# Increased blood lactate levels during exercise and mitochondrial DNA alterations converge on mitochondrial dysfunction in schizophrenia

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**Abstract**

*Background:* Mitochondrial dysfunction and an elevation of lactate are observed in patients with schizophrenia (SZ). However, it is unknown whether mitochondrial dysfunction is associated with the presence of mitochondrial DNA (mtDNA) alterations and comorbid clinical conditions. We aimed to identify systemic mitochondrial abnormalities in blood samples of patients with SZ that may have a high impact on the brain due to its high bioenergetic requirements.

*Methods:* Case/control study between 57 patients with SZ and 33 healthy controls (HCs). We measured lactate levels at baseline, during 15 minutes of exercise (at 5, 10 and 15 min.) and at rest. We also evaluated the presence of clinical conditions associated with mitochondrial disorders (CAMDs), measured the neutrophil to lymphocyte ratio (NLR, a subclinical inflammatory marker), and analyzed mtDNA variation and copy number.

*Results:* Linear models adjusting for covariates showed that patients with SZ exhibited higher elevation of lactate than HCs during exercise but not at baseline or at rest. In accordance, patients showed higher number of CAMDs and lower mtDNA copy number. Interestingly, CAMDs correlated with both lactate levels and mtDNA copy number, which in turn correlated with the NLR. Finally, we identified 13 putative pathogenic variants in the mtDNA of 11 participants with SZ not present in HCs, together with a lactate elevation during exercise that was significantly higher in these 11 carriers than in the noncarriers.

*Conclusions:* These results are consistent with systemic mitochondrial malfunctioning in SZ and pinpoint lactate metabolism and mtDNA as targets for potential therapeutic treatments.

Keywords: mitochondria; schizophrenia; lactate; mitochondrial DNA; comorbidity

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## 1. Introduction

Schizophrenia (SZ) is a spectrum disorder with a strong genetic load interacting with environmental factors involved in the disease onset and progression. Variations in the nuclear genome, both low-risk common single nucleotide variants (SNPs) and high-risk relatively rare copy number variants (CNVs), are involved in the disease, although the specific set of genetic factors remains unknown (Kirov et al., 2014; Ripke et al., 2014). Notably, the study of the mitochondrial DNA (mtDNA) in states of health and disease has been neglected compared to the efforts dedicated to the study of the nuclear genome, especially in psychiatric disorders (Pesole et al., 2012; Wallace, 2017). Notwithstanding, several lines of evidence suggest a mitochondrial dysfunction in the etiopathology of SZ, including altered mitochondrial metabolism (Ben-Shachar, 2017; Holper et al., 2019; Ni et al., 2019; Rajasekaran et al., 2015; Rosenfeld et al., 2011), deficits in bioenergetic metabolites (Chouinard et al., 2017), and a reduction in mitochondrial number in several brain regions (Roberts, 2017). Consequently, mitochondrial abnormalities have been proposed to play a critical role in altered brain functioning in SZ (Konradi and Öngür, 2017; Pei and Wallace, 2018), in addition to other mitochondria-associated systemic abnormalities, such as inflammation, redox dysregulation and oxidative stress (Fournier et al., 2014; Kim et al., 2017; Kirkpatrick and Miller, 2013; Müller, 2018).

Mitochondria are cellular organelles that generate most cell energy through oxidative phosphorylation in the mitochondrial respiratory chain. These organelles contain their own genome, the mtDNA, consisting of a maternally inherited double circular DNA molecule of 16 569 base pairs (bp) that encodes the 37 genes necessary to synthesize 13 crucial polypeptides of the mitochondrial respiratory chain (Verge et al., 2011). Unlike nuclear DNA, mtDNA is present in multiple copies per cell, and mtDNA copy number has been associated with an array of phenotypes, including neuropsychiatric symptoms (Malik and Czajka, 2013). Altogether, more than 250

variants in mtDNA have been reported to cause mitochondrial disorders (Chinnery, 2014), while many others have been associated with different health conditions (Schon et al., 2012). Interestingly, patients with primary mitochondrial disorder have shown blood lactate elevation with a diagnostic sensitivity between 34 and 62% and a specificity between 83 and 100% (Parikh et al., 2015).

Lactate is constantly produced during normal metabolism and exercise in diverse cells under fully aerobic conditions; classically, it was considered a consequence of oxygen deficits. The lactate anion is transported between tissues (i.e., from skeletal muscle to heart) and between cells (i.e., from astrocytes to neurons) by several lactate shuttles and also between the cytosol and mitochondria (Brooks, 2018; Chen et al., 2016). An increase in lactate occurs because the flux through glycolysis overcomes the use of pyruvate in the mitochondria, and the lactate stress test, which measures increased lactate during physical exercise, has proven useful for evaluating oxidative metabolism in patients with mitochondrial disorders (Finsterer et al., 2000, 1998). Of note, increased lactate and decreased glutathione levels were the first abnormalities associated with energy generation in SZ reported 1934 (Looney and Childs, 1934).

The present study explored the presence of systemic mitochondrial abnormalities in SZ by assessing blood lactate levels during exercise, the occurrence of clinical conditions commonly associated with mitochondrial disorders (CAMDs), mtDNA variation and the mtDNA copy number.

## 2. Methods and materials

### 2.1. Study design, participants and ethical considerations

This cross-sectional study included 57 patients with SZ according to DSM-IV-TR criteria and 33 HCs without a personal or family history of psychotic or affective disorders in either first- or second-degree relatives. All participants provided written

informed consent after fully understanding the benefits and risks of participation. The study was approved by the Hospital de Sant Joan Clinical Research Ethics Committee and was conducted according to the criteria set by the declaration of Helsinki. The exclusion criteria for participants (both patients and HCs) were the presence of diabetes mellitus, acquired immunodeficiency syndrome, cardiac abnormality or altered electrocardiogram, seizures, and renal or liver pathology that could alter lactate measures. Moreover, these individuals were not under pharmacological treatment consisting of valproic acid, glucocorticoids, anesthetics, salicylates, or oral contraceptives that could alter mitochondrial function. Participants were not consuming drugs such as cannabis, hallucinogens, cocaine, or opioids before or during the study, and they did not have alcohol abuse or dependence. They were evaluated with basic biochemistry and blood counts to exclude individuals with significant alterations. Because muscular exercise greatly influences the anaerobic threshold, only participants with moderate physical activity or no activity were accepted. Information on participants is shown in Table 1.

## 2.2. Clinical Assessments

All participants were assessed with the Spanish adaptation of the Schedules for Clinical Assessment in Neuropsychiatry (SCAN) to confirm or exclude psychiatric diagnosis (Vazquez-Barquero et al., 1994). Additionally, the psychiatrist completed a previously used questionnaire focused on CAMDs through direct interview (Valiente-Pallejà et al., 2018; Verge et al., 2012). CAMDs are grouped into the following categories: headaches, bowel function, soft tissues and fatigue, nervous system, ears and eyes, endocrine and heart and blood vessels (Table 2). Sociodemographic and anthropometric data, physical examination, medication and drug consumption data were also obtained. The exercise practices and habits of participants during the last

week were obtained with the International Physical Activity Questionnaire (IPAQ) and were calculated as metabolic equivalent of task (MET)-min/week (Craig et al., 2003). MET is a physiological measure expressing the energy cost of physical activities and is defined as the ratio of the metabolic rate (related to energy consumption) during a specific physical activity to a reference metabolic rate. All patients were diagnosed with SZ using the Operational Checklist for Psychotic Disorders (OPCRIT) computer algorithm after administration of the SCAN. The severity of psychotic symptoms in patients with SZ was measured with the Positive and Negative Syndrome Scale (PANSS) (Peralta and Cuesta, 1994).

### 2.3. mtDNA copy number quantification

Total DNA was extracted from peripheral blood mononuclear cells using Gentra® PureGene reagent (Qiagen, Barcelona, Spain) according to the manufacturer's instructions and was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). We measured three mtDNA regions, the *MT-ND1* and *MT-ND4* genes and the *MT-7S* region, by qPCR in triplicate as previously described (Torrell et al., 2013; Valiente-Pallejà et al., 2018). *MT-ND1* is rarely deleted in mitochondrial disorders, *MT-ND4* is located in the major arc, where most mtDNA deletions characterized in humans are located, and *MT-7S* is a noncoding region located in the D-loop that contains essential elements for mtDNA replication and transcription (Torrell et al., 2013).

### 2.4. mtDNA-targeted next-generation sequencing and pathogenicity prediction of variants

Total DNA was used to amplify mtDNA in two fragments of 8,338 and 8,647 bp using previously described primers (Gunnarsdóttir et al., 2011), and the purified PCR

products were mixed in equimolar ratios for the sequencing protocol using an Ion Torrent Personal Genome Machine (PGM, Fisher Scientific, Madrid, Spain) according to the manufacturer's user guide. The obtained mtDNA sequences were analyzed using the MToolBox pipeline, using the revised Cambridge Reference Sequence (rCRS, **NC_012920**) as a reference for human mtDNA (Calabrese et al., 2014). Further details of this multiparametric workflow for mtDNA variant prioritization have been previously described (Valiente-Pallejà et al., 2018). Briefly, this method aligns each sample-specific reconstructed contig against the related macrohaplogroup-specific consensus sequence to recognize, via a prioritization process, rare or private mitochondrial variants that deserve further clinical investigation. The prioritization process consists of several steps that exploit various annotation resources such as biological databases and pathogenicity prediction software. At the end of the process, this method reports the rare or private variants identified, the nucleotide variability, a disease score based on several predictors of pathogenicity and information about the variants associated with clinical outcomes present in public databases together with data on homoplasmy and heteroplasmy (Diroma et al., 2016; Santorsola et al., 2016). Homoplasmy refers to a cell that has a uniform collection of mtDNA—either completely normal mtDNA or completely mutant mtDNA—and heteroplasmy refers to a cell with different proportions of normal and mutant mtDNA. The mean ± standard deviation coverage obtained was 159.5 ± 178.9.

The selection criteria for the pathogenicity prediction of variants were as follows: nonsynonymous variants were selected based on a disease score >0.4311 and nucleotide variability <0.0026, while rRNA and tRNA variants with an RNA prediction score between 0.35 and 1 or nucleotide variability ≤0.0097 were selected as previously described (Diroma et al., 2016; Santorsola et al., 2016). The heteroplasmy level cutoff was set at 0.60, the threshold at what pathogenic mutations usually manifest (Valiente-Pallejà et al., 2018). However, this threshold depends on the mutation type, cell type

and tissue distribution and can vary between 60% and 90% (Nissanka and Moraes, 2020).

Datasets of mtDNA sequences and clinical data from participants are available at the European Genome-phenome Archive (EGA, https://ega-archive.org) with the study reference number EGAS00001003269.

## 2.5. Lactate stress test

Fasting participants underwent venipuncture with a BD Nexiva™ closed catheter system (BD Medical, Madrid, Spain) without a tourniquet prior to the initiation of cycling between 8:00 and 9:00 a.m. The first blood samples were collected for DNA analyses, blood counts, basic biochemistry, and basal lactate determination. Then, participants were asked to pedal for 15 min. at a consistent power of 30 watts (W) and 60 rotations per min. (rpm) on an electronically braked bicycle ergometer (Optibike, Ergoline, Madrid). Blood samples were collected at 5, 10, and 15 min. after cycling began and at 15 min. after stopping. Blood pressure and heart rate were monitored during all the exercise. Lactate levels were determined before cycling (baseline), during cycling (5, 10, and 15 min.), and at rest (30 min.) using a GEM® Premier™ 3000 Analyzer (Instrumentation Laboratory, New York, USA). The percent increase in lactate (%Δ lactate) at 5, 10, 15 and 30 min. was calculated as follows: %Δ lactate= ((lactate at each time point - basal lactate) / (basal lactate)) x 100.

## 2.6. Statistical analyses

Data were processed using R software (37). Sample homogeneity was tested with analysis of variance (ANOVA) and $\chi^2$ tests. Spearman correlations were used to explore the relationships among continuous variables and were visualized using the

corrplot package. We conducted multiple linear regression analysis to identify differences between patients with SZ and HCs in lactate levels (0, 5, 10, 15, 30 min) and mtDNA copy number, including covariates. A general linear model was used to estimate marginal means, adjusted means controlled for covariates and lactate levels and mtDNA copy number. We did not correct for multiple comparisons because the analyses were exploratory in nature, and we considered statistical significance when $p<0.05$.

## 3. Results

### 3.1 CAMDs

The frequencies of participants presenting CAMDs were higher in the SZ group than in the HC group when evaluating bowel function, nervous system and heart and blood vessels ($p=0.009$, $p=0.024$ and $p=0.001$, respectively). In addition, the number of CAMDs present in each individual was significantly higher in patients with SZ than in HCs ($p<0.001$) (Table 2).

### 3.2. mtDNA copy number

We evaluated whether the mtDNA copy number showed differences between SZ and HCs in a linear model including the following as covariates: age, sex, body mass index (BMI), number of cigarettes/day, units of alcohol/day, neutrophil to lymphocyte ratio (NLR), number of CAMDs and physical activity performed over the last week before the blood extraction. The mtDNA copy number was higher in HCs than in SZ (*MT-7S*, $p<0.001$; *MT-ND1*, $p=0.002$; *MT-ND4*, $p=0.011$) for all regions evaluated (Figure 1).

The three mtDNA regions assessed showed high correlations among them: *MT-ND1* with *MT-7S* (ρ=0.94, p<0.001), *MT-ND1* with *MT-ND4* (ρ=0.85, p<0.001) and *MT-7S* with *MT-ND4* (ρ=0.85, p<0.001). In addition, the mtDNA copy number of the three mtDNA regions positively correlated with units of alcohol/day (ρ>0.3, p<0.01) and negatively with the number of cigarettes/day (ρ<-0.35, p<0.001), number of CAMDs (ρ<-0.2, p<0.05), BMI (ρ<-0.2, p<0.05) and NLR (ρ-0.4, p<0.001). Only *MT-ND4* copy number negatively correlated with age (ρ<-0.3, p<0.05), basal lactate (ρ<-0.2, p<0.05) and the Positive and Negative Syndrome Scale (PANSS) general (ρ-0.3, p<0.05) and total (ρ<-0.3, p<0.05) scores. Conversely, no correlation was observed between mtDNA copy number and antipsychotic doses of chlorpromazine equivalents (Figure 2). Regarding pharmacological medication, being under anticholinergic treatment was associated with a lower mtDNA copy number, but no significant effect was observed in the use of antidepressants, mood stabilizers or benzodiazepines (Supplementary Table 1). Overall, these results indicate that mtDNA copy number decreases with aging, weight gain, tobacco consumption, inflammatory processes and anticholinergic use and increases with alcohol consumption.

### 3.3. mtDNA pathogenic mutations

By using the prioritization workflow of the MToolBox pipeline and applying the selection criteria, we identified 19 putative pathogenic variants with heteroplasmy levels >0.60 (Supplementary Table S2 and Supplementary Figure S1). Eleven patients with SZ (19.3%) carried at least one putative pathogenic mtDNA variant above the heteroplasmy threshold; four HCs (12.1%) also carried putative pathogenic variants (summarized in Supplementary Table S2). None of the 13 variants present in patients with SZ was found in HCs. Two variants carried by one HC and one patient with SZ (m.745 A>G and m.1192 C>T, respectively) are reported to confer increased sensitivity

to ototoxic drugs or nonsyndromic deafness in the MITOMAP database. However, none of these individuals suffered from deafness at the time of the study. Finally, a specific mtDNA haplogroup was assigned to each participant (Supplementary Table S3), and 44%, 17%, 10%, 9% and 2% of participants belonged to common European haplogroups H, U, J, T and V, respectively. The distribution of mtDNA putative pathogenic variants did not differ between grouped haplogroups ($\chi^2$=1.362, p=0.968), indicating that they were not overrepresented in a specific mtDNA lineage in our sample.

### 3.4. Lactate levels

To indirectly investigate the oxidative phosphorylation capacity in our sample, we measured venous lactate levels at baseline, during physical exercise (5 min., 10 min. and 15 min.) and at rest, 15 min. after the exercise (30 min.) and calculated the %Δ lactate from baseline. The %Δ lactate levels during the exercise and at rest were highly increased in patients with SZ compared to HCs but not at baseline (Figure 3). Linear regression models at each time point of lactate determination confirmed that %Δ lactate levels were much higher in patients with SZ than in HCs at 5, 10, and 15 min. and nearly at 30 min., despite the fact that basal lactate levels were very similar between the two groups. We included the following covariates in the analyses: age, sex, BMI, the number of CAMDs, number of cigarettes/day, units of alcohol/day, physical activity performed last week, *MT-ND1* copy number and the presence of a putative pathogenic variant with heteroplasmy >0.60). Because the mtDNA copy number assessment was highly correlated among the three mtDNA regions, only the *MT-ND1* region was included in the analyses. The linear models were significant at 5, 10 and 15 min. and, remarkably, in all these models, SZ diagnosis and age were significantly associated with the %Δ lactate (Table 3).

To assess whether the 13 mtDNA putative pathogenic variants present in the 11 patients had an impact on lactate levels, we compared %Δ lactate between the SZ carriers and the remaining individuals (both patients and HCs) who were noncarriers. In particular, the %Δ lactate was significantly higher in these 11 patients with SZ at 5 min. (p=0.02) and 10 min. (p=0.01) and was almost significant at 15 min. (p=0.06) (Supplementary Figure S2).

We also evaluated the relationship between lactate and the rest of the variables (Figure 2). Basal lactate levels were positively correlated with BMI (ρ=0.27, p=0.01) and negatively correlated with %Δ lactate at 5 min. (ρ=-0.26, p=0.02), 10 min. (ρ=-0.26, p=0.02), 15 min. (ρ=-0.23, p=0.03) and 30 min. (ρ=-0.24, p=0.03). A negative correlation between basal lactate levels and *MT-ND4* copy number was observed (ρ=-0.24, p=0.03). During exercise, %Δ lactate was positively correlated with age, the number of CAMDs and the NLR, showing that higher lactate levels were associated with the presence of comorbid clinical conditions and inflammation. In contrast, physical activity performed over the last week before performing the lactate stress test was negatively correlated with %Δ lactate, which indicates that the %Δ lactate in individuals who perform physical exercise is lower than in individuals who do not (Figure 2). No relationships were observed between current antipsychotic medication and lactate levels at any time point (Figure 2). Additionally, we did not identify significant differences in lactate levels between patients who were under anticholinergic, mood stabilizing or benzodiazepine treatment and those who were not (Supplementary Table 4).

## 4. Discussion

### 4.1. Elevated lactate during exercise in SZ

In the present study, we measured lactate levels as a biomarker of mitochondrial metabolic function at different time points during moderate physical exercise. Lactate formation and subsequent distribution throughout the body by the cell-to-cell lactate shuttles is the mechanism whereby different tissues and cell types coordinate intermediary metabolism depending on the energy demand (Goodwin et al., 2007). Here, patients with SZ showed higher lactate levels than HCs during exercise but not at baseline or after rest, suggesting that when energy requirements are increased, patients with SZ show poorer mitochondrial respiratory oxidative capacity, even though the involvement of other mitochondrial mechanisms related to oxygen supply, pyruvate dehydrogenase activity or the tricarboxylic acid cycle could not discounted (Adeva-Andany et al., 2014). Recently, a central role of mitochondrial functions in SZ, especially oxidative stress, has been proposed based on a leukocyte proteomic approach that identified alterations in energy metabolism, immunity, oxidative stress and apoptosis in first-episode drug-naïve psychotic patients, in line with the results identified in our study (Jiang et al., 2019).

### 4.2. Lactate levels correlated with comorbid clinical conditions

Lactate levels were positively correlated with the number of CAMDs. This finding was in accordance with a systemic mitochondrial dysfunction, which was more pronounced in those subjects with more clinical comorbid conditions. The mitochondrial dysfunction has been associated with chronic systemic inflammation present in SZ (Morris and Berk, 2015) and, interestingly, we observed a positive correlation between %$\Delta$ lactate and the NLR inflammatory marker, in accordance with this hypothesis. Increased brain lactate levels in SZ have been reported in the cerebrospinal fluid (Regenold et al., 2009) and in brain postmortem tissue (Sullivan et al., 2019) and in vivo by magnetic resonance spectroscopy (Rowland et al., 2016). In the brain, lactate utilization at

synapses is supposedly impaired in SZ, ultimately triggering cognitive and negative symptoms (Sullivan et al., 2018). In fact, a mechanism based on lactate production in astrocytes and its transport to neurons (i.e., the “astrocyte-neuron lactate shuttle”) has been deemed a pathophysiological substrate in SZ (Sullivan et al., 2018). This idea was mainly proposed because the disturbance of this bioenergetic coupling in rodents disrupts synaptic transmission, resulting in impaired working memory performance and long-term memory formation (Sullivan et al., 2018). Dysfunctional synapses and bioenergetic deficits can be primary or secondary causes of SZ, and psychotic symptoms may be the result of a mild bioenergetic brain defect (Pei and Wallace, 2018; Wallace, 2017). We propose that the high levels of blood lactate in SZ that we observed during exercise might parallel altered energy metabolism occurring in the brain.

### 4.3. High number of CAMDs in patients with SZ

Previous clinical studies have revealed that up to 50-70% of patients affected by a mitochondrial disorder caused by a mtDNA mutation show evidence of, or will develop, a psychiatric disorder; however, little attention has been paid to the specific psychiatric manifestations of mitochondrial disorders (Rosebush et al., 2017). From the opposite point of view, patients with SZ frequently suffer from several CAMDs, such as epilepsy, gastrointestinal symptoms, metabolic disturbances and cardiovascular diseases (Kritharides et al., 2017; Schoepf et al., 2014; Virtanen et al., 2017). The present study confirmed that a high number of patients with SZ presented alterations, with the most frequently observed comorbidities in the digestive, nervous and cardiovascular systems. Indeed, patients with SZ presented twice the number of CAMDs compared to HCs, which is in accordance with the hypothesis that some patients with psychiatric illnesses have undiagnosed mitochondrial disorders (Rosebush et al., 2017). Overall,

these results put forward that mitochondrial dysfunction may be an underlying mechanism involved in both the brain abnormalities and the other comorbidities reported in SZ (Gonçalves et al., 2014; Rajasekaran et al., 2015; Rosebush et al., 2017).

### 4.4. Low mtDNA copy number is a hallmark of SZ

In our study, we revealed significantly lower mtDNA content in blood samples of patients with SZ than HCs, thus confirming previous results in first-episode antipsychotic-naïve patients (Z. Li et al., 2015). Reduced mtDNA copy number has been observed with aging, use of clozapine, risperidone and psychosis severity in patients with psychotic disorders (Kumar et al., 2018) and with poorer outcomes in cognitive performance, physical strength and self-rated health (Mengel-From et al., 2014). Our results reinforce the hypothesis that low mtDNA copy number may be a pathophysiological hallmark of SZ, as described in other neurodevelopmental disorders (Valiente-Pallejà et al., 2018). Moreover, we identified significant correlations between mtDNA copy number and lifestyle habits, such as smoking and alcohol use. The results in tobacco use were in accordance with a recent study demonstrating that smoking reduces the mtDNA copy number in blood (Wu et al., 2019). We also show that the convergence of clinical conditions in distinct organs, and with inflammation as well, is correlated with low mtDNA copy number in the blood, indicating that these two features are likely associated. In this field, mtDNA has been demonstrated to be crucial in the underlying mechanism involved in the uncontrolled inflammation present in many chronic diseases (Zhong et al., 2018). Finally, high scores in the general psychopathology scale of the PANNS were associated with low mtDNA copy number, suggesting a correlation with disease severity. Regarding psychotropic medication, clozapine and risperidone have been demonstrated to reduce the mtDNA copy number

(Kumar et al., 2018); however, we did not identify any correlation between chlorpromazine equivalents and mtDNA copy number, suggesting that antipsychotic medication may not have a direct relationship with mtDNA copy number in our sample. Similarly, being treated with antidepressants, mood stabilizers and benzodiazepines seems to be unrelated to mtDNA copy number. However, the effects of anticholinergic treatment, which have been revealed to have protective effects against anoxia via mitochondria (Wang et al., 2017), need to be further studied, as we identified lower mtDNA copy numbers in patients taking anticholinergics.

### 4.5. Putative mtDNA pathogenic variants are associated with increased lactate levels

Although some studies have examined the role of the mtDNA sequence in SZ, it was not properly investigated in depth until the development of next-generation sequencing analyses, which allow the determination of the heteroplasmy levels. We selected putative pathogenic variants with heteroplasmy levels above 0.60 given that for most pathogenic mutations, the disease manifests at the heteroplasmic threshold in the range of 0.60 - 0.95 (Nissanka and Moraes, 2020). We found a slightly higher number of mtDNA putative pathogenic mutations in SZ (19.2%) than in HC (12.1%) individuals. Only one variant, m.9909T>C, corresponding to the amino acid change Phe235Leu at the *MT-CO3* gene, present in a patient with SZ has been previously associated with psychiatric disorders. In the Human Mitochondrial Genome Database (https://www.hmtdb.uniba.it/) (Rubino et al., 2012), 17 out of 85 sequenced patients with a psychiatric disorder (SZ, schizoaffective disorder or bipolar disorder), all of Italian origin, showed a nucleotide change at this position (Bertolin et al., 2011), reinforcing its involvement in psychiatric disorders. Research in this field is scarce, and the results are inconsistent. Some authors demonstrated that common mtDNA variants modified the risk of developing several complex traits, including SZ (Hudson et al.,

2014), although recent studies have excluded the roles of common mtDNA variations (Gonçalves et al., 2018) and heteroplasmy (H. Li et al., 2016) in SZ. We added evidence of the involvement of these genetic factors, meriting additional studies to confirm its pathogenicity, especially m.9909T>C. In addition, the fact that blood lactate levels were increased in the SZ carriers of putative mtDNA mutations compared to the rest is in accordance with mtDNA sequence variants implicated in the mild bioenergetic brain defect.

It is worth mentioning that we determined a heteroplasmy level cutoff of 0.60; however, it cannot be ruled out that mtDNA variants at lower levels in blood may present high heteroplasmy levels in the brain.

### 4.6. Limitations

Some limitations of this study must be noted. First, our sample is based on patients with a long evolution of SZ instead of unmedicated antipsychotic-naïve patients. Thus, we could only exclude the possibility that distinct medications and doses influenced lactate levels but not the presence of medication per se. Second, we evaluated mtDNA and lactate variations in blood samples, even though brain tissue is the target for SZ symptoms. However, we selected blood samples in the context of systemic rather than organ-specific mitochondrial dysfunction. Although no differences in physical activity were observed between patients with SZ and HCs, patients could have deficits in fitness or muscle tone, especially because of the lack of exercise throughout disease evolution. This issue could explain, at least in part, the differences in %$\Delta$ lactate. Third, several factors may alter the accuracy of the mtDNA copy number including the presence of mitochondrial sequences in the nuclear genome, the use of inappropriate nuclear primers, dilution bias and template preparation (Malik and Czajka, 2013), which we have already considered in this study. Finally, although the

mtDNA variants were not overrepresented in a single haplogroup, some may be specific to our population, and the results may not be generalizable until further studies with larger samples of other populations are conducted.

## 4.7. Conclusions

In summary, this study describes higher lactate levels during exercise and the presence of higher a number of CAMDs together with a lower mtDNA copy number in patients with SZ. Additionally, during exercise, lactate levels were greater in patients with SZ who were carriers of a putative mtDNA pathogenic mutation than in the remaining participants. Finally, lactate levels and the mtDNA copy number were correlated with the number of CAMDs and the NLR inflammatory biomarker. Together, our results are consistent with a systemic mitochondrial dysfunction in SZ and target lactate metabolism and mtDNA for potential therapeutic treatments.

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**Legends to Figures**

**Figure 1.** Estimated marginal means of mtDNA copy number in our sample.

∗: p<0.05; ∗∗: p<0.01; ∗∗∗: p<0.001.

**Figure 2.** Correlations between all the variables studied.

*Percentage of lactate increases (%Δ) at 5, 10, and 15 minutes and at rest. MT-7S cn, MT-ND1 cn and MT-ND4 cn:* copy numbers of the *MT-7S, MT-ND1 and MT-ND4* genes; N CAMDs: number of clinical conditions associated with mitochondrial disorders; BMI: body mass index; Alcohol: standard drink units/day; Tobacco: number of cigarettes/day; IPAQ: physical activity of the last seven days measured with the International Physical Activity Questionnaire; Medication: current medication in mg equivalent to chlorpromazine doses/day; PANNS-P: Positive and Negative Syndrome Scale, positive score; PANNS-N: PANSS, negative score; PANNS-G: PANSS, general score; PANSS-T: PANSS, total score; NLR: neutrophil to lymphocyte ratio.

The strength and the direction of the correlation between each pair of variables are displayed by color intensity (red and blue for positive and negative correlations, respectively). Legend for Pearson's coefficients ($\rho^2$) is shown below the graph.

∗: p<0.05; ∗∗: p<0.01; ∗∗∗: p<0.001.

**Figure 3.** Basal lactate levels and %Δ lactate in the study groups during the lactate stress test.

∗: p<0.05; ∗∗∗: p<0.001.

**Table 1.** Descriptive characteristics of the sample.

| | Normal values | **SZ** N=57 | **HC** N=33 | p |
|---|---|---|---|---|
| **Epidemiological and biological data** | | | | |
| Sex, males, n (%) | | 44 (77%) | 21 (64%) | 0.166 |
| Age, years | | 43.9 ± 12.3 | 39.8 ± 12.1 | 0.125 |
| BMI, kg/m$^2$ | 18.5 – 24.9 | 27.8 ± 5.0 | 24.0 ± 3.7 | **<0.001** |
| Systolic pressure, mm Hg | < 120 | 125.6 ± 14.5 | 125.0 ± 16.7 | 0.858 |
| Diastolic pressure, mm Hg | < 80 | 76.7 ± 10.9 | 76.7 ± 11.6 | 0.997 |
| Creatinine (mg/dl) | 0.7 – 1.3 | 0.84 ± 0.21 | 0.79 ± 0.15 | 0.244 |
| Creatinine kinase (IU/l) | 25 – 170 | 113.9 ± 57.9 | 122.6 ± 65.5 | 0.545 |
| AST (IU/l) | < 32 | 22.4 ± 12.7 | 19.5 ± 6.0 | 0.214 |
| ALT (IU/l) | < 33 | 29.5 ± 25.8 | 19.3 ± 21.0 | 0.064 |
| Total protein (g/dl) | 6 – 8 | 6.7 ± 0.4 | 6.7 ± 0.3 | 0.793 |
| Albumin (g/dl) | 3.4 – 4.7 | 4.5 ± 0.3 | 4.6 ± 0.2 | 0.050 |
| Hematocrit (%) | 36 – 46 | 43.0 ± 3.7 | 42.0 ± 3.8 | 0.268 |
| NLR | 0.78 – 3.53 | 2.3 ± 1.1 | 1.7 ± 1.1 | **<0.001** |
| **PANSS score** | | | | |
| PANSS positive | < 7 | 16.6 ± 6.9 | - | |
| PANSS negative | < 7 | 25.1 ± 5.8 | - | |
| PANSS general | < 16 | 27.4 ± 10.4 | - | |
| PANSS total | < 30 | 69.2 ± 18.8 | - | |
| **Illness progression** | | | | |
| Age at onset | | 19.9 ± 3.9 | - | |
| Illness duration | | 23.9 ± 11.7 | - | |
| **Pharmacological treatment** | | | | |
| Benzodiazepines | | 28 (49%) | - | |
| Anticholinergics | | 12 (21%) | - | |
| Antidepressants | | 11 (19%) | - | |
| Mood stabilizers | | 19 (33%) | - | |
| Antipsychotics[a], mg/day | | 416.0 ± 305.0 | - | |
| **Drug use** | | | | |
| Alcohol, standard drink/day | | 0.02 ± 0.13 | 0.64 ± 0.70 | **<0.001** |
| Smoking, cigarettes/day | | 12.2 ± 11.5 | 1.7 ± 4.5 | **<0.001** |
| **Physical activity** | | | | |
| IPAQ-total[b], MET-min/week | | 3031 ± 3539 | 3003 ± 3030 | 0.970 |

SZ: schizophrenia; HC: healthy controls; M: male; F: female; N: number; AST: aspartate aminotransferase; ALT: alanine aminotransferase; NLR: neutrophil to lymphocyte ratio; PANSS: Positive and Negative Syndrome Scale; IPAQ: International Physical Activity Questionnaire; MET: metabolic equivalent of task.

Data are presented as means ± SDs or numbers of subjects (%). Significant p values are shown in boldface.

[a] Current antipsychotic medication is shown as equivalents to chlorpromazine doses.

[b] IPAQ-total= 8 x (minutes of vigorous activity/day x 7 days/week) + 4 x (minutes of moderate activity/day x 7 days/week) + 3.3 x (minutes of activity/day x 7 days/week).

**Table 2.** Number of subjects presenting CAMDs.

| **CAMDs category** | **SZ** N=57 | **HCs** N=33 | p |
|---|---|---|---|
| Headaches, n (%) | 14 (25%) | 5 (15%) | 0.292 |
| Bowel function, n (%) | 28 (49%) | 7 (21%) | **0.009** |
| Soft tissues and fatigue, n (%) | 18 (32%) | 6 (18%) | 0.166 |
| Nervous system, n (%) | 15 (26%) | 2 (6%) | **0.024** |
| Ears and eyes, n (%) | 17 (30%) | 8 (24%) | 0.569 |
| Endocrine, n (%) | 6 (11%) | 3 (9%) | 1.000 |
| Heart and blood vessels, n (%) | 26 (46%) | 7 (21%) | **0.021** |
| Number of conditions/individual, mean ± SD | 3.5 ± 1.7 | 1.6 ± 1.3 | <**0.001** |

CAMDs: conditions associated with mitochondrial disorders; SZ: schizophrenia; HCs: healthy control. Significant p values are shown in boldface.

Conditions included in each CAMD category. Headaches: headaches, migraine; Bowel function: constipation, diarrhea, abdominal pain, nausea; Soft tissues and fatigue: kinetosis, severe fatigue, fibromyalgia, dysautonomia, arthritis, muscular weakness; Nervous system: intellectual disability, seizures, attention deficit hyperactivity disorder, learning disability, autism, anxiety disorder, bipolar disorder, depressive disorder and panic disorder; Ears and eyes: deafness, vision alterations; Endocrine: hypercholesterolemia, kidney disease, cancer, hypoglycemia, hypothyroidism, diabetes; Heart and blood vessels: heart disease, stroke, hypertension.

**Table 3**

**Table 3.** Linear models for predicting basal lactate and %Δ lactate during the lactate stress test.

| | **Basal lactate** | | | **%Δ lactate at 5 min** | | | **%Δ lactate at 10 min** | | | **%Δ lactate at 15 min** | | | **%Δ lactate at rest** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Adj.$R^2$=0.093; F=1.801 p=**0.069** | | | Adj.$R^2$=0.237; F=3.425 p=**0.001** | | | Adj.$R^2$=0.281; F=4.013 p**<0.001** | | | Adj.$R^2$=0.200; F=2.887 p=**0.003** | | | Adj.$R^2$=0.049; F=1.391 p=0.196 | | |
| | Beta | t | p | Beta | t | p | Beta | t | p | Beta | t | p | Beta | t | p |
| SZ | -0.103 | -0.564 | 0.575 | 28.741 | 2.484 | **0.015** | 38.718 | 2.770 | **0.007** | 46.006 | 2.786 | **0.007** | 24.714 | 2.132 | **0.036** |
| Sex | 0.094 | 0.656 | 0.514 | 15.219 | 1.676 | 0.098 | 16.697 | 1.546 | 0.126 | 14.773 | 1.142 | 0.257 | 0.960 | 0.103 | 0.918 |
| Age | 0.007 | 1.473 | 0.145 | 0.773 | 2.558 | **0.013** | 1.143 | 3.160 | **0.002** | 1.167 | 2.696 | **0.009** | 0.584 | 1.894 | 0.062 |
| BMI | 0.039 | 3.041 | **0.003** | -0.600 | -0.732 | 0.467 | -0.860 | -0.858 | 0.394 | -1.662 | -1.424 | 0.159 | -0.523 | -0.624 | 0.535 |
| NLR | -0.050 | -0.854 | 0.396 | 3.599 | 0.978 | 0.331 | 6.917 | 1.503 | 0.137 | 7.818 | 1.451 | 0.151 | 3.817 | 1.004 | 0.319 |
| IPAQ | 0.000 | 0.722 | 0.472 | -0.001 | -0.927 | 0.357 | -0.003 | -2.092 | **0.040** | -0.002 | -1.131 | 0.262 | 0.000 | -0.240 | 0.811 |
| CAMDs | 0.016 | 0.405 | 0.687 | 3.448 | 1.387 | 0.170 | 1.516 | 0.511 | 0.611 | 0.791 | 0.228 | 0.820 | 0.413 | 0.169 | 0.866 |
| Tobacco | 0.003 | 0.425 | 0.672 | -0.441 | -1.154 | 0.252 | -0.419 | -0.924 | 0.358 | -0.768 | -1.461 | 0.148 | -0.804 | -2.189 | **0.032** |
| Alcohol | -0.012 | -0.087 | 0.931 | 5.996 | 0.688 | 0.493 | 2.406 | 0.233 | 0.816 | -2.385 | -0.199 | 0.843 | 0.038 | 0.004 | 0.996 |
| *MT-ND1* cn | -0.032 | -1.242 | 0.218 | 0.478 | 0.297 | 0.767 | 1.789 | 0.930 | 0.355 | 1.904 | 0.839 | 0.404 | 1.777 | 1.129 | 0.263 |
| mtDNA ppv | 0.083 | 0.530 | 0.597 | 8.611 | 0.865 | 0.390 | 8.298 | 0.705 | 0.483 | 9.613 | 0.699 | 0.487 | 2.477 | 0.257 | 0.798 |

SZ: schizophrenia diagnosis; BMI: body mass index; NLR: neutrophil to lymphocyte ratio; IPAQ: physical activity in the last 7 days measured with the International Physical Activity Questionnaire; CAMDs: number of conditions associated with mitochondrial disorders; Tobacco: consumption measured as number of cigarettes/day; Alcohol: consumption as standard units of alcohol/day; *MT-ND1* cn: mitochondrial NADH dehydrogenase subunit 1 gene copy number; mtDNA ppv: having or not a putative pathogenic variant in the mitochondrial DNA with heteroplasmy≥60%.
Significant p values are shown in boldface.

**Figure 1**

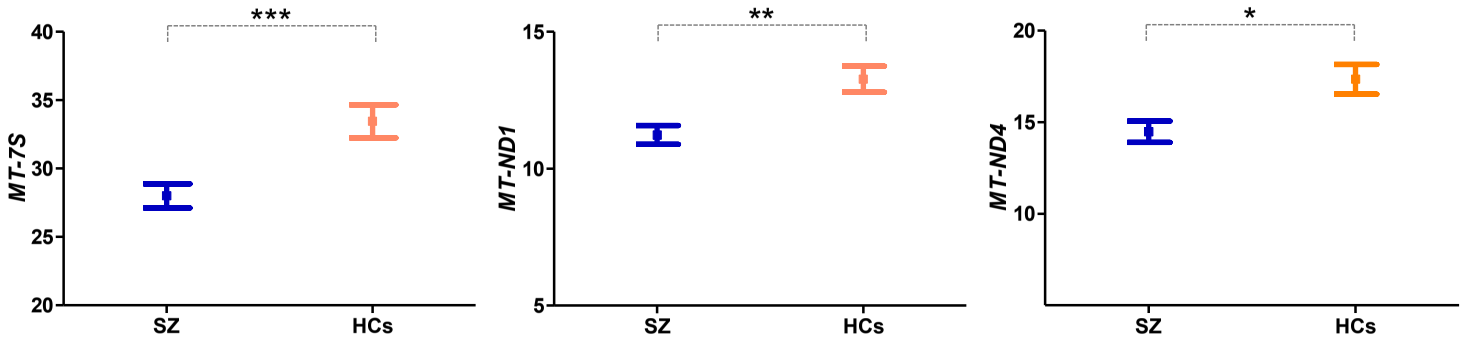

***
40
35
30
25
20
MT-7S
SZ
HCs
**
15
10
5
MT-ND1
SZ
HCs
*
20
15
10
MT-ND4
SZ
HCs

**Figure 2**

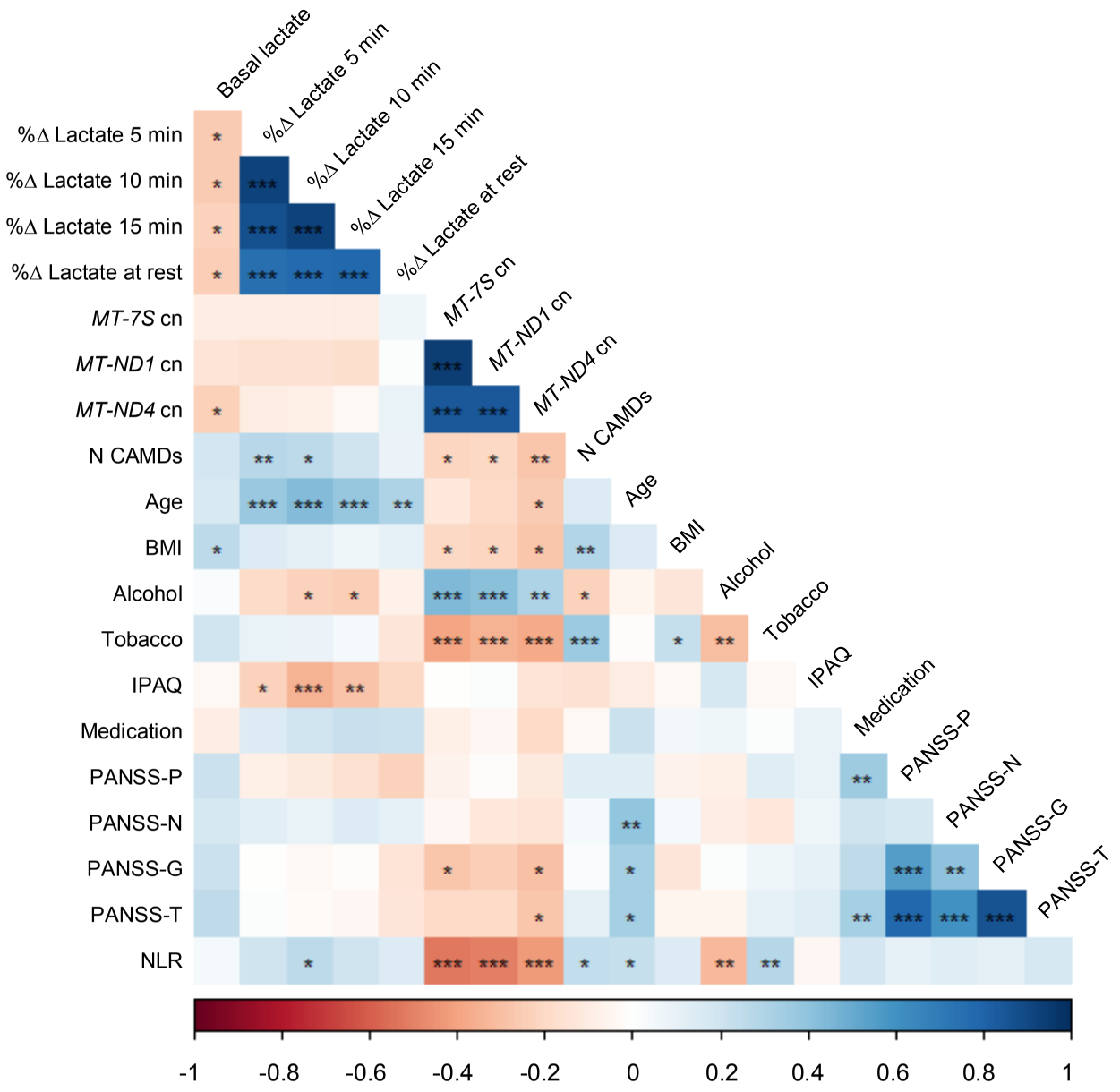
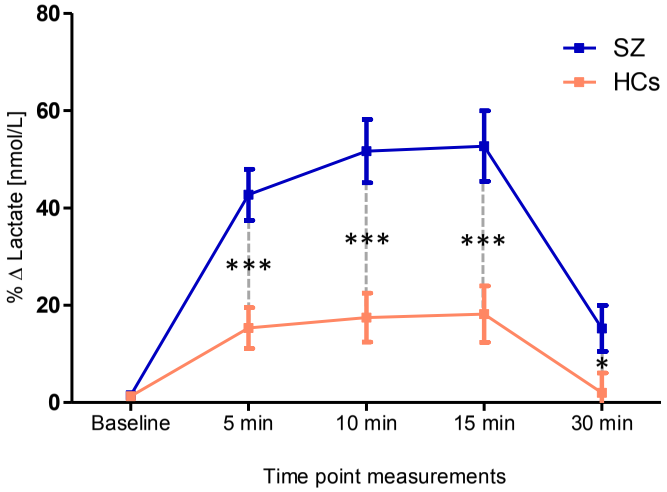

Basal lactate
%Δ Lactate 5 min
%Δ Lactate 10 min
%Δ Lactate 15 min
%Δ Lactate at rest
MT-7S cn
MT-ND1 cn
MT-ND4 cn
N CAMDs
Age
BMI
Alcohol
Tobacco
IPAQ
Medication
PANSS-P
PANSS-N
PANSS-G
PANSS-T
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

**Figure 3**


80
60
40
20
0
% Δ Lactate [nmol/L]
SZ
HCs
***
***
***
*
Baseline
5 min
10 min
15 min
30 min
Time point measurements

## Contributors

LM, EV, YA and GM designed the study and wrote the protocol. AV-P, HT, YA and EV obtained the clinical, biochemical and genetic data. GM analyzed the mtDNA sequences using the MToolBox and conducted the statistical analyses. AV-P wrote the durst draft of the manuscript and managed the literature searches. All the authors contributed to and approved the final version of the manuscript.

*Role of the Funding Source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## Declaration of Competing Interest

The authors have no conflicts of interest to report, financial or otherwise.

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