TITLE PAGE

FULL TITLE: A Proteomic Analysis of the Myocardium in Hypertrophic Obstructive Cardiomyopathy.

RUNNING TITLE (36 characters): Proteome of Hypertrophic Cardiomyopathy

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ABSTRACT

Background: Hypertrophic cardiomyopathy (HCM) is characterized by a complex phenotype that is only partly explained by the biological effects of individual genetic variants. The aim of this study was to use proteomic analysis of myocardial tissue to explore the post genomic phenotype.

Methods: Label-free proteomic analysis was used initially to compare protein profiles in myocardial samples from eleven patients with HCM undergoing surgical myectomy with control samples from six healthy unused donor hearts. Differentially expressed proteins of interest were validated in myocardial samples from 65 unrelated individuals [HCM (n=51), controls (n=7) and aortic stenosis (n=7)] by the development and use of targeted multiple reaction monitoring (MRM) based triple quadrupole mass spectrometry.

Results: In this exploratory study, 1586 proteins were identified with 151 proteins differentially expressed in HCM samples compared to controls ($p<0.05$). Protein expression profiling showed that many proteins identified in the initial discovery study were associated with metabolism, muscle contraction, calcium regulation and oxidative stress. Proteins down-regulated in HCM versus controls included creatine kinase M-type, Fructose-bisphosphate aldolase A and phosphoglycerate mutase ($p<0.001$). Proteins up-regulated in HCM included lumican, carbonic anhydrase 3, desmin, alpha actin skeletal and four and half LIM domain protein 1 ($p<0.01$). Myocardial lumican concentration correlated with left atrial area (rho 0.34, $p=0.015$), late gadolinium enhancement (LGE) on cardiac magnetic resonance imaging ($P=0.03$) and the presence of a pathogenic sarcomere mutation ($p=0.04$).

Conclusion: The myocardial proteome of HCM provides supporting evidence for dysregulation of metabolic and structural proteins. The finding that lumican is raised in HCM hearts provides insight into the myocardial fibrosis that characterizes this disease.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is a common inherited disease caused mainly by dominantly inherited mutations in genes encoding cardiac sarcomere proteins [1]. The disease phenotype is characteristically diverse, ranging from no clinically detectable abnormality to extreme hypertrophy and death from ventricular arrhythmia, heart failure and stroke. The biological effects of individual genetic variants only partly explain this complex clinical spectrum and much still needs to be learnt about the downstream pathways that lead to disease development and progression.

Global proteome studies have provided mechanistic insights into many human cardiovascular diseases including heart failure [2], aortic aneurysm [3], atherosclerosis [4], atrial fibrillation [5] and inflammatory dilated cardiomyopathy (DCM) [6], but there have been few proteomic studies in HCM. In this study we perform comparative whole tissue proteomics using myocardial tissue removed at the time of septal myectomy. Our aim was to identify dysregulated proteins that provide insight into disease pathogenesis.

METHODS:

The National Research Ethics Committee approved the study (REC Ref. no. 04/0035 and REC 11/LO/0913) and written informed consent was obtained from all patients prior to surgery. Detailed methods are available in Supplementary Material: Methods. The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure.

RESULTS

Study cohort: The clinical characteristics of patients included in the Phase 2 study are shown in Table 1. The demographics and genetic characteristics of patients included in the Phase 1 and 2 studies are shown in Supplementary Table 1. Genetic testing was performed in 47 of the 52 HCM patients. Patients (n=6) with a variant of uncertain significance (VUS) were excluded from the sub-group analysis by genotype in the Phase 2 study. Two patients included in the Phase 1 study had genetic variant in *MYBPC3* initially classified as pathogenic downgraded to a VUS at the time of final analysis.

Identification and classification of proteins: A total of 1586 proteins were identified across all heart samples, 722 of which were detectable across both disease and control groups (Supplementary Table 2A: Protein List (see

excel file). After excluding proteins with <1 unique peptide, the core proteome consisted of 531 proteins (Supplementary Table 2B (see excel file)). Principal component analysis demonstrated that the control samples were clustered with separation from the HCM samples, indicating the HCM tissue had a distinctly different proteome (Figure 1).

Proteomic differential expression analysis: A statistically significant difference ($p<0.05$) was found between HCM and controls for 151 proteins; 56 proteins were upregulated and 95 downregulated in HCM compared with controls. The fold change was >1.3 for 125 proteins and >1.5 for 79 proteins (Supplementary Table 2B (see excel file)).

Gene ontology and pathway analysis. The core proteome dataset was submitted for gene ontology and IPA® pathway analysis. Functional annotation clustering using PANTHER-GO Biological process analysis showed that many proteins identified in the Phase 1 study were associated with muscle contraction and metabolism (Supplementary Table 2C: Gene ontology (see excel file)). Proteins involved in actin cytoskeletal signalling, calcium signalling, and Integrin linked kinase (ILK) signalling pathways were upregulated, whereas proteins involved in oxidative phosphorylation and mitochondrial dysfunction pathways were downregulated (Figure 2 and Supplementary Table 2D: Canonical pathways (see excel file)). Upregulated biological functions specifically associated with the heart included cardiac hypertrophy, arrhythmia, dilatation and heart failure (Supplementary Supplementary Table 2E: Biological functions (see excel file)).

Validation of expression proteomic data using a targeted MRM-MS assay. The 33 proteins included in the validation assay are listed in Supplementary Table 3. The direction, fold change and significance level across the pilot, Phase 1 and Phase 2 studies is shown. Using quantitative MS/MS eight proteins were found to be significantly altered between the HCM and control groups. The MRM transitions are shown in Supplementary Table 4. Actin, alpha skeletal muscle (ACTS, P68133), Four and a half LIM domains protein 1 (FHL1, Q13642), Desmin (DES, P17661), Carbonic anhydrase 3 (CA3, P07451) and Lumican (LUM, P51884) were elevated in HCM versus control ($p<0.01$). Fructose-bisphosphate aldolase A (ALDOA, P04075), Creatine kinase M-type (CKM, P06732) and phosphoglycerate mutase (PGAM2, P15259 and PGAM1, P18669) were reduced in HCM versus control ($p<0.01$).

Structural proteins: The percentage of cardiac α-actin (expressed as a percentage of total skeletal and cardiac actin) was lower in HCM (54 ± 3 %) than both controls (65 ± 7 %; p<0.05) and AS (61 ± 8 %, p=0.016). FHL1 was higher in HCM (326 ± 113 pmol/l) compared with controls (111 ± 90, p=0.0002) and aortic stenosis (154 ± 139, p=0.0031). Desmin was higher in HCM (211 ± 83 pmol/l) compared with controls (128 ± 36, p=0.011) and AS samples (119 ± 80, p=0.0051) (Figure 3).

Metabolic proteins: Creatine kinase M-type was higher in controls (738 ± 154 pmol/l) than HCM (458 ± 109, p=0.0002) and AS (476 ± 176, p=0.0175). Fructose-bisphosphate aldolase A was lower in HCM (154 ± 44 pmol/l) than controls (201 ± 62, p=0.0425) but there was no difference with AS samples. The peptide chosen for validation of phosphoglycerate mutase exists in both the b-type (PGAM1) and m-type (PGAM2) sub-units selected because the mb-isozyme predominates in cardiac muscle.

Lumican: The discovery of significantly elevated levels of lumican was a novel finding and considered to be relevant to disease mechanisms. Therefore, two peptides were chosen for inclusion in the Phase 2 study. There was a linear correlation between both peptides (r^2=0.96, p<0.01) indicating they are good markers for whole protein quantitation (Figure 4A). Lumican concentration was higher in HCM (283 ± 176 pmol/l) than controls (133 ± 25, p=0.003) and lower than AS (544 ± 311, p=0.026) (Figure 4B). Lumican concentration correlated with left atrial area on echocardiogram (rho 0.34 (95%CI 0.06-0.57), p=0.015) (Supplementary Figure 4) but there was no correlation with age, sex, body mass index, glomerular filtration rate, C-reactive protein, N-terminal pro brain natriuretic peptide (NT-proBNP), left ventricular (LV) wall thickness or cardiopulmonary by-pass time. Lumican concentration was higher in 16 patients with a pathogenic sarcomere mutation (366 ± 230 pmol/l) than 24 patients (243 ± 124, p=0.041) with no pathogenic variant identified. Of the patients that underwent cardiac magnetic resonance imaging (MRI), lumican was higher in those with LGE in the septum (n=20, 317 ± 151 pmol/l) compared to those without LGE (n=12, 208 ± 121 pmol/l, p=0.032) (Figure 4D).

Carbonic anhydrase 3: CA3 concentration was higher in HCM (108 ± 129 pmol/l) than controls (21 ± 7, p=0.003) but there was no significant difference between AS and HCM (Figure 5A). There was a positive correlation between % skeletal actin and CA3 (r=0.271, p=0.04) and levels of FHL1 and CA3 (r=0.352, p=0.07). There was no difference between patients with and without a pathogenic sarcomere mutation (Figure 5B). To be certain this protein was myocardial in origin, the increased expression of CA3 was confirmed by immunohistochemistry

where it was found to be localized to the cytoplasm of the cardiomyocytes with considerable heterogeneity in expression both between samples and within the same sample (Figure 5C). In patients with HCM (n=51) and aortic stenosis (n=7), CA3 levels correlated with serum NT-pro BNP (rho=0.33 (95% CI 0.07-0.54), p=0.013, n=58) and left atrial area (rho=0.30 (95% CI 0.04-0.52), p=0.023, n=58). There was no correlation with age or time on cardiopulmonary bypass, both potential confounders of underlying myocardial metabolic and acid-base status. No significant difference in CA3 was found in patients with late gadolinium enhancement on cardiac MRI compared to those without.

DISCUSSION

This is the first unbiased label-free comparative whole tissue proteomic characterization of myocardium from patients with HCM. Several observations are compatible with the known pathophysiology of the disease, while others provide new insights into cellular processes that might be involved in cardiac dysfunction. While there are limitations to studying whole heart homogenate, this was an exploratory study to generate testable hypotheses. To date, there has only been one global proteomic study in a murine model of HCM expressing a *TNNI3* gene mutation (Gly203Ser) which showed more than two-fold changes in 34 proteins involved in energy production, calcium handling and cardiomyocyte structure[7]. There have been several small transcriptomic studies, which corroborate many of our findings including increased expression of markers of secondary hypertrophy and changes in proteins associated with energy metabolism, the extracellular matrix and inflammation [8-10].

Altered myocardial metabolism: The normal myocardium depends on oxygen for high-energy phosphate production via oxidative phosphorylation [11]. A switch in energy substrate preference from carbohydrates to fatty acids is a hallmark of the transition from fetal to adult cardiac metabolism [12]. We observed a mixed pattern of changes in glycolytic enzymes, but since several of these proteins catalyze bidirectional reactions it was not possible to ascertain whether glycolysis or gluconeogenesis were up- or downregulated (functional assays would be necessary to confirm this) [13]. Enzymes responsible for the metabolism of medium and long chain fatty acids including long chain fatty acid CoA ligase 1, very long chain specific acyl CoA dehydrogenase and medium chain specific acyl CoA dehydrogenase were all down regulated. The mitochondrial trifunctional enzyme subunit beta

which catalyzes the final step of beta-oxidation, was also reduced. Overall these findings suggest that beta-oxidation is down-regulated in HCM. The reduction of creatine kinase observed in both HCM and AS (Figure 3) is consistent with previous reports of impaired high-energy phosphate metabolism in these diseases [14, 15]. In the Phase 1 study, there was also a significant reduction of ADP ATP translocase 1 in HCM compared with controls and many of the sub-units of ATP synthase (Supplementary Data File 2) which supports the notion of an overall decrease in energy flux within the cell as down-regulation of cytoplasmic creatine kinase causes a decline in ATP transfer into the mitochondria and thus a reduction in energy delivered to the myofibrils [16].

Mitochondria generate energy in the form of adenosine triphosphate (ATP) to support intracellular processes including contraction of myofilaments[17]. Mitochondrial dysfunction is a recognized feature of many diseases, including HCM, but it is unclear whether it is a primary or secondary phenomenon[18]. Pathway analysis found oxidative phosphorylation was downregulated (Figure 2) with decreased expression of multiple protein sub units from Complex I, III, IV and V in HCM compared with normal controls. This finding is in keeping with evidence from animal and human studies suggesting that HCM is characterized by a reduction in the concentration of high-energy phosphates in the myocardium [19, 20] and with the hypothesis that energy deficiency is a fundamental driver of the HCM phenotype [21].

Citrate synthase activity is frequently used as a measure of mitochondrial enrichment [22]. We found no difference in expression of citrate synthase (CISY, O75390) in HCM compared with controls (Supplementary Data File 2) implying that the observed changes in the electron transport chain were not due to fewer mitochondria. As there was no difference in expression of Complex II, which is encoded entirely by nuclear DNA, another possible hypothesis is that perturbations in mitochondrial DNA (mtDNA) contribute to the reduced expression of protein sub units making up the electron transport chain. This idea has been considered in patients with congenital heart disease where mtDNA depletion has been attributed to reduced mtDNA replication in hypertrophied and failing right ventricles [23].

Novel findings

Four and a half LIM domains protein 1

FHL1 is strongly expressed in the developing outflow tract and to a lesser extent in myocardium. In mouse models of dilated and hypertrophic cardiomyopathy, cardiac ventricular expression of FHL1, but not of related

proteins FHL2 or FHL3, is upregulated [24]. The FHL1 protein has also been shown to be significantly upregulated in human and animal models of pulmonary hypertension where its expression is dependent on hypoxia-inducible transcription factor[25]. FHL1 interacts with myosin-binding protein C contributing to sarcomere assembly [26]and also Talin-1 although the function of this interaction is not understood[25]. A recent transcriptomic study suggested FHL1 may be a genetic modifier in HCM [27] but the mechanism and role of increased FHL1 expression requires further investigation.

Lumican

Lumican, a small leucine-rich proteoglycan (SLRP) was elevated in HCM and AS samples and correlated with myocardial fibrosis determined non-invasively using cardiac MRI. SLRPs regulate the kinetics, assembly, spatial organization, and stabilization of collagen fibrils and influence tissue fibrosis through interaction with transforming growth factor-β and possibly via proinflammatory pathways[28]. Lumican mRNA has previously been identified in human heart and appears to be significantly upregulated in HCM and DCM compared with controls [29]. In a mouse model, left ventricular lumican protein levels were shown to increase after aortic banding and fell after de-banding implying that production can be altered in response to external stimuli[30]. Stimulation of cardiac fibroblasts with recombinant glycosylated lumican, causes an increase in type I collagen, a decrease in activity of the collagen-degrading enzyme matrix metalloproteinase-9, and an increase in the phosphorylation of fibrosis-inducing SMAD3 [31]. The stimulus to increased lumican production in HCM is unclear, but the direct link with molecules known to be important for cardiac remodeling and fibrosis and mechanical stress is of relevance to HCM where wall stress is increased by left ventricular outflow obstruction, asymmetric hypertrophy and cardiomyocyte disarray. Furthermore, the correlation we found with sarcomere mutations and LGE suggests it may be important in disease pathogenesis.

Carbonic anhydrase 3

The presence of carbonic anhydrase (CA) in heart muscle is well established with cardiac ventricular myocytes expressing different isoforms in the cytosol (CA2), mitochondria (CA5), sarcolemma (CA4, CA14) and sarcoplasmic reticulum membrane (CA4, CA9, CA14)[32]. However the CA3 isoform has previously been reported at very low levels in the myocardium [33, 34]. In this study we have demonstrated by two methods that CA3 is over

expressed in HCM but whether this is a response to cardiac hypertrophy in general remains to be determined. The positive correlation with heart failure markers (NT-pro BNP and left atrial size) and other proteins (skeletal actin and FHL1) known to be upregulated in pathological cardiac hypertrophy and heart failure suggests this isoform may not be disease specific. In the heart, cellular or mitochondrial acidosis can impair contractility by reducing calcium sensitivity and compromising energetics. By transporting carbon dioxide out of cells into nearby capillaries acidosis is prevented and intracellular pH maintained. CA3 has not been specifically studied in human hearts but there is emerging evidence that isoforms CA2 and CA4 are activated in both hypertrophic and dilated failing human hearts [35]. Carbonic anhydrase inhibition has been shown to prevent and revert cardiomyocyte hypertrophy in cultured myocytes[36]. Further studies are required to understand whether over expression of CA is a protective response or whether CA activity could or should be pharmacologically modulated.

Limitations: As drug refractory patients with outflow tract obstruction requiring surgery, represent an advanced stage of HCM we cannot extrapolate our findings to early disease. The control samples were obtained from unused donor hearts, some of whom suffered catastrophic neurologic injury which could have altered the tissue proteome. Nonetheless, the inclusion of AS samples as a positive control group in the Phase 2 study gave further insight into whether novel findings might be disease specific or related to general downstream effects observed in hypertrophy and heart failure.

Global proteomic studies of human heart tissue are complicated by factors such as disease state, tissue and genetic heterogeneity, medical co-morbidities and drug therapy. Approaches to overcome these problems include laser capture microdissection, use of animal models and induced pluripotent stem cell (iPSC) lines. To date none of these studies have been undertaken in HCM. The whole tissue homogenate approach we used is similar to the approach that has been used in the 2-dimensional gel electrophoresis myocardial protein databases and comparative DCM studies[37]. Whilst multiple sub-cellular fractionation steps would have enhanced organelle isolation, it would still be difficult to reliably identify the cell of origin (e.g. myocyte, fibroblast or endothelial cell). We found no difference in expression of citrate synthase implying that changes were not just due to fewer mitochondria. After protein extraction and centrifugation, the insoluble pellet

(containing extracellular proteins) was retained for further work. Whilst we acknowledge the inherent limitations we believe this study still serves as an important basis to examine the HCM proteome

Although pathway analysis tools offer an insight into the underlying biology, there are inherent limitations in using a list of differentially expressed proteins (or genes) to make firm conclusions about disease mechanisms. As further experimental evidence is generated, the annotation knowledge base will grow, and it is likely pathway and network analysis will provide better understanding of the downstream effects of altered proteins. While the increased or decreased expression of a protein does not necessarily determine whether pathways are up- or downregulated, the similarity between our results and previous transcriptomic studies provides supporting evidence at protein level for an altered pattern of metabolic and structural proteins in HCM.

CONCLUSION

The myocardial proteome of HCM provides supporting evidence for dysregulation of metabolic and structural proteins as well as proteins associated with muscle contraction, calcium regulation and oxidative stress. The discovery that lumican is raised in HCM hearts provides insight into the myocardial fibrosis that characterizes this disease.

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DISCLOSURES:

None. The funders played no role in the performance or writing of the study and the views expressed are those of the authors.

DATA SHARING:

All data and metadata underlying the findings reported in this manuscript will be deposited in the PRIDE public repository.

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Table 1. Characteristics of 65 patients included in the Phase 2 study. Continuous variables are expressed as mean ± standard deviation, and categorical variable as number (percentage). P values are calculated using t-test or Chi-squared test to compare means and proportions between subjects in the aortic stenosis and hypertrophic cardiomyopathy (HCM) groups.

	Control	Aortic Stenosis	HCM	P value
Number of patient samples	7	7	51	-
Male (%)	2 (29%)	3 (43%)	28 (55%)	NS
Age (years)	47 ± 10	69 ± 10	51 ± 14	<0.05
BMI (kg/m^2)	-	27 ± 5	30 ± 5	NS
Sinus rhythm (%)	-	6 (86 %)	50 (98 %)	NS
Paroxysmal AF (%)		0 (0 %)	8 (16 %)	-
NSVT (%)	-	0 (0 %)	7 (14 %)	-
NT-proBNP (pmol/l)	-	92 ± 132	221 ± 267	NS
eGFR (ml/min)	-	88 ± 27	82 ± 20	NS
CRP (mg/l)	-	2.1 ± 2.7	4.6 ± 6.7	NS
Hb (g/dl)	-	14.3 ± 1.7	13.7 ± 1.6	NS
Hypertension (%)	-	3 (43 %)	17 (33 %)	NS
Coronary disease (%)	-	1 (14 %)	1 (2 %)	NS
Diabetes (%)	-	1 (14 %)	3 (6 %)	NS
Smoking (%)	-	2 (29 %)	9 (18 %)	NS
Family history of HCM (%)	-	0 (0 %)	16 (31 %)	-
Family history of SCD (%)	-	0 (0 %)	6 (12 %)	-
History of syncope (%)	-	0 (0 %)	13 (26 %)	-
Medication				
Beta-blocker		4 (57 %)	38 (75 %)	NS
Calcium-blocker		1 (14 %)	13 (26 %)	NS
Disopyramide		0 (0 %)	24 (47 %)	-
ACE or ARB		3 (43 %)	3 (6 %)	<0.05

Warfarin	1 (14 %)	12 (24 %)	NS
Amiodarone	0 (0 %)	7 (14 %)	NS
Statin	4 (57 %)	11 (22 %)	<0.05
Echocardiogram			
Max LV wall thickness (mm)	11 ± 1	19 ± 4	<0.05
LA size (mm)	40 ± 6	45 ± 7	NS
LVOT/AV gradient (mm Hg)	72 ± 10	91 ± 38	-
Moderate MR (%)	0 (0 %)	30 (59 %)	-
RVH (%)	0 (0 %)	11 (22 %)	-
Operative details -			
Aortic cross-clamp time (min)	61 ± 17	75 ± 32	NS
Cardiopulmonary bypass time (min)	90 ± 27	115 ± 63	NS

AV aortic valve, BMI body mass index, NSVT non-sustained ventricular tachycardia, VF ventricular fibrillation, RVH right ventricular hypertrophy, NT-proBNP N-terminal pro brain natriuretic peptide, eGFR estimated glomerular filtration rate, CRP C-reactive protein, Hb haemoglobin, HCM hypertrophic cardiomyopathy, SCD sudden cardiac death, ACE angiotensin converting enzyme inhibitor, ARB angiotensin receptor blocker, LA left atrium, LV left ventricular, LVOT left ventricular outflow tract, MR mitral regurgitation, RVH right ventricular hypertrophy.

FIGURE LEGENDS

Figure 1. Principal component analysis shows hypertrophic cardiomyopathy samples segregate from controls. The score plot for the two component PCA model shows (t1) and (t2) account for 12.7% and 8.8% of the variation respectively. The control (green circles) and hypertrophic cardiomyopathy (red circles) myocardial samples show distinct clustering

Figure 2. The top ten a) upregulated and b) downregulated biological pathways in hypertrophic cardiomyopathy. Bar charts show canonical pathways altered in hypertrophic cardiomyopathy compared to controls as indicated by Ingenuity pathways analysis (IPA; Ingenuity Systems, www.ingenuity.com). The pathways are indicated on the y-axis. On the x-axis, the significance score (negative log of P-value calculated using Fisher exact test) for each pathway is indicated by the bars. The proteins identified as enriched in each pathway are listed in Supplementary data file 2, Worksheet D.

Figure 3. Validation of differentially expressed proteins in the Phase 1 cohort as determined by MRM-mass spectrometry. Scatter plots show concentration of selected proteins in the Phase 2 cohort in HCM (n=51) compared with control (n=7) and aortic stenosis (AS) samples (n=7). The contribution of cardiac alpha actin to the total amount of alpha actin is expressed as a percentage. Significant differences are shown with p-values (Mann Whitney U test).

Figure 4. Validation and relevance of lumican in hypertrophic cardiomyopathy (HCM). A) The response of peptide 1 (NIPTVNENLENYYLEVNQLEK) and peptide 2 (SLEYLDLSFNQIAR), relative to the internal standard analyzed by UPLC-MS/MS in the study cohort showed an excellent correlation (equation y=0.9171x -0.0006, R^2 = 0.96. The x and y-axes indicate the ratio of the area under the curve for each peptide compared to the internal standard. B) Scatter plot showing concentration of lumican in HCM (n=51) compared with control (n=7) and aortic stenosis samples (n=7). Significant differences are shown with p values (Mann Whitney test). C) Myocardial lumican concentration was significantly higher in 16 patients with a sarcomere gene mutation (366 ± 230 pmol/l) compared with 24 patients with no genetic variants identified (243 ± 124 pmol/l, p=0.041). D) Myocardial lumican concentration in 32 patients with HCM stratified by presence of late gadolinium enhancement (LGE) in the septum on cardiac magnetic resonance imaging.

Figure 5. Validation and relevance of carbonic anhydrase 3 in hypertrophic cardiomyopathy. A) Scatter plot showing concentration of carbonic anhydrase 3 (CA3) in HCM (n=51) compared with control (n=7) and aortic stenosis (AS) samples (n=7). Significant differences are shown with p values (Mann Whitney test). NS indicates not significant. B) CA3 expression in myocardial samples from 7 controls and 40 patients with HCM, separated by genetic sub-groups (sarcomere mutation n=16, nil mutation n=24). C) Histological validation of CA3 in myocardium. Positive staining for CA3 (1:1000 dilution, AbCam ab118428) was visualized using a Leica BondMax Refine detection kit (DS9800). Brown (diaminobenzidine) staining indicates the presence of CA3 in a) skeletal muscle (positive control), b) normal heart, c) HCM heart (all x 4 magnification). Localization of CA3 within cytoplasm of cardiomyocytes at d) low (x 4), e) medium (x 10) and f) high (x 20) magnification.