Mitochondrial DNA and Alzheimer's Disease: A First Case-control Study of the Tunisian Population

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Abstract

Background: Alzheimer's disease (AD) is the most common neurodegenerative disorder in humans and presents a major health problem throughout the world. The etiology of AD is complex, and many factors are implicated, including mitochondria. Mitochondrial alteration has been proposed as a possible cause of AD. Therefore, several studies have focused on finding an association between inherited mitochondrial DNA variants and AD onset.

Methods: In this study, we looked, for the first time, for a potential association between mitochondrial haplogroups or polymorphisms and AD in the Tunisian population. We also evaluated the distribution of the major genetic risk factor for AD, the apolipoprotein E epsilon 4 (*APOE* ϵ 4), in this population. In total, 159 single-nucleotide polymorphisms (SNPs) of mitochondrial DNA haplogroups were genotyped in 254 individuals (58 patients and 196 controls). An additional genotyping of *APOE* ϵ 4 was performed.

Results: No significant association between mitochondrial haplogroups and AD was found. However, two individual SNPs, A5656G ($p = 0.03821$, OR = 10.46) and A13759G ($p = 0.03719$, OR = 10.78), showed a significant association with AD. *APOE* 4 was confirmed as a risk factor for AD ($p = 0.000014$).

Conclusion: Our findings may confirm the absence of a relation between mitochondrial haplogroups and AD and support the possible involvement of some inherited variants in the pathogenicity of AD.

Introduction

Alzheimer's disease (AD; MIM 104300) is the major cause of dementia and the most common neurodegenerative disorder (Bekris et al. 2010; Bi et al. 2015). AD is manifested by a progressive memory loss and a decline in cognitive function (van der Walt et al. 2004; Bi et al. 2015). According to the age of onset, two forms of AD can be distinguished: early-onset AD (EOAD) and late-onset AD (LOAD). About 1–6% of AD cases are of the early-onset type, with 60% of them involving familial aggregation (Campion et al. 1999; Brickell et al. 2006).

Most AD cases have the LOAD phenotype, also called sporadic AD. Sporadic AD is considered a multifactorial disease with a non-Mendelian (complex) transmission. To date, AD pathogenesis is not well understood; only some risk factors are known to be associated with this disease. AD is caused by both genetic and non-genetic factors. The genetic cause is still not known, especially because patients with the LOAD phenotype show no mutations in presenile genes and amyloid precursor protein (APP) (Fesahat et al. 2007; Rezazadeh et al. 2019). The apolipoprotein E (*APOE*) ϵ 4 allele is regarded as the strongest risk factor (Mahley, Weisgraber, and Huang 2006; Santoro et al. 2010a; Fachal et al. 2015; Neu et al. 2017). Besides mutations in the *APOE* ϵ 4 allele, genetic variations in more than 20 susceptibility genes have been identified by genome-wide association studies and next-generation sequencing. These genes are involved in many biological functions, such as immune response, synapse function, and lipid metabolism. Each of these genes has a small effect on the risk of developing AD (Coppede and Migliore 2015; Seshadri et al. 2010; Antúnez et al. 2011; de Rojas et al. 2021; Jansen et al. 2019; Bis et al. 2020).

The lack of a clear and established etiology of AD has prompted the emergence of the “mitochondrial hypothesis” for explaining AD. Mitochondrial abnormalities have been found in the brain and other tissues of AD patients, reinforcing the potential association between mitochondrial dysfunction and neurodegeneration (Swerdlow and Khan 2004; Swerdlow, Burns, and Khan 2010; Swerdlow 2007; Perez Ortiz and Swerdlow 2019). In addition, a relationship between a maternal history of AD and an increased risk of AD onset has been found, supporting the mitochondrial theory of AD (Honea et al. 2012). In that regard, over 20% of inherited LOAD cases have a maternal history, and the maternal influence seems to be more powerful than the paternal one (Mosconi et al. 2010; Ridge et al. 2012).

Mitochondrial DNA (mtDNA) is highly polymorphic and has more mutations than nuclear DNA. These mutations are dispersed across the whole mtDNA. The mutation rate in mtDNA is 10 times higher than that in nuclear DNA (Maruszak et al. 2006; Takasaki 2009). In addition to uniparental transmission, mammalian mtDNA does not undergo recombination, so the evolution of mtDNA shows stable variations that are inherited from the same ancestor and that are grouped into specific lineages called mitochondrial haplogroups (Torroni et al. 1996; Mancuso, Filosto, et al. 2008). Every haplogroup is composed of a combination of characteristic single-nucleotide polymorphisms (SNPs) or signature mtDNA mutations, representing the major branch points in the human phylogenetic tree (van Oven and Kayser 2009). That is, every haplogroup has accumulated mutations over time, leading to the divergence of maternal lineages. Consequently, haplogroups tend to be specific for populations and continents (Torroni et al. 2006). In these haplogroups, some additional mutations may have arisen, creating young branches in the human mtDNA tree. As a result, haplogroups can be divided into smaller groups called sub-haplogroups (Santoro et al. 2010b). The classification of sub-haplogroups is still in progress because of the increase in complete mtDNA sequences of many populations from different ethnic groups (Mancuso et al. 2009). According to the latest classification, individuals with haplogroup L are sub-Saharan Africans, with seven major families (L0, L1, L2, L3, L4, L5, L6) (Mancuso et al. 2009). East Asian populations have haplogroups A, B, C, D, and E, while haplogroups H, I, J, K, T, U, V, W, and X are specific to Eurasian populations (Torroni et al. 2006). Thanks to a huge number of studies on mitochondrial genome diversity, specific mitochondrial haplogroups have been identified for many populations (Ienco et al. 2011; Wang and Brinton 2016). A hypothesis was proposed that some mitochondrial haplogroups are not neutral and are involved in many mechanisms. Some haplogroups may have been selected during evolution as an adaptation to particular environmental conditions (Bilal et al. 2008). Accordingly, several studies have explored the association of mitochondrial haplogroups with parameters such as longevity (Bilal et al. 2008), age-related diseases (Coto et al. 2011), and multifactorial diseases such as AD (van der Walt et al. 2005). For example, in the Italian population, sub-haplogroup H5 was described as a risk factor for AD (Santoro et al. 2010b), while in the Japanese population, the haplogroups G2a, B4c1, and N9b1 seem to be associated with an increased risk of AD (Takasaki 2009). However, no definitive results can confirm or prove the involvement of mtDNA in AD onset. The results cannot be generalised to other world populations because of the differences in mitochondrial haplogroups from different regions. In this context, it would be interesting to study the association between haplogroups or polymorphisms of mtDNA and AD in populations founded by mixtures, such as the Tunisian population.

Tunisia is located in northwest Africa. It is bordered by Algeria to the west and southwest, by Libya to the southeast, and by the Mediterranean Sea to the north and east. The settlement history of Tunisia goes back to prehistoric times. The country has successively witnessed the flowering of the Aterian civilisation (20,000 YBP), the Iberomaurusian civilisation (12,000 YBP), and the Capsian civilisation (9,000 YBP). The Berbers, the original people of Tunisia, are a heterogeneous group of humans who have spread uniformly across North Africa for about 4000 years. This Berber population has been modified by the arrival of the Phoenicians, Roman Byzantines, Arabs and Bedouins, Andalusians, Ottomans, and French. Genetic studies conducted on the Tunisian population targeting mtDNA have shown a significant diversity of the mitochondrial haplogroup landscape in relation to the country's rich history of complex and eventful settlements (Fadhlaoui-Zid et al. 2004; Cherni et al. 2005; Cherni et al. 2009; Ennafaa et al. 2009; Ennafaa et al. 2011; Loueslati et al. 2006). The Tunisian populations host a Eurasian component represented by haplogroups originating from Europe and the Middle East (H, V, U5, J, T, R0a), with a frequency of about 70%; a sub-Saharan component represented by L haplogroups and their subtypes, with a global frequency of 25%; and a North African component represented by haplogroup U6 and its subtypes, with an overall frequency of around 5%. In Tunisia, these three components vary in their frequency from one group to another, reflecting the mosaic character of the Tunisian population (Frigi et al. 2017). This mix of West European, Middle Eastern, and sub-Saharan mtDNA lineages is highly informative for our study, especially the presence of the sub-Saharan haplogroups, which is an advantage over previously studied populations in relation to AD. This rich population encouraged us to investigate the involvement of the Tunisian mtDNA genome in AD. First, we evaluated mitochondrial haplogroups, and then we chose to simplify the research by focusing on some single mitochondrial mutations instead of a set of SNPs. We adopted this approach because polymorphisms in mtDNA can affect encoded proteins, inducing changes in mitochondrial respiratory activity and increasing the number of free radicals, resulting in early apoptosis (Mancuso et al. 2009).

Materials And Methods

Samples

DNA samples from 254 subjects (58 AD patients and 196 healthy controls) were recruited from the Department of Neurology, University Hospital of Fattouma Bourguiba, Monastir, Tunisia; the Department of Neurology, University Hospital of Mahdia, Tunisia; and the Alzheimer Family Assistance Center in Tunisia (AFA center). The control group was comparable in age, gender, and ethnicity to the patient group.

Written informed consent was obtained from all participants or their representatives. All participants were unrelated over three generations. For each participant, age, demographic information, and family and medical histories were collected. In addition, for AD patients, a detailed clinical assessment was carried out, including a Mini Mental State Examination (MMSE), a standard brain MRI, neuropsychological tests, and laboratory studies. All AD patients were included after at least 2 years of follow-up.

Diagnoses of AD were made according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA) for possible or probable AD (McKhann et al. 1984).

Controls were selected after passing an MMSE test and according to the following criteria: no family member with AD or any type of dementia or psychiatric disorder, a mentally healthy status, and no history of alcohol or drug use.

mtDNA genotyping

Genomic DNA was extracted from fresh blood (2 ml) by the standard phenol-chloroform protocol. Then, genotyping was performed on the Axiom 815K Spanish Biobank Array (Thermo Fisher). This array has polymorphic sites (SNPs) covering the whole genome, including variations with common and low frequencies and 159 mtDNA sequence polymorphic sites. A data set of mitochondrial SNPs, dispersed across the coding region and representing the most common branch of the mtDNA phylogeny, was obtained. Genotyping was performed in the Spanish National Center for Genotyping (CeGEN, Santiago de Compostela, Spain). The genotyping was conducted according to the manufacturer's instructions (Axiom™ 2.0 Assay Manual Workflow). We performed a quality control (QC) for the plates and then for the samples using the Affymetrix Power Tool (APT) 1.15.0 software and according to Axiom's data analysis instructions. Only samples with a call rate above 97% were considered. The data passed through many QC checks, including checks for discordant sex, duplicated samples, and call rate (samples with a call rate <0.95 were eliminated), and underwent an identity by descent (IBD) analysis. Duplicated and genetically related individuals (PIHAT >0.1875) were excluded. Individuals above six standard deviations from the European population mean were regarded as outliers of the population and removed from our cohort. Then, the data of 159 mitochondrial SNPs were extracted from the whole array (**Supporting Information, table s1**). Haplotyping and the construction of a phylogenetic tree were done using HaploGrep 2 following the updated nomenclature proposed in PhyloTree Build 17 (van Oven and Kayser 2009).

mtDNA HVS-I sequencing

The amplification of HVS-I (16024–16383) was done for 64 samples (25% of our cohort). The HVS-I region of mtDNA was amplified by using specific primers (Vigilant et al. 1989):

-L15996 (5'-CTCCACCATTAGCACCCAAAGC-3')

-H16401 (5'-TGATTTCACGGAGGAGGATGGTG-3')

The amplified product was purified with the QIAGEN kit. The sequencing reaction was performed using the BigDye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (Applied Biosystems). The sequencing products were run in the automatic sequencer ABI377 (Applied Biosystems). The obtained HVS-I sequences were checked manually and then aligned to the revised Cambridge Reference Sequence

(rCRS; (Anderson et al. 1981; Andrews et al. 1999) using BioEdit version 7.2.5. As with the previous results obtained by the array, the haplotyping of the sequences was done using HaploGrep2.

Statistical analyses

Descriptive statistics—means, frequencies, and standard deviations (SDs)—were calculated. For continuous measures, the comparison of the mean between patients and controls was performed using Student's t-test. Odds ratios (ORs) were calculated with 95% confidence intervals.

Mitochondrial sub-haplogroups, APOE genotypes, and gender and genotype frequencies were compared between AD patients and controls using Pearson's chi-square test or Fisher's exact tests with SPSS. A p-value of 0.05 was considered significant with 95% confidence intervals (CIs). The obtained results from HaploGrep2 showed a large variability in mitochondrial sub-haplogroups (52 subgroups). mtDNA sub-haplogroups were grouped into 41 sub-haplogroups according to their phylogenetic relationships. The association between mitochondrial SNPs and AD was tested using Fisher's exact test provided by PLINK 1.9.

Since we observed very low frequencies of some mitochondrial sub-haplogroups, which might disturb the analysis of our results, we decided to reduce the number of haplogroups by eliminating all sub-haplogroups with a frequency of less than 0.5%.

We grouped every sub-haplogroup into the haplogroup it belongs to. Thus, the new classification contains a group called H*, which includes H4, H5, H9, H11, and H30. Another group called J* includes J1, J1b, and J1c. The group U* includes U1, U4, U5, and U7, and N* includes N1 and N9a. After this classification, some heterogenous haplogroups with low frequencies remained, which we classified into "others". This group contains haplogroups X2, X3, M73a, I2a, W, and G. This step reduced the number of sub-haplogroups from 41 to 27 haplogroups or sub-haplogroups. Then, each of the sub-haplogroups was evaluated separately to look for a significant association. The comparisons between each mitochondrial sub-haplogroup were conducted using Bonferroni's adjustments.

APOE genotyping

The *APOE* status was also examined in our study. ApoE genotyping was performed using data of two SNPs (rs7412 and rs429358) from the array. SNP genotypes for both polymorphisms were combined for each sample. The classification of the participants' genotypes was based on the presence of APOE alleles. *APOE* genotypes were determined according to Table 1 (Koleck et al. 2014). Patients and controls were classed into two groups, taking the presence of the *APOE4* allele as a criterion. The first group (*APOE4+*) had at least one copy of APOE4. The second group (*APOE4-*) did not carry any copy of the *APOE4* allele.

Table 1
Apolipoprotein E (APOE) status depending on the genotyping of two SNPs : rs7412 and rs429358 .

ApoE genotype	rs7412	rs429358
$\epsilon 2/\epsilon 2$	TT	TT
$\epsilon 3/\epsilon 3$	CC	TT
$\epsilon 2/\epsilon 3$	CT	TT
$\epsilon 2/\epsilon 4$	CT	CT
$\epsilon 3/\epsilon 4$	CC	CT
$\epsilon 4/\epsilon 4$	CC	CC
This table is extracted from (Koleck et al. 2014).		

Web Resources

mtDB: Human Mitochondrial Genome Database: www.genpat.uu.se/mtDB

PLINK 1.9: www.cog-genomics.org/plink/

HaploGrep 2: <https://haplogrep.i-med.ac.at>

Results

This study included 254 Tunisian subjects (mean age 70.72 ± 10.17); 196 samples were controls (mean age 69.52 ± 9.94), and 58 samples were AD patients (mean age 78.53 ± 9.33). Females (mean age 71.69 ± 10.28) represented 55.5% of the cohort, and males (mean age 69.52 ± 9.94) 45.5% (Table 2).

Table 2

A detailed description of the study participants: gender, age, and *APOE4* status, together with the p-value of each parameter

		AD patients (N=58)	Controls (N=196)	p-value*
Age (years)	(mean ± SD)	78.53 ± 9.33	68.41 ± 9.23	p < 0.0001
Gender	Female	46.6%	58.2%	
	Male	53.4%	41.8%	
APOE4 status	APOE4+	46.6%	18.5%	p < 0.0001
	APOE4-	53.4%	81.5%	
Female	Number	(N = 27)	(N = 114)	
Age (years)	(mean ± SD)	80.25 ± 10.88	69.66 ± 9.05	
APOE4 status	APOE4+	48.1%	83.3%	p < 0.0001
	APOE4-	51.9%	16.7%	
Male	Number	(N = 31)	(N = 82)	
Age (years)	(mean ± SD)	77.03 ± 7.60	66.68 ± 9.24	
APOE4 status	APOE4+	54.8%	79%	p < 0.0001
	APOE4-	45.2%	21%	
Abbreviations: AD: Alzheimer’s disease, SD: standard deviation, N: number				
*A p-value of 0.05 was considered significant with 95% confidence intervals (CIs).				

mtDNA haplogroup landscape in the global cohort

The hierarchical survey of the genotyped polymorphisms in the mitochondrial genome allowed the classification of our cohort into 52 haplogroups and sub-haplogroups, revealing a high level of diversity in our samples (**Supporting Information, Table S2**). In order to verify the assigning of the sub-haplogroups, we chose to sequence the mitochondrial region HVS-I. The sequencing was carried out for 82 samples (32% of our cohort). The obtained HVS-I haplotypes confirmed the previous classification based on the array results. The results are summarised in **Table S3 (Supporting Information)**. Most haplogroups (70.5%) belonged to Eurasian lineages (H, T, U, K, N, V, I, W, J, and X). Sub-Saharan lineages (haplogroup L and subtypes) represented 22.8%. In contrast, the North African component, represented by the U6 and M1 lineages, comprised only 6.7% of our global cohort (AD and Controls) (Table 3).

Table 3
Frequencies of mitochondrial sub-haplogroups in AD patients and controls

Sub- haplogroups	AD patients			Controls		
	(N = 58)			(N = 196)		
	N	%	SE	N	%	SE
H*	3	5.1	0.028	6	3	0.012
H1	4	6.8	0.033	13	6.6	0.017
H2a	13	22.4	0.054	27	13.7	0.024
H3	3	5.1	0.028	11	5.6	0.016
H6	3	5.1	0.028	7	3.5	0.013
J*	1	1.7	0.016	2	1	0.007
J2	1	1.7	0.016	2	1	0.007
J2b	0	0	0	4	2	0.01
K1a	3	5.1	0.028	13	6.6	0.017
L0	0	0	0	2	1	0.007
L1	3	5.1	0.028	10	5.1	0.015
L2	4	6.8	0.033	10	5.1	0.015
L3b	0	0	0	13	6.6	0.017
L3e	1	1.7	0.016	9	4.5	0.014
L3f	1	1.7	0.016	5	2.5	0.011
M13	1	1.7	0.016	2	1	0.007
M73a1	1	1.7	0.016	6	3	0.012
N*	0	0	0	2	1	0.007
T1	5	8.6	0.036	10	5.1	0.015
T2	1	1.7	0.016	9	4.5	0.014
U*	0	0	0	4	2	0.01
U3	0	0	0	3	1.5	0.008
U5b	3	5.1	0.028	3	1.5	0.008

H* includes H4, H5, H9, H11, and H30. J* includes J1, J1b, and J1c. U* includes U1, U4, U5, and U7. N* includes N1 and N9a. "others" contains the haplogroups X2, X3, M73a, I2a, W, and G. Abbreviations: AD: Alzheimer's disease, N: number, SE: standard error.

Sub- haplogroups	AD patients (N = 58)			Controls (N = 196)		
U6	1	1.7	0.016	13	6.6	0.017
V	1	1.7	0.016	3	1.5	0.008
X2	2	3.4	0.023	5	2.5	0.011
Others	3	5.1	0.028	2	1	0.007
H* includes H4, H5, H9, H11, and H30. J* includes J1, J1b, and J1c. U* includes U1, U4, U5, and U7. N* includes N1 and N9a. "others" contains the haplogroups X2, X3, M73a, I2a, W, and G. Abbreviations: AD: Alzheimer's disease, N: number, SE: standard error.						

The most represented Eurasian lineage was haplogroup H, with a frequency of 35.4%; this branch contained many sub-haplogroups: H2a (15.7%), H1 (6.7%), H3 (5.5%), H6 (3.9%), H4 (0.8%), H5 (0.8%), H11 (0.8%), H30 (0.8%), and H9 (0.4%). In addition to haplogroup H, many other Eurasian lineages were observed: U (10.6%), T (9.8%), K (6.3%), J (3.9%), V (1.6%), I (0.4%), N (0.8%), and W (0.4%). Furthermore, our data contained the North African-specific lineages U6 (5.5%) and M1 (1.2%) and the Middle Eastern sub-haplogroups T2 (3.9%), T1 (5.9%), U7 (0.4%), J1b (0.4%), and U1 (0.4%). Sub-Saharan lineages were represented by the following sub-haplogroups: L0 (0.8%), L1 (5.1%), L2 (5.5%), L3b (5.1%), L3e (3.9%), and L3f (2.4%).

Comparison between the two cohorts: AD patients and controls

The comparison of the distribution of mitochondrial haplogroups between the AD and the control cohort was first carried out by considering the 27 sub-haplogroups described previously in the two cohorts. No difference was found between the two cohorts ($p = 0.774$). Moreover, by comparing each of the 27 sub-haplogroups separately between the two cohorts, we did not detect any significant differences between the AD and the control cohort, suggesting the absence of an association between a particular lineage and AD in the Tunisian population (Table 4). Then, we grouped the haplogroups and sub-haplogroups into 10 lineages (Tables 5 and 6) in the two cohorts. None of these haplogroups showed an association with AD, although some haplogroups showed differences in their distribution between the two cohorts, such as haplogroup H ($p = 0.0906$, OR= 1.6758), which was more frequent in AD patients (44.5%) than in controls (32.4%), and the haplogroups L ($p = 0.1296$, OR= 0.551) and U6 ($p = 0.1506$, OR= 0.247), which, on the contrary, were more frequent in controls (24.8%, 6.6%) than in AD patients (15.3%, 1.7%) (Figure 1).

Table 4
distribution of sub-haplogroups in AD patients and controls, together with the chi-square test (χ^2) results

Sub-haplogroups	AD patients		Controls			
	(N = 58)		(N = 196)			
	N	%	N	%	χ^2	p-value*
H*	3	5.1	6	3	0.585	0.4442
H1	4	6.8	13	6.6	0.003	0.9572
H2a	13	22.4	27	13.7	2.551	0.1102
H3	3	5.1	11	5.6	0.021	0.8834
H6	3	5.1	7	3.5	0.307	0.5795
J*	1	1.7	2	1	0.191	0.6625
J2	1	1.7	2	1	0.191	0.6625
J2b	0	0	4	2	1.174	0.2787
K1a	3	5.1	13	6.6	0.171	0.6792
L0	0	0	2	1	0.582	0.4454
L1	3	5.1	10	5.1	0.000	1
L2	4	6.8	10	5.1	0.248	0.6182
L3b	0	0	13	6.6	4.018	0.0450
L3e	1	1.7	9	4.5	0.942	0.3319
L3f	1	1.7	5	2.5	0.126	0.7226
M13	1	1.7	2	1	0.191	0.6625
M73a1	1	1.7	6	3	0.286	0.5925
N*	0	0	2	1	0.582	0.4454
T1	5	8.6	10	5.1	0.984	0.3213
T2	1	1.7	9	4.5	0.942	0.3319
U*	0	0	4	2	1.174	0.2787
U3	0	0	3	1.5	0.877	0.3491

H* includes H4, H5, H9, H11, and H30. J* includes J1, J1b, and J1c. U* includes U1, U4, U5, and U7. N* includes N1 and N9a. "others" contains the haplogroups X2, X3, M73a, I2a, W, and G.

*A p-value of 0.05 was considered significant with 95% confidence intervals (CIs).

Sub-haplogroups	AD patients		Controls			
	(N = 58)		(N = 196)			
U5b	3	5.1	3	1.5	2.547	0.1105
U6	1	1.7	13	6.6	2.066	0.1506
V	1	1.7	3	1.5	0.012	0.9138
X2	2	3.4	5	2.5	0.137	0.7111
Others	3	5.1	2	1	3.947	0.0470
H* includes H4, H5, H9, H11, and H30. J* includes J1, J1b, and J1c. U* includes U1, U4, U5, and U7. N* includes N1 and N9a. "others" contains the haplogroups X2, X3, M73a, I2a, W, and G.						
*A p-value of 0.05 was considered significant with 95% confidence intervals (CIs).						

Table 5
distribution of haplogroups in AD patients and controls, together with the chi-square test (χ^2) results

Sub-haplogroups	AD patients (N = 58)		Controls (N = 196)		χ^2	p-value*
	N	%	N	%		
H	26	44.5	64	32.4	2.863	0.0906
J	2	3.4	8	4	0.043	0.8353
K1a	3	5.1	13	6.6	0.171	0.6792
L	9	15.3	49	24.8	2.298	0.1296
M	2	3.4	8	4	0.043	0.8353
T	6	10.3	19	9.6	0.025	0.8749
U	3	5.1	10	5	0.001	0.9756
U6	1	1.7	13	6.6	2.066	0.1506
V	1	1.7	3	1.5	0.012	0.9138
X2	2	3.4	5	2.5	0.137	0.7111
Others	3	5.1	2	1	3.947	0.0470
Abbreviations: AD: Alzheimer's disease, N: number						
"others" contains the haplogroups X2, X3, M73a, I2a, W, and G.						
*A p-value of 0.05 was considered significant with 95% confidence intervals (CIs).						

Table 6
distribution of haplogroups in AD patients and controls, together with the odds ratio (OR) results

	AD patients (N = 58)		Controls (N = 196)			
	N	%	N	%	OR	p-value*
H	26	44.5	64	32.4	1.6758	0.1175
J	2	3.4	8	4	0.8393	1
K1a	3	5.1	13	6.6	0.7678	0.7719
L	9	15.3	49	24.8	0.551	0.1556
M	2	3.4	8	4	0.8393	1
T	6	10.3	19	9.6	1.0749	1
U	3	5.1	10	5	1.0145	1
U6	1	1.7	13	6.6	0.247	0.2008
V	1	1.7	3	1.5	1.1287	1
X2	2	3.4	5	2.5	1.3643	1
Others	3	5.1	2	1	5.2909	0.0799
Abbreviations: AD: Alzheimer's disease, OR: odds ratio, N: number						
"others" contains the haplogroups X2, X3, M73a, I2a, W, and G.						
*A p-value of 0.05 was considered significant with 95% confidence intervals (CIs).						

To obtain a clear and global picture of the composition of mitochondrial haplogroups in our samples, we focused on grouping all haplogroups according to their ethnic lineages: North African, sub-Saharan, and Eurasian lineages. The major component in our population was the Eurasian lineages (70.5%), followed by the sub-Saharan lineages (22.8%) and the North African lineages (6.7%).

Almost the same profile of these three principal lineages was observed when we compared the mitochondrial haplogroups separately between patients and controls. Again, the dominant component belonged to the Eurasian lineages, with percentages of 67.3% and 81% for controls and AD patients, respectively. Sub-Saharan haplogroups were present in 25% of the control group and in 15.5% of AD patients. Finally, the North African lineages were found in 3.5% of AD patients and 7.7% of controls (Figures 2).

The frequencies of mitochondrial haplogroups were compared separately for gender between AD patients and controls. The results showed no significant differences between both groups: $p = 0.439$ for males and $p = 0.515$ for females.

Search for an association between mtDNA SNPs and AD

The second part of our investigation was focused on individual mtDNA SNPs without considering haplogroups. We analysed all 159 mitochondrial SNPs in order to test a possible association with AD. The association was tested using Fisher's exact test. Of all the mitochondrial polymorphisms studied, only two SNPs showed a significant result among patients: A5656G ($p = 0.03821$, OR = 10.46) and A13759G ($p = 0.03719$, OR = 10.78). The mitochondrial SNP A13759G is located in NADH dehydrogenase subunit 5. This SNP appears in the branch point of two sub-haplogroups, H11 and j2a2, according to the human phylogenetic tree (see the phylogenetic tree appendix). The mitochondrial SNP A5656G is located in a non-coding region separating two structural genes of transfer RNAs (tRNAs): tRNA (Ala) and tRNA (Asn).

APOE study

Since *APOE* is the major genetic risk factor of AD and became a routine examination in all genetic AD studies, we evaluated the allele frequencies with the covariates gender and age.

APOE4+ subjects (with one or two copies of *APOE4*) represented 13.2% of our total cohort. Of all AD patients, 46.6% are *APOE4+*, and 53.4% are *APOE4-*. The effect size (OR) for *APOE4* was 0.39. It is important to mention that only 4.3% of our samples presented the $\epsilon 2$ allele of *APOE*, while a large part of the samples (82.5%) presented the $\epsilon 3$ allele.

The comparison between AD patients and controls without taking gender into consideration showed a significant correlation for *APOE4* carriers ($p = 0.000014$). We conducted a separate comparison according to gender to evaluate *APOE4* values in AD patients and controls. The results showed that *APOE4* carriers had a significant p-value, which confirmed our previous findings (Table 2).

Discussion

Sporadic AD is a complex disease with many risk factors, including genetic, environmental, and lifestyle factors (higher education, reading, taking care of family, smoking, etc.) (Bhardwaj et al. 2017). Whether mitochondrial polymorphisms are also risk factors is largely unknown. Recent studies suggest that mitochondrial mutations and other disturbances have an important effect on the pathogenesis of AD. Many mitochondrial haplogroups have been linked to AD as factors capable of conferring increased or decreased risk of AD. Given the role of mitochondria in cell death, their implication in neurodegenerative disorders, including AD, seems plausible. Multiple studies have been conducted to inspect the potential causative relation between mtDNA and AD. In this study, we evaluated the possible association between AD and mitochondrial haplogroups or polymorphisms in the Tunisian population by genotyping

mitochondrial polymorphisms dispersed across the whole mitochondrial genome, using a cohort of 58 AD patients and 196 controls comparable in gender, age, and ethnicity.

To our knowledge, this investigation is the first case-control study related to AD and mtDNA in Tunisia, home to an admixed population from North Africa. The results showed that none of the observed mitochondrial haplogroups in our population had a significant association with AD. However, we observed that two mitochondrial variants, A5656G and A13759G, were statistically associated with AD.

In this study, we explored Tunisian mtDNA in relation to AD. The results showed a large variety of haplogroups, creating a mosaic structure composed of 41 haplogroups in our cohort (254 individuals only) and representing a high level of genetic diversity in the Tunisian population. This genetic diversity in Tunisia can be explained by a heterogeneous gene pool caused by invasions from different populations: Phoenicians, Byzantines, Arabs, Romans, Ottomans, Vandals, Andalusians, etc. Most of these populations have left a genetic imprint on the Tunisian population. In addition, the arrival of thousands of Europeans (from France, Italy, Spain, Austria, Belgium, Greece, Russia, and Malta) in Tunisia during the colonisation (about 1881–1921) further enriched the genetic pool (Cherni et al. 2016). As has been noted in previous studies, the long and rich history of Tunisia created a specific genetic pool. This genetic heterogeneity was observed both in different Tunisian regions and in populations of the same ethnic background. In our study, we found the same haplogroups as previously described in other studies (Elkamel et al. 2018; Costa et al. 2009). The major haplogroups found belonged to the West Eurasian lineages (H, T, U, K, N, V, I, W, J, X), with a predominance of the sub-haplogroup H2a. In general, haplogroup H is the most frequent European lineage in North Africa. The highest frequencies were found in Berber- and Arab-speaking Moroccans (37% and 34%, respectively) (Rando et al. 1998). Approximately the same percentage was found in our study, as 35.4% of our cohort had haplogroup H. Some previous studies have reported an association between haplogroup H and AD. For example, in Asturias (northern Spain), a significant association was found between haplogroup H and sporadic AD (Coto et al. 2011). In Italy, a specific sub-haplogroup of H (H5) was identified as a risk factor for AD (Santoro et al. 2010b). Despite the predominance of sub-haplogroup H2a in our population, we did not find any significant value for an association with AD.

The Eurasian lineage U was also present in our population (10.6%). This mitochondrial haplogroup has a large distribution in Europe and southwest Asia (Sahakyan et al. 2017). In Caucasian Americans, haplogroup U ($p = 0.007$) showed a differential effect in AD patients according to gender; it had a protective effect in females, and it increased the risk of developing AD in males (van der Walt et al. 2004). The North African-specific lineage U6 was present (5.5%) and constituted, together with the North African haplogroup M1, 6.7% of our population.

In agreement with Frigi et al. (2017), who described the Tunisian genetic pool by studying 15 Tunisian population groups distributed across the country, our cohort contained two North African haplogroups, M1 and U6, with a percentage of 7.1%. For the sub-Saharan lineages (haplogroup L), we found almost the

same percentage (22.8%) as Frigi et al. (2017) (23%). Sub-Saharan haplogroup L1 showed an increased risk of developing dementia in African Americans with lower plasma levels of A β (Tranah et al. 2014).

Mitochondrial haplogroup T was among the Eurasian lineage in our cohort (9.8%) and was presented by both sub-haplogroups T1 and T2. In conflicting reports, mitochondrial haplogroups U and T have been implicated as conferring both a decreased and an increased risk of developing AD (Ridge and Kauwe 2018). In our case, the results showed no huge difference between patients and controls regarding haplogroup T (T1 +T2), which was present in 10.3% of patients and 9.6% of controls.

In many studies, evidence was found for an association between some specific haplogroups and AD, either as a risk factor or as a protective one. In our cohort, we have a mix composed of North African, sub-Saharan, and Eurasian lineages. This mixed population facilitates the evaluation of mitochondrial haplogroups in relation to AD. In our cohort, we have almost all mitochondrial haplogroups that were indicated in previous studies as being associated with AD. However, in our case, none of the haplogroups had a significant association with AD. These findings support the idea that haplogroup implication in AD is not strong enough to explain the risk of AD. Our study is in agreement with previous studies showing no definitive association of AD with a specific mitochondrial haplogroup (van der Walt et al. 2005; Elson et al. 2006; Mancuso et al. 2007; Mancuso et al. 2009; Fachal et al. 2015; Ridge and Kauwe 2018; Monzio Compagnoni et al. 2020; Zhunina et al. 2020). In fact, investigating the implication of mitochondrial haplogroups in AD pathology is an alternative way of determining the involvement of the mitochondrial genome. It is an approach to analysing the frequencies of mitochondrial polymorphisms that could affect AD pathology by altering mitochondrial respiration and causing overproduction of reactive oxygen species (ROS). This means that a group of individuals or a population with the same mitochondrial genotype has the same predisposition to apoptotic processes (Mancuso, Orsucci, et al. 2008). The analysis of the distribution of haplogroups in controls and AD patients is a way to determine whether a particular mitochondrial haplogroup is associated with the risk of AD by being more vulnerable to oxidative stress than other mitochondrial haplogroups.

However, the real involvement of mtDNA and its haplogroups in AD is still under discussion. Two theories are possible. The first one is that oxidative stress and mitochondrial dysfunction are responsible for neurodegenerative pathologies and have a primary role in AD. The second theory is that neurodegenerative pathologies are the consequence of cell death and that the decline in mitochondrial function is caused by other factors (Mancuso et al. 2006). To our knowledge, no previous findings, except one study (Coto et al. 2011), were replicated to date.

Classically, it has been difficult to study the direct implication of mtDNA mutations in AD patients, and many studies have produced non-conclusive results (Elson et al. 2006). Therefore, genetic investigations started to focus on single polymorphisms rather than haplogroups (Mancuso et al. 2009). Since some mitochondrial mutations were identified in association with AD (Shoffner et al. 1993), we changed the strategy from haplogroups to mitochondrial SNPs. Our findings showed two large effects: A5656G ($p =$

0.03821, OR = 10.46) and A13759G ($p = 0.03719$, OR = 10.78); both SNPs were not mentioned before as a risk factor for AD.

According to the human phylogenetic tree, A13759G is located on the branch point of two sub-haplogroups, H11 and j2a2. A13759G is located in subunit 5 of the *NADH dehydrogenase* locus. This enzyme belongs to complex I (NADH dehydrogenase, NDU) of the electron transport chain (ETC) and represents one of the entry enzymes of cellular respiration and contributes to the production of ATP. The *NADH dehydrogenase* gene was mentioned before in relation to AD. In 1992, Lin et al. identified two mutations in the mtDNA of AD patients, located in subunit 2 of the *NADH dehydrogenase* gene, but these results were not confirmed by other studies (Lin et al. 1992; Grazina et al. 2006). Recently, a meta-analysis was carried out by Holper et al. (2019) of both complex I and complex IV activities in many disorders, including AD. They provided strong evidence for impairments in both enzymes' activities in the blood and some brain regions. These mitochondrial enzymes are well documented in AD and seem to be affected by amyloid β peptide ($A\beta$) accumulation in mitochondria, inducing neural toxicity and inhibiting mitochondrial enzymes, finally leading to mitochondrial dysfunction (Giachin et al. 2016; Coskun et al. 2012; Cenini et al. 2016). Thus, it is clear from the literature that complex I has been implicated in neurodegenerative diseases, including AD, but no previous findings were found regarding A13759G in association with AD.

The second positive association was with A5656G. This polymorphism is the characteristic SNP of sub-haplogroup U5b1. Ridge et al. (2013) showed that four clades (U5B1, U5B1B2, K1A1B, and K1A1B2A1) were associated with different endophenotypes of AD patients. Our results confirm and extend these previous studies, proving that sub-haplogroup U5b1 is probably implicated in AD. Furthermore, our findings show that the A5656G polymorphism may be the main factor in this sub-haplogroup's involvement in AD. This SNP is located in the non-coding region separating two structural genes of tRNAs: tRNA (Ala) and tRNA (Asn). Interestingly, a similar transition in position 3302 has been reported to disturb the processing of primary transcripts, causing a functional alteration (Bindoff et al. 1993). Thus, the mechanism is still not clear, but it seems probable that this polymorphism located between two tRNA genes might cause a functional defect (Zsurka et al. 1997). This theory is plausible because the A5656G transition was found in many diseases: tubulointerstitial nephritis (Zsurka et al. 1997), occipital stroke (Finnilä, Hassinen, and Majamaa 2001), and Parkinson's disease (Vives-Bauza et al. 2002).

An increased mutation frequency was observed in the brains of AD patients (Chang et al. 2000). Also, it has been suggested that mutations in mtDNA might change the age of AD onset and contribute to the process of neurodegeneration (Grazina et al. 2006). Unfortunately, only a few SNPs were described as being linked to AD risk; an example is the variant at position 4336 in the tRNA (Gln) gene (Shoffner et al. 1993; Egensperger et al. 1997). The associated SNPs (A5656G and A13759G) found in our study were not mentioned before as a risk factor for AD in previous studies. Since mtDNA encodes for mitochondrial subunits, transfer RNAs, and ribosomal RNAs, it is logical to think that mutations or clusters in the mtDNA might alter mitochondrial metabolism and lead to AD (Maruszak et al. 2009). However, oxidative stress can explain the high level of mitochondrial mutations.

In other studies, brains of elderly subjects and AD patients showed an increased mutation rate in comparison with young subjects, indicating that mutations in mtDNA are not specific to the pathogenesis of AD. Mutations are also observed in ageing (Lin et al. 2002), so it is unclear whether an increased mutation rate is caused by AD or ageing.

At the same time, evidence of maternal transmission of AD was mentioned in many studies. Honea et al. (2012) reported that persons with a maternal history of dementia showed an increased presence of a biomarker of AD compared with persons with paternal AD or no history of AD. Since mtDNA is maternally transmitted, it is logical to think about the implication of mtDNA in the pathogenesis of AD.

In view of all these hypotheses, many studies have been undertaken to understand the role of mtDNA in AD. Unfortunately, there are no conclusive results regarding the exact role of mtDNA in AD pathogenesis. Mutations in mtDNA can be a primary cause of AD, or the damages observed in mitochondria might be the result of AD.

The association of mutations in mtDNA with AD is still under discussion. An issue in this discussion is the potential involvement of nuclear DNA in mitochondrial variants in the pathogenicity of AD. No research has been conducted to evaluate the possibility of an association between mitonuclear genes and mtDNA in influencing the risk of AD (Andrews et al. 2019).

Finally, in the second part of our study, we found an association of the *APOE* $\epsilon 4$ allele ($p = 0.000014$) with AD, confirming previous findings indicating that the *APOE* $\epsilon 4$ allele is a major genetic risk factor for AD in Tunisia. The frequencies of observed genotypes were 4.3%, 82.5%, and 13.2% for the *APOE* alleles $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$, respectively. These values are almost the same as those reported by previous studies on the *APOE* allele frequencies in the Tunisian population (Smach et al. 2008).

The *APOE* gene is the strongest genetic risk factor for AD (R 2019; Corder et al. 1993). This gene encodes the apolipoprotein E protein, which is implicated in cholesterol and lipid transport. Several hypotheses have been put forward to explain the relation between *APOE* and AD risk. One idea is that there is a connection between *APOE* and mitochondrial dysfunction. This hypothesis was based on the observation that the apolipoprotein E4 isoform is differently folded compared with other isoforms. This alternative folding makes it more susceptible to proteolysis, leading to a small peptide. The resulting fragments constitute toxic bioactive elements that enter the cytosol and lead to cell death by disturbing the mitochondrial energy balance (Mahley, Weisgraber, and Huang 2006; Swerdlow et al. 2017).

In addition, the interaction of *APOE* $\epsilon 4$ and mtDNA could be of interest for AD pathology. This idea was put forward in a cognitive study on the association of parental dementia with AD. The results revealed that non-demented *APOE* $\epsilon 4$ carriers with an AD-affected mother showed less memory performance than individuals with an AD-affected father with a higher rate of brain atrophy (Debette et al. 2009). This finding could point to the possibility of an interaction between *APOE* $\epsilon 4$ and certain mitochondrial regions or haplogroups in modulating AD risk. Indeed, several previous reports have mentioned that some mitochondrial haplogroups (U and K) seem to neutralise the harmful impact of *APOE* $\epsilon 4$ (Carrieri et al.

2001). One study proposed that some mitochondrial haplogroups have an influence on allele 4 of *APOE*, the major risk factor of AD. In particular, haplogroups K and U have been suggested to neutralise the effect of *APOE4* (Carrieri et al. 2001). This hypothesis could be investigated in future studies with a larger number of samples of the Tunisian population.

Conclusion

Recently, the implication of mtDNA in AD has been widely studied. Despite many findings indicating the presence of some possible risk factors for AD, such as mitochondrial haplogroups or SNPs, none has been proven so far. This difficulty of obtaining conclusive results might be due to differences between populations in the frequencies of mitochondrial polymorphisms, as caused by the variety of ethnic groups. Therefore, in our case, it was interesting to study the association of mitochondrial haplogroups with AD at the population level. However, we did not find any associated haplogroup. Then, in the second part of our investigation, we looked for the association of specific mitochondrial SNPs with AD and found two significant associations (A5656G and A13759G). This finding provides additional information on the role of mtDNA in the risk of AD and may contribute to the diagnostic process. Finally, our results were based on a small sample, so future investigations need to replicate our results in larger samples with the sequencing of the whole mtDNA.

Declarations

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Author's Contribution

Conceptualization Lotfi Cherni and Nesrine Ben Salem; Data curation, Nesrine Ben Salem, Sami Boussetta Itziar de Rojas, Sonia Moreno-Grau and Laura Montreal; Formal analysis Nesrine Ben Salem and Sami Boussetta; Investigation, Nesrine Ben Salem ; Methodology, Nesrine Ben Salem and Sami Boussetta; Project administration, Lotfi Cherni and Agustín Ruiz; Diagnostic of patients and controls, Mokni Narjes, Mahmoud Imene, Younes Samia, Nizar Daouassi, Frih-Ayed Mahboubba and Afef Hammami ; Supervision, Lotfi Cherni; Validation, Lotfi Cherni, Agustín Ruiz and Amel Ben Ammar Elgaaiied; Visualization, Nesrine Ben Salem; Writing – original draft, Nesrine Ben Salem; Writing – review & editing, Lotfi Cherni Agustín Ruiz and Sonia Moreno-Grau.

All authors read and approved the final manuscript.

Compliance with ethical standards

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Conflicts of Interest/competing interests

The authors declare there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

Ethical approval

This retrospective chart study involving human participants was in accordance with the ethical standards of the Ethics Committee for Research in Life Sciences and Health of the ISBM (CER-SVS/ISBM) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

All procedures performed in the present work were approved by Ethics Committee for Research in Life Sciences and Health of the ISBM (CER-SVS/ISBM) , Monastir ,Tunisia .

Consent to participate

Informed consent was obtained from all individuals according to the ethical standards of the Ethics Committee for Research in Life Sciences and Health of the ISBM (CER-SVS/ISBM) and the 1964 Helsinki Declaration.

Consent for publication

Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

Availability of data and material

Not applicable

Code availability

Not applicable

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Figures

Figure 1

The distribution of Sub-haplogroups frequencies in patients and controls H* includes H4, H5, H9, H11, and H30. J* includes J1, J1b, and J1c. U* includes U1, U4, U5, and U7. N* includes N1 and N9a. "others" contains the haplogroups X2, X3, M73a, I2a, W, and G. Orange bars denote patients, and bleu bars indicate controls

Figure 2

The frequency Distribution of Mitochondrial Haplogroups in: Studied population (AD patients and controls), Alzheimer's patients (AD) and in Controls All sub-haplogroups observed in our cohort are grouped according to their ethnic lineages in three groups: North African (indicated in grey), sub-Saharan (indicated in orange), and Eurasian lineages (indicated in bleu). (a) the distribution of Mitochondrial Haplogroups in the studied population in general. (b) the distribution of Mitochondrial Haplogroups in the group of AD patients. (c) the distribution of Mitochondrial Haplogroups in the group of controls. The major observed component was the Eurasian lineages, followed by the sub-Saharan lineages and the North African lineages.

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