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Mitochondrial DNA sequence variation is associated with free-living activity energy expenditure in the elderly

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Abstract

The decline in activity energy expenditure underlies a range of age-associated pathological conditions, neuromuscular and neurological impairments, disability, and mortality. The majority (90%) of the energy needs of the human body are met by mitochondrial oxidative phosphorylation (OXPHOS). OXPHOS is dependent on the coordinated expression and interaction of genes encoded in the nuclear and mitochondrial genomes. We examined the role of mitochondrial

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The authors declare no conflict of interest

genomic variation in free-living activity energy expenditure (AEE) and physical activity levels (PAL) by sequencing the entire (~16.5 kilobases) mtDNA from 138 Health, Aging, and Body Composition Study participants. Among the common mtDNA variants, the hypervariable region 2 m.185G>A variant was significantly associated with AEE (p=0.001) and PAL (p=0.0005) after adjustment for multiple comparisons. Several unique nonsynonymous variants were identified in the extremes of AEE with some occurring at highly conserved sites predicted to affect protein structure and function. Of interest is the p.T194M, *CytB* substitution in the lower extreme of AEE occurring at a residue in the Qi site of complex III. Among participants with low activity levels, the burden of singleton variants was 30% higher across the entire mtDNA and OXPHOS complex I when compared to those having moderate to high activity levels. A significant pooled variant association across the hypervariable 2 region was observed for AEE and PAL. These results suggest that mtDNA variation is associated with free-living AEE in older persons and may generate new hypotheses by which specific mtDNA complexes, genes, and variants may contribute to the maintenance of activity levels in late life.

Keywords

metabolic rate; energy expenditure; mitochondria; mtDNA; oxidative phosphorylation; DNA sequencing

1.0 Introduction

Activity energy expenditure (AEE) decreases with age [1, 2] and this decline is associated with an increased risk of mortality, disability, neuromuscular and neurological impairments, and a range of age-associated pathological conditions [3]. Higher free-living AEE is strongly associated with lower risk of mortality among older adults [4]. Manini et al. [4] showed that for every 287 kcal/d in free-living activity energy expenditure (approximately 1 1/4 hours of activity per day), there is approximately a 32% lower risk of mortality. Higher levels of physical activity are associated with reductions in coronary heart disease [5], cancer incidence [6], falls [7], and physical disability [8]. It is unknown, however, why energetic decline occurs and how AEE protects older adults from physical disability, disease and premature mortality. The factors that determine energy balance vary between persons and are to some extent genetically determined [9-14]. The heritability for AEE is 72% [14] and genetic factors explain 30-47% [11, 13] of the variance in resting metabolic rate.

Mitochondrial oxidative phosphorylation (OXPHOS) supplies the vast majority (90%) of the energy needs of the human body. Mitochondrial OXPHOS is a highly efficient system dependent upon the coordinated expression and interaction of genes encoded in both the nuclear and mitochondrial genomes. OXPHOS enzyme activities decline with age in human and primate muscle [15-17], liver [18], and brain [19, 20] and correlate with the accumulation of somatic mitochondrial DNA (mtDNA) deletions [21-46] and base substitutions [47-51]. During the lifetime of an individual, mtDNA undergoes a variety of mutation events and rearrangements that may be important factors in the age-related decline of somatic tissues [52-56]. The progressive and gradual accumulation of mtDNA mutations has been hypothesized to account for the decrease in scope of activity affiliated with the reduced function of cells and organs that accompany the aging process [57].

Hundreds of genes responsible for mitochondrial assembly, metabolism, growth, and reproduction are distributed throughout the nuclear and mitochondrial genomes [58, 59]. This includes the ~100 nuclear- and mitochondria-encoded polypeptide genes for five OXPHOS complexes [58, 59]. The mtDNA contains the highest density of bioenergetic genes, including 13 OXPHOS genes that encode protein components of complexes I, III, IV,

and V [60]. The mtDNA is a circular double-stranded DNA molecule of 16,569 bases that does not recombine, is maternally inherited [61], and has a unique organization in that its genes lack introns, intergenic spaces, and 5′ and 3′ noncoding sequences. Impaired mitochondrial function resulting from mtDNA and/or nuclear DNA variation is likely to contribute to an imbalance in cellular energy homeostasis, increase in oxidative stress, and accelerate or inappropriately terminate senescence and aging.

The evolution of human mtDNA is characterized by the emergence of distinct lineages (haplogroups) associated with the major global ethnic groups. It is clear that European Ancestry is linked with energy expenditure [62], but in a recent effort we identified specific major African and European haplogroups that had significantly different resting metabolic rate (RMR) and total energy expenditure (TEE) [63]. Both RMR and TEE were significantly elevated in the major European haplogroup N compared to the major African haplogroup L and significant heterogeneity was observed within the African and European lineages [63]. These results demonstrate that mtDNA variants underlying specific haplogroups affect human RMR and TEE and therefore motivate the additional investigation mtDNA sequence-level associations with free-living activity energy expenditure.

While it is clear that AEE levels are associated with environmental factors, mtDNA mutations could have implications for the degree to which physical activity is performed daily. For example, individuals who harbor certain mtDNA mutations would be unable to effectively optimize mitochondria's ability to rephosphorylate ATP for cellular activities. Research seeking to identify genetic factors that contribute to complex phenotypes such as metabolic rate must be sensitive to the various ways in which genes and genetic perturbations operate. For example, it is now recognized that common genetic variants play a much smaller role in mediating phenotypic expression and disease risk than previously thought [64-67] and that collections of rare variants are likely to influence normal ranges of phenotypic expression in important ways [66, 68-74]. Since human mtDNA has a mutation rate that is 10-20 times higher than that of nuclear DNA [75-77] and up to one-third of sequence variants found in the general population may be functionally important [59], it is possible that the majority of variation that impacts function is rare in frequency and only detectable by direct sequencing [78]. Different loci may exhibit different relationships between allele frequency and functional effect. In addition, some genes may harbor functional alleles at higher frequencies, whereas other genes may have only private functional variants. Indeed, it may be that the simultaneous effect of all mtDNA mutations combined are responsible for the gross physiological and pathological changes associated with the decline in scope of activity observed in aged tissue. Following our previous results wherein heterogeneity in TEE was observed among European mitochondrial lineages [63], we sequenced the entire mitochondrial genome in these subjects to examine specific mtDNA variants and aggregate sequence variation associated with differences in AEE.

2.0 Materials and Methods

We examined the role of mtDNA sequence variation in metabolic rate and energy expenditure by sequencing the entire mtDNA from 138 participants from the Health, Aging, and Body Composition Study. The role of individual variants was first assessed in these phenotypes with an emphasis on nonsynonymous (NS) substitutions at the extremes of free-living AEE. *In-silico* methods were employed to examine mtDNA nucleotide conservation and predict the functional implications of NS substitutions on amino acid protein sequences. We then examined the collective effects of variants within genes or genomic regions using several rare variant burden tests and assessed singleton burden.

2.1 Participants

Participants were part of the Health, Aging and Body Composition (Health ABC) study, a prospective cohort study of 3,075 community-dwelling black and white men and women living in Memphis, TN, or Pittsburgh, PA, and aged 70-79 years at recruitment in 1996-1997. To identify potential participants, a random sample of white and all black Medicare-eligible elders, within designated zip code areas, were contacted. To be eligible, participants had to report no difficulty with activities of daily living, walking a quarter of a mile, or climbing 10 steps without resting. They also had to be free of life-threatening cancer diagnoses and have no plans to move out of the study area for at least 3 years. The sample was approximately balanced for sex (51% women) and 41% of participants were black. Participants self-designated race/ethnicity from a fixed set of options (Asian/Pacific Islander, black/African American, white/Caucasian, Latino/Hispanic, do not know, other). The study was designed to have sufficient numbers of black participants to allow estimates of the relationship of body composition to functional decline. All eligible participants signed a written informed consent, approved by the institutional review boards at the clinical sites. This study was approved by the institutional review boards of the clinical sites and the coordinating center (University of California, San Francisco).

2.2 Metabolic Rate and Energy Expenditure

In 1998-1999, free-living activity energy expenditure was assessed in 302 high-functioning, community-dwelling older adults (aged 70-82 years) from the Health ABC study [4]. The present sequencing study is focused on 138 Health ABC participants of European genetic ancestry with measured free-living AEE. Briefly, RMR was measured via indirect calorimetry on a Deltatrac II respiratory gas analyzer (Datex Ohmeda Inc, Helsinki); detailed procedures have been described elsewhere [79]. TEE was measured using what is considered the gold-standard and involves a 2-point doubly-labeled water technique that has been previously described [80]. Free-living activity energy expenditure was expressed in two ways [81]. AEE was calculated as [(total energy expenditure*0.90) – resting metabolic rate], removing energy expenditure from the thermic effect of meals that is estimated at 10% of TEE and subtracting energy devoted to basal metabolism. Physical activity level (PAL) is another method for expressing energy expenditure due to physical activity and was calculated as a ratio of TEE and RMR (TEE/RMR). The division of TEE by RMR, a major determinate of which is lean mass, adjusts for differences in body composition (in part reflecting weight and sex) [2]. The PAL formula was adopted by the Food and Agriculture Organization, the World Health Organization, and the United Nations University [82] and these agencies have developed physical activity level categories (sedentary: 1.40-1.69; active, 1.70- 1.99; vigorous activity, 2.00-2.40). AEE and PAL are highly correlated in the current analysis (r=0.87) but we provide results for both energy expression types since these offer different advantages (e.g., simplicity of expression and inherent control for differences in body composition, respectively).

2.3 Mitochondrial DNA sequencing

MtDNA extracted from platelets was sequenced with the Affymetrix Mitochondrial Resequencing Array 2.0 (MitoChip, Affymetrix, Santa Clara, CA). The MitoChip interrogates the forward and reverse strands of the 16.5 kb mitochondrial genome for a total of ~30 kb sequence, enables the detection of known and novel mutations and has redundant probe tiling for detecting the major human mitochondrial haplotypes and known disease-related mutations. Built-in redundancy via independent probe sets also allows a test of within-chip reproducibility. Briefly, the entire mitochondrial genome was first amplified in two long-range PCR reactions using LA PCR Kit (Takara Bio U.S.A., Madison, WI) for each sample using two sets of overlapping primers. Mitochondrial fragments were amplified and prepared for array hybridization according to the Affymetrix protocol for GeneChip

CustomSeq Resequencing Array. The resulting PCR products were assessed qualitatively by 1% agarose gel electrophoresis and purified using a Clonetech Clean-Up plate (Clonetech, Mountain View, CA). The purified DNA was quantified by PicoGreen and for selected samples, confirmed by NanoDrop measurements. The amplicons were pooled at equi-molar concentrations. Chemical fragmentation was performed and products were confirmed to be in the size range of 20-200 bp by 20% polyacrylamide gel electrophoresis with SYBR Gold staining. The IQ-EX control template, a 7.5 kb plasmid DNA, was used as a positive control. The samples were labeled with TdT and hybridized to the array in a 49°C rotating hybridization oven for 16 hours. Finally, streptavidin phycoerythrin (SAPE), and then antibody staining was performed. The microarrays were processed in the GeneChip Fluidic Station and the GeneChip Scanner. Signal intensity data was output for all four nucleotides, permitting quantitative estimates of allelic contribution. The allelic contribution was assessed using the raw data from the individual signal intensities by deriving the ratio of expected allele (REA), which is the log ratio of the raw signal intensity of the expected allele at any site (as defined by the mtDNA reference sequence) to the average raw signal intensity of the other three alleles, at each site for every individual. DAT files with raw pixel data were generated and used as input for grid alignment. CEL files generated from DAT files were analyzed in batches using GSEQ. Samples with call rates of less than 95% were discarded. For samples passing initial filtering, ResqMi 1.2 [83] was used for re-analysis of bases originally called as “N” by GSEQ. Analysis was performed using custom Perl scripts. Data was extracted from gene regions as defined by NCBI annotations for the revised Cambridge Reference Sequence (rCRS; NC_012920.1).

2.4 Analysis of Individual Variants

Rare sequence variants (minor allele frequency [MAF] <5%) were identified from 48 participants in the extremes (± 1SD from the mean) of free-living energy expenditure (AEE < 401 kcal/d vs. 907 kcal/d). These included rare variants from the OXPHOS coding regions (both NS and synonymous [S]), ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and each of the three hypervariable (HV) regions. Several *in-silico* methods were employed to examine mtDNA nucleotide conservation (PhastCons [84] and PhyloP [85]) for all variants and to predict the potential functional consequences of NS substitutions on amino acid protein sequences (Sorting Intolerant From Tolerant ’ (SIFT) [86, 87], MutPred [88], and PolyPhen2 [89]). The potential effects of NS substitutions on *CytB*, *COI*, *COII*, and *COIII* were examined with the PyMOL molecular visualization system (v1.4) using the bovine mitochondrial bc1 complex structure with antimycin bound (PDB 2A06, 2.28 Å resolution) [90] and complex IV reduced (PDB 2EIJ, 1.9 Å resolution) and oxidized (PDB 2DYR, 1.8 Å resolution) structures [91, 92]. Mammalian complexes I and V (F_0 subunit) are not currently available for molecular modeling of mitochondrial encoded proteins.

For individual common mtDNA sequence variants (MAF ≥ 5%) we compared differences in RMR, TEE, AEE and PAL for each allele (mtDNA is single-copy) using a generalized linear model in R (v 2.12.0). All analyses were adjusted for age, sex, lean mass, and 10 eigenvectors of mitochondrial genetic ancestry derived from principal component analysis (PCA, calculated using SAS version 9.1, SAS Institute Inc, Cary, NC). The first 10 eigenvectors account for 59% of the variance in the mtDNA sequence dataset. The cumulative variance explained by eigenvectors did not substantially increase after the 10th PC. Mitochondrial PCA has been shown to outperform haplogroup-stratified or adjusted association analyses with no loss in power for the detection of true associations [93]. Additionally, correlation between nuclear and mitochondrial PCs was limited and adjustment for nuclear PCs had no effect on mitochondrial analysis [93].

2.5 Analysis of Aggregated Variants

The joint effects of all mitochondrial variants within each gene on energetic measures of interest were evaluated using several rare variant burden tests. Pooled associations of all sequence variants were run using VT test [94] in R and included the T1 (1% MAF threshold) [95], T5 (5% MAF threshold) [95], WE (weighted-sum) [96], and VT (variable threshold) approaches [97]. Energetic measures were adjusted for covariates of age at exam, sex and study site using residuals from linear regression and then normalized to Z scores prior to conducting analyses. We applied these approaches to the four energetic traits and computed statistical significance for each test using 10,000 independent simulations. Variant aggregations were tested across the following regions: 1) the individual OXPHOS complexes; 2) all rRNAs combined; 3) all tRNAs combined; and 4) each of the three HV regions.

Singletons are variants occurring in single participants that can be quantified to identify genes or genetic regions that harbor significantly higher mutation burdens between groups (e.g. cases vs. controls or phenotype extremes) and possibly play a role in the etiology of a particular disease or trait. Fisher's exact tests were used to compare the total number of singleton variants between participants with little activity (PAL<1.70) and moderate to high activity (PAL≥1.70) for: 1) the entire mitochondrial genome; 2) the individual OXPHOS complexes; 3) the individual genes encoding OXPHOS complexes; 4) all tRNAs combined; 5) all rRNAs combined; and 6) each of the HV regions.

3.0 Results

A total of 135 Health ABC participants yielded sequence data of sufficient quality for analysis. Of these, 63 were men and 72 were women, with mean (SD) age of 73.4 (2.9) years. Six participants were missing doubly-labeled water measurements resulting in a sample size of 129 for analyses involving TEE, AEE and PAL. Sequencing of 16,544 mtDNA bases (positions 12-16,555) from 135 participants yielded a cumulative total of 449 variants including: 56 common (MAF ≥5%), 160 low frequency variants (MAF 1-5%), and 233 singletons. The 10 duplicate samples had >98% sequence concordance (the majority of discordant calls resulted from positions successfully called in one but called as "N" in another). The within-chip error rate was 0.0028%, which is comparable to previously published rates of 0.0025% and 0.0021% [98, 99].

3.1 Individual Variants

We identified a large number of unique OXPHOS, rRNA, tRNA and HV region variants that are unique to individuals at the high and low ends of the AEE distribution with some occurring at sites that are highly conserved and predicted to affect protein structure or function (Tables 1, S1 and S2). While the focus of this analysis was to identify variants at the extremes of AEE, additional variants were also unique to the participants with intermediate AEE levels (Table S3). Most substitutions were unique to single individuals including six *CytB* NS substitutions unique to high and low AEE. Of these, several were predicted to significantly affect function: p.T61A; p.D171N; p.I338V; and p.N374D and, and/or to be highly conserved: p.A191T; p.T194M; and p.N374D. Examining the structural model of bovine cytochrome bc1 complex identified the p.A191T, *CytB* and p.T194M, *CytB* substitutions as occurring in a potentially functionally relevant site (Figure 1). Some substitutions observed in multiple samples were consistently unique to high (p.T533M, *ND5*) or low (p.I338V, *CytB*) AEE levels. Two additional variants in the HV2 region were observed in multiple samples that were consistently unique to high (m.200A>G) or low (m. 263G>A) AEE levels.

Removing common variants found to be in complete LD (r^2=1) yielded 47 "independent" SNPs with minor allele frequency (MAF) ≥5%. Among the 47 "independent" variants, the m.185G>A was significantly associated with AEE (p=0.001) and PAL (p=0.0005) after adjustment for multiple comparisons (adjusted p=0.001). AEE and PAL values among the 7 carriers of the m.185G>A variant allele were 937.9 (174.3 SD) and 1.93 (0.18), respectively. This compares with AEE and PAL values of 637.3 (452.4 SD) and 1.66 (0.20), respectively, among the common allele carriers of the m.185G>A variant allele.

3.2 Aggregated Variants

Significant pooled effects (p≤0.01 due to multiple test correction) across the HV2 region were observed for free-living AEE and PAL using the T5, WE, and VT methods [97] but not the T1 method (Table 2). No statistically significant associations for RMR and TEE were observed for pooled HV2 effects (Table 2). Pooled associations for variants across the OXPHOS complexes, rRNAs, and tRNAs were not observed.

A higher burden of singleton variants among sedentary participants was observed across the entire mtDNA (p=0.004), with nominal differences in OXPHOS complex I (p=0.045), *ND4* (p=0.015) and *COI* (p=0.012) when compared with active participants (Figure 2). The frequency of singletons across the entire mtDNA and complex I was 30% higher in sedentary versus active participants. The frequency of singleton variants in the *ND4* and *COI* genes was 2-3 times higher in sedentary versus active participants. By contrast, the proportion of singleton variants in the *ND4L* (p=0.03) and *COII* genes (p=0.03) was 10 times higher in the active group when compared with the sedentary group (Figure 2).

4.0 Discussion

We examined the role of mtDNA sequence variation in AEE and PAL and identified a single HV2 region variant that was significantly associated with both measures and a large number of highly conserved and potentially functional variants that are unique to individuals at the high and low ends of the AEE distribution. Among these are variants that have been implicated in mitochondrial several diseases, including: Leber's Hereditary Optic Neuropathy (LHON); mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS); and mitochondrial cardiomyopathy. Because sequence variants do not work in isolation [100], we also considered how multiple sequence variants and accumulations of singletons are associated with energy expenditure.

Among the six *CytB* NS substitutions unique to high and low AEE levels, several were predicted to significantly affect function and/or to be highly conserved. Of particular interest are the p.A191T, *CytB* and p.T194M, *CytB* substitutions which are unique to participants with low AEE. Both are located in the Qi binding pocket of complex III, where quinone is reduced by cytochrome b [101]. The p.T194M, *CytB* variant occurs at a residue that is noted to undergo significant conformational changes upon contact with antimycin A, a pharmacological inhibitor of the Qi site [101]. In the presence of antimycin A, complex III produces high quantities of superoxide indicating that inhibition at this site blocks electron transfer (from cytochrome b to quinone at Qi) causing a buildup of semiquinone at the Qo site. This buildup results in increased ROS production from complex III [102]. The structure of bovine cytochrome bc1 complex was also used to predict whether specific *CytB* NS substitutions occur in functionally relevant sites. The p.N374D, *CytB* substitution occurs near Lysine (311, 375, and 378) and Serine (310, 314, and 370) residues and may be potentially involved in polar interactions with these neighboring sites. The p.D171N, *CytB* substitution which is located on the outer core of protein is a risk factor for LHON [103-110]. Complex III is the ETC enzyme responsible for oxidizing ubiquionol and transferring electrons to cytochrome c through the cytochrome b mediated Q cycle. During

the process of electron transfer through complex III, a net of 4 protons are pumped out of the mitochondrial matrix increasing PMF. The resulting reduced cytochrome c then transports the electrons downstream to complex IV. If the mutations identified by sequencing lead to dysfunction in cytochrome b, the result may be a backup of electrons in the upstream OXPHOS components resulting in ROS production and insufficient ATP supply [111].

Among the complex I NS substitutions identified in the extremes of AEE, several are predicted to affect function including two that are considered possible risk factors for LHON: p.I57M, *ND2* [112] and p.Y159H, *ND5* [105, 113]. The p.I57M, *ND2* substitution is predicted to cause a gain of a catalytic residue and a gain of disorder. In addition, the p.M1T, *ND1* substitution is a risk factor for MELAS [114] and the m.3308T>C mutation that encodes this substitution may alter the hydrophobicity and antigenicity of the N-terminal peptide of *ND1* [114]. Other substitutions are predicted to result in the loss of stability p.I96T, *ND3*, loss of a catalytic residue p.T533M, *ND5*, and the gain of a catalytic residue p.I100V, *ND5*. Complex I is a large multi-subunit, membrane-bound protein which serves as the major entry point for most electrons into the electron transport chain (ETC). This process involves the electron transfer from NADH to quinone and contributes to the generation of mitochondrial proton motive force (PMF, potential energy for ATP generation) through the pumping of 4 protons. In eukaryotes, the mitochondrial genome encodes the 7 most hydrophobic subunits of complex I (*ND1*-*ND6* and *ND4L*) [115, 116]. These proteins comprise a large portion of the membrane domain in complex I and are thought to be essential to both quinone binding and proton translocation.

Among the complex V NS substitutions identified in the extremes of AEE are p.P10S, *ATP8* and p.M42T, *ATP8*. Complex V is a multisubunit complex consisting of two functional domains, F_1 and F_0. The F_0 domain is embedded in the mitochondrial inner membrane and is in part encoded by the mtDNA *ATP6* and *ATP8* genes. Complex V is the site of ATP synthesis, a process that consumes membrane potential by allowing protons to flow back down their electro-chemical gradient into the mitochondrial matrix, resulting in ATP production. Defects in complex V are associated with ATP synthase deficiency and it has been proposed that mutations in *ATP6* and *ATP8* are associated with reduced complex V assembly and impaired ATP synthase function [117, 118] . Potentially, modification of the function of these integral components of the ETC could alter the efficiency of ATP production or result superoxide production through a backup of electrons on the upstream ETC components.

In addition to the m.185G>A variant that was was significantly associated with elevated AEE and PAL (after adjustment for multiple comparisons), two variants in the HV2 region were observed in multiple samples that were consistently unique to high (m.200A>G) or low (m.263G>A) AEE levels. While it is not clear how these HV2 variants are associated with AEE, it is possible that this variation is involved in regulating mtDNA copy number [119]. The functions of the HV2 region include: priming site for mtDNA replication; the heavy-strand origin encoding 12 of the 13 OXPHOs genes; three conserved sequence blocks; and two transcription factor binding sites [120]. In a previous study the HV2 m.295C>T variant was found to increase both mtDNA transcription and copy number [119]. This particular mtDNA variant defines Caucasian haplogroup J and cybrids (experimental hybrid cells containing mtDNA from different sources placed in a uniform nuclear DNA background) containing haplogroup J mtDNA had a greater than 2-fold increase in mtDNA copy number compared with cybrids containing haplogroup H mtDNA [119]. The m.185G>A variant identified herein is commonly observed in sub-haplogroup J1c. Among the 7 carriers of the m.185G>A variant allele in this study, five are from haplogroup J and the other two are from haplogroups H and V. Not all haplogroup J participants in this study carried the variant m. 185G>A allele. The impact of haplogroup J-related regulatory region mutations on mtDNA

replication or stability may partially account for several observations that haplogroup J is over-represented in long-lived people and centenarians from several populations [121-123]. Several variants in the tRNA and rRNA regions were observed in samples that were consistently unique to high or low AEE levels. The mitochondrial tRNAs and rRNAs are critical for protein synthesis and mitochondrial assembly. The m.8348A>G (tRNA Lys) variant that is unique to a participant with extremely low AEE has also been identified as a risk factor for cardiomyopathy [124].

As collections of variants within genes or genomic regions are likely to influence phenotypes in important ways [66], examining the combined effect of rare variants may also reveal the role of specific genes in disease etiology. Across the entire mtDNA and complex I specifically we observed a significant 30% higher singleton burden among sedentary participants when compared to those defined as active. In addition, the singleton burden for *ND4* and *COI* genes was twice as high in sedentary participants whereas the proportion of singleton variants in the *ND4L* and COII genes was 10 times higher in the active group. Complex I is a major contributor to cellular reactive oxygen species (ROS) production [125]. Inhibition of complex I leads to increased generation of ROS, decreased ATP levels, and induction of apoptosis [126-128], all of which could play a major role in reducing AEE. Dysfunction in complex I has been linked to multiple diseases and mitochondrial pathologies including tumorigenesis [129], Parkinson's disease [130], and aging [131] (through a ROS dependent or a ROS independent mechanism). Complex IV transfers electrons from cytochrome c to oxygen, creating water. Through this process it translocates 4 protons contributing to the ATP generating proton motive force. Defects in complex IV are associated with Leigh Syndrome, hypertrophic cardiomyopathy, and myopathy [132].

Analytic approaches that test the combined effect of multiple variants have been used to resolve genetic associations for several complex traits [133-136] including the role of rare mitochondrial variants in disease [137]. We evaluated several approaches including the allele-frequency threshold approach (1% or 5%) [95], a weighted-sum approach [96], and the variable-threshold approach [97]. Significant variant burden effects in the HV2 region were observed for free-living energy expenditure. Rare variant burden in HV2 was associated with AEE and PAL but not with RMR or TEE, suggesting that this variation is most important for physical activity and volitional exercise [138]. Our results also suggest that HV2 variation under the 5% allele-frequency threshold, but not under the 1% allele-frequency threshold is associated with AEE and PAL, though this finding may be due to a lack of statistical power. Both weighted-sum and variable-threshold approaches, however, suggest that HV2 variation is associated with AEE and PAL.

This study had a number of strengths, including: complete mtDNA sequencing allowing for an unbiased assessment of mitochondrial genomic variation; a well-characterized population-based longitudinal cohort with energetics measured using state of the art methods; an analytic approach that includes both aggregated and accumulated sequence variants; and *in silico* prediction and structural modeling that allowed for detailed interpretation of sequence-based findings. Some weaknesses are also acknowledged, including: small sample size and low power to detect an effect of individual variants. It is possible that the mtDNA variants identified in this study may not be causally related to the energetic phenotypes thus the lack of a replication cohort is also a limitation.

In summary, there is little understanding of genetic factors that contribute to an individual's daily activity levels and here we identify a number of potentially functional mtDNA variants and collections of sequence variants that contribute to free-living activity energy expenditure. These results may help to uncover specific mitochondrial functions that explain age-related declines in activity but also maintenance of high activity energy levels in the

elders. While the 13 mtDNA-encoded OXPHOS genes are essential to mitochondrial energy production and are considered the most functionally important [60], hundreds of nuclear DNA-encoded and dozens of mtDNA-encoded bioenergetics genes are distributed throughout both genomes [58, 59]. We have shown that nuclear genomic European Ancestry in African Americans is strongly associated with higher RMR [139]. Future studies of mitochondrial genetic variation will therefore need to account for a complex set of interactions involving the nuclear and mitochondrial genomes [140]. Since the 13 mtDNA-encoded OXPHOS genes are essential to mitochondrial energy production [60] , the coding region variation identified in this study might be related to ROS production at OXPHOS complexes I and III, ATP generation efficiency through the collective impairment of the respiratory chain [102, 141] or through apoptosis [128]. Individual and collective variation in the HV2, tRNA and rRNA regions may affect mitochondrial function by affecting the rate or efficiency of mitochondrial biogenesis (increase in mitochondrial number and/or mass). An important aspect of mitochondrial biogenesis is rate of turnover, which is thought to decline with age [142]. Impaired ability to turnover may allow for defective mitochondria to accumulate, especially in older, postmitotic cells lead to impaired respiratory capacity [143]. It is known that mitochondrial biogenesis is affected by pharmacologic agents [144-149], natural compounds such as resveratrol [150] and behavioral interventions such as caloric restriction and exercise [151-154]. However, identifying mitochondrial genetic variants that are associated with free-living activity energy expenditure generates new hypotheses about additional molecular targets (*e.g.* Qi binding pocket of complex III) or mechanisms (*e.g.* mitochondrial protein synthesis and assembly) that may be involved in human energetics.

Supplementary Material

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Highlights

- Examining the role of human mitochondrial sequence variation in free-living activity energy expenditure.
- Several highly conserved and potentially functional variants in OXPHOS genes are unique to participants in the extremes of activity energy expenditure.
- Collective sequence variation across OXPHOS complex I and hypervariable region 2 are associated with activity energy expenditure.

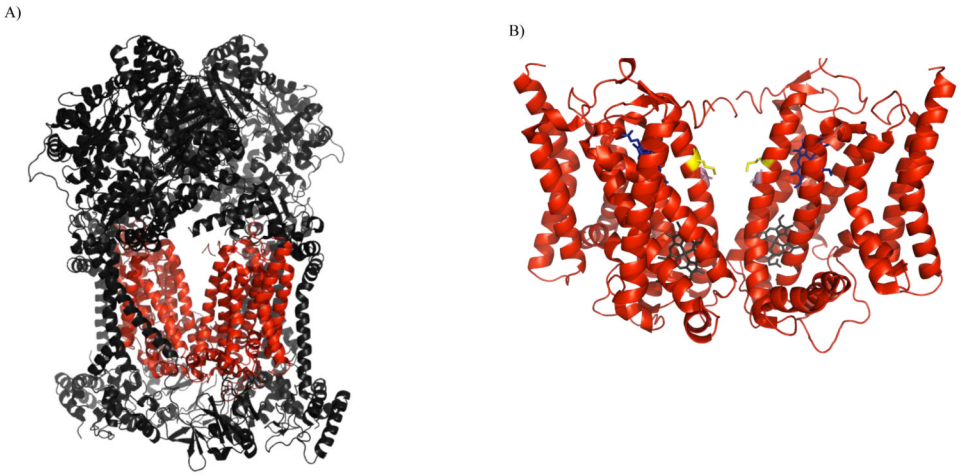

Figure 1.

Structure of the dimeric bovine cytochrome bc1 complex at 2.28 Å resolution (PDB2a06) [90]. A) Cytochrome bc1 complex with mtDNA-encoded *CytB* (red) and nDNA-encoded subunits (gray) indicated. B) Close-up of *CytB* dimer indicating p.A191T (Purple) and p.T194M (yellow) positions located in the Qi binding pocket of complex III, where quinone is reduced by *CytB* [101]. The b_L heme (blue) adjacent to the Qo site and b_H heme (grey) adjacent to the Qi site are also indicated. The T194M variant occurs at a residue that undergoes significant conformational changes upon contact with antimycin A, a pharmacological inhibitor of the Qi site [101]. In the presence of antimycin A, complex III produces high quantities of superoxide indicating that inhibition at this site blocks electron transfer from cytochrome b to quinone causing a buildup of semiquinone resulting in increased ROS production [102].

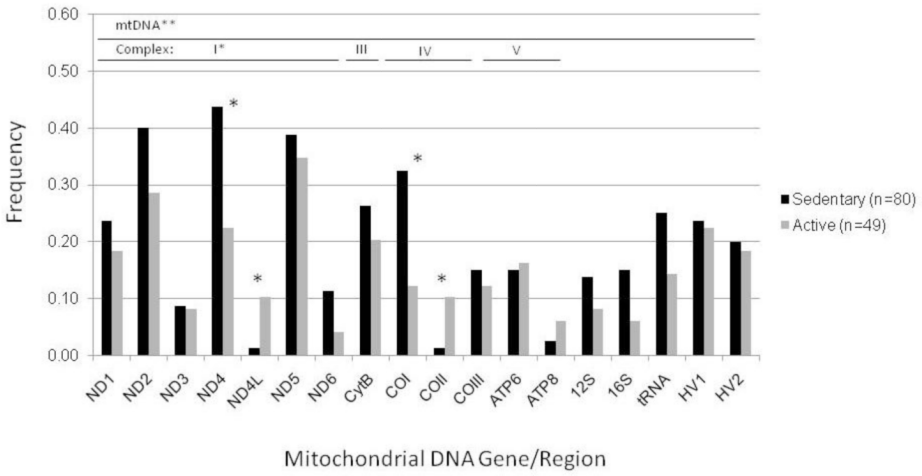

Figure 2.

Frequency of mtDNA singleton variants unique to sedentary or active Health ABC Study participants.

[1]Sedentary, physical activity level <1.7

[2]Active, physical activity level ≥1.7

Sedentary vs. Active, Fisher's Exact Test P-value <0.05*, <0.01**.

Table 1

Detailed analysis of nonsynonymous substitutions unique to sedentary (AEE < 401 kcal/d) and active (AEE > 907 kcal/d) Health ABC Study participants. Significant prediction (SIFT, PolyPhen) and conservation (PhastCons, PhyloP) scores are indicated in bold.

Complex	**I**																**III**						**IV**		**V**	
Gene	**ND1**				**ND2**			**ND3**	**ND4**		**ND5**					**ND6**	**CytB**						**COI**	**COIII**	**ATP8**	
Nt. position	**3308t**	**3593t**	**3943a**	**4172t**	**4501c**	**4640a**	**4890a**	**10345t**	**11065a**	**11172a**	**12634a**	**12811t**	**12952g**	**13676a**	**13934c**	**14180t**	**14927a**	**15257g**	**15317g**	**15326g**	**15758a**	**15866a**	**7080t**	**9300g**	**8393c**	**8490t**
AA Position	**M1**	**V96**	**I213**	**L289**	**S11**	**I57**	**I141**	**I96**	**L102**	**N138**	**I100**	**Y159**	**A206**	**N447**	**T533**	**Y165**	**T61**	**D171**	**A191**	**T194**	**I338**	**N374**	**F393**	**A32**	**P10**	**M42**
A EE kcal/day																										
230	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
291	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-
307	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-
316	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-
332	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
341	-	-	-	-	-	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-
351	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-
366	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-
381	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
391	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
396	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-
935	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
956	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-
985	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-
1010	-	-	-	-	-	-	V	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	L	-	-	-
1042	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	T
1042	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1048	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-
1061	-	-	-	-	F	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1081	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-
1141	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-
PhastCons	**0.69**	**0.00**	**0.97**	**0.12**	0.00	0.00	0.00	0.00	0.00	**0.23**	**0.95**	0.00	0.00	**0.11**	0.00	**0.69**	**0.16**	**0.99**	**0.67**	0.00	1.00	1.00	**0.89**	0.00	**0.03**	0.00

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Complex	**I**																**III**						**IV**		**V**	
Gene	**ND1**				**ND2**			**ND3**	**ND4**		**ND5**					**ND6**	**CytB**						**COI**	**COIII**	**ATP8**	
Nt. position	**3308t**	**3593t**	**3943a**	**4172t**	**4501c**	**4640a**	**4890a**	**10345t**	**11065a**	**11172a**	**12634a**	**12811t**	**12952g**	**13676a**	**13934c**	**14180t**	**14927a**	**15257g**	**15317g**	**15326g**	**15758a**	**15866a**	**7080t**	**9300g**	**8393c**	**8490t**
AA Position	**M1**	**V96**	**I213**	**L289**	**S11**	**I57**	**I141**	**I96**	**L102**	**N138**	**I100**	**Y159**	**A206**	**N447**	**T533**	**Y165**	**T61**	**D171**	**A191**	**T194**	**I338**	**N374**	**F393**	**A32**	**P10**	**M42**
A EE kcal/day																										
PhyloP	**2.23**	**1.17**	**4.00**	**2.05**	−1.19	−3.05	−0.07	−1.16	−8.02	**2.08**	**4.39**	−0.08	−0.20	**1.23**	−0.27	**1.69**	**0.77**	**2.74**	**3.85**	−1.08	1.81	1.91	**2.43**	−0.29	−0.09	−0.72
SIFT	**0.00**	**0.03**	1.00	**0.00**	0.17	**0.00**	1.00	0.29	**0.00**	**0.08**	**0.00**	0.71	0.62	**0.05**	1.00	**0.03**	**0.02**	**0.04**	0.11	0.83	**0.00**	**0.00**	0.25	**0.00**	**0.10**	0.75
PolyPhen	0.13	0.04	0.00	**0.99**	0.00	**0.55**	**0.82**	0.00	**1.00**	**0.98**	**0.98**	0.00	0.00	0.09	0.00	**0.91**	0.00	0.00	0.00	0.00	0.00	**0.83**	0.00	0.00	**0.90**	0.07

Table 2

Rare variant burden tests of associations across hypervariable region 2 for metabolic rate and energy expenditure in the Health ABC Study.

	N	P_{T1}	P_{T5}	P_{WE}	P_{VT}
RMR[1]	135	0.64	0.86	0.86	0.90
TEE[2]	129	0.20	0.14	0.20	0.32
AEE[3]	129	0.09	0.01	0.02	0.03
PAL[4]	129	0.13	0.006	0.01	0.02

P-values for T1 (1% allele-frequency threshold), T5 (5% allele-frequency threshold), WE (weighted), and VT (variable threshold), analyses are displayed. A significance level of $p \leq 0.01$ is used after multiple testing correction ($\alpha=0.05$) for 9 mtDNA regions, based on 10,000 independent simulations

[1]Resting metabolic rate (RMR) was measured via indirect calorimetry.

[2]Total energy expenditure (TEE) was measured using the 2-point doubly-labeled water technique.

[3]Activity energy expenditure (AEE) was calculated as [(TEE*0.90) – RMR].

[4]Physical activity level (PAL) was calculated as TEE/RMR.