding cassette (ABC) transporter activity via the TLR4-induced and NF-κB-mediated release of CSF2 [64]. A similar activation of NF-κB in endothelial cells via TLR2, TLR3 and TLR4 may further worsen the condition [50, 51]. Moreover, patients with T2DM presented accelerated platelet aggregation correlated with the release of phosphorylated HSP27 from platelets induced by thrombin receptor-activating protein (TRAP) activation of Akt and p38 MAP kinase [65, 66].

3.2. HSP60

Till now, extracellular HSP60 has not been linked to any specific function. What has been explored so far is mostly related to its release mechanism. It has been shown that HSP60 is released into the extracellular space via the exosomal pathway, with most of the HSP60 tightly bound to (as opposed to embedded in) the exosomal membrane, rather than housed in the lumen of the exosomes. Moreover, evidence indicates that exosomal HSP60 is at least in part ubiquitinated (but not poly-ubiquitinated, i.e. not marked for degradation), which might act as a signal for the sorting of HSP60 to exosomes [12]. This ties in with its presence in cancer and T2DM, although the significance of its role in the aetiology or disease progression have not been well investigated.

When looking at the cancer context, tumours often tend to present HSP60 in the cell membrane [67] as well as secreted via exosomes [68]. It is hypothesised that cellular stress results in ubiquitination and possibly other post-translational modifications on cytosolic HSP60, which lead to its localisation in the cell membrane and consequently internalisation via lipid rafts, accumulation in multivesicular bodies and release into the extracellular space via the exosomal pathway [12]. Once secreted (either alone or in conjunction with other biomolecules), it then fulfils an as-yet unspecified but probably immunomodulatory extracellular function [69, 70].

Bioinformatic analysis of colorectal cancer (CRC) pointed at the HSP60 gene as one of the best indicators for diagnosis [71] and proteomic studies have corroborated this finding [72] giving it diagnostic and prognostic value. Similarly, HSP60 has also been found to be linked to Crohn's disease and ulcerative colitis [73], two conditions with a high risk for CRC development, probably having a pro-inflammatory role in the remodelling of the colonic mucosa via a TLR4-ERK-dependent mechanism [74].

Extracellular HSP60 is also thought to play a role in diabetes, as stresses associated with diabetes result in the expression of HSP60 on the cell surface as well as its extracellular release, such that it has been detected in both the serum and the saliva of T2DM patients [75, 76]. Moreover, T2DM patients with cardiovascular disease were associated with higher levels of circulating HSP60 compared to control subjects without cardiovascular disease [77]. Extracellular HSP60 has been associated with the severity of atherosclerosis and has been proposed as a biomarker for coronary heart disease [78, 79].

3.3. HSP70

Extracellular HSP70 has been shown to have important immunostimulatory properties, activating macrophages, monocytes, dendritic cells (DCs) and natural killer (NK) cells, by acting either as a cross presenter of immunogenic peptides via major histocompatibility complex (MHC) antigens, as a chaperone stimulating both innate and adaptive immunities, or as a stimulator and target for innate immune responses mediated by NK cells [35, 80, 81]. In contrast, some studies have shown that it can also have anti-inflammatory effects by activating both immunosuppressive regulatory T cells (Tregs) and Siglec receptors that block the inflammatory process by interacting with TLRs [82]. Moreover, extracellular HSP70 bound to vesicle membranes has been shown to induce an immunosuppressive effect [27], supporting the notion that HSP70 fulfils different roles depending on the composition, source and effector of the vesicles it is associated with.

Apart from immunity, extracellular HSP70 has been implicated in a wide array of conditions including cancer, diabetes, chronic inflammation, cardiovascular disease, hypertension, preeclampsia, Alzheimer's disease (inhibiting amyloid β aggregation) and ischemia [3, 83, 84].

When it comes to the cancer setting, serum HSP70 levels have been correlated with treatment response and tumour volume [85], making extracellular HSP70 a potential biomarker for cancer [86] both as a candidate biomarker for tumour detection and monitoring clinical outcome of radiotherapy [87], as well as a prognostic marker, such as in CRC, associated with rapid disease progression and poor survival [88]. In some contexts, extracellular HSP70 has even shown potential in discriminating between infection or inflammation and cancer (e.g. chronic hepatitis, liver cirrhosis and hepatocellular carcinoma) [89]. Extracellular HSP70 has been found to increase MMP9 expression by activating NF-κB and AP-1 and that the subsequent increase in pro-MMP9 secretion results in enhanced cell motility and invasiveness [90]. HSP70 was also isolated from the surface of tumour-derived exosomes [26], in which setting it can interact with myeloid-derived suppressor cells, so as to suppress T-cell activation and promote cancer development [27]. Extracellular HSP70 has also been used as a cancer vaccine, such that immunisation of mice with a vaccine made of HSP70-peptide complexes extracted

from fusions between DCs and radiation-enriched tumour cells resulted in a T-cell-mediated immune response against radioresistant tumour cells [91].

In vitro experiments of diabetes have shown that extracellular HSP70 plays a role in diabetic nephropathy in T2DM by promoting inflammation in the proximal tubule cells via a TLR4-NF-κB pathway. HSP70 release induced by the albumin in the proximal tubule cells triggered the overexpression of the inflammatory cytokines monocyte chemoattractant protein' 1 (MCP-1), tumour necrosis factor alpha (TNF-α) and macrophage inflammatory protein 2 (MIP2) [92]. Similar results were obtained in diabetic mice where TLR4 deletion or HSP70 inhibition reduced albuminuria and markers of inflammation and tubular injury [92]. Further supporting these findings, patients with T2DM with albuminuria showed higher serum HSP70 levels [93] as well as an association between urinary HSP70 levels and albuminuria [94]. Serum HSP70 was also found to be higher in patients with diabetic retinopathy, together with HIF-1α compared with subjects without [95] and correlated well with asymmetric dimethylarginine (ADMA) and C-reactive protein (CRP) levels in T2DM patients compared with healthy controls [96].

An inverse association has been reported between levels of HSP70 with the presence and severity of cardiovascular disease [97–100]. Moreover, an inverse correlation was found between HSP70 levels and the risk of future development of atherosclerosis in subjects with established hypertension [101]. Extracellular Hsp70 levels have also been inversely correlated with the risk of cardiovascular disease [97, 101, 102] and the severity and survival after chronic heart failure [103].

3.4. GRP78

Extracellular GRP78 has been documented [104, 105], but it has been studied much more extensively at the cell surface than in the extracellular space or in circulation. GRP78 could be detected in plasma as both full-length and C-terminus fragments [106]. GRP78 is secreted from cells via exosomes, and the release appears to be at least partly controlled by acetylation since the use of histone deacetylase (HDAC) inhibitors could block GRP78 release, causing aggregation in the ER. Suppression of HDAC6 activity leads to GRP78 acetylation, which is then bound to vacuolar protein sorting 34 (VPS34), a class III phosphoinositide-3 kinase, preventing GRP78 from being sorted into multivesicular bodies [107]. Since it has been shown that ER stress can actively promote the expression of GRP78 on the cell surface, and that over-expression of GRP78 can result in similar cell surface localisation, independent of ER stress [102], this might also hold true for extracellular release of GRP78. Once in the plasma membrane GRP78 binds to a wide selection of proteins, which in turn causes signalling cascades through multiple pathways that can result both in cell survival and cell death [108, 109], however the potential interaction or competition of extracellular GRP78 has not been explored. Interestingly, HSP40 (DnaJ) seems to be involved in GRP78 cell surface localisation and silencing of the murine homolog, MTJ-1 abolished cell surface localisation of GRP78 [110], but so far its possible involvement in extracellular release instead has not been investigated.

When looking at cancers, extracellular GRP78 is not commonly investigated; however, some tumours secrete significant levels of GRP78 into the tumour microenvironment [105], and in one study, extracellular GRP78 was identified exclusively in the sera of 28% of gastric cancer

patients but not in healthy controls [111]. It is speculated that ER stress and activation of the unfolded protein response (UPR), an evolutionarily conserved mechanism in which survival or apoptotic pathways are activated in response to ER stress, induce GRP78 in tumour cells leading to increased secretion of GRP78, and by binding to cell surface receptors of endothelial cells, extracellular GRP78 activates ERK and AKT pathways [105].

Useful inferences could be made by looking at cell surface GRP78 which is expressed significantly in human tumours and generally associated with cell proliferation, cell survival, angiogenesis and metastasis [112]. Cell surface GRP78 interacts with α2-macroglobulin, a plasma protease inhibitor, through its amino-terminal domain-activating the PI3K/Akt, ERK1/ERK2 and p38 MAPK pathways, promoting cell proliferation and cell survival via Akt and NF-kB signalling cascades, by inducing the UPR [105, 113, 114]. Moreover, interaction of cell surface GRP78 with teratocarcinoma-derived growth factor 1 (TDGF1; Cripto-1), a small, glycosylphosphatidylinositol (GPI)-anchored protein, modulates activin-A, activin-B, nodal and transforming growth factor-b (TGF-b)-dependent signalling of several ligands via the MAPK/PI3K and Smad2/3 pathway and promotes cell proliferation, downregulates E-cadherin (which decreased cell adhesion) and promotes pro-proliferative responses to activin-A and nodal [115, 116]. Of particular interest is that specifically on the surface of cancer cells but not healthy cells, GRP78 interacts via its amino-terminal domain with extracellular prostate apoptosis response 4 (Par-4), which together with tumour necrosis factor-related apoptosis-inducing ligand or Apo 2 ligand (TRAIL/Apo2L) mediates apoptosis via an extrinsic apoptotic pathway (dependent on ER stress and the Fas-associated death domain (FADD)/caspase-8/caspase-3 pathway) [117]. Similarly, plasminogen kringle 5 (K5), an angiogenesis inhibitor, interacts with cell surface GRP78 via the carboxy-terminal domain, on hypoxic and cytotoxic stressed tumour cells, mediating anti-angiogenic and pro-apoptotic activity following the internalisation of GRP78 by the scavenger receptor low-density lipoprotein receptor-related protein 1 (LRP1) and activation of p38 mitogen-activated protein kinase [118, 119].

The isolation of a tumour-specific variant of GRP78 containing an O-linked carbohydrate moiety with a molecular weight of 82 kDa opens up numerous therapeutic possibilities not only of targeting tumours by specific variants of GRP78 [120] but also of searching for the presence of tumour-specific variants in circulation, as a diagnostic marker.

Once again in the context of diabetes, extracellular GRP78 is poorly investigated. However, data from cell surface expression of GRP78 indicates that the extracellular counterpart might play some role in the cardiovascular complications linked to T2DM. GRP78 has been detected on microparticles shed from activated endothelial cells indicating that GRP78 expression may be involved in regulating thrombosis [121]. Expression of cell surface GRP78 in arterial atherosclerotic lesions negatively regulates the initiation of the tissue factor(TF)-mediated coagulation cascade [122, 123], attenuating procoagulant activity similar to the effect observed from the binding of K5 to cell surface GRP78 on stimulated endothelial cells [119]. Atherosclerotic lesions also present an increase in truncated cadherin (T-cadherin) expression, which interacts with cell surface GRP78, similar to the interaction on vascular endothelial cells [124] and on endothelial cells during tumour angiogenesis [125], promoting cell survival and indicating that this interaction plays a role in vascular tissue remodelling related to stress.

3.5. HSP90

As with most other HSPs, extracellular HSP90 has been mainly studied in relation to inflammation and immunity [126]. However, no specific roles, processes or mechanisms have been elucidated yet.

In the context of cancer, extracellular HSP90 (mainly not only HSP90a but also HSP90b) is known to be involved in tumour cell migration, invasion and metastasis [127–131]. Serum levels of extracellular Hsp90a were significantly higher in the patient groups with tumour burden, with a positive correlation with tumour malignancy and metastasis [132]. The interaction of extracellular Hsp90 with the LRP1 receptor as well as HER-2 activates AKT1/AKT2 (in the phosphatidylinositol-3-kinase (PI3K) signalling pathway) and ERK1/ERK2 signalling cascades giving rise to increased cell migration, supporting growth and survival [128, 133, 134]. AKT activation is sustained by the phosphorylation of the receptor tyrosine kinase ephrin type-A receptor 2 (EPHA2), which is a downstream product of the interaction between LRP1 and extracellular Hsp90 [135]. Also, critical for cell migration is the presence of extracellular HSP90 for the interaction between Src and integrin β1 at focal adhesion points between the cell and ECM [130]. The interaction of extracellular HSP90 with TLR4 also signals through Src, and this transactivates the epithelial growth factor receptor (EGFR), which increases cell migration [136]. It has also been shown that extracellular HSP90 can have a role in ECM remodelling or stabilisation via its direct interaction with fibronectin [137]. Work in colorectal cancer cells showed that extracellular Hsp90 promotes epithelial-to-mesenchymal transition (EMT) via an LRP1-NF-κB pathway [138], whilst exposure of prostate cancer cells to extracellular Hsp90 promoted EMT via a process requiring both matrix metalloprotein 9 (MMP9) and ERK activity [139]. Extracellular Hsp90 was also shown to interact with MMP2 [140]. The activation of ERK by extracellular Hsp90 has also been shown to increase expression of the polycomb repressor complex methyltransferase enhancer of zeste homologue 2 (EZH2), bringing about the epigenetic repression of E-cadherin [141], further supporting the EMT process.

Extracellular HSP90 has not been studied much in the context of diabetes, with the majority of studies investigating HSP90 inhibition in general and thus focusing on intracellular mechanisms whilst not excluding effects by extracellular HSP90. In response to oxidative stress, vascular smooth muscle cells secrete HSP90a, and the stimulation of these cells by HSP90a induces MAPK activity [142]. Similarly, endothelial cells also secrete HSP90 upon activation, and this stimulates angiogenesis [143]. Experiments in diabetic rats have shown that annexin II on endothelial cells interacts with extracellular HSP90a, modulating plasminogen activation to plasmin [144]. Furthermore, HSP90 levels were found to be higher in the serum of patients with atherosclerosis [145]. Exosomes collected from cultured fibrocytes contained HSP90a (among other biomolecules) and enhanced cellular migration and proliferation as well as secretion of type I collagen (COL1) and type III collagen (COL3) and expression of α-smooth muscle actin (α-SMA) [146]. Inhibition of total HSP90 disrupts the IKK complex [147] and JAK2 protein stability [148], blocking the activity of the transcription factors NF-kB [149] and STAT [150], respectively, together with a downregulation in the expression of pro-atherogenic cytokines and chemokines. Dysregulated NF-kB and STAT pathways contribute to diabetic nephropathy [150, 151] and atherosclerosis [152, 153]. The inhibition of HSP90 thus

modulates inflammation and oxidative stress, improving diabetes-associated renal damage and atheroprogression [154], insulin sensitivity [155], high-fat-diet-induced renal failure [156] and diabetic peripheral neuropathy [157].

4. Conclusion

The need to identify biomarkers for complex systemic and chronic diseases is pressing, with an increasing push towards the successful development of therapies aimed at modulating serum levels, blocking receptor binding or inhibiting signalling cascades. HSPs hold great potential as therapeutic targets for those conditions with underlying mechanisms involving accumulation of misfolded or damaged proteins, oxidative stress, altered mitochondrial bioenergetics or dysregulated apoptosis, particularly as a result of their non-chaperoning functions. Studies presented herein suggest that circulating HSP levels may be exploited as biomarkers of such conditions, with cancer and cardiovascular complications linked to T2DM being the contexts used to exemplify.

A major limitation of most studies performed on extracellular HSPs is that their functions and roles in disease have not been elucidated yet. As a result the biochemistry and signalling are investigated very poorly, such as testing for a single downstream product of a complex cascade which can be affected by multiple inputs. Similarly, the PTMs on extracellular HSPs are still in their majority obscure both in abundance and functional significance. Studies conducted retrospectively, on single HSPs in isolation, using small patient groups and without adjustment for confounding effects offer a very poor analysis of the predictive power of HSPs for early diagnosis or prognostic assessment. Thus, in future research, it is important to take into consideration that HSPs do not work in isolation, but act within a network, rather than just detect changes in the total extracellular expression levels of individual HSPs and analyse changes in both total HSP and specific PTMs within groups of chaperone proteins that are functionally relevant to either the development of or resultant from the progression of the condition under investigation. Furthermore, this needs to be performed in large cohorts of well-characterised patients, with prospective validation of promising biomarker panels, if the intent is really their application in a clinical setting.

Conflict of interest

The author declares no conflict of interest.

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