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Short title: CARDIOMYOPATHY GENES IN POLYGENIC CARDIAC HYPERTROPHY

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Author contribution

PRP, LMDD, FJC and SBH conception and study design; PRP, FZM, GL and PL performed experiments; PRP analyzed data; PRP interpreted results; PRP prepared figures; PRP drafted manuscript; PRP, FZM, GL, PL, LMDD, FJC and SBH edited and revised manuscript; PRP, FZM, GL, PL, LMDD, FJC and SBH approved final version of manuscript.

Abstract

Hypertrophic cardiomyopathy thickens heart muscles reducing functionality and increasing risk of cardiac disease and morbidity. Genetic factors are involved, but their contribution is poorly understood. We used the hypertrophic heart rat (HHR), a unique normotensive polygenic model of cardiac hypertrophy and heart failure to investigate the role of genes associated with monogenic human cardiomyopathy. We selected 42 genes involved in monogenic human cardiomyopathies to study: 1) DNA variants, by sequencing the whole-genome of 13-week old HHR and age-matched normal heart rat (NHR), its genetic control strain; 2) mRNA expression, by targeted RNA-sequencing in left ventricles of HHR and NHR at five ages (2-days old, 4-, 13-, 33- and 50-weeks old) compared to human idiopathic dilated data; and 3) microRNA expression, with rat microRNA microarrays in left ventricles of 2-days old HHR and age-matched NHR. We also investigated experimentally validated microRNA-mRNA interactions. Whole-genome sequencing revealed unique variants mostly located in non-coding regions of HHR and NHR. We found 29 genes differentially expressed in at least one age. Genes encoding desmoglein 2 (*Dsg2*) and transthyretin (*Ttr*) were significantly differentially expressed at all ages in the HHR, but only *Ttr* was also differentially expressed in human idiopathic cardiomyopathy. Lastly, only two microRNAs differentially expressed in the HHR were present in our comparison of validated microRNA-mRNA interactions. These two microRNAs interact with five of the genes studied. Our study shows that genes involved in monogenic forms of human cardiomyopathies may also influence polygenic forms of the disease.

Keywords: DNA sequencing, gene expression, microRNA, cardiac hypertrophy, cardiomyopathy

Introduction

Cardiovascular disease (CVD) is the main cause of death and morbidity worldwide, killing approximately 17.5 million people in 2012 (23, 37). Both genetic and environmental factors contribute to CVD. The most common genetic contributions are considered to be polygenic, although the exact number and nature of genes involved has been difficult to determine as new genes are yet to be identified with the advancement of technology (16). However, less common monogenic causes of CH have been well characterized in terms of the causative DNA variants and pathophysiology (10, 26). Variation of the expression of genes involved in monogenic CH might provide clues to the causes of polygenic etiology of CH.

Hypertrophic cardiomyopathy (HCM) is the most common inherited form of CVD, affecting one in 500 adults and is the major cause of heart failure and sudden death in young people (21, 25, 26). The condition is characterized by the asymmetric thickening of the cardiac wall, heart failure and risk of sudden death. A variety of mutations in genes coding sarcomere and cardiac filament proteins account for over 88% of familial HCM (5, 26, 31). Mutations in two genes that encode myosin heavy chain 7 (*MYH7*) and cardiac myosin binding protein C (*MYBPC3*) are the most common causes of monogenic HCM (31, 39). Familial dilated cardiomyopathies form another important group of monogenic CVD characterized by CH and heart failure, for which mutations in genes encoding sarcomeric proteins account for almost half of the known forms (15).

The study of genes involved in monogenic hypertrophy and failure have led to an understanding of disease mechanisms and might also provide explanations for more common polygenic forms of heart failure.

We have developed and characterized the hypertrophic heart rat (HHR), a unique polygenic normotensive model of spontaneous ventricular hypertrophy,

cardiac failure and premature death (13). Compared with their genetic control strain, the normal heart rat (NHR), HHR begin life with fewer cardiomyocytes that develop cellular hypertrophy leading to cardiac enlargement and heart failure (29).

Our aim was to study genes previously associated with human monogenic forms of dilated and hypertrophic cardiomyopathies in the polygenic etiology of cardiac hypertrophy in the HHR. We combined analyses of RNA expression and DNA sequence variation to identify those genes that might be of importance in the polygenic setting.

Materials and methods

Sample collection

The HHR and NHR strains have been described in detail elsewhere (13). Two-day-old HHR and NHR (n=8 HHR, n=9 NHR) were euthanized by decapitation. At 4-, (n=10 HHR, n=10 NHR); 13-, (n=10 HHR, n=11 NHR); 33- (n=7 HHR, n=9 NHR) and 50-weeks of age (n=12 HHR, n=10 NHR) rats were euthanized using a lethal dose of pentobarbitone (Lethobarb). Hearts were removed and left ventricles (LV) were immediately dissected from atria. Cardiac weight indexes (CWI, mg/g) were calculated as the total heart weight (mg) relative to total body weight (g) of each animal (ref 28 and Figure 1).

The five age groups investigated represent different developmental stages in their life. Prior to hypertrophy (2-day-old), during the development of hypertrophy (4-weeks), early hypertrophy (13-weeks), established hypertrophy (33-weeks), hypertrophy complicated by heart failure (50-weeks-old). This study was approved by the Animal Ethics Committees of the University of Melbourne and Deakin University, and ratified at Federation University Australia.

DNA and RNA extraction

DNA from LV was extracted using PureLink® Genomic Extraction kit (ThermoFisher Scientific). RNA from LV was extracted using miRNeasy kit (Qiagen). DNA was quantified by spectrophotometry using NanoDrop® 2000 and RNA was quantified by fluorescence using Qubit™ 3.0 Fluorometer and the RNA high sensitivity assay kit (ThermoFisher Scientific).

Genes investigated

For these focused studies we selected 42 genes (Table 1) involved in monogenic forms of familial cardiomyopathies (5, 15, 31, 39). Physiologically, most of these genes are involved in growth and contractility, regulation of mechanical-stress, calcium channels and a variety of muscle development pathways, mainly for cardiac filaments and sarcomere assembly (5, 31).

DNA sequence variants in HHR were identified according to methods detailed previously (30). Briefly, we sequenced the whole-genome of one male 13-week-old NHR and one age-matched HHR. Variants were analyzed according to GATK best practices (35) and functional annotation was performed using SnpEff software (4). Results were stored in a database developed “in-house” and then we identified unique single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) in the NHR and HHR (30).

mRNA expression of the genes listed in Table 1 was measured using Targeted RNA Expression (TREx) custom panel in the MiSeq Desktop sequencer (Illumina®) and analyzed using MSR: Targeted RNA v2.4.60.8 on Illumina® BaseSpace. False discovery rate (FDR) was set as <0.1.

microRNA (miRNA) arrays were conducted using the Agilent rat microRNA microarray kit 16.0 in left ventricles of 2 days old male HHR and age-matched NHR (n=4/group). The data obtained has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database with series

accession number GSE38710. Differentially expressed miRNAs were identified using Partek Genomics Suit v6.6 with FDR set as <0.05.

In silico investigations

We combined *in silico* approaches to explore a link between gene expression and DNA sequencing data from the HHR and NHR and human studies available online.

We investigated the possibility that miRNAs might be involved in regulating mRNA expression pre- and post-transcriptionally using three algorithms individually (miRWalk 2.0, (7, 8) miRanda (3) and TargetScan (11)) as a comparative platform to predict possible miRNA binding sites within the sequence in and around each gene of interest. We also used miRWalk 2.0 to investigate experimentally validated miRNA-mRNA interactions and evaluate which miRNAs targeted the genes under investigation.

As DNA methylation can modulate gene expression by compacting DNA sequences, we investigated if any of the DNA sites were differentially methylated in CVD using the Disease Meth database 2.0 (38).

Gene expression in human hearts

We also investigated the mRNA expression of the genes listed identified in HHR in human cardiac samples from the dataset “heart failure arising from different etiologies” in the repository Gene Expression Omnibus (GEO) reference series GSE1145 (2, 9) (n=11 control hearts and n=15 idiopathic dilated hearts). We then determined the expression of the genes investigated using the GEO tool GEO2R.

Results

DNA Sequence Analyses

DNA sequencing in and around the 42 genes of interest revealed greater number of unique DNA variants discovered in the HHR (compared to the rat reference genome)

with 851 SNPs and 491 InDels, as opposed to 383 SNPs and 316 InDels in the NHR (Table 1). We found no evidence of DNA sequence variation in 6 genes in the NHR (namely *Csrp3, Emd*, *Gla*, *Mylk2*, *Pln* and *Taz*) and 3 genes in the HHR (*Emd*, *Myoz2* and *Tpm1***,** Table 1).

In both the HHR and NHR, most unique variants were located in intergenic or intronic regions. However, we found 11 unique synonymous SNPs in exonic regions in the HHR and 2 in the NHR. These SNPs were located in genes encoding actinin alpha 2 (*Actn2*), desmin (*Des*), ryanodine receptor 2 (*Ryr2*), troponin T type 2 (*Tnnt2*) and vinculin (*Vcl*) in the HHR; and *Ryr2* and *Vcl* in the NHR. Interestingly, only one unique non-synonymous missense variant (gGt>gTt) in an exonic region was found in the *Vcl* gene in the NHR changing the aminoacid from glycine to valine.

Cardiac RNA Expression Analyses

The relative fold changes (FC) in mRNA expression in the HHR relative to NHR were never greater than 4-fold for any of the 42 genes under investigation. We found that 29 of the 42 genes showed significant differential expression for at least one age (Figure 2, 3 and 4).

Genes differentially expressed were not consistent throughout the age groups, possibly reflecting developmental stage-specific regulation. At 2 days old, only four genes were differentially expressed (Figure 2a). In contrast, at 4 weeks of age, we observed 50% of genes differentially expressed, the highest prevalence of all the age groups (Figure 2b). Most of the differences at this age presented increased expression. For some genes (such as *Gla*, *Jup*, *Lamp2* and *Pln*) differential expression was evident only at 4 weeks of age. The differential expression of other genes (such as *Actc1*, *Cav3* and *Fhl2*) became first evident at 4 weeks of age and then persisted throughout adulthood. Differences in the expression of other genes (such as *Tnnt2*, *Myoz2* and *Myl3*) appeared at 4 weeks of age, but

disappeared in later adulthood. Still other genes (such as *Ankrd1*) did not show differential expression until later adulthood (Figure 2, 3 and 4).

Two genes, those encoding desmoglein 2 (*Dsg2*) and transthyretin (*Ttr*) were significantly differentially expressed at all ages in the HHR compared with NHR. *Dsg2* was underexpressed in the hearts of HHR at all ages sampled, whereas *Ttr* was significantly downregulated at day 2 in the neonatal period but significantly upregulated at all subsequent ages corresponding to the development of cardiomyocyte hypertrophy (Figure 2). *Ttr* also showed greater expression in adult human idiopathic cardiomyopathy (Figure 4).

The analysis of human idiopathic cardiomyopathy revealed that 16 orthologous genes differentially expressed of the 42 genes investigated. We found only 9 of those 16 genes were also differentially expressed in at least one rat age.

Predicted miRNA binding sites

We also predicted possible miRNA binding sites within and around each gene region. Potentially, there are over 220,000 miRNA binding sites in the gene coding regions alone and almost 54,000 in the promoter regions (Table 2). However, our comparison of validated miRNA-mRNA interactions to miRNAs differentially expressed in HHR compared to NHR in our microarray data found two miRNAs (miR-34a-5p and miR-17-5p) upregulated in the HHR (Table 3). Interestingly, those two miRNAs interact with five of the genes investigated, plakophilin 2 (*Pkp2*), RNA binding motif protein 20 (*Rbm20*), ryanodine receptor 2 (*Ryr2*), tropomyosin 1 (*Tpm1*) and vinculin (*Vcl*) (14, 19, 24). Although not statistically significant, the gene expression of *Pkp2*, *Ryr2*, *Tpm1* and *Vcl* is downregulated in 2 days old HHR (FC=0.93, 0.95, 0.89 and 0.88, respectively).

Other analyses

Scans of published GWAS (using GWASdb v2 (22)) data also indicated the presence of SNPs found in the 42 genes investigated that are associated with CVD traits in humans (Table 4).

Surprisingly no methylation profiles have been reported in human heart related diseases for any of the genes in our dataset.

Discussion

The HHR and their NHR genetic control are derived from the spontaneously hypertensive rat (SHR) and Fisher 344 rat and provide a unique model of left ventricular hypertrophy independent of high blood pressure and heart failure. Although HHR and NHR have a polygenic background, it is not unreasonable to presume that genes best known for their major mutations causing human cardiac and failure might also encompass DNA variants with a more subtle quantitative impact on cardiac structure and function. Here we sought to determine whether any of the 42 genes implicated in Mendelian human hypertrophic and dilated cardiomyopathies might also be relevant to the polygenic hypertrophy of HHR – either as DNA sequence variants or abnormal gene expression patterns. We had the advantage with our life course approach to also examine the ontogeny of the expressions of these genes and their relationships with the developmental stages of hypertrophy in the HHR.

Molecular genetic studies have identified over 1400 mutations responsible for inherited cardiomyopathies and genetic testing is offered to identify genetic causes in families or assess family members at risk (26, 34). However, emerging data suggests that compound mutations have an additive role causing a gene dosage effect influencing the severity and progression of HCM (17, 20, 27). Such interactions at the level of monogenic cardiomyopathic disease provide general

support for the hypothesis that interaction between other DNA variants in the same genes might influence polygenic cardiac hypertrophy.

Among the sequence variants identified in our sequencing analyses we found 11 synonymous SNPs in exonic regions in the HHR and one nonsynonymous SNP in the NHR. It is well established that nonsynonymous mutations may impact aminoacid sequences and therefore human health (18). However, synonymous variants which had previously been regarded as “silent” mutations and thought to have no effect on disease phenotype, have been reported to also cause changes in protein-protein interactions. Importantly, studies suggest that these synonymous mutations contribute to human disease risk and other complex traits (32). The synonymous variants we discovered (Table 1) might result in changes in protein-protein interactions and explain changes in gene expression (Figure 1) due to imbalanced availability of tRNA caused by codon bias (12).

Interestingly, HHR are born with smaller and fewer cardiomyocytes than NHR. We believe that this reduced endowment is important for the subsequent development of cardiomyocyte hypertrophy, which becomes evident in early adolescence (4 weeks, Figure 1), when increased pressure and volume loads in the growing animals place proportionately greater stress on the fewer individual myocytes (29). By 13 weeks of age, both cardiac and cardiomyocyte hypertrophy are established (ref 13 and Figure 1), but in the end this is counterproductive, as the enlarged cells do not function efficiently and gradually deteriorate, leading to heart failure (evident as early as 30 weeks of age) and premature death (towards 50 weeks of age) (13, 29). These pathophysiological phenotypic changes can provide context for the developmental stage-specific changes in gene expression we observed.

Our analysis of gene expression showed that at 2 days old, when HHR hearts are smaller than NHR (29), the genes encoding cardiac filaments and sarcomeric proteins are generally underexpressed (Figure 2). Whether this is a cause or effect of the fewer, smaller cells is not possible to say from these data. As the cells begin to hypertrophy and throughout the rest of their lives, most of these genes are overexpressed. These findings demonstrate that the left ventricle undergoes changes in gene expression that could be related to the pathophysiology of the hypertrophy (36). Interestingly, the switch of the *Ttr* gene from underexpressed in neonates to overexpressed in adults suggest stage-specific regulation. *Ttr* encodes a protein for exosome production and high protein levels in serum have been previously associated with lung cancer (6) and heart failure caused by accumulation of transthyretin amyloid fibrils in the heart (33). Additionally, the genes for cardiac alpha actin 1 (*Actc1*), caveolin-3 (*Cav3*) and four and a half LIM domains 2 (*Fhl2*) are upregulated in adult HHR and have been associated with cardiomyopathies in mice and humans (1, 28). Conversely, the persistent underexpression of *Dsg2* suggests a constitutive difference between HHR and NHR that appears independent of the pathophysiological changes with age.

Interestingly, the majority of genes identified in HHR were not differentially expressed in the human samples (Figure 2). This might reflect the nature of the diseases in the human repository that is composed primarily of samples from human idiopathic dilated cardiomyopathy rather than Mendelian human hypertrophic cardiomyopathy. Furthermore, as we were unable to establish the age of the human samples, we cannot make direct comparisons to our rat data to further our understanding of gene expression patterns with the progression of HCM.

In order to provide a comprehensive perspective about the processes involved in this pathology, we investigated DNA methylation profiles and SNPs

published in GWAS data of the genes investigated. We were unable to find any relevant methylation data supporting further analyses as the majority of the studies available was performed in a variety of cancers and only few and individual studies are available in CVD. However, two SNPs found in the GWAS data located in the cardiac troponin T2 (*TNNT2*) gene are associated with cardiac troponin-T levels and directly correlated to heart failure.(40) These challenges in merging DNA, RNA and epigenome data highlight the importance of comprehensive studies publicly available.

Our findings provide evidence of the involvement of monogenic genes in polygenic hypertrophic but might only account for part of the genes responsible for this disease. The prevalence of rare variants and unidentified genes responsible for HCM are yet to be elucidated. Furthermore, the interaction mechanisms used by these genes deserve more attention and might aid professionals in determining diagnostics and prognosis of the disease.

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Disclosures

None.

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Figure legends

Figure 1. The hypertrophic heart rat (HHR) has an enlarged heart when compared to its genetic control, the normal heart rat (NHR). Cardiac weight index (CWI, mg/g) of HHR is represented as a percentage difference to NHR (normalized to 100%) at 2-day old, 4-, 13-, 33- and 50-week old (n=7-12 per group per age). *P<0.05; **P<0.01; ***P<0.001.

Figure 2. Heart mRNA expression of genes differentially expressed in at least one age group. There are four and 21 genes differentially expressed in A) neonatal and B) 4 weeks old hypertrophic heart rats (HHR), respectively. HHR fold change relative to normal heart rat (NHR) is shown. Genes not differentially expressed are shown in open boxes. *P<0.05; **P<0.01; ***P<0.001.

Figure 3. Heart mRNA expression of genes differentially expressed in at least one age group. There are 14 and 13 genes differentially expressed in A) 13 weeks old and B) 33 weeks old hypertrophic heart rats (HHR), respectively. HHR fold change relative to normal heart rat (NHR) is shown. Genes not differentially expressed are shown in open boxes. *P<0.05; **P<0.01; ***P<0.001.

Figure 4. Heart mRNA expression of genes differentially expressed in at least one age group. There are ten and nine genes differentially expressed in A) 50 weeks old hypertrophic heart rats (HHR) and B) human idiopathic dilated cardiomyopathy (DCM), respectively. HHR fold change relative to normal heart rat (NHR) is shown. DCM patients fold change relative to healthy is shown in humans. Genes not differentially expressed are shown in open boxes. *P<0.05; **P<0.01; ***P<0.001.

Tables

Table 1. Variants found in each gene investigated unique to each strain classified according to type.

Gene ID[a]	Gene name	Frequency in patients		Rat Genome			HHR[c]		NHR[d]	
		Hypertrophic	Dilated	Chr[b]	Location	Strand	SNP[e]	InDel[f]	SNP[e]	InDel[f]
Abcc9	ATP-binding cassette, subfamily C member 9	n/d[g]	0.6%	4	241,019,980 - 241,139,051	-	4	10	2	9
Actc1	cardiac actin alpha 1	<1%	rare	3	112,080,853 - 112,086,389	-	3	2	1	1
Actn2	actinin alpha 2	rare	0.9%	17	68,050,946 - 68,143,522	-	61	42	58	39
Ankrd1	ankyrin repeat domain 1	rare	1.9%	1	262,038,137 - 262,046,691	+	none	5	2	2
Casq2	calsequestrin 2	rare	n/d	2	223,945,611 - 224,001,893	+	11	8	5	12
Cav3	caveolin 3	0.6%	n/d	4	207,683,202 - 207,699,176	+	none	2	none	1
Cryab	crystallin, alpha B	n/d[g]	0.7%	8	53,776,100 - 53,779,780	+	3	6	2	8
Csrp3	cysteine and glycine-rich protein 3	rare	0.3%	1	105,206,459 - 105,225,635	-	1	1	none	none
Ctf1	cardiotrophin 1	n/d[g]	n/d[g]	1	206,186,511 - 206,191,930	+	2	3	none	3
Des	desmin	n/d[g]	0.3-2.1%	9	82,325,835 - 82,333,549	+	34	7	none	1
Dsc2	desmocollin 2	n/d[g]	n/d[g]	18	11,625,847 - 11,658,036	-	9	4	2	3
Dsg2	desmoglein 2	n/d[g]	2.3%	18	15,353,348 - 15,411,825	-	5	3	24	4

Emd	emerin	n/d[g]	n/d[g]	1	152,192,993 - 152,196,004	-	none	none	none	none
Fhl2	four and a half LIM domains 2	n/d[g]	2.1%	9	49,591,185 - 49,664,022	-	1	5	none	3
Gla	galactosidase, alpha	1-2% in men	n/d[g]	X	105,295,029 - 105,306,686	-	none	1	none	none
Jup	junction plakoglobin	n/d[g]	n/d	10	88,073,764 - 88,100,700	-	1	10	1	6
Lama4	laminin, alpha 4	n/d[g]	1.1%	20	45,786,892 - 45,926,468	+	37	24	101	17
Lamp2	lysosomal-associated membrane protein 2	rare	n/d[g]	X	124,809,053 - 124,852,509	-	2	4	1	3
Lmna	lamin A/C	n/d[g]	6-7.5%	2	207,245,237 - 207,265,928	-	24	11	3	11
Mybpc3	myosin binding protein C	20-42%	0.2-4%	3	86,649,264 - 86,667,484	+	none	3	none	3
Myh6	myosin heavy chain 6	rare	4.3%	15	37,492,599 - 37,516,786	-	1	4	none	3
Myh7	myosin heavy chain 7	20-40%	4.2-6.3%	15	37,512,803 - 37,544,317	-	2	none	none	1
Myl2	myosin light chain 2	<5%	n/d[g]	12	41,831,137 - 41,835,806	-	6	13	5	7
Myl3	myosin light chain 3	1-2%	n/d[g]	8	118,370,030 - 118,376,218	+	none	4	3	4
Mylk2	myosin light chain kinase 2	rare	n/d[g]	3	154,789,177 - 154,800,843	+	none	1	none	none
Myoz2	myozenin 2	rare	n/d[g]	2	246,542,267 - 246,569,008	-	none	none	2	none
Nexn	nexilin	n/d[g]	1%	2	276,129,684 - 276,161,434	-	2	5	2	2
Pkp2	plakophilin 2	n/d[g]	n/d[g]	11	91,966,047 - 92,031,277	-	2	4	2	4

Pln	phospholamban	rare	rare	20	36,390,879 - 36,400,626	+	1	none	none	none
Prkag2	protein kinase, AMP-activated, non-catalytic subunit gamma 2	<1%	n/d[g]	4	6,577,007 - 6,816,813	+	2	4	none	5
Psen2	presenilin 2	n/d[g]	1%	13	103,521,460 - 103,547,174	-	1	0	2	2
Rbm20	RNA binding motif protein 20	n/d[g]	1.9%	1	281,783,646 - 282,003,053	+	12	21	3	12
Ryr2	ryanodine receptor 2	rare	n/d[g]	17	67,285,205 - 67,704,766	-	518	218	122	106
Sgcd	sarcoglycan delta	n/d[g]	rare	10	31,878,904 - 32,285,036	-	26	34	15	24
Taz	tafazzin	n/d[g]	n/d[g]	1	152,161,153 - 152,169,569	-	1	none	none	none
Tmem43	transmembrane protein 43	n/d[g]	n/d[g]	4	187,412,090 - 187,427,232	-	3	2	2	1
Tnnc1	troponin C type 1	rare	0.4%	16	7,220,777 - 7,223,730	+	0	0	0	0
Tnnt2	troponin T type 2	3-10%	2.9%	13	57,711,369 - 57,729,182	+	48	13	3	4
Tpm1	tropomyosin 1	<5%	0.6-1.9%	8	77,147,580 - 77,174,392	-	none	none	none	2
Ttn	titin	rare	14.1-20%	3	70,138,896 - 70,408,647	-	24	12	6	10
Ttr	transthyretin	1-10%	n/d[g]	18	15,307,563 - 15,316,780	-	none	1	8	1
Vcl	vinculin	rare	rare	15	3,433,521 - 3,522,441	-	4	4	6	2
Total							851	491	383	316

Legend: [a]ID, identification; [b]Chr, chromosome; [c]HHR, hypertrophic heart rat; [d]NHR, normal heart rat; [e]SNP, single nucleotide polymorphism; [f]InDel, insertion/deletion; [g]n/d, not described.

Table 2. Predicted number of microRNA binding sites in or around the genes of interest.

	Region			
Gene	Promoter	5'UTR*	CDS†	3'UTR*
Abcc9	1255	3618	19131	8848
Actc1	1394	290	1126	1721
Actn2	1195	1296	6071	3848
Ankrd1	1265	407	1016	625
Casq2	1332	835	2678	2594
Cav3	1443	240	1103	1411
Cryab	1356	487	871	116
Csrp3	1260	332	772	620
Ctf1	1371	106	1231	1607
Des	1379	137	1194	907
Dsc2	1161	1345	4605	2698
Dsg2	1289	222	1982	1224
Emd	1249	209	946	458
Fhl2	1319	2240	7695	3197
Gla	1271	89	1354	6
Jup	1280	2251	25961	14746
Lama4	1233	2439	16253	3119
Lamp2	1202	732	3485	4589
Lmna	1279	976	5076	1977
Mybpc3	1362	137	2025	592
Myh6	1314	118	5753	234

Myh7	1341	461	5832	427
Myl2	1330	205	646	472
Myl3	1197	293	819	482
Mylk2	1403	256	1552	858
Myoz2	1235	344	844	933
Nexn	1242	1939	7475	7584
Pkp2	1345	234	3398	2367
Pln	1024	254	215	1062
Prkag2	1293	3224	12154	12185
Psen2	1212	1818	4518	2407
Rbm20	1262	41	2014	1835
Ryr2	1270	565	5045	1941
Sgcd	1216	1623	3963	9768
Taz	1191	1139	3286	3161
Tmem43	1362	286	1218	1263
Tnnc1	1370	41	567	347
Tnnt2	1299	2222	5604	2586
Tpm1	1302	4249	15719	14114
Ttn	1117	648	28263	4702
Ttr	1287	224	750	435
Vcl	1295	396	7084	6209
Total	53802	38968	221294	130275

Legend: UTR*, untranslated region; CDS†, coding DNA sequence.

Table 3. Differentially expressed microRNAs experimentally validated to interact with genes under investigation.

miRNA*	MIMATid	Count	Genes	p-value	FDR†	Mean Ratio (HHR‡/NHR§)
hsa-miR-34a-5p	MIMAT0000255	3	PKP2, TPM1, VCL	0.000117868	0.00977691	1.83
hsa-miR-17-5p	MIMAT0000070	2	RBM20, RYR2	0.0000029	0.00076469	1.10

Legend: miRNA*, microRNA; FDR†, false discovery rate; HHR‡, hypertrophic heart rat; NHR§, normal heart rat.

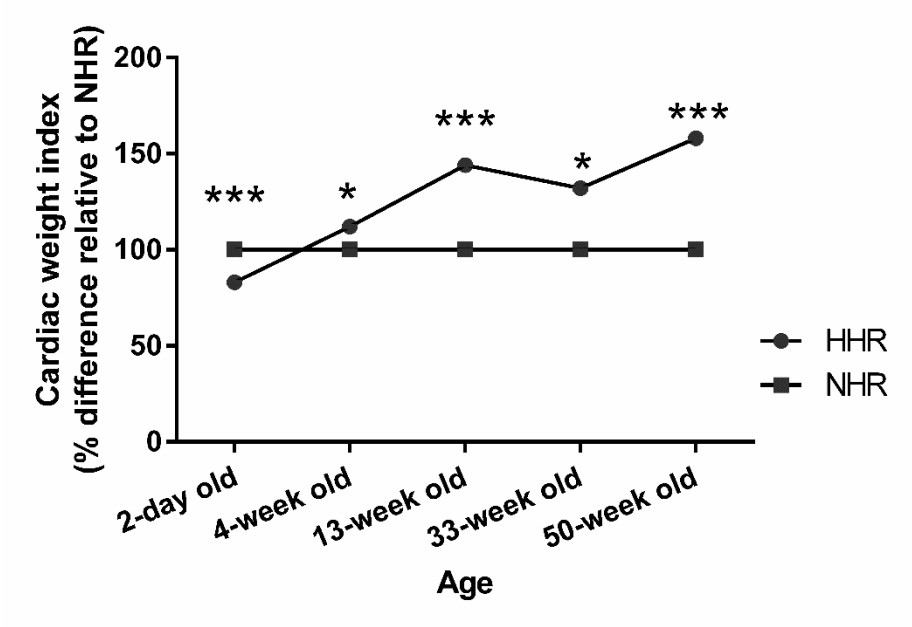
Table 4. Single nucleotide polymorphisms found in genes investigated associated with traits related to cardiovascular disease in human genome wide association studies.

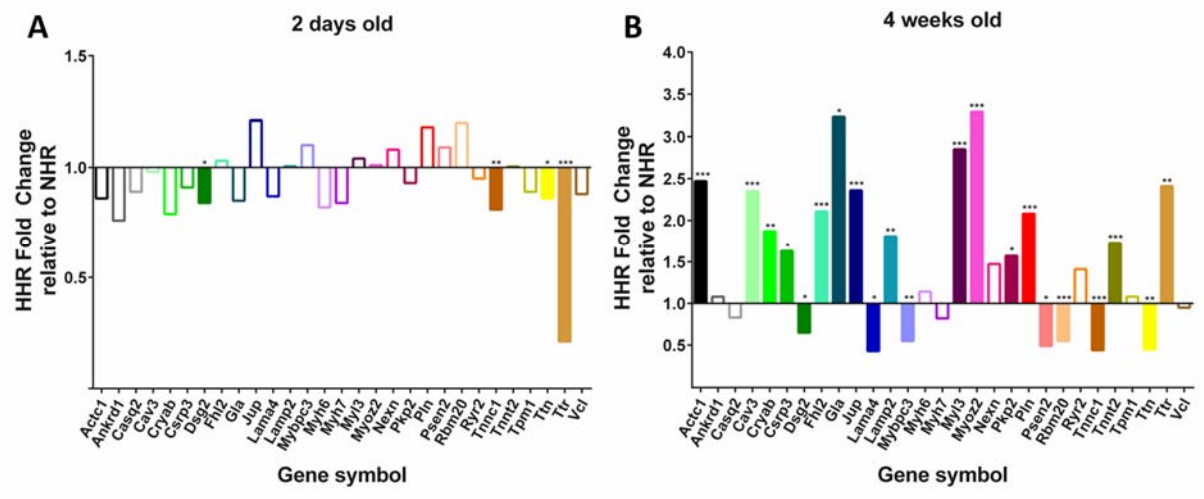
Trait	SNP count	Genes
Aortic root size	1	*VCL*
Atrial fibrillation	2	*RYR2, TTN*
Blood pressure	5	*RBM20, SGCD, RYR2, PRKAG2*
Blood pressure (response to angiotensin II receptor blocker)	3	*ABCC9, RYR2*
Blood pressure, CVD* RF† and other traits (body mass index, waist to hip ratio, renin activity and aldosterone concentration in plasma, BNP‡ levels in plasma, alcohol consumption)	9	*TTN, PRKAG2, MYH6, LAMA4*
Body mass index	4	*RYR2, PKP2, ACTN2*
Cardiac troponin-T levels	2	*TNNT2*
Cardiovascular disease	1	*RYR2*
Chronic kidney disease	1	*PRKAG2*
Coronary artery calcification	8	*PRKAG, SGCD, RYR2, MYBPC3, MYH6, RYR2*
Coronary artery disease	1	*VCL*
Coronary heart disease	2	*VCL, TNNT2*
ECG§ dimensions, brachial artery endothelial function, treadmill exercise responses	3	*PRKAG2, RYR2*

Electrocardiographic conduction measures	1	*RYR2*
Electrocardiographic traits	1	*MYH6*
Glomerular filtration rate	1	*PRKAG2*
Health and aging, CVD and cancer age of onset	1	*TTN*
Heart failure	1	*SGCD*
Heart rate	2	*CRYAB, MYH6*
Height	1	*DSC2*
Hypertension	2	*SGCD*
Hypertension (early onset hypertension)	2	*ACTN2*
Multiple complex diseases	26	*FHL2, RYR2, LMNA, CSRP3, PRKAG2, SGCD, RBM20, DSC2, ACTN2, LAMA4, MYLK2, MYOZ2*
Myocardial infarction	2	*CASQ2*
Obesity-related traits	7	*ABCC9, RYR2, PKP2*
Red blood cell traits	13	*PRKAG2, CTF1*
Resting heart rate	1	*MYH6*
Sudden cardiac arrest	1	*RBM20*
Triglycerides	5	*MYBPC3, VCL, RYR2, TNNT2, CSRP3*

Ventricular conduction	1	*CASQ2*
Waist circumference	1	*RYR2*

Legend: CVD*, cardiovascular disease; RF†, risk factors; BNP‡, brain natriuretic peptide; ECG§, electrocardiogram.


Cardiac weight index
(% difference relative to NHR)
200
150
100
50
0
***
*
***
*
***
2-day old
4-week old
13-week old
33-week old
50-week old
Age
HHR
NHR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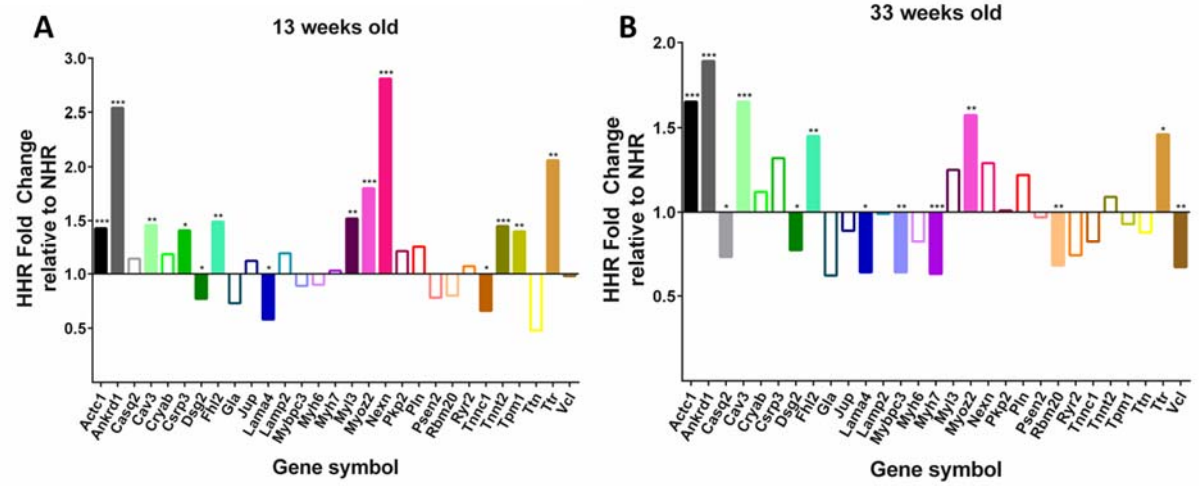
2 days old
HHR Fold Change relative to NHR
Gene symbol
B
4 weeks old
HHR Fold Change relative to NHR
Gene symbol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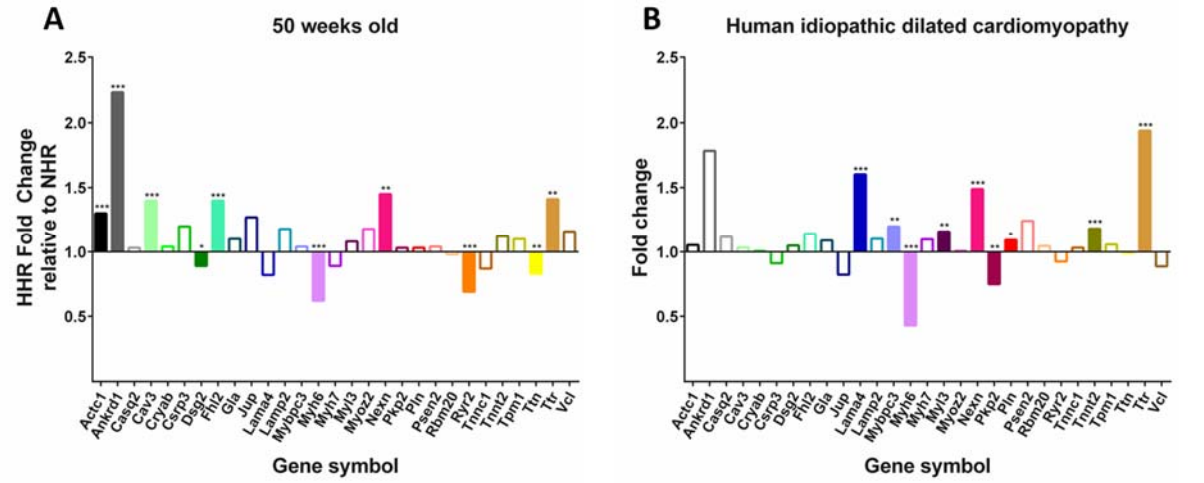
HHR Fold Change relative to NHR
Gene symbol
B
33 weeks old
HHR Fold Change relative to NHR
Gene symbol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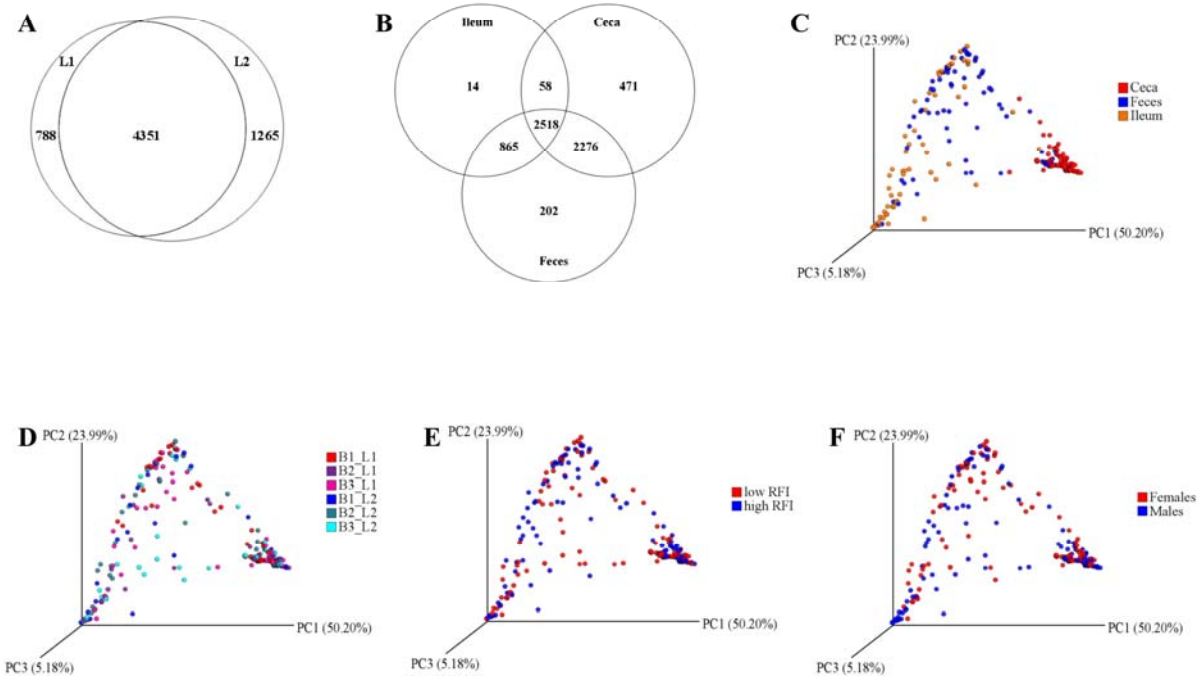
50 weeks old
HHR Fold Change relative to NHR
Gene symbol
B
Human idiopathic dilated cardiomyopathy
Fold change
Gene symbol

Figure 1


A
L1
L2
788
4351
1265
B
Ileum
Ceca
Feces
14
58
471
2518
865
2276
202
C
PC2 (23.99%)
PC1 (50.20%)
PC3 (5.18%)
Ceca
Feces
Ileum
D
PC2 (23.99%)
PC1 (50.20%)
PC3 (5.18%)
B1_L1
B2_L1
B3_L1
B1_L2
B2_L2
B3_L2
E
PC2 (23.99%)
PC1 (50.20%)
PC3 (5.18%)
low RFI
high RFI
F
PC2 (23.99%)
PC1 (50.20%)
PC3 (5.18%)
Females
Males