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Benign and conserved DNA variants m.8860A>G and m.8701A>G indicating mitochondrial genetic drift in Pakistani population

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ABSTRACT

Background: Mitochondria are vital subcellular organelles that orchestrate the intricate process of oxidative phosphorylation (OXPHOS), generating adenosine triphosphate (ATP) as the primary energy molecule fueling cellular activities. During our research on mitochondrial mutations in breast cancer patients, we identified two notable single nucleotide polymorphisms (SNPs) present in both cancer patients and control individuals from the Pakistani population.

Materials and methods: DNA was extracted from the blood samples of 30 individuals, and *MT-ATP8* and *MT-ATP6* were amplified using PCR with specific primers. Purified PCR products were sequenced and analyzed for mutations using SnapGene and BioEdit. Bioinformatics tools, Consurf and PolyPhen-2, were used to analyze the genetic variants and their impact on protein function and stability.

Results: The analysis revealed two significant mutations in *MT-ATP6* gene i.e., m.8860A>G (found in all 30 out of 30 samples) which results in the variant p.(Thr112Ala) and m.8701A>G (found in 13 out of 30 samples) which results in the variant p.(Thr59Ala). PolyPhen-2 analysis reveals the benign nature of both mutations, suggesting that the sequence variants are unlikely to cause any adverse effects on protein structure and function.

1. Introduction

The interplay between diet, environmental factors, and mitochondrial DNA (mtDNA) variation serves as a raw material for evolution via reshaping human genetic diversity. Mitochondria are subcellular organelles playing an essential role in the process of OXPHOS, producing ATP that powers all cellular processes (Daniels et al., 2020). Mitochondria has an autonomous, compact circular genome spanning 16,569 base pairs (bp), it is responsible for encoding 13 proteins, 22 transfer RNAs (tRNA), and 2 ribosomal RNAs (rRNA), the protein-coding genes of mtDNA are involved in the mitochondrial respiratory chain which uses the transfer of electrons, generated by the Krebs cycle to produce ATP.

The regulation of complexes involved in the respiratory chain is influenced by both the nuclear and mitochondrial genomes. However, mtDNA plays the predominant role in encoding the assembly of the core complexes (Bertram et al., 2006).The OXPHOSprocess involves five enzyme complexes located within the inner mitochondrial membrane. These complexes include multiple subunits, each playing a critical role. Complex I, known as NADH dehydrogenase, comprises several subunits including ND1-ND6.Complex II comprises of four subunits; SDHA, SDHB and two subunits SDHC and SDHD that anchor the complex to inner mitochondrial membrane (Hadrava Vanova et al., 2020). Cytochrome b subunit forms part of Complex III while Complex IV comprises of cytochrome *c* oxidase subunit. Complex V, or ATP synthase enzyme, constitutes subunits 6 and 8. These complexes synergize to generate ATP, the cell's main energy currency molecule (Rusecka et al., 2018).

ATP synthase is a crucial enzyme involved in OXPHOS by playing its role in catalyzing the synthesis of ATP from ADP and phosphate. The Human Complex V encodes for 29 proteins of 18 types, two of which are *MT-ATP6*: Mitochondrially Encoded ATP Synthase Membrane Subunit 6 and *MT-ATP8*: Mitochondrially Encoded ATP Synthase Membrane Subunit 8 (MT-ATP6 and MT-ATP8) (Protasoni and Zeviani, 2021).

Mitochondrial genetics diverges from the basic Mendelian laws of inheritance as it is strictly maternally inherited. Due to its close proximity with Reactive Oxygen Species (ROS) and the lack of efficient mismatch repair mechanism mtDNA is more susceptible to DNA damage (Vadakedath et al., 2023).

Abbreviations: mtDNA, Mitochondrial DNA; MT-ATP6, ATP synthase enzyme subunits 6; MT-ATP8, ATP synthase enzyme subunits 8; OXPHOS, Oxidative phosphorylation; CHD, Congenital heart disease.

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Mitochondrial Haplogroups are groups of related haplotypes that have similar mtDNA single nuclear polymorphisms (SNPs) that were passed down from a common ancestor. Through the processes of Natural selection, genetic drift and founders effect, mutations passed down from maternal lineage accumulate over time to form distinct haplogroups (Saeb and Al-Naqeb, 2016).

In nature, the balance of macronutrients in diet acts as a strong selection force both within and across populations. The relative amounts of macronutrients in a diet can affect both allele frequency and population migration patterns (Aw et al., 2018). Based on scientific evidence dietary changes greatly influence mitochondrial genetics (Ordovas, 2008; Unckless et al., 2015; Kyriazis et al., 2022).

The study of genetic variations among a population is essential for the understanding of region-specific evolutionary inputs, potential health implications and for informing targeted healthcare strategies. During our research on mitochondrial mutations in breast cancer patients, we identified two notable single nucleotide polymorphisms (SNPs) present in both cancer patients and control individuals from the Pakistani population. The incredible consistency of these sequence variantsacross all samples is indicative of the region-specific environmental conditions and dietary patterns. In addition there may be other factors that shape the allele frequency which include genetic drift, natural selection and gene flow (Chen et al., 2019). This short communication aims to report the population-specific SNPs identified, emphasizing the need for further studies to unveil its potential implications for health and disease.

2. Methodology

The study sampling design involved the collection of blood samples along with relevant clinical and demographic data from individuals in the Punjab region, Pakistan. A total of 30 samples were used in the study, comprising 25 from breast cancer patients and 5 from control individuals. The control group consisted of healthy individuals of both genders, with ages ranging from 23 to 30 years. All control participants were of Pakistani ethnicity, ensuring a consistent demographic background for comparison. The study is conducted with the approval of KAM School of Life Sciences, Forman Christian College (A Chartered University) Research Ethics Committee (Permission dated: 25/09/2023, No.ERC-135-2023) and Institutional Review Board (Permission dated 02-10-2023, No.IRB-513/10-2023).

After obtaining informed consent from the participants, blood samples were collected and used for genomic DNA extraction following the standard TRIzol® Chloroform protocol. Gene-specific primers targeting mitochondrial complex (MLCOMV) genes (*MT-ATP 6* and *MT-ATP 8*) were used for PCR amplification using Phusion™ High-Fidelity DNA Polymerase, employing the PCR mixture compositions specified in the ThermoFisher catalog (ThermoFisher, Cat#F53). PCR amplifications were done using the ML-Com-V-Fl (5′ CTAGAGCCCACTGTAAAGCTAA 3′) and ML-Com-V-R1 (5′ CGAAAGCCTATAATCACTGTGC 3′) primers. The PCR products were sent to Macrogen Inc. (South Korea) for Sanger sequencing and sequencing was done using ML-Com-V-F1 primer. Macrogen uses standard sequencing protocol involving PCR and gel electrophoresis along with fluorophore labelled ddNTPs that cause chain termination and result in DNA fragments of varying sizes. These DNA fragments are separated using Gel electrophoresis and laser is used to scan the gel, determining the DNA sequence (Standard Sequencing-Macrogen, 2024).

Multiple sequence alignment of the results was performed using BioEdit, specifically employing ClustalW. The input was sequencing data files, which were aligned against the mtDNA reference sequence obtained from UCSC genome browser, enabling the identification of mutations. ConSurf was used to study the evolutionary conserved amino acids within the *MT-ATP 6* gene of ATP Synthase protein. ConSurf uses multiple sequence alignment of homologues to assign each amino acid a conservation score which is visually depicted through a color-coded scale, the scores (8–9) represent conserved amino acids (Ben Chorin et al., 2020).

SnapGene was utilized to assess the sequence quality of the identified sequence variants by studying the chromatogram sequencing peaks. To study the impact of mutations within *MT-ATP 6* on the structure and function of ATP Synthase, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) was used. In addition, PyMol software (https://pymol.org/2/) was used to visualize and analyze the protein's three-dimensional structure.

3. Results

3.1. DNA sequencing and analysis

Multiple sequence alignment for sequencing data was performed, using the reference sequence from UCSC genome browser (GRCh38/hg38) for *MT-ATP 8* and *MT-ATP 6* genes. ClustalW analysis revealed two DNA variants of interest. The first sequence variant was identified (*MT-ATP6*: m.8860A>G p.(Thr112Ala)) at nucleotide position m.8860, where an adenine (A) was substituted by guanine (G), resulting in an amino acid change from threonine (Thr112) to alanine (Ala112). This mutation was consistently present across all 30 samples, regardless of whether they were from the control group or breast cancer patients, hence, this mutation was observed in 100 % of the samples. The presence of the benign mutation across all samples, irrespective of breast cancer patients and controls, indicates that the variant is a common polymorphism among the Pakistani population rather than being a disease-specific mutation.

The second variant of interest (*MT-ATP6*: m.8701A>G p.(Thr59Ala)) occurred at nucleotide position m.8701, with a similar A to G substitution, also resulting in the same amino acid change from threonine (Thr59) to alanine (Ala59). However, this benign mutation was only found in 13 out of the 30 participants. Among these 13 participants, 2 were from the control group and 11 were breast cancer patients (Fig. 1).

The recurring benign mutation from Threonine to Alanine, an essential to non-essential amino acid is indicative of the genomic adaptation that the people of Pakistan have acquired over time, this might be due to environmental selection pressures or dietary patterns that have led to metabolic modifications within the population. The impact of this metabolic modification may or may not confer a selective advantage to the population depending on the multifaceted and complex nature of molecular genetics. The findings are summarized in Table 1.

3.2. In silico analysis and structural visualization of identified genetic variants

ConSurf analysis for the study of amino acid conservation of MT-ATP 6 gene revealed that the amino acid Thr112 had a score of 7 indicating that it is highly conserved among species. Having a high evolutionary conservation suggests that the amino acid Thr112 plays an essential role in the ATP Synthase structure, function or stability and any mutation at the position might adversely affect the protein. On the other hand, amino acid Thr59 had a score of 1, suggesting an evolutionary divergence of the residue across species and carrying a less critical role in the function of ATP Synthase. Therefore the genetic variant Thr59Ala can be considered benign or non-deleterious.

PolyPhen-2 analysis was conducted to predict the functional impact on the protein MT-ATP 6, of the identified genetic variants at nucleotide positions m.8860 and m.8701. PolyPhen-2 assesses the impact of a mutation by assigning a prediction score and a color-coded representation of the results, ranging from (0.1–1.0), colors green to red, with the range (0.0–0.15) green as benign, (0.15–0.85) yellow as possibly damaging and (0.85–1.0) and the color red coding for the mutation to be probably damaging to the protein (Adzhubei et al., 2013). The analysis revealed a PolyPhen-2 score of 0.000 and 0.002 respectively, suggesting that the mutations are benign. This score indicates a low probability in

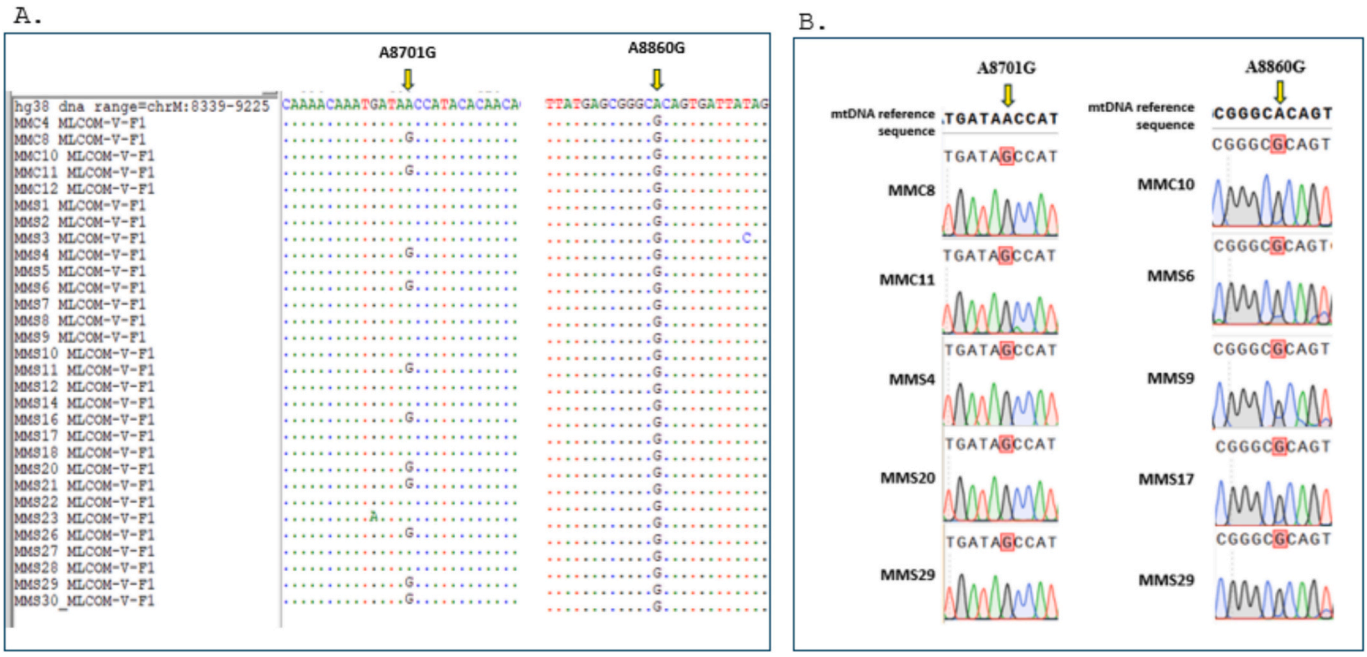

Fig. 1. A. Sequencing alignment of 30 samples, including 5 control and 25 breast cancer samples, highlighting DNA variants with A to G conversions at positions m.8701 and m.8860. B. Sequence chromatogram of represented samples.

Table 1
Details of sequence variants identified in *MT-ATP6* gene.

Gene	Protein	Locus	Nucleotide change	Amino acid change	Frequency in samples ($n = 30$)
MT-ATP6	ATP synthase	m.8860	A > G	Thr112Ala	30 (100 %)
MT-ATP6	ATP synthase	m.8701	A > G	Thr59Ala	13 (43.3 %)

causing significant functional changes in the protein, making the benign mutation well tolerable to ATP Synthase.

PyMol software was used for the visualization of the three-dimensional structure of ATP synthase protein (PDB: 8H9F). The visualization changes to reflect the substitution of threonine (a polar amino acid) with alanine (a non-polar amino acid) with a significant difference as Threonine has a hydroxyl group (-OH) which can participate in hydrogen bonding and Alanine, being smaller and non-polar, lacks this group. There is no noticeable change in the protein secondary, tertiary or quaternary structures as the helical structure of the protein is still intact at the site of mutation, suggesting that the benign mutations at Thr112Ala and Thr59Ala do not destabilize the structure of ATP Synthase (Fig. 2).

4. Discussion

In our primary research focused on analyzing the genetic variations in mitochondrial complex V, we identified two ubiquitous non-synonymous mutations in *MT-ATP6*: m.8860 A>G p.(Thr112Ala) and *MT-ATP6*: m.8701 A>G p.(Thr59Ala), in breast cancer patients as well as control. The *MT-ATP6* is one of the complex V genes, forms a part of membrane F_o domain of the ATP synthase and is involved in the proton translocation, contributing to its rotational mechanism (Lai et al., 2023).

Due to the heteroplasmic nature of mtDNA, multiple sequence variants of mtDNA coexist in a single mitochondria. Consequently, Sanger sequencing chromatograms may show multiple nucleotide peaks at a single position, indicating the presence of more than variant (Irwin et al., 2009). However, in our study we obtained clear call for single nucleotide at each position suggesting that the heteroplasmy was below the detection threshold of Sanger sequencing in our samples. The analysis of sequencing data revealed m.8860A>G and m.8701A>G as two high frequency variants in *MT-ATP6* gene in a cohort of Pakistani population. The m.8860A>G and m.8701A>G variants in *MT-ATP6* gene lead to Thr112Ala and Thr59Ala amino acid substitutions, respectively. The PolyPhen-2 analysis indicates that these variants are benign and have no impact on the protein structure as they were positioned in relatively non-conserved stretch of protein. However, the transition from an uncharged, polar threonine to a hydrophobic alanine residue at both the positions within the transmembrane region of ATPase 6 protein could potentially affect the ATP synthase efficiency.

Of note is the frequency of m.8860A>G, which was present in all the samples, including controls whereas m.8701A>G was observed in only 43.3 % of the study subjects. It is noteworthy that m.8860A>G and m.8701A>G polymorphisms in *MT-ATP6* gene have been reported earlier, therefore these are not novel sequence variants (Kraja et al., 2019; Dirican et al., 2022). The MITOMAP database also reports a high occurrence rate of m.8701A>G and m.8860A>G variants in major mtDNA lineages, with the maximum frequency of 99.2 % and 99.3 % in L haplogroup (mitomap.org).

The m.8701A>G variant has been associated with altered mitochondrial matrix pH and intracellular Ca^{+} levels (Gonzalez, 2021). Moreover, a study on Chinese pedigree of consanguineous marriage revealed that m.8701A>G may act as an inherited risk factor for matrilineal transmission of hypertension and can potentially contribute to the pathophysiology of cardiovascular disorders (Zhu et al., 2016).

A study on 200 cardiac congenital heart disease (CHD) patients predicted that m.8860A>G (T112A) affects the structure of ATPase 6 protein and this variant was deleterious and probably damaging (Pdel score:0.85) (Heidari et al., 2022). Interestingly, m.8860A>G variant was also detected in a Chinese family ($n = 15$) being studied for mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) and maternally inherited diabetes. However, the variant

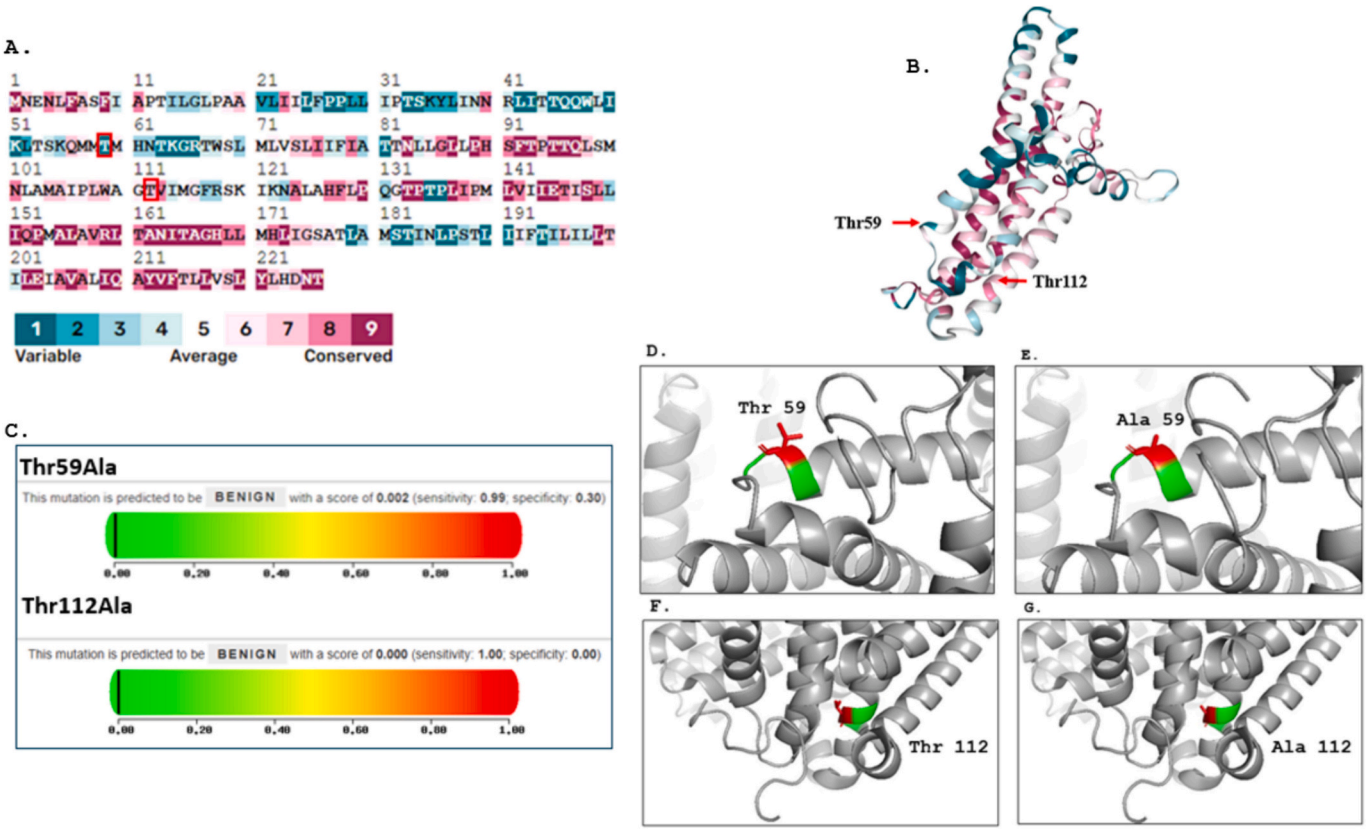

Fig. 2. A. ConSurf results showing the evolutionary conservation of Amino acids encoding for MT-ATP 6 gene of ATP synthase protein. Highlighted regions in red boxes show the site of genetic variationThr59 and Thr112 respectively. The scoring chart below shows the degree of conservation of amino acids. B. Three-dimensional structure of MT-ATP6, with highlighted regions showing the site of SNP, the color coding represents the degree of conservation according to the key on the left. C. PolyPhen-2 results show prediction scores and the benign nature of the identified mutations. D. Three-dimensional representation of MT-ATP 6 region of ATP synthase protein structure illustrating structural alterations. The protein backbone is depicted in grey, while side chains are highlighted in green, amino acid at position 59 marked in red depicts the native protein conformation with threonine residue. E. Mutated protein configuration with alanine substitution, shown in red. F. Amino acid at position 112 marked in red represents the native protein conformation with threonine residue. G. Mutated protein configuration with alanine substitution at amino acid position 112. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was not strictly limited to the maternal line as it was also present in non-maternally related individuals within the family (Li et al., 2015).

Since deleterious mutations are eliminated over time by purifying selection, they are relatively rare in a specific population. Whereas, benign or synonymous mutations retained by adaptive selection could be enriched in a topographical context. Stewart et al., provides strong experimental evidence for the purifying selection against the non-synonymous mutations in the protein-coding genes during the maternal transmission of mutated mtDNA in mouse model (Stewart et al., 2008). An excess of non-synonymous substitutions in *MT-ATP6* gene have previously been observed for haplogroup associated mutations as well as individual polymorphism (Elson et al., 2004). However, the ubiquitous retention of the observed non-synonymous mutation (m.8860A>T) in the Pakistani population could imply that the pattern could be due to less-intense selective pressure on the ATP6 gene, allowing the mutation to accumulate over time and persist in mtDNA pool. Although m.8860A>G has been documented as variant in disease-specific context in other populations, but it is reported for the first time in our study as ubiquitous in Pakistani population (Fadhl and Abdulkarim, 2021; Mani et al., 2019; Satiyarti et al., 2020; Liu et al., 2017; Amer et al., 2020).

In the phylogenetics analysis of human mtDNA-coding region genes, over half of the amino acid replacement involves the codon for threonine and valine. The excessive mutation involving threonine codons could be due to the adaptive correlation with the higher mutability frequency in the mitochondrian-encoded $tRNA^{Thr}$. Moreover, a comparison between amino acid substitutions in mtDNA-encoded proteins revealed that the substitutions between threonine and alanine are overrepresented in human population (Kivisild et al., 2006). Theoretically, the aberrant amino acid replacement patterns observed in various populations could be due to diet. Earlier, it was considered that genetic drift is the cause of region-specific variations in the mtDNA coding sequences in indigenous population. However, recent studies suggest that the regional distribution of mtDNA lineages (haplotypes) is also influenced by climatic selection and dietary factors (Ruiz-Pesini et al., 2004). Furthermore, selection pressure in response to regional dietary regimes has significantly influenced the global human genomic diversity (Fan et al., 2016).

In Pakistan, the dietary pattern is primarily characterized by the high intake of grains, particularly wheat and rice. The essential amino acids, including threonine and valine, must be taken in diet and are abundant in lentils, meats, cottage cheese, peanuts and fish but deficit in grains. The T>A substitution in *MT-ATP6* underscores the adaptation in protein structure to prioritize non-essential residue, potentially due to insufficient availability of essential amino acids in the local diet. The dietary constraints coupled with other evolutionary factors might contribute to the region-specific variations in mitochondrial genome.

Our observation supports the hypothesis that certain mtDNA variants facilitate individuals to adapt to various geographical and nutrient-deficit conditions, enabling the regional enrichment of certain mtDNA lineages. The observed variants in mtDNA could play an essential role in determining the energy utilization and metabolic efficiency of the Pakistani population. As the subjects in our study were primarily from the province of Punjab, future research with a diverse and broader cohort could highlight the linkage between regional dietary practices

and mitochondrial genome variations.

CRediT authorship contribution statement

Mishal Tariq: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sheza Javaid:** Writing – review & editing, Visualization, Software. **Fatima Mohsin:** Writing – review & editing, Visualization, Software. **Gull e lalah Saleem:** Writing – review & editing, Resources, Data curation. **Muhammad Mustafa:** Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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