

Copy number variants implicate cardiac function and development pathways in earthquake-induced stress cardiomyopathy.

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1 **Abstract**

2 The pathophysiology of stress cardiomyopathy (SCM), also known as takotsubo
3 syndrome, is poorly understood. SCM usually occurs sporadically, often in
4 association with a stressful event, but clusters of cases are reported after major
5 natural disasters. There is some evidence that this is a familial condition. We
6 have examined three possible models for an underlying genetic predisposition to
7 SCM. Our primary study cohort consists of 28 women who suffered SCM as a
8 result of two devastating earthquakes that struck the city of Christchurch, New
9 Zealand, in 2010 and 2011. To seek possible underlying genetic factors we
10 carried out exome analysis, Cardio-MetaboChip genotyping array analysis and
11 array comparative genomic hybridization on these subjects. The most striking
12 finding from these analyses was the observation of a markedly elevated rate of
13 rare, heterogeneous copy number variants (CNV) of uncertain clinical
14 significance (in 12/28 subjects). Several of these CNVs clearly impacted on
15 genes of cardiac relevance including *RBFOX1*, *GPC5*, *KCNRG*, *CHODL*, and
16 *GPBP1L1*. There is no physical overlap between the CNVs, and the genes they
17 impact do not fall into a clear pathophysiological pathway. However, the
18 recognition that SCM cases display a high rate of unusual CNV, and that SCM
19 predisposition may therefore be associated with these CNVs, offers a novel
20 perspective and a new approach by which to understand this enigmatic
21 condition.

22

23 **Introduction**

24 Stress cardiomyopathy (SCM), also known as "broken heart syndrome" or
25 takotsubo syndrome,^{1, 2} is a condition that captures widespread public interest.

26 The cardiomyopathy is distinctive and the precipitating emotional event is
 27 typically clearly defined, however the mechanism for the cardiomyopathy and
 28 links between the psychological event and the physical illness are not
 29 understood.
 30
 31 Sporadic cases of SCM are estimated to account for 1-5% of acute coronary
 32 syndrome presentations.³⁻⁵ Predominantly the condition occurs in post-
 33 menopausal women,^{6; 7} and because of this, 5-10% of female presentations with
 34 suspected acute coronary syndrome are attributed to SCM.⁸⁻¹¹ Although SCM
 35 can be fatal, the symptoms are commonly transient and patients generally have
 36 a good prognosis and recover well over a period of days to weeks.¹² In classic
 37 descriptions the cardiomyopathy has a typical pattern but a number of variations
 38 are now widely recognised and it is increasingly apparent that cases can be
 39 quite heterogeneous.¹³
 40
 41 SCM occurring in clusters around the time of major disasters such as
 42 earthquakes, floods and bushfires is also well recognised.^{6; 14-16} Due to the large
 43 impact these events have upon hospital resources and medical infrastructure, it
 44 is rare for such clusters of SCM to be studied in any depth. This was made clear
 45 in reports from the Great East Japan Earthquake.¹⁷ In the Canterbury (New
 46 Zealand) earthquake sequence of 2010 and 2011 the two main events
 47 precipitated large case clusters of SCM.¹⁷⁻²² Unusually for a major natural
 48 disaster, the tertiary hospital in Christchurch continued to function, allowing the
 49 collection of a relatively large homogenous cohort of cases which have been
 50 followed over several years.¹⁸⁻²³ Most research around this disorder has focused

51 on sporadic SCM associated with heterogenous triggers.^{4; 24-26} Although the
52 presentation of earthquake-associated SCM (EqSCM) appears to be similar to
53 that of sporadic cases, a key difference is the homogenous nature of the trigger.

54

55 Various mechanisms have been postulated for takotsubo cardiomyopathy,
56 including that the syndrome arises from stunning of the heart muscle
57 (myocardium) as a result of either ischemia from spasm of the coronary arteries,
58 or from the direct effect of catecholamines (dopamine, adrenaline or
59 noradrenaline) on cardiac myocytes.^{4; 24; 27; 28} Despite suggestive

60 pathophysiological observations and theories, most authors conclude that the
61 aetiology of SCM is poorly understood, and we do not yet have satisfactory
62 explanations for the origins of this condition.^{27; 29-32 26; 32} Some retrospective
63 case series have suggested that the incidence of SCM is increased in patients
64 with anxiety conditions, but in our studies we did not find any correlation with
65 psychiatric or anxiety disorders.^{19; 23}

66

67 Amongst the models that may be proposed for SCM aetiology, it is worth
68 considering the possible contribution of genetic factors. Many forms of
69 cardiomyopathy have genetic origins.^{33; 34} Hypertrophic cardiomyopathy is the
70 most common form of familial heart disease and a leading cause of sudden
71 cardiac death. It is inherited in an autosomal dominant Mendelian manner with
72 variable expressivity and age-related penetrance.³³ These cardiomyopathies
73 show considerable genetic heterogeneity, with cases now attributed to some
74 1400 mutations in 11 genes, all of which contribute to cardiac sarcomere
75 function. Familial dilated cardiomyopathy is also frequently attributable to an

76 underlying genetic predisposition and at least 50 genes have now been
77 implicated, with most eliciting disease as dominant mutations.³⁴
78
79 Evidence for genetic contributions to SCM are not as strong as for other
80 cardiomyopathies. However, there are several examples of familial occurrence
81 of SCM involving siblings³⁵⁻³⁷ or mother-daughter pairs,³⁸⁻⁴² and a large Swedish
82 study of SCM identified three families in which several close relatives developed
83 the condition.⁴³ The overall rarity of SCM would suggest that these familial
84 clusters are significant, and it is quite possible that more overt familial
85 relationships in this disorder are obscured by the simultaneous requirement for
86 two key circumstances (in most cases): post-menopausal status and
87 environmental exposure to a sudden major stressful event. Occasional cases of
88 SCM occur in younger women or males, and a proportion of patients report no
89 preceding stressor,⁴⁴ suggesting that an intrinsic pathogenic mechanism is
90 involved. The recurrence of SCM in some patients, including one Christchurch
91 EqSCM case,²² also implies a biological vulnerability.
92
93 These observations have prompted consideration of genetic susceptibility to this
94 condition.^{39; 42; 45; 46} Until recently, genetic studies were restricted to candidate
95 gene analysis in case series of sporadic SCM patients,⁴⁷⁻⁵¹ but these have
96 yielded mainly negative findings. One candidate gene study reported a
97 significant difference in the frequency of a *GRK5* polymorphism in cases,⁵² but
98 this has not been replicated and past history of single gene association studies
99 suggests it is unlikely to be meaningful.⁵³ More recently, another candidate gene
100 study has implicated estrogen receptor genes as potential risk factors for

101 SCM.⁵⁴ In an effort to capture genome-wide data, exome sequencing⁵⁵⁻⁵⁸ was
 102 recently applied to a sample of sporadic SCM cases.⁴² Although this analysis did
 103 not reveal any difference in allele frequency or burden between SCM cases and
 104 population controls (28 adults with normal echocardiograms), it was noted that
 105 two thirds of the cases carried a rare deleterious variant within at least one gene
 106 of a large set of adrenergic pathway genes, and 11 genes harboured a variant in
 107 two or more cases. However, the significance of these rare variants remains
 108 unclear.

109

110 In this study, we set out to explore the role of genetic factors in predisposition to
 111 EqSCM. We specifically tested three discrete hypotheses for potential genetic
 112 contributions to risk of SCM: (i) an essentially Mendelian hypothesis that rare
 113 genetic variants in one or a few key genes cause predisposition, which was
 114 tested by whole exome sequencing (WES); (ii) that SCM was a complex
 115 disorder with genetic contributions from multiple common variants, which was
 116 tested using the Cardio-MetaboChip genotyping array; and (iii) that rare copy
 117 number variants (CNV) impacting on relevant genes contribute to risk, which
 118 was tested by array comparative genomic hybridization (aCGH).

119

120 **Material and Methods**

121 **Cases**

122 The September 2010 earthquake of magnitude 7.1 on the Richter scale (Mw
 123 7.1) in Christchurch (New Zealand) triggered eight cases of EqSCM, and the
 124 shallow highly destructive quake (Mw 6.3) that followed in February 2011
 125 triggered 21 cases over four days. One woman presented after both quakes²²,

126 and one was in hospital during the initial quake. Enrolment of this latter
127 participant was delayed, and her sample was available for aCGH analysis
128 (n=28) but not for Cardio-Metabolome analysis (n=27). The steps leading to
129 recruitment of our EqSCM cohort are detailed elsewhere^{20; 21}, but briefly, our
130 study commenced the day of the first earthquake with the creation of a register
131 of prospectively identified earthquake stress cardiomyopathy cases. As our
132 hospital was still functioning we could build a cohort with first-world data from
133 complete single centre capture. After the second earthquake the study was
134 extended.²⁰⁻²²

135

136 Inclusion criteria: i) Meeting modified Mayo criteria for stress cardiomyopathy
137 and admitted to Christchurch Hospital within one week of either the September
138 2010 or February 2011 earthquake; ii) age over 18; iii) informed consent given.
139 Exclusion criteria: i) unable to understand English sufficiently to be able to
140 complete questionnaires.

141

142 All participants were recruited with informed consent, including discussion of the
143 possibility of incidental findings from genetic analyses, and return of such
144 findings after consultation with a medical geneticist. The Southern Health and
145 Disability Ethics Committee (New Zealand) approved this study.

146

147 *DNA extraction*

148 Peripheral blood samples were obtained from consenting participants. Genomic
149 DNA was extracted from 3 mL peripheral blood using NucleoMag extraction kits
150 (Machery-Nagel GmbH, Düren, Germany) on a KingFisher™ Flex Magnetic

151 liquid-handling robot (Thermo Fisher Scientific, Inc, Waltham, MA). DNA was
152 quantified by analysis with the NanodropTM (ThermoFisher), and, where
153 appropriate, the Tapestation 4200 system (Agilent Technologies).

154

155 *Exome analysis*

156 We applied WES to a subset (24 of 28) EqSCM cases. The exome capture and
157 sequencing was carried out in two batches of 12, during 2012-13 (New Zealand
158 Genomics Limited, Dunedin, New Zealand). DNA was processed with Illumina
159 TruSeq sample preparation and exome enrichment kits (which capture ~62Mb of
160 genomic DNA), and sequencing (100bp paired-end reads) was carried out on an
161 Illumina HiSeq2000 system. Good quality sequence was obtained across all
162 exomes, with very few unassigned reads, and greater than 20 million sequence
163 reads per sample at mean quality scores (Phred) of Q37. Raw read data were
164 aligned to the GRCh37 human reference genome using the Burrows-Wheeler
165 Aligner (BWA),⁵⁹ and processed through the Broad GATK pipeline.⁶⁰ The
166 alignment process included removal of reads from duplicate fragments,
167 realignment around known indels, and recalibration of all base quality scores.
168 Joint variant calling was performed with GATK's HaplotypeCaller. This included
169 *de novo* assembly at each potential variant locus. Variants were annotated and
170 analysed using Ingenuity Variant Analysis (IVA) software (QIAGEN, Redwood
171 City, CA, USA), MutationTaster2,⁶¹ SnpEff,⁶² SeattleSeq annotation server,⁵⁵
172 and Galaxy (via usegalaxy.org).⁶³ Allele frequencies and additional annotations
173 were drawn from 1000 Genomes project,⁶⁴ NHLBI GO Exome Sequencing
174 Project (ESP), Seattle, WA (URL: <http://evs.gs.washington.edu/EVS/>), ClinVar,⁶⁵
175 and Exome Aggregation Consortium (ExAC), Cambridge, MA (URL:

176 <http://exac.broadinstitute.org>). Promising gene variants were inspected by
177 Sanger sequence analysis on the appropriate genomic DNA samples.

178

179 *Cardio-MetaboChip Analysis*

180 Three groups were genotyped using the Illumina Cardio-MetaboChip: 27 out of
181 28 female Christchurch EqSCM cases, 133 heart-healthy controls from the
182 Canterbury Healthy Volunteers Study (HVOLs, 54 F / 79 M),⁶⁶ and 157 patients
183 recruited for an ongoing study of premature coronary heart disease and
184 consented for genotyping (CHD, 64 F / 93 M). DNA samples were run on the
185 Cardio-MetaboChip and scanned on the Illumina® iScan platform by
186 AgResearch Limited (Invermay, New Zealand).

187

188 Quality control with summary analysis of allele and genotype frequencies,
189 Hardy-Weinberg equilibrium tests, and missing genotype rates were performed
190 with PLINK version 1.07 software.⁶⁷ SNPs with a minor allele frequency of <0.05
191 and those that failed the Hardy-Weinberg equilibrium test ($p < 0.001$) were
192 excluded from the analysis, leaving 141,095 SNPs in the analysis (Table 1).

193 Three samples from the CHD Study were also removed after analysis of
194 relatedness. Principal Component Analysis (PCA, Eigenstrat 4.2) was
195 performed on an independent subset of almost 50,000 SNPs; the first principal
196 component explained 6% of the variation, subsequent components all less than
197 0.5%, and matched self-reported ethnicity (visual inspection). Hence the first
198 principal component was subsequently included as a factor in the logistic
199 regression. Logistic regression was performed to evaluate differences in SNP
200 minor allele frequencies between groups, adjusted for ethnicity and gender,

201 using an additive genetic model (R 3.01 software⁶⁸). P values were adjusted for
202 false discovery rate (FDR) using the Benjamini Yekutieli method.⁶⁹ Pathway
203 analysis was performed for the leading 100 SNPs in each pairwise group
204 comparison, using MetaCore from GeneGo (Thomson Reuters).

205

206 *CNV detection and analysis*

207 Array comparative genomic hybridisation (aCGH) was undertaken on 28
208 EqSCM cases to examine structural variants in the cohort. For this analysis, we
209 used either the Nimblegen 135k oligo array (CGX12) (Roche NimbleGen Inc,
210 Madison, WI, USA), capable of genome-wide screening for CNV to a resolution
211 of 10kb in well-categorised pathogenic genomic regions, and 50kb elsewhere or
212 the Agilent 180k HD oligo array (Sureprint G3 Human 4x180k) (Agilent
213 Technologies, Santa Clara, CA, USA), which has a similar resolution.

214

215 Pooled reference DNA samples (catalogue numbers G147A and G152A) were
216 purchased from Promega (Madison, WI, USA). EqSCM case and reference
217 DNA samples (0.5-1 µg each) were labelled with Cy3 and Cy5 dyes
218 respectively, purified, hybridized, and washed according to Nimblegen and
219 Agilent protocols. Microarrays were scanned on a GenePix 4000B laser scanner
220 (Axon Instruments, CA, USA) or a G2600D Agilent SureScan microarray
221 scanner (Agilent Technologies). Data was processed using Nimblescan (Roche
222 Nimblegen Inc) or Cytogenomics software (Agilent Technologies) with the
223 default algorithms and analysis settings, but with a 5 probe minimum calling
224 threshold. All arrays passed QC metrics for derivative log ratio spread (DLRS)
225 values of <0.2. CNV data was visualised and interpreted using Genoglyphix

226 software (Perkin Elmer) and NCBI genome browser software (genome build
227 hg19 (GRCh37)). The EqSCM aCGH data were assessed against many CNV
228 databases including Genoglyphix Chromosome Aberration Database (containing
229 over 14,000 validated variants from 50,000 samples) (Perkin Elmer, Waltham,
230 MA, USA),⁷⁰ DECIPHER,⁷¹ and the Database of Genomic Variation (DGV,
231 containing CNV data from over 35,000 unaffected individuals).⁷² CNVs were
232 classified as thought to be benign (TBB), uncertain clinical significance (UCS),
233 or clinically significant (CS) using an evidence-based approach⁷³⁻⁷⁶ which
234 included database comparisons (frequency in cases/controls and relation to
235 phenotype), gene content, gene function and dosage sensitivity. A broad
236 summary of our CNV interpretation algorithm is depicted in Figure S1. Rare
237 CNVs are defined as those that occur at a frequency of $\leq 1\%$. We classified our
238 rare CNV frequency using larger DGV studies containing >1000 individuals.⁷⁷⁻⁸¹
239

240 **Results**

241 *Exome Analysis*

242 To test the potential for an essentially Mendelian predisposition to EqSCM, WES
243 was carried out on 24 of the 28 Christchurch EqSCM cases. Several
244 approaches to analysis of the identified variants were used, all of them
245 hypothesising the involvement of gene variants with a low population minor
246 allele frequency (MAF), that were over-represented in the EqSCM cohort. We
247 carried out various iterations of filtering using variant allele frequency data
248 derived from large population databases (1000 Genomes; NHBLI Exome
249 Sequencing Project), followed by careful manual inspection of remaining
250 variants. For example, excluding all variants present in these databases with an

251 allele frequency > 3%, and selecting for any present in at least 4/24 EqSCM
252 exomes, identified variants in 131 genes, none of which proved to be convincing
253 on closer analysis. We also carried out ranking of gene variants by predicted
254 functional impact using various approaches.^{61; 62; 82} Once again, none of the
255 variants identified in these analyses proved to be significantly enriched amongst
256 our EqSCM exomes.

257

258 Mitochondrial DNA reads can be recovered from exome data⁸³. We carried out
259 manual inspection of BAM files of mitochondrial DNA for our exome data
260 compared with non-disease control exomes⁸⁴. No unusual variants were
261 detected in mitochondrial sequences of the EqSCM samples.

262

263 Finally, the 11 genes listed in Figure 2 of Goodloe et al (2014),⁴² as well as a
264 gene recently proposed to play a role in SCM, *BAG3*,⁴⁶ were carefully examined
265 for presence of any rare variants in the EqSCM dataset. None of the previously
266 identified variants,^{42; 46} and no other convincing rare variants in these genes,
267 were detected.

268

269 *Cardio-MetaboChip Analysis*

270 To test the possibility of a more complex, polygenetic basis to SCM risk,
271 involving multiple variants of small effect size, Cardio-MetaboChip analysis was
272 carried out. The Cardio-MetaboChip data for 27 EqSCM cases and 133 heart-
273 healthy controls were compared by logistic regression (adjusted for ethnicity and
274 gender, additive genetic model), first performed for pairwise comparisons across
275 groups. No SNPs reached statistical significance of <0.05 after adjusting for

276 false discovery rate (FDR) when comparing either the EqSCM and HVOLs, or
 277 the EqSCM and CHD samples. To investigate whether the top 100 of these
 278 SNPs mapped to gene pathways that might assist in understanding potential
 279 disease mechanisms underlying SCM, pathway analysis of the leading 100
 280 SNPs in the EqSCM versus HVOLs pairwise comparison was performed in
 281 MetaCore. Disease Biomarker Pathway analysis identified Myocardial Ischemia
 282 as the third most enriched pathway (FDR-adjusted $p=1.3e^{-2}$), featuring 11 SNP
 283 loci on our list out of 886 pathway objects, including annexin V, ANRIL,
 284 COL4A1, dynein, HXK4, nectin-2, PPAR-gamma, prolidase, Tcf(Lef), UGT, and
 285 VEGFR-2 .

286

287 *aCGH Analysis*

288 To test for potential involvement of CNVs in SCM, we applied aCGH to all
 289 cases. Of the 28 EqSCM cases examined by aCGH, twelve (42%) showed
 290 evidence of large, rare heterozygous CNVs classified as being of unclear clinical
 291 significance (Table 2), meaning that insufficient evidence is available for
 292 unequivocal determination of clinical significance.⁷³ Of these CNVs, seven were
 293 deletions and six were duplications. All of the CNVs were different, and there
 294 was no physical overlap between the various CNVs. Each of these rare CNVs
 295 encompasses one or more genes, or their immediate upstream regulatory
 296 regions, and many of the genes included within the CNVs have functions of
 297 cardiac relevance. A full list of all CNVs detected in the cohort is presented in
 298 Table S1.

299

Three cases (EqSCM 01, 06 and 19) harboured deletions very likely to impact genes of high relevance to cardiomyopathy or cardiac function. In EqSCM 01, intragenic deletion of *RBFOX1* results in a single copy loss of one exon used by the majority of transcripts predicted for the gene (Figure 1). This exon contains the start methionine for the RBFOX1 protein, meaning the gene is most likely rendered non-functional. *RBFOX1* is an important RNA-binding protein mediating the incorporation of microexons into many transcripts associated with neurological patterning and tissue development,^{85; 86} particularly in the brain, heart and muscles. Intragenic deletions in *RBFOX1* have been observed in a range of conditions, including occasional cases with cardiac defects.^{87; 88; 89} Furthermore, RBFOX1-mediated RNA splicing was also recently shown to be an important regulator of cardiac hypertrophy and heart failure⁹⁰.

In the second case (EqSCM 06, Figure. 2), a heterozygous deletion encompassed exon 2 and the majority of intron 2 of the *Glypican 5 (GPC5)* locus. *GPC5* encodes a cell surface proteoglycan, which binds to the outer surface of the plasma membrane in the cardiovascular system and displays diverse functions including blood vessel formation after ischemic injury and proliferation of smooth muscle cells during atherogenesis.⁹¹ *GPC5* was also implicated by GWAS as a protective locus for sudden cardiac arrest,⁹² and other glypicans (GPC3, 4 and 6) have been associated with cardiac dysfunction.⁹³ This case (EqSCM 06) also harbours a duplication on 4q21.21 involving the *ANXA3* gene, which encodes a member of the annexin family, annexin A3. Members of this calcium-dependent phospholipid-binding protein family have a range of functions in the regulation of cellular growth and signal transduction

325 pathways. Annexin A6 for example is the most abundant annexin expressed in
326 the heart and its overexpression in mice has been shown to cause physiological
327 alterations in contractility leading to dilated cardiomyopathy, while Annexin A6
328 knockout has been found to induce faster changes in Ca^{2+} transience and
329 increased contractility.^{94; 95} Alterations in expression and activity of annexins A5
330 and A7 have also been found to be associated with regulation of Ca^{2+} handling
331 in the heart.⁹⁶ The function of annexin A3 is not fully understood, however it has
332 been shown to play a role in endothelial migration and vascular development.⁹⁷
333

334 The third case (EqSCM 19), contained a deletion at chr13q14.3. This region
335 harbours at least 10 genes (*DLEU2*, *TRIM13*, *KCNRG*, *MIR16-1*, *MIR15A*,
336 *DLEU1*, *DLEU1AS-1*, *ST13P4*, *DLEU7AS-1*, *DLEU7*, and *RNASEH2B-AS1*),
337 including several non-coding RNAs (DLEU genes and micro-RNA genes) and a
338 gene (*KCNRG*) encoding a protein involved in the regulation of voltage-gated
339 potassium channel activity. The micro-RNA genes mir-16-1 and mir-15a in this
340 interval have been implicated in a range of cardiovascular phenotypes, including
341 a role for mir-15a in postnatal mitotic arrest of cardiomyocytes.⁹⁸⁻¹⁰⁰ Two further
342 chromosome 13 duplicated CNVs of approximately 150kb were classified as
343 uncertain significance - one involving LINC00346 and ANKRD10 and the other
344 containing ENOX1, postulated to affect vascular development based on
345 zebrafish expression patterns¹⁰¹ (Table 2).

346
347 Beyond these three cases, cardiac or relevant neurological impacts appeared
348 likely for many of the other rare CNVs identified in EqSCM cases (Table 2,
349 Figure 2, Table S1), several of which are discussed below.

350

351 A 96kb heterozygous deletion in EqSCM 03 disrupts all predicted transcripts of
352 the chondrolectin gene (*CHODL*), a membrane bound C-type lectin involved in
353 muscle organ development, whose protein product is detected in heart and
354 skeletal muscle by immunohistochemistry.¹⁰² In addition to this CNV, this patient
355 carries a 1.94Mb duplication at 22q11.25, a locus containing 45 genes or
356 miRNAs, associated with learning difficulties.^{103; 104} This individual, who
357 exhibited a degree of cognitive impairment, had consented for clinically relevant
358 findings to be forwarded to their General Practice clinician, who subsequently
359 recommended genetic counselling for this individual.

360

361 A 455kb duplication within EqSCM 04 at 1p34.1 affects the genes *GPBP1L1*,
362 *TMEM69*, *IPP*, *MAST2*, and *PIK3R3*. Smaller rare duplications in this region
363 have been reported by the DGV database¹⁰⁵ but none span the genes within this
364 CNV. *GPBP1L1* is widely expressed in many tissues, including heart muscle¹⁰⁶
365 and predicted to be involved in transcriptional regulation. *TMEM69*, a gene of
366 unknown function, is most strongly expressed in heart tissue.¹⁰⁶ *IPP* is a
367 transcription factor with a 50 amino acid Kelch repeat known to interact with
368 actin, while *MAST2* contains a PDZ domain and is another gene highly
369 expressed in heart and skeletal muscle.¹⁰⁶ The protein product of *PIK3R3*,
370 phosphoinositide-3-kinase regulatory subunit 3, acts downstream of G-protein-
371 coupled receptors in cardiac function,¹⁰⁷ and is also a target for isoproterenol,
372 which can trigger SCM-like conditions in humans and rodents.^{12; 108-110}

373

374 Duplication of a long non-coding RNA (LOC101928358), the 3' segment of
 375 *COL4A5* and the entire *IRS4* gene at Xq22.3 (9 probes, 113kb) was identified
 376 within case EqSCM 05. *IRS4* is an insulin receptor molecule expressed in heart
 377 and skeletal muscle cells¹¹¹ and other tissues such as brain, kidney and liver.¹¹²
 378 Duplication of *IRS4* may be of functional significance, although copy number
 379 increases on the X chromosome of females may be counteracted to a degree by
 380 random X inactivation. Of note is a Genoglyphix Chromosome Aberration
 381 Database (GCAD⁷⁰) case 52414 with a phenotype of low muscle tone, which
 382 has an identical duplication at this locus (as well as a 1p33 deletion). With
 383 regard to *IRS4*, Schreyer et al. (2003)¹¹¹ found a more restricted tissue
 384 distribution than *IRS1* and *IRS2*, in primary human skeletal muscle cells and rat
 385 cardiac muscle and isolated cardiomyocytes. Although *IRS4* protein function is
 386 still relatively unknown, the role of IRS proteins in general, acting as mediators
 387 of intracellular signalling from insulin and insulin-like growth factor 1 receptors,
 388 implicates *IRS4* in cell growth and survival.¹¹³ It is interesting to note that PI 3-
 389 kinase (PI3K) signalling in HEH293T cells depends on *IRS4*, and that the IRS
 390 proteins relay signals from receptor tyrosine kinases to downstream
 391 components of signalling pathways,¹¹¹ which we note is a connection with the
 392 *PIK3R3* gene duplicated in one of our other cases, EqSCM 04.
 393
 394 EqSCM 10 harboured a 130kb duplication of *NLRP7*, *NLRP2*, *GP6* and *RDH13*
 395 at 19q13.42. One similar DGV duplication has been seen in this area
 396 (nsv1062047¹⁰⁵), but otherwise duplicated CNVs are generally much smaller
 397 and rare. *NLRP2* and *NLRP7* are genes that encode members of the NACHT,
 398 leucine rich repeat, and PYD containing (NLRP) protein family. These proteins

are implicated in the activation of pro-inflammatory caspases. Recessive mutations in *NLRP2/7* in humans are associated with reproductive disorders.¹¹⁴ Another gene in this duplicated cluster associated with disease is *GP6*, a platelet membrane glycoprotein, involved in collagen-induced platelet aggregation and thrombus formation, which is expressed at high levels in heart, kidney and whole blood.¹⁰⁶ A *GP6* SNP (c.13254TC) has been implicated in recurrent cardiovascular events and mortality.¹¹⁵ Another study involving this SNP,¹¹⁶ found that hormone replacement therapy (HT) reduced the hazard ratio (HR) of CHD events in patients with the *GP6* 13254TT genotype by 17% but increased the HR in patients with the TC+CC genotypes by 35% (adjusted interaction $P < 0.001$). The authors found that in postmenopausal women with established CHD, the *GP6* polymorphism, and another in *GP1B*, were predictors of CHD events and significantly modified the effects of HT on CHD risk.¹¹⁶

Duplication of *PPL2*, *YPEL1* and *MAPK1* on chromosome 22q11.21 (180kb) observed in EqsCM 11, does not appear in the DGV catalogue of CNVs in healthy individuals, and the consequences of overexpression of these genes, miRNAs or regulatory sequences are unknown. One of the affected genes, *MAPK1* (previously named *ERK* or *ERK2*), may constitute a link to another kinase intracellular signalling pathway – the RAF-MEK-ERK kinase cascade, which in mice and human has an established role in the induction of cardiac tissue hypertrophy.¹¹⁷ Although not the kind of left ventricular enlargement seen in SCM, subtle copy number variation at *MAPK1* may influence signalling through this pathway. Another duplication in EqsCM 11 involving the 3' half of *TSPAN7*, a member of the tetraspanin protein superfamily (Xp11.4) was noted

424 as rare, and a similar (though larger) duplication was recently observed in a
425 patient with Rolandic epilepsy.¹¹⁸

426

427 An agenic duplication 50kb upstream from *NRG3* (10q23.1) was seen in case
428 EqSCM 15. This CNV could conceivably disrupt upstream regulatory regions of
429 *NRG3*, which encodes an important ligand for the transmembrane tyrosine
430 kinase receptor ERBB4. *NRG3* has been shown to activate tyrosine
431 phosphorylation of its cognate receptor, ERBB4, and is thought to influence
432 neuroblast proliferation, migration and differentiation by signalling through
433 ERBB4. *NRG3* is a strong candidate gene for schizophrenia, and neuregulin
434 molecules and their receptors are involved in rat cardiac development and
435 maintenance.¹¹⁹

436

437 Finally, the two rare deletions observed on chromosome 13 (13q21.33 and
438 13q33.1) in case EqSCM 17, fall into largely uncharacterised areas of the
439 genome. The first is agenic, although there is a prediction of a spliced EST in
440 the NCBI database, and the second occurs as two 50kb blocks within the
441 *ITGBL1* gene. *ITGBL1* is most strongly expressed in aorta.^{120; 121}

442

443

444 **Discussion**

445 *Monogenic and polygenic models of risk*

446 The Christchurch earthquakes repeatedly exposed the entire population of the
447 city, approximately 350,000 people, to major stress and life disruption. Almost all
448 patients presenting with EqSCM were post-menopausal females, consistent with

449 other reports.¹²² We set out to explore three categories of genetic contributions
450 to SCM predisposition, using WES to explore Mendelian models of risk, Cardio-
451 MetaboChip analysis to test for polygenic risk factors, and aCGH analysis to
452 evaluate the role of genomic structural variants.

453

454 Extensive analysis of the WES data did not yield any apparent enrichment of
455 rare, damaging variants within exome regions amongst the EqSCM cases.
456 Therefore, it seems unlikely that point mutations or small insertion-deletion
457 (indels) in a single gene underlie predisposition to earthquake SCM. A limitation
458 of this analysis is that it would have been unable to detect regulatory mutations,
459 or other important variants, not included or well represented within the captured
460 exome regions. Whole genome sequencing may therefore be warranted to
461 further test the hypothesis of Mendelian underpinnings of SCM, as this approach
462 could identify any regulatory variants not obtained with WES, and due to the
463 absence of a DNA capture step, would also provide more uniform coverage of
464 exons.

465

466 In a second approach, we explored the alternative hypothesis of polygenic risk
467 alleles of small effect size using a case-control association study, with
468 genotypes generated by the Cardio-MetaboChip. This chip allowed genotyping
469 of ~200,000 SNPs previously identified through genome - wide association
470 studies (GWAS) for risk of metabolic, atherosclerotic and cardiovascular
471 diseases and traits.¹²³ The traits covered by the panel of genetic variants on the
472 chip include myocardial infarction (MI) and coronary heart disease (CHD), type 2
473 diabetes (T2D), T2D age diagnosed, T2D early onset, mean platelet volume,

474 platelet count, white blood cell, HDL cholesterol, LDL cholesterol, triglycerides,
475 total cholesterol, body mass index, waist hip ratio (BMI adjusted), waist
476 circumference (BMI adjusted), height, percent fat mass, fasting glucose, fasting
477 insulin, 2-hour glucose, HbA1c, systolic blood pressure, diastolic blood pressure
478 and QT interval. This analysis did not yield variants of genome-wide
479 significance in the SCM cases compared to either healthy controls or patients
480 with coronary disease. Exploratory pathway analysis suggested that the EqSCM
481 cases carried a greater burden of SNPs that mapped to a myocardial ischemia
482 pathway compared to the healthy controls, although this must be interpreted
483 with caution as our small sample set meant very limited statistical power. Two
484 limitations of this analysis were the relatively constrained content of the Cardio-
485 MetaboChip, which is less able to provide a rich dataset of genome-wide SNP
486 genotypes than the chips commonly used for GWAS, and the relatively small
487 cohort of cases available for study. Recruitment of a much larger SCM cohort
488 with a view to a well-powered GWAS with a more extensive genotyping chip
489 would therefore be a worthwhile future goal to more fully explore possible
490 polygenic underpinnings of this disorder. We note the recent publication of a
491 preliminary GWAS on 96 SCM cases and 475 healthy controls,¹²⁴ and believe
492 extension of this approach to larger cohorts is an important goal.

493

494 *Involvement of copy number variants*

495 CNVs have been implicated in many diseases since the recognition a decade
496 ago of their widespread distribution through the genome.¹²⁵⁻¹²⁷ Of note, rare
497 CNVs are implicated in autism, epilepsy, schizophrenia, developmental delay
498 and intellectual disability.^{105; 128-132} Cardiac conditions which involve CNVs

include congenital left-sided heart disease,^{133; 134} congenital heart disease,^{135; 136}
some cases of long QT syndrome,¹³⁷ and Tetralogy of Fallot.¹³⁸ Our final
analysis, therefore, was to explore the potential involvement of CNV in risk of
EqSCM, using aCGH analysis of all cases. Results from this analysis were
striking, with 42% of EqSCM cases having a rare CNV of unclear clinical
significance. The CNV detection rate for diagnostic aCGH in childhood
developmental disorders such as autism, developmental delay and intellectual
disabilities, is approximately 20-30%.¹³⁹⁻¹⁴¹ A recent report of a large New
Zealand aCGH case series (5,300 pre- and post-natal tests) reported CNVs in
28.3% of these clinically-selected cases.¹⁴² Our observation of a rate of 42% for
the EqSCM case series is significantly greater ($P < 0.02$) than rates for the
enriched case cohorts normally referred for clinical aCGH testing¹³⁹⁻¹⁴². The
CNVs detected in EqSCM cases were all different, and there were no physical
overlaps between them. This situation is similar to the pattern of CNVs seen in
other conditions, including rolandic epilepsies¹¹⁸ and congenital heart
disease.^{133; 135; 136} Many of the CNVs we observed are likely to impact genes of
potential relevance to physiological processes implicated in SCM.

We have taken a relatively conservative approach to categorising CNVs, in
terms of rarity and predicted functional significance. For example, we did not
include two CNVs located at 9p24.3, involving individuals EqSCM 14 and 28 - a
deletion and duplication, respectively. These CNVs encompassed a region
including the large isoform of *DOCK8* gene. *DOCK8* encodes a protein
implicated in the regulation of the actin cytoskeleton,¹⁴³ and *DOCK8* mutations
cause autosomal recessive hyper-IgE syndrome.¹⁴⁴ One reported case of a

524 homozygous 129kb deletion in this region was associated with Graves's disease
 525 and aortic aneurysm.¹⁴⁵ However, several deletions of *DOCK8* are recorded in
 526 the DGV for unaffected individuals, therefore the CNVs in EqSCM 14 and 28
 527 were categorised as TBB (thought to be benign). The approximately 160kb
 528 duplication in EqSCM 28 was larger than the 44kb deletion in EqSCM 14, and it
 529 encompassed a second gene, *KANK1*. A small number of similar duplications
 530 have been recorded in the DGV, and therefore we did not consider this to be
 531 pathogenic. However, it is of interest that we see two relatively rare CNVs at this
 532 locus in our small EqSCM cohort.

533

534 Of the twelve EqSCM cases with rare CNVs, we consider that three (EqSCM 01,
 535 06 and 19) contain CNVs that affect genes of high relevance to cardiomyopathy
 536 or cardiac function. The remaining candidate CNVs are also strong candidates
 537 with potential functional relevance. In one of our most highly-ranked candidate
 538 CNV containing cases (EqSCM 01) a large genomic deletion removes an exon
 539 of *RBFOX1* which contains the start codon used by the majority of transcripts
 540 predicted for the gene. A recent report by Gao *et al.* (2016) provided strong
 541 functional data that would support our hypothesis of this gene's involvement as
 542 a susceptibility locus for SCM⁹⁰. Their work with mouse models has shown
 543 *RBFOX1* deficiency in the heart promoted pressure overload-induced heart
 544 failure, and induction of *RBFOX1* over-expression in these murine pressure-
 545 overload models, substantially attenuated cardiac hypertrophy and pathological
 546 manifestations⁹⁰. The haploinsufficiency seen in EqSCM 01 at the *RBFOX1*
 547 locus may, in concert with other environmental or genetic factors, contribute to
 548 SCM through reduced global RNA splicing changes in the heart.

549 Conclusion

550 Beginning with a cohort of 28 SCM cases triggered by two major earthquakes
551 that caused extensive death and damage in Christchurch (New Zealand), we
552 carried out exploratory analyses of three models for genetic predisposition to
553 this disorder. Using WES and Cardio-MetaboChip genotyping analyses we did
554 not detect an obvious role for exonic mutations in a monogenic model, or SNPs
555 in a polygenic model, for SCM risk. However, our analysis of copy number
556 variation in SCM cases revealed a high rate of occurrence of CNV categorised
557 as of uncertain clinical significance. Most of the CNV we detected in SCM cases
558 were rare, or not previously seen (Table 2).

559
560 These observations lead us to propose that SCM is a copy number variant
561 disorder, whereby haploinsufficiency of genes overlapping deletions or over-
562 expression of duplicated genes leads to relatively subtle modification of cardiac
563 or adrenergic physiology, such that these individuals are at increased risk of
564 suffering SCM when exposed to specific environmental triggers. Although no
565 obvious single pathway relationships between the genes affected by these
566 CNVs is apparent, most of the CNVs encompass loci relevant to cardiac
567 function or cardioneuronal development.

568
569 In order to confirm whether SCM predisposition does indeed arise from CNVs,
570 four key areas for future work need to be pursued. First, more widespread
571 analysis is required of CNVs in many SCM cases. This would confirm whether
572 our observation of a high rate of CNV in EqSCM also prevails in sporadic cases,
573 and it will broaden the catalog of affected genes, helping to discern underlying

574 signalling networks and physiological processes. In addition, with increasing
 575 numbers of cases, physical overlaps between CNVs in different individuals
 576 should become apparent, pinpointing key genomic regions for more intensive
 577 analysis. Second, the inheritance patterns of these CNVs must be established. It
 578 is unclear what proportion are *de novo* versus inherited from either parent.
 579 Third, there is a clear need for detailed physiological and gene expression
 580 analyses on appropriate cells, including cardiomyocytes, derived from SCM
 581 cases. Given the diversity of CNV seen in our SCM cases, this goal would most
 582 effectively be achieved by generation of induced-pluripotent stem cell (iPSC)
 583 lines from many patients and appropriate controls.^{146; 147} Finally, although our
 584 data implicate CNV as a significant genetic factor underlying SCM risk, it would
 585 seem wise to pursue an effective GWAS strategy to identify other genetic
 586 contributors to SCM and build on the initial study in this area.¹²⁴ International
 587 initiatives to collate SCM cases¹³ should therefore ensure that consented DNA is
 588 available to provide appropriately large numbers of well phenotyped cases and
 589 controls to facilitate this goal.

590

591 Finally, we hope our observations implicating CNV in this unique case series of
 592 EqsCM will stimulate further studies of copy number variation in other SCM
 593 cohorts, and lead to an improved understanding of this perplexing and intriguing
 594 condition.

595

596 **Supplemental Data**

597 Supplemental Data include one figure and one table and can be found with this
 598 article online at....

599

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610

611

612 **Web Resources**

- 613 1000 Genomes project, <http://www.internationalgenome.org/>
- 614 ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>
- 615 Database of Genomic Variation (DGV), <http://dgv.tcag.ca/dgv/app/home>
- 616 DECIPHER, <https://decipher.sanger.ac.uk/>
- 617 Exome Aggregation Consortium (ExAC), <http://exac.broadinstitute.org>
- 618 Galaxy, <https://galaxyproject.org/>
- 619 GTex, <https://www.gtexportal.org/home/>
- 620 HUGO Gene Nomenclature Committee, <http://www.genenames.org>
- 621 MutationTaster2, <http://www.mutationtaster.org/>
- 622 NHLBI GO Exome Sequencing Project (ESP),
- 623 <https://esp.gs.washington.edu/drupal/>
- 624 SeattleSeq annotation server, <http://evs.gs.washington.edu/EVS/>
- 625

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1144 **Figure titles and legends**

1145

1146 **Figure 1.** CNV detected in EqSCM case 01. **A:** Chromosomal location of the
 1147 CNV at the RBFOX1 locus of chromosome 16. **B:** Enlargement of the fifteen
 1148 probe deletion (139kb, delimited by vertical green lines and blue shading)
 1149 illustrating loss of the fMet-containing exon (pale blue vertical bar) for three
 1150 major RBFOX1 isoforms. DGV track of known CNVs shown at bottom of figure,
 1151 beneath the genes2 and regions of interest tracks. Graphical views from
 1152 Genoglyphix (PerkinElmer) software.

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1155 **Figure 2.** Genome wide distribution of CNVs. CNVs detected in 12 (of 28)
 1156 EqSCM individuals by aCGH analysis. Numbers beside arrows relate to EqSCM
 1157 patient number. Red arrows denote deletions, blue arrows duplications. Note
 1158 that EqSCM 03, EqSCM 06, EqSCM 11 carry two rare CNVs, while EqSCM 19
 1159 contains three.

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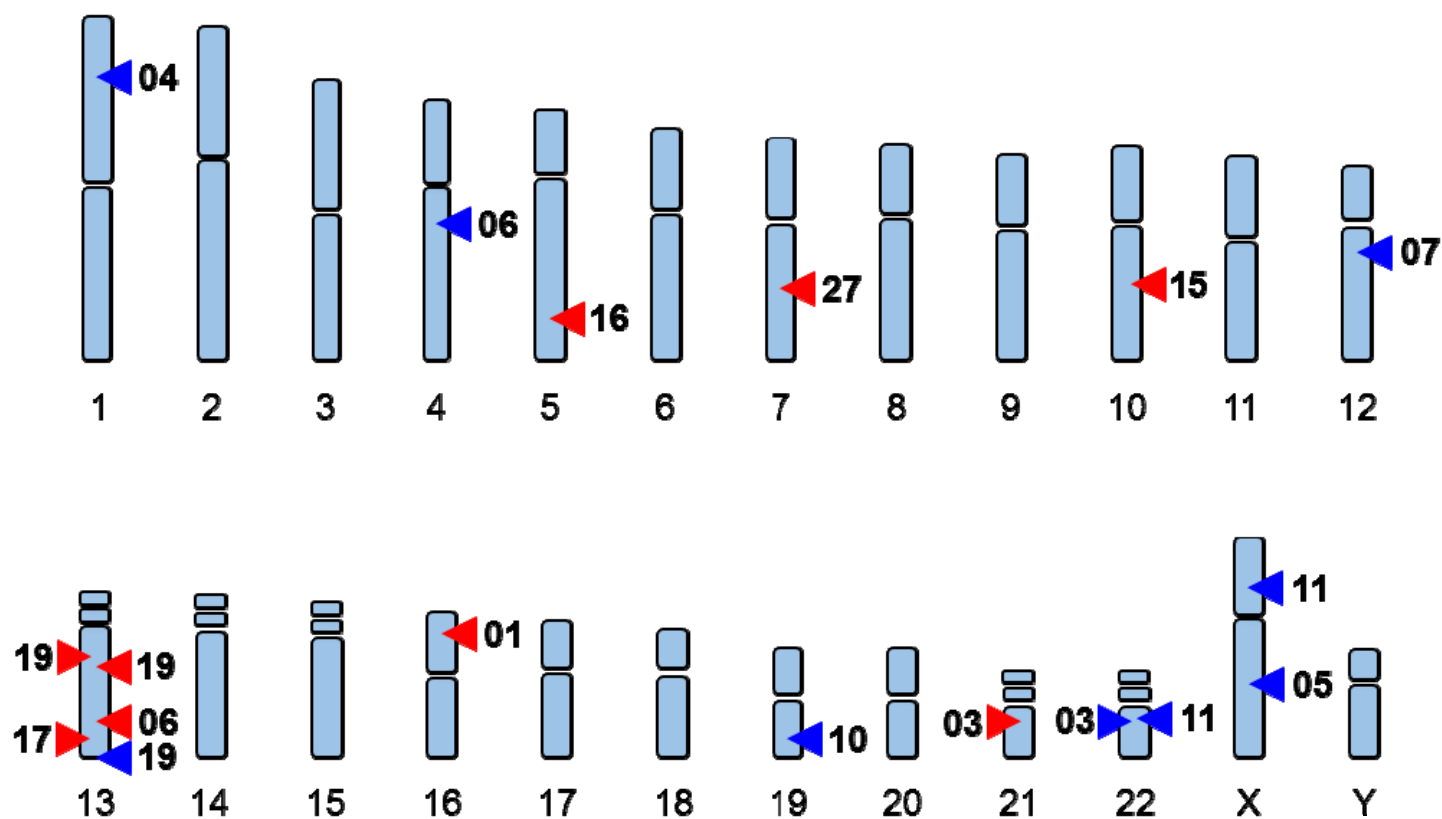
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Figure 2.



1183 **Table titles and legends**

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1185 **Table 1.** SNP markers removed from Cardio-MetaboChip analysis in quality
1186 control

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Stage of analysis	Number of SNPs	Comments
Assayed	196,725	
No genotype	10,927	
Mono allelic	28,367	
MAF <0.004	44,424	SNPs with only 1 minor allele found
Missing > 0.02 of samples	36,884	
HWE fail	130	HWE_P <1e-20 and 1.5x more heterozygotes than expected
Total Removed	65,622	Note that SNPs could fail on two or more of the criteria above
SNPs remaining in analysis	131,103	

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Table 2. Rare CNVs detected in a cohort of 28 EqSCM cases.

Case ID	Prediction*	Location	Del/Dup	Key genes**	Size	Frequency	Location
EqSCM 01	UCS	16p13.2	DEL	RBFOX1**	139kb	1/20000	HG19 chr16:7,054,481-7,193,526
EqSCM 03	UCS	21q21.1	DEL	CHODL**	96kb	1/9000	HG19 chr21:19,537,178-19,633,275
	CS	22q11.26.1	DUP		2Mb		HG19 chr22:23,055,148-24,991,669
EqSCM 04	UCS	1p34.1	DUP	IPP** PIK3R3 (+3 others)	455kb	1/7000	HG19 chr1:46,144,479-46,599,815
EqSCM 05	UCS	Xq22.3	DUP	IRS4** COL4A5**	113kb	1/2000	HG19 chrX:107,896,435-108,009,609
EqSCM 06	UCS	13q31.5	DEL	GPC5**	207kb	1/5000	HG19 chr13:92,075,673-92,283,600
	UCS	4q21.21	DUP	FRAS1 ANX3** LINC01094	329kb	1/29000	HG19 chr4:79,281,048-79,610,796
EqSCM 07	UCS	12q13.13	DUP	17 Keratin genes	411kb	0	HG19 chr12:52,657,396-53,069,013
EqSCM 10	UCS	19q13.42	DUP	GP6**	130kb	1/15000	HG19 chr19:55,439,927-55,570,442
EqSCM 11	UCS	22q11.21	DUP	YPEL1** (+ 2 others)	180kb	0	HG19 chr22:22,008,249-22,189,094
	UCS	Xp11.4	DUP	TSPAN7**	140kb	1/1000	HG19 chrX:38,485,991-38,626,762
EqSCM 15	UCS	10q23.1	DUP	upstream NRG3**	78kb	0	HG19 chr10:83,506,502-83,585,097
EqSCM 17	UCS	13q33.1	DEL	ITGBL1**	148kb	0	HG19 chr13:102,148,514-102,296,766
EqSCM 19	?CS	13q14.2	DEL	KCNRG (+ 6 others)	886kb	0	HG19 chr13:50,585,186-51,452,033
	UCS	13q34	DUP	LINC00346, ANKRD10	146kb	0	HG19 chr13:111,385,673-111,532,564
	UCS	13q14.11	DUP	ENOX1**	149kb	0	HG19 chr13:89,219,432-89,359,036
EqSCM 27	UCS	7q31.1	DEL	LRRN3** IMMP2L	105kb	1/12000	HG19 chr7:110,744,611-110,849,681

*UCS = unclear clinical significance, ?CS = potential clinical significance. **Genes with clear cardiovascular association. Frequency calculations are based on larger (>1000 individuals) studies included in the DGV. A full list of all CNVs detected for each individual is presented in Supplementary Table 1.

Supplemental data

Table S1: Complete CNV detection list of EqSCM cases in this study. (Separate Excel spreadsheet).

Figure S1: CNV interpretation algorithm (Separate word file doc)