Mitochondrial DNA deletions are frequent in leukocytes of subjects with intellectual disability

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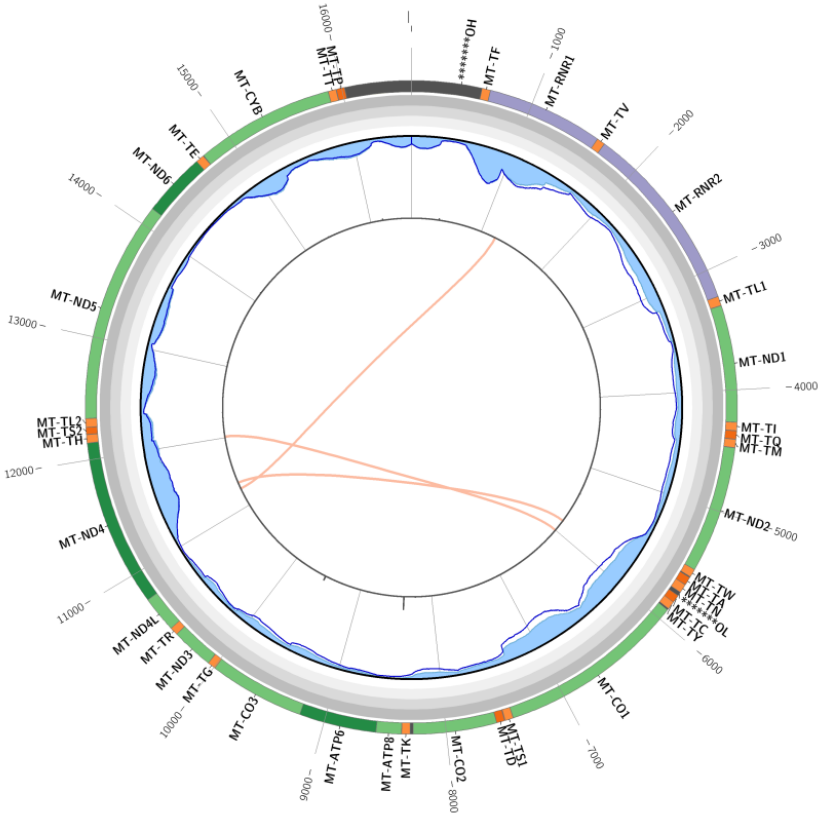
FINAL DEGREE PROJECT

Bachelor's degree in Biotechnology



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1. Information of the center

This project has been carried out within the line of research "Mitochondrial DNA and mental health" at the Hospital Universitari Institut Pere Mata (www.peremata.com) and the Rovira i Virgili University (www.urv.cat). I have been working in the research group "Genetics and Environment in Psychiatry, GAP", whose responsible is Elisabet Vilella. The GAP is a research group consolidated by the Catalan Agency of Universities and Research of the Generalitat de Catalunya (AGAUR 2014 SGR 995), by the Roviria i Virgili University (URV) and is part of the Strategic Area of Neurosciences and Mental Health of the Pere Virgili Health Research Institute (IISPV). All the researchers on the team are members of CIBERSAM. This research initiated the study of the involvement of mitochondrial DNA (mtDNA) in schizophrenia following observations of excessive maternal transmission in a number of parent-child patients (Martorell et al, 1999). The development of 3 competitive projects PI06/1586, PS09/01052, PI12/01885, allowed us to deeply study this molecule in several psychiatric disorders and in the intellectual disability, with relevant contributions in this field.

The present study has analyzed mtDNA samples from patients attended by several facilities of the Institut Pere Mata, a mental health services entity that manages a wide network of publicly owned devices: from the specialized services of the outpatient network for adults and for children and young people under 18, to the resources for hospitalization and other specialties. The mitochondrial DNA sequences were obtained at the Center of Omic Sciences (http://omicscentre.com) in Reus and the analyses of the data was performed at the Unit of Psychiatry in the Faculty of Medicine and Health Sciences at the URV.

This study has been performed in the framework of the research Project PI18/00514, financed by the Instituto de Salud Carlos III, with Lourdes Martorell as the Principal Investigator (PI) and Gerard Muntané as Co-PI, and has been sent for peer review on May, 6th 2020, to the Journal of Medical Genetics.

2. Abstract

Background: Mitochondria are cellular organelles which the main function is the production of energy in ATP form. Mitochondrial dysfunction has been reported in many neurodevelopmental disorders, including autism spectrum disorder (ASD), intellectual disability (ID) and schizophrenia (SCZ). Mitochondrial DNA (mtDNA) rearrangements, like deletions, may have a great impact in the mitochondrial correct function leading to oxidative phosphorylation system (OXPHOS) deficiency and, finally, driving to mitochondrial dysfunction.

Methods: MtDNA of leukocytes from 85 individuals with ID, 97 patients with ID and ASD (ID-ASD), 56 individuals with SCZ and 39 healthy controls (HC) was analyzed by next-generation sequencing in combination with the eKLIPse bioinformatic tool to detect and quantify mtDNA rearrangements.

Results: Results showed that mtDNA deletions in blood were frequent in individuals with ID and ID-ASD (17.7% and 9.3%, respectively), whereas such deletions were not common in both SCZ (1.8%), and HC (2.6%). Also, the removal of the origin of replication of the heavy strand (O_H) was more detrimental than the deletion of the origin of replication of the light strand (O_L), which was strongly correlated with ID severity and higher CARS scores in ID and ID-ASD patients. Nevertheless, heteroplasmy levels in leukocytes were low, not higher than 5% across all samples.

Conclusions: ID severity and CARS score were associated with the presence of large mtDNA deletions in blood and positively correlated with the lack of the O_H. Further studies evaluating the involvement of mitochondrial rearrangements in post-mitotic tissues in neurodevelopmental disorders, especially ID, will help to clarify whether these mtDNA deletions detected in blood are present in higher heteroplasmy levels in these tissues.

Keywords: mitochondrial DNA; deletion; autism spectrum disorder; intellectual disability; schizophrenia

3. Introduction

3.1. Neurodevelopmental disorders

Neurodevelopmental disorders encompass a group of disorders in which the development of the central nervous system is disturbed. This includes developmental brain dysfunction, which normally manifests as neuropsychiatric disorders, impaired motor function, learning, language or non-verbal communication (Ahn & Hwang, 2017).

The amount of people suffering different psychiatric illness has risen in the past few decades. This event may be the consequence of better and more accurate diagnosis of the disorders, the rising of awareness of mental health and the advances made in the research field. Genetic contribution in mental disorders is very relevant. Heritability for most of the psychiatric disorders usually varies from 30% to 80%, being 80% for autism spectrum disorder (ASD) and schizophrenia (SCZ). Furthermore, different mental diseases share diverse genetic factors, which may suggest the existence of a common genetic background. Therefore, taking into account the genetic contribution, a service of genetic counseling could give the patients and their families a better understanding of the disorder and help them to adapt to that specific situation, which has been demonstrated to be beneficial for patients and their families (Martorell et al., 2019).

Some studies have found that several genes are inactivated in more than one psychiatric disorder, with crossovers between intellectual disability (ID), attention deficit and hyperactive disorder (ADHD), ASD and SCZ, among others. There is also a high correlation between age and an increasing risk of developing a neuropsychiatric disease, which is consistent with the etiology of mitochondrial diseases, where mutations in the mitochondrial DNA (mtDNA) accumulate during the developmental stage and the aging phase (Pei & Wallace, 2018). Furthermore, medical conditions commonly associated with mitochondrial disorders, are common in individuals with ID, ASD and SCZ, thus, pointing to a possible role of mitochondria in the development of these disorders (Pei & Wallace, 2018).

3.1.1. Autism Spectrum Disorder (ASD)

ASD encompasses a set of heterogeneous neurodevelopmental conditions that have common features such as, deficits in social communication and interaction, and unusual restricted, repetitive behavior, activities and interests (Croen et al., 2015). Its prevalence in the population stands between 1% and 2% and it is up to 4 times more common in males than it is in females (Lai et al., 2014). Furthermore, previous studies with twin siblings suggest that ASD has high inheritability (higher than 80%) (Lai et al., 2014). In addition, most of the major medical conditions, like gastrointestinal problems and immune dysregulation are common in ASD

population (Croen et al., 2015; Lai et al., 2014; Valiente-Pallejà et al., 2018). Interestingly, ID is also more common in individuals suffering from ASD (Croen et al., 2015) and the risk of developing psychiatric conditions is higher in individuals with ASD. Also, an association between ASD and SCZ has been suggested (Croen et al., 2015). Therefore, studying the genetic basis of the disorder is very relevant, which could allow to have a better understanding of the underlying mechanisms involved in the development of the disease and to identify specific molecular targets. This, in consequence, will enable to provide better and more effective patient care and treatment.

On the one hand, increasing the insight into disease heterogeneity and being able to stratify ASD population into different subgroups based on diverse parameters (genetics, environmental factors, behavior and development, comorbidities, …) could bring clarity to the understanding of the differences within individuals with ASD. On the other hand, we also have the need to understand that heterogeneity in ASD may probably be part of a broader heterogeneity among neurodevelopmental and health conditions (Lombardo et al., 2019).

3.1.2. Intellectual disability (ID)

ID is defined as a neurodevelopmental syndrome that entails limitations in both, intellectual and adaptive functioning, which originates in early childhood and before the age of 18, and lasts for the rest of the lifespan of the individual (Srour & Shevell, 2014; Vasudevan & Suri, 2017). The prevalence of global developmental delay and ID in the worldwide population is around 2% to 3%. Equal to ASD, ID affects more men than women (Srour & Shevell, 2014). It can occur isolated, being ID the unique clinical feature, known as non-syndromic intellectual disability. When it happens in combination with other conditions, physical or neurological, it is referred as syndromic intellectual disability (Srour & Shevell, 2014). Moreover, individuals with ID have a higher risk for diverse comorbidities, which may include epilepsy, psychiatric illness or sensory impairments among others (Srour & Shevell, 2014).

Nowadays, up to half of the cases of individuals with ID have been identified to have a genetic basis. This genetic origin implicates around 450 different nuclear genes. Besides the nuclear genome, mutations and defects in the mitochondrial genome may give rise to ID, with a characteristic maternal inheritance (Srour & Shevell, 2014). For this reason, an accurate diagnosis based in genetic features would be advantageous for improved treatment and management of the condition and associated comorbidities.

3.1.3. Schizophrenia (SCZ)

SCZ, is one of the most complex and severe psychiatric disorders. It is manifested with delusions, hallucinations, self-neglect, social withdrawal and paucity of speech, among others. The worldwide prevalence is approximately 1% and the onset age is usually at late adolescence (Ben-Shachar, 2017). Studies support the hypothesis that SCZ is, in fact, a polygenic disorder (Hjelm et al., 2015). Therefore, deficits in numerous genes could lead to a particular disease phenotype.

It has been described that individuals with female relatives with this condition have a greater risk of developing the disorder compared to those with affected male relatives which might be related with the maternal inheritance of mtDNA (Verge et al., 2011).

Since there is evidence of genetic contribution to the disorder, being able to identify mutations, variants or rearrangements regarding both the nuclear and mitochondrial genome would be beneficial. This might be helpful to provide a better and more specific treatment based on the patient's genetic background.

In summary, these neurodevelopmental syndromes are caused by the interactions of genetic and environmental factors and, collectively, have up to 3% prevalence worldwide (Ben-Shachar, 2017; Pei & Wallace, 2018; Verge et al., 2011). A huge number of nuclear variants have been associated with ID, ASD and SCZ; however, defects in the mtDNA can also lead to such disorders, but studies in this field are scarce (Pei & Wallace, 2018).

3.2. Mitochondrial DNA (mtDNA)

As mentioned, mitochondria might play an important role in the development of neurodevelopmental disorders. Mitochondria are considered the powerhouse of the cell since its main function is to produce most of the cellular energy in the form of ATP (Nissanka & Moraes, 2020; Pei & Wallace, 2018). In addition, it has many other roles, such production of reactive oxygen species (ROS), control calcium homeostasis in the cell and regulation of its intrinsic apoptotic pathway and the redox status of the cell (Pei & Wallace, 2018). Depending on the energy requirements , there can be hundreds to thousands of copies in each cell to fulfill its specific energy needs (Nissanka & Moraes, 2020; Pei & Wallace, 2018).

Interestingly, mitochondria also have their own genome as shown in Figure 1, called the mitochondrial DNA (mtDNA), which is a circular, 16,569 base pair (bp) long and double-stranded DNA molecule, which follows a uniquely maternal inheritance, in contrast with the nuclear DNA (Lott et al., 2013). It encodes 37 genes, among them, 13 are essential polypeptides for the

oxidative phosphorylation system (OXPHOS), 22 are tRNAs and two are rRNAs involved in mitochondrial DNA replication and protein synthesis. MtDNA also includes a non-coding region known as the displacement loop (D-loop), which is crucial for its replication, as one of the 2 origins of replication, the one for the heavy strand (O_H), is located at the D-loop, at nucleotide 407. The other one, for the light strand (O_L) is located at nucleotide 5747. Their location defines the mtDNA major and minor arcs and a broad range of deletions have been described in both regions (Nissanka & Moraes, 2020; Rygiel et al., 2016; Verge et al., 2011).

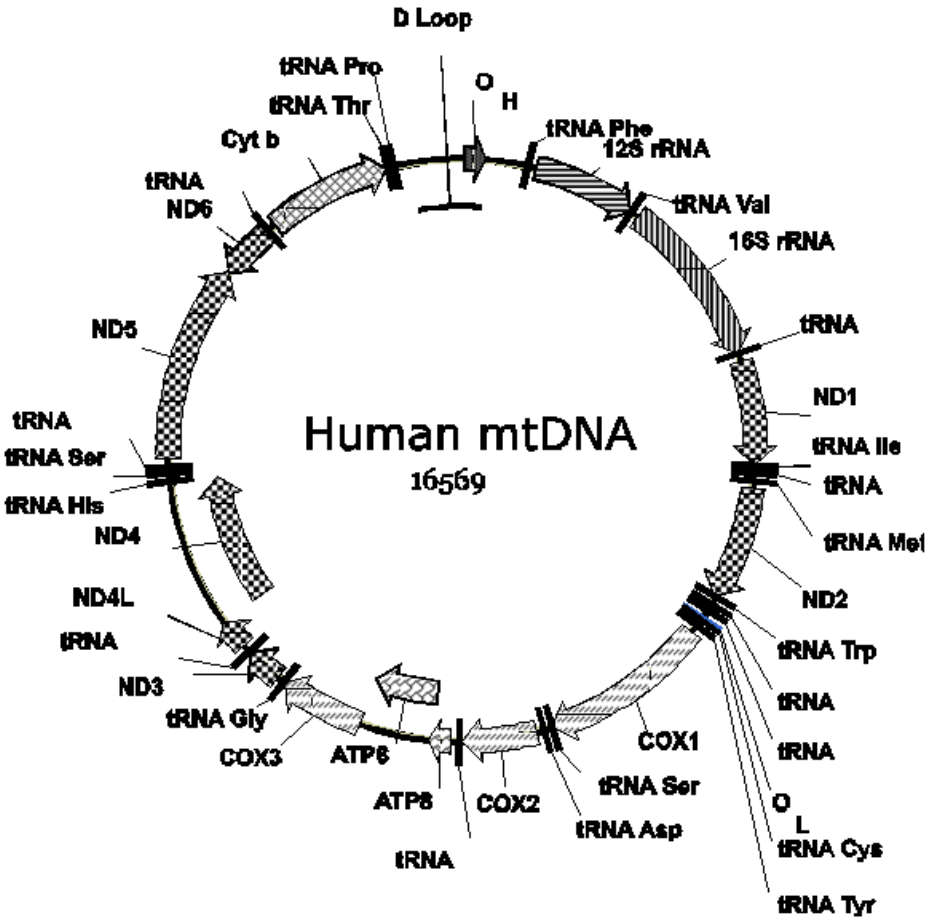

Figure 1. The map of human Mitochondrial DNA. O_H and O_L, origins of replication of heavy and light strand, respectively; ND1-ND6, subunits of NADH dehydrogenase (ETC complex I) subunits 1 through 6; COX1-COX3, subunits of cytochrome oxidase subunits 1 through 3 (ETC complex IV), ATP6 and ATP8, subunits 6 and 8 of mitochondrial ATPase (complex V), Cyt b, cytochrome b (complex III) (Shokolenko et al., 2011).

One of the particularities of mtDNA is its mutation rate, which is 10 to 20 times higher than that of the nuclear genome (Nissanka & Moraes, 2020; Verge et al., 2011). This may be a

consequence of the higher concentration of ROS or the absence of mitochondrial histones, unlike the nuclear DNA, which is protected by histones (Verge et al., 2011). Mutations can be classified in single-nucleotide variants (SNVs), indels (short insertions and deletions) and major rearrangements, that englobe deletions and duplications of larger fragments of the mtDNA. SNVs are commonly inherited (El-Hattab & Scaglia, 2016; Verge et al., 2011), while large-scale mtDNA deletions are usually sporadic, they are associated with many mitochondrial disorders and accumulate in post-mitotic tissues during the aging process (Taylor et al., 2014). The mechanisms underlying mtDNA deletions remain unknown; however, around 90% of the deletions reported are flanked by short, perfect or imperfect repeat sequences (Persson et al., 2019). While mechanisms of mtDNA deletions formation are not known there is evidence that suggests that the formation of mtDNA deletions may be consequence of the repair mechanism of damaged mtDNA (Nissanka et al., 2019).

Additionally, the number of mtDNA copies in a cell may vary and the mtDNA molecules might all be identical or, in contrast, different mtDNA species may co-exist in a cell due to the polyploid nature of mtDNA. These two situations are, respectively, named homoplasmic and heteroplasmic state (Nissanka & Moraes, 2020). Heteroplasmy is defined by the presence of both mutant and normal mtDNA molecules simultaneously, and it may vary between tissues within the same individual. The rate of heteroplasmy is defined by the load of mutant mtDNA relative to normal mtDNA. On the other hand, homoplasmy refers to the state in which all mtDNA molecules are identical, either 100% mutant or entirely normal (Nissanka & Moraes, 2020; Pei & Wallace, 2018; Verge et al., 2011).

The brain is the organ with the highest energy demands. Consequently, it has a high dependence on the mitochondria to fulfill its energy requirements and it is the number one energy source in this tissue. It has also been proposed the involvement of mitochondria in brain development and regulation of neuronal activity (Ben-Shachar, 2017; Valenti et al., 2014). Considering the brain's high energy demands and the role of mitochondria in the developing brain, mitochondrial dysfunction could have severe consequences in brain function.

Several studies have reported mitochondrial malfunction in some neuropsychiatric and neurodevelopmental disorders, such ASD, ID and SCZ, among others (Ben-Shachar, 2017; Pei & Wallace, 2018; Valenti et al., 2014; Verge et al., 2011). There has also been demonstrated that neuronal functions are quite sensitive to alterations in mitochondrial function (Valenti et al., 2014). Since evidence of mitochondrial dysfunction has been demonstrated in patients with ID, ASD and SCZ, the analysis of the mtDNA could bring clarity to the issue.

Sequence variants have been studied more widely than major rearrangements due to lack of proper technology to study deletions and duplications in the mtDNA. However, nowadays, thanks to the next-generation sequencing technology, the possibility to detect and quantify mtDNA deletions is now a reality (Goudenège et al., 2019; Hjelm et al., 2019; Taylor et al., 2014). The use of massive parallel sequencing tools allows to analyze precisely mtDNA as it has been done previously (Valiente-Pallejà et al., 2018). Nevertheless, the detection of major rearrangements is still challenging, especially those at very low heteroplasmic levels. Therefore, to solve this inconvenience different novel bioinformatics tools and pipelines have been developed, enabling the detection and quantification of mtDNA rearrangements, even at extremely low heteroplasmies (Goudenège et al., 2019; Hjelm et al., 2019).

4. Interests and hypothesis

The hypothesis of this research project is that patients with neurodevelopmental disorders will carry significantly more mtDNA deletions compared to the healthy control (HC) group and that the presence of these deletions may account for some of the comorbidities or phenotypic features.

It is interesting to carry out this kind of projects since mtDNA is usually left aside when investigating the genetic origins of neurodevelopmental disorders. In the future it may be necessary to take the mitochondrial genome into account to have a better knowledge of the genetic basis and the underlying mechanisms involved in the development of neurodevelopmental diseases, as well as to provide more specific and personalized treatments to the affected individuals.

5. Objectives of the study

We argue that the examination of the mtDNA may be crucial to identify mechanisms underlying neurodevelopmental disorders. The main objective of this work was to study whether mtDNA deletions are present in blood samples of individuals with ID, ASD, and SCZ, as well as in HCs; and to investigate whether patients with neurodevelopmental disorders carry more mtDNA rearrangements than the HC group.

6. Materials and methods

6.1. Participants and ethical considerations

Informed consent approved by the local ethics committee was provided to participants from the same geographical region and ethnic background. Personal medical histories and physical examinations were obtained as previously described (Valiente-Pallejà et al., 2018, 2020). The sample consisted of four different diagnostic groups: 56 individuals that had been diagnosed with SCZ, 85 subjects with severe or profound ID, 97 individuals with severe or profound ID who also fulfilled the criteria for the diagnosis of ASD (referred as ID-ASD in our study), and 39 HC (Table 1).

Table 1: Characteristics of the sample

	ID N=85	**ID-ASD** N=97	**SCZ** N=56	**HC** N=39	Compared groups	P value
Sex						
Male, N (%)	56 (65.9)	58 (59.8)	43 (76.8)	22 (56.4)	HC *vs.* ID	0.416
Female, N (%)	29 (34.1)	39 (40.2)	13 (23.2)	17 (43.6)	HC *vs.* ID-ASD	0.865
					HC *vs*. SCZ	0.060
Age in years (mean, SD)	52.1 (10.9)	40.8 (8.4)	44.1 (12.3)	42.3 (13.2)	HC *vs*. ID	**<0.001**
					HC *vs*. ID-ASD	0.452
					HC *vs*. SCZ	0.499
BMI in kg/m^2 (mean, SD)	27.4 (5.3)	25.0 (4.8)	27.9 (4.9)	24.2 (3.5)	HC *vs*. ID	**0.001**
					HC *vs*. ID-ASD	0.328
					HC *vs*. SCZ	**<0.001**
Tobacco consumption in c/d (mean, SD)	0.7 (3.0)	0.1 (1.0)	12.0 (11.6)	4.0 (7.5)	HC *vs*. ID	**0.001**
					HC *vs*. ID-ASD	**<0.001**
					HC *vs*. SCZ	**<0.001**
Chromosomal alterations, N (%)	9 (10.6)	34 (35.1)	-	-	ID *vs*. ID-ASD	**<0.001**
ID Severity						
Severe, N (%)	50 (58.8)	15 (15.8)	-	-	ID *vs*. ID-ASD	**<0.001**
Profound, N (%)	35 (41.2)	80 (84.2)	-	-		
CARS score (mean, SD)	22.0 (4.6)	42.4 (6.0)	-	-	ID *vs*. ID-ASD	**<0.001**

ID: intellectual disability; ASD: autism spectrum disorder; SCZ: schizophrenia; HC: healthy control; N: number of cases; BMI: body mass index; c/d: cigarettes smoked per day; CARS: Childhood Autism Rating Scale.

Significant differences are indicated in boldface (P value <0.05).

6.2. MtDNA-targeted next-generation sequencing and prediction of rearrangements

Total DNA from individuals was obtained from peripheral blood mononuclear cells. The entire mtDNA was amplified in two or three overlapping fragments and sequenced in an Ion Torrent Personal Genome Machine (PGM, Fisher Scientific, Madrid, Spain), according to the manufacturer's user guide (Valiente-Pallejà et al., 2018, 2020). The samples that did not reach our inclusion criteria of a minimum of 85% mean coverage of the mtDNA were discarded and a total of 277 were included in the analyses.

Altogether, we analyzed 277 mtDNA samples with an average read depth of 392 using eKLIPse, a sensitive bioinformatic tool for the detection and quantification of mitochondrial DNA deletions and breakpoints from next generation sequencing data (Goudenège et al., 2019). The method allows for the detection of deletions, even at extremely low heteroplasmy levels, as below as 0.5% of mutant load (Goudenège et al., 2019), which might be expected when using blood samples (Hjelm et al., 2019; Taylor et al., 2014). It enables to detect single deletions as well as multiple deletions within the same sample, and minimizes the false-positive deletion detection because of the minimal requirements of the reciprocal bidirectional BLAST, which is the deletion filtering module, of both perfect upstream match extension and soft-clipping sequences (Goudenège et al., 2019). There are different parameters that can be modified depending on the sequencing quality. The parameters used in eKLIPse to analyze the metadata are shown in Table 2. The down-sampling (-downcov) option was disabled to improve the detection of low frequency and multiple deletions. Furthermore, the minimum coverage percentage (-cov) was increased from 70% to 90% to increase the minimum percent of identity between the analyzed sequences and the reference sequence, to obtain more reliable results. As most of the samples in the SCZ had a lower read length, we adjusted some of the parameters for this group. We reduced minimal soft-clipping length (-scsize) to >15bp, and upstream mapping length (-mapsize) to 10, since the mean length of the reads for this study group was shorter than 150 bp. Increasing this parameter did not result in any change of the results for the samples with longer read lengths. However, SCZ and HC samples were analyzed using the same parameters when comparing between both groups. Deleted mtDNA size (-mitosize) was lowered from 1000 to 100 to be able to detect sublimons.

Table 2. eKLIPse parameters used in the analysis of the samples of each study group.

Parameter	Description of the parameter	ID	ID-ASD	SCZ	HC
-scsize	Soft-clipping minimal length	25	25	**15**	**15**/25
-mapsize	Upstream mapping length	20	20	**10**	**10**/20
-downcov	Downsampling read number (0=disable)	0	0	0	0
-minq	Read quality threshold	20	20	20	20
-minlen	Read length threshold	100	100	100	100
-shift	Breakpoint sliding-window size	5	5	5	5
-minblast	Minimal number of BLASTs per breakpoint	1	1	1	1
-bilateral	Filter unidirectional BLAST	True	True	True	True
-mitosize	Remove deleted mtDNA less than	1000/**100**	1000/**100**	1000/**100**	1000/**100**
-id	BLAST % identity threshold	80	80	80	80
-cov	BLAST % coverage threshold	70/**90**	70/**90**	70/**90**	70/**90**
-gapopen	BLAST cost to open a gap	0	0	0	0
-gaptext	BLAST cost to extend a gap	2	2	2	2

Default values are shown, and modified parameters are indicated in boldface.

At the end of the process, we obtained for each sample: the predicted deletions and breakpoints positions, deletion load, results of the BLAST analysis and the enclosing repeats, and the total frequency of each deletion per gene, as well as a circos plot, which a graphical way of reporting the results of the analysis.

Deletions are reported according to the revised Cambridge Reference Sequence (rCRS) of the human mitochondrial genome (GenBank sequence NC_012920). Breakpoints were corrected using the MitoBreak 'Classifier tool' (Damas et al., 2014b), which fixes the location of the breakpoints when identified in direct repeats. It assigns the breakpoint to the 3' nucleotide of the direct repeat for standardization purposes as done before (Hjelm et al., 2019). We also examined whether the deletions we found had previously been reported in MitoBreak (Damas et al., 2014b) and/or Splice-Break databases (Hjelm et al., 2019).

Datasets of mtDNA sequences and clinical data from participants are available from the European Genome-phenome Archive (EGA, https://ega-archive.org) under the reference numbers EGAS00001002750 and EGAS00001003269.

6.3. Statistical analyses

The normality of continuous variables was carried out by the Kolmogorov–Smirnov test. Chi square tests were performed to compare the frequency of individuals with deletions between study groups. We also used this test to contrast the number of subjects with nuclear chromosomal alterations and the ID severity subtypes between ID-ASD and ID groups. Student's *t*-tests were used to compare ID severity, age, body mass index (BMI) and the number of cigarettes smoked per day between each disease group and the HC group. The non-parametric Mann-Whitney *U* test was used to compare ID severity and CARS score between individuals with and without the O_H deleted. Data were processed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY).

7. Results

Among the 277 sequenced samples we identified a total of 30 unique deletions (Table 3, Figure 2, Figure 3 and Figure 4), since none of them was present in more than one subject. In our sample, the presence of a deletions was neither associated with the age of the individual (t=-1.774, p=0.087), gender (χ^2=0.0007, p=0.932), BMI (t=1.002, p=0.317) and tobacco consumption (t=0.768, p=0.443).

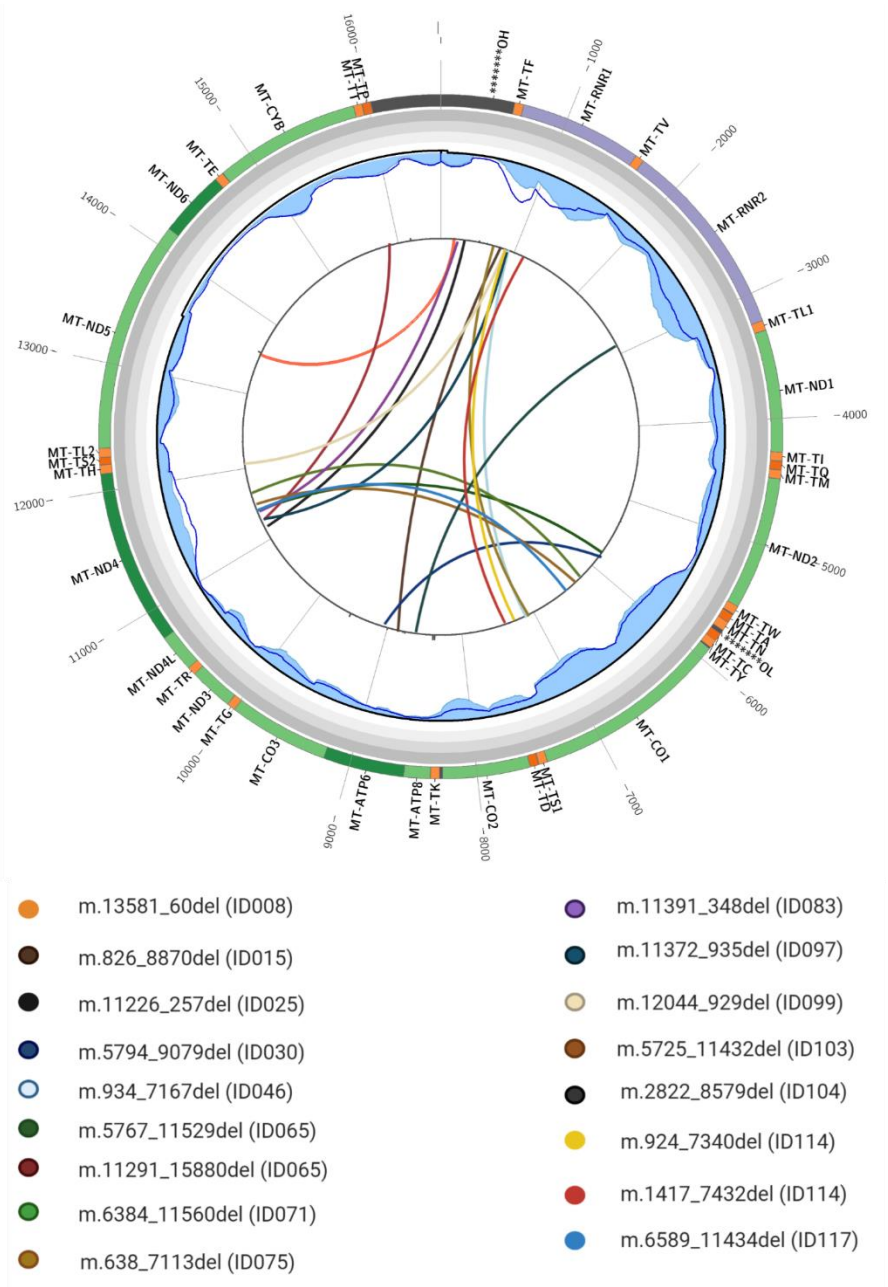

Figure 2. MtDNA deletions detected in the ID study group using the eKLIPse bioinformatics tool.

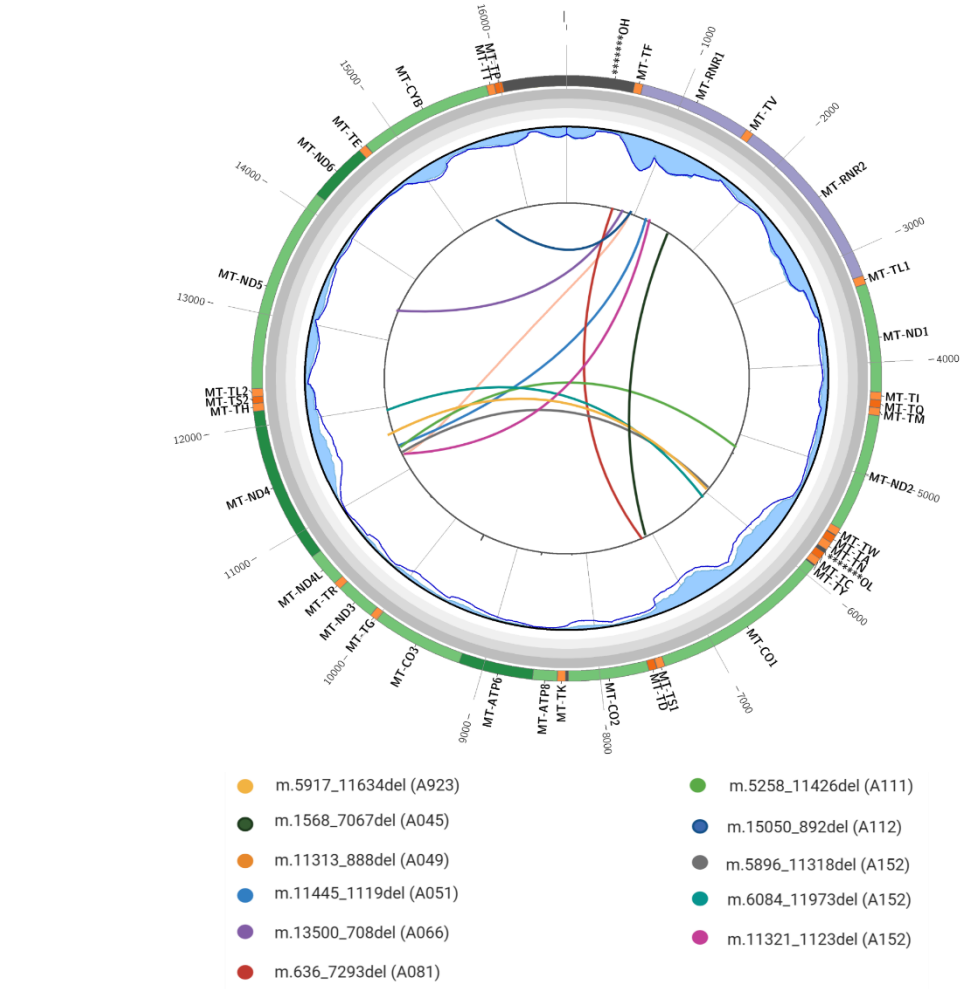

Figure 3: MtDNA deletions detected in the ID-ASD study groups using eKLIPSe.

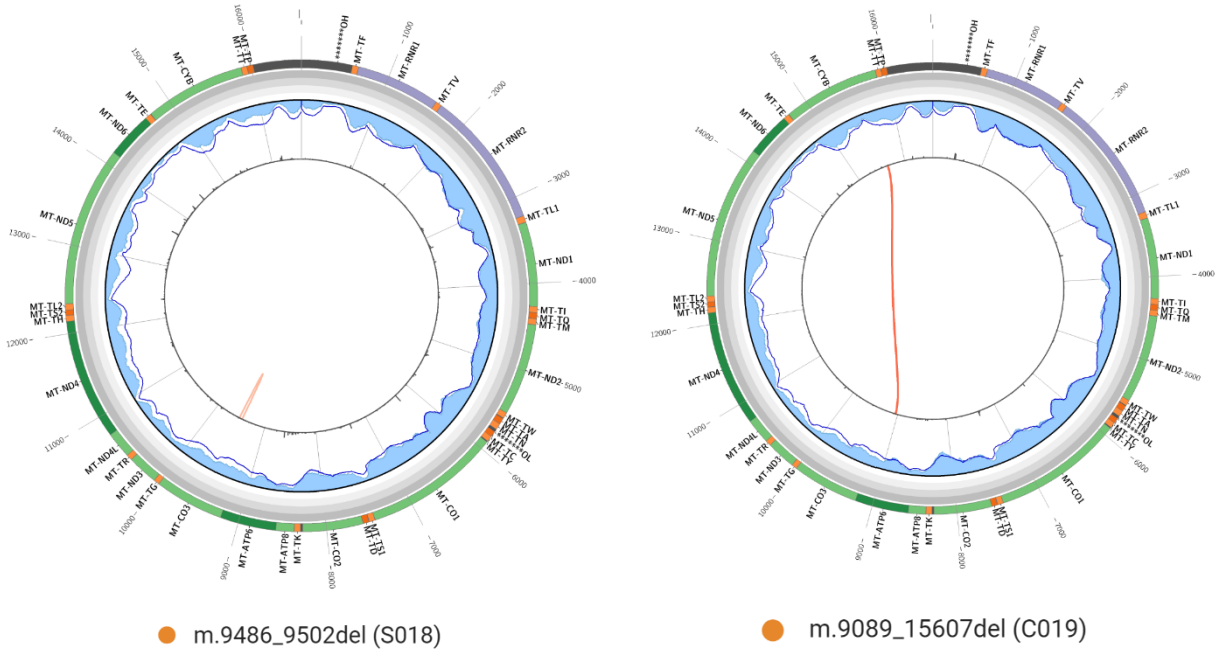

Figure 4: MtDNA deletions found in the SCZ (left) and the HC (right) individuals using eKLIPse.

Fifteen individuals in the ID group and nine in the ID-ASD group carried at least one deletion. As shown in Table 3 and Figure 4, there was only one subject in both the SCZ and the HC group harboring deletions (a single 15 bp microdeletion and a 6517 bp deletion, respectively). Therefore, the percentage of individuals presenting mtDNA deletions were as follows: 17.7% in the ID group, 9.3% in the ID-ASD group, 1.8% in the SCZ group and 2.6% in the HC group. We report significant differences between the ID and HC group (χ^2=4.153; p = 0.042). Notably, there were two subjects with ID harboring two deletions and one ID-ASD patient who carried three deletions (Table 3). The heteroplasmy levels of the deletions varied between 0.03% and 4.54% across all samples. Furthermore, 2 of the deletions identified had been previously reported.

The mean deletion size in the ID group was 5532 ± 1170bp, and almost 71% of the deletions were flanked by repeats. Similarly, in the ID-ASD group, the average size of the deletions was 5481 ± 1274 bp and 55% of them were found in repeats. Furthermore, we identified a 15 bp microdeletion placed in a 7 bp direct repeat in a patient with SCZ. The deletion identified in the HC individual was 6517 bp long and not located in repeats.

The gene most deleted in the ID group was *MT-COX1*, which was partially or totally removed in 70% of the mtDNA molecules carrying deletions. *MT-TC*, *MT-TY* and *MT-ND4* genes were also deleted in more than 53% of mtDNA deleted species within the ID individuals with deletions. By contrast, in the ID-ASD group, the *MT-TF* and *MT-RNR1* were the most frequently deleted, being the latter affected in 78% of the mtDNA molecules harboring deletions from subjects in the ID-ASD group. *MT-COX1*, *MT-ND4*, *MT-CYB*, *MT-TT*, *MT-TP* genes and the D-Loop where totally or partially removed in more than 55% of the mtDNA deleted molecules from individuals in the ID-ASD group. Even though there are differences between the genes that tend to be affected in each group, we did not find statistically significant differences.

Table 3. Characteristics of the mtDNA deletions identified in the study groups by using the eKLIPse bioinformatics tool.

Study Group	Subject	5' bkp	3' bkp	Corrected bkps	Frequency (%)	Repeats	Deletion size (bp)
ID	ID008	13581	60	SAME	4.94	none	3047
	ID015	817	8861	826-8870	0.78	818-CAGTGATTA-826 <> 8861-CAGTGATTA-8869	8043
	ID025	11226[2]	257	SAME	1.11	none	5599
	ID030	5793[2]	9078	5794-9079	0.35	5794-T-5794 <> 9078-T-9078	3284
	ID046	934	7167	SAME	0.77	none	6232
	ID065	5767	11529	SAME	0.75	none	5761
	ID065	11291	15880	SAME	0.74	11290-C-11290 <> 15880-C-15880	4588
	ID071	6383	11559	6384-11560	0.10	6384-G-6384 <> 11559-G-11559	5175
	ID075	637	7112	638-7113	0.94	638-C-638 <> 7112-C-7112	6474
	ID083	11390	347	11391-348	0.36	11391-G-11391 <> 347-G-347	5525
	ID097	11371	934	11372-935	0.32	11372-G-11372 <> 934-G-934	6131
	ID099	12044	929	SAME	0.13	none	5453
	ID103	5725	11432[1]	SAME	0.12	5723-AA-5724 <> 11431-AA-11432	5706
	ID104	2821[2]	8578	2822-8579	0.77	2822-C-2822 <> 8578-C-8578	5756
	ID114	922	7338	924[2]-7340	0.35	923-AA-924 <> 7338-AA-7339	6415
	ID114	1417[1]	7432	SAME	0.05	1416-A-1416 <> 7432-A-7432	6014
	ID117	6583	11428	6589-11434[1]	0.08	6584-CCCCAT-6589 <> 11428-CCCCAT-11433	4844
ID-ASD	A023	5917[1]	11634	SAME	0.12	none	5716
	A045	1565	7067	1568-7070	0.75	1566-CAT-1568<>7067-CAT-7069	5501
	A049	11313	888	SAME	0.88	none	6143
	A051	11445	1119	SAME	0.32	none	6242
	A066	13500	708	SAME	0.48	none	3776
	A081	634	7291	636-7293	0.23	635-CA-636 <> 7291-CA-7292	6656
	A111	5256	11424	5258-11426[1]	0.46	5257-AA-5258 <> 11424-AA-11425	6167
	A112	15047	889	15050-892	0.03	15048-GCC-15050 <> 889-GCC-891	2410
	A152	5896[2]	11318	SAME	0.16	none	5421
	A152	6074[1]	11963	6084[1,2]-11973[2] [4]	0.46	6075-GTCACAGCCC-6084 <> 11963-GTCACAGCCC-11972	58884
	A152	11316	1118	11321-1123	0.38	11317-ATCAA-11321 <> 1118-ATCAA-1122	6370
SCZ	S018	9479	9495	9486[1]-9502[1] [3]	0.68	9480-TTTTTCT-9486 <> 9495-TTTTTCT-9501	153
HC	C019	9089	15607[1,2]	SAME	1.71	none	6517

ID: intellectual disability; ASD: autism spectrum disorder; SCZ: schizophrenia; HC: healthy control; bkp: breakpoint; bp: base pair.

1: breakpoint reported in MitoBreak; 2: breakpoint reported in Splice-Break; 3: deletion reported in MitoBreak; 4: deletion reported in Splice-Break.

Regarding the origins of replication, almost half of the subjects with deletions in the ID group did not have any of the replication origins deleted, and the O_L and the O_H were removed in 40% and 13% of the mtDNA deleted molecules of subjects with ID, respectively. As expected, no individuals showed deletions for both origins of replication. The ID-ASD group showed a lower percentage (11%) of individuals with none of the origins of replication deleted. Up to 56% of the mtDNA deleted molecules in the ID-ASD group lacked the O_H and 33% had the O_L removed. As expected, no individual lacked from replication origins in the detected mtDNA deleted species. None of the origins of replication was affected neither in the SCZ subject nor in the HC individual. Finally, as ID severity and CARS score were available from both the ID and the ID-ASD groups, we pooled together the 24 individuals with ID carrying mitochondrial deletions (15 ID and 9 ID-ASD). Interestingly, ID severity and CARS scores were significantly higher in the 7 subjects with the O_H deleted than in the 17 subjects who maintained intact this mtDNA region ($U=24.5$, $p=0.009$; and $U=20.5$, $p=0.013$, respectively) independent of the group of study that they belonged to.

8. Discussion

Most studies in the field of neurodevelopmental conditions have been focused on the analysis of the nuclear DNA, leaving aside the mitochondrial genome. Nonetheless, mitochondrial dysfunction has been described previously in several psychiatric disorders, including ASD, ID and SCZ (Hjelm et al., 2015; Valiente-Pallejà et al., 2018; Varga et al., 2018; Verge et al., 2011), which indicates the need to assess the presence of mtDNA alterations in subjects presenting these clinical conditions.

Recently, many informatics tools have been developed enabling the analysis of major mtDNA rearrangements, like deletions (Bosworth et al., 2017; Goudenège et al., 2019; Hjelm et al., 2019; Taylor et al., 2014). Here we used the eKLIPse tool, which is capable of detecting and quantifying rearrangements with a very low heteroplasmy level and with high fidelity (Goudenège et al., 2019). Since the performances of the bioinformatics tools are conditioned by the sequencing quality and coverage depth, the filtering process allowed us to make sure that the limit of detection was the lowest it can be, and therefore, we would be able to detect rearrangements even at very low frequency. We also reduced the deleted mtDNA length threshold (-mitosize) from 1000 to 100, so we may detect sublimons, which are rearranged mtDNA fragments present at very low levels (Kajander et al., 2000).

The present study showed that mtDNA deletions are frequent in blood samples of individuals presenting ID. We discovered that 17.7% of individuals with ID carried at least one mtDNA deletion in blood, whereas only one HC individual out of 39 had part of the mtDNA removed. Such large deletions were not present in the SCZ group and only one individual harbors a mtDNA microdeletion of 15 bp. Interestingly, this deletion was located in a highly conserved region of the *MT-COX3* gene and was previously reported (Keightley et al., 1996), and present in the MitoBreak database. Regarding the presence of deletions, the difference between ID and HC was statistically significant, but we did not find significant differences when the ID-ASD group and HC groups were compared. However, the percentage of ID-ASD individuals with mtDNA deletions was nearly four times that in the HC group. This result is in accordance with a previous study, that found a higher number of ASD individuals that harbored deletions compared to the HC group (Varga et al., 2018).

The fact that a healthy subject presented a deletion is not unusual, since there are studies that have reported the presence of deletions in HCs previously (Behar et al., 2008). MtDNA deletions are usually sporadic and might be found in individuals who do not have mitochondrial dysfunction or any mitochondrial disorders, since it does not only depend on the presence of

the rearrangement but also the heteroplasmy level and its presence in other tissues (Behar et al., 2008; Damas et al., 2014a; El-Hattab & Scaglia, 2016; Verge et al., 2011).

Single deletions are more common, since they are usually sporadic; while multiple deletions are usually inherited, frequently caused by defects in nuclear genes, such as *POLG* and *SLC25A4*, implicated in the maintenance of the mtDNA (Damas et al., 2014a). In our sample, most individuals carried single deletions; however, two subjects with ID harbored two deletions each and one subject in the ID-ASD group carried three different species of deleted mtDNA, which is in accordance with a previous study where multiple deletions were much less common than single deletions (Rygiel et al., 2016). Remarkably, 2 of the deletions identified had been previously reported in Mitobreak (Damas et al., 2014b) and Splice-Break (Hjelm et al., 2019), being one of them present in the patient with ID-ASD who carried three distinct mtDNA deletions.

As mentioned, the ID and ID-ASD groups harbored large deletions, and did show strong variation, which is consistent with the size of the deletions reported in previous studies (Hjelm et al., 2019; Varga et al., 2018). Furthermore, 71% of the deletions in the ID group were located in repeats, while 55% of the deletions found in the ID-ASD group were also flanked by repeats, which is compatible with previous studies (Damas et al. , 2014a; Hjelm et al., 2019; Sadikovic et al., 2010; Taylor et al., 2014). Consequently, this agrees with the fact that breakpoints tend to locate within or contiguous to sequences that are homologous, that are normally perfect or imperfect repeats (Damas et al., 2014a). Numerous breakpoints found in deletions have been reported before, either in MitoBreak as well as in Splice-Break, as shown in Table 3 (Damas et al., 2014b; Hjelm et al., 2019).

Noteworthy, despite the frequencies of the deletions are low (between 0.03 and 4.54%), we should consider that blood samples were used in this study, a tissue that usually displays very low heteroplasmy levels of deletions, usually not higher than 1% (Hjelm et al., 2019; Taylor et al., 2014; Wang et al., 2013). This may be attributed to the fact that post-mitotic tissues, such as brain or muscle, tend to accumulate deletions, while self-renewing tissues, like blood, tend to lose them (Hjelm et al., 2019; Volmering et al., 2016). Usually, some mutations undergo negative selection in blood, like m.3243 A>G mtDNA mutation in MELAS syndrome, which might be explained following the Darwinian principle of natural selection (Rahman et al.,2001). Therefore, the low mutant load of deleted mtDNA molecules in blood is very common and could be justified by the same principle.

MT-COX1, which encodes a member of OXPHOS, was found to be removed partially or totally in most of the mtDNA molecules with deletions in ID individuals. Mitochondrial genes *MT-TC*, *MT-TY* and *MT-ND4* were also deleted in up to 53% the mtDNA deleted species. The latter is usually removed as shown by different studies (Damas et al., 2014a). *MT-COX1* encodes for a member of OXPHOS and it has been shown that mutations in genes encoding for OXPHOS could lead to OXPHOS deficiency, leading to defects in the respiration chain (Pei & Wallace, 2018; Rygiel et al., 2016). In contrast, in the ID-ASD group the genes that were deleted the most, in 78% of the individuals with mtDNA deletions, were *MT-TF* and *MT-RNR1*, discordant with the fact that they are normally preserved (Damas et al., 2014a). Mitochondrial genes *MT-COX1* and *MT-ND4* among others were also affected in more than half of the individuals who harbored deletions in the ID-ASD group, consistent with the fact that MT-ND4 is commonly deleted (Damas et al., 2014a).

Generally, the lack of origins of replication is unusual since mitochondrial DNA molecules do not replicate efficiently without them within the cell (Damas et al., 2012), but a review of previous studies have shown that around 9.6% of the deletions remove a replication origin (Damas et al., 2014a). It has also been suggested that missing the O_H is probably more detrimental than lacking the O_L (Damas et al., 2014a). However, a recent study found that up to 24.4% of the most common deletions they detected lacked the O_L (Hjelm et al., 2019). In the present study, we found that 40% of the individuals in the ID group with mtDNA deleted species lacked the O_L and 13% had the O_H removed. Conversely, in the ID-ASD group, we unveiled that more than the 55% of the individuals that harbored deletions had the O_H missing, while 33.3% had the O_L removed. No individuals lacked both origins of replication, as expected. The O_H was deleted more frequently in the ID-ASD group than in the ID group. To explain this observation, we demonstrated that patients without the O_H had significantly more severe ID, regardless of the diagnostic group. We also demonstrated that individuals with the O_H removed had significantly higher CARS scores, independent of the diagnostic groups, which is in accordance with the idea that missing the O_H is more critical than lacking the O_L (Damas et al., 2014a). As most of the deleted mtDNA species that we have identified lack one replication origin, this may also account for the low heteroplasmy level of the deletions. Considering that mtDNA molecules that are missing an origin of replication have a limited replication and proliferation (Damas et al., 2014a), this could be a justification of why they have not expanded more widely. Furthermore, 100% of the deletions removed part of the major arc, which is consistent with previous studies where approximately 90% of the deletions lacked part of the major arc (Damas et al., 2012; Damas et al., 2014a; Krishnan et al., 2008).

Our study found a higher tendency of harboring mitochondrial deletions in individuals with ID and patients with ID who also fulfilled the criteria for the diagnosis of ASD (ID-ASD) when compared to heathy individuals.

Despite we did not find differences between individuals with SCZ and the HC group, we still found one subject in the SCZ group who harbored a microdeletion of 15 bp in the mitochondrial DNA. This deletion had been previously reported (Keightley et al., 1996) and is present in the MitoBreak dataset (Damas et al., 2014b). This deletion locates in a highly conserved region of the *MT-COX3* gene, which encodes the cytochrome C oxidase subunit III and it was first reported in a patient with severe isolated COX deficiency and recurrent myoglobinuria. The deletion was also detected in skeletal muscle and leukocytes, but heteroplasmy levels differed considerably between tissues. The mutant load in the muscle reached levels of 92% while blood heteroplasmy level was 0.7%, equal to that in the patient with SCZ in this study (Keightley et al., 1996). This suggests that even though the frequency level of the mutation in blood is very low, the presence and heteroplasmy levels of the same deletion may vary within tissues. This study showed that the same deletion was present in the two different tissues and that it accumulated in a much higher rate in post-mitotic tissues, such as muscle, than self-renewing tissues, like blood. Therefore, since we used blood samples to analyze the presence of mtDNA deletions, we could not rule out the presence of the deletion in other post-mitotic tissues within the same individual with SCZ (Keightley et al., 1996; Taylor et al., 2014). Furthermore, mtDNA pathogenic mutations often have clinical consequences when they surpass a certain heteroplasmy level (threshold effect), although clinical phenotypes have also been associated with mtDNA mutations (pathogenic variants or deletions) at low frequencies (Leung et al., 2018).

9. Limitations

The main limitation of this study was that we used blood samples and sequenced the mtDNA of leukocytes. As mentioned, blood is a self-renewing tissue and, in consequence, tends to lose the mtDNA deleted molecules. However, obtaining blood is an easy procedure, less invasive, more ethical and much less disturbing for the individuals. In addition, the presence of deletions in blood, even at low heteroplasmy levels, may be indicative of the presence of deletions in post-mitotic tissues, such as brain or muscle.

Moreover, although the size of our sample was small, we were able to analyze samples of unrelated individuals with different neurodevelopmental disorders and healthy controls. Furthermore, the patients were from the same geographic and genetic origin as the individuals from the HC group.

10. Conclusions

Our study showed that the presence of large mtDNA deletions in blood samples was more common in the ID-ASD and ID group than in control subjects.

No differences were observed between the HC and the SCZ group.

The heteroplasmy levels of the deletions were low, when using blood samples. A strong positive correlation between the deletion of O_H and ID severity and CARS score, in both ID and ASD groups was identified.

The presence of large mtDNA deletions in blood samples from individuals with ID urges to investigate other post-mitotic tissues, like brain, to determine whether deletions are present with higher heteroplasmy levels.

11. Self-assessment

In the beginning, when I started this internship in the research group "Genetics and Environment in Psychiatry, GAP" I did not know it would be such a satisfying and amazing experience.

At first, during the first couple of weeks I learned how to use the eKLIPse bioinformatics tool. I was able to learn from David Goudènege (the developer of eKLIPse) how it worked, what the parameters meant and he even helped me with the interpretation of results that were obtained with eKLIPse and offered me all the help I needed with the pipeline when I needed to fix errors or had doubts about it.

Furthermore, I was able to work with metadata obtained from next-generation sequencing techniques, which was my very first time working with it. I learnt to interpret the data that was held in a BAM file. I also used different informatics tools, such as Integrative Genomics Viewer (IGV) and Galaxy, which is an open source, web-based platform for data intensive biomedical research; in order to analyze the quality of the metadata.

Once I was able to run all the samples with the correct parameters and had obtained the results, I started reading the bibliography and started learning about mitochondrial DNA; its functions and involvement in many different diseases and its importance in the energetic metabolism. I also realized that mitochondrial DNA is much more important than what I thought and its fundamentat role in neuropsychiatric disorders.

Overall, I have learnt to work independently, to be resolutive on my own and to think out of the box when it was needed. I have learnt to do research work without even stepping into a laboratory and that bioinformatics may as well be the future of the biomedical research field.

Finally, I think this experience has made me grown as a person but also as a researcher and has helped me to elect what I would like to do in the future.

12. References

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Jo, Leire Torres Egurrola , amb DNI 79125812-Q, sóc coneixedor de la guia de prevenció del plagi a la URV Prevenció, detecció i tractament del plagi en la docència: guia per a estudiants (aprovada el juliol 2017) (http://www.urv.cat/ca/vidacampus/serveis/crai/que-us-oferim/formacio-competencies-nuclears/plagi/) i afirmo que aquet TFG no constitueixen cap de les conductes considerades com a plagi per la URV.

Tarragona, 07 de junio de 2020

Leire Torres

(signatura)