

# Heat shock proteins as modulators and therapeutic targets of chronic disease: an integrated perspective

Running Title: Heat shock proteins in health and chronic disease

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49 **Abstract**

50 Many heat shock proteins (HSPs) are essential to survival as a consequence of their role as  
51 molecular chaperones, and play a critical role in maintaining cellular proteostasis by  
52 integrating the fundamental processes of protein folding and degradation. HSPs are arguably  
53 amongst the most prominent classes of proteins that have been broadly linked to many human  
54 disorders, with changes in their expression profile and/or intracellular/extracellular location  
55 now being described as contributing to the pathogenesis of a number of different diseases.  
56 Although the concept was initially controversial, it is now widely accepted that HSPs have  
57 additional biological functions over and above their role in proteostasis (so called ‘protein  
58 moonlighting’). Most importantly, these new insights are enlightening our understanding of  
59 biological processes in health and disease, and revealing novel and exciting therapeutic  
60 opportunities. This theme issue draws on therapeutic insights from established research on  
61 HSPs in cancer and other non-communicable disorders, with an emphasis on how the  
62 intracellular function of HSPs contrasts with their extracellular properties and function, and  
63 interrogates their potential diagnostic and therapeutic value to the prevention, management  
64 and treatment of chronic diseases.

65  
66 **1. Introduction**

67 The most extensively studied heat shock proteins (HSPs) are the molecular chaperones that  
68 function intracellularly in an ATP-dependent manner and include heat shock protein 60  
69 kDa/heat shock protein 10 kDa (HSP60/HSP10; chaperonins) (HSPD/HSPE); HSP40 (DNAJ),  
70 HSP70 (HSPA); HSP90 (HSPC); HSP100; and HSP110 (HSPH) families. The expression of  
71 many of these HSP is regulated by heat shock transcription factors (HSF), of which HSF1 is  
72 the best studied. Increasing evidence now suggests that these molecular chaperones also  
73 have biological properties in the extracellular environment which may be independent of their  
74 chaperone functions. In addition to ATP, the molecular chaperone activity of the major HSPs  
75 is regulated by a cohort of non-substrate accessory proteins, known as co-chaperones. Co-  
76 chaperones are a diverse group of chaperone regulatory proteins which are required, to a  
77 greater or lesser degree, by certain chaperones. HSP90, for example, has over 20 co-  
78 chaperones that fine tune its function and adapt it to the different stages of the protein folding  
79 pathway. Some HSP families, such as HSP40, include members having both chaperone and  
80 co-chaperone activity.

81  
82 A particularly lively area relates to the evolving insight into the therapeutic potential of targeting  
83 HSPs in cancer, and their value as an exciting class of molecular target. Although HSPs and  
84 their transcription factors have been the subject of sustained interest in the field of cancer  
85 biology, more recently they have been attracting interest in many other chronic conditions such  
86 as diabetes, obesity, autoimmune disease, neurodegeneration, muscular dystrophies,  
87 psychiatric disorders and chronic heart failure. These studies are revealing that although  
88 increased levels of intracellular HSPs may be beneficial for acute conditions, such increases  
89 can be detrimental for certain chronic conditions, as exemplified by acute and chronic heart  
90 conditions. The contribution of extracellular HSPs to chronic disease is poorly understood.  
91 Increased levels of extracellular HSPs appear to be detrimental by enhancing inflammation  
92 pathways, and hence for conditions such as diabetes a reduction in the ratio of extracellular  
93 to intracellular HSPs is beneficial. In contrast, extracellular HSPs can also be beneficial to  
94 certain autoimmune conditions as a consequence of their ability to engage with, and recruit  
95 the immunomodulatory activity of regulatory T cell populations. Although the reported  
96 dichotomies in functionality of HSPs would appear to be counter-intuitive and has been the  
97 subject of great debate and counter-arguments, one needs to consider the context and the  
98 temporal nature of disease and its control. What is clear from current knowledge is that HSPs  
99 play important biological roles under physiological, stressful and disease conditions.

100  
101 The articles in this theme issue highlight how insights (both anticipated and unanticipated) into  
102 the biological function of HSPs in cancer have revealed new therapeutic options for the

103 treatment of the disease. The issue also explores how the intracellular function (ATP-rich  
104 context) of HSPs contrasts with their extracellular function (ATP-poor context), and their  
105 potential diagnostic and therapeutic value to the prevention, management and treatment of  
106 chronic diseases. Here we integrate and critique the content of this theme issue, addressing  
107 HSP moonlighting in the context of their contrasting intracellular and extracellular roles.  
108

## 109 **2. Heat shock proteins and protein moonlighting**

110 Although the finding that exposure to a non-physiological temperature (37°C *versus* 26°C)  
111 induced a new puffing pattern in the polytene chromosomes of *Drosophila* [1] was interesting,  
112 the author could not have anticipated the significance and broad reach of this finding,  
113 especially given that the ‘biological relevance of the findings were unclear’ and it proved  
114 difficult to publish the findings. However, over 50 years later, we continue to appreciate the  
115 importance of this heat shock response (HSR) to the maintenance of cellular homeostasis and  
116 protection against a multitude of physical, chemical and biological stressors that exist in the  
117 environment [2].  
118

119 As the protein folding paradigm and molecular chaperone functions of HSPs were developing  
120 in the late 1980s and 1990s, it became apparent that some of these proteins were also present  
121 on the surface of cells or in the extracellular fluids. This contradicted the established dogma  
122 that these proteins were exclusively intracellular and so it took time for the data to be accepted,  
123 the findings to gain traction with the scientific community and for this new field of extracellular  
124 HSPs to be accepted and become established. Interest in the biological role(s) and functions  
125 of these proteins grew, as did interest into the potential capacity of extracellular HSPs to  
126 influence biology and physiology. As discussed in this issue, it was shown that the treatment  
127 of cells with purified HSPs resulted in cell activation similar to that induced by pro-inflammatory  
128 cytokines. Despite controversy surrounding the possibility that at least some of the pro-  
129 inflammatory effects of HSPs might be due to contaminants of the preparations that have been  
130 used [3, 4], there is also a wealth of evidence from a number of settings which argues against  
131 this concept [5].  
132

133 A new paradigm that at least some HSPs are secreted proteins [6] with pro- (HSP60, HSP70,  
134 HSP90) or anti-inflammatory (HSP10, thioredoxin, HSP27, BiP) actions of importance in  
135 human diseases such as cancer, coronary heart disease, diabetes and rheumatoid arthritis  
136 [7], to name but a few, has therefore arisen. In addition to having direct effects on cells, HSPs  
137 can bind peptides and present them to T cells to modulate immune responses, and this might  
138 have implications in a number of disease settings, including cancer [8]. It has become  
139 apparent that HSP70 can be present in a membrane expressed form. The significant  
140 diagnostic, therapeutic and imaging potential of this finding, and the progress which has been  
141 made in exploiting membrane HSP70-based theranostics (i.e. combining diagnostic and  
142 therapeutic capabilities into a single agent; a key element of Precision Medicine) for the  
143 management and treatment of patients with cancer, is considered in detail by Multhoff and  
144 colleagues in this issue.  
145

146 Taken together, the findings that HSPs can be present in the extracellular and cell-associated  
147 compartments have led to the establishment of a new paradigm which designates these  
148 proteins as ‘moonlighting proteins’ (proteins with more than one function) that have the  
149 capacity to ‘escape’ from cells and interact with different cell types to elicit a range of biological  
150 effects. These proteins can even act as receptors for inflammatory mediators called  
151 ‘inflammogens’ [9]. Support for this new paradigm comes from a number of studies that are  
152 highlighted by Pockley in this issue, and a large number of studies that have, and continue to  
153 reveal, the presence of a number of HSPs in the bodily fluids of man and animals [10]. The  
154 first two contributions in this issue provide a critical overview of extracellular HSPs (by  
155 Pockley) and the biology of protein moonlighting (by Jeffery).  
156

### 157 **3. Intracellular versus extracellular heat shock proteins in cancer**

158 The initiation, progression and metastasis of cancer have all been shown to be accompanied  
159 by multiple cellular insults arising from both intracellular and extracellular sources. Internal to  
160 the cancer cell, the high expression of oncogenic proteins (many of which are mutated),  
161 altered cellular metabolism, aneuploidy and genomic instability all contribute to its  
162 characteristic stressed phenotype. Moreover, during cancer development, cells are exposed  
163 to altered extracellular conditions that can include hypoxic, acidotic, mechanical and nutrient  
164 deprived microenvironments, further stimulating the cancer cell to engage highly conserved  
165 survival pathways such as the HSR. Consistent with the knowledge that cancer cells are  
166 exposed, both internally and externally, to major proteotoxic insults that challenge cellular  
167 homeostasis and survival, it is not surprising to find that cancers constitutively express high  
168 levels of HSP family members. In fact, tumour cells have become to be regarded as addicted  
169 to HSPs (e.g. HSP90) as well as their transcriptional regulators (e.g. HSF1).

170  
171 Increased expression of many HSPs, including HSP27 (HSPB1), HSP72 (HSPA1A, HSPA1B)  
172 and HSP90 (HSP90AA1, HSP90B1), have been shown in a wide variety of cancer types such  
173 as breast, prostate, lung and melanoma, and are associated with poor patient outcomes.  
174 Moreover, HSF1, the master regulator of the HSR has also been shown to be increased in  
175 expression and constitutively activated in many cancers. The parallel molecular, genetic and  
176 pharmacological investigations that have been performed in relation to HSPs and their  
177 signalling and transcriptional regulation, has further confirmed their importance to the growth  
178 and progression of many tumour types (reviewed by Calderwood and colleagues in this issue).  
179 For example, the work in targeting and developing HSP90 inhibitors has confirmed the  
180 importance of HSP90 to cancer signalling and oncogene driven growth (reviewed by Neckers  
181 and colleagues in this issue). In a similar manner, the HSR has been shown to be an integral  
182 part of the oncogenic network, working through the actions of HSF1 to maintain cancer cell  
183 survival and function (reviewed by Dai in this issue). Interestingly, it has been shown that  
184 within the oncogenic context, the expression of HSF1 is indispensable for the growth and  
185 survival of cancer cells, while its loss in non-transformed cells has little to no effect [11].

186  
187 HSF1 and many of the HSPs have been shown to play fundamental roles in many aspects of  
188 the cancer cell phenotype associated with the hallmarks of cancer [12] including sustained  
189 proliferative signalling, evading growth suppression, replicative immortality, angiogenesis,  
190 resisting cell death and supporting invasion and metastasis [13]. Moreover, they are also  
191 involved in a number of the more recently identified hallmarks of cancer such as the  
192 deregulation of cellular energetics, genome instability, avoiding immune destruction and  
193 enabling tumour-promoting inflammation. The wide-ranging actions of the HSPs and HSF1  
194 are not limited to the cancer cells themselves, but have also been shown to play important  
195 roles for accessory cell function within the tumour microenvironment such as the cancer  
196 associated fibroblasts (CAFs) and tumour associated macrophages (TAMs), ultimately  
197 contributing to cancer cell growth and progression [14].

198  
199 Although it was originally proposed that the actions of HSPs were primarily intracellular to  
200 cancer cells and other cells of the tumour microenvironment, it is now evident that their  
201 presence and functionality are also very important to many molecules and processes external  
202 to the cell. For example, HSP90 $\alpha$  (HSP90AA1) is known to exist outside the cell, termed as  
203 eHSP90, and has been shown to interact with a number of client proteins, including matrix  
204 metalloproteinase 2 (MMP2) through which it enhances the migration and invasion of cancer  
205 cells (reviewed by Neckers and colleagues, and by Calderwood and colleagues in this issue).  
206 It has been shown that the functions of extracellular HSPs can have both anti-tumour or pro-  
207 tumour effects, ranging from anti-tumour or pro-tumour immunomodulation (HSP90, HSP72,  
208 HSC70, HSP60, HSP27), suppression or promotion of tumour cell proliferation (GRP78,  
209 HSP20, HSP27), as well as promotion of cancer cell invasion (HSP90, GRP75, HSP27) and  
210 angiogenesis (HSC70)[15-20]. Moreover, co-chaperones of HSP90, such as the  
211 HSP70/HSP90 organising protein (HOP), HSP40 and p23 have also been shown to be

212 extracellular, and similar to their role internal to the cell, are in complex with HSP90 to illicit  
213 extracellular functions such as MMP-2 activation and cancer cell invasion and migration [17,  
214 21].

215

216 Our increasing knowledge of the unique roles of HSPs and their co-chaperones external to  
217 the cell is leading to novel approaches for the therapeutic targeting of cancers. For example,  
218 cell surface HSP70 is currently being used as a target of novel therapies that include  
219 nanoparticle-based treatments for cancer (reviewed by Multhoff and colleagues in this issue),  
220 and cell-impermeable HSP90 inhibitors are being examined as to their efficacy in inhibiting  
221 cancer migration and invasion (reviewed by Multhoff and colleagues in this issue). Therefore,  
222 our increased understanding of the actions of extracellular HSPs will not only lead us to a  
223 better understanding of the biology of cancer and its progression, but will also reveal further  
224 therapeutic opportunities for the treatment of advanced cancers.

225

#### 226 **4. Intracellular *versus* extracellular heat shock proteins in chronic diseases**

227 Much of the research into the function of HSPs in chronic disease has been focussed on  
228 cancer. However, it is also clear that HSPs are involved in many other chronic conditions, from  
229 neurological and muscle-wasting disorders to obesity and post-traumatic stress. This range of  
230 chaperonopathies highlights the important and central role which these proteins play in  
231 maintenance of correct cellular function.

232

233 Findings from experimental, pharmacological or exercise studies on changes to HSP72  
234 expression levels suggest that the manipulation of the extracellular to intracellular ratio of HSP  
235 levels represents a useful avenue for the prevention and treatment of diabetes (reviewed by  
236 Geiger and colleagues in this issue). For example, there is evidence that exercise promotes  
237 the release of extracellular HSP72 from certain human cells (brain, [22]; epithelium, [23];  
238 immune system, [24]; muscle and adipose tissue, [25]). However, long-term exercise  
239 promotes a decrease in extracellular HSP72 and an increase in intracellular skeletal muscle  
240 HSP72 (reviewed by Geiger and colleagues in this issue). In fact, it is now apparent that the  
241 balance of extracellular (pro-inflammatory) *versus* intracellular (anti-inflammatory) HSP72  
242 appears to be a determining factor for the extent of tissue inflammation and hence the  
243 pathology associated with diabetes. It is hypothesised that interventions that lower the  
244 extracellular to intracellular HSP72 ratio are potentially beneficial in the context of diabetes  
245 progression [26]. Hence, carefully constructed exercise regimes that favourably modulate this  
246 HSP72 ratio may serve as powerful therapeutic interventions for the prevention and  
247 management of diabetes. However, more detailed studies on extracellular HSPs and the  
248 effects of exercise are needed, particularly the contribution of different tissues to extracellular  
249 HSP expression levels, and the biochemical and physiological mechanisms of action of these  
250 HSPs.

251

252 HSPs, and HSP72 in particular, also play an important role in muscle function. HSP90, HSP72,  
253 and HSP27 all have a pro-myogenic role in muscle development, albeit via distinct  
254 mechanisms. HSPs are also differentially expressed in the muscle progenitor pool that  
255 differentiates to give rise to new muscle tissue. HSP72 is the most widely studied HSP in this  
256 context and is required for muscle repair after acute injury. Both intracellular and extracellular  
257 HSP72 contribute to this process, with extracellular HSP72 functioning primarily via the  
258 activation of the immune response. Interestingly, many of the effects of HSP72 knockout on  
259 muscle regeneration involve the immune response, which suggests that, given that  
260 extracellular HSP72 arises from intracellular HSP72, the extracellular functions of HSP72 are  
261 more important in this context. Indeed, injection of extracellular HSP72 has been shown to  
262 ameliorate many of the effects of muscle injury in HSP72 null mice [27]. With respect to  
263 disease, over-expression of intracellular HSP72 had a positive effect and led to improvements  
264 in body strength and endurance, diaphragm health, normalised muscle force and reduced  
265 markers of muscle damage in a mouse model of Duchenne Muscular Dystrophy [28]. HSP72  
266 also has a positive effect on muscle function in the context of muscle immobilisation,

267 suggesting that over-expression of this protein may be a therapeutic approach for a range of  
268 muscle wasting conditions. Although it has not been demonstrated, it is likely that at least  
269 some of the described functions of HSP72 in these conditions are attributed to the extracellular  
270 function.

271  
272 In addition to a role in muscle-related immune responses, experimental models have provided  
273 evidence that both intracellular and extracellular HSPs also have a protective function in  
274 autoimmune diseases. The application of exogenous extracellular recombinant HSPs and the  
275 experimental co-induction of endogenous intracellular HSPs have been shown to lead to  
276 production of disease protective regulatory T (Treg) cells ([29]; reviewed by van Eden in this  
277 issue). This has stimulated research into the development of therapeutic HSP-based peptide  
278 vaccines for the restoration of immune tolerance in inflammatory diseases.

279  
280 There is emerging evidence for increased expression of extracellular HSP70, HSP90, and  
281 certain associated co-chaperones (e.g. BAG-3) in heart failure, and that their functions are  
282 complementary and independent of their intracellular isoforms. The important therapeutic and  
283 diagnostic considerations of these findings are reviewed by Willis and colleagues in this issue.  
284 Current findings suggest that therapeutic strategies involving the increase of HSP levels may  
285 be applicable in the context of acute heart conditions (e.g. acute myocardial  
286 infarction/ischemic reperfusion injury), but not chronic heart conditions (e.g. hypertension).  
287 Indeed, the pharmacological enhancement of intracellular HSP function has been shown to  
288 provide protection against experimental myocardial infarction [30]. With respect to chronic  
289 heart conditions, extracellular and intracellular HSPs exert different effects. For example, a  
290 decrease in the expression of intracellular HSP70 promotes cardiomyocyte hypertrophy and  
291 dysfunction while protecting animals from cardiac fibrosis development, whereas inhibition of  
292 extracellular HSP70 has been shown to improve hypertension-induced hypertrophy and  
293 fibrosis [31]. In the context of chronic heart disease, there are some parallels in the findings  
294 for extracellular HSP90 and extracellular HSP70. For example, the decrease in fibronectin  
295 levels, collagen production and the associated TGF $\beta$  signalling pathway via the inhibition of  
296 extracellular HSP90 [32, 33] has implications for the fibrosis-related pathology of chronic heart  
297 conditions. Although there is great promise for extracellular HSP70 and HSP90 as diagnostic  
298 markers of chronic heart disease, a deeper understanding of the mechanism(s) of action of  
299 extracellular HSP70 and HSP90 and its co-chaperones is required before effective prevention  
300 and treatment can be achieved.

301  
302 HSPs are also important in the context of neurodegeneration and neurological dysfunction  
303 leading to psychiatric diseases. HSP40s are the largest and most diverse of the HSPs and  
304 changes in different HSP40 isoforms all give rise to different, but related forms of  
305 neurodegeneration (reviewed by Cheetham and colleagues in this issue). Although these  
306 HSP40 isoforms share structural features such as the J domain, they also contain a number  
307 of unique functional domains (particularly since most of the isoforms associated with disease  
308 are the more diverse type III HSP40/DNAJC). The redundancy between isoforms in some  
309 contexts can also explain why it is possible to ameliorate the disease consequences of a  
310 mutation or deficiency of one isoform via over-expression of another. For example,  
311 overexpression of DNAJA1 can suppress aggregation of polyQ ataxin associated with  
312 neurodegeneration [34]. Interestingly, there are no neurological disorders associated with  
313 mutations in type I HSP40s like DNAJA1, presumably because many of these proteins are  
314 essential and loss of function cannot therefore be tolerated. With respect to psychiatric  
315 disorders, the co-chaperone FKBP51, acting via HSP90, is both a causative agent and  
316 biomarker for various forms of the disease (reviewed by Blair and colleagues in this issue).  
317 Increased levels of FKBP51 lead to glucocorticoid resistance by retarding the recruitment of  
318 glucocorticoid receptor (GR) to the nucleus and perturbing signalling via the hypothalamic-  
319 pituitary-adrenal (HPA) axis that culminates in a poor stress coping phenotype (reviewed by  
320 Blair and colleagues in this issue). Specific single nucleotide polymorphisms that result in  
321 methylation changes which alter levels of FKBP51 may be a risk or prognostic factor for

322 anxiety or suicide risk [35, 36]. This suggests that modulation of FKBP51 levels may be a  
323 relevant therapeutic strategy. However, in the context of both HSP40-related  
324 neurodegeneration and FKBP51-related psychiatric disorders, we have limited understanding  
325 of the relative contribution of intracellular *versus* extracellular forms of the relevant HSPs due  
326 to a paucity of data. Certainly, it is known that both HSP70 and HSP90 are extracellular and  
327 therefore it is at least theoretically possible that co-chaperones of these two proteins (HSP40  
328 and FKBP51) also exist in functional extracellular forms. In these examples, what we do know  
329 is that disease is usually associated with a change in the levels of a particular HSP. For  
330 example, mutations or deletions in the HSP40 isoform DNAJC29 is one of the most common  
331 causes of ataxia [37]. In some instances, the change in HSP levels are associated with  
332 missense mutations, deletions or splicing changes, while in other cases levels change in  
333 response to the environment (such as age-induced increases in FKBP51 levels which are  
334 associated with psychiatric disorders).

335

## 336 **5. Conclusion**

337 Fundamental insights into how HSPs give rise to disease will be an important component of  
338 therapeutic targeting of these proteins. However, many knowledge gaps remain and need to  
339 be addressed. Importantly, with cancer and autoimmune disease being the exceptions, there  
340 is limited insight into the role played by extracellular HSPs in chronic diseases such as  
341 neurodegeneration or psychiatric disorders. In addition, while much is known about the  
342 mechanism of action of specific intracellular HSP networks, such as the HSP90-HOP-HSP70  
343 or HSP70-HSP40 complexes, the genesis and function of these HSP complexes in the  
344 extracellular milieu is poorly understood and raises many fundamental questions that need to  
345 be answered before therapeutic applications can be properly developed. Like the HSPs they  
346 regulate, co-chaperones like HOP appear to also be secreted via exosomes [38]. However, it  
347 is not known if HOP is secreted together with HSP90 and HSP70 as a functional complex, or  
348 if it is secreted separately and then forms a complex with the HSPs [39]. Therefore, the major  
349 questions that need to be answered for these extracellular HSP complexes and many other  
350 extracellular HSPs include the following:

351

- 352 1. What is the origin of extracellular HSPs, and which isoforms are structurally and  
353 functionally distinct from their intracellular counterparts, and which isoforms are  
354 derived from their intracellular counterparts?
- 355 2. Which isoforms of extracellular HSPs are encoded by separate genes and which are  
356 encoded by splice variants of the same gene?
- 357 3. Are there always receptors associated with extracellular HSPs?
- 358 4. As a general principle, is the ratio of extracellular to intracellular HSP levels important  
359 for cellular and physiological homeostasis?
- 360 5. What stimuli, mechanisms and pathways are required for the secretion of extracellular  
361 HSPs?
- 362 6. Do extracellular HSPs function as molecular chaperones, is their activity regulated by  
363 extracellular co-chaperones and what defines extracellular client proteins?

364

365 While there is much work to be done before we can more fully define the true biological role,  
366 therapeutic potential and significance of extracellular HSPs, we can draw inspiration from  
367 Hippocrates who stated: 'That which drugs fail to cure, the scalpel can cure. That which the  
368 scalpel fails to cure, heat can cure. If the heat cannot cure, it must be determined to be  
369 incurable'.

370

371

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374

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393

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