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Stress activated signalling impaired protein quality control pathways in human hypertrophic cardiomyopathy

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ABSTRACT

Hypertrophic cardiomyopathy (HCM) is a complex myocardial disorder with no well-established disease-modifying therapy so far. Our study aimed to investigate how autophagy, oxidative stress, inflammation, stress signalling pathways, and apoptosis are hallmark of HCM and their contribution to the cardiac dysfunction. Demembranated cardiomyocytes from patients with HCM display increased titin-based stiffness ($F_{passive}$), which was corrected upon antioxidant treatment. Titin as a main determinant of $F_{passive}$ was S-glutathionylated and highly ubiquitinated in HCM patients. This was associated with a shift in the balance of reduced and oxidized forms of glutathione (GSH and GSSG, respectively). Both heat shock proteins (HSP27 and α-ß crystalline) were upregulated and S-glutathionylated in HCM. Administration of HSPs in vitro significantly reduced HCM cardiomyocyte stiffness. High levels of the phosphorylated monomeric superoxide anion-generating endothelial nitric oxide synthase (eNOS), decreased nitric oxide (NO) bioavailability, decreased soluble guanylyl cyclase (sGC) activity, and high levels of 3-nitrotyrosine were observed in HCM. Many regulators of signal transduction pathways that are involved in autophagy, apoptosis, cardiac contractility, and growth including the mitogen-activated protein kinase (MAPK), protein kinase B (AKT), glycogen synthase kinase 3ß (GSK-3ß), mammalian target of rapamycin (mTOR), forkhead box O transcription factor (FOXO), c-Jun N-terminal protein kinase (JNK), and extracellular-signal-regulated kinase (ERK1/2) were modified in HCM. The apoptotic factors cathepsin, procaspase 3, procaspase 9 and caspase 12, but not caspase 9, were elevated in HCM hearts and associated with increased proinflammatory cytokines (Interleukin 6 (IL-6), interleukin 18 (IL-18), intercellular cell adhesion molecule-1 (ICAM1), vascular cell adhesion molecule-1 (VCAM1), the Toll-like receptors 2 (TLR2) and the Toll-like receptors 4 (TLR4)) and oxidative stress (3-nitrotyrosine and hydrogen peroxide (H_2O_2)). Here we reveal

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stress signalling and impaired PQS as potential mechanisms underlying the HCM phenotype. Our data suggest that reducing oxidative stress can be a viable therapeutic approach to attenuating the severity of cardiac dysfunction in heart failure and potentially in HCM and prevent its progression.

1. Introduction

Hypertrophic cardiomyopathy (HCM) is a complex myocardial disorder characterized by asymmetrical ventricular hypertrophy, hypercontractile systolic function, and impaired relaxation. HCM is the most common cardiomyopathy with a prevalence of 1:500–1:1000 [1]. It can be acquired or of genetic disorder, whereby more than 70% of the cases are familial [1]. HCM has been associated with heart failure and high incidence of sudden cardiac death in young populations. The genetic analysis revealed an autosomal dominant inheritance with over 1400 mutations in sarcomeric proteins [1]. The enhanced Ca^{2+}-sensitivity of contraction is known to be the main characteristic feature of HCM and considered as the primary pathological mechanism [2,3]. Functional studies on the effects of HCM mutations on Ca^{2+}-sensitivity provided evidence on the linkage between the altered myofilament Ca^{2+}-response and hypercontractility, impaired relaxation, and ventricular arrythmia observed in HCM patients.

Previous studies reported disturbances in the physiological *protein quality control system* (PQS) and its contribution to HCM pathophysiology [4,5]. PQS maintains the myocardial and mitochondrial protein homeostasis by regulating protein synthesis, folding, assembly, trafficking, and clearance of misfolded/damaged proteins. The main cellular signalling pathways through which PQS maintains a healthy proteostasis are heat shock proteins (HSPs), autophagy/lysosomal and the ubiquitin-proteasome systems (UPS) [6].

Heat shock proteins (HSPs) are highly conserved chaperons that are expressed in both healthy and stressed cells. They correct misfolded proteins preventing the formation of insoluble aggregates. In response to oxidative stress and inflammation, HSPs are upregulated and translocated to sarcomeric proteins thereby protecting cardiomyocytes and stabilizing their structure [7]. When HSPs fail to refold the abnormally folded proteins (for instance oxidized or mutant proteins), they preserve the proteins in soluble form, enabling the subsequent clearance of these proteins by protein degradation pathways [8]. Together with HSPs, the co-chaperons BCL2-associated athanogene 3 (BAG3) and carboxy-terminus of HSP70-interacting protein (CHIP) are key mediators of PQS mechanisms.

Under conditions of oxidative stress and an imbalance between ROS generation and antioxidant defence pathways, ROS are found to suppress autophagy leading to the accumulation of ubiquitinated proteins and subsequently to cardiac fibrosis and hypertrophy [9].

Activation of phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway plays a pivotal role in regulating autophagy and hypertrophy [10]. The downstream glycogen synthase kinase 3ß of the AKT pathway (GSK-3ß) was found to be inactivated in HCM animal models and associated to the initiation of pathological hypertrophy [11,12]. Further downstream effectors of AKT pathway including mTOR and the Forkhead box O (FOXO) transcription factor are involved in transcription, translation, and degradation of many regulatory proteins. Previous studies showed that elevated level of PI3K and the sustained activation of AKT resulted in dysregulated FOXO 1 and c-Jun N-terminal protein kinase (JNK) signalling, cardiac hypertrophy, impaired mitochondrial energy metabolism, and increased apoptosis. However, it is unknown whether they contribute to the development of HCM [13,14]. Therefore, our study aimed to investigate how autophagy, oxidative stress, inflammation, stress signalling pathways, and apoptosis are hallmarks of HCM and their contribution to the cardiac dysfunction.

2. Materials and methods

2.1. Online methods

A detailed description of the methods is provided in the online supplementary methods.

2.2. Human heart tissues

Left ventricle (LV) tissue was obtained during heart transplantation surgery from end-stage heart failure patients (NYHA class III or IV; $n = 10$), hypertrophic cardiomyopathy (all male and average age, 52 years). All hearts presented with hypertrophic cardiomyopathy and obtained during cardiac transplantation surgery. LV tissue from non-failing donor hearts (n = 10; male; average age, 40 years) served as reference, non-failing cardiac LV tissue was obtained from donor hearts for which no suitable transplant recipient was found. Samples were obtained after informed consent and with approval of the local Ethics Committee (St Vincent's Hospital of Sydney, Australia, Human Research Ethics Committee; File number: H03/118; Title: Molecular Analysis of Human Heart Failure) and 20–6976 BR. The investigation conforms to the principles outlined in the Declaration of Helsinki.

2.3. Force measurements on isolated cardiomyocytes

Force measurements were performed on single de-membranated cardiomyocytes (n = 20–24/5–6 heart/group) as described before [15,16]. $F_{passive}$ was recorded over the sarcomere length (SL) range between 1.8 and 2.3 μm and was measured before/after GSH and/or recombinant human αB-crystallin or HSP27 or HSP70.

2.4. Duolink in situ PLA (proximity ligation assay (PLA)) technology

The in situ Duolink® PLA technique was used to visualize protein-protein interaction in fixed human LV tissue according to the Duolink® PLA protocol. The slides with the fixated, WGA stained and permeabilized heart samples were incubated with the primary antibodies. Following the amplification reaction, DAPI was added, and the samples were analysed by confocal laser scanning microscopy.

2.5. Immunofluorescence imaging

The frozen LV tissue slides were fixed, blocked, and stained with sequence-specific anti-titin antibodies, followed by appropriate secondary antibodies Cy3 and FITC. Immuno-stained samples were analysed by confocal laser scanning microscopy.

2.6. Protein analysis by western blot

End-stage human heart failure tissue was analysed and separated by 1,8% SDS-PAGE and western blots for titin or using 8 and 15% SDS–PAGE and western blot for small proteins and the phosphorylation of small proteins [15]. All signals of small proteins were normalized to GAPDH stained on the same blots. Titin signals were indexed to PVDF staining.

2.7. Quantification of tissue NO and sGC

The concentration of NO and sGC in LV tissue samples were assessed by means of a colorimetric assay kit providing a measure of total nitrate/

nitrite [15,17].

2.8. Quantification of tissue oxidative stress, inflammatory response, inflammation, inflammasomes

Myocardial levels (n = 8–10 LV sample/group) of oxidative stress and inflammatory markers were tested with enzyme-linked immunosorbent assay (ELISA) and colorimetric assay kits according to manufacturer's instructions and as previously described [18].

2.9. Statistical analysis

Data are given as median with interquartile range. For statistical analysis of two groups of parametric data Student's *t*-test was used, for non-parametric data Mann-Whitney test was used. For analysis of parametric data comparing more than two groups one-way ANOVA was used. *P*-values were corrected for multiple comparisons by the Tukey method. For analysis of proportions Fisher's exact test was used. Analysis was performed using GraphPad Prism 8. P-values are two-sided and considered statistically significant if $P < 0.05$.

3. Results

3.1. Increased myocardial stiffness in HCM myocytes is linked to oxidative stress

Myocardial dysfunction observed in human HCM is tightly coupled with impaired mechanical properties of cardiac myofibrils, often due to alterations in thin- and thick-filament proteins [3]. However, it is currently unknown whether changes in titin and $F_{passive}$ exist in HCM. In demembranated cardiomyocytes Ca^{2+}-independent passive force ($F_{passive}$) Fig. 1A was significantly elevated at SL 2.0 to 2.3 μm compared to controls, this increase was restored at SL 2.2 μm or higher upon treatment with GSH, while $F_{passive}$ of control cardiomyocytes remained unaltered, indicating the contribution of oxidative stress and potentially titin oxidation to the diastolic dysfunction observed in HCM. These changes were associated with reduced GSH levels in HCM myocardial samples compared to controls suggesting the imbalance of oxidants and antioxidants in HCM hearts (Fig. 1D).

We found a significant increase in total titin glutathionylation and a high level of titin ubiquitination in HCM samples compared to human non failing hearts (Fig. 1C). In addition, using the Duolink proximity ligation assay (PLA) and confocal microscopy, we revealed a glutathionylation of titin in HCM heart tissues (Fig. 1E-H), which further validates a direct oxidative stress related effect on titin-based myocardial stiffness.

3.2. In vitro administration of heat shock proteins reduces passive relaxation of HCM cardiomyocytes

We have tested the ability of HSP27, α-ß crystallin and HSP70 to reverse the elevated $F_{passive}$ in HCM samples. Increased $F_{passive}$ of HCM cardiomyocytes could be corrected by α-ß crystallin treatment, whereas no effects on $F_{passive}$ of the control group were detected (Fig. 2A). A significant increase of the expression level of α-ß crystallin and HSP27 was observed in HCM samples compared to controls (Fig. 2B, C).

Furthermore, HSP27 phosphorylation was significantly increased in HCM hearts compared to controls (Fig. 2D). Confocal microscopy of immunostained HCM tissue demonstrated glutathionylation of HSP27 and α-ß crystalline (Fig. 2E-H).

Administration of HSP70 significantly reduced $F_{passive}$ of HCM cardiomyocytes at SL of 2.2 μm or higher (Supplementary Fig. 1A), though the HSP70 expression level was comparable between the two groups (Supplementary Fig. 1B). Confocal imaging of immunostained HCM tissue using the Duolink fluorescence confirmed the S-glutathionylation of HSP70 as well (Supplementary Fig. 1C, D). We found a significant reduction in protein levels of both BAG3 and CHIP in HCM hearts compared to controls (Supplementary Fig. 1E, F).

3.3. Decreased nitric oxide (NO) level and soluble guanylyl cyclase (sGC) activity in HCM hearts

In human HCM tissues we found that both monomeric and dimeric endothelial nitric oxide synthase (eNOS) levels were unchanged compared to controls (Fig. 3A, B), however, the phosphorylated and hence activated eNOS was significantly increased in HCM hearts (Fig. 3C). Of note, higher phosphorylation of monomeric eNOS increases the production of superoxide, higher phosphorylation of dimeric eNOS increases the production of NO. Interestingly, NO bioavailability was significantly decreased in HCM samples (Fig. 3D), resulted in reduced sGC activity compared to controls (Supplementary Fig. 2A). The reduced NO level and sGC activity could however be due to increased 3-nitrotyrosine (Supplementary Fig. 2B) in HCM patients compared to controls.

3.4. Autophagy and hypertrophy-related regulators of signalling pathways in HCM hearts

Next, we analysed the protein levels and the phosphorylation status of AKT and the mitogen-activated protein kinase (MAPK). Both were significantly upregulated and hyperphosphorylated in HCM compared to controls, suggesting a high activation of these kinases (Fig. 3E-H). Both phosphorylation and protein levels of GSK-3ß, the downstream kinase of AKT, were unchanged in HCM samples compared to controls (Fig. 3I, J). Furthermore, we found significant increase in the nuclear factor of activated T cells (NFAT) expression and phosphorylation in HCM compared to controls (Fig. 3K, L).

We also evaluated further downstream targets of AKT and MAPK pathways including mTOR, FOXO, and JNK, which were all hyperphosphorylated in HCM hearts compared to controls (Fig. 3M-O). However, the protein level of ubiquitin was unchanged in HCM samples compared to controls (Fig. 3P).

Since the extracellular-signal-regulated kinase 1/2 (ERK1/2) pathway plays a pivotal role in cardiomyocytes growth, we checked its expression level and phosphorylation and found both to be elevated in HCM myocytes compared to controls (Supplementary Fig. 2C, D). In addition, GSK-3α was hyperphosphorylated (Supplementary Fig. 2E).

3.5. Cathepsin and caspases-mediated apoptosis in HCM hearts

As shown in (Fig. 4A-D), procaspase 3, procaspase 9, and caspase 12 were significantly elevated in HCM myocytes, while the active caspase 9 level was unchanged. These results indicate a comparable caspase 9-dependent occurrence of apoptotic events in HCM and donor hearts. We then aimed to identify alterations of apoptotic pathways in HCM and hence determined the expression levels of cathepsin, a protease that activates caspases and triggers the release of proapoptotic factors. Indeed, cathepsin was upregulated in HCM hearts compared to controls (Fig. 4E).

3.6. Elevated levels of pro-inflammatory and oxidative stress markers in HCM hearts

Finally, we assessed oxidative stress level by measuring the hydrogen peroxide (H_2O_2) concentration which was significantly increased in HCM hearts (Fig. 4L). We then assessed pro-inflammatory cytokine levels (intercellular cell adhesion molecule-1ICAM1, vascular cell adhesion molecule-1 VCAM1, Interleukin 6 (IL-6), interleukin 18 (IL-18), the Toll-like receptors 2 (TLR2), and the Toll-like receptors 4 (TLR4)), all of which were significantly elevated in HCM tissues compared to controls (Fig. 4F, G, H, I, J, K), confirming high levels of inflammation and oxidative stress.

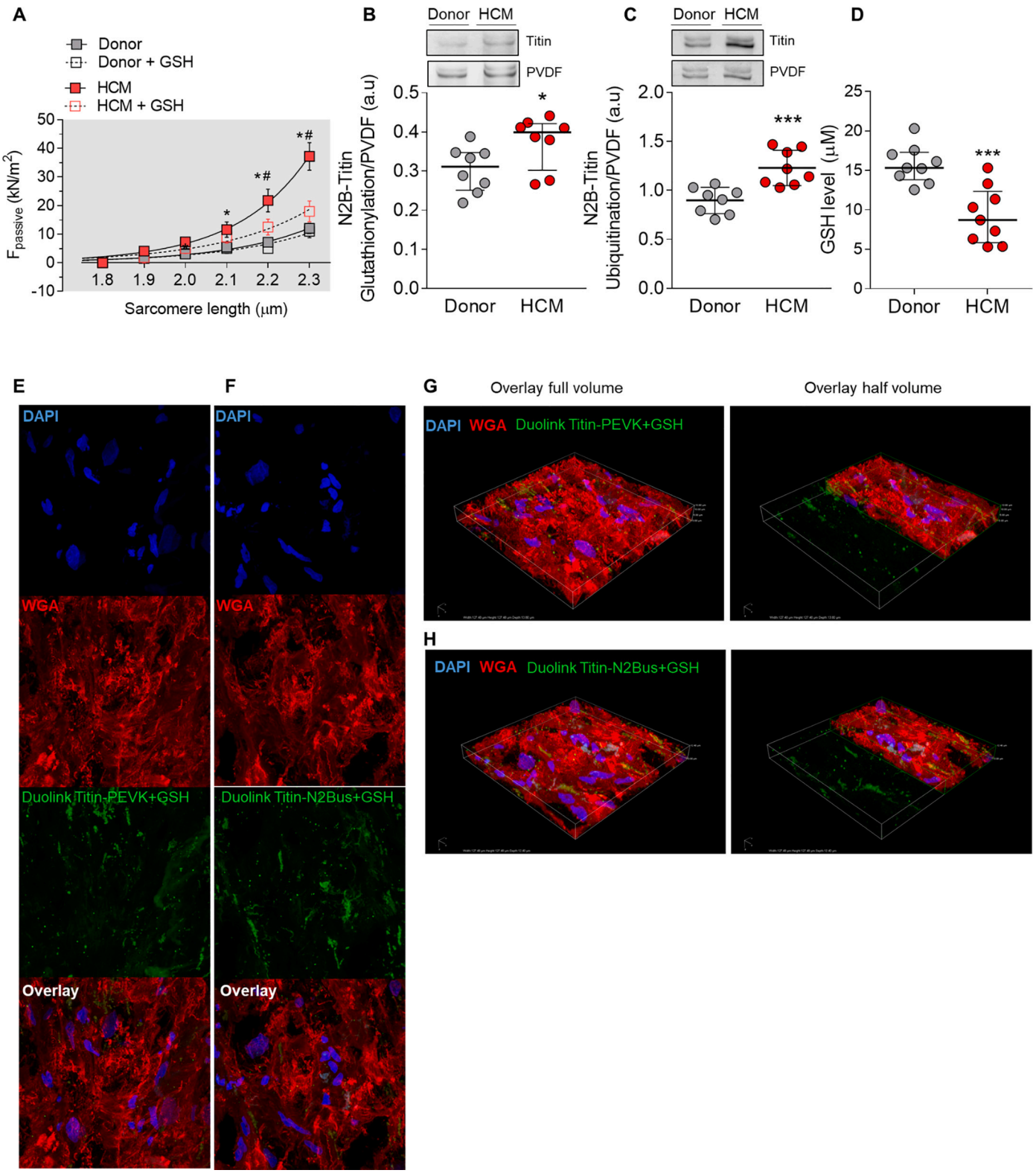

Fig. 1. Altered cardiomyocyte passive stiffness and titin oxidation in human HCM hearts. A. Passive stiffness of HCM cardiomyocytes and non-failing hearts (controls) before and after reduced glutathione (GSH) treatment at sarcomere length 1.8–2.3 μm. B. S-glutathionylation of N2B titin. C. Ubiquitination of N2B titin. D. Reduced glutathione (GSH) concentration levels. E. and F. Representative immunofluorescence confocal images (maximum intensity projection of z-stacks) of cardiomyocytes stained with DAPI (blue), WGA (red), and Duolink in Situ detection of GSH and titin interaction (Green) in human HCM hearts. G. and H. Representative images of Duolink in Situ detection of GSH and titin interaction in a z-stack in human HCM hearts. Curves are second-order polynomial fits to the (mean ± SEM; n = 20–24/4–5 cardiomyocytes/heart). *$P < 0.05$ controls vs. HCM, ***$P < 0.0001$, and $^{\#}P < 0.05$ controls after GSH treatment vs. HCM after GSH treatment. One-way ANOVA was used. P-values were corrected for multiple comparisons by the Tukey method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

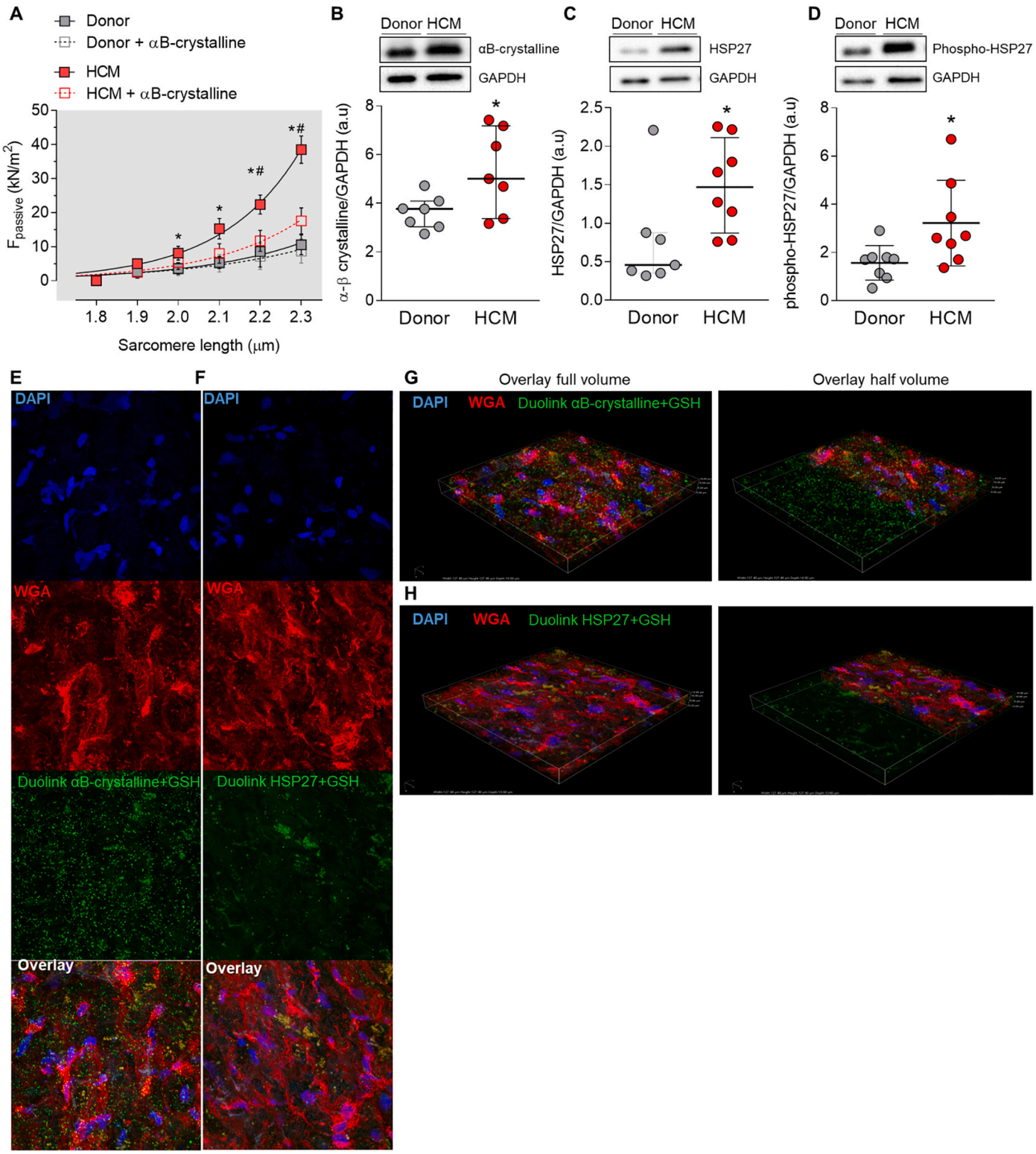

Fig. 2. Altered cardiomyocyte passive stiffness and small HSPs oxidation in human HCM hearts.A. Passive stiffness of HCM cardiomyocytes and non-failing hearts (controls) before and after α-ß crystallin treatment at sarcomere length 1.8–2.3 μm. B. α-ß crystallin protein levels. C. HSP27 protein levels D. Total HSP27 phosphorylation. E. Representative immunofluorescence confocal images (maximum intensity projection of z-stacks) of cardiomyocytes stained with DAPI (blue), WGA (red), and Duolink in Situ detection of GSH and α-ß crystallin interaction (Green) in human HCM hearts. F.Representative immunofluorescence confocal images (maximum intensity projection of z-stacks) of cardiomyocytes stained with DAPI (blue), WGA (red), and Duolink in Situ detection of GSH and HSP27 interaction (Green) in human HCM hearts. G. Representative images of Duolink in Situ detection of GSH and α-ß crystallin interaction in a z-stack in human HCM hearts. H. Representative images of Duolink in Situ detection of GSH and HSP27 interaction in a z-stack in human HCM hearts. Curves are second-order polynomial fits to the (mean ± SEM; n = 20–24/4–5 cardiomyocytes/heart). *$P < 0.05$ controls vs. HCM, and $^{\#}P < 0.05$ controls after α-ß crystallin treatment vs. HCM after α-ß crystallin treatment. One-way ANOVA was used. P-values were corrected for multiple comparisons by the Tukey method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

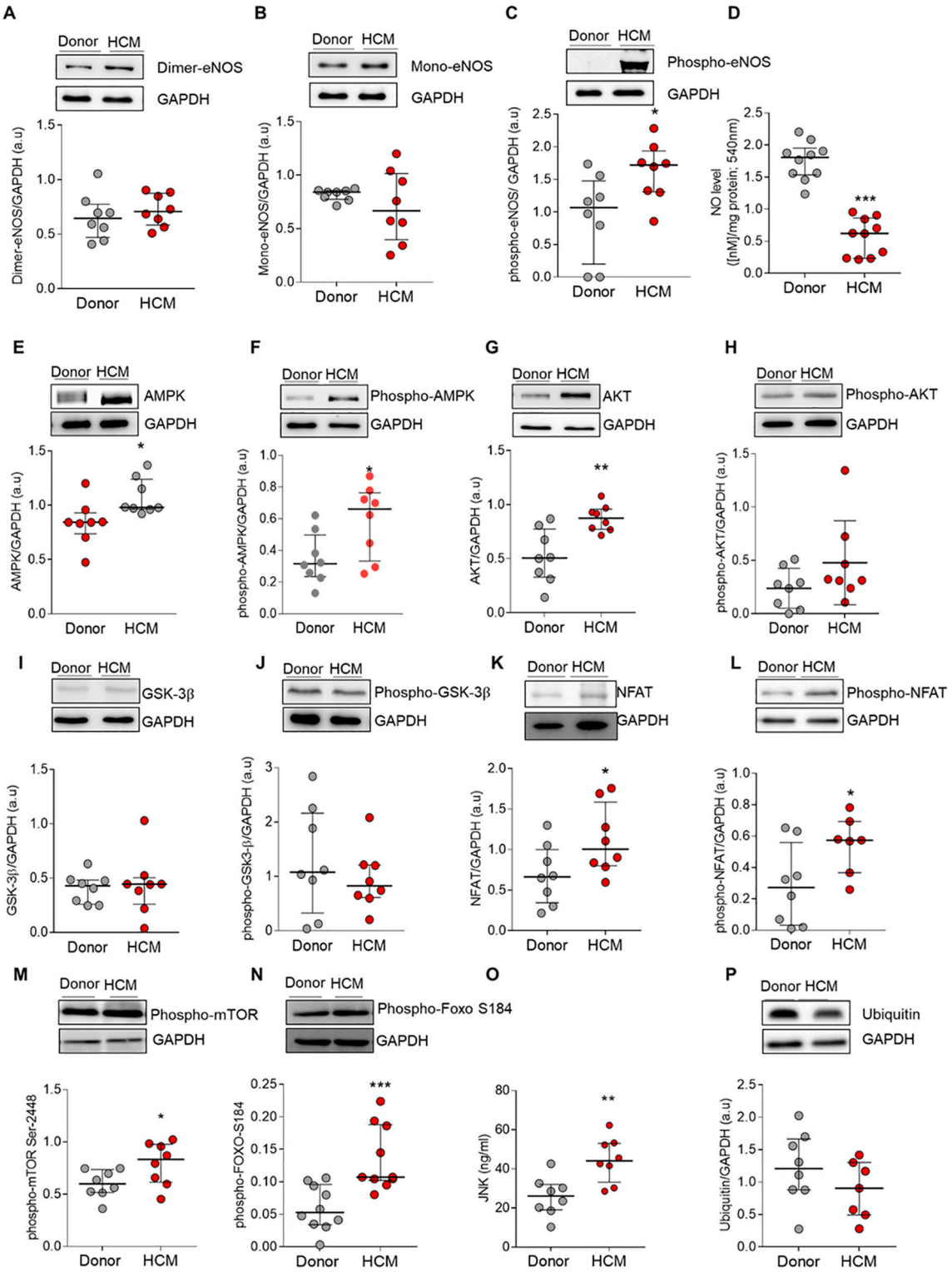

Fig. 3. NO-sGC-cGMP, AMPK and AKT pathways components in controls and human HCM cardiomyocytes. A. Dimeric eNOs protein levels. B. Monomeric eNOS protein levels. C. monomeric eNOs phosphorylation. D. Nitric oxide (NO) bioavailability. E. 5′ adenosine monophosphate-activated protein kinase (AMPK) protein levels F. Total 5′ adenosine monophosphate-activated protein kinase (AMPK) phosphorylation. G. Protein kinase B (AKT) protein levels. H. Total protein kinase B (AKT) phosphorylation I. Glycogen synthase kinase 3ß (GSK-3ß) protein levels. J. Total glycogen synthase kinase 3ß (GSK-3ß) phosphorylation. K. Nuclear factor of activated T-cells (NFAT) protein levels. L. Total nuclear factor of activated T-cells (NFAT) phosphorylation. M. Site specific phosphorylation of mammalian target of rapamycin (mTOR) at Ser 2448. N. Site specific phosphorylation of Forkhead box O (FOXO) transcription factor at Ser 184. O. c-Jun N-terminal protein kinase (JNK) concentration. P. Ubiquitin protein levels. Data are given as median with interquartile range; $n = 8$ samples/group. *P < 0.05/**P < 0.001/***P < 0.0001 controls vs. HCM.

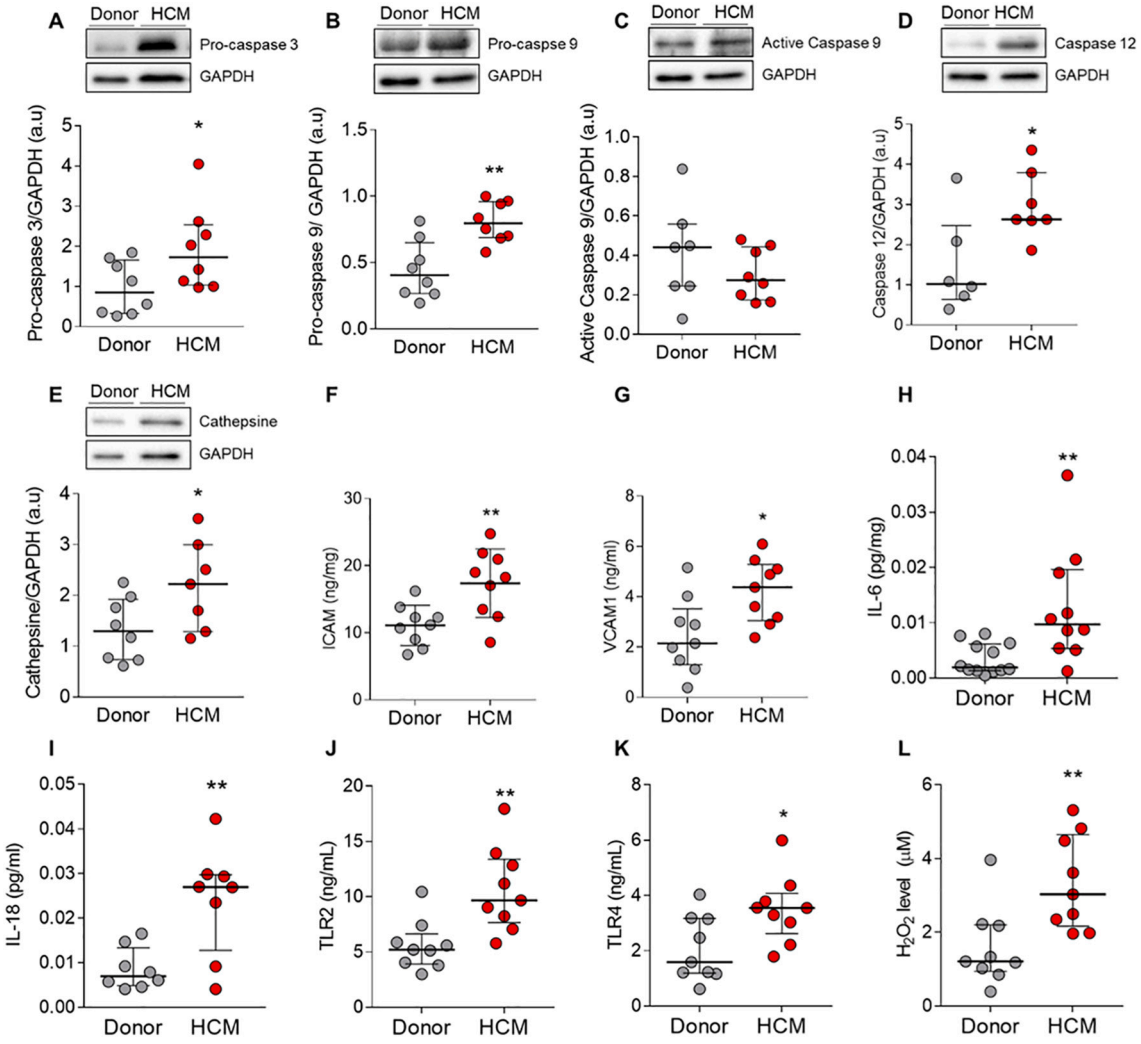

Fig. 4. Apoptotic factors, proinflammatory cytokines and myocardial stress in controls and human HCM cardiomyocytes.A. Procaspase 3 protein levels. B. Procaspase 9 protein levels. C. Active caspase 9 protein levels. D. Caspase 12 protein levels. E. Cathepsin protein levels F. Intercellular cell adhesion molecule-1 (ICAM-1) concentration levels. G. Vascular cell adhesion molecule-1 (VCAM-1) concentration levels. H. Interleukin 6 (IL-6) concentration levels I. Interleukin 18 (IL-18) concentration levels. J. The Toll-like receptors 2 (TLR2) concentration levels. K. The Toll-like receptors 4 (TLR4) concentration levels. L. Hydrogen peroxide (H_2O_2) concentration levels. Data are given as median with interquartile range; n = 8 samples/group. *P < 0.05/**P < 0.001controls vs. HCM.

4. Discussion

In the present study we provide evidence of 1) increased myocardial stiffness which can be partially attributed to increased oxidative stress, 2) dysregulated PQS as shown by the upregulation of HSPs, ubiquitin-proteasome and autophagy markers, 3) increased levels of apoptotic factors, 4) alterations of signalling pathways involved in post translational modifications of sarcomeric proteins, metabolic stress, and hypertrophy induction.

4.1. Increased titin-based myocardial stiffness in HCM hearts

HCM is associated with altered mechanical properties of the myocardium [2,19]. Sequeira et al., have recently provided a comprehensive investigation of the involvement of thin- and thick-filament components in human HCM [2,19], whereby demonstrated increased myofilament Ca^{2+}-sensitivity in HCM hearts. However, it remains unclear from the previous study whether changes in $F_{passive}$ also occur in HCM. Here we provide evidence that myocardial $F_{passive}$ is significantly increased in human HCM and associated with increased oxidative stress. We found decreased GSH levels in HCM hearts, while treatment with GSH reversed the elevated $F_{passive}$ in HCM cardiomyocytes suggesting an imbalanced redox state in HCM.

Titin is the key determinant of myocardial stiffness. Titin elasticity contributes to the length-dependent activation of contraction described in the Frank-Starling law [7,20], a mechanism shown to be highly affected by titin regulation and post translational modifications. In our study, titin was S-glutathionylated and highly ubiquitinated in HCM hearts. The contribution of titin post-translational modifications to elevated myocardial stiffness has been previously shown [18,21–26]. Accumulation of ubiquitinated titin might produce defects in the ubiquitin-proteasome system in HCM and might also affect its elasticity and thus modulate myocardial stiffness and thereby contributing to the HCM phenotype.

4.2. HSPs and autophagy pathways in HCM

HSPs and their co-chaperons are well established as crucial players in PQS through their pivotal roles in correcting misfolded proteins, directing the aberrant proteins to degradation, and inhibiting apoptosis [27].

Both HSP 27 and α-ß crystallin co-localize with titin at the *Z*-disc and I-band titin thereby providing protection against Ig domain unfolding, aggregation, and subsequent high myocyte stiffness [17,28]. Our results

show that α-ß crystallin can restore the elevated $F_{passive}$ in HCM cardiomyocytes. In addition, we found increased protein levels and hyperphosphorylation of HSP27 in HCM hearts. Overexpression of protein kinase D-mediated hyper-phosphorylation of HSP27 has been described as a cytoprotective mechanisms against oxidative stress in HCM [29–31]. Upon phosphorylation, HSP27 dissociates into lower-molecular-weight oligomers that inhibit apoptosis [32,33]. However, we have previously reported an altered HSP27 localized away from the Z-disk and A-band in HCM [29], hence HSPs may fail to exert their cytoprotective effect in HCM, perhaps because both are S-glutathionylated in HCM tissue, which might induce the translocation leading to protein aggregation and a subsequent increase in cardiomyocyte stiffness.

4.3. Dysregulated signalling pathways in HCM

A study by Franssen et al. 2016, demonstrated the contribution of oxidative stress to the reduced NO-dependent signalling, increased cardiomyocyte stiffness and hypertrophy in HFpEF [17]. In the HCM human tissues, a significant increase in the phosphorylated and hence activated monomeric eNOS, and diminished NO bioavailability and sGC activity is observed. Additionally, we detected high levels of 3-nitrotyrosine in HCM patients, which is known to be a marker of peroxynitrite formation. The uncoupling of eNOS is evident in oxidative stress-induced endothelial dysfunction, this pathological process results in switching the eNOS dimer to a superoxide anion-generating monomer. The subsequent downregulation of the NO-sGC-cGMP-PKG pathway may lead to a hypophosphorylation of sarcomeric proteins, including titin, thereby increasing the myocardial stiffness [17,26]. Since the NO-cGMP pathway supresses cardiac hypertrophy by inhibiting calcineurin-NFAT signalling [34], its downregulation might also contribute to hypertrophy.

PI3K/AKT/mTOR and AMPK signalling pathways have been linked to metabolic stress, autophagy, and hypertrophy. Both regulate ROS homeostasis and apoptosis via their downstream effectors mTOR and FOXO. Furthermore, BAG3 induces eNOS release by activating the PI3/AKT pathway [35], while CHIP increases both mTOR and AKT activation, and decreases AMPK activation in $CHIP^{-/-}$ mouse hearts during cardiac hypertrophy [36]. Our study showed upregulation and hyperphosphorylation of AKT and MAPK in HCM hearts, suggesting activation of both kinases in HCM .

Coordination of MAPK and NFAT signalling regulates the hypertrophic response [37], in HCM hearts both were significantly increased suggesting that GSK-3ß preserved its active status and that the hypertrophic growth must have been promoted via the involvement of other relevant effectors. NFAT expression signalling is well known to regulate the hypertrophic response independent of phosphorylation of NFAT, while phosphorylated NFAT leads to inhibition of its nuclear translocation and thus attenuating cardiac hypertrophy, so both NFAT and phosphorylated NFAT are regulating the hypertrophic response independently. NFAT signalling initiates the hypertrophic response through a mechanism involving only a few of direct effectors. This activates downstream targets of NFAT and coordinate the hypertrophic response. As previously discussed, the attenuation of NO-cGMP signalling caused by oxidative stress, prevents the NO-dependent regulation of the calcineurin-NFAT pathway, which might contribute to the development of cardiac hypertrophy [34].

We also evaluated further downstream signalling molecules of AKT and MAPK pathways like FOXO, and JNK, which were either hyperphosphorylated or high in concentration respectively in HCM hearts. Under metabolic stress, AMPK facilitates FOXO acetylation and nuclear translocation leading to the transcription of antioxidant genes, however, AKT dependent phosphorylation of FOXO leads to its export from the nucleus to the cytoplasm [38]. AMPK is highly activated in HCM, this is potentially a concequence of an increase in the AMP/ATP ratio which triggers AMPK activation to phosphorylate downstream targets switching off those energy (ATP)-utilizing pathways that are not essential for cell survival and switching on catabolic energy-generating pathways [39]. AMPK is a cellular energy sensor that monitors the ratio of AMP/ATP. During myocardial ischaemia activation AMPK occurs, resulting in an activation of glucose uptake and glycolysis, along with an increase in fatty acid oxidation. This activation of AMPK possibly increases energy production and inhibits apoptosis, thereby protecting the heart during the ischaemic stress [19,40–42].

Hyperactivation of ERK1/2, and the subsequent increase in protein synthesis are associated with the development of concentric hypertrophy in response to hypertrophic stimuli [43,44]. In line with these studies, we found increased expression and phosphorylation of ERK1/2 in HCM hearts. Hence, the altered ERK1/2 activity might play an important role in protein homeostasis in HCM.

4.4. Cardiomyocyte apoptosis in HCM

We found a significant elevation in apoptotic and pro-apoptotic factors in HCM cardiomyocytes. Multiple cellular events can induce apoptosis, such as oxidative stress and ROS production, inflammation and the upregulation of distinct cytokines, sustained growth stimulation in adult cardiomyocytes, and hyperactivation of ß-adrenergic signalling pathways induced by activated myocyte stretch [45–48]. Our results provide evidence for the association between these events and HCM, in addition, the impaired PQS and proteotoxicity might have induced apoptotic responses as observed in HCM hearts. Furthermore, phosphorylation of HSPs is reported to protect the cell against apoptosis [49], indeed, we found highly phosphorylated HSPs in HCM, which might potentially affect the ability of HSPs to protect against apoptosis.

4.5. Inflammation and oxidative stress in HCM

In the present study, we show that pro-inflammatory cytokines and oxidative stress parameters are increased in HCM hearts. Oxidative stress and inflammation modulate signalling pathways that are crucial for cardiac function [50], namely AKT, ERK1/2, c-Jun and NO-sGC-cGMP pathways. Moreover, pro-oxidant agonists, such as angiotensin II (Ang II) and tumor necrosis factor- α (TNF-α) induce the expression of pro-inflammatory molecules.

Of note, by attenuation of NO-sGC-cGMP signalling, ROS induce endothelial dysfunction and contribute to the increased titin based-myocardial stiffness and hence cardiomyocyte dysfunction. Furthermore, ROS modulate post translational modifications of other proteins involved in excitation-contraction coupling such as cardiac myosin, myosin-binding protein C (cMyBP-C), and troponin I (cTnI), hence oxidative stress can be correlated to the impaired mechanical properties observed in HCM.

Possible targets through which oxidative stress might also affect PQS include HSPs. The latter are known to protect the cell from oxidative stress. In our study, and despite of the increased protein levels of HSPs in HCM myocytes, we found high levels of S-glutathionylation suggesting that HSPs can be potential substrates for oxidation leading to a direct oxidative stress-mediated impairment of their cytoprotective function. Such an effect that might result in the accumulation of toxic proteins and induction of apoptosis.

5. Study limitations

Our study lacks the in vivo studies that can support the ex-vivo findings, it would be of great interest to have an experimental model for the in vivo characterization for a translational perspective. Our study used transplanted hearts instead of myocardial tissue/biopsies from patients and control subjects due the limited availability, which may trigger processes such as apoptosis or elicit a switch in transcription/translation of proteins. An inclusion of an experimental HCM animal model could support understanding and confirming the ex-vivo findings

and could partally restrain this limitations. As HCM is clearly multi-causal disease, we believe the pathogenicity of HCM will depend partially on the PQS regulation.

6. Conclusion and clinical relevance

The results presented in this study suggest that metabolic stress and impaired PQS are potential mechanisms underlying the initiation and progression of the HCM phenotype. Under oxidative stress conditions, several regulators of signal transduction pathways are modified by high levels of ROS. This leads to altered translation and transcription of essential genes involved in cardiac contractility, growth, autophagy, and apoptosis. A potential mechanism linking the mechanical disturbances in HCM to oxidative stress is the diminished NO-sGC-PKG pathway and the subsequent alterations in protein phosphorylation leading to increased myocardial stiffness and diastolic dysfunction. The diastolic dysfunction may also be attributed to impaired PQS. Failure of HSPs and their co-chaperons in maintaining a balanced proteostasis might lead to the accumulation of toxic proteins and the induction of aggregation, apoptosis, fibrosis, and progression of cardiac dysfunction.

7. Future therapeutic approaches

Collectively, our data suggest that PQS, oxidative stress, and inflammation are a hallmarks of HCM and could be a viable therapeutic approach to attenuating the severity of cardiac dysfunction and prevent its progression. One of the interventions that improves PQS is through compounds that increase HSP expression [51], however, HSPs can be targeted by oxidative stress leading to the loss of their cytoprotective function. A recent study by us demonstrated the therapeutic benefits of empagliflozin in reversing the oxidative stress mediated attenuation of NO-sGC-PKG pathway in heart failure [18]. Shende et.al, reported the antiproteotoxic effects of mTOR inhibitors by reducing protein synthesis and increasing autophagy, consequently reversing cardiac hypertrophy [52]. Therefore, both strategies may constitute potential therapeutic intervention that merit further investigations in HCM. Taken together, strategies that target the disturbed signalling pathways and their downstream effectors may hold promise to novel drug discoveries.

Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

Author contributions

RH, HB, SZ, MH, MS, SD, NM, KG, MT, MJ, SP, SB, MS, ML, VS, AK carried out experiments, analysed the data, and revised the manuscript. RH, HB wrote the manuscript. CDR, AM, SS provided human samples and revised the manuscript. KJ, TG, HGM revised the manuscript. NH designed the study, funded the project, supervised the project/all experiments, carried out experiments, analysed the data, wrote the manuscript with edits from other authors.

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All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2021.09.009.

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