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Keywords: chagas; cardiomyopathy; mitochondria; haplogroups; variants; copy-number



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Article

Mitochondrial DNA Haplogroups and Variants Predispose to Chagas Disease Cardiomyopathy

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Abstract: Cardiomyopathies are major causes of heart failure. Chagas disease (CD) is caused by the parasite *Trypanosoma cruzi*, and it is endemic in Central, South America. Thirty percent of the cases evolve into chronic cardiomyopathy (CCC) with worse prognosis as compared with other cardiomyopathies. In vivo bioenergetic analysis and ex vivo proteomic analysis of myocardial tissues highlighted worse mitochondrial dysfunction in CCC, and previous studies identified nuclear-encoded mitochondrial gene variants segregating with CCC. Here, we assessed the role of the mitochondrial genome through mtDNA copy number variations and mtDNA haplotyping and sequencing from heart or blood tissues of severe, moderate CCC and asymptomatic/indeterminate Chagas disease as well as healthy controls as an attempt to help decipher mitochondrial-intrinsic genetic involvement in Chagas disease development. We have found that mtDNA copy number was significantly lower in CCC than in heart tissue from healthy individuals, while blood mtDNA content was similar among asymptomatic Chagas disease, moderate and severe CCC patients. MtDNA haplogrouping study has indicated that African haplogroups were over represented in the Chagas subject groups in comparison with Brazilian healthy individuals. The European lineage is associated to protection against cardiomyopathy and the macro haplogroup H is associated with increased risk towards CCC. By mitochondria DNA sequencing, 84 mtDNA-encoded protein sequence pathogenic variants were associated with CCC. Among them, two variants were associated to left ventricular non-compaction and two to hypertrophic cardiomyopathy. The finding that mitochondrial protein-coding SNPs and mitochondrial haplogroups associate with risk of evolving to CCC is consistent with a key role of mitochondrial DNA in the development of Chronic Chagas disease Cardiomyopathy.

Keywords: chagas; cardiomyopathy; mitochondria; haplogroups; variants; copy-number

1. Introduction

Cardiomyopathies are a heterogeneous group of myocardial diseases associated with mechanical or electrical dysfunction that usually exhibit inappropriate ventricular hypertrophy or dilation. Dilated cardiomyopathy is one of the most common causes of heart failure (1) and the most common indication for heart transplantation worldwide, with an estimated prevalence of 40 cases per 100 000 individuals and an annual incidence of 7 cases per 100 000 individuals. Dilated cardiomyopathy occurs more often among people in the third or fourth decade of life. Indeed, patients with dilated cardiomyopathy generally appear asymptomatic in the early stages, but symptoms gradually emerge with age as the heart function starts to decline due to progressive loss of function in the cardiomyocytes, impairing normal heart function (2, 3).

It is now also admitted that adult males seem to be more frequently affected than adult females (2, 3). Although most cases of dilated cardiomyopathy are considered idiopathic, approximately 20 to 35%, or potentially up to 50% of patient cases, could be attributed to the genetic basis, called familial dilated cardiomyopathy (4). More than 100 genes have been associated to dilated cardiomyopathies (5-8). Myocardial energy deprivation is a main feature of cardiomyopathies and heart failure, and multiple evidences indicate this is caused by mitochondrial dysfunction. Because the heart is an organ requiring a high energy demand, regulation of mitochondrial metabolism plays an important role in the pathogenesis of dilated cardiomyopathies. During the first stages of dilated cardiomyopathies, increased mitochondrial proliferation acts as a compensating mechanism for maintaining energy supply (9). However, the number of mitochondria declines during dilated cardiomyopathy progression leading to a reduction in ATP, decreased contractility and increased reactive oxidative species (ROS) all of which resulting in diastolic dysfunction and heart failure (10). In many cases mitochondrial cardiomyopathies have an underlying genetic component resulting in mitochondrial respiratory chain deficiencies (11-13), including fatty acid oxydation (11) or cardiolipin synthesis defects (14-16) and alterations of mitochondrial dynamics (11). Mitochondria are under dual genome control, where a small fraction of mitochondrial proteins are encoded by mitochondrial DNA (mtDNA), while more than 99% of them are encoded by nuclear DNA (nDNA). Mutations in over 250 mitochondria-related nDNA or mtDNA genes have been associated with mitochondrial

disease, resulting in mitochondrial dysfunction leading to insufficient energy production required to meet the needs for organs with high energy requirements (17).

Cardiac involvement occurs in mitochondrial diseases, with cardiomyopathies being one of the most frequent cardiac manifestations found in these disorders occurring in 20–40% of patients. Mitochondrial cardiomyopathies can vary in severity from asymptomatic status to severe manifestations including heart failure, arrhythmias, and sudden cardiac death (11). MtDNA polymorphisms that have occurred over evolutionary time have permitted a distribution of mtDNA variants into mitochondrial haplogroups inherited from the maternal lineage, and these haplotypes may influence mitochondrial function (18).

Chagas disease (CD) is caused by the parasite *Trypanosoma cruzi*, and it is endemic in Central, South America, and Mexico. The clinical course of the disease comprises an acute phase, mostly asymptomatic, and a chronic phase, where 60% of the patients remain asymptomatic. Forty percent of the cases evolve into gastrointestinal disease and chronic cardiomyopathy with varying degrees of severity including refractory heart failure (19). Antiparasitic treatment with benznidazole or nifurtimox is most effective in the acute phase and prevents progression to chronic phase when given to children. However, these treatments had multiple adverse effects (20). Heart failure due to Chronic Chagas Cardiomyopathy (CCC) may have a worse prognosis with 50% shorter survival when compared to other cardiomyopathies of different etiologies such as ischemic cardiomyopathy and idiopathic dilated cardiomyopathy (21, 22). The major histopathological feature attending dilated cardiomyopathy in CCC is the presence of a diffuse myocarditis, with intense cardiomyocyte damage and hypertrophy, and significant fibrosis, in the presence of very scarce *T. cruzi* forms. The myocardial inflammatory infiltrate is thought to play a major role in disease development and progression (23, 24). The myocardial cytokine production profile suggests an IFN γ /TNF α Th1 type response, with interferon γ -induced chemokines (25-29). Moreover, our group has previously demonstrated that CCC myocardium presents a unique gene expression profile, distinct from other noninflammatory dilated cardiomyopathies, with a large number of interferon-gamma inducible genes among differentially expressed RNA (30, 31). The proteomic analysis of myocardial tissues revealed that CCC display a specific global protein expression profile. Pathway analysis of proteins differentially expressed in CCC highlighted mitochondrial dysfunction, cardiac hypertrophy, fibrosis, mitochondrial energy metabolism, fatty acid metabolism, the involvement of Creatine Kinase System and ATP Synthase Complex, variations of the mitochondrial membrane potential (mitochondrial $\Delta\Psi_m$) (32-35). IFN- γ and TNF- α treatment of the human cardiomyocyte cell line AC16 induces a dose-dependent reduction of mitochondrial $\Delta\Psi_m$ and mtDNA copy number, suggesting a possible inflammatory origin for mitochondrial dysfunction in CCC (33). IFN- γ and TNF- α treatment of AC-16 cardiomyocytes' mitochondria also induce an increased nitro-oxidative stress profile. The inhibition of STAT1/NF- κ B/NOS2 axis and activation of AMPK, NRF2 and SIRT1 signaling pathways promoted protective effects in the IFN- γ /TNF- α -induced impairment of mitochondrial $\Delta\Psi_m$ (36). This finding is especially crucial since the mitochondrial $\Delta\Psi_m$ is the OXPHOS proton motive force that drives ATP production through ATP synthase is an essential mechanism for contraction and survival of cardiac cells (37). Gene and protein expression analyses in cytokine-treated AC-16 cardiomyocytes confirmed decreased levels of mitochondrial proteins involved in ATP generation, ion import and mitochondrial structural maintenance proteins and the overexpression of proteins involved in ATP catabolism and mitochondrial transition pore.

About 30% of Chagas disease patients develop CCC, suggesting the participation of modifier genes in differential disease progression. This was reinforced by the discovery of familial aggregation of cases of CCC in endemic area settings (38). Genetic susceptibility to CCC may result from functionally relevant genetic variants that lead to variations in the intensity of the innate or acquired immune response and in inflammatory cytokines and chemokines involved in the pathogenesis of the disease (39-41). Based on whole exome sequencing on multiple familial cases of Chagas disease, we have previously identified heterozygous, pathogenic variants linked to CCC in all tested families on 22 distinct nuclear-encoded genes, from which 20 were mitochondrial or inflammation-related - most of the latter involved in proinflammatory cytokine production (42). So, mitochondrial

dysfunction and inflammation may be genetically determined in CCC, driven by rare genetic variants which increase mitochondrial susceptibility to IFN- γ -induced damage in the myocardium. Mutations in the mitochondrial genome accumulate over time in organs suffering mitochondrial dysfunction, resulting in decreased energy production and reactive oxygen species (ROS) overproduction. Defective copies of mtDNA might accumulate over time, raising the overall heteroplasmy level of deleterious mutations. Heteroplasmy is the concurrence of both normal (wt) and abnormal (mutant) mtDNA within one cell. In general, the level of mitochondrial dysfunction is commensurate with the mutational load of mtDNA within the tissue or cell.

In this paper, we assessed the role of the mitochondrial genome through mtDNA copy number variations and mtDNA haplotyping and sequencing from heart or blood tissues of severe, moderate CCC and asymptomatic/indeterminate Chagas disease as well as healthy controls as an attempt to help decipher mitochondrial-intrinsic genetic involvement in Chagas disease development.

2. Materials and Methods

2.1. Patients and Tissue Collection

Human left ventricular free wall heart tissue samples were obtained from patients with end-stage heart failure CCC at the time of heart transplantation (n = 34). These CCC patients underwent serological diagnosis of *T. cruzi* infection and standard electrocardiography and echocardiography, and tissues were subject to histopathological assessment (43). Biopsies from controls (n = 6) were obtained from healthy hearts of organ donors having no suitable recipient. The protocol was approved by the institutional review boards of the University of São Paulo School of Medicine and INSERM (French National Institute of Health and Medical Research). Written informed consent was obtained from all patients. All experimental methods comply with the Helsinki Declaration.

2.2. Heart tissue and blood DNA isolation

Heart tissue samples were crushed twice with lysis buffer. After proteinase K treatment, DNA was extracted with QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. For each subject, 5 to 15 ml of blood were collected into EDTA tube. Genomic DNA was isolated with FlexiGene DNA kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations.

2.3. Mitochondrial DNA copy number quantification

The Quantitative PCR (qPCR) was conducted using SYBR Green/ROX qPCR Master Mix (2x) (Thermo Scientific), 1 ng of template DNA and primers for mitochondrial-encoded genes NADH: ubiquinone oxidoreductase subunit 1 (MT-ND1), cytochrome c oxidase subunit 1 (MT-COXI). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. The qPCR reactions were done in QuantStudio 12K (Applied Biosystem) with the following cycling program, 1 cycle of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 50°C for 2 min, 95°C for 15s, and 60°C for 1 min. The ratio of mtDNA/nDNA was calculated using the formula $2^{x2^{\Delta CT}}$ (44). The primer sequences were: MT-ND1forward: 5' ATACCCATGGCCAACCTCCT 3', MT-ND1 reverse: 5' GGGCCTTTGCGTAGTTGTAT 3'; MT-COXI forward: 5' TCCACTATGTCCTATCAATA 3', MT-COXI reverse: 5'GGTGTAGCCTGAGAATAG 3'; GAPDH forward: 5' CCCTGTCCAGTTAATTTC 3', GAPDH reverse: 5' CACCCTTTAGGGAGAAAA 3'.

2.4. Mitochondrial haplogroup determination

Mitochondrial haplogroups were determined according to MITOMASTER tool (<https://www.mitomap.org/foswiki/bin/view/MITOMASTER/WebHome>) which used HaploGrep2 with Phylotree 17 for haplogroup determination. Mitochondrial DNA genotyping was assessed using four different methods:

PCR amplicon sequencing: The genomic DNA was amplified by PCR. The primer sequences were: HmtL15997: 5' CACCATTAGCACCCAAAGCT 3', HmtH112: 5' ACAGATACTGCGACATAGGG 3'. PCR amplifications were carried out with GoTaq polymerase (Promega, Charbonnières-les-Bains, France) and 1µM of each primer. 50µl reactions were carried out on Eppendorf thermocycler. The PCR products were loaded on agarose gel and purified with QIAEXII gel extraction kit (Qiagen) before Sanger sequencing. Sanger sequencing of the PCR amplicon was done with the same primers.

Direct sequencing: Hi-SNPseq, provided by CD Genomics, combines multiplex PCR and high-throughput sequencing (CD Genomics, Shirley, USA). Multiplex PCR amplification was done with 110 site-specific primers in two single tubes. These two reactions comprise the entire library covering the whole mtDNA sequence. Then, the amplicon pooled samples were purified to remove polymerase, primers, and short by-products. Different samples were distinguished by different barcoded primers. After mixing the samples, high-throughput sequencing was performed (pair ends 150). The sequences were aligned on the reference rCRS genome.

Whole Exome sequencing: Library preparation (Agilent SureSelect Human All Exon V6) and sequencing steps were commissioned to Genewiz (Leipzig, Germany). Raw BCL files generated by the sequencer were converted to fastq files for each sample using bcl2fastq v.2.19. Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.38. The sequences were aligned on the reference rCRS genome.

Whole genome sequencing: For the Brazilian reference population (45), high-coverage WGS data were aligned on the human reference hg38 genome. For each sample, variant calling was done using the Genome Analysis Toolkit 4 (GATK4). Based on the mitochondria variants a mitochondria specific bam file.

2.5. Variant annotations

Sequencing data analysis was performed using a homemade nextflow pipeline that integrates all the classical NGS steps for molecular diagnosis. Mapping and quality control was done using BWA (46) and FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) respectively. Base recalibration, base quality score recalibration and duplicate marking processed with elPrep (47). Variant calling combines the results of GATK Mutect2 (48) in mitochondrial mode and Strelka2 (49). Variant annotation and prioritization were performed using an in-house tool KerMit that integrates various mitochondrial dedicated tools and databases like MITOMAP, HmtDB, ClinVar, MitoTip and HaploGrep for haplogroup prediction. Mitochondrial deletions were searched using eKLIPse (50).

2.6. Statistical analysis

All the statistical tests have been made using R 3.6.3. A first comparison on the presence/absence of genetic variants between two groups was carried out using the chi2 and fisher tests. A second analysis, based on allelic frequency, was done with a Kruskal Wallis test between all groups, and then with a Dunn test comparing phenotypes two by two using the rstatix R package. All the plots have been done with the ggplot2 R package.

Differences between groups are evaluated by statistical tests (Chi2 test with Yate's correction, Fisher's exact test, Kruskal Wallis test) using GraphPad Prism 9.3.0. Asterisk and the sharp sign (*) indicate p -value <0.05, (** p -value <0.001, (***) p -value <0.0001.

3. Results

3.1. MtDNA copy number is reduced in heart of Chagas patients

The quantification of mtDNA copy number was evaluated on heart tissue samples from end stage patient heart tissues (n=33) and on healthy hearts of organ donors having no suitable recipient (n=6) (Supplementary Table S1). MtDNA copy number using (MT-ND1) was significantly lower in CCC ((MT-ND1 39%; p = 0.0034) than control samples (Figure 1A) indicating a reduction of mtDNA copy number. Mitochondrial DNA (MT-COX1) gave a similar result (37%; p = 0.0019) (Figure 1B).

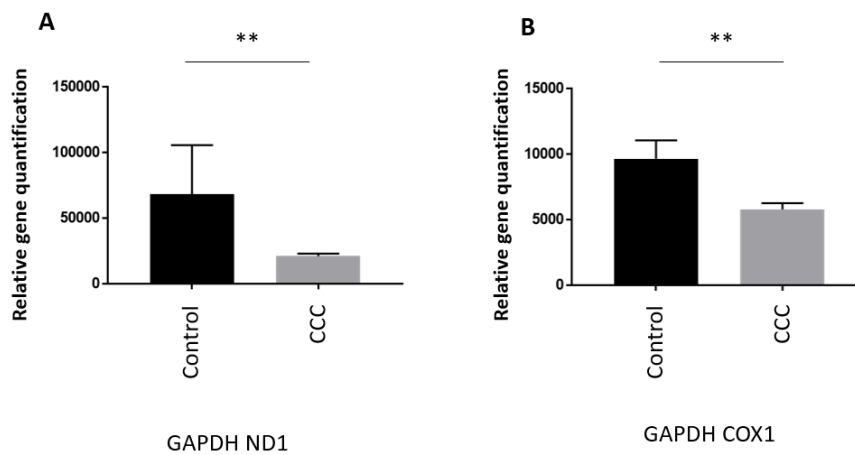


Figure 1. Mitochondrial DNA quantification in heart tissues. We performed qPCR with primers for mitochondrial encoded genes NADH: ubiquinone oxidoreductase subunit 1 (MT-ND1), cytochrome c oxidase subunit 1 (MT-COXI) and endogenous nuclear-encoded control gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative quantification of mitochondrial DNA was calculated by determining the $\Delta Ct = Ct(\text{endogenous nuclear-encoded control gene}) - Ct(\text{mitochondrial encoded genes})$ and then using the formula $2^{\Delta Ct}$.

The mtDNA copy number was also evaluated in DNA extracted from blood of asymptomatic subjects (n=209), moderate CCC patients (n=235) and severe CCC patients (n=168) (Supplementary Table S2). The age of the individuals which ranges from 40 to 60 years old, was similar among the three groups (Figure 2A). As shown on Figure 2B and 2C, the mtDNA content (and therefore mitochondrial mass) was similar between the three groups. So, the decrease of the mitochondrial mass detected on heart biopsies seems to be tissue specific.

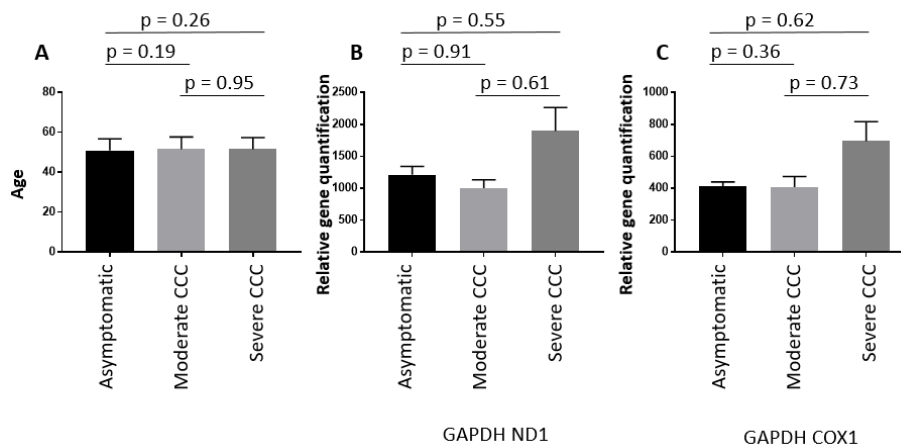


Figure 2. Mitochondrial DNA quantification in blood DNA samples.

This quantification was done on blood DNAs from patients between 40 and 60 years old. These patients were either asymptomatic for Chagas disease, or developed chronic cardiomyopathy stratified as moderate or severe based on left ventricular ejection fraction values [moderate CCC ($0.40 \leq EF \leq 1$) and severe CCC ($0 \leq EF < 0.40$)].

Human mtDNA haplogroups and their distributions have been extensively investigated across different nations and geographical regions, predominantly to define population origins and genetic structure. The rate at which mtDNA mutates is known as the mitochondrial molecular clock. The entire mtDNA was sequenced from 26 end stage heart tissue samples and 6 healthy hearts of organ donors. Table 1 describes the mitochondrial haplogroup lineage distribution between both groups.

Table 1. Haplogroup distribution in heart tissue samples.

Haplogroup lineage	End stage heart tissue samples (CCC)	Healthy hearts of organ donors (control)
African	19 (73%)	4 (67%)
European	4 (15%)	0 (0%)
Native American/Asian	2 (8%)	1 (17%)
Eurasian	1 (4%)	1 (17%)

This distribution suggests that African and European lineages may be enriched in end stage CCC patients compared to healthy controls. However, due to the limited size of our study group, the difference was not statistically significant.

In order to investigate this haplogroup distribution, a larger collection of blood DNA was then investigated. It included CCC patients (n=321), asymptomatic subjects (n=38) and we had access to a census-based sample of elderly individuals from São Paulo (Brazil) (Supplementary Table S3). This latest reference population includes 1171 healthy individuals (45). For each of them, the WGS was available and mitochondrial haplogrouping was assessed in a group of 289 individuals selected randomly from this cohort. The three main lineages were African, European and Native American/Asian (Figure 3). Chagas individuals (asymptomatic or with Chronic Chagas Cardiomyopathy) were compared to healthy controls, percentage of African lineage in healthy individuals reached 27% whereas this percentage was 55% in the Chagas patient groups. The Chi-square test with Yates' correction was significant ($p<0.0001$) with a relative risk of 2.004. This may imply that Chagas patients have a higher African mother lineage than healthy elderly individuals from the state of São Paulo, Brazil.

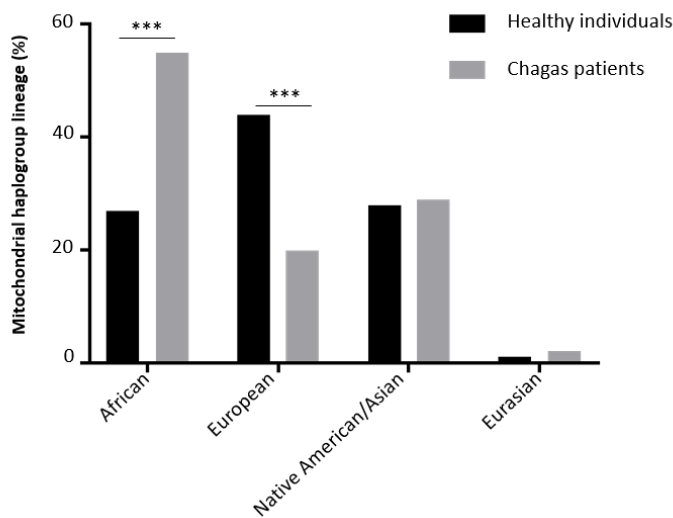


Figure 3. Mitochondrial haplogroup distribution between the healthy Brazilian reference population and the Chagas cohort.

The percentage of European lineages in healthy individuals reached 44% whereas this percentage was 18% in the Chagas subject groups. The Chi-square test with Yates' correction gave a significant p value ($p<0.0001$) for a relative risk of 0.5292 (Figure 3).

We then stratified the Chagas subject group, in the asymptomatic/indeterminate subjects, the moderate CCC ($0.40\leq EF\leq 1$) and severe CCC ($0\leq EF<0.40$) patients. The percentage of each lineage in each subgroup is shown on Figure 4. The African lineage was more represented in the asymptomatic sub-group than the CCC patient sub-group (71% vs 53%) ($p=0.04$). Similarly, the European lineage was overrepresented in the CCC patient subgroup (7.9% vs 18.7%) but this difference was not significant ($p=0.12$).

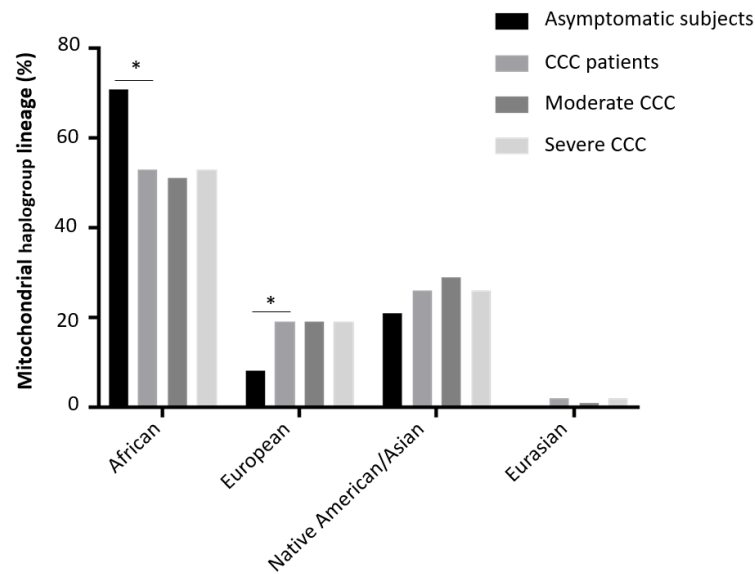


Figure 4. Mitochondrial haplogroup distribution among the Chagas cohort.

Focusing on African sub-haplogroups, the distribution was compared between ASY subjects, CCC patients, moderate CCC patients and severe CCC patients (Figure 5). This African sub-haplogroup distribution was not significantly different between the study groups (Supplementary Table S4). However, the percentage of L0 sub-haplogroup in ASY individuals reached 11% whereas this percentage was 4% in the CCC patient group. The percentage of L3 sub-haplogroup in ASY individuals reached 22% whereas this percentage was 33% in the CCC patient group. It may suggest that the L0 sub-haplogroup has a protective effect whereas the L3 sub-haplogroup may act as a disease mark.

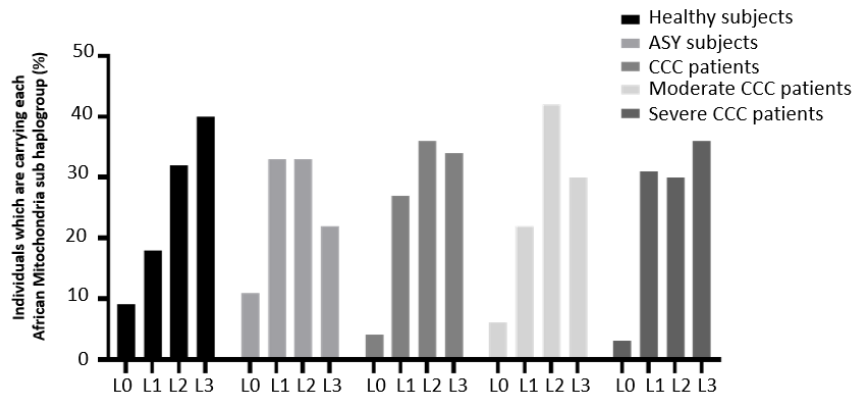


Figure 5. African mitochondrial sub-haplogroup distribution in the Chagas cohort.

In the same way, the European sub-haplogroup distribution was similar between the CCC study groups (Figure 6). However, when we compared the frequency of the European H sub-haplogroup, we detected some trends of association Table 2.

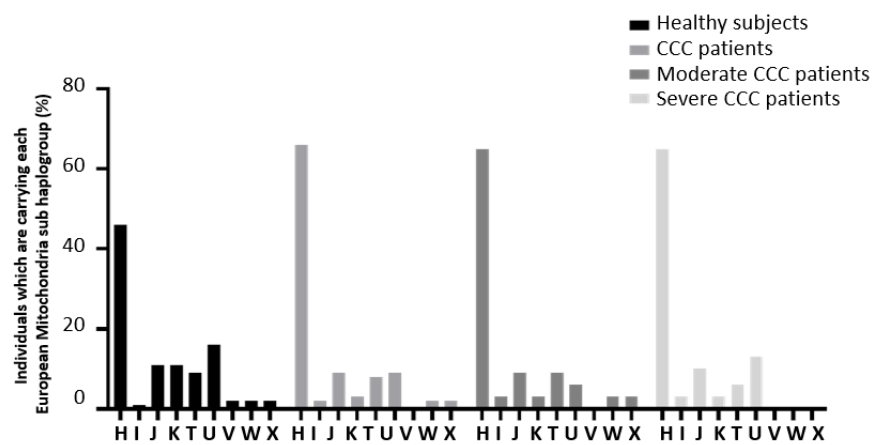


Figure 6. European mitochondrial sub-haplogroup distribution in the Chagas cohort.

Table 2. European haplogroup H association study.

Groups	p Value	Relative Risk
Healthy individuals versus chagas patients	0.01	0.76
Healthy individuals versus moderate chagas cardiomyopathy patients	0.06	0.85
Healthy individuals versus severe chagas cardiomyopathy patients	0.07	0.86

Whole mtDNA sequencing was performed on 112 samples (Supplementary Table S5). It included 38 asymptomatic subjects and 74 CCC patients. Among the CCC patients, 34 patients suffered from moderate cardiomyopathy whereas 36 patients had a severe cardiomyopathy.

Sequences obtained from on 112 samples were aligned according to the Cambridge Reference Sequence (rCRS). 12429 variants were detected. Three analyses were done. First of all, we compared the number of carriers of each variant between each group (ASY vs CCC; ASY vs Moderate CCC; ASY vs Severe CCC and Moderate CCC vs Severe CCC) (Supplementary Table S6). We also compared the heteroplasmy levels between the three groups (Kruskal Wallis between ASY, moderate CCC and severe CCC). Finally, we compared the heteroplasmy levels between the groups (two by two: ASY vs CCC; ASY vs Moderate CCC; ASY vs Severe CCC and Moderate CCC vs Severe CCC) (Supplementary Table S7). All these tests were corrected for multiple testing. 712 variants showed significant results in at least one test (adjusted p value ≤ 0.05).

We annotated the associated variants, based on available information in VEP ClinVar, MitoMap and MitImpact databases (Supplementary Table S8 and S9). These variants are either located in intergenic region (n=220; 30.8%) or in coding region (n=493; 69.2%). Among these variants, only 84 variants change the protein sequence (Table 3). These protein sequence variants are enriched in a limited number of genes (MT-ND5 (n=21), MT-CYB (n=15), MT-ATP6 (n=11), MT-CO3 (n=8), MT-CO1 (n=7), MT-ND4 (n=6), MT-ATP8 (n=5), MT-ND6 (n=4) MT-ND4L (n=2), MT-ND1 (n=2), MT-CO2 (n=2), MT-ND2 (n=1)). The pathogenicity of each variant was defined with MitImpact which aggregate 24 databases (Clinvar, PolyPhen2, SIFT, FatHmW, FatHm, PROVEAN, MutatiOSSessor, EFIN_SP, EFIN_HD, CADD, VEST, PANTHER, PhDSNP, SP, MutationTaster, SNPDryad, Condel, COVEC_WMV, MtoolBox, APOGEE, DEOGEN2, PolyPhen2 transf, SIFT transf, MutatiOSSessor transf) (51). All the 84 protein modifier variants were classified as pathogenic in at least one database. 16 protein modifier variants were classified as pathogenic in more than 10 databases.

Table 3. Annotation of the 84 protein modifier variants.

Pos	Ref	Alt	Csq	Gene symbol	dbSNP ID	Uniprot name	Uniprot ID	AA pos	AA ref	AA alt	Described as pathogenic in the following databases.
3565	A	C	RNA	MT-ND1		NU1M	P03886	87	T	P	PolyPhen2, SIFT, PROVEAN, CADD, PhDSNP, SP, COVEC_WMV, MtoolBox
3579	A	C	RNA	MT-ND1		NU1M	P03886	91	M	I	CADD, PhDSNP, MutationTaster, SNPDryad, Condel
5262	G	A	RNA	MT-ND2		NU2M	P03891	265	A	T	CADD, PANTHER, Condel,
6571	C	T	RNA	MT-CO1		COX1	P00395	223	A	V	PROVEAN, EFIN_SP, CADD, PhDSNP, MutationTaster, Condel, PolyPhen2 transf
6873	C	T	RNA	MT-CO1		COX1	P00395	324	L	F	PolyPhen2, SIFT, MutatiOSSessor, EFIN_SP, EFIN_HD, CADD, PANTHER, PhDSNP, MutationTaster, COVEC_WMV, MtoolBox, MutatiOSSessor transf
6899	G	C	RNA	MT-CO1	rs1556423194	COX1	P00395	332	M	I	Condel, PolyPhen2 transf, SIFT transf
6909	G	T	RNA	MT-CO1		COX1	P00395	336	A	S	SIFT, CADD, PhDSNP
6915	G	A	RNA	MT-CO1	rs1603220687	COX1	P00395	338	V	M	Condel, PolyPhen2 transf, SIFT transf
7008	G	T	RNA	MT-CO1		COX1	P00395	369	D	Y	PolyPhen2, SIFT, FatHmm, PROVEAN, MutatiOSSessor, EFIN_SP, EFIN_HD, CADD, PANTHER, PhDSNP, SP, MutationTaster, SNPDryad, COVEC_WMV, MtoolBox, APOGEE, MutatiOSSessor transf
7072	T	C	RNA	MT-CO1	rs1603220760	COX1	P00395	390	M	T	PolyPhen2, SIFT, EFIN_HD, CADD, PhDSNP, SP, MutationTaster, COVEC_WMV, MtoolBox
7868	C	T	RNA	MT-CO2	rs1556423357	COX2	P00403	95	L	F	PROVEAN, CADD, PANTHER, PhDSNP, Condel, PolyPhen2 transf
8021	A	G	RNA	MT-CO2	rs1603221261	COX2	P00403	146	I	V	Condel, PolyPhen2 transf
8401	A	C	INT	MT-ATP8		ATP8	P03928	12	M	I	Condel, SIFT transf
8405	A	G	INT	MT-ATP8	rs1603221462	ATP8	P03928	14	T	A	Condel, PolyPhen2 transf
8527	A	G	RNA	MT-ATP6	rs878853003	ATP6	P00846	1	M	V	PolyPhen2, MtoolBox

8558	C	G	RNA	MT-ATP8		ATP8	P03928	65	P	A	PolyPhen2, FatHmm, PROVEAN, CADD, SP, COVEC_WMV, MtoolBox
8558	C	G	RNA	MT-ATP8		ATP6	P00846	11	A	G	CADD, PANTHER, PhDSNP
8566	A	G	RNA	MT-ATP6	rs3020563	ATP6	P00846	14	I	V	SP, Condel
8568	C	T	RNA	MT-ATP8	rs1603221589	ATP8	P03928	68	S	F	PolyPhen2, FatHmm, EFIN_HD, CADD, SP, MtoolBox
8618	T	C	RNA	MT-ATP6	rs28358885	ATP6	P00846	31	I	T	Condel
8632	T	C	RNA	MT-ATP6	rs1603221654	ATP6	P00846	36	Y	H	PANTHER, Condel, PolyPhen2 transf
8677	A	C	RNA	MT-ATP6		ATP6	P00846	51	K	Q	Condel, PolyPhen2 transf
8714	C	T	RNA	MT-ATP6	rs1603221724	ATP6	P00846	63	T	I	FatHmm, PANTHER, Condel
8795	A	C	RNA	MT-ATP6		ATP6	P00846	90	H	P	PolyPhen2, SIFT, PROVEAN, CADD, PANTHER, PhDSNP, SP, SNPDryad, COVEC_WMV, MtoolBox
8854	G	A	RNA	MT-ATP6	rs386829055	ATP6	P00846	110	A	T	SIFT, CADD, PANTHER, PhDSNP, Condel
8860	A	G	RNA	MT-ATP6	rs2001031	ATP6	P00846	112	T	A	PROVEAN, PhDSNP, SP, Condel, PolyPhen2 transf
8870	T	C	RNA	MT-ATP6	rs1556423560	ATP6	P00846	115	M	T	Condel, PolyPhen2 transf
8875	T	C	RNA	MT-ATP6	rs201123510	ATP6	P00846	117	F	L	Condel, PolyPhen2 transf
9261	A	C	RNA	MT-CO3		COX3	P00414	19	T	P	PolyPhen2, PROVEAN, EFIN_SP, CADD, PhDSNP, SP, SNPDryad, MtoolBox
9325	T	C	RNA	MT-CO3	rs879000531	COX3	P00414	40	M	T	Condel, PolyPhen2 transf
9717	C	G	RNA	MT-CO3		COX3	P00414	171	L	V	Condel, PolyPhen2 transf
9861	T	C	RNA	MT-CO3	rs878853060	COX3	P00414	219	F	L	Condel, PolyPhen2 transf, SIFT transf
9877	T	A	RNA	MT-CO3		COX3	P00414	224	M	K	MutationTaster, Condel
9880	T	A	RNA	MT-CO3		COX3	P00414	225	F	Y	Condel, PolyPhen2 transf
9896	A	C	RNA	MT-CO3		COX3	P00414	230	K	N	Condel, PolyPhen2 transf
9924	T	G	RNA	MT-CO3		COX3	P00414	240	W	G	PolyPhen2, PROVEAN, Mutatiossessor, EFIN_HD, CADD, VEST, PhDSNP, SP, SNPDryad, COVEC_WMV, MtoolBox
10677	G	A	RNA	MT-ND4L	rs1603222944	NU4LM	P03901	70	E	K	FatHmm, PROVEAN, Mutatiossessor, EFIN_SP, CADD, PANTHER, PhDSNP, SP, MutationTaster, SNPDryad, Condel, MtoolBox, Mutatiossessor transf

10750	A	G	RNA	MT-ND4L	rs372297272	NU4LM	P03901	94	N	S	PROVEAN, PhDSNP, MutationTaster, Condel
10775	G	A	RNA	MT-ND4	rs879015842	NU4M	P03905	6	V	I	Condel, PolyPhen2 transf, SIFT transf
10808	C	T	RNA	MT-ND4	rs2068723560	NU4M	P03905	17	L	F	CADD, Condel, MtoolBox
10866	T	C	RNA	MT-ND4	rs1603222994	NU4M	P03905	36	I	T	Condel
10972	A	C	RNA	MT-ND4		NU4M	P03905	71	W	C	PolyPhen2, SIFT, FatHmm, PROVEAN, Mutatiossessor, EFIN_SP, EFIN_HD, CADD, PhDSNP, SP, SNPDryad, COVEC_WMV, MtoolBox, APOGEE, Mutatiossessor transf
11129	A	G	RNA	MT-ND4	rs1603223122	NU4M	P03905	124	T	A	Condel, SIFT transf
11963	G	A	RNA	MT-ND4	rs201803948	NU4M	P03905	402	V	I	Condel, PolyPhen2 transf, SIFT transf
12341	C	A	RNA	MT-ND5		NU5M	P03915	2	T	N	Condel, PolyPhen2 transf
12346	C	T	RNA	MT-ND5		NU5M	P03915	4	H	Y	Condel, PolyPhen2 transf, SIFT transf
12368	C	T	RNA	MT-ND5		NU5M	P03915	11	T	I	FatHmm, PROVEAN, Condel, PolyPhen2 transf
12403	C	T	RNA	MT-ND5		NU5M	P03915	23	L	F	Condel, PolyPhen2 transf
12802	A	G	RNA	MT-ND5		NU5M	P03915	156	S	G	Condel
13034	T	C	RNA	MT-ND5		NU5M	P03915	233	L	P	PolyPhen2, FatHmm, PROVEAN, Mutatiossessor, EFIN_SP, EFIN_HD, CADD, PANTHER, PhDSNP, SP, MutationTaster, SNPDryad, COVEC_WMV, MtoolBox, APOGEE, Mutatiossessor transf
13118	T	A	RNA	MT-ND5		NU5M	P03915	261	I	N	PolyPhen2, FatHmm, PROVEAN, Mutatiossessor, EFIN_SP, CADD, PANTHER, PhDSNP, SP, SNPDryad, COVEC_WMV, MtoolBox, APOGEE, Mutatiossessor transf
13145	G	A	RNA	MT-ND5		NU5M	P03915	270	S	N	Condel
13226	A	C	RNA	MT-ND5		NU5M	P03915	297	D	A	PolyPhen2, FatHmm, PROVEAN, Mutatiossessor, EFIN_SP, CADD, PANTHER, PhDSNP, SP, MutationTaster, SNPDryad, COVEC_WMV, MtoolBox, APOGEE, Mutatiossessor transf
13303	C	T	RNA	MT-ND5		NU5M	P03915	323	H	Y	PANTHER, PhDSNP, Condel, SIFT transf
13466	G	A	RNA	MT-ND5		NU5M	P03915	377	S	N	FatHmm, PANTHER, Condel

13476	A	T	RNA	MT-ND5		NU5M	P03915	380	L	F	PolyPhen2, FatHmm, PROVEAN, Mutatiossessor, EFIN_SP, EFIN_HD, CADD, PANTHER, PhDSNP, SP, SNPDryad, COVEC_WMV, MtoolBox, APOGEE, Mutatiossessor transf
13517	A	T	RNA	MT-ND5		NU5M	P03915	394	H	L	PANTHER, Condel, PolyPhen2 transf
13600	T	A	RNA	MT-ND5		NU5M	P03915	422	Y	N	PolyPhen2, FatHmmW, FatHmm, PROVEAN, Mutatiossessor, EFIN_SP, CADD, PANTHER, PhDSNP, SP, SNPDryad, COVEC_WMV, MtoolBox, APOGEE, DEOGEN2, Mutatiossessor transf
13614	A	T	RNA	MT-ND5		NU5M	P03915	426	M	I	Condel
13618	C	T	RNA	MT-ND5		NU5M	P03915	428	L	F	Condel, MtoolBox
13651	A	G	RNA	MT-ND5	rs1569484594	NU5M	P03915	439	T	A	CADD, Condel, MtoolBox
13763	C	A	RNA	MT-ND5		NU5M	P03915	476	S	Y	PolyPhen2, PROVEAN, CADD, PANTHER, SP, Condel, MtoolBox, SIFT transf
13804	G	A	RNA	MT-ND5	rs1603224360	NU5M	P03915	490	A	T	PolyPhen2, PROVEAN, CADD, PhDSNP, SP, SNPDryad, COVEC_WMV, MtoolBox
13816	A	C	RNA	MT-ND5		NU5M	P03915	494	T	P	PolyPhen2, FatHmm, PROVEAN, Mutatiossessor, CADD, PANTHER, PhDSNP, SP, SNPDryad, COVEC_WMV, MtoolBox, Mutatiossessor transf
13886	T	C	RNA	MT-ND5	rs28359182	NU5M	P03915	517	L	P	PhDSNP, Condel
14170	A	T	RNA	MT-ND6		NU6M	P03923	168	I	M	PolyPhen2, MtoolBox
14172	T	G	RNA	MT-ND6		NU6M	P03923	168	I	L	PolyPhen2, CADD, MtoolBox
14280	A	G	RNA	MT-ND6		NU6M	P03923	132	S	P	FatHmm, PhDSNP, Condel
14562	C	T	RNA	MT-ND6		NU6M	P03923	38	V	I	Condel
14757	T	C	RNA	MT-CYB	rs1603224859	CYB	P00156	4	M	T	Condel, PolyPhen2 transf
14793	A	G	RNA	MT-CYB	rs2853504	CYB	P00156	16	H	R	PhDSNP, SP, Condel, PolyPhen2 transf
14798	T	C	RNA	MT-CYB	rs28357681	CYB	P00156	18	F	L	Condel, PolyPhen2 transf
14871	T	C	RNA	MT-CYB	rs28660155	CYB	P00156	42	I	T	PROVEAN, PhDSNP, Condel, PolyPhen2 transf
14873	C	A	RNA	MT-CYB		CYB	P00156	43	L	I	Condel
14881	C	G	RNA	MT-CYB		CYB	P00156	45	I	M	PolyPhen2, FatHmm, PANTHER, PhDSNP, SP, MtoolBox

15077	G	A	RNA	MT-CYB	rs201943501	CYB	P00156	111	E	K	PolyPhen2, PROVEAN, CADD, PhDSNP, SNPDryad, MtoolBox
15596	G	A	RNA	MT-CYB	rs160322536 9	CYB	P00156	284	V	I	Condel
15617	G	A	RNA	MT-CYB	rs155642462 5	CYB	P00156	291	V	I	PolyPhen2, CADD, PhDSNP, SP, COVEC_WMV, MtoolBox
15664	C	A	RNA	MT-CYB	rs160322541 4	CYB	P00156	306	I	M	CADD, Condel
15699	G	A	RNA	MT-CYB		CYB	P00156	318	R	H	PolyPhen2, FatHmm, PROVEAN, Mutatiossessor, EFIN_HD, CADD, PANTHER, PhDSNP, SNPDryad, COVEC_WMV, MtoolBox, Mutatiossessor transf
15725	C	T	RNA	MT-CYB	rs160322543 8	CYB	P00156	327	L	F	PANTHER, Condel
15734	G	A	RNA	MT-CYB	rs386829259	CYB	P00156	330	A	T	PhDSNP, Condel
15777	G	A	RNA	MT-CYB	rs879182710	CYB	P00156	344	S	N	CADD, PhDSNP, Condel
15812	G	A	RNA	MT-CYB	rs200336777	CYB	P00156	356	V	M	EFIN_SP, MutationTaster, Condel

Among these 712 variants, 70 of them were associated to diseases (Supplementary Table S9). The 70 variants include 40 protein modifier variants. Among them, 2 were associated to Left Ventricular Non-Compaction disease and 2 to hypertrophic cardiomyopathy.

4. Discussion

We have found that mtDNA copy number in heart tissue was significantly lower in CCC than in healthy individuals, while blood mtDNA content was similar among asymptomatic Chagas disease, moderate and severe CCC patients. While mitochondrial haplogroup lineages of severe CCC patients and healthy heart donors were predominantly African, the percentage of African lineage was much higher among Chagas disease patients from the peripheral blood cohort than healthy individuals from the census-based São Paulo reference population. Conversely, the percentage of the European lineage was under-represented among the Chagas disease cohort than the reference population. The African lineage was over represented in the asymptomatic subgroup when compared with the CCC patient subgroup. Regarding European major haplogroups, H haplogroup was more prevalent among Chagas patients than in the reference population. Whole mtDNA sequencing disclosed that 84 CCC-specific variants were non-synonymous pathogenic changes, some of them previously associated with heart disease.

Here, we have shown that heart from end stage CCC patients display lower mitochondrial DNA copy number than heart tissues from organ donors having no suitable recipient. This result was also consistent with our previous findings that CCC showed even lower mtDNA copy number than other non-inflammatory cardiomyopathies (36) and heart proteomic analysis done on myocardial tissue (32-35). The finding that mtDNA copy number in blood is comparable among Chagas disease clinical groups indicates that the mtDNA copy number variation is tissue specific, and therefore mitochondrial dysfunction detected in CCC hearts is tissue-specific. Indeed, mtDNA copy number variation is depending on cell-type (52). mtDNA copy number variations have been associated to multiple diseases including cardiovascular diseases such as Myocardial Infarction (53), cardiomyopathy, heart failure, and arrhythmias (53). However, our data indicate that blood mtDNA copy number is not a useful clinical biomarker for CCC.

Cardiac mitochondria provide the most ATP in the heart. Therefore, mitochondrial dysfunction is pivotal in heart diseases because of energy supply shortage and increased production of reactive oxidative species (ROS). So, maintaining optimal mitochondrial homeostasis is essential for cardiomyocytes. The total mitochondrial content is mainly controlled by biogenesis and mitochondria-specific autophagy for degradation (54). Damaged mitochondria are normally removed by autophagy/lysosome machinery (55). Several studies described the importance of autophagy in parasite invasion (56). The most characterized mechanism of autophagy is the ATG5-, ATG7-, and LC3-mediated conventional autophagy pathway (57), recent studies have identified an Atg5/Atg7-independent pathway called alternative autophagy (58). In chronic Chagas patients, autophagy activity was not completely investigated. However, based on bulk RNA-seq we investigated the genetic deregulations present in the moderate and severe stages of CCC. Several genes, associated to mitophagy, are differentially expressed in CCC bulk tissues (such as ATG7, LC3, FIP200 and BECN1) (59). Severe CCC is associated to a myocardial inflammatory infiltrate and to an IFN γ /TNF α -rich Th1 type response (22-28). This exacerbated response will lead to ROS/NOS overproduction triggering mitochondrial damage and cardiomyocyte apoptosis. Defective mitophagy or autophagy may be associated to mtDNA release into the cytoplasm, outside of the cell, or into circulation. Circulating mtDNA enhances expression of type I interferons, and by NLRP3 inflammasome, which promotes the activation of pro-inflammatory cytokines Interleukin-1 β and Interleukin-18 (60). These trigger inflammatory responses in cardiomyocytes and may induce myocarditis, and dilated cardiomyopathy (61).

MtDNA haplogrouping study indicates that African haplogroups were over represented in the Chagas subject groups in compared to healthy individuals. Even if it is not statistically significant, the L0 African sub-haplogroup is more frequent in ASY individuals than in CCC patient groups. The L3 African sub-haplogroup is less frequent in ASY individuals than in the CCC patients. This

sub-haplogroup distribution among the groups is consistent with the association of RNR1-921T/C which is a key marker of the L3 African sub-haplogroup. We cannot conclude that the African haplogroup is directly associated with an increased risk of developing severe chronic chagasic cardiomyopathy. It can be simply stated that individuals with African ancestry have a greater chance of lower socioeconomic status that exposes them to parasitic diseases such as Chagas disease. Therefore, the percentage of patients with CCC in the African ethnic group increases. Conversely, the reverse is observed for the European haplogroup. Our results suggest that European macro haplogroup H is associated with increased risk towards CCC. This result agrees with previous work on dilated and hypertrophic cardiomyopathy (62-64). Significantly, intrinsic mitochondrial function has been reported in skeletal muscle myocytes from individuals carrying haplogroup H as compared to haplogroup U (18). This may imply that different haplogroups display distinct mitochondrial function profiles. In the case of Haplogroup H, it is possible to hypothesize that increased OXPHOS is associated with increased ROS production, which may lead to mitochondrial dysfunction and heart damage in CCC patients.

The two main types of mitochondrial DNA mutation are large deletion and point mutations, mostly maternally transmitted, with some de novo mutations have also been reported. Here, no deletion was associated to Chagas disease. The finding of 84 mtDNA-encoded protein sequence pathogenic variants associated with CCC may indicate a role of mtDNA variants in CCC pathogenesis. At this level, the genetic contribution of each of them to the phenotype is undefined. It has been reported that mtDNA variants affect the expressivity of nDNA mutations, leading to experimental cardiomyopathy (65). The functional characterization is still an important bottleneck in mitochondrial diseases. Indeed, in the databases on mitochondrial variants, a very small proportion of these variants have been functionally characterized. Thus, for the vast majority of variants, indications of their functional implications remain putative. Moreover, as no clear variant is associated to disease here, may suggested an additive effect of the mtDNA variants. These studies are complicated by the fact that these variants have been shown to have incomplete penetrance (66). In addition, to complicate matters, mitochondrial disorders are clinically heterogeneous diseases associated with germline mutations in mitochondrial DNA (mtDNA) and/or nuclear DNA-encoded (nDNA) genes, with impaired mitochondrial structure and function. Therefore, mutations at the mtDNA or nDNA levels can have a variety of pathogenesis outcomes. We can thus postulate a synergic effect between these mtDNA variants and nuclear DNA mitochondrial variants previously associated to CCC (42). The description and the characterization of these variants is essential to define a susceptibility polygenic score analysis including all the associated variants.

When the proportion of harmful mtDNA variants reaches a critical level of heteroplasmy, the defects that can give rise to disease. The description of associations of pathogenic variants to multiple diseases, raise the concept of mitochondria genome editing technology. Gene editing technologies, commonly used for nuclear variants, are not so obvious to apply to mitochondria variants. The first approach leads to decrease the amount of variant mtDNA in mitochondria by specifically targeting and cleaving the mutant mtDNA molecules. Double-strand breaks in mutated mtDNA induce the rapid degradation of the linearized molecule. The residual mtDNA, mostly wild-type, replicates and the heteroplasmy level decreases. The critical point in this approach is the efficiency of the nucleases (mitoREs, mtZFNs, mitoTALENs) (67-69). The development of mitochondria targeted CRISPR/Cas9 systems has been hampered by the lack of efficiency to import guide RNA (gRNA) into the mitochondrial matrix (70, 71). The second approach does not try to decrease the heteroplasmy level but simply to correct the mutated base (72). This concept has been recently described by Kar et al. (73).

There is no cure available for mitochondrial diseases owing to the different genes and phenotypes associated with the cause of such disorders. Nevertheless, few symptomatic treatments have been proven by clinical trials as palliative therapies in the last decade. A mitochondrial cocktail, i.e. a combination of vitamins, cofactors, nutrients and antioxidants, may alleviate symptoms, limit disease progression, and overcome mitochondrial toxins. In the short term, specific dietary restrictions have been shown to ameliorate mitochondrial health in patients with mitochondrial

disorders. Thus, high-carbohydrate diet increases oxidative stress, perhaps for individuals with impaired oxidative phosphorylation (74). The ketogenic diet (high-fat diet) has been shown to be beneficial for patients with pyruvate dehydrogenase deficiency, but not for pyruvate carboxylase deficiency and in the treatment of fatty acid oxidation disorders (75). Individuals with disorders of the respiratory chain could be treated with agents that enhance electron transport and substrate delivery and bypass of its components. For example, CoQ10 supplementation and have been shown to reduce high lactate levels after exercise and increase oxygen consumption (76, 77). A diet rich in vitamins and amino acids can be used as a source of redox agents and intracellular buffers for ATP (78). For stroke-like episodes, myopathy, diabetes and lactic acidosis a treatment using natural NO precursors (e.g. arginine and citrulline) was found to restore NO production (74, 79, 80).

Our results are consistent with our previous data based on proteomic, transcriptomic and genetic analyses (27, 33, 35, 36, 42, 59, 81-83). Mitochondrial dysfunction is clearly related to the compromise of mitochondria's ability to make appropriate levels of ATP and to an enhanced formation of reactive oxygen species (ROS). This dysfunction may be the result of mtDNA or nDNA variants, but may also occur as a response to aging and various disease and environmental stresses, leading to the development of cardiomyopathies (CMs), ROS accumulation and cell death. Mitochondrial dysfunction can have a direct deleterious effect on the contractile capacity of myocardial cells. Defects in mitochondrial regulation of calcium homeostasis can alter mechanical function and electrical conduction (84). Moreover, pathogenic inflammatory responses produced by damaged mitochondria can particularly trigger the atherosclerotic process. ROS generated in the mitochondria may lead to target-organ damage, dysfunction, hypertrophy, and inflammation. Hypertension and cardiac fibrosis are associated with depletion and inactivation of the key mitochondrial deacetylase, sirtuin 3, which is involved in the control of key metabolic steps (85-87).

The finding that pathogenic mitochondrial protein-coding SNPs and mitochondrial haplogroups associate with risk of evolving to CCC is consistent with a key role of mitochondrial DNA in the development of Chronic Chagas disease Cardiomyopathy and are in line with previous reports of nuclear DNA-encoded mitochondrial gene variants. These may interact and increase mitochondrial susceptibility to IFN- γ -induced damage in the CCC myocardium, leading to ventricular dysfunction.

5. Conclusions

This section is mandatory, with one or two paragraphs to end the main text.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Heart tissue samples used for mitochondria quantification. Table S2: Blood DNAs samples used for mitochondria quantification. Table S3: Blood samples used for haplogroup analysis. Table S4: Statistics on African sub-haplogroup distribution. Table S5: Clinical information on patients for whom the whole mitochondria genome has been sequenced (n=106). Table S6: Carrier analysis performed on 12429 Variants. Table S7: Heteroplasmy level test between the three groups (ASY, moderate CCC and severe CCC) and heteroplasmy level analyses between the groups (two by two: ASY vs CCC; ASY vs Moderate CCC; ASY vs Severe CCC and Moderate CCC vs Severe CCC). Table S8: Variant annotation. Table S9: List of the variants associated to diseases.

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