

# A genetic polymorphism that is associated with mitochondrial energy metabolism increases risk of fibromyalgia

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## Abstract

Alterations in cellular energy metabolism have been implicated in chronic pain, suggesting a role for mitochondrial DNA. Previous studies reported associations of a limited number of mitochondrial DNA polymorphisms with specific pain conditions. In this study, we examined the full mitochondrial genomes of people with a variety of chronic pain conditions. A discovery cohort consisting of 609 participants either with or without a complex persistent pain conditions (CPPCs) was examined. Mitochondrial DNA was subjected to deep sequencing for identification of rare mutations, common variants, haplogroups, and heteroplasmy associated with 5 CPPCs: episodic migraine, irritable bowel syndrome, fibromyalgia, vulvar vestibulitis, or temporomandibular disorders. The strongest association found was the presence of the C allele at the single nucleotide polymorphism m.2352T>C (rs28358579) that significantly increased the risk for fibromyalgia (odds ratio [OR] = 4.6,  $P = 4.3 \times 10^{-4}$ ). This relationship was even stronger in women (OR = 5.1,  $P = 2.8 \times 10^{-4}$ ), and m.2352T>C was associated with all other CPPCs in a consistent risk-increasing fashion. This finding was replicated in another cohort (OR = 4.3,  $P = 2.6 \times 10^{-2}$ ) of the Orofacial Pain: Prospective Evaluation and Risk Assessment study consisting of 1754 female participants. To gain insight into the cellular consequences of the associated genetic variability, we conducted an assay testing metabolic reprogramming in human cell lines with defined genotypes. The minor allele C was associated with decreased mitochondrial membrane potential under conditions where oxidative phosphorylation is required, indicating a role of oxidative phosphorylation in pathophysiology of chronic pain. Our results suggest that cellular energy metabolism, modulated by m.2352T>C, contributes to fibromyalgia and possibly other chronic pain conditions.

**Keywords:** Mitochondria, Deep sequencing, Single nucleotide polymorphism, Chronic pain, Fibromyalgia, Inner membrane potential

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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## 1. Introduction

One in 3 adults in America lives with chronic pain.<sup>22,49</sup> In the majority of cases, pain is reported in multiple body locations and is associated with other nonpainful bodily symptoms. These comorbid disorders are often idiopathic, as such no identifiable structural pathology or biochemical aberration can be associated with the reported pain, and are commonly accompanied with dysregulation of the central, peripheral, and/or enteric nervous systems.<sup>11,12</sup>

Recently, mitochondrial dysfunction has been shown to contribute to pain perception and chronic pain conditions.<sup>16,48,59</sup> This organelle is found in most eukaryotic cells, including most human cells. Copy numbers for mitochondria vary greatly among cell types, with each mitochondrion containing a few to thousands of copies of its own mitochondrial DNA (mtDNA).<sup>41,67</sup> Mitochondria fuse and divide in an ongoing, dynamic process in response to various cell stimuli and needs.<sup>52</sup> They are often referred to as the “energy powerhouses of the cell” because they generate most of the cell’s chemical energy in the form of adenosine triphosphate (ATP).<sup>56</sup> The mitochondrion’s genome (mtDNA) is haploid and is exclusively inherited from the maternal line.<sup>18</sup> Mitochondria play key roles in neuronal transmission and plasticity,<sup>24,58</sup> immune function,<sup>40,68</sup> and the ability to modulate a cell’s fate.<sup>23,55,70</sup> Mitochondria-related diseases generally result in abnormalities in tissues of neuronal and muscular origin, likely because these tissues have high and fluctuating energy requirements.

Several studies have shown a link between energy metabolism and chronic pain,<sup>16,48,59</sup> suggesting several pathways through which mitochondrial dysfunction can increase or inhibit neuropathic and inflammatory pain. These include the mitochondria's critical functions such as energy metabolism and metabolism of reactive oxygen species. Furthermore, mtDNA may drive some of these dysfunctions. Despite comprising only 37 genes, mtDNA has both high-frequency inherited polymorphisms and occurrences of new mtDNA mutations. Yet, due to several unique characteristics of mitochondrial genetics, each polymorphism can be either heteroplasmic (abnormal and wild-type coexist in the same cell) or homoplasmic (in which all mtDNA is affected). Furthermore, mtDNA mutations generally demonstrate marked variability in terms of clinical expression, organ systems affected, severity, age of onset, and natural history of disease. This complicates studying the role of mtDNA in the context of chronic diseases. Despite these challenges, several preliminary studies have found associations of candidate polymorphisms in mtDNA with irritable bowel syndrome (IBS), nonspecific abdominal pain, migraine, and cyclic vomiting syndrome.<sup>7,34,61,64,72</sup>

These previous studies, however, were often small, targeted a specific genetic group of people (often mtDNA haplotype H) as well as specific pain conditions (eg, only patients with IBS), and focused on a limited number of polymorphisms. This current study aimed to examine the full mitochondrial genetic makeup from a wide variety of people affected by chronic pain in the largest sample reported to date.

## 2. Materials and methods

### 2.1. Study approval

The primary study, Complex Persistent Pain Conditions (CPPC): Unique and Shared Pathways of Vulnerability, was approved by the institutional review boards of the University of North Carolina and McGill University. The replication study, conducted using data and samples from the Orofacial Pain: Prospective Evaluation and Risk Assessment (OPPERA) cohort, was approved by institutional review boards of the 4 recruitment sites (the University of Florida, the University of North Carolina at Chapel Hill, the University of Maryland, and the University at Buffalo), the data coordinating center at Battelle Memorial Institute, and by McGill University.

### 2.2. Discovery cohort

The CPPC cohort<sup>57</sup> included participants enrolled in a cross-sectional study of overlapping pain conditions conducted at the University of North Carolina at Chapel Hill. A total of 848 study participants were enrolled, of which 752 had high-quality DNA to perform mitochondria deep-sequencing. After quality control filters were applied, 609 participants had simultaneous clinically assessed phenotypes with genomic data available for association studies (**Table 1**). Subjects were aged 18 to 64 years, and included both sexes (86% female) and major ethnic and racial groups (69% Caucasian as determined by participant self-report). Subjects had at least 1 of the 5 index CPPCs (episodic migraine [EM, 263 subjects], IBS, 221 subjects, fibromyalgia [FM, 96 subjects], vulvar vestibulitis [VVS, 100 subjects], or temporomandibular disorders [TMDs, 172 subjects]), or were otherwise healthy controls with none of these conditions (237 subjects). Each pain condition was classified by study clinicians using validated protocols: EM was classified after an examination with a neurologist, IBS was classified according to ROME-II criteria,<sup>36</sup>

**Table 1**

**Complex persistent pain condition study demographic and patient characteristics.**

|                 | Males (N)    | Females (N)  | Total (N)    | F/M   | P     |
|-----------------|--------------|--------------|--------------|-------|-------|
| <b>CPPC</b>     |              |              |              |       |       |
| EM              | 18           | 245          | 263          | 2.3x  | <0.01 |
| TMD             | 10           | 162          | 172          | 2.7x  | <0.01 |
| IBS             | 20           | 201          | 221          | 1.7x  | 0.02  |
| FM              | 2            | 94           | 96           | 7.8x  | <0.01 |
| VVS             | 0            | 100          | 100          | N/A   | N/A   |
| ANY             | 30           | 342          | 372          | 1.9x  | <0.01 |
| CTL             | 57           | 180          | 237          | 0.5x  | <0.01 |
| <b># CPPCs</b>  |              |              |              |       |       |
| 0               | 57           | 180          | 237          | 0.5x  | <0.01 |
| 1               | 19           | 123          | 142          | 1.1x  | 0.90  |
| 2               | 3            | 49           | 52           | 2.7x  | 0.10  |
| 3               | 7            | 115          | 122          | 2.7x  | <0.01 |
| 4               | 1            | 39           | 40           | 6.5x  | 0.04  |
| 5               | 0            | 16           | 16           | N/A   | N/A   |
| <b>Ancestry</b> |              |              |              |       |       |
| Cauc            | 55           | 366          | 421          | 1.1x  | 0.53  |
| Af-Am           | 25           | 117          | 142          | 0.8x  | 0.28  |
| Other           | 7            | 39           | 46           | 0.9x  | 0.83  |
| <b>Age (y)</b>  |              |              |              |       |       |
|                 | 34.6 (±11.9) | 36.2 (±11.6) | 36.0 (±11.6) | 1.05x | 0.61  |
| min/Max         | 18/61        | 18/64        | 18/64        |       |       |
| <b>Counts</b>   |              |              |              |       |       |
|                 | 87           | 522          | 609          |       |       |

Distribution of CPPCs and characteristics in males and in females. Participants can report more than one chronic pain conditions, therefore the counts in each sex is higher than the total number of individuals in the cohort. All *P*-values obtained using exact Fisher test, except for age, using Welch two-sample unequal variance. Complex persistent pain conditions were: EM, episodic migraine; TMD, temporomandibular disorders; IBS, irritable bowel syndrome; FM, fibromyalgia; VVS, vulvar vestibulitis; ANY, any of preceding; CTL, controls. Number of CPPCs (#) in any study participant range from 0 to 5, inclusively. Ancestries were: Caucasians, Cauc; African Americans, Af-Am; and others.

FM was classified using ACR-1990 criteria,<sup>69</sup> and TMD was classified using RDC-TMD criteria.<sup>14</sup> Women were classified as VVS cases if they reported provoked pain on contact in the genital region; or having been told by a gynecologist that they have VVS; or both. Women who endorsed generalized pain and/or itching in the genital area for 3 months or more were excluded from VVS cases. We performed Principal Component Analyses of the phenotype matrix (1 = unaffected, 2 = affected) using the R statistical package's function "prcomp."

### 2.3. Sequencing of mitochondrial DNA

Whole blood was collected by venipuncture and genomic DNA was extracted using the NucleoSpin Tissue kit (Macherey & Nagel, Duren, Germany), diluted to 20 ng/μL, and aliquoted to 25 μL per sample. High-coverage (>100x) mitochondria DNA sequencing (mtSeq) from whole-blood fractions was performed to determine allele content and assess heteroplasmy levels at each genomic position. The high coverage was made possible using the Ovation Human Mitochondrion Target Enrichment System (NuGEN, San Carlos, CA). Deep sequencing was performed at the University of Toronto on an Illumina 2500 instrument (Illumina, San Diego, CA), with data converted to FASTQ using Illumina's CASAVA software. Length of reads was 101 nucleotides, single-ended.

### 2.4. Bioinformatics

Deep-sequencing reads were trimmed using Trimmomatic v0.32,<sup>5</sup> using aggressive trimming command-line option:

“-phred33 ILLUMINACLIP:adapters.fa:1:30:6 LEADING:20 TRAILING:20 SLIDINGWINDOW:5:20 MINLEN:22.” Reads were then mapped to the human genome version GRCh38/hg38, which consists of the revised Cambridge Reference Sequence (rCRS).<sup>2</sup> The alignment of reads was done using the Bowtie v2 aligner,<sup>29</sup> chosen for its ability to perform local alignments (in contrast to Bowtie v1, which performs end-to-end global alignment only), with the following command-line arguments: “-very-sensitive-local -k 10.” The circular nature of mtDNA was not an issue because of the very deep sequencing coverage. Polymerase chain reaction duplicates were removed using scripts provided by NuGEN. Binary alignment map flags for secondary alignments were converted into primary alignments because Bowtie randomly assigns one of the equally scored alignments as primary, and downstream bioinformatics tool only considers primary alignments. BAM files were analyzed using MitoSeek,<sup>21</sup> which provided counts of alleles for each genomic position. Sequencing quality controls included: Phred scores  $\geq 30$ ,  $\geq 10$  counts, and  $\geq 95\%$  same allele (sequencing errors and low levels of heteroplasmy were tolerated). Sequencing data were transformed into genotyping data with allele counts provided by MitoSeek. Genotyping quality controls included: Hardy-Weinberg equilibrium  $P$ -value  $\geq 1 \times 10^{-4}$  (ie, not peculiar), genotyping rate per single nucleotide polymorphism (SNP)  $\geq 98\%$ , genotyping rate per individual  $\geq 98\%$ , minor allele frequency  $\geq 5\%$ , and required that in an individual, a position had to feature at least 95% of the same nucleotide, thus allowing for parsimonious amounts of heteroplasmy levels.

A total of 752 samples were deep sequenced at a read length of 101 nucleotides. The total number of sequenced reads per sample was: minimum 50 K, maximum 11.9 M, mean 2.0 M, and SD 1.1 M. After the removal of polymerase chain reaction duplicates, the ratio of number of mapped reads to reads sequenced was: mean 55.2% and SD 7.8%. The ratio of number of reads mapped to mitochondria vs total number mapped, including nuclear DNA: mean 81.4% and SD 2.8%. No single read aligned on mtDNA was reported aligned elsewhere on nuclear DNA, indicating perfect specificity of sequenced reads for mitochondria studies. Mapped alignment length in nucleotides on mitochondria (CIGAR “M” symbol): mean 81.0 and SD 25.6. The average nucleotide coverage was (Figure S1A, available at <http://links.lww.com/PAIN/B105>): mean 3913x and SD 3383x; in the 100 nucleotides from 5' and 3' ends of the chromosome (Figure S1B, available at <http://links.lww.com/PAIN/B105>): mean 799x and SD 595x. We used the Bowtie2 program that was able to analyze the circular configuration of the mitochondrial chromosome with the help of long reads, deep sequencing of mtDNA-enriched samples, and the ability to perform a local alignment (Bowtie1 performs end-to-end alignment only). Reads spanning the control region were either mapped at the 5' or the 3' end of the linearized chromosome sequence, whichever end yielded better alignment scores. The coverage is on par in quality with previous studies on mitochondria.<sup>65,71</sup>

## 2.5. Statistics

Mitochondria-wide association analyses were conducted using PLINK v1.9,<sup>46</sup> with CPPCs as phenotypes, and sex, age, as well as the first 2 principal genetic components as covariables. The mitochondrial control region (rCRS nucleotides 1-576 and 16,024-16,579), known to be hypervariable, was excluded from association analyses. Sites with less than 95% of the same allele were coded as “0” (“undefined genotype”) for input to PLINK. The chromosome designation “26” for mitochondria was also used to

instruct PLINK that the genotyping data are haploid-based. Separate association tests were conducted for each CPPC using logistic regression models in which cases were defined as subjects with the relevant CPPC and controls were subjects who did not have that CPPC. Here, we followed recommendations to object to rely on “supercontrols” for association testing because people considered cases for one CPPC can also be subjects to other CPPCs, just as the control subjects.<sup>45</sup> An additional linear regression model tested for associations with the total number of CPPCs (ie, ranging from 0 to 5). A position was tested if its minor allele frequency was at least 5% (ie, not a rare variant). A sex-stratified analysis was performed for each comparison. A principal component analysis (PCA)-based approach that considered correlated SNP alleles in linkage disequilibrium was used for determining statistical significance, and Bonferroni correction was applied for multiple testing based on the estimated number of effective SNPs. The Genetic Type I error calculator was used to estimate the effective number of SNPs<sup>32</sup> as an alternative to mitochondrial haplogroup assignment.

Haplogroup assignments were determined from the deep sequencing by reconstructing each individual's mtDNA sequence, in which the original rCRS sequence was adjusted to MitoSeek's major allele base call. Haplogroup assignments were performed using HAPLOFIND<sup>63</sup> and HaploGrep 2,<sup>66</sup> and results were congruent between the 2 methods. In African Americans, 71.9% were classified as haplogroup L, 7.8% as H, and 5.9% as U. In Caucasians, 38.8% were in H, 16.0% in U, and 10.2% in T. In the “other” ancestry group, haplogroups B (22.2%), A (18.5%), and D (14.8%) were the most common. Results were consistent with known high levels of Caucasian admixture among African Americans, and Native Americans among Hispanics, who constituted much of the “other” group. These proportions are in line with the findings from the 1000 Genomes Project about the distribution of mitochondrial haplogroups in the U.S. population.<sup>50</sup> We performed haplogroup-based association tests with CPPC by comparing individuals of one haplogroup to those of all other haplogroups. Haplogroups tested featured at least 30 individuals, and were: H ( $n = 177$ ), L ( $n = 137$ ), U ( $n = 79$ ), J ( $n = 51$ ), T ( $n = 47$ ), and K ( $n = 32$ ). Age and sex were used as covariables.

Rare variant association tests were performed using the SKAT-O approach.<sup>31</sup> Single nucleotide polymorphisms were pooled by genes or by pathways. Tested genes comprised all 13 mitochondrial protein-coding genes, 22 transfer RNAs, and small (12S rRNA) and large (16S rRNA) ribosomal subunits. Tested pathways pertained to the oxidative phosphorylation complexes, and were<sup>33</sup>: Complex I = [MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5, MT-ND6], Complex III = [MT-CYB], Complex IV = [MT-CO1, MT-CO2, MT-CO3], and Complex V = [MT-ATP6, MT-ATP8]. Age, sex, and the first 2 principal genetic components were used to define the SKAT test null model.

Association tests with heteroplasmy levels were conducted as follows: for each genomic position, the distribution of heteroplasmy odds ratio (OR) in subjects with a CPPC was contrasted against the distribution of those without. In each individual, the heteroplasmy OR was established from a  $2 \times 2$  Fisher table composed of the observed major and minor allele counts, and estimated counts of major and minor alleles from 0.1% heteroplasmy baseline levels, which could be attributed to sequencing error, deep-sequencing read misalignment, etc. The OR was calculated using the observed minor allele count to that expected, given the background of observed and estimated major allele counts. Sequencing depth modulated the statistical

significance of the ORs, but here we performed the tests based on effect size only, while making sure that genomic positions with marked differences in heteroplasmy levels would correspond to deeply sequenced positions, ie, with several thousand-fold coverage on average. We performed logistic tests for CPPC as a function of heteroplasmy ORs, using age, sex, and first 2 genetic principal components as covariables. Because the OR is self-normalized, there was no need to account for sample size factors (sample-wide sequencing depth).

## 2.6. Visualization

Graphics were plotted using the R statistical package, version 3.5.2 (2018-12-20).<sup>47</sup>

## 2.7. Replication cohort

The replication case–control cohort included 1754 female subjects selected from the OPPERA study,<sup>38</sup> of whom 53 were fibromyalgia cases, whereas 1701 were noncases based on self-reported item in the Medical History questionnaire in OPPERA (Fibromyalgia/Chronic Fatigue Syndrome). Cases were defined as those that answered “yes” to the question: “did you have this fibromyalgia in the past or have it now?” Cases and controls were not excluded if they had other CPPCs. (Supplementary Table S1, available at <http://links.lww.com/PAIN/B105>). Genotyping was performed by the Center for Inherited Disease Research (Baltimore, MD) using the Illumina HumanOmni 2.5 Exome Bead Chip platform (Illumina). Genetic data cleaning was accomplished by the Genetic Analysis Center at the University of Washington following their established pipeline.<sup>30</sup> The genotyping array included SNP m.2352T>C, probed through exm2216242.<sup>57</sup> Association tests used as covariables age, sex, and the first 2 genetic principal components to account for population stratification.

## 2.8. Cell culture

Twenty B lymphoblast cell lines were obtained from the NHGRI sample repository for Human Genetic Research through the Coriell Institute (Camden, NJ). Of these cell lines, 10 had the major allele at m.2352T>C, whereas 10 had the minor allele at this position. All cell lines were derived from women of African American or Western African ancestries; 16 females were of African American ancestry living in the United States (1000 Genomes Project population code ASW), whereas 4 were from Western Africa (YRI). These ancestries were selected over other ancestries because they carry significantly higher minor allele content at m.2352T>C and women were selected over men because they showed stronger association with pain phenotypes. The cell lines were maintained according to the supplier's protocol. Briefly, cells were cultured in maintenance medium of RPMI 1640 (GIBCO; Thermo Fisher Scientific, Waltham, MA) supplemented with 15% fetal bovine serum (GE Healthcare Bio-Sciences, Marlborough, MA) and 200 mM glutamine (GIBCO). B lymphoblast density was kept between 0.2 and  $1 \times 10^6$  cells/mL.

## 2.9. JC-10 staining

Each B lymphoblast line was washed and resuspended at  $0.5 \times 10^6$  cells/mL in either RPMI 1640 without glucose supplemented with 4.5g/L glucose (Sigma-Aldrich, St-Louis, MO), 15% dialyzed fetal bovine serum (GIBCO), and 200 mM glutamine, or RPMI 1640 without glucose supplemented with 4.5g/L galactose

(Sigma-Aldrich), 15% dialyzed fetal bovine serum, and 200 mM glutamine. Cells were incubated for 24 hours at 37°C with 5% CO<sub>2</sub> in a humidified chamber. Based on optimization experiments,  $1 \times 10^5$  cells were stained in 200  $\mu$ L JC-10 staining solution (AbCam, Cambridge, United Kingdom) for 30 minutes at 37°C according to manufacturer's instructions. Control samples were depleted for JC-10 aggregates by a mitochondrial uncoupling agent: 10  $\mu$ M carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma-Aldrich).  $3 \times 10^4$  events per sample were acquired on an LSR-Fortessa SORP (BD BioSciences, Franklin Lakes, NJ) using excitation at 488 nm and 530/30 nm detection filter for the JC-10 monomers, and excitation at 561 nm and 582/15 nm detection filter for the JC-10 aggregates.

## 2.10. Flow cytometry data analysis

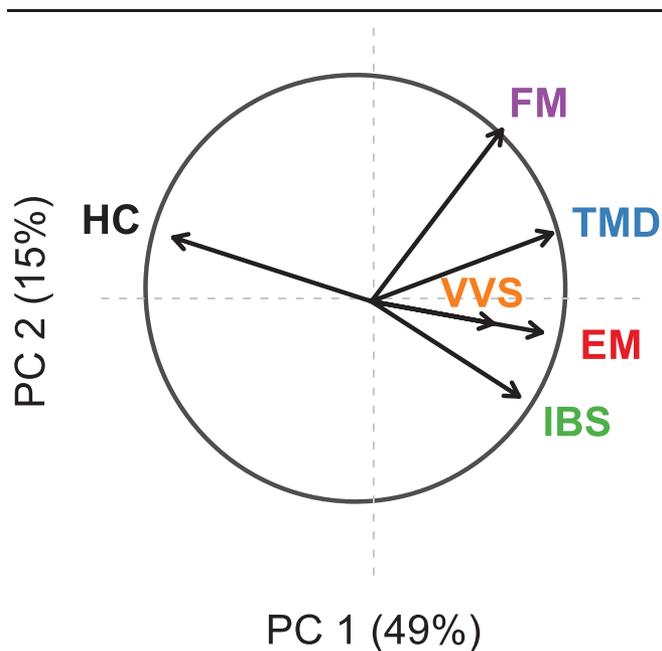
Preliminary cleaning of flow cytometry data was performed using a time-gate to exclude anomalies from abrupt changes in the flow rate, followed by exclusion of debris and doublets based on forward and side scatter parameters. Approximately 5000 live B lymphoblasts were selected per sample for unsupervised clustering analysis. Mean fluorescent intensity signals from JC-10 monomers and polymers were normalized (mean = 0, SD = 1) across all the 40 samples (2 conditions  $\times$  [10 samples with the reference allele + 10 samples with the alternative allele]). Density-based spatial clustering of applications with noise was performed using the DBSCAN R-package.<sup>15</sup> Two clusters were identified: cluster A (high in JC-10 aggregate signal) and cluster B (high in JC-10 monomer signal). Linear modelling was performed with the ratio of cluster B to cluster A as the dependent variable, and the presence of minor allele and culture media (glucose or galactose) as independent variables. The cluster ratio served as a marker for the number of cells with low mitochondrial membrane potential ( $\Delta\psi_m$ ) compared to healthy cells. FlowJo (FlowJo, LLC; Ashland, OR) and R version 3.6.0 were used for the analyses.

## 3. Results

### 3.1. Discovery cohort sample characteristics

A total of 848 participants were enrolled in the CPPC cohort. The pairing of samples with epidemiological data with those with deep sequencing of mitochondria data resulted in 609 matched samples (**Table 1**). The cohort was composed of individuals with at least one CPPC (61.1%; controls 38.9%), and most were female (85.7%), of predominantly Caucasian (69.1%) or African American (23.3%) ancestries, and aged between 18 and 64 years (mean  $36.0 \pm 11.6$ ).

Complex persistent pain conditions were: EM (43.2%), TMDs (28.2%), IBS (36.3%), fibromyalgia (FM, 15.8%), and VVS (16.4%). Women were more likely to have each CPPC compared to men: from 1.7x for IBS to 7.8x for FM (VVS, by definition, affects women only). Most individuals with one or more CPPC had comorbid conditions: 142 (23.3%) individuals had only one CPPC, whereas 230 (37.8%) had 2 or more CPPCs. Principal component analysis of the phenotype matrix distinguished, as expected, the health status against the chronic pain conditions (**Fig. 1**). This major axis (PC 1) contributed to as much as 49% of the variance in the matrix. The second major axis (PC 2) explained 15%; at opposite ends of the spectrum were fibromyalgia and IBS. The principal component eigenvectors extracted from the phenotype matrix were useful surrogates for an association study with a simplified endophenotype underlying all CPPCs,<sup>3</sup> while enabling consideration of all CPPCs into one association study,



**Figure 1.** Correlation wheel of CPPCs with the first 2 principal components of the phenotype matrix. Complex persistent pain conditions are: EM, episodic migraine; TMD, temporomandibular disorders; IBS, irritable bowel syndrome; FM, fibromyalgia; VVS, vulvar vestibulitis. HC stands for healthy controls. Percent variance explained by each principal component shown in parentheses. CPPC, complex persistent pain condition.

thus circumventing the need for sample overlap correction in a meta-analysis.

### 3.2. Association with complex persistent pain conditions

First, we performed association tests between each of the 5 CPPCs and allelic polymorphic content along the mitochondrial chromosome (Fig. 2). We also tested for association with any CPPC, and the number of CPPCs.

The most significantly associated mitochondrial position with a CPPC after correction for effective number of SNPs was position 2352 with fibromyalgia (OR = 5.1,  $P = 2.8 \times 10^{-4}$ , F only; OR = 4.62,  $P = 4.3 \times 10^{-4}$ , F + M) (Fig. 2D; Supplementary Table S2, available at <http://links.lww.com/PAIN/B106>). This position corresponds to SNP m.2352T>C (rs28358579) that is located in the large mitochondrial ribosomal subunit (16S rRNA) encoded by the MT-RNR2 gene. The 16S rRNA locus also hosts a peptide-coding gene, humanin, found to have neuroprotective<sup>60</sup> and antiapoptosis<sup>20</sup> properties, and in which m.2352T>C lies in its 5'UTR. That SNP was also significantly associated with VVS (OR = 4.6,  $P = 1.4 \times 10^{-3}$ ) (Fig. 2E) and with the number of CPPCs (beta = +0.83,  $P = 1.3 \times 10^{-4}$ , F only; beta = +0.68,  $P = 3.8 \times 10^{-4}$ , F + M) (Fig. 2G). Consistently, the presence of the C allele increased risk with almost a unit increase in number of CPPCs with the risk allele (beta close to +1). The latter associations were slightly stronger in female only populations.

A detailed account of the relationships between SNP m.2352T>C, fibromyalgia status, and ancestry is presented in Figure 3. The percentage of subjects with fibromyalgia was similar in Caucasians (15.9%) and African Americans (17.6%) (Fig. 3A). The C minor allele was present in 32.5% of cases, whereas the T major allele was present in only 14.8% (Fig. 3B). Only 9 (<1.5%) individuals featured heteroplasmy levels above 5% for SNP m.2352T>C (Fig. 3B; allele "O"). The C minor allele

was predominantly found in participants of African American ancestry (Fig. 3C): about 24% in African Americans, whereas only about 1% in Caucasians.

We next examined each CPPC for the effect of SNP m.2352T>C as secondary analyses (Fig. 4; Supplementary Table S3, available at <http://links.lww.com/PAIN/B107>). First, we recapitulated the initial findings for FM in both CPPC and OPPERA cohorts in a forest plot (Fig. 4A). Then, we observed a consistent, nominally significant ( $P < 0.05$ ) increased risk for all other CPPC with the presence of the C allele, notably, for EM (OR = 2.5,  $P = 1.9 \times 10^{-2}$ ) (Fig. 4B), for TMDs (OR = 2.6,  $P = 2.1 \times 10^{-2}$ ) (Fig. 4C), and with number of CPPC (NB: beta = +0.7,  $P = 3.8 \times 10^{-4}$ ) (Fig. 4D). The association with any CPPC was also nominally significant in females only (ANY: OR = 3.0,  $P = 2.0 \times 10^{-2}$ ) (Fig. 4E), but highly significant in VVS (OR = 4.6,  $P = 1.4 \times 10^{-3}$ ) (Fig. 4F).

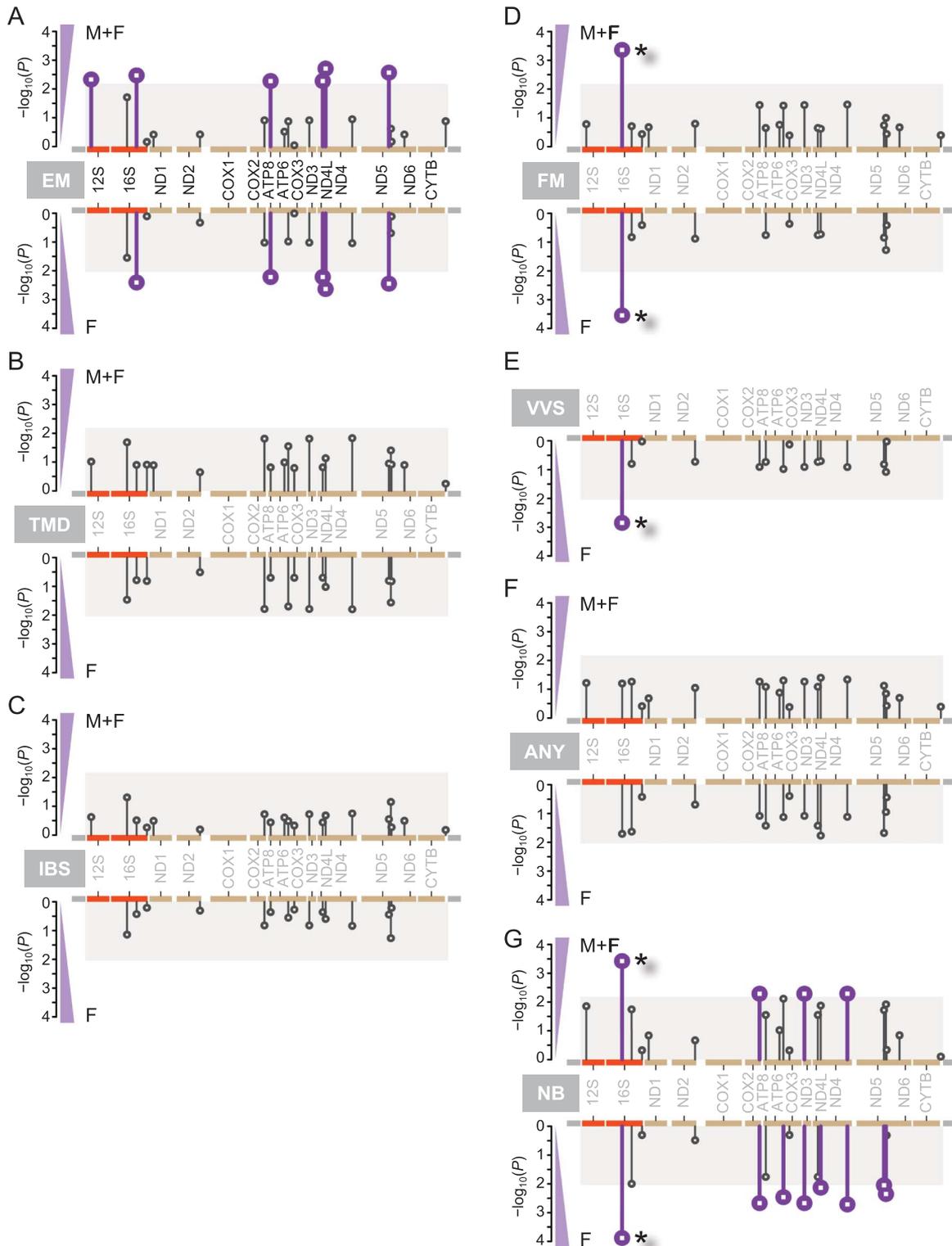
We next used principal component analysis of the phenotype matrix, which provided for an eigenvector associated with the largest eigenvalue (Fig. 1). The eigenvector PC 1 was used as a quantitative phenotype for association with m.2352T>C, to estimate the contribution of the polymorphism to chronic pain states at large. We found that the SNP's effect (beta) was positive, implying that the minor allele conferred significantly increased risk for the presence of pain (PC 1: beta = +0.93,  $P = 1.4 \times 10^{-3}$ ; Fig. 4G). Again, this effect was even stronger in women (PC 1: beta = +1.14,  $P = 4.7 \times 10^{-4}$ ; Fig. 4G).

Haplotype-based tests were performed to explore association between CPPC and maternal-lineage ancestry. We tested the most represented haplotypes H, L, U, J, T, and K. No association results were deemed significant at the false discovery rate 10% level (Supplementary Table S4, available at <http://links.lww.com/PAIN/B108>). We also performed rare variant-based tests with SKAT-O. Single nucleotide polymorphisms were pooled by genes or by oxidative phosphorylation protein complexes comprising one or multiple genes. Again, no association results were significant at the FDR 10% level (Supplementary Table S5, available at <http://links.lww.com/PAIN/B109>).

Finally, we capitalized on the very deep mtDNA sequencing to assess impact of heteroplasmy levels with respect to CPPC status (Supplementary Table S6, available at <http://links.lww.com/PAIN/B110>). Each time, differences in heteroplasmy levels between cases and controls were minimal (FDR > 10%). Overall, heteroplasmy levels were significantly associated with the presence of all CPPC at multiple positions, with higher levels in control subjects than in cases. Notably, in association with any CPPC, (ANY) position m.6412A>G in the MT-CO1 gene (beta = -0.84,  $P = 2.1 \times 10^{-7}$ ) is a nonsynonymous mutation AAU to AGU, coding a change from asparagine to serine.

#### 3.2.1. Replication of m.2352T>C in the Orofacial Pain: Prospective Evaluation and Risk Assessment cohort

We next tested our finding for replication in an independent cohort: The OPPERA Study.<sup>38</sup> Although the cohort was focused on the study of TMD, it contained self-reported data on fibromyalgia status and other CPPCs. Furthermore, the ancestry structure of OPPERA was similar to the discovery cohort. Female subjects were predominantly of Caucasian (61.3%) and African American (23.2%) ancestries, and aged between 18 and 44 years (mean  $27.7 \pm 7.7$ ). They were partitioned into 52 (3%) cases for fibromyalgia and 1660 controls (nonfibromyalgia), for a total of 1712 individuals. Women of African American ancestry comprised 8 (2%) cases and 402 controls (Supplementary Table S1, available at <http://links.lww.com/PAIN/B105>).



**Figure 2.** Mitochondria-wide association studies of CPPCs. Shown are Manhattan plots, tracking association  $P$ -value ( $P$ ) along the mitochondrial chromosome, in everyone (top) and in females only (bottom). The mitochondrial chromosome shown is the linearized version with annotated genomic features: rRNA (orange), protein coding genes (tan), control region or D-loop (gray). Vertical bars indicate tested positions, with minor allele frequency  $\geq 5\%$ . Gray boxes indicate areas outside of statistical significance, whereas vertical purple bars highlight significance. Significant associations with position m.2352T>C marked with a star (\*). (A) Episodic migraine (EM). (B) Temporomandibular disorders (TMD). (C) Irritable bowel syndrome (IBS). (D) Fibromyalgia (FM). (E) Vulvar vestibulitis (VVS). (F) Presence of any CPPC (ANY). (G) Number of CPPCs (NB). CPPC, complex persistent pain condition.

We found that fibromyalgia status was associated significantly with SNP m.2352T>C in women (OR = 4.3,  $P = 2.6 \times 10^{-2}$ ), thus replicating our initial finding. The polymorphism had even stronger effect size in a smaller population of African American

women (OR = 7.6,  $P = 1.4 \times 10^{-2}$ ; **Fig. 4A**; Supplementary Table S7, available at <http://links.lww.com/PAIN/B111>). Thus, overall, we found that m.2352T>C consistently increased risk for fibromyalgia in both the discovery and replication cohorts (**Fig.**

| ancestry  | has FM |     |       |       |
|-----------|--------|-----|-------|-------|
|           | yes    | no  | total | % yes |
| Caucasian | 67     | 354 | 421   | 15.9  |
| Afr-Am    | 25     | 117 | 142   | 17.6  |
| Other     | 4      | 42  | 46    | 8.7   |
| Total     | 96     | 513 | 609   | 15.8  |

| allele | has FM |     |       |       |
|--------|--------|-----|-------|-------|
|        | yes    | no  | total | % yes |
| 0      | 0      | 9   | 9     | 0.0   |
| C      | 13     | 27  | 40    | 32.5  |
| T      | 83     | 477 | 560   | 14.8  |
| Total  | 96     | 513 | 609   | 15.8  |
| % C    | 13.5   | 5.3 |       |       |

| ancestry  | allele |    |     |       |      |
|-----------|--------|----|-----|-------|------|
|           | 0      | C  | T   | total | % C  |
| Caucasian | 5      | 4  | 412 | 421   | 1.0  |
| Afr-Am    | 3      | 34 | 105 | 142   | 23.9 |
| Other     | 1      | 2  | 43  | 46    | 4.3  |
| Total     | 9      | 40 | 560 | 609   | 6.6  |

**Figure 3.** Relationship between fibromyalgia, ancestry, and allele content at SNP m.2352T>C in females. (A) Fibromyalgia in different ancestral groups. (B) Allelic distribution in fibromyalgia patients. (C) Allelic distribution in different ancestral groups. Allele "0" stands for discarded samples due to heteroplasmy.

4A; Supplementary Table S7, available at <http://links.lww.com/PAIN/B111>) with very robust effect size.

### 3.2.2. Replication of previous work

Several previous studies have suggested a role for mtDNA polymorphisms, such as m.16519T>C and m.3010G>A, in various chronic pain-related disorders such as IBS, migraine, and cyclic vomiting.<sup>4,7,61,62,72</sup> In our study, these 2 SNPs exhibited heteroplasmy levels greater than 5% in about 5% of samples, and so were excluded from primary and secondary analyses after quality control for genotyping rate. Heteroplasmy results showed nominal association between dosage of A at position 3010 with migraine ( $P = 0.03$ ) or presence of any CPPC ( $P = 0.04$ ), with controls displaying greater levels of heteroplasmy. At position 16,519, dosage of C was nominally associated with TMD ( $P = 0.02$ ), IBS ( $P = 0.03$ ), or presence of any CPPC ( $P = 0.05$ ), with controls displaying greater levels of heteroplasmy here too.

### 3.2.3. Effect of m.2352T>C on mitochondrial membrane potential

The mitochondrial genome encodes 37 genes, including 2 rRNAs, 22 tRNAs, and 13 polypeptides, which are required for oxidative phosphorylation as part of the electron transport chain (ETC). The mitochondrial rRNAs and their assembled ribosomes are solely responsible for translating these ETC proteins. Because the m.2352T>C polymorphism is situated in the mitochondria's large ribosomal subunit 16S rRNA, it could potentially impact the translation of ETC transcripts, and consequently oxidative phosphorylation.

To test the functional effects of the m.2352T>C polymorphism, we used B lymphoblast cell lines with known genotypes from female individuals of African American or West African ancestry from the NHGRI sample repository. The obtained cell lines were from populations enrolled in the 1000 Genome Project,

with available genotypes, sex, age, and ancestry, but no pain phenotypes. We identified 10 individuals who carried the T (major) allele, and 10 other individuals with the C (minor) allele at m.2352, for a total of 20.

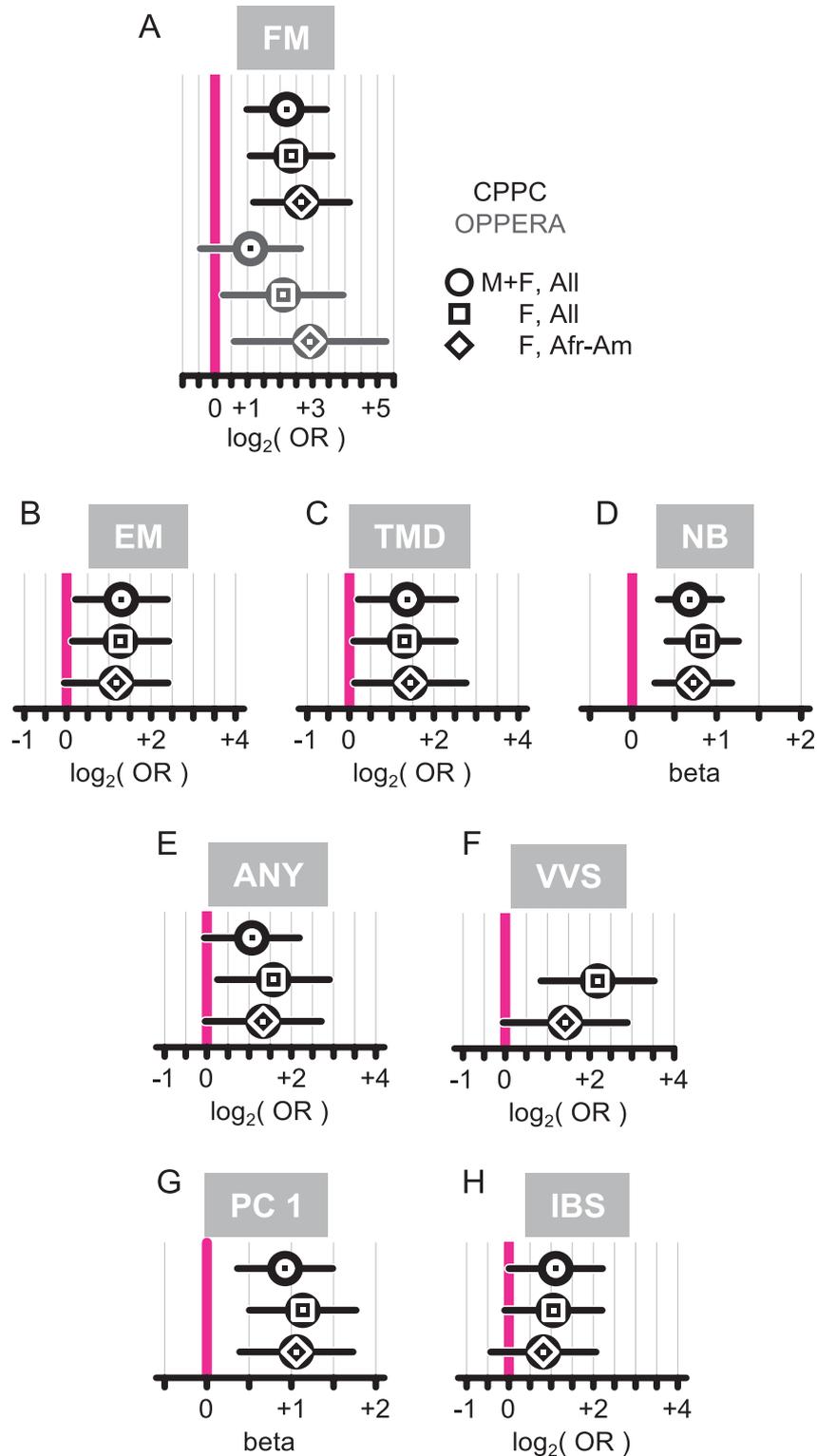
We tested the effect of the SNP on oxidative phosphorylation in B lymphoblasts by growing cells in either glucose-containing media that allowed energy production by glycolysis and oxidative phosphorylation or in galactose-containing media, which forces the cells to use oxidative phosphorylation for ATP production.<sup>13,51</sup> Increased oxidative phosphorylation results in a greater mitochondrial membrane potential ( $\Delta\psi_m$ ) due to a corresponding increase in the transport of protons across the inner mitochondrial membrane due to the ETC.<sup>44,73</sup> Thus,  $\Delta\psi_m$  is correlated with oxidative phosphorylation. The staining of both genotypic variants of cell lines with JC-10 dye was used to assess mitochondrial membrane potential by measuring fluorescence. The JC-10 dye is present as either a J-monomer in cells with low  $\Delta\psi_m$  or as aggregates in mitochondria when  $\Delta\psi_m$  is increased. These 2 forms of the dye have different excitation and emission spectra, and the ratio of fluorescence was used as a proxy for  $\Delta\psi_m$ . Furthermore, FCCP reagent, which uncouples the ETC and therefore decreases  $\Delta\psi_m$ , was used as a negative control to specifically deplete JC-10 aggregate fluorescent signal (561 nm excitation laser, 582/15 nm detection filter) without significantly affecting JC-10 monomer staining (488 nm excitation, 530/30 nm detection filter). As expected, there was much lower JC-10 aggregate staining in FCCP-treated cells (**Fig. 5A**, FCCP-treated cells).

For the experimental samples, the majority of the lymphoblasts formed 2 discrete clusters when JC-10 monomer was plotted against JC-10 aggregate signal (**Fig. 5A**). Both of these clusters had more JC-10 aggregate staining than the FCCP-treated control cells, indicating that our assay captured gross changes in  $\Delta\psi_m$ . Also, the presence of JC-10 aggregate staining in untreated lymphoblasts indicated that they were largely a viable population because a collapse of  $\Delta\psi_m$  is recognized as an early hallmark of apoptosis. Our assay also captured more subtle changes in  $\Delta\psi_m$  because cells that were grown in galactose-containing media showed an increase in JC-10 aggregate staining compared to cells grown in glucose ( $P = 1.0 \times 10^{-2}$ ) (data not shown). The increase in  $\Delta\psi_m$  in galactose-containing media was expected because the cells were reliant on oxidative phosphorylation.

Because the overlap in distributions on both axes did not allow for adequate conventional flow cytometric analysis, an unbiased cluster analysis was performed to determine that the population of live cells contained subpopulations, cluster A (high  $\Delta\psi_m$ ), and cluster B (low  $\Delta\psi_m$ ) (**Fig. 5B**). There were no obvious differences in cluster A or cluster B between B lymphoblasts with the minor or major alleles when grown in glucose-containing media. However, when grown in galactose-containing media, the B lymphoblasts with the minor allele showed a significant increase in the number of cells with a higher JC-10 monomer signal and a lower JC-10 aggregate signal (cluster B, low  $\Delta\psi_m$ ) than B lymphoblasts with the major allele ( $P = 3.8 \times 10^{-2}$ ) (**Fig. 5C**). This indicated that the cells with the minor allele had decreased  $\Delta\psi_m$  under conditions where oxidative phosphorylation was required.

## 4. Discussion

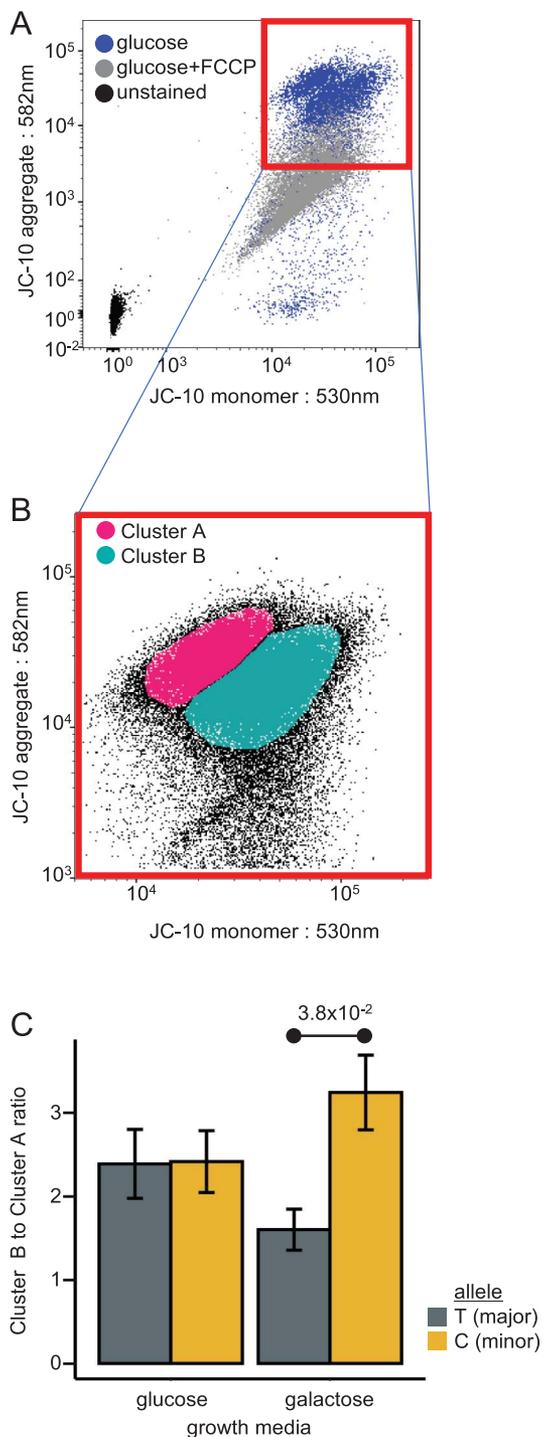
Although previous studies have unmasked the role of mitochondria in chronic pain, our analysis is the first to examine the full mitochondrial genetic makeup of people affected by a panoply of chronic pain conditions. It is also the largest sample size reported



**Figure 4.** Forest plots for the association of m.2352T>C with CPPCs. Odd ratios (log<sub>2</sub> scale) or betas with 95% confidence intervals shown. CPPC discovery data in black, whereas OPPERA replication data in gray. Population stratifications are: everyone (circle), females only (square), or African American females (lozenge). (A) Fibromyalgia (FM). (B) Episodic migraine (EM). (C) Temporomandibular disorders (TMD). (D) Number of CPPCs (NB). (E) Presence of any CPPC (ANY). (F) Vulvar vestibulitis (VVS). (G) Principal component 1 (PC 1). (H) Irritable bowel syndrome (IBS). CPPC, complex persistent pain condition; OPPERA, Orofacial Pain: Prospective Evaluation and Risk Assessment.

in the literature to date involving mitochondrial genetics and chronic pain. Because we generated mitochondrial sequencing data of a very high density in the CPPC cohort, we were able to

test different modalities of the mitochondrial genetic contribution to chronic pain, such as rare mutations, common variants, haplogroups, and heteroplasmy.



**Figure 5.** Cellular mitochondrial assay for functional characterization of SNP m.2352T>C using JC-10 staining. (A) Fluorescence scatter plot showing JC-10 aggregate intensity (582 nm channel) as a function of JC-10 monomer intensity (530 nm channel). Plotted data from JC-10 stained B lymphoblast cells either without treatment (blue) or treated with mitochondrial uncoupling agent, FCCP (10  $\mu$ M) (gray). Background signal shown by cells left unstained for JC-10 (black). (B) Representation of the 2 cell clusters of live B lymphoblasts, A and B, generated by density-based spatial clustering of applications with noise (DBSCAN) on a fluorescence scatter plot showing JC-10 aggregate intensity (582 nm channel) as a function of JC-10 monomer intensity (530 nm channel). (C) The ratio of cells in cluster B, which has lower  $\Delta\Psi_m$ , to cluster A, which has higher  $\Delta\Psi_m$ . Ratio measurements performed in glucose- and galactose-containing growth media. In each medium, ratio measurements were performed for B lymphoblasts with the T (major) and C (minor) alleles. Error bars represent mean  $\pm$  SEM. 10 biological replicates for each treatment group.

The most robust association results have been obtained for the common polymorphic variants. We found that SNP m.2352T>C was associated with an increased risk for fibromyalgia in the presence of the alternative C allele. The replicated genetic effect size of the C allele on the disease risk (OR 5.1 and 4.3 in discovery and replication cohorts, respectively) is impressive and has little precedence within the field of common diseases. The relatively high minor allelic frequency of the associated allele makes our result even more unique, as an inverse relationship between an SNP's frequency and disease OR is predicted by population genetics and is observed in a daily fashion with the outpouring of genome-wide association study results.<sup>17,43</sup> In the sex-specific stratified analysis, the association was significant only in women but not in men, although it is difficult to be sure that the identified effect is truly sex-specific because of the limited number of male fibromyalgia patients. Furthermore, the minor allele of this SNP was abundant in participants of African American ancestry and much rarer in other ancestry groups. However, for the majority of pain phenotypes, the effect size of the C allele was stronger in a mixed population than in the African American population (Figs. 4F–H), suggesting that the effect of the C allele is not race-specific.

Although our primary screen identified SNP m.2352T>C most significantly associated with FM, we observed other significant associations with VWS and number of CPPCs, and noticed nominal associations with all other CPPCs in secondary analyses, in a consistent risk-associated fashion. The association was observed in EM, TMDs, IBS, and number of CPPCs. Association with the global pain phenotype through the first principal component was also significant.

This SNP was not previously documented to be associated with a disease or trait in the Online Mendelian Inheritance in Man (OMIM) catalog,<sup>1</sup> nor is its clinical significance reported in the ClinVar resource.<sup>28</sup> This might be because genome-wide association studies are mainly conducted in people of European ancestry, although this shortcoming is now being addressed.<sup>42</sup> Furthermore, the m.2352T>C SNP is not routinely assessed because it is missing from popular genotyping arrays, including those used by large genetic studies such as the UK Biobank project and 23AndMe. We attempted, but failed, to unambiguously impute the SNP using the large database of complete human mitochondrial sequences (MITOMAP<sup>37</sup>), indicating low linkage disequilibrium with neighboring genotyped SNPs. Thus, our results suggest that including the m.2352T>C SNP into future genotyping platforms will benefit the research field of mitochondrial genetics.

The m.2352T>C SNP is situated in mitochondria's 16S rRNA gene, the large subunit of the ribosome<sup>25</sup> (gene MT-RNR2). It is also situated in the 5'UTR of the humanin gene, a peptide with antiapoptotic and neuroprotective properties, although there is uncertainty whether this is a transcribed protein-coding gene, or if it is a nuclear pseudogene of the mitochondrial MT-RNR2 gene.<sup>20,35,39</sup> The alternative allele may affect the stability of the transcript or its secondary structure of one or both corresponding RNAs, as well as the translation level of humanin. It is also possible that m.2352T>C polymorphism alters ribosomal translation speed or decoding fidelity. Unfortunately, the crystal structure of the large ribosomal subunit of human mitochondria does not display enough electron density to unambiguously resolve the position of m.2352 (chain A of PDB code 3J7Y<sup>6</sup>), hampering the deciphering of its role.

To test if the polymorphic variant affects overall cellular function, we conducted an assay in which mitochondria were presented with alternative energy sources while monitoring the

mitochondrial membrane potential. The mitochondrial membrane potential is a consequence of electron transport in mitochondria, which is necessary for ATP synthesis. It also has non-ATP-producing functions such as cell viability.<sup>73</sup> Although limited variation in the mitochondrial membrane potential is common, prolonged changes can affect mitochondrial function.<sup>73</sup> We found that cells carrying the minor allele compared to the major allele were more likely to be in the cell cluster with increased JC-10 monomer staining when grown in galactose, rather than in glucose. This result suggests that the presence of the minor allele decreased the mitochondrial membrane potential under conditions where oxidative phosphorylation is required. Although the exact mechanism is unknown, our results indicate that SNP m.2352T>C impacts oxidative phosphorylation, thus potentially linking oxidative phosphorylation with the development of chronic pain conditions. Our results are concordant with previous findings on an association between impaired mitochondrial metabolism and fibromyalgia<sup>9</sup> and a decreased level of coenzyme Q10, an essential electron carrier in the mitochondrial respiratory chain, in the blood of fibromyalgia patients.<sup>8</sup> These relationships are of particular interest because they are indicative of potential therapeutic targets.<sup>10</sup>

To our surprise, we did not find any significant associations when we tested the contribution of rare variants to pain states using the SKAT-O approach, neither by combining their effects on genes nor pathways. By contrast, such associations have been found for Complex I of the OXPHOS pathway and cancer,<sup>33</sup> mitochondria-wide rare variants and schizophrenia,<sup>19</sup> or specific, combined gene-based analyses with various metabolic traits.<sup>27</sup> Furthermore, haplogroup-based tests did not produce any significant results with pain-related phenotypes.

When we tested heteroplasmy levels, the coexistence of multiple alleles at the same genomic locus in a given individual, we found overall small but significantly elevated heteroplasmy levels at defined mitochondrial genomic loci in control subjects compared to those with any one of the CPPC. This indicated that nucleotide diversity might be beneficial regarding protection from painful conditions. We could not find a cohort to test this finding for replication. However, our results are in line with previous findings that have shown that mitochondrial heteroplasmy is widespread and tolerated in healthy subjects despite its pathogenicity under specific circumstances.<sup>71</sup> Moreover, the relationship between heteroplasmy level and pathogenicity has been demonstrated to display nonlinear behavior, as for the case with m.3243A>G,<sup>26</sup> in which distinct cellular consequences can be observed dependent on increasing minor allele dosage. The diversity hypothesis has been shown fruitful for cell survival in other cellular contexts.<sup>53,54</sup>

This study has many strengths, including (1) deep-sequencing of mitochondrial-enriched DNA fragments to determine with high accuracy the complete mitochondrial genetic makeup of people affected by chronic pain. (2) A large sample size compared to other studies of the mitochondria's genetic role in pain. (3) Inclusion of human subjects with diverse mtDNA haplogroups, in particular H and L. (4) Inclusion of several pain conditions previously unstudied with respect to mtDNA. (5) Assessment of all participants by medical experts for each of the 5 conditions providing reliable, high-quality phenotyping. It also has several weaknesses. (1) Although the sample size of our study is the largest published yet, the results indicated that the cohort is underpowered to detect low effect size for common SNPs, and combined effects for grouped rare variants. (2) We have assessed the association between heteroplasmy and CPPCs, but could not find an appropriate cohort to replicate our findings. (3) We used B

lymphoblast cell lines for our functional assays to test the allelic effect on oxidative phosphorylation. The B lymphoblasts were used as a cell model for measuring allelic-dependent mitochondrial membrane potential because these were the only cell lines with fully characterized genotypes available; the cells contributing to pathophysiology of pain states, such as neurons, would be better choice. (4) Great care has been put into selecting individuals from the NHGRI sample repository such that the subjects with both the T and C alleles of m.2352 were randomized, but we cannot rule out the possibility that other SNPs in high linkage disequilibrium are the true effectors of the observed modulation of the mitochondria membrane potential.

In conclusion, our results suggest that the m.2352T>C polymorphism has a strong clinical effect on the risk of fibromyalgia and possibly other chronic pain conditions. Prevalence of the SNP was elevated in participants of African American ancestry, whereas almost absent in those of Caucasian ancestry. Using a cellular assay, we identified differences in mitochondrial functions in B lymphoblast cells from individuals with defined allelic variants at that SNP position. We show that the SNP allele is associated with lower mitochondrial inner membrane potential during oxidative phosphorylation. This implies that decreased cellular energy metabolism may contribute to chronic pain, although the exact mechanisms still need to be identified. Taken together, our findings suggest a novel pathway for the development of treatments for chronic pain patients directed at detecting and restoring mitochondrial dysfunction.

### Conflict of interest statement

The authors have no conflicts of interest to declare.

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Author contributions: M.A.L. van Tilburg, R.G. Boles, and L. Diatchenko designed the analytical plan and experiments. W.E. Whitehead, D.A. Zolnoun, I. Tchivileva, and W. Maixner performed clinical assessments and diagnostics for CPPCs. A.G. Nackley, S.B. Smith, and G.D. Slade were responsible for human subject collection and samples processing. M. Parisien, S. Khoury, and V. Verma performed bioinformatics analyses. G.L. Drury, J. Smith-Voudouris, and A.-J. Chabot-Doré performed cellular assays. M.A.L. van Tilburg, M. Parisien, R.G. Boles, and L. Diatchenko interpreted the results and wrote the initial manuscript. All the authors read and edited the final manuscript.

## Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at <http://links.lww.com/PAIN/B105>, <http://links.lww.com/PAIN/B106>, <http://links.lww.com/PAIN/B107>, <http://links.lww.com/PAIN/B108>, <http://links.lww.com/PAIN/B109>, <http://links.lww.com/PAIN/B110>, and <http://links.lww.com/PAIN/B111>.

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