

1 **Transcriptomic analysis of cardiomyocyte extracellular vesicles in hypertrophic**
2 **cardiomyopathy reveals differential snoRNA cargo**

3 Victoria James*¹, Zubair A. Nizamudeen¹, Daniel Lea¹, Tania Dottorini¹, Terri L.
4 Holmes², Ben B. Johnson², Kenton P. Arkill³, Chris Denning⁴, and James G.W. Smith*²

5 1: School of Veterinary Medicine and Science, University of Nottingham, NG7 2RD,
6 UK. 2: Faculty of Medicine and Health Sciences, Norwich Medical School, University
7 of East Anglia, NR4 7UQ, UK. 3: School of Medicine, University of Nottingham,
8 Nottingham, NG7 2UH, United Kingdom 4: Biodiscovery Institute, University of
9 Nottingham, NG7 2RD, UK.

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11 * To whom correspondence should be addressed.

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13 Email: Victoria.James@nottingham.ac.uk

14 School of Veterinary Medicine and Science, University of Nottingham, NG7 2RD, UK

15 Email: J.G.Smith@uea.ac.uk

16 Faculty of Medicine and Health Sciences, Norwich Medical School, University of East
17 Anglia, NR4 7UQ, UK.

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23 **Abstract**

24 Hypertrophic cardiomyopathy (HCM) is characterised by increased left ventricular wall
25 thickness that can lead to devastating conditions such as heart failure and sudden
26 cardiac death. Despite extensive study, the mechanisms mediating many of the
27 associated clinical manifestations remain unknown and human models are required.
28 To address this, human induced pluripotent stem cell (hiPSC) lines were generated
29 from patients with a HCM associated mutation (*c.ACTC1^{G301A}*) and isogenic controls
30 created by correcting the mutation using CRISPR/Cas9 gene editing technology.
31 Cardiomyocytes (hiPSC-CMs) were differentiated from these hiPSCs and analysed at
32 baseline, and at increased contractile workload (2 Hz electrical stimulation). Released
33 extracellular vesicles (EVs) were isolated and characterised following a 24 hour culture
34 period and transcriptomic analysis performed on both hiPSC-CMs and released EVs.
35 Transcriptomic analysis of cellular mRNA showed the HCM mutation caused
36 differential splicing within known HCM pathways, and disrupted metabolic pathways.
37 Analysis at increasing contraction frequency showed further disruption of metabolic
38 gene expression, with an additive effect in the HCM background. Intriguingly, we
39 observed differences in snoRNA cargo within HCM released EVs, that specifically
40 altered when HCM hiPSC-CMs were subjected to increased workload. These
41 snoRNAs were predicted to have roles in post translational modifications and
42 alternative splicing, processes differentially regulated in HCM. As such, the snoRNAs
43 identified in this study may unveil mechanistic insight into unexplained HCM
44 phenotypes and offer potential future use as HCM biomarkers or as targets in future
45 RNA-targeting therapies.

46

47

48 **Introduction**

49 Hypertrophic cardiomyopathy (HCM) is the most commonly inherited heart disease,
50 with a prevalence now considered to be greater than 1 in 500 in the general population
51 [1]. Clinically, HCM can be characterised by increased left ventricular wall thickness
52 (>15 mm for adults) that is not solely explained by abnormal loading conditions [2].
53 Histologically, the hearts of HCM patients consist of enlarged cardiomyocytes, with
54 alignment disarray, and the presence of interstitial fibrosis [3]. Genetically, HCM is an
55 autosomal-dominant disorder mainly caused by mutations in genes encoding for
56 contractile and structural proteins of the cardiac muscle sarcomere apparatus [4].

57 The penetrance of the HCM phenotype is variable, with mutation carriers presenting
58 with symptoms that range from fully asymptomatic to fatal cardiac dysfunction [5].
59 Insights into the molecular mechanism behind HCM have advanced significantly, and
60 it is now clear that an HCM mutations can alter myofilament Ca^{2+} sensitivity leading to
61 the abnormal contractility, energy depletion, and the enhanced susceptibility to
62 arrhythmia [6]. Yet the mechanisms leading to secondary HCM phenotypes, such as
63 myocyte hypertrophy, myocardial disarray, and interstitial fibrosis, are not fully
64 explained. It remains unclear how mutations in genes encoding sarcomeric proteins
65 only expressed in cardiomyocytes, give rise to phenotypes that encompass multiple
66 cardiac cell populations.

67 Extracellular vesicles (EVs) have been known to play roles in cell signalling for many
68 years, but more recently, focus on smaller EVs (which include exosomes) has
69 increased. Exosomes and smaller EVs usually between 30-150nm in size, have the
70 ability to transmit bioactive molecules between cells [7,8]. Blood contains vast

71 amounts of exosomes released by many cell types, and the contribution of this
72 complex signalling within the cardiovascular system is now increasingly investigated
73 [9]. Circulating EVs have been found to contain a broad spectrum of small non-coding
74 RNAs (snRNAs), including small nucleolar RNAs (snoRNAs) [10-12]. These snoRNAs
75 are known to have a diverse range of functions, ranging from 2'-O-methylation and
76 pseudouridylation of RNAs, through nucleolytic processing of rRNAs to the synthesis
77 of telomeric DNA [13]. Studies have identified snoRNA involvement in cardiometabolic
78 disease, including in lipid metabolism [14], diabetes mellitus [15], and doxorubicin
79 cardiotoxicity [16]. Additionally, snoRNAs have also been shown to regulate splicing
80 in normal cardiac development [17] and can alter splicing in some congenital heart
81 diseases (tetralogy of Fallot) [18]. Interestingly, the knockdown of specific snoRNAs in
82 mice models and rat cardiomyoblasts has been shown to reduce heart size and cell
83 growth [19]. This suggests that snoRNAs may play a role in the regulation of healthy
84 cardiac development, however a regulatory role for snoRNAs in hypertrophy
85 cardiomyopathy has not yet been reported.

86 The heart is a multi-cellular organ, with intercellular communication between
87 cardiomyocytes, cardiac fibroblasts, vascular smooth muscle cells, and endothelial
88 cells necessary to maintain normal cardiac function. EV signalling is involved in
89 dynamic and two-way interactions that occur between cardiac cell populations, with it
90 is becoming clear that this signalling plays important roles in cardiovascular disease
91 [20]. Cardiomyocyte exosomes are known to have a specific proteomic signature, with
92 the presence of sarcomeric proteins (including cardiac actin) highlighting their
93 myocardial origin [21]. In health, cardiomyocytes release cardioprotective exosomes,
94 suppressing cardiac fibrosis [22]. Exercise has been shown to induce the secretion of
95 cardiac exosomes containing anti-fibrotic miRNAs in rats [23], potentially preventing

96 fibrosis accompanying exercise induced cardiac hypertrophy. During damage,
97 cardiomyocytes-derived and cardiac fibroblast-derived exosomes have been shown
98 to induce hypertrophy and fibrosis [24,25]. However, it is still unclear how sarcomeric
99 mutations change the EV profile and the potential signalling roles these play in HCM.

100 Here, we aimed to investigate whether cardiomyocytes displayed abnormal EV
101 signalling profiles during HCM. We further sort to determine whether this profile was
102 altered during periods of increase work. To achieve this we utilised an isogenic pair of
103 human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), with or
104 without the *c.ACTC1*^{G301A} mutation. This model of HCM [26] was chosen for study as
105 it had previously been shown to recapitulate many key disease phenotypes including
106 abnormal contractility, Ca²⁺ sensitivity/ handling, arrhythmogenesis, and hypertrophic
107 brain natriuretic peptide (BNP) signalling.

108 Using this isogenic model we were successfully able to isolate and perform
109 transcriptomic analysis on EVs from wild type (WT) and HCM cardiomyocytes, both at
110 baseline and increased contraction frequency. Comparisons between the HCM and
111 WT EV transcripts identified multiple functional pathways affected by the presence of
112 the disease mutation. Transcriptomic analysis of cellular mRNA showed increasing
113 contraction frequency had a large effect on metabolic gene expression, which was
114 amplified in the HCM background. Analysis of small RNA (<200 nucleotides) showed
115 the HCM hiPSC-CMs altered their loading of specific snoRNAs into EVs, and
116 responded to increased contraction frequency with a greater fold change in EV release
117 rate, and changes in small RNA profile. The snoRNAs identified in HCM EVs at
118 baseline and during increased work, may have potential signaling roles in post
119 translational modifications and alternative splicing. These may provide future be used
120 as biomarkers or therapeutic targets.

121

122 **Materials and Methods**

123 **hiPSC lines and culture**

124 Isogenic hiPSC lines with or without the *c.ACTC1*^{G301A} mutation were generated as
125 previously described in detail [26,27]. All cell culture experiments were performed in a
126 type II Biological Safety Cabinet, and cells were incubated in a humidified incubator at
127 37 °C and 5% CO₂. hiPSCs were routinely maintained in E8 medium (Life
128 Technologies) on 1:100 MT (Corning)-coated plastic ware (Nunc). Medium was
129 changed every day and cells were passaged with TrypLE (Life Technologies) every 3
130 days, and used between passages 20-30.

131

132 **hiPSC-CM differentiation and electrical stimulation**

133 Cardiomyocyte differentiation was performed by seeding hiPSCs on MT (Corning)-
134 coated plastic ware at approximately 20-40 thousand cells / cm². Fresh E8 was added
135 the next day and the pre-conditioning step of hiPSC was performed the day after, by
136 adding a MT overlay (MT diluted 1:100 in StemPro™34- Serum Free Medium (SP34,
137 Gibco, supplemented with 1ng/ml BMP4 (R&D). Approximately 16h later, medium was
138 replaced by SP34 supplemented with 8ng/ml Activin A (ActA, LifeTechnologies) and
139 10 ng/ml BMP4. 48h later, medium was changed by RPMI supplemented with B27
140 without insulin (-INS, LifeTechnologies) and KY0211 (R&D) and XAV939 (R&D), both
141 at 100 μM. These small molecules were added again two days later, in RPMI
142 supplemented with B27 with insulin (+INS, LifeTechnologies) instead. Thereafter,
143 medium was changed every 2-3 days by fresh RPMI+B27+INS until day 15 of
144 differentiation, when hiPSC-CM were replated, and kept in RPMI+B27+INS for

145 approximately 10 days when electrical stimulation and exosome isolation assays were
146 performed. Electrical pacing of the hiPSC-CMs was performed with conditions of 1 V
147 / 2 Hz, impulse duration of 2 ms, using carbon electrodes (IonOptix) for 24 hours.

148 **EV isolation and characterisation**

149 The conditioned media was centrifuged at 300xg for 10 minutes, 200xg for 10 minutes
150 and 10,000xg for 30 minutes at room temperature. The resulting supernatant was
151 concentrated by Vivaspin-20 (100 MWCO) protein concentration column (Sigma
152 Aldrich) following the manufacturers directions to a total volume of 500µl.

153 The concentrated supernatant was applied to a qEVoriginal (70nm) size exclusion
154 chromatography (SEC) column and EVs isolated according the manufacturers
155 direction. The resulting EVs were subject to Western blot analysis of tetraspanin
156 marker CD9, cytosolic marker Alix and a golgi marker negative control GM130. Briefly,
157 EVs and cells were resuspended in RIPA buffer supplemented with protease inhibitor
158 cocktail (Promega). Protein concentration was determined by the Qubit protein assay
159 (Invitrogen), and 20µg of protein per EV sample and 10µg per cell sample were
160 separated by SDS-PAGE on 10% polyacrylamide gels (BioRad). Proteins were
161 transferred to nitrocellulose membrane using the BioRad Transblot Turbo System.
162 Membranes were blocked for 1 hour in 5% (w/v) bovine serum albumin (BSA) in TBST.
163 Primary antibodies were incubated for 18 hours at 4C at a 1:1000 dilution in 3% (w/v)
164 BSA in TBST as follows: CD9 (D801A), Alix (3A49) and GM130 (D6B1) (Cell
165 Signalling). Membranes were washed three times in TBST for 10 minutes, followed by
166 a 60 minute incubation with either HRP-conjugated anti-rabbit or anti-mouse
167 secondary antibody (Cell Signaling) at room temperature. Membranes were washed

168 three times in TBST for 10 minutes, followed by incubation with Clarity ECL (BioRad)
169 and visualisation using the BioRad GelDoc Go Imaging System.

170 The size and concentration of EVs was determined by electrophoresis and Brownian
171 motion analysis using laser scattering microscopy and the resulting EVs were also
172 characterised by nanosight and transmission electron microscopy and the Nanosight
173 LM10 (Malvern Panalytical).

174 Finally, EVs were visualised by transmission electron microscopy for ultrastructural
175 characterisation. EV SEC elutes were fixed at a 1:1 ratio in 4% (v/v)
176 paraformaldehyde (PFA) in cacodylate buffer for 18 hours. The fixed EVs were seeded
177 onto 0.1% poly- L-Lysine treated carbon/formvar slot grids (EM resolution) and
178 embedded for 30 minutes at room temperature. The grids were washed twice with
179 distilled water and stained with 1% aqueous uranyl acetate and left to air dry.
180 Transmission electron microscopy was carried out at 100KEV (FEI Tecnai Biotwin-12)
181 with a side mount megaview III camera.

182

183 **RNA isolation and bioinformatics analysis**

184 RNA was extracted using the miRNeasy miRNA isolation mini kit (Qiagen). RNA was
185 subject prepared for sequencing using the NEBNext library preparation kits for the cell
186 lines and the NEBNext Low Input library preparation kit for the EV samples (New
187 England Biolabs). The RNAseq was carried out at the Barts Genome Centre (Queen
188 Mary University London) using the NextSeq 500 High Output Run (150 cycles) for all
189 samples. The sequencing data was analysed using a pipeline consisting of FastQC
190 for quality control, Skewer [28] for read trimming, HiSat2 [29] for read alignment (to
191 GRch38 build), StringTie [30] for transcript quantification and DESeq2 [31] and

192 Snakemake [32] to calculate differential expression. Pathway analysis was
193 conducted using PANTHER V16.0 Classification System (<http://www.pantherdb.org>).
194 Volcano plots generated using VolcanoR
195 (<https://huygens.science.uva.nl/VolcanoR/>).

196 **Results**

197 **Transcriptomic analysis of HCM hiPSC-CM model**

198 Skin-punch biopsies were previously obtained from a 48-year old male patient carrying
199 the c.*ACTC1*^{G301A} HCM-causing mutation and reprogrammed to hiPSCs [26]. Isogenic
200 wild-type controls were generated for these hiPSCs using a footprint-free PiggyBac
201 based CRISPR/Cas9 strategy [27]. Differentiation of these hiPSC lines to high purity
202 (>90% α -actinin⁺) cardiomyocytes (hiPSC-CMs) produced an *in-vitro* model in which
203 the mutant cells displayed characteristic HCM phenotypes when compared to the
204 isogenic control line [26,33]. A summary of these HCM phenotypes is shown in
205 Supplementary Figure 1, with HCM hiPSC-CMs displaying increased arrhythmias,
206 hypercontractility, calcium dysregulation, and inefficient energy metabolism.

207 To determine if the HCM phenotypes seen within the cellular model could be analysed
208 at an expression level, transcriptomic analysis was performed on the cellular mRNA.
209 Here we found 127 genes were dysregulated between the WT and HCM hiPSC-CMs
210 (Supplementary Figure 1B). Of these, 70% of genes were associated with controlling
211 metabolic processes, indicating the background presence of the disease mutation
212 leads to global alterations in metabolic processes, consistent with the observed energy
213 depletion phenotype. Importantly, pathway analysis confirmed the disease mutation
214 had led to disruption of known HCM pathway genes (Figure 1). Interestingly, multiple
215 genes within this pathway were shown to have isoforms with increased and decreased

216 expression, indicating the HCM mutant lines may alter the expression of alternative
217 spliced variants.

218 Together this analysis confirmed the HCM phenotypes seen within the cellular model
219 corresponded with altered transcript expression, and this could be identified through
220 bioinformatics analysis. The presence of the c.*ACTC1*^{G301A} mutation had altered
221 genes within the defined HCM pathway (KEGG pathway hsa05410), with differential
222 isoform expression, as well as the dysregulation of global metabolic processes.

223

224 **Altered EV signaling in HCM**

225 Following the confirmation of characteristic HCM cellular gene expression in the
226 mutant hiPSC-CMs, we next sort to determine whether there was altered transcript
227 cargo within extracellular vesicles (EVs) released by HCM hiPSC-CM. EVs released
228 into culture medium by hiPSC-CMs were isolated after 24 hours using size exclusion
229 chromatography (SEC). These physical properties were characterized using
230 nanoparticle tracking analysis (NTA), transmission electron microscopy and western
231 blots to determine the quality of the EV isolations and that the profiles corresponded
232 to exosomes and smaller EVs (Supplementary Figure 2).

233 The isolated EVs were processed for transcriptomic analysis (Figure 2). Biological
234 process enrichment analysis was conducted using Panther to determine the number
235 of genes per function pathway present in EVs produced from either the WT and HCM
236 hiPSC-CMs (Figure 2B). Of the functional pathways identified, catalytic activity and
237 binding were the two pathways with the highest numbers of transcripts identified.
238 Binding molecules have direct interactions with other molecules, and molecules with
239 catalytic activity are involved in enzymatic reactions following the required binding. It

240 was observed that EVs from HCM hiPSC-CMs had an increase in the number of
241 transcripts in the catalytic activity pathway, and a decrease in the number of transcripts
242 in the binding pathway compared to EVs from WT hiPSC-CMs. Detailed analysis of
243 the catalase activity pathways identified 'activity acting on a protein' as the pathway
244 with the greatest increase within the EVs from hiPSC-CMs compared to EVs WT
245 hiPSC-CMs (Figure 2C). Similarly, the detailed analysis of the binding pathways
246 identified 'activity acting on a protein' as the pathway with the greatest decrease within
247 the EVs from hiPSC-CMs compared to EVs WT hiPSC-CMs (Figure 2D). The specific
248 genes and their functions identified in these two pathways are detailed in the
249 supplementary figures 3 and 4. Notably genes associated with aspects calcium
250 signaling such as *CAMK2D*, *ATP2B4*, and *CAPN1*, and cardiac specific structural
251 genes such as *TNNT2*, *TPM1*, *TPM3*, and *TPM4*, were found within these pathways.
252 Small RNA cargo analysis also identified differential levels of snoRNAs between WT
253 and HCM hiPSC-CMs, and these were predicted to have roles in post translational
254 modifications (PTMs) and alternative splicing (Supplementary Figure 5). In total, 12
255 snoRNAs were identified including 10 SNORDs (SNORD6, SNOTRD116-23,
256 SNORD116-25, SNORD116-29, SNORD18A, SNORD42A, SNORD43, SNORD58C,
257 SNORD60, and SNORD 101) and 2 SNORAs (SNORA3B and SNORA20).

258 Following the comparison of transcripts within the HCM hiPSC-CM released EVs
259 compared to WT hiPSC-CM released EVs, we subsequently compared the transcripts
260 enriched within the EVs to their parental cell lines. Here we found that 34.9% of
261 enriched transcripts are independent of the disease mutation, whilst 65.1% are
262 mutation dependent (Figure 3A). Comparison of the enriched EV small RNA (Figure
263 3B) and enriched EV total mRNA (Figure 3C) to the corresponding parental cell line,
264 identified common transcripts between the WT and HCM EVs, including many

265 snoRNAs (such as enriched SNORD123 and depleted SNORD105) and cytochromes
266 P450 genes (CYPs), that were enriched within EVs independent of the disease
267 mutation (such as CYP11B1 and CYP17A1).

268 Pathway enrichment analysis of the mRNAs found to be mutation dependent and
269 therefore unique to either WT hiPSC-CMs or HCM hiPSC-CMs EVs identified many
270 potential functions of the enriched mRNAs within multiple pathways (Figure 3D).
271 Notably, HCM hiPSC-CM EVs show an increased presence in unique transcripts
272 associated with EGF and FGF signaling, 5-HT receptor mediated signaling,
273 angiotensin receptor signaling, beta 1 and beta 2 adrenergic signaling, and G protein
274 receptor signaling. HCM hiPSC-CM EVs also showed a reduction in the presence of
275 unique transcripts associated with cadherin signaling.

276 Together these data showed we were able to isolate and perform transcriptomic
277 analysis on the cargo of HCM and WT hiPSC-CMs EVs. Comparisons between the
278 HCM and WT EVs transcripts identified 'catalytic activity on a protein' and 'protein
279 binding' as the two functional pathways most affected by the presence of the disease
280 mutation. Comparisons of either the HCM or the WT EVs and their parental cell lines
281 showed that the majority (around two thirds) of enriched transcripts were dependent
282 of the disease mutation. Of these, pathway enrichment analysis of the mRNAs found
283 to be mutation dependent identified many signaling pathways that may be influenced
284 by the altered HCM EV transcripts. Small RNA analysis showed the HCM hiPSC-CMs
285 altered their loading of specific snoRNAs into EVs, potentially altering splicing and
286 PTMs in recipient cells.

287

288 **The altered response to increased cardiac contraction in HCM**

289 Many of the most damaging phenotypes associated with HCM only present during
290 periods of increased stress and cardiac work. We next sort to determine if we could
291 identify differential responses between the WT and HCM hiPSC-CMs when subjected
292 to an increased contraction frequency of 2 Hz, compared to the baseline 1 Hz.
293 Transcriptomic analysis of cellular mRNA showed that electrically stimulating the
294 hiPSC-CMs at 2 Hz, simulating a moderate- vigorous physical intensity, had a large
295 effect on gene expression (Figure 4A). The presence of both the mutation and 2 Hz
296 pacing appeared to create an additive effect, suggesting each factor alters the
297 abundance of independent sets of genes (Figure 4B). Of the 66 genes that
298 demonstrated altered expression as a result of 2 Hz pacing stimulation regardless of
299 the disease mutation, 40 were identified within the macromolecule metabolic process
300 (including the MAP kinases MAPK3 and MAPK12), indicating altered metabolic
301 expression as a result of the increased contraction frequency (Figure 4C). The additive
302 effect of the 2 Hz pacing in the presence of the disease mutation was observed by the
303 altered expression of 1058 genes independently of these two factors. GO enrichment
304 analysis of these genes identified many potential biological processes were altered by
305 the combined effect of these two factors (Figure 4D). These included genes associated
306 with processes such as biogenesis and morphogenesis (including contractile myosins
307 MYL2, MYH3, and MYH6), cardiac and circulatory development (including the cardiac
308 transcription factors TBX1 and TBX18), and cellular communication and signaling
309 (including BMP2 and NOTCH2).

310 Following the cellular comparisons, EVs released by both WT and HCM hiPSC-CMs
311 during 2 Hz pacing were isolated to determine if RNA changes could also be detected
312 within signaling vesicles. Interestingly, when EV release was quantified through the
313 use of NTA, it was observed that release rates differed between WT and HCM hiPSC-

314 CMs. HCM hiPSC-CMs increased the release of EVs 1.5-fold during 2 Hz stimulation,
315 compared to WT hiPSC-CM which only responded with a 1.2-fold change (Figure 5A).
316 Size analysis showed modest changes in EV size distribution as a result of 2 Hz
317 stimulation, indicating an increase in release corresponds with a decrease in EV size
318 (Figure 5B). A breakdown of this change in EV size distribution is shown in
319 Supplementary Figure 2c.

320 Small RNA analysis of EV cargo following 2 Hz stimulus did not identify any changes
321 in small RNA cargo in the EVs released from WT hiPSC-CMs when subjected to 2 Hz
322 stimulation. However, comparing these WT EVs to the 2 Hz stimulated HCM hiPSC-
323 CM EVs did identify differences in small RNA cargo (Figure 5C). SNORD96A,
324 SNORD73A, and ZN3241.1 were increased in 2 Hz stimulated HCM hiPSC-CM EVs
325 compared to 2 Hz stimulated WT hiPSC-CM EVs Furthermore, and in contrast the WT
326 line, it was shown that 2 Hz stimulation was sufficient to alter small RNA cargo in the
327 HCM hiPSC-CM EVs, including snoRNAs SNORD3A and SNORA12 (Figure 5D).
328 These small RNAs were predicted to have roles in post translational modifications and
329 alternative splicing, indicating the HCM response to increased contraction frequency
330 may further alter these processes.

331 Together, these data show that the response to an increased contraction frequency is
332 altered in HCM hiPSC-CMs. Electrical stimulation alters metabolic pathways in WT
333 hiPSC-CMs, but has an additive effect on many other biological pathways in HCM
334 hiPSC-CMs. Additionally, the cargo and release of EVs in response to the increased
335 contraction frequency was significantly altered in HCM hiPSC-CMs. HCM hiPSC-CMs
336 respond with a greater fold change in EV release rate compared to WT hiPSC-CMs
337 and with a differential profile of loaded small RNAs, with potential signaling roles in
338 PTMs and alternative splicing.

339

340 **Discussion**

341 In this study we present transcriptomic analysis of an isogenic hiPSC-CM model of
342 HCM. These data highlight some key cellular pathways altered in hypertrophic
343 cardiomyocytes, as well as altered signaling during HCM pathogenesis. It should be
344 noted that over 1400 mutations across over 25 genes have been associated with HCM
345 progression [34]. We have recently shown that different HCM-associated mutations
346 can cause differences in disease signaling pathways, and as such a ‘one-size fits all’
347 classification of HCM underestimates the complexity of the disease [6]. Future studies
348 are therefore required to determine if the pathways reported for p.ACTC1-E99K
349 mutant also contribute in alternative HCM mutants, especially those shown to have
350 phenotypic differences such as p.β-MHC-R453C [33].

351

352 **Transcriptomic analysis of HCM cardiomyocytes**

353 The variable penetrance of HCM and its manifold molecular mechanisms has hindered
354 the development of efficient treatment options [6]. To reconcile the lack of consistency
355 in the abnormalities that occur in HCM, a unifying model has been proposed in which
356 HCM dysfunction is due to an increased energy demand that results from inefficient
357 sarcomeric ATP utilization [35]. This model is known as the ‘*energy depletion model*’,
358 where the increased energy demand compromises the capacity of the cardiomyocyte
359 to maintain sufficient energy levels in subcellular compartments responsible for
360 contraction and critical homeostatic functions (such as Ca²⁺ re-uptake). Isogenic
361 hiPSC-CMs offer the only human model system that accounts for genetic background
362 differences in order to unveil deeper mechanistic insights. Our isogenic hiPSC-CM

363 model supports the theory that single sarcomeric mutations can lead to a global
364 alteration of cardiomyocyte metabolic processes. We found that of the 127 genes that
365 were dysregulated between the WT and HCM hiPSC-CMs (Figure 1A), 70% were
366 associated with controlling metabolic processes, indicating that this was the major
367 cellular pathway altered, in agreement with the energy depletion model of HCM.

368 Pathway analysis confirmed the disease mutation had led to disruption of known HCM
369 pathway genes (Figure 1B). Interestingly, we observed that for multiple genes within
370 this pathway there was a switch in isoform expression, with specific isoforms
371 increasing, whilst others within the same gene decreasing. This indicated the HCM
372 mutant lines may differentially regulate alternative splicing. The role of splicing in HCM
373 is becoming an area of increased focus [36]. Proteomic analysis of primary tissue from
374 the failing hearts of 16 HCM patients has recently revealed a common pattern of
375 altered sarcomeric proteoforms compared to heart tissues from non-failing donors
376 [37]. Despite the complexity and heterogeneity of these HCM samples, (including
377 differences in disease-causing mutations, genetic background, age, and gender), this
378 study observed consistent alterations in PTMs and changes in isoform expression
379 within the HCM samples. Additionally, HCM mutations have been shown to cause
380 aberrant splicing in an *in-vitro* model, where minigenes were transfected into HEK293
381 cells [38]. However, it has been acknowledged that this is not an ideal system, as the
382 mechanisms controlling splicing decisions are known to be influenced by chromatin
383 structure and therefore are cell-type specific. In the present study, we used an isogenic
384 hiPSC-CMs, accounting for differences in genetic backgrounds and physiological
385 relevance of cell-type specific chromatin structure. In agreement with previous studies
386 we found the presence of a sarcomeric HCM mutation altered splicing in multiple
387 pathways, including the known HCM pathway.

389 **Transcriptomic analysis of HCM EVs**

390 Although our understanding of the molecular mechanisms the underlie primary HCM
391 phenotypes is advancing rapidly, the link between sarcomeric gene defects and
392 'extended' HCM phenotypes (such as myocardial disarray, interstitial fibrosis, mitral
393 valve abnormalities, and microvascular remodeling), remains less defined [39]. The
394 presentation of these phenotypes indicates the involvement of other cell lineages and
395 therefore a role in aberrant signaling between mutant cardiomyocytes and healthy
396 neighboring population. The regulatory roles of cardiomyocyte-derived EVs through
397 signaling to neighboring populations such as fibroblasts [40] and endothelial cells [41],
398 in addition to ability to act upon themselves [42], is becoming clearer.

399 Here we show that cardiomyocytes alter their EV cargo when HCM sarcomeric
400 mutations are present. Comparisons of mRNA transcripts within EVs released from
401 HCM and WT identified 'catalytic activity on a protein' and 'protein binding' as the two
402 functional pathways most affected by the presence of the disease mutation. The
403 presence of calcium signaling genes, such as *CAMK2D*, *ATP2B4*, and *CAPN1*, is of
404 particular note as abnormal calcium handling is common throughout HCM mutations.
405 However, evidence suggests different HCM mutations lead to mutation-specific
406 alterations in calcium handling [43], and as such the differences seen in our cells and
407 their EV cargo may be specific to the *c.ACTC1^{G301A}* mutation.

408 Comparisons of either the HCM or the WT EVs and their parental cell lines showed
409 that 65.1% of enriched transcripts are mutation dependent, showing the extent of the
410 mutation effect on EV signaling. Pathway enrichment analysis of the EV mRNAs found
411 to be mutation dependent, identified many potential signaling functions, with the

412 greatest change observed in the cadherin signaling pathway (Figure 3D). Cadherins
413 have previously been implicated in the regulation of hypertrophy with the modulation
414 of cadherin-mediated adhesion inducing hypertrophy cardiomyocytes and linked to
415 early onset HCM [44]. Additionally, a study that profiled the mRNA cargo of serum-
416 derived exosomes from patients with heart disease also identified altered cadherin
417 signaling [45]. This same study also identified the presence of over 200 differentially
418 expressed lncRNAs within the serum-derived heart disease exosomes, with functions
419 unknown.

420 One finding of particular note, was the presence of differential levels of snoRNAs
421 between WT and HCM hiPSC-CM EVs (Supplementary Figure 5). As previously
422 stated, studies have identified snoRNA involvement in cardiometabolic disease,
423 including in lipid metabolism [14], diabetes mellitus [15], and doxorubicin cardiotoxicity
424 [16]. Interestingly, snoRNAs have also been shown to regulate splicing in normal
425 cardiac development [17] and can alter splicing in some congenital heart diseases
426 (tetralogy of Fallot) [18]. The snoRNAs identified in the present study as differentially
427 regulated in HCM EVs were predicted to have roles in both PTMs and alternative
428 splicing, offering a potential mechanistic link between aberrant signaling and the
429 presentation of these processes in HCM.

430 Notably, multiple paralogues of SNORD116 were detected with increased abundance
431 (>2fc) in HCM hiPSC-CM EVs compared to WT hiPSC-CM EVs (Supplementary
432 Figure 5). The SNORD116 locus is encoded on chromosome 15 as a ~57,000 bp
433 cluster consisting of 30 paralogues of SNORD116, each roughly 100 bp in length [46].
434 Although SNORD116 expression is detected at highest levels in brain tissue, it is also
435 found at lower levels in other tissues including heart, ovary, prostate, thyroid and
436 kidney [47,48]. SNORD116 is a disease-associated snoRNA, with all deletions and

437 mutations reported for Prader-Willi syndrome (PWS) resulting in a loss of expression
438 of this cluster [49]. PWS is a complex multisystem disorder that is the most common
439 cause of life-threatening obesity in humans [50]. A link between PWS and an
440 increased prevalence of congenital defects has been established, with congenital
441 heart defects being the most common of these [51]. This raises the interesting
442 possibility that SNORD116 may contribute to congenital heart disease and exploring
443 its role within HCM EVs merits further investigation.

444 Of the 10 snoRNAs with increased abundance in HCM hiPSC-CM EVs, one other
445 apart from SNORD166 has previously been reported in human disease. SNORD43 is
446 one of four snoRNAs that has been reported as dysregulated in cancer [52]. Its
447 potential roles in directing 2'-O-ribose methylation in ribosome biogenesis may
448 contribute to disease signalling [13], although more investigation is needed to
449 determine if this is contributing to the HCM phenotype. Although there is evidence that
450 2'-O-ribose methylation may contribute to heart disease, with a specific set of snoRNAs
451 found at the 14q32 locus, guiding these RNA modifications in associated heart failure
452 [53].

453

454 **Transcriptomic changes in HCM during increased work**

455 Sudden death remains the most visible and damaging complication associated with
456 HCM [54]. Intense exercise represents a major risk factor in sudden death and as
457 such, young athletes with HCM are often prevented from strenuous competition [55].
458 In the present study, we subjected HCM cardiomyocytes to 2 Hz electrical stimulation
459 to model an increased contraction workload that commonly precedes sudden death.
460 We observed that 2 Hz pacing stimulation altered the expression of 66 genes

461 regardless of the disease mutation, with 40 of these identified within the
462 macromolecule metabolic process (Figure 4C). The altered metabolic expression in
463 response suggested that our model was successful in subjecting the cardiomyocytes
464 to increased energy demand and they were responding accordingly. Interestingly, the
465 presence of the disease mutation has a substantial additive effect, altering the
466 expression of a further 1058 genes. This additive effect may be responsible for the
467 abnormal HCM response to an energy depleted state. HCM cardiomyocytes
468 responded to the increased workload with altered gene expression profile in processes
469 involved in biogenesis and morphogenesis, cardiac and circulatory development, and
470 cellular communication and signaling.

471 The altered cellular communication and signaling gene expression observed,
472 translated through to differences in EV signaling. Whereas WT hiPSC-CMs increased
473 EV release rate by 1.2 fold during 2 Hz stimulation, HCM hiPSC-CMs had a greater
474 response of 1.5 fold ($P < 0.0001$), suggesting a role for exosome signaling in the HCM
475 energy depletion response. Analysis of these 2 Hz stimulated exosomes revealed a
476 notable difference in small RNA cargo during this HCM signaling response. Following
477 2 Hz stimulation, the small RNA cargo only changed within HCM hiPSC-CMs EVs and
478 not WT EVs. Although the identities of these snoRNAs differed to those identified at
479 baseline contraction frequency, the functional roles were similarly predicted to regulate
480 PTMs and alternative splicing. As such, the change in EV snoRNA signaling may
481 indicate a role for PTMs and alternative splicing in HCM signaling at baseline and
482 during a further energy depletion state.

483 In conclusion, our data support previous findings that HCM mutations disrupt normal
484 metabolic activities, and this worsens disproportionately in response to increased
485 workload. Additionally, we found sarcomeric mutations caused differential splicing

486 within known HCM pathways. Intriguingly, we observed differences in snoRNA cargo
487 within HCM released EV signals, which specifically altered in HCM mutant and not WT
488 cardiomyocyte EVs, when workload was increased. These snoRNAs were predicted
489 to regulate PTMs and splicing, pathways previously suggested to be common
490 throughout HCM mutants. Therefore further investigation is warranted to determine
491 whether snoRNAs play a common role in the convergence of HCM phenotypes. The
492 therapeutic potential of RNA-targeting in cardiovascular disease is currently being
493 explored in multiple clinical trials, including both antisense oligonucleotides (ASO) and
494 small interfering RNAs (siRNAs) therapies [56]. As such, the identification of snoRNAs
495 as targets in the treatment common HCM phenotypes opens a large field of potential
496 future therapies.

497

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502

503 **Author Disclosure Statement**

504 Authors have no competing financial interests.

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