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Neither cardiac mitochondrial DNA variation nor copy number contribute to congenital heart disease risk

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Abstract

The well-established manifestation of mitochondrial mutations in functional cardiac disease (e.g., mitochondrial cardiomyopathy) prompted the hypothesis that mitochondrial DNA (mtDNA) sequence and/or copy number (mtDNAcn) variation contribute to cardiac defects in congenital heart disease (CHD). MtDNAcns were calculated and rare, non-synonymous mtDNA mutations were identified in 1,837 CHD-affected proband-parent trios, 116 CHD-affected singletons, and 114 paired cardiovascular tissue/blood samples. The variant allele fraction (VAF) of heteroplasmic variants in mitochondrial RNA from 257 CHD cardiovascular tissue samples was also calculated. On average, mtDNA from blood had 0.14 rare variants and 52.9 mtDNA copies per nuclear genome per proband. No variation with parental age at proband birth or CHD-affected proband age was seen. mtDNAcns in valve/vessel tissue (320 ± 70) were lower than in atrial tissue (1,080 ± 320, p = 6.8E−21), which were lower than in ventricle tissue (1,340 ± 280, p = 1.4E−4). The frequency of rare variants in CHD-affected individual DNA was indistinguishable from the frequency in an unaffected cohort, and proband mtDNAcns did not vary from those of CHD cohort parents. In both the CHD and the comparison cohorts, mtDNAcns were significantly correlated between mother-child, father-child, and mother-father. mtDNAcns among people with European (mean = 52.0), African (53.0), and Asian haplogroups (53.5) were calculated and were significantly different for European and Asian haplogroups (p = 2.6E−3). Variant heteroplasmic fraction (HF) in blood correlated well with paired cardiovascular tissue HF (r = 0.975) and RNA VAF (r = 0.953), which suggests blood HF is a reasonable proxy for HF in heart tissue. We conclude that mtDNA mutations and mtDNAcns are unlikely to contribute significantly to CHD risk.

Congenital heart disease (CHD) is the leading cause of mortality from birth defects, affecting about 1% of live births.[1] An increased recurrence risk of CHD in families and the identification of pathogenic *de novo* variants provides strong evidence for the role of genetic variation in the etiology of CHD. Statistical analysis of 2,645 parent-proband trios demonstrated that *de novo* mutations in more than 440 genes likely contribute to approximately 8% of CHD cases,[2,3] but only a fraction of these genes have been identified.

Because non-nuclear mitochondrial genes are critical to cellular respiration, pathogenic mutations in mtDNA often cause the dysfunction of tissue with high energy consumption. Cardiac muscle is a well-established tissue type for the manifestation of mitochondrial disease; an estimated 20%–40% of mitochondrial diseases have an associated cardiomyopathy.[4] During early cardiac development, the heart requires functioning mitochondria to produce ATP and as a signaling node to control myocyte function and differentiation.[5,6] Additionally, mitochondrial defects have been observed in a hypoplastic left heart syndrome mouse model,[7] and the nuclear mitochondrial gene *LONP1* (MIM: 605490) has been reported as a candidate CHD-associated gene.[8] In this study, we considered the hypothesis that variation in mitochondrial DNA (mtDNA) copy number or sequence could also

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contribute to perturbation of cardiac morphogenesis that leads to CHD.

To test for the presence or absence of an association between abnormal mtDNA and CHD, we assessed mtDNA copy number (mtDNAcn)—the number of mitochondrial genomes per nuclear genome—and defined rare non-synonymous mutations in the mtDNA from whole-genome sequencing (WGS) data of 1,837 CHD-affected proband-parent trios and 116 CHD-affected probands (without parent WGS), all recruited as part of the Pediatric Cardiac Genome Consortium (PCGC, Table S1). Here, we considered only variants encoded by the mitochondrial genome as opposed to nuclear encoded mitochondrial genes. For comparison, we assessed the mitochondrial genomes of 1,638 mother-unaffected sibling pairs from the Simons Simplex Collection of the Simons Foundation Autism Research Initiative[9,10] (SFARI, Table S1).

Due to the large number mitochondrial genomes per cell, many variants are heteroplasmic (i.e., two alleles are present in the same tissue).[11] The heteroplasmic fraction (HF) corresponds to the ratio of a variant allele to the total number of mitochondrial genomes. The variation of HF in different tissues from the same patient is poorly understood. Because HF in blood cells is frequently used as a proxy for HF in more disease-affected tissues, further analyses of the relationships between HF in different tissues are required. In this study, we performed analyses on WGS of 114 cardiovascular tissue samples paired with blood samples from the CHD-affected probands (PCGC) to identify the extent of HF variation between blood and heart tissue (Table S2).

mtDNA sequences were characterized by whole genome sequence analyses of blood, saliva, and cardiac tissues.[12] We calculated mtDNA copy numbers using the fastMitoCount program in the mitoAnalyzer software package.[13,14] mtDNAcns increased dramatically with age in atrial and ventricular tissue, while they remained relatively constant in valve and vessel tissue (Table S3). Given that our cohort is comprised of young individuals (mean age = 3 ± 4.8 years, max age = 18 years), we employed a linear model to describe the correlation between mtDNAcn and affected individual age, with the caveat that this linear rate would likely not hold true across a larger age range. Mean rates of change in atrial and ventricular tissue were 47 (r = 0.66; p = 8.4E−7; Figure 1A) and 35 (r = 0.70; p = 7.5E−7; Figure 1B) mtDNAcn/year, respectively, while the correlation in valve and vessel samples was not significant (p = 0.48; Figure 1C). We also note that mtDNAcns are higher in ventricle tissue than atrial tissue, which are higher than valve/vessel tissue with respective y-intercepts of 1,220, 980, and 330. Saliva-derived samples had approximately 3.5-fold more mtDNA copies per cell (n = 114; mean = 185 ± 80) than blood-derived samples (n = 5,373; mean = 52 ± 14; t test p = 3.9E−34). Therefore, comparisons of mtDNAcn between probands and parents were restricted to sequences from blood-derived DNA to avoid confounding issues of copy number variation between tissue types. Additionally, some low blood sample volumes were processed separately, and mtDNAcn was found to differ by DNA extraction procedure. 5,373 DNA samples were extracted using protocol 1 as described in the supplemental methods section and in previous publications, while 149 samples were extracted by an alternate procedure (protocol 2 in the supplemental methods section) which affected the ratio of mtDNA

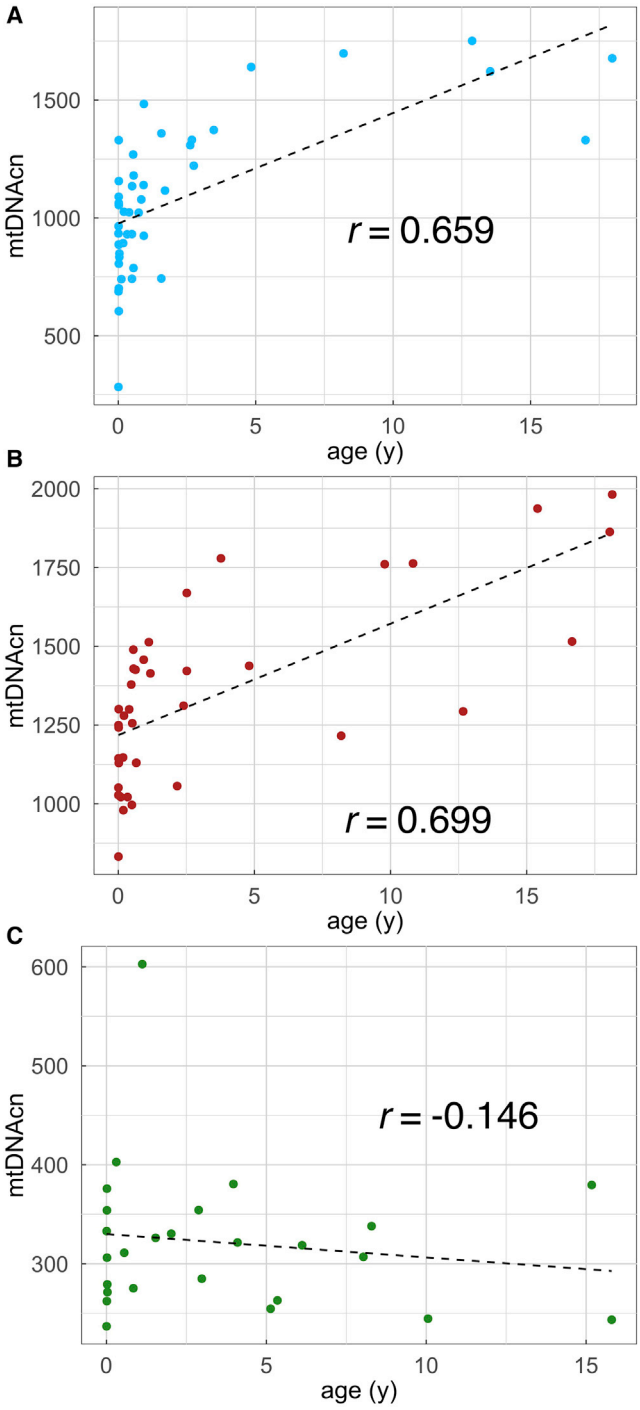

Figure 1. mtDNAcn versus age in cardiovascular tissue
mtDNAcn plotted against age for (A) atrial (n = 45), (B) ventricular (n = 39), and (C) valve/vessel tissues (n = 26) with Pearson correlation values (*r*) reported.

to nuclear DNA captured; these samples were excluded from the mtDNAcn analysis.

To test the hypothesis that mtDNA copy number varied with age or sex, we compared the mtDNA copy number within the total cohort. mtDNA copy number was not significantly influenced by subject age (Figure 2; n_{pro} = 1,830; n_{mo} = 1,764; n_{fa} = 1,745; $r = -0.02$; p = 0.20) or sex (t test p = 0.86). mtDNAcn was lowest in individuals with European haplogroups (n = 3,668; mean = 52.0) followed by individuals with African haplogroups (n = 488; mean = 53.0) and individuals with Asian haplogroups (n = 1,216; mean = 53.5); the only significant difference was between individuals with European and Asian haplogroups (t test p = 2.6E−3). mtDNAcn has previously been shown to have a high degree of heritability.[13] In our cohort, CHD proband mtDNAcn was correlated with maternal mtDNAcn (slope = 0.20, r = 0.260, p = 1.5E−57, Figure S2A), as was proband and paternal mtDNAcn (slope = 0.16, r = 0.198, p = 1.5E−33, Figure S2C). Maternal and paternal mtDNAcn in the CHD cohort were also correlated (slope = 0.54; r = 0.531; p = 5.8E−33; Figure S2E), suggesting that these correlations are a result of environmental factors and/or shared parental ancestry. All three relationships were also observed among SFARI cohort trios, though the correlation between parents was not as pronounced (unaffected sibling-mother: slope = 0.29, r = 0.313, p = 1.1E−38, Figure S2B; unaffected sibling-father: slope = 0.30, r = 0.343, p = 1.5E−46, Figure S2D; mother-father: slope = 0.14, r = 0.156, p = 2.0E−10, Figure S2F).

We used the MToolBox v.1.0 pipeline[15] to realign WGS reads to the mitochondrial Reconstructed Sapiens Reference Sequence (RSRS)[16] and perform variant calling. From the MToolBox predicted haplogroups, simplified haplogroups were assigned to the CHD and SFARI subjects, with L, L0, L1, L2, L3, L4, L5, and L6 corresponding to African ancestry, A, B, C, D, E, F, G, M, N, O, P, Q, S, Y, and Z corresponding to Asian ancestry, and H, HV, I, J, K, R, T, U, V, W, and X corresponding to European ancestry. The distributions of CHD-affected probands' haplogroups and SFARI unaffected siblings' haplogroups were similar (Figure S3), though the CHD cohort had more African and Asian haplogroups (CHD: 8.9% African, 24.1% Asian, 67.1% European; SFARI: 3.9% African, 11.9% Asian, 84.2% European). As a second comparison cohort, we used the CHD-affected probands' fathers, who do not contribute to the mitochondrial genome of their offspring. The CHD-affected probands' fathers' haplotype distribution was indistinguishable from the CHD-affected probands' haplotype distribution (9.7% African, 22.0% Asian, 68.2% European).

Mitochondrial DNA variants of 48,882 individuals, reported by Mitomap in the Mitobank (revision r86), were used to determine population frequencies.[17] We defined a variant as rare if it fulfills two criteria: (1) the variant is found in < 0.01% of the Mitomap population, and (2) the variant is found in zero Mitomap individuals of the same simplified haplogroup. Insertions, deletions, and variants in the mitochondrial hypervariable segments (HVS-I: chrM:16,024–16,383; HVS-II: chrM:57–372; HVS-III: chrM:438–574)[16,18] were excluded for this analysis due to the high false-positive rate in those variant types. Additionally, to avoid inaccurate variant assignments caused by misaligned reads, individuals with mtDNAcn < 10 (n = 9) were excluded. The heteroplasmic fractions (HFs) of rare variants in CHD-affected probands and their mothers were remarkably similar, as were the heteroplasmic fractions observed in SFARI unaffected siblings and mothers. 279 rare variants were identified in CHD-affected probands (226 with HF > 0.5; Table S4) and 227 rare variants were identified in SFARI unaffected siblings (184 with HF > 0.5; Table S5), among 1,945 probands in the CHD cohort and 1,638 SFARI unaffected siblings; 105 of the CHD variants and 81 of the variants in SFARI unaffected siblings with HF > 0.1 did not appear in Mitobank, including 8 variants that were present in both cohorts. The number of rare variants per CHD-affected proband was indistinguishable from the number of rare variants per SFARI subject for non-synonymous rare variants with HF > 0.5 in the child, and still no significant increase was found in CHD-affected probands after limiting to variants with HF at least 10% higher in the child than in the mother. This remained true if rare synonymous variants were also considered (HF > 0.5 [p = 0.8] or for HF > 0.1 [p = 0.7]). The distribution of rare variants in CHD-affected probands, stratified by functional consequence (damaging missense, Dmis) or gene function (tRNA, or D-loop), were indistinguishable from the distribution of rare, stratified variants in SFARI unaffected siblings or CHD-affected fathers (Table S6). We performed additional comparisons of variant frequency in CHD-affected probands with specific lesions (i.e., atrial septal defects, conotruncal defects, hypoplastic left heart syndrome, heterotaxy, and tetralogy of Fallot) and the frequency of variants among SFARI

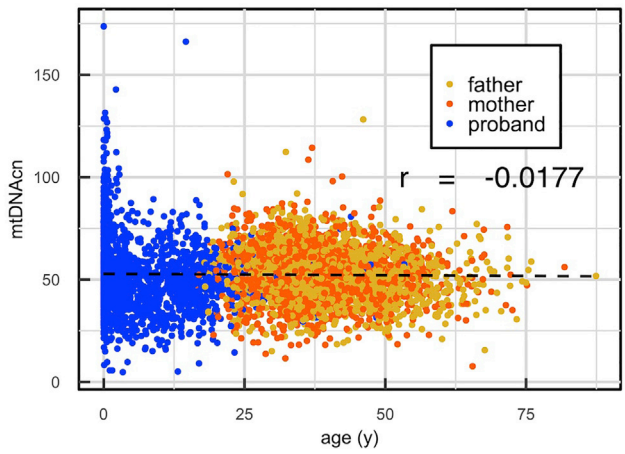

Figure 2. mtDNAcn versus age in blood
mtDNAcn plotted against age for probands (blue; n = 1,830), mothers (red; n = 1,764), and fathers (gold; n = 1,745). Only samples from whole blood were included, with Pearson correlation values (r) reported.

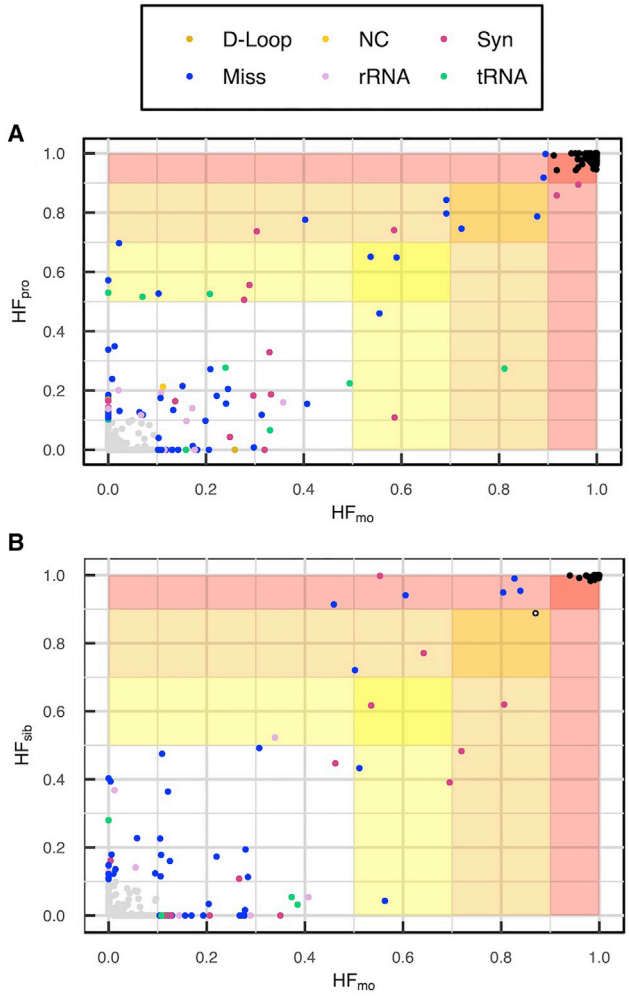

Figure 3. HF in children versus mothers
Variant HF in children is plotted against the maternal HF for variants discovered in either the child or the mother for (A) CHD and (B) SFARI. Variants are colored by classification as the control region (D-Loop), non-coding regions (NC), synonymous (Syn), missense (Miss), ribosomal RNA (rRNA), or transfer RNA (tRNA). Variants with HF < 0.1 in both child and mother are considered effectively homoplasmic wild type and are displayed in gray, while those with HF > 0.9 in both child and mother are considered effectively homoplasmic mutant and are displayed in black. The previously unreported variant 16469G is displayed in white.

cohort subjects but no lesion was significantly enriched for rare non-synonymous mutations.

Four of the CHD-affected probands were found to have variants at HF > 0.5 that were previously confirmed to be associated with disease (two with 1555G, one with 14484C, and one with 11778A). 1555G is reported as causing deafness and autism spectrum intellectual disability and may be anti-atherosclerotic; 14484C has been reported to cause Leber's hereditary optic neuropathy (LHON [MIM: 535000]); and 11778A has been reported to cause LHON and progressive dystonia (MIM: 500001). None of the probands with these mutations reported signs of these conditions, though the probands were likely too young to present symptoms of the diseases at time of enrollment. Three SFARI unaffected siblings were found to have disease-associated variants with HF > 0.5 (two with 14484C and one with 1555G).

Because mitochondria are inherited maternally, probands and their fathers should not share rare variants. However, two rare variants with HF > 0.1 are shared by the proband and his/her father. These two probands are offspring of either a first cousin or second cousin marriage and all three family members share the same haplogroup.

Among 5,603 CHD-affected and 4,914 SFARI participants (probands and parents), we identified three previously unreported sub haplotypes. One variant—16469G, (white in Figure 3; Table S4)—was not observed in Mitomap individuals but was found in all subjects with haplogroup A2u1 (n = 6; 2 CHD-affected probands/mothers, 1 SFARI unaffected sibling/mother) and was absent in all other individuals. A missense variant in the *MT-CYB* gene (MIM: 516020), 14903G, was found in all individuals with the haplogroup D1i2 (n = 4; 2 CHD-affected probands/mothers) and was absent in all other individuals. A synonymous variant in the *MT-CO1* (MIM: 516030) gene, 6017G, was found in all individuals with haplogroup H56a1 (n = 4; 3 SFARI unaffected siblings, 1 CHD-affected father). This variant was present in one individual with haplogroup L1c in Mitomap as well as the mothers of the three SFARI unaffected siblings, who were identified as having haplogroups H2a2a1, H1e1a6, and U2e1a.

To test whether variant HF differed between blood and cardiac tissue, we calculated variant HF using blood and tissue-derived DNA from 114 individuals with CHD sequenced as part of the Trans-Omics for Precision Medicine (TOPMed) Program. No variants were found to be unique to either blood or tissue for these individuals and Figure 4 shows that the HF in blood correlates well with the HF in tissue ($r = 0.975$; $p = 6.5E{-}12$). We performed a similar mitochondrial variant analysis using MToolBox on mitochondrial reads extracted from RNA-seq data for 257 cardiovascular tissue samples (Table S3) from CHD-affected probands and, as a control, mitochondrial reads extracted from WGS and RNA-seq data for 205 right atrial (RA) and 263 left ventricle (LV) tissue samples from the Genotype-Tissue Expression (GTEx) project.[19] MToolBox output for RNA-seq data was filtered to include only variants in protein coding regions. Low-depth sites (DP < 100) were found to occur near the end of transcripts and in regions where reads commonly misaligned to nuclear mitochondrial sequences (NUMTs; see supplemental methods);[20,21] these sites were removed to avoid skewed variant allele fractions (VAFs). Variants found in 5 or more unrelated samples with HFs consistently below 70% were assumed to be alignment errors as opposed to true heteroplasmic variants arising in independent lineages. Cardiovascular RNA-VAF was compared to blood-derived DNA-HF for CHD-affected and GTEx subjects (Figure 5). For both cohorts there is a strong

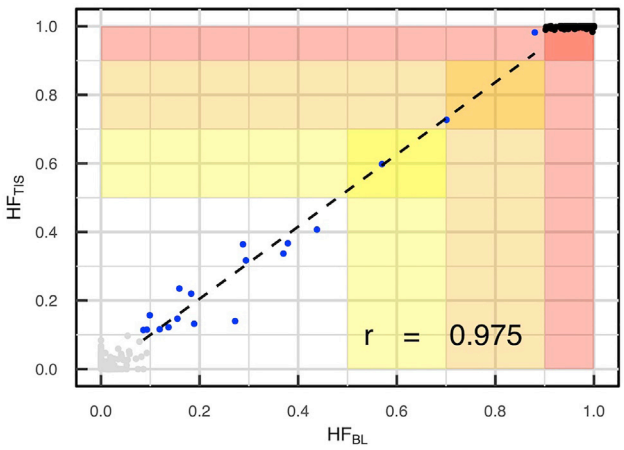

Figure 4. HF in blood versus tissue
Variant HF in tissue was plotted against variant HF in blood for 114 individuals with CHD. Heteroplasmic variants are colored in blue, those with HF < 0.1 in both blood and tissue are considered homoplasmic wild type and are displayed in gray, while those with HF > 0.9 in both blood and tissue are considered homoplasmic mutant and are displayed in black. The Pearson correlation (*r*) was calculated including only heteroplasmic variants.

correlation ($r_{CHD} = 0.953$; $r_{GTEx} = 0.951$) between blood HF and cardiovascular RNA VAF for heteroplasmic variants. These results strongly suggest that, in most cases, (1) blood HF is representative of the HF in a range of cardiovascular tissue (as was also indicated by the direct comparison of HF in blood and tissue DNA) and (2) HF is a reasonable predictor for the VAF in RNA.

In summary, rare mitochondrial variants are not enriched in the CHD cohort compared to SFARI, and there was no deviation from the expected mtDNAcn among CHD-affected probands. We therefore conclude that neither mitochondrial copy number variation nor mitochondrial gene variants are likely to contribute significantly to CHD risk or to act as genetic modifiers for CHD.

Data and code availability

Mitochondrial copy numbers were calculated using the fastMitoCalc program in the mitoAnalyzer software package. Mitochondrial variants were identified using the MtoolBox package. Population frequencies of variants were found in the Mitobank. CHD whole-genome sequencing was either done as part of the NIH Gabriella Miller Kids First Program or the Trans-Omics for Precision Medicine (TOPMed) program and are available via controlled access from dbGaP (dbGaP:phs001138.v1.p2 and dbGaP:phs001735.v2.p1, respectively). CHD RNA-seq data are also available via controlled access from dbGaP (dbGaP:phs000571.v6.p2). Additional data files may be obtained from the authors upon request.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.ajhg.2022.03.011.

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Declaration of interests

The authors declare that they have no competing interests.

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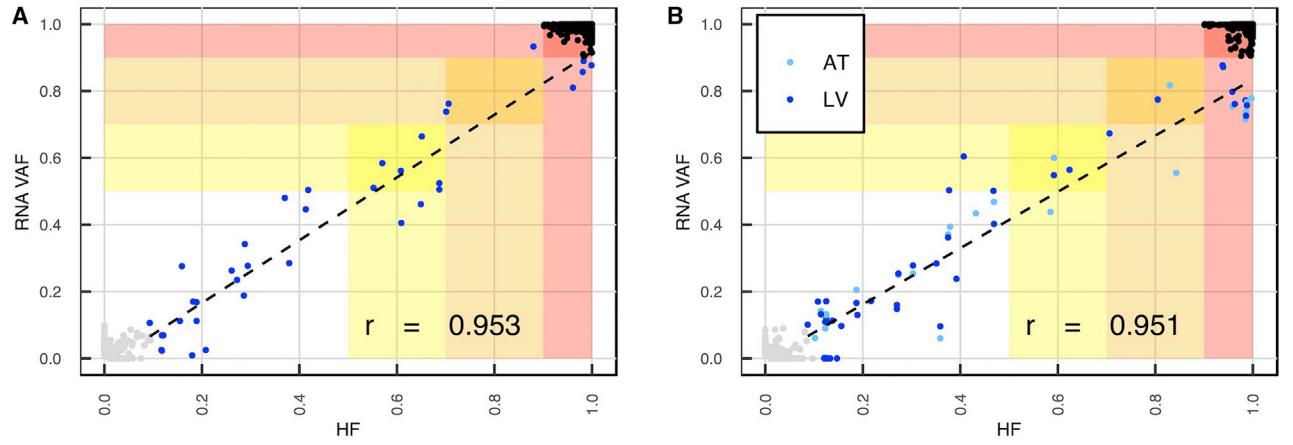

Figure 5. Cardiovascular RNA VAF versus blood HF
The VAF is plotted against HF of variants discovered in either the blood mtDNA or the cardiovascular mtRNA in (A) CHD-affected probands and (B) GTEx subjects. Variants with both VAF and HF < 0.1 considered effectively homoplasmic wild type and are displayed in gray, while those with both VAF and HF > 0.9 are considered effectively homoplasmic mutant and are displayed in black. The Pearson correlations (*r*) were calculated including only heteroplasmic variants.

Web resources

mitoAnalyzer, https://lgsun.irp.nia.nih.gov/hsgu/software/mitoAnalyzer/index.html
MitoBank, https://mitomap.org/foswiki/bin/view/MITOMAP/Mitobank
MToolbox, https://github.com/mitoNGS/MToolBox
OMIM, https://www.omim.org/

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