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Mitochondrial DNA Copy Number in Peripheral Blood Is Reduced in Type 2 Diabetes Patients with Polyneuropathy and Associated with a *MIR499A* Gene Polymorphism

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Our aim was to evaluate in a cohort of 125 Italian patients with type 2 diabetes (T2D), who underwent neurological evaluation, the possible differences in the number of mitochondrial DNA copies (mtDNA) comparing positive and negative patients for cardiovascular autonomic neuropathy (CAN) or diabetic peripheral neuropathy (DPN) and comparing them with healthy controls. We also investigated a possible correlation of the number of mtDNA copies with the polymorphism rs3746444 of the *MIR499A*. T2D patients show a decrease in the number of mtDNA copies compared to healthy controls ($p = 2 \times 10^{-10}$). Dividing the T2D subjects by neurological evaluation, we found a significant mtDNA decrease in patients with DPN compared with those without ($p = 0.02$), while no differences were observed between subjects with and without CAN. Furthermore, the homozygous variant genotype for the polymorphism rs3746444 of *MIR499A* correlates with a decrease in the number of mtDNA copies, particularly in T2D patients ($p = 0.009$). Our data show a decrease in the number of mtDNA copies in subjects with T2D and suggest that this decrease is more evident in patients who develop DPN. Furthermore, the association of the variant allele of *MIR499A* with the number of mtDNA copies allows us to hypothesize a possible effect of this polymorphism in oxidative stress.

Keywords: diabetic neuropathy, mtDNA, microRNA, polymorphism, oxidative stress

Introduction

The mitochondrion, an organelle responsible for the production of ATP, is the main protagonist for cellular metabolism. Mitochondria form a complex dynamic network, known as mitochondrial dynamics, involved in the respiratory capacity and cell response to stress (Lin *et al.*, 2018). Mitochondria generate energy as electrons are passed from donors at lower to acceptors at higher redox potential. Although most electrons are eventually passed to molecular oxygen, 2% of molecular oxygen is not completely reduced to water and, therefore, is the primary site for the potential overproduction of reactive oxygen species (ROS) (Sivitz and Yorek, 2010). Oxidative stress is one of the pathogenic mechanisms underlying numerous diseases, including type 2 diabetes (T2D).

T2D is well known to be a progressive disorder characterized by both pancreatic beta-cell dysfunction and insulin resistance in insulin-sensitive tissues. Hyperglycemia and T2D are also directly related to oxidative stress. In fact, hyperglycemia induces ROS production and consequently oxidative stress. Moreover, a high production of ROS and a subsequent change in redox state and cellular homeostasis have been described in T2D and considered main pathogenetic mechanisms of micro- and macrovascular diabetic complications (Rovira-Llopis *et al.*, 2017). Indeed, a prolonged increased ROS production, induced by insulin resistance and hyperglycemia, in turn impairs protective mechanisms such as the endothelial production of nitric oxide and promotes pathogenic signaling pathways, including mitochondrial dysfunction (Shah and Brownlee, 2016; Rovira-Llopis *et al.*, 2017).

Several studies have suggested that mitochondria play an important role in insulin secretion in pancreas beta cells as well as insulin action in peripheral tissues (Maechler and Wollheim, 2001). Mitochondrial DNA (mtDNA) is

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vulnerable to oxidative stress, resulting in both qualitative and quantitative changes. MtDNA content was found to decrease in skeletal muscle (Antonetti *et al.*, 1995) and peripheral blood leukocytes (Lee *et al.*, 1998; Xu *et al.*, 2012; Al-Kafaji *et al.*, 2018) of patients with diabetes and was also found associated with a phenotype related to insulin resistance (Gianotti *et al.*, 2008).

Diabetic neuropathy (DN) is a common complication of T2D leading to increased morbidity and mortality. The DN is heterogeneous in terms of clinical manifestations, pattern of neurological involvement, pathological alterations, and underlying mechanisms (Tesfaye *et al.*, 2010). However, the most common forms of DN are diabetic peripheral neuropathy (DPN) and cardiovascular autonomic neuropathy (CAN), which affect 30% and 20% patients, respectively, with a prevalence that reaches values of more than 50% in those with higher age and longer disease duration (Spallone *et al.*, 2011; Ziegler *et al.*, 2014).

It is known that hyperglycemia is the main risk factor of DN, which leads to increased oxidative stress, mitochondrial dysfunction, and cell death in the affected organs, including peripheral nerves. Changes of mitochondrial number and size have been observed in human Schwann cells of diabetic patients (Kalichman *et al.*, 1998) and in neurons of mice DN models (Vincent *et al.*, 2010). A study, using mitochondria of cultured dorsal root ganglia neurons (DRGs) exposed to high glucose as *in vitro* model of DN, has shown that hyperglycemia was able to promote mitochondrial fragmentation and reduce mitochondrial number (Leinninger *et al.*, 2006a).

The excess of mitochondrial fission is an early and important event in neurodegenerative diseases and several studies suggest that mitochondrial fission could be enhanced by the oxidative stress and hyperglycemia (Leinninger *et al.*, 2006b; Edwards *et al.*, 2010). Fission is regulated by at least two proteins: a large GTPase, dynamin-like protein 1 (Drp1), and a small molecule, Fis1, and indeed the DRGs with mitochondrial dysfunction induced by hyperglycemia presented an increase in the expression of Drp1 (Leinninger *et al.*, 2006b). It is known that Drp1 activation is also regulated by calcineurin (CnA). Several studies have demonstrated that miR-499a targets CnA and inhibits its expression, attenuating Drp1 activity (Wang *et al.*, 2011; Chua *et al.*, 2016).

The expression of miR-499a was increased at circulating level after acute myocardial infarction (Xin *et al.*, 2016) and it has been observed that an overexpression of this miRNA is able to enhance the glycogen and improve insulin signaling by Phosphatase and Tensin Homolog (PTEN) inhibition (Wang *et al.*, 2015). Recently, we have shown that the variant allele of rs3746444 in the *MIR499A* increases the susceptibility to both forms of neuropathy. In particular, we observed that carriers of the GG genotype had a greater risk of developing both DPN (odds ratio [OR] = 6.56 and $p = 0.037$) and CAN (OR = 16.08 and $p = 0.002$), and the correlation with several neurological parameters seemed to indicate a dose-dependent effect of the G allele, related to the severity of neuropathy (Ciccacci *et al.*, 2018).

In consideration of the above, we have decided to investigate the amount of mtDNA in peripheral blood of 125 patients with T2D, evaluated for CAN and DPN, and in 61 healthy subjects. We then analyzed the possible correlations between the number of mtDNA copies and the rs3746444 SNP genotype in *MIR499A* gene.

Materials and Methods

Patients recruitment

We collected blood samples from 125 patients with T2D, recruited among the patients attending the diabetic clinic of the Policlinico Tor Vergata in Rome (Italy), who underwent neurological evaluation. The inclusion criteria were a diagnosis of T2D and age between 18 and 80 years. The exclusion criteria included presence of peripheral or autonomic neuropathies from causes other than diabetes, conditions potentially responsible for autonomic dysfunction, severe comorbidities (such as malignancies, recent cardiovascular events, heart failure, advanced renal failure, or liver disease), advanced peripheral arterial disease, severe psychiatric disorders, or any other condition preventing understanding of the questionnaires. The control population of the study consisted of 61 ethnically and age-matched (mean age 59.26 years) healthy unrelated blood donors, enrolled at University of Rome Tor Vergata.

The study was approved by Ethics Committee of the University Hospital of Rome Tor Vergata (Approval No. 2936/2017). All participants provided written informed consent.

A complete clinical history was recorded regarding diabetes, comorbidity, cardiovascular disease, and any potential cause of polyneuropathy. Height, weight, waist circumference, casual blood pressure, and blood glucose at the moment of neurological assessment were measured. Routine laboratory and neurological assessments performed on all patients were described in a previous article (Ciccacci *et al.*, 2014). Dyslipidemia was considered present if total cholesterol was >200 mg/dL, and/or triglycerides >150 mg/dL and/or high-density lipoprotein (HDL) cholesterol <40 mg/dL in men and <50 mg/dL in women.

The diagnosis of DPN was based on the presence of two abnormalities among neuropathic symptoms, signs, vibration, and thermal perception thresholds, whereas CAN diagnosis was based on four cardiovascular reflex tests by defining early CAN in the case of at least one abnormal test and confirmed CAN with ≥2 abnormal tests.

mtDNA copy number and genotyping

Genomic and mtDNA was extracted from peripheral blood using standard procedures and the Qiagen Blood DNA Mini Kit. DNA quality and concentration were evaluated by NanoDrop ND-1000 Spectrophotometer (Euro-Clone). MtDNA copy number analysis was performed as described by Rooney (Rooney *et al.*, 2015). In brief, 5 ng of total cellular DNA was used as input for quantitative PCR (qPCR). Primers amplifying a nuclear DNA region (hemoglobin subunit beta [HGB]) and a mtDNA region (NADH dehydrogenase, subunit 1, ND1) were taken from literature (Xing *et al.*, 2008).

qPCR reactions were performed with 7500 real-time instrument (Applied Biosystems) with the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. qPCR was performed in a final volume of 14 μL, containing 7 μL of 1 × SYBR Green MasterMix (Applied Biosystems; Foster City, CA), 2 μmol of each primer, and 1 μL (5 ng) of DNA template. Each reaction was performed in triplicate. The Ct values for HGB gene and

mitochondrial ND1 gene were concurrently determined in each sample during the same qPCR run. The mitochondrial copy number in leukocytes of each subject was calculated by the equation $(2\times2^{(Ct(HGB)-Ct(ND1))})$ (Rooney *et al.*, 2015). Genotyping data were obtained from our previous study on the same patients' cohort (Ciccacci *et al.*, 2018).

Statistical analysis

The analysis of variance (ANOVA) test was used to compare mean mtDNA copy number levels among the different phenotypic and genotypic groups and to perform adjustment for sex, age, diabetes duration, body mass index (BMI) and HbA1c. A p-value ≤0.05 was considered as significant. All statistical analyses were performed by the SPSS program, version 25 (IBM Corp, Armonk, NY).

Results

One hundred twenty-five participants with T2D (77 men) included in the study had a mean age of 63.6 ± 7.9 years, a diabetes duration of 12.5 ± 9.1 years, a BMI of 31.0 ± 6.0 kg/m^2, and HbA1c of 7.3% ± 1.5% (56.15 ± 16.40 mmol/mol) (Table 1). All patients underwent neurological assessment: among 125 participants, 63 (50.4%) satisfied the diagnostic criteria for DPN, 38 (30.9%) of those for early CAN, and 15 (12.2%) for confirmed CAN (Table 1).

The amount of mtDNA was analyzed in peripheral blood of 125 T2D patients and 61 healthy subjects. As shown in Figure 1, in patients with T2D the mean count of mtDNA copy number was lower compared to the healthy subjects ($p=2\times10^{-10}$) (Fig. 1).

Table 1. Clinical and Anthropometric Characteristics of the 125 Patients with Type 2 Diabetes Examined in This Study

Females/males	48/77
Age (years)	63.6 ± 7.88
Disease duration (years)	12.5 ± 9.1
BMI (kg/m^2)	31.0 ± 6.0
Insulin treated (%)	17.6
HbA1c (%)	7.3 ± 1.5
HbA1c (mmol/mol)	56.15 ± 16.40
Total cholesterol (mg/dL)	170.0 ± 39.7
HDL cholesterol (mg/dL)	46.7 ± 13.8
Triglycerides (mg/dL)	161.2 ± 261.0
eGFR (mL/min)	96.35 ± 39.47
With microalbuminuria (%)	22.0
With dyslipidemia (%)	90.4
Casual systolic blood pressure (mmHg)	138.4 ± 18.6
Casual diastolic blood pressure (mmHg)	79.3 ± 13.2
With hypertension (%)	83.1
With peripheral arterial disease (%)	12.1
With diabetic retinopathy (%)	28.3
With cardiovascular disease (%)	16.8
Current smokers (%)	56.7
Regular physical activity (%)	62.2
Alcohol consumption (%)	35.8
Patients with DPN (%)	50.4
Patients with early CAN (%)	30.9
Patients with confirmed CAN (%)	12.2

BMI, body mass index; CAN, cardiovascular autonomic neuropathy; DPN, diabetic peripheral neuropathy; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein.

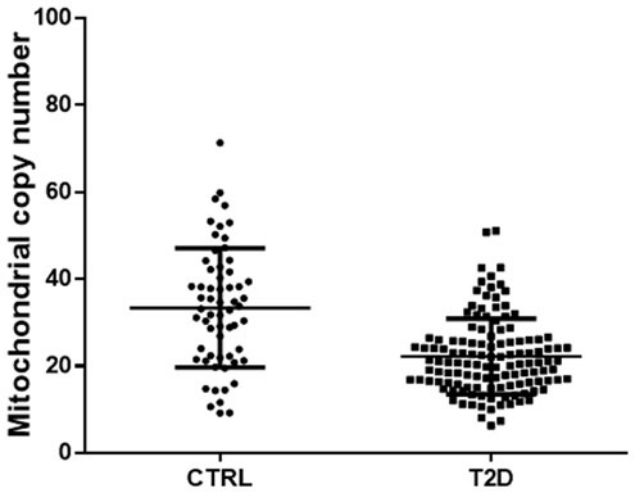

FIG. 1. Comparison of mtDNA copy number between T2D patients and healthy controls ($p=2\times10^{-10}$). mtDNA, mitochondrial DNA; T2D, type 2 diabetes.

Then, we analyzed the subgroups of patients according to the presence or absence of DPN, CAN, or at least one form of DN. We found a significant decrease of mtDNA copy number in patients with DPN compared to those without ($p=0.02$). This difference in mtDNA copies was statistically significant also after correction for sex, age, diabetes duration, BMI, and Hbg ($p=0.04$) (Table 2). Moreover, in Figure 2, it is possible to observe a highly significant linear decrease in mtDNA copy number in the groups of healthy subjects, T2D patients without DPN and T2D with DPN ($p=4\times10^{-6}$) (Fig. 2). On the contrary, no difference was observed between subjects with and without early CAN or confirmed CAN or DN in general (Table 2).

To verify the possible relationship between rs3746444 polymorphism in the *MIR499A* gene and mtDNA copy number, we compared the distribution of mtDNA amount in the different genotypic classes for *MIR499A* SNP, in the whole cohort of subject analyzed (Table 3). As shown in Table 3, the variant homozygous genotype for the polymorphism rs3746444 of the *MIR499A* is associated with a

Table 2. Comparison of Mitochondrial DNA Copy Number Between Subgroups of Type 2 Diabetes Patients

Diabetic sub-groups	*mtDNA copy number*	p	P*adj**
With DPN ($n=63$)	20.43 ± 7.35	**0.02**	**0.04**
Without DPN ($n=62$)	23.96 ± 9.61		
With early CAN ($n=38$)	22.27 ± 8.71	0.9	0.55
Without early CAN ($n=85$)	22.07 ± 8.75		
With confirmed CAN ($n=15$)	21.44 ± 8.37	0.74	0.89
Without confirmed CAN ($n=108$)	22.23 ± 8.78		
With DN ($n=70$)	20.95 ± 8.01	0.07	0.17
Without DN ($n=55$)	23.75 ± 9.34		

mtDNA copy number are reported as mean ± standard deviation. Significant differences are reported in bold.

*Padj = p-values have been corrected for sex, age, diabetes duration, BMI and HbA1c.

DN, diabetic neuropathy; mtDNA, mitochondrial DNA.

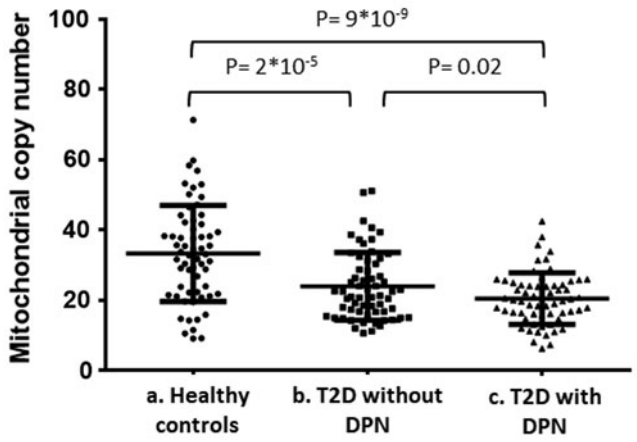

FIG. 2. Comparison of mtDNA copy number between subgroups of T2D patients and healthy controls. Comparison among three groups (a, b, c), $p = 4 \times 10^{-6}$.

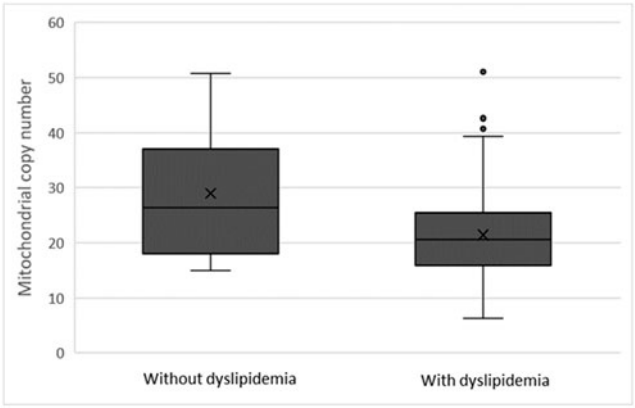

FIG. 3. Comparison of mtDNA copy number between subgroups of T2D patients with and without dyslipidemia ($p = 0.004$).

decrease in the number of copies of mtDNA, compared to the group of wild-type and heterozygous genotype considered together ($p = 0.028$). This difference in mtDNA copy number among genotypic classes is more evident in patients with T2D ($p = 0.009$).

Finally, we verified whether the mtDNA copy number was associated with any clinical or metabolic parameters. We observed a lower mtDNA content in T2D patients with dyslipidemia ($p = 0.004$) (Fig. 3). Furthermore, the mtDNA content was not correlated with age and sex in T2D patients.

Discussion

Mitochondrial dysfunction is central to the pathogenesis of diabetes and its vascular complications, including DN. Indeed, it is known that hyperglycemia, the main risk factor for DN, leads to increased oxidative stress and produces mitochondrial dysfunction with deregulation between mitochondrial fission and fusion. DPN and CAN are the most common forms of DN, and a better understanding of the molecular mechanisms underlying their pathogenesis could help develop targeted treatment. The copy number of mtDNA reflects the abundance of mitochondria and may change according to the cell energy requirements, as well as the physiological or environmental conditions. A number of studies have described a decrease in mtDNA content in diseases associated with oxidative stress, including T2D, but no study so far has investigated the mtDNA copy number in subjects with DN (Han and Chen, 2013; Cenini *et al.*, 2019).

In our study, we observed that the mtDNA copy number was significantly decreased in the peripheral blood of patients with DPN compared with those without DPN and the healthy controls, while no difference was found between patients with and without early CAN or confirmed CAN or DN in general. Therefore, this correlation seems to be specific for DPN. Our results confirm and extend data obtained by Al-Kafaji *et al.* (2018) who had observed that the amount of mtDNA progressively declined with the severity of diabetic nephropathy. This effect seems to be attributable to the chronic high level of oxidative stress in insulin resistance and diabetes (Shah and Brownlee, 2016). The increased ROS and inflammation, owing to mitochondrial injury, contribute to ongoing nervous system dysfunction (Feldman *et al.*, 2019).

Previously, we observed that the homozygous variant genotype of rs3746444 SNP in *MIR499A* was associated with a higher risk of developing DN (Ciccacci *et al.*, 2018). In a recent study, we also observed a lower expression of miR-499a in patients with DPN ($p = 0.05$) and with general DN ($p = 0.045$) compared to those negative for these neurological complications, but we did not observe correlations

Table 3. Mitochondrial DNA Copy Number in Different Classes of Genotypes for rs3746444 SNP in *MIR499A*

MIR499A genotypic classes	*All subjects*	*mtDNA copy number*	p	*T2D patients*	*mtDNA copy number*	p	*Healthy controls*	*mtDNA copy number*	p
AA	($n = 97$)	26.36 ± 11.42	0.09	($n = 65$)	22.43 ± 8.26	**0.03**	($n = 32$)	34.35 ± 12.85	0.24
AG	($n = 64$)	26.44 ± 12.43		($n = 52$)	23.07 ± 9.18		($n = 22$)	34.42 ± 15.13	
GG	($n = 15$)	19.45 ± 10.18		($n = 8$)	14.43 ± 5.29		($n = 7$)	25.18 ± 11.71	
Dominant model									
AA	($n = 97$)	26.36 ± 12.23	0.52	($n = 65$)	22.43 ± 8.26	0.74	($n = 32$)	34.35 ± 12.85	0.54
AG + GG	($n = 89$)	25.26 ± 9.21		($n = 60$)	21.92 ± 9.21		($n = 29$)	32.18 ± 14.74	
Recessive model									
AA + AG	($n = 171$)	26.40 ± 11.80	**0.028**	($n = 117$)	22.71 ± 8.65	**0.009**	($n = 54$)	34.38 ± 13.69	0.09
GG	($n = 15$)	19.45 ± 10.18		($n = 8$)	14.43 ± 5.29		($n = 7$)	25.18 ± 11.71	

Significant associations are reported in bold.
T2D, type 2 diabetes.

between the polymorphism rs3746444 of miR-499a and the expression of the miRNA (Ciccacci *et al.*, 2020). In this study, we highlighted an association between this genotype and a mtDNA copy number decrease.

MiR-499a is abundantly expressed in heart, muscle, and central nervous system, and it has been described as regulating insulin resistance. Mir-499a expression was increased at a circulating level after acute myocardial infarction, and it has also been described the association of *MIR499A* A/G rs3746444 SNP with ischemic stroke and postmyocardial infarction prognosis. Mir-499a targets the gene of CnA and inhibits its expression, and consequently, the CnA-mediated Drp1 activation and the resultant mitochondrial fission and apoptosis, as shown in rat and human cardiomyocytes (Wang *et al.*, 2011; Chua *et al.*, 2016).

Rs3746444 SNP is located on miR-499a-3p mature and the variant allele might affect its ability to suppress Drp1 and mitochondrial fission. Indeed, a recent *in vitro* study demonstrated that in cells transfected with pri-miR-499a carrying the variant allele, a minor reduction of its target, CnA, was observed than that in those transfected with pri-miR-499a carrying the wild-type allele (Ding *et al.*, 2018). The correlation between the rs3746444 homozygous variant genotype and reduced mtDNA copy number could also explain the higher DN risk associated with the variant allele of this miRNA gene, which we had described in our previous article (Ciccacci *et al.*, 2018).

Furthermore, we described a lower mtDNA content in T2D patients with dyslipidemia. This finding is not surprising because dyslipidemia plays a meaningful role in the pathogenesis of DN, acting in synergism with hyperglycemia (Vincent *et al.*, 2013). An excess of long-chain fatty acids in T2D can lead to an excess of acetyl-CoA, as a product of mitochondrial beta-oxidation, and consequently an excess of acylcarnitines upstream.

Accumulated acylcarnitines in Schwann cells and DRG neurons are released and might trigger flux of Ca^{2+} into the axon, inducing changes in mitochondrial trafficking and apoptosis and impairing the mitophagy clearance pathways (the retrograde transport mechanism of damaged mitochondria to the soma), contributing to axonal degeneration (Feldman *et al.*, 2017). Moreover, several studies suggested that complex lipids that are upregulated in dyslipidemia such as cardiolipins, phosphatidic acid, diacylglycerols, and phosphatidylethanolamines could regulate mitochondrial morphology and interact with fusion and fission proteins (Ha and Frohman, 2014).

In conclusion, our data show that the decrease in the number of mtDNA copies (already observed in subjects with T2D) is more evident in patients who develop DPN. Furthermore, we describe for the first time the association of the variant allele of *MIR499A* gene with the number of mtDNA copies. This observation allows us to hypothesize a possible effect of this polymorphism in oxidative stress, specifically in mitochondrial fission. These results, if further supported by functional studies, would provide further evidence about the importance of the role played by oxidative stress and by the microRNAs in the development of DN.

Author Disclosure Statement

No competing financial interests exist.

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