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Mitochondrial DNA copy number is modulated by genetic variation in the signal transducer and activator of transcription 3 (*STAT3*)

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ABSTRACT

The regulation of mitochondrial DNA (mtDNA) copy number not only is critical for the maintenance of the normal mitochondrial function but has a strong clinical significance. A recent report revealed that the signal transducer and activator of transcription 3 (*STAT3*) is involved in the regulation of the mitochondrial function and is required for the optimal function of the electron transport chain. In this study, we explored whether gene variants in the *STAT3* influence the leukocyte mtDNA copy number. Clinical data and blood samples were collected from 179 subjects (aged 52.8 ± 0.9 years). Mitochondrial DNA quantification using nuclear DNA (nDNA) as a reference was carried out by a real-time quantitative polymerase chain reaction method; results are presented as the mtDNA/nDNA ratio. We selected 3 tag single nucleotide polymorphisms showing a minor allele frequency greater than 10% (rs2293152 C/G, rs6503695 C/T, and rs9891119 A/C), representing 24 polymorphic sites of the *STAT3* ($r^2 > 0.8$). We observed a significant association between mtDNA/nDNA ratio and both rs6503695 and rs9891119, adjusted by age and homeostasis model assessment index. The proportion of the total variance of the mtDNA/nDNA ratio accounted for by the rs6503695 and rs9891119 genotypes was 4.7% and 6.53%, respectively. Common variation in the *STAT3* may influence mtDNA copy number.

1. Introduction

The human mitochondrial genome is a double-stranded circular molecule of DNA encompassing 16 569 base pairs (bp) in length that encodes 12S and 16S rRNAs, 22 tRNAs, and 13 polypeptides. Mitochondria are involved in the respiratory electron transport chain and adenosine triphosphate synthesis; thus, it is not surprising that the number of mitochondria and mitochondrial DNA (mtDNA) molecules per mitochondrion

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varies according to the different cell types and the specific tissue energetic demands. On average, a single cell can contain from 200 to 2000 mitochondria [1]; and although the number of mitochondria in specific cell types varies considerably, within a given cell type, the number is closely regulated [2]. In addition, despite differences in the number of mitochondrial genome copies per cell (ranging mostly from 1000-10 000 [3]) that were previously reported, Tang et al [4] showed that mammalian cells maintain a constant mass of mtDNA, suggesting that mtDNA copy number is a tightly controlled process.

The control of mitochondrial biogenesis is extremely complex; and in addition to the mitochondrial RNA polymerase, several nuclear genes are involved in the maintenance of the mtDNA content, for instance, mitochondrial transcription factor A (*TFAM*), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PPARGC1A*), and nuclear respiratory factor 1 (*NRF1*) [5]. Moreover, it was recently reported that the peroxisome proliferator-activated receptor gamma (*PPARG*) activation promotes biogenesis of functional mitochondria [6]. As well, *PPARG* activation increases the expression of the messenger RNA (mRNA) of mitochondrial uncoupling protein–1, a member of the family of mitochondrial anion carrier proteins [7]. In addition, *PPARG* is required for the biologic function of *PPARGC1A* on mitochondrial biogenesis [8].

Interestingly, a recent report from Wegrzyn and coworkers [9] revealed that the signal transducer and activator of transcription 3 (*STAT3*) plays a key role in the regulation of the mitochondrial function and provided evidence that *STAT3* not only is required for the optimal function of the electron transport chain, but is a modulator of the mitochondrial respiration. These observations are consistent with the finding that *STAT3* also resides within the mitochondria [9]. Actually, the authors stated that *STAT3* exerts its actions not as a transcription factor that regulates nuclear gene expression, but through its localization in the mitochondria.

The regulation of the mtDNA copy number not only is critical for the maintenance of the normal mitochondrial function but has a strong clinical significance, as many syndromes and diseases are associated with depletion of mtDNA content. In fact, it is suggested that even in the absence of detectable mtDNA mutations, abnormal amounts of mtDNA, either depletion or elevation, can be indicative of mitochondrial dysfunction [10]. Although mtDNA mutations, often founded as substitution, deletion, or duplication of mtDNA bases, are widely associated with different mitochondrial, metabolic, cardiovascular, and neurologic disorders, there is also some evidence about the role of genetic variation in nuclear genes contributing to the regulation of the mtDNA copy number [11-13]. Nevertheless, the published evidence is mostly restricted to the role of genetic polymorphisms of the *TFAM*.

Based on the hypothesis that polymorphisms in nuclear and mitochondrial genes might regulate the mtDNA copy number and the fact that *STAT3* not only is required the optimal mitochondrial function but also resides within the mitochondria, we explored whether common gene variants in the *STAT3* influence the mtDNA content. We further investigated the role of functional gene variants in 2 additional genes involved in the regulation of mitochondrial biogenesis and mtDNA copy number, such as *TFAM* and *PPARG*.

2. Subjects and methods

2.1. Subjects

A sample of 179 subjects, aged 52.8 ± 0.9 years, was included in our study in a cross-sectional fashion. Health examinations included anthropometric measurements, a questionnaire on health-related behaviors, and biochemical determinations. After a 30-minute rest in a quiet room, the systolic and diastolic arterial blood pressure was measured while the subject was in sitting position. The blood pressure values were the means of 3 different measurements. Body mass index was calculated as weight/height^2 (kilograms per square meter) and was used as the index for relative weight. In addition, trained staff assessed waist and hip circumferences.

All participants were asked to fast for at least 8 hours, and blood was drawn from subjects who had lain in supine resting position for at least 30 minutes. Serum insulin, total cholesterol, high-density lipoprotein and low-density lipoprotein cholesterol, triglycerides, and plasma glucose were measured by standard clinical laboratory techniques. Homeostasis model assessment (HOMA) was used to evaluate the insulin resistance index and was calculated as fasting serum insulin (microunits per milliliter) × fasting plasma glucose (millimoles per liter)/22.5.

2.2. Quantification of mtDNA

Nucleic acids were extracted from white blood cells from a blood sample by a standard method as previously described [14]. An assay based on real-time quantitative polymerase chain reaction (PCR) was used for both nuclear DNA (nDNA) and mtDNA quantification using SYBR Green as a fluorescent dye (Invitrogen, Buenos Aires, Argentina) as we previously described [15]. Briefly, the primer sequences for mtDNA, mtF3212 (5′CACCCAAGAACAGGGTTTGT3′) and mtR3319 (5′ TGGCCATGGGTATGTT-GTTAA3′), and those for nDNA for loading normalization, 18S rRNA gene 18S1546F (5′TAGAGG-GACAAGTGGCGTTC3′) and 18S1650R (5′CGCTGAGCCAGTCA-GTGT3′), were reported previously by Bai et al [10].

The PCR profile was 1 cycle of 95°C for 2 minutes followed by 35 cycles (95°C 15 seconds and 60°C 1 minute). Real-time quantitative PCR was carried out in a BioRad iCycler (Bio-Rad Laboratories, Hercules, CA). The calculation of DNA copy number involved extrapolation from the fluorescence readings in the mode of background subtracted from the BioRad iCycler according to Rutledge [16].

The evaluation of the specificity of the amplified (108 bp) region of mtDNA was assessed as we previously described [15].

The 2 target amplicon sequences (mtDNA and nDNA) were visualized in agarose 2% and purified by Qiagen Qiaex II gel extraction kit (Tecnolab, Buenos Aires, Argentina), and dilutions of purified amplicons were used as the standard curve. The interassay variation coefficient was less than 20%. The results were presented as the mtDNA/nDNA ratio.

2.3. *STAT3* gene variants

To assess the contribution of *STAT3* variants to the mtDNA content in peripheral white blood cells, we selected tag single

Table 1 – Clinical and biochemical characteristics of the genotyped subjects

Variables	Mean ± SE
No. of subjects	179
Age (y)	52.8 ± 0.9
Sex (male/female)	54/125
Smoking habit (cigarettes/d)	1.44 ± 0.4
Physical activity (h/wk)	1.42 ± 0.2
Drinking habits (g alcohol/d)	1.05 ± 0.4
BMI (kg/m^2)	32.3 ± 2.04
Waist-hip ratio	0.89 ± 0.006
SBP (mm Hg)	122.9 ± 1.4
DBP (mm Hg)	76.6 ± 1.1
Fasting plasma glucose (mmol/L)	5.41 ± 0.15
Fasting plasma insulin (pmol/L)	77.4 ± 4.9
HOMA index	2.77 ± 0.2
Total cholesterol (mmol/L)	5.43 ± 0.14
HDL cholesterol (mmol/L)	1.20 ± 0.05
LDL cholesterol (mmol/L)	3.09 ± 0.14
Uric acid (mmol/L)	240 ± 26
Triglycerides (mmol/L)	1.98 ± 0.12

All measurements are in SI units. BMI indicates body mass index; SBP, systolic arterial blood pressure; DBP, diastolic arterial blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

nucleotide polymorphisms (SNPs) by using an aggressive tagging approach to capture alleles of interest and the phase II genotyping data from the HapMap project for whites (CEU data set) with a minor allele frequency (MAF) of at least 0.10 and a minimum r^2 of 0.8.

The *STAT3* gene contains 24 exons and spans more than 75.17 kilobases (kb) in chromosome 17q21 at location 37 718 869-37 794 039. To diminish the burden of genotyping the complete number of variants of the *STAT3* (the HapMap B35 full set database includes 28 polymorphic sites with MAF >0.05), we selected 3 tag SNPs showing a MAF greater than 10% (rs2293152 C/G located in the intron 13, rs6503695 C/T located in the intron 2, and rs9891119 A/C located in the noncoding region near the gene promoter) encompassing 68.55 kb of the gene. The 3 *STAT3* tag SNPs represent 24 polymorphic sites with an r^2 greater than 0.8 considering the HapMap project data [17]. Genotyping was performed by a high-throughput genotyping method as previously described [18]. The PLINK software was used for testing Hardy-Weinberg equilibrium, linkage disequilibrium (LD) measures, and imputing haplotypes [19].

2.4. *PPARG and TFAM variants*

Genotyping for *PPARG* (pro12Ala) and *TFAM* (rs1937) polymorphisms was performed by Hot-start PCR-based restriction fragment length polymorphism analysis using a Robocycler 96 thermal cycler (Stratagene, La Jolla, CA) and molecular biology grade reagents unless otherwise indicated.

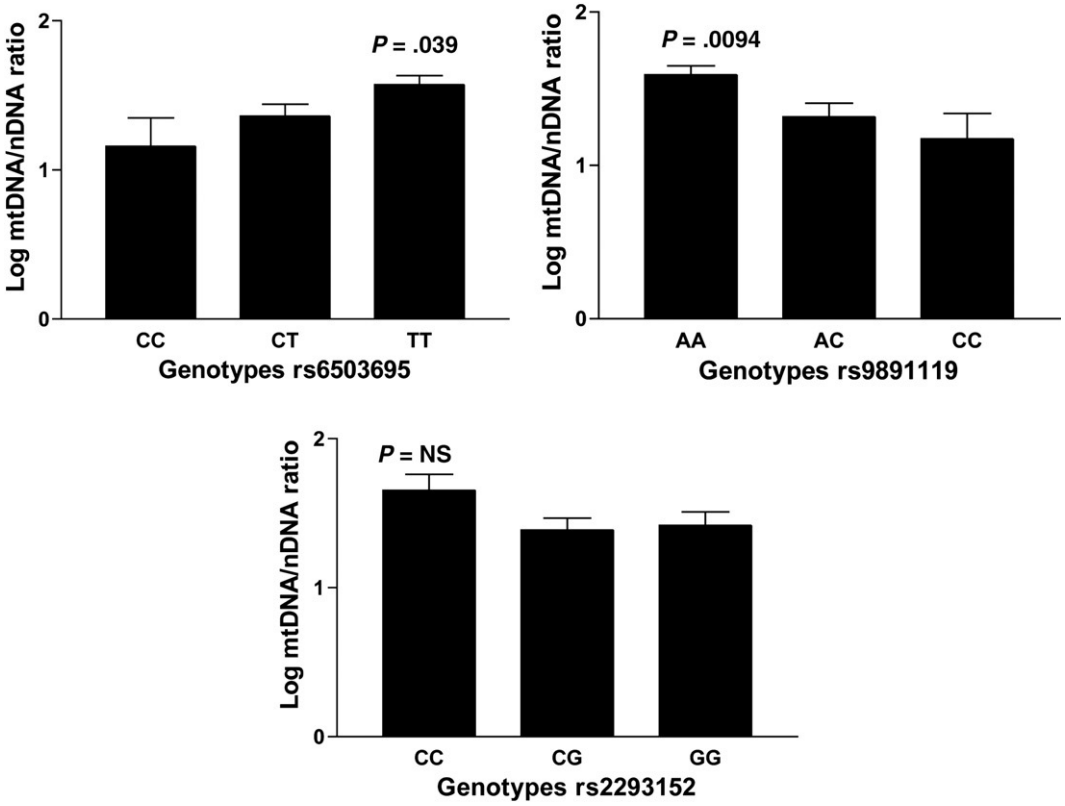


Fig. 1 – Association test results for *STAT3* gene variants and peripheral blood leukocyte mtDNA copy number. Results are expressed as mean ± SE. P stands for the level of statistical significance using ANCOVA for the log-transformed mtDNA/nDNA ratio as a dependent variable, genotype as a categorical factor adjusted by age, and log HOMA as a covariate. NS indicates not significant.

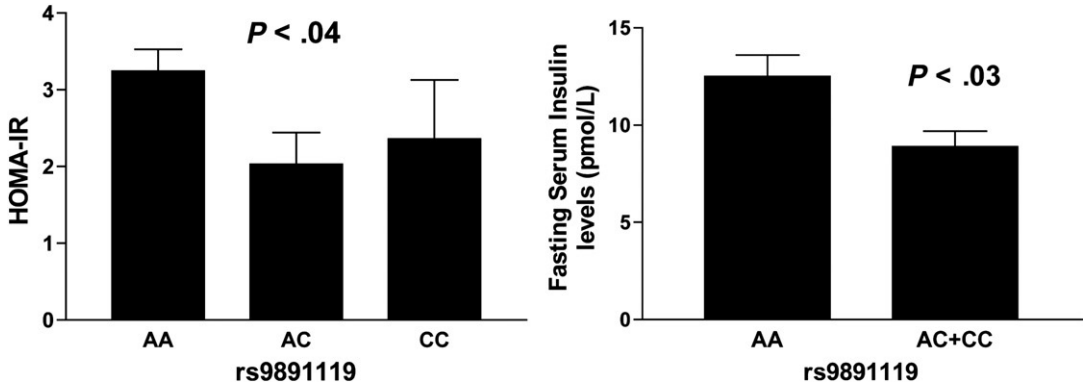


Fig. 2 – Association test results for *STAT3* rs9891119 genotypes and HOMA of insulin resistance (left panel) and plasma fasting insulin levels (right panel). Results are expressed as mean ± SE. *P* stands for the level of statistical significance using ANCOVA.

The most commonly studied *PPARG* variant is the coding-nonsynonymous pro12Ala (rs1801282); thus, it was chosen in our study. In addition, the minor ala allele is associated with decreased binding affinity to the cognate promoter element and reduced ability to transactivate responsive promoters [20]. Primers to detect Pro12Ala variant were 5′-GCC AAT TCA AGC CCA GTC-3′ and 5′-GAT ATG TTT GCA GAC AGT GTA TCA GTG AAG GAA TCG CTT TCC G-3′; the variant was genotyped using digestion with *Bst*UI (New England Biolabs, Ipswich, MA).

We selected for genotyping in our study the rs1937, a missence SNP (Ser12Thr) located in exon 1 of the *TFAM* gene, because this SNP is the only coding-nonsynonymous variant validated by cluster, frequency, and the HapMap. In addition, sequence analysis of a part of *TFAM* exon 1 confirmed SNP rs1937 (G>C), predicting the amino acid change S12T in the mitochondrial signal peptide sequence [21]. Primers to detect the rs1937 polymorphism were 5′ TAGGAGGGGCAGAAAGTGA 3′ and 5′ CGGGTTCCAGTTGTGATTG 3′, and genotyping used digestion with *Dde*I (New England Biolabs, Ipswich, MA).

2.5. *Statistical analysis*

Quantitative data were expressed as mean ± SE. Because the mtDNA/nDNA ratio does not have a normal distribution, we assessed differences in this variable according to genotypes as categorical factors by analysis of covariance (ANCOVA) after a log transformation and using age and HOMA index as cofounders owing to the well-known effect of these variables on mitochondrial DNA content. Univariate and haplotype analyses were performed by linear regression as implemented in the PLINK software. We used the CSS/Statistica program package, StatSoft V 6.0 (Tulsa, OK) to perform these analyses.

3. Results

A summary of the clinical features, anthropometric variables, and laboratory findings of the participants is shown in Table 1.

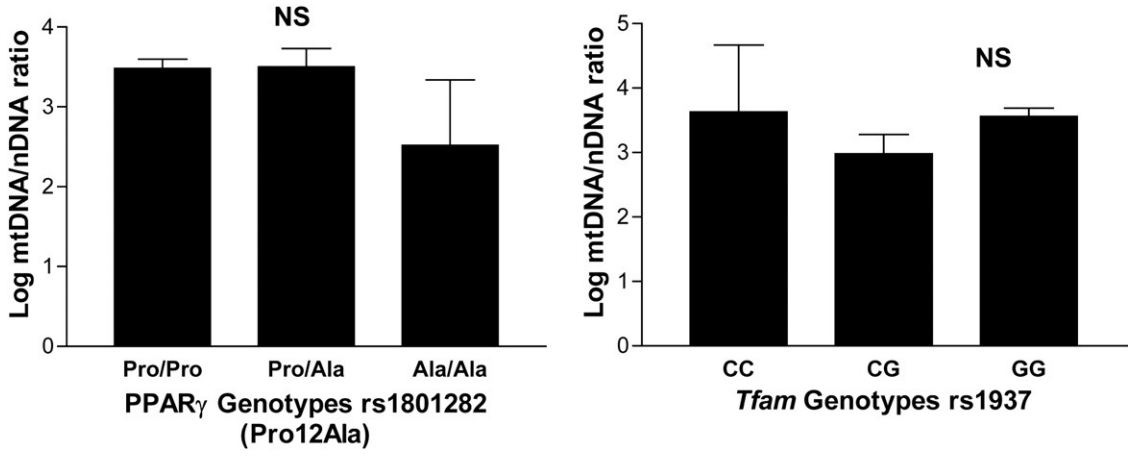


Fig. 3 – Association test results for PPARγ and *TFAM* gene variants and peripheral blood leukocyte mtDNA copy number. Results are expressed as mean ± SE. *P* stands for the level of statistical significance using ANCOVA for the log-transformed mtDNA/nDNA ratio as a dependent variable, genotype as a categorical factor adjusted by age, and log HOMA as a covariate.

STAT3 genotype frequencies were in Hardy-Weinberg equilibrium. Statistical analysis of the log-transformed mtDNA/nDNA ratio of individuals classified according to genotypes of *STAT3* tag SNPs showed a significant association between mtDNA/nDNA ratio and SNPs rs6503695 and rs9891119, either unadjusted (rs6503695; β: –0.4852 ± 0.1871, $P < .011$ and rs9891119; β: –0.5546 ± 0.174, $P < .0018$, which remained significant even after considering the most conservative multiple testing correction of Bonferroni $P < .05/3 = .016$) or adjusted by HOMA and age as covariate (Fig. 1); the lower mtDNA content was observed in those homozygous for either the rs6503695 C or rs9891119 C allele. The proportion of the total variance of the mtDNA/nDNA ratio accounted for by the rs6503695 and rs9891119 genotypes was 4.7% and 6.53%, respectively. These associations are not entirely surprising because of the relative linkage of both SNP rs6503695 and SNP rs9891119 (r^2: 0.778). Although we found no significant association between mtDNA content and the rs2293152 variant (Fig. 1), the relatively common haplotype composed of the rs2293152 G, rs6503695 C, and rs9891119 C variants (frequency: 19%) was significantly associated with decreased mtDNA content (β: –0.6672, $P < .0015$) and a trend toward the opposite for the haplotype composed of the rs2293152 C, rs6503695 T, and rs9891119 A variants (frequency: 45%, β: 0.307, $P < .07$). To further evaluate the potential physiologic significance of our findings, we examined whether *STAT3* variation correlated with any clinical or biochemical parameter in our sample. Interestingly, we observed that the rs9891119 genotypes were also significantly associated with HOMA of insulin resistance index (Fig. 2, left panel). Moreover, those homozygous for the rs9891119 A allele show significantly higher levels of plasma fasting insulin in comparison with carriers of the C allele (Fig. 2, right panel).

In addition, the variants of *PPARG*, rs1801282 (pro12Ala) and *TFAM*, rs1937 were evaluated in the whole sample; and genotype frequencies were in Hardy-Weinberg equilibrium. When we studied the mtDNA content in relation with the aforementioned variants of these candidate genes, we found no significant differences in the mtDNA/nDNA ratio among the genotypes (Fig. 3).

4. Discussion

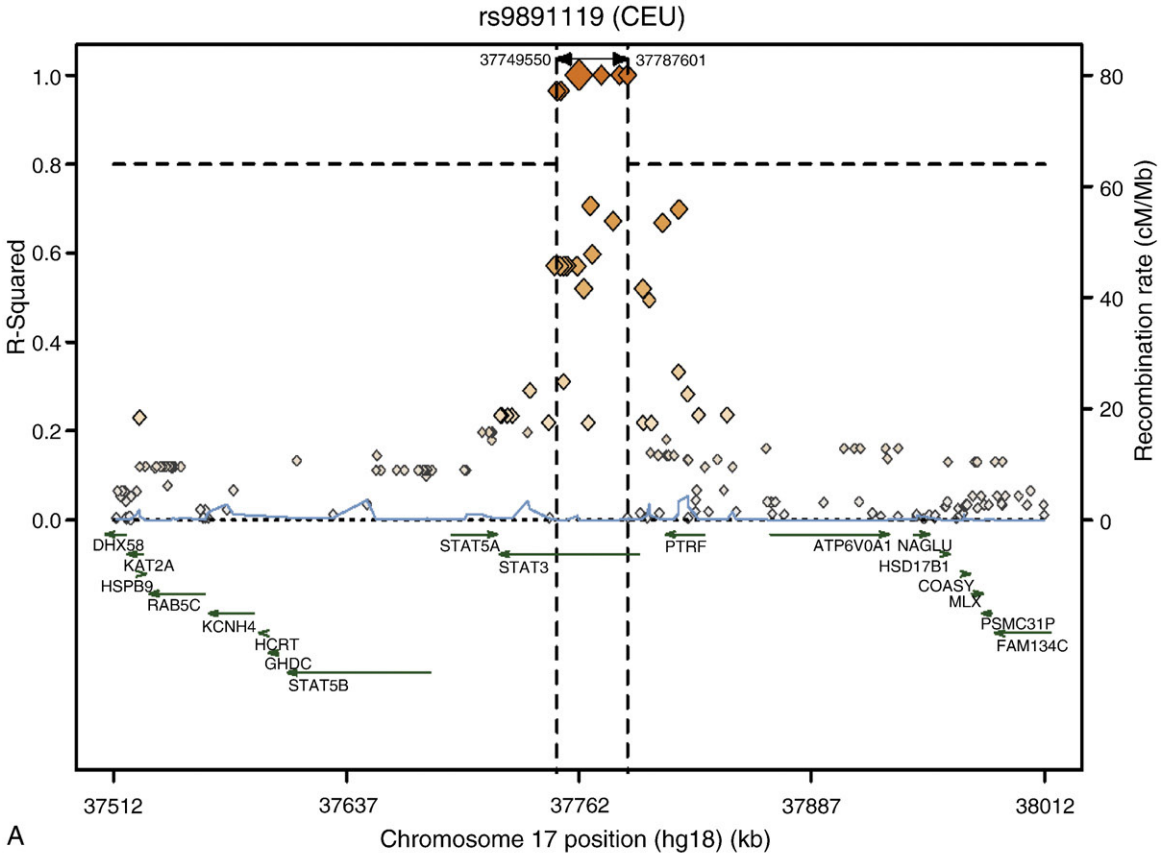
The main conclusion of this study is that common variation in the *STAT3* may be implicated in the regulation of mtDNA copy number. The SNPs we found to be strongly associated with the mtDNA/nDNA ratio accounted for a substantial proportion of the total variance (4.7%-6.53%), as the genetic contribution to the phenotypic variance in complex traits is much less on average. To our knowledge, the involvement of the *STAT3* gene variation as a modulator of the mtDNA content has never been previously reported.

STAT3, a transcription factor also known as an acute-phase response factor, is involved in several cellular process, such as cell life and differentiation, innate immune response, hepatic glucose homeostasis and carbohydrate metabolism, cardiomyocyte resistance to inflammation and acute injury, and leptin actions on energy balance, among others [22]. STAT3 protein is widely expressed and is located in the cytoplasm and translocated to the nucleus after activation by tyrosine phosphorylation. It is now known that STAT3 is also present in the mitochondria of cultured cells and primary tissues, including the liver and heart [9]. Thus, STAT3 has an additional property independent of its classic role in the nucleus, as a regulator of the metabolic function in mitochondria. Interestingly, it was shown that *STAT3* is capable of binding to the regulatory D-loop region of human mtDNA upon activation [23]; and the D-loop region is responsible for the control of replication and transcription of mtDNA. Thus, it sounds reasonable to speculate that *STAT3* may modulate the mtDNA content. It is worth mentioning, however, that previous experimental studies in Stat3$^{-/-}$ pro-B cells suggested that neither mtDNA content nor mitochondrial concentrations of mitochondrial-encoded RNAs were affected by the absence of Stat3 [9,24]. Thus, the discrepancies between the experimental data and our findings deserve further investigation.

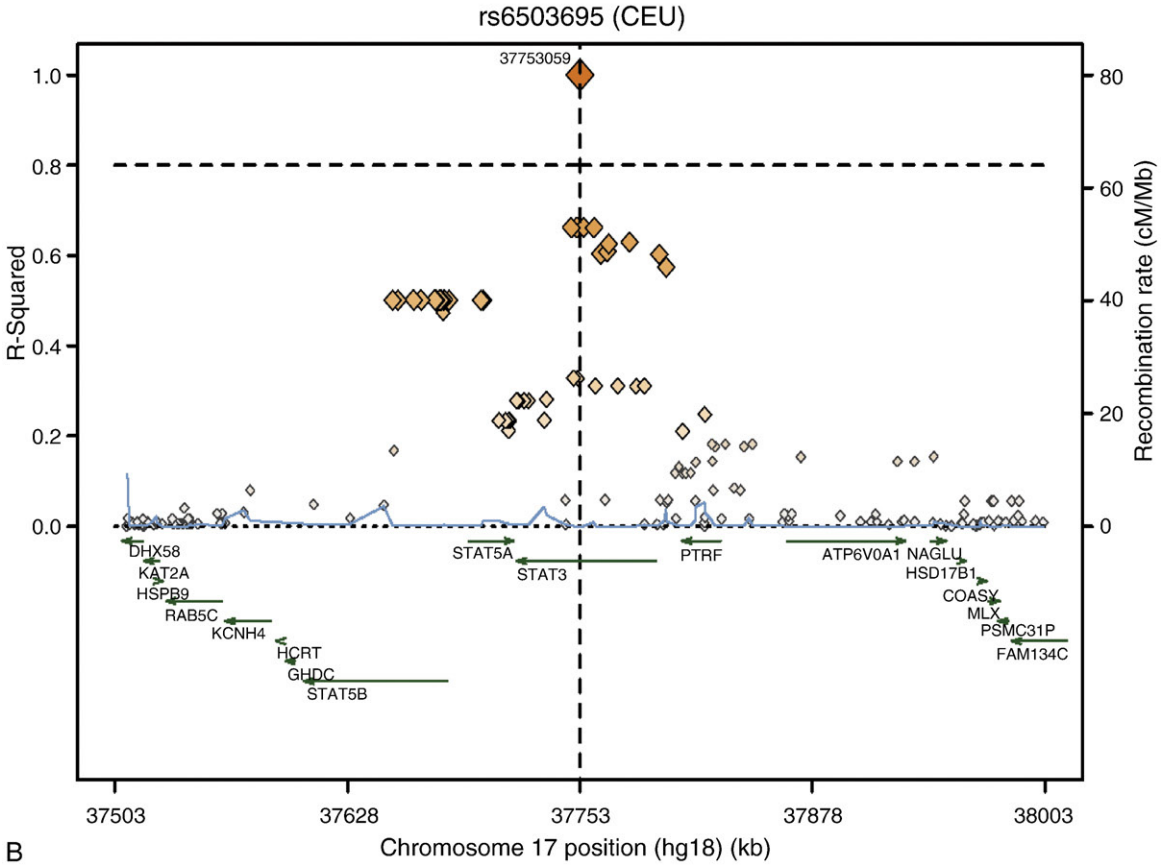
Furthermore, several pieces of evidence can be linked together to support our results and explain how *STAT3* and mtDNA content may be biologically related. First, mtDNA depletion may contribute to tumorigenesis, as mtDNA copy number is diminished in many types of cancers, including ovarian, gastric, hepatocellular, and renal cell and carcinoma [25]; and *STAT3* is an oncogene persistently active in a high proportion of all human cancers [26]. In addition, mitochondrial STAT3 is involved in altered glycolytic and oxidative phosphorylation activities of cancer cells [24]. Second, mtDNA depletion was associated with metabolic syndrome and insulin resistance [15,27,28]; and *STAT3* gene variation was associated with nonalcoholic fatty liver diseases in patients with metabolic syndrome [17]. Third, we showed that mtDNA/nDNA ratio was significantly lower in both extremes of abnormal fetal growth [29]; and *STAT3* is involved in early embryogenesis and postnatal survival and growth [30,31]. Furthermore, aging and life span are closely correlated with mtDNA damage and content [32]; and *STAT3* has been associated with age-related heart failure in mice [33]. Finally, there are a convincing number of studies showing that highly active antiretroviral therapy produces a depletion of mtDNA content. Surprisingly, nelfinavir disrupts *STAT3* signaling by blocking interleukin-6–induced phosphorylation of STAT3 and inhibited STAT3 DNA binding activity [34]. Our findings about a significant association between *STAT3* variation and metabolic parameters of insulin resistance may add some clues about the putative physiologic impact of our results. In fact, a recent report showed that *STAT3* signaling is mechanistically related to the development of hepatic insulin resistance and dyslipidemia [35].

Until now, the interaction between the mitochondrial and nuclear genomes in terms of regulation of mitochondrial

Fig. 4 – Regional LD plots for the 2 significantly associated SNPs of the *STAT3*, rs9891119 (A) and rs6503695 (B), at chromosome 17 (250 kb) with mtDNA. The SNPs are plotted with their proxies (shown as diamonds) (based on HapMap data for CEU) as a function of genomic location, annotated by the recombination rate across the locus and nearby genes. The regional association plots were performed by the SNAP server, available at http://www.broad.mit.edu/mpg/snap/http://www.broad.mit.edu/mpg/snap/.


rs9891119 (CEU)
37749550
37787601
R-Squared
Recombination rate (cM/Mb)
DHX58
KAT2A
HSPB9
RAB5C
KCNH4
HCRT
GHDC
STAT5B
STAT5A
STAT3
PTRF
ATP6V0A1
NAGLU
HSD17B1
COASY
MLX
PSMC31P
FAM134C
37512
37637
37762
37887
38012
Chromosome 17 position (hg18) (kb)

A


rs6503695 (CEU)
37753059
R-Squared
Recombination rate (cM/Mb)
DHX58
KAT2A
HSPB9
RAB5C
KCNH4
HCRT
GHDC
STAT5B
STAT5A
STAT3
PTRF
ATP6V0A1
NAGLU
HSD17B1
COASY
MLX
PSMC31P
FAM134C
37503
37628
37753
37878
38003
Chromosome 17 position (hg18) (kb)

B

biogenesis has been mostly focused on some particular nuclear genes. For instance, previous evidence showed that *TFAM* controls the mitochondrial copy number by recognition of mtDNA sequence motifs, which are involved in the mtDNA transcription and replication machineries [36]. Afterward, in addition to the *STAT3* variants, we also evaluated whether 2 functional polymorphisms of the genes *TFAM* and *PPARG* were associated with mtDNA content. We did not observe any significant association between leukocyte mtDNA content and these variants even after adjustment for potential confounders such as age and HOMA index. Although we were unable to find a significant association between *PPARG* and *TFAM* polymorphisms and mtDNA content, we do not exclude the possibility that a novel or untyped variant in linkage equilibrium with the selected SNPs in the previously mentioned genes may be involved in the regulation of mtDNA content.

As a final comment, it is noteworthy that the *STAT3* tag SNPs genotyped in our study represent another 24 gene variants [17] (including 3 SNPs in the 3′UTR region and 21 intronic SNPs); thus, they may be not necessarily the causal variant. Although our study does not answer the question of how these SNPs exert their effect on mtDNA content, it is important to note that analyzing the functional effect of SNPs will not always explain the mechanism by which the SNP alleles cause the phenotypic changes. In particular, because any alteration in DNA sequence may have an effect other than gene expression and protein function, SNPs in the 3′ can influence RNA half-life and ribosomal translation of mRNA; and intronic SNPs may interfere with the mRNA processing [37]. Moreover, the variants can modulate the function of other genes on different chromosomes. For instance, annotation of nearby SNPs in LD (proxies) with the rs9891119 and rs6503695 variants based on HapMap data shows that although the putatively associated variants are in the *STAT3* locus, they also represent some other variants, despite having a low r^2, of 2 nearby loci (*PTRF*, RNA polymerase I and transcript release factor and *STAT5A*, signal transducer and activator of transcription 5A) (Fig. 4).

Finally, the STAT3 variants identified in our study were found in association with other human diseases [38-40], suggesting that they or their tagged SNPs may have any affect on STAT3 protein or transcription. Moreover, the rs6503695 is in high LD with the rs4796793, a variant located in the *STAT3* promoter region that may have functional effects on gene expression by affecting transcription factor binding sites activity [41].

In summary, the mechanism underlying the role of the *STAT3* gene variation in the regulation of mtDNA copy number remains under investigation. Nevertheless, our findings may open new avenues of investigation toward understanding the impact of *STAT3* polymorphisms on mtDNA dynamics and its influence on the susceptibility of human diseases.

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