

Identification of Haplogroups and Molecular Markers in Skeletal Samples Excavated from the Ancient City of Resulođlu (Uđurludađ, orum)

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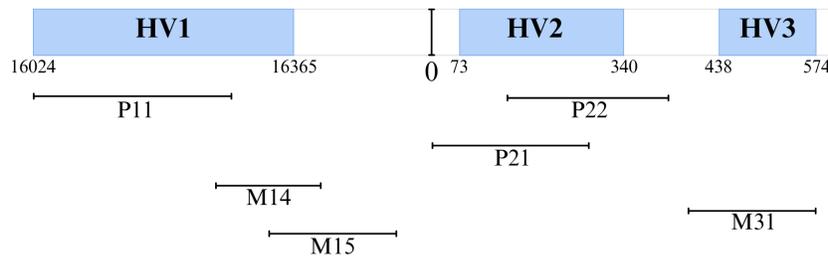
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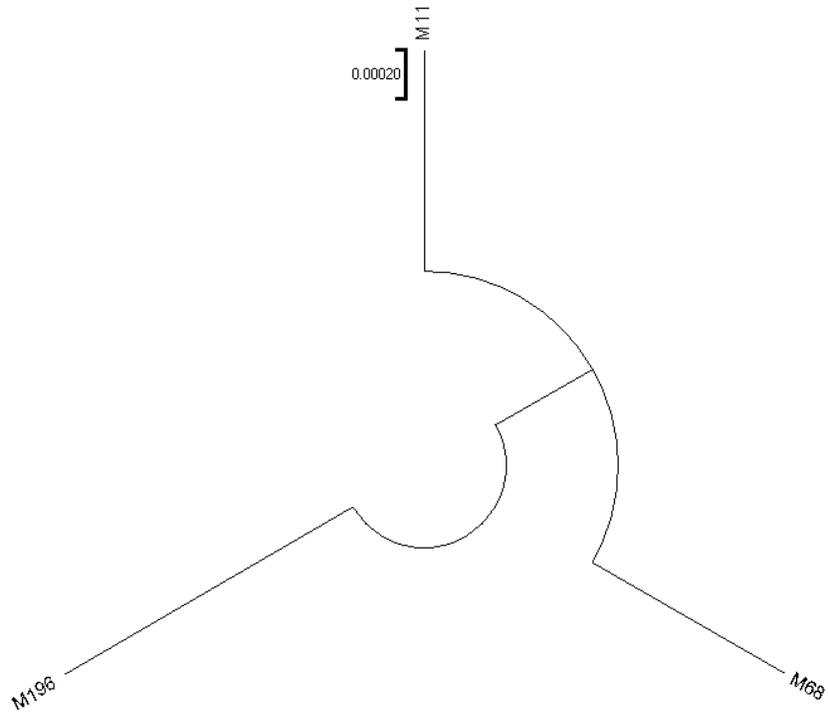
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Abstract

Mitochondrial DNA analyses were carried out in order to determine the haplogroups of 6 individuals obtained from the cemetery of Resulođlu and dated to the Early Bronze Age (EBA III). The individuals to be included in the bioinformatics analysis were evaluated according to their sequence quality and it was decided that 3 individuals could be used in further analysis. Using bioinformatics tools, it was determined that three individuals belonged to the T2e+152, H32 and JT haplogroups. These results support a link between the origins of the present-day European population and the farmers of the Anatolian Neolithic period. Furthermore, a detailed analysis of single nucleotide polymorphisms revealed T16189C and C150T mutations in the two of three individuals, which are associated with the risk of melanoma and cervical cancer - HPV infection. These molecular findings are consistent with the health profiles of the excavated skeletons, which indicate that the community struggled with infectious and metabolic diseases. The entire study was carried out in the Ancient DNA and Metagenomics Research Laboratory of the Department of Molecular Biology and Genetics, Istanbul University. Keywords: Ancient DNA, Mitochondrial DNA, Single Nucleotide Polymorphism, Haplogroup







Identification of Haplogroups and Molecular Markers in Skeletal Samples Excavated from the Ancient City of Resulođlu (Uđurludađ, Çorum)

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Mitochondrial DNA analyses were carried out in order to determine the haplogroups of 6 individuals obtained from the cemetery of Resulođlu and dated to the Early Bronze Age (EBA III). The individuals to be included in the bioinformatics analysis were evaluated according to their sequence quality and it was decided that 3 individuals could be used in further analysis. Using bioinformatics tools, it was determined that three individuals belonged to the T2e+152, H32 and JT haplogroups. These results support a link between the origins of the present-day European population and the farmers of the Anatolian Neolithic period. Furthermore, a detailed analysis of single nucleotide polymorphisms revealed T16189C and C150T mutations in the two of three individuals, which are associated with the risk of melanoma and cervical cancer - HPV infection. These molecular findings are consistent with the health profiles of the excavated skeletons, which indicate that the community struggled with infectious and metabolic diseases. The entire study was carried out in the Ancient DNA and Metagenomics Research Laboratory of the Department of Molecular Biology and Genetics, Istanbul University.

Keywords: Ancient DNA, Mitochondrial DNA, Single Nucleotide Polymorphism, Haplogroup

Introduction

The Resulođlu settlement and cemetery, located northwest of the village of Resulođlu (Kaleboynu) in the Uđurludađ district of the province of Çorum, in the north of Central Anatolia, dates to the second half of the 3rd millennium BC and is one of the rare cemeteries that can be systematically investigated. According to surface finds in the southeast, north and northwest of the cemetery, it is dated to the last period of the Early Bronze Age (EBA III) (Atamtürk & Duyar, 2009). Carbon 14 samples taken from different parts of the settlement yielded dates of 2500/2400-2100/2050 BC (Dardeniz & Yıldırım, 2022).

Human mitochondrial DNA, a circular double-stranded structure of 16569 nucleotides and 37 genes, encodes 13 protein subunits of the electron transport chain, 2 rRNAs and 22 tRNAs, in addition to a non-coding control region called the D-loop (displacement loop), which is responsible for transcription and regulation of mtDNA (Taanman, n.d.). This region contains three short sequences (HV1, HV2, HV3), the so-called hyper-variable control region (HVR), which show high population-level variation compared to other regions of the genome (Brandstätter et al., 2004; Krebs et al., 2018). HV regions, which are used in ancient DNA (aDNA) analysis due to their comparably high levels of polymorphism, form geographic

patterns according to the mutations they contain. Groups of gene sequences from a common ancestor with the same SNPs (single nucleotide polymorphisms) are called haplogroups (Carelli et al., 2006).

Mitochondrial DNA (mtDNA), which is easier to obtain than the nuclear genome, is preferred in molecular anthropology studies due to its high copy number in eukaryotic cells (Pakendorf & Stoneking, 2005). However, the fact that mtDNA shows maternal inheritance (Manfredi et al., 1997), does not undergo recombination (Ingman et al., 2000), and the mutation rate of the non-coding d-loop region is quite high (Carelli et al., 2006) are the main factors that have led to the widespread use of the mitochondrial genome in aDNA studies (Pakendorf & Stoneking, 2005).

In addition, it has long been hypothesized that the functional diversity of mitochondria, which play an important role in energy metabolism, initiation of apoptosis, and generation of reactive oxygen species (ROS), may influence the development and progression of cancer (Carew & Huang, 2002). It is thought that mutations or inherited polymorphisms in mtDNA can alter the encoded protein subunits of respiratory chain complexes, leading to altered ROS production and accelerating a series of events, including impaired respiratory chain activity. Further ROS production activates a vicious cycle of oxidative stress that may play a role in tumor initiation and progression (Birch-Machin, 2006; Ishikawa et al., 2008; Modica-Napolitano et al., 2007).

Somatic mtDNA mutations have been found in numerous malignancies, including breast, ovarian, endometrial, prostate, colon, gastric, thyroid, renal, hepatocellular, esophageal, pancreatic, and brain tumors (Carew & Huang, 2002; Chatterjee et al., 2006; Kulawiec et al., 2009; Penta et al., 2001). In addition, the D-loop region (nucleotides 16024-516) has been identified as a mutational hotspot in human cancers (Parsons et al., 1997; Yoneyama et al., 2005a). There is strong evidence that genetic instability in the D-loop region plays a role in carcinogenesis by affecting mtDNA copy number and gene expression (Lee et al., 2004).

In our study, the DNA of tooth samples obtained from skeletons excavated in Resuloğlu (Uğurludağ, Çorum) was isolated and the HV1, HV2 and HV3 regions of the d-loop region of the mitochondrial genome were sequenced and bioinformatics analysis was performed. Using the obtained data, mitochondrial haplogroups were determined with the help of MITOMASTER and hereditary diseases with possible mitochondrial origin were identified.

Materials And Methods

The aim of this study was to amplify and sequence the HV1, HV2, and HV3 regions of the mtDNA D-loop region extracted from the skeletons excavated in the ancient city of Resuloğlu (Uğurludağ/Çorum) and to perform molecular analyses based on these sequences.

In this study, the appropriate clothes of the person who would be in the sterile room in the laboratory were sterilised with UV and the study was started using sterile mask and cap. Subsequently, tooth samples from six different individuals were cleaned with bleach and treated with 100% ethanol for 30 minutes, followed by three washes with pure water. The samples were then exposed to UV light in both directions for a total of 30 minutes. After surface sterilization, the root portions of the teeth were cut with a sterile-tipped Dremel and pulverized with liquid nitrogen in a sterile environment. Prior to DNA isolation, a decalcification protocol was used to remove calcium from the powdered samples by treating them with a solution of 0.5M EDTA (pH: 7.5) at a ratio of 10 ml EDTA per 10 g tooth powder. After this process, DNA isolation

was performed using the Genomic DNA Isolation Kit (LOT: 0721-OY-1464) from HİBRİGEN, with the amounts of certain solutions in the tissue procedure optimized according to the sample amounts. The concentration and purity of the isolated DNA was determined using Thermo Scientific Nanodrop 2000 spectrophotometer. In addition to the ancient individuals, DNA was also isolated from the researcher who conducted the study in the laboratory for control purposes using the HİBRİGEN Saliva DNA Isolation Kit (LOT: MG-TDNA-01).

To accomplish amplification of the entire D-loop region of mitochondrial DNA from the isolated DNA samples, six specific primers were designed using the NCBI Primer Blast online program. The amplified region (amplicon) generated by the primers was determined using the Snapgene program, and the primer sequences were constructed (Table 1).

Table 1. List of primers used in PCR.

OLIGONAME	5'-3'
P11F	CCCAAAGCTAAGATTCTAAT
P11R	CTTTGGAGTTGCAGTTGATG
P21F	CACCCTATTAACCACTCACG
P21R	GCTGTGCAGACATTCAATTGTT
P22F	TATTTATCGCACCTACGTTCA
P22R	CTGGTTAGGCTGGTGTTAGG
M14F	ACCCCTCACCCACTAGGATA
M14R	GAGGATGGTGGTCAAGGGA
M15F	CCTCAGATAGGGGTCCCTTG
M15R	GGGAACGTGTGGGCTATTTA
M31F	TCTTTTGGCGGTATGCACTTT
M31R	GTGTCTTTGGGGTTTGGTTG

The positions of the PCR amplified amplicons on the d-loop HV regions of the human mitochondrial genome are shown below (Figure 1).

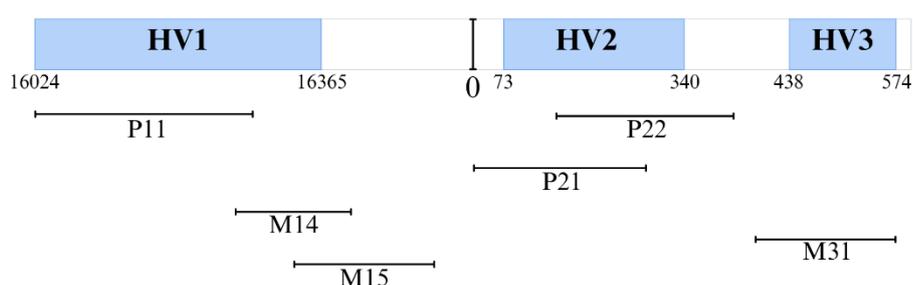


Figure 1. Positions of targeted amplicons on mtDNA with designed primers.

PCR experiments were performed separately for each amplicon according to a modified protocol of Kim et al. (2013) based on the T_m temperatures of the primers (Kim et al., 2013). The reactions were performed according to the specifications listed in Table 2.

Table 2. The performed PCR protocol.

	Time	Loop	Tempature
Initial denaturation	30 seconds	1	94°C
Denaturation	1 minute	40	94°C
Annealing	1 minute		P11 - 55°C-51°C
			P21 - 57°C
			P22 - 61°C-55°C
		M14 - 59°C	
		M15 - 61°C	
		M31 - 58°C	
Extension	1 minute		72°C
Final extension	10 minutes	1	72°C

The sequencing of the amplified D-loop region amplicons was performed by BM Laboratuvar Sistemleri (BMLabosis BM Lab. Sist. Ltd. Şti.) using the Sanger sequencing method. The resulting Sanger sequence data were analysed according to quality scores and the poor quality ends of the sequences were trimmed in SnapGene software (www.snapgene.com). After trimming, the sequence data were aligned to the d-loop region of human mitochondrial DNA obtained from the NCBI database (NCBI Reference Sequence: NC_012920.1) using the NCBI Blast software (Altschul, et al., 1990). Subsequently, the gaps were filled according to the d-loop region and a continuous sequence was obtained. Haplogroup and molecular marker identification was performed on the processed sequence data following these analyses using the MITOMASTER software (Brandon et al., 2009).

Results

Prior to the determination of haplogroups and molecular markers, the suitability of the raw sequence data obtained for analysis was assessed. After evaluating the usability of the sequence data, it was concluded that only the amplicon sequences obtained with primers P11, M14 and P21 from PCR studies performed with DNA isolated from M68 and M11 individuals could be used. It was also concluded that the amplicon sequences obtained with primers P11, M14, M15, P21 and P31 from the PCR reaction run with the ancient DNA obtained from M196 individual could be used. In the PCR run using DNA isolated from the control individual, the amplicon sequences obtained with primers P11, P13, P22 and M31 were considered suitable for further analysis.

The mitochondrial genome analysis revealed the following haplogroups of the three ancient individuals from six samples: Sample M68 was assigned to haplogroup H32, M196 to JT and M11 to T2e+152 (Table 3). Three of the six individuals from which raw sequence data were obtained, M7, M17 and M172, were not included in the analysis since the quality scores of the sequence data were very low. The analyses also revealed that the haplogroup of the control individual was H2a2a. In addition to the haplogroups identified, various molecular markers were also detected in individuals (Table 3).

Table 3. Haplogroups and descriptive variations.

Individual	Haplogroup	Variations
M68	H32	A73ATG, T152C
M196	JT	A73G, G16049GG, T16126C, T16189C
M11	T2e+152	A73G, C150T, T152C, T16126C, A16254AAAA(=C16251CAAA)
Control	H2a2a	A263G, C315CC, G16255GA

To demonstrate the relationships between these ancient individuals, the MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura et al, 2021) was used to construct the phylogenetic tree using the Maximum Likelihood method (Figure 2). The Maximum Likelihood method is an estimation method that selects the most probable parameters within the statistical model used (Rossi, Richard J. 2018).

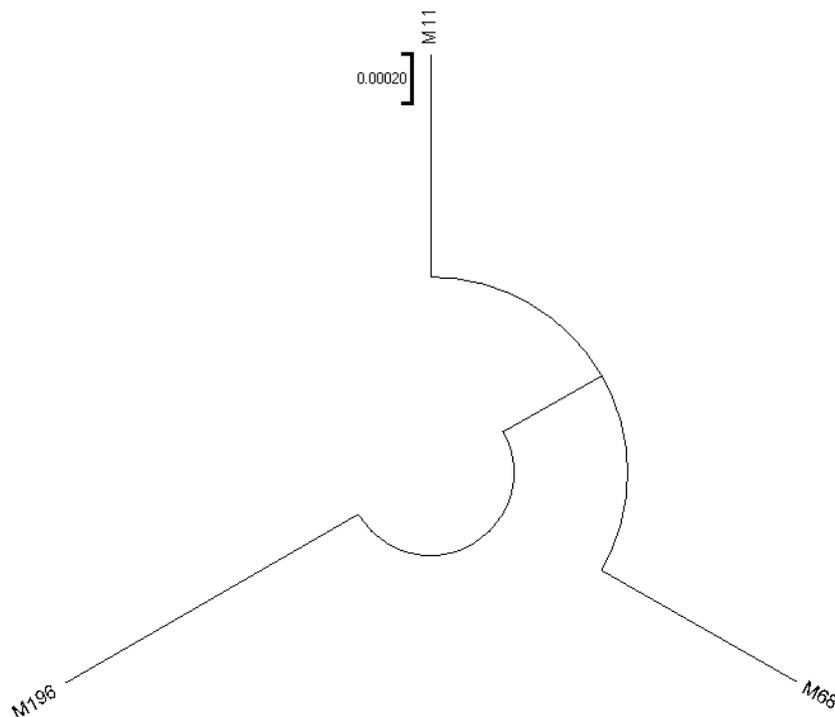


Figure 2. Phylogenetic tree constructed using the maximum likelihood method.

Discussion

During the spread of humans from Africa to other continents, rapid changes in mtDNA at the level of small founder populations, founder events, and genetic drift shaped haplotype frequencies, resulting in haplogroups and sub-haplogroups restricted to specific geographic areas and/or populations. Most likely, with the exception of haplogroups U5 and V, all mtDNA haplogroups common in Europe (H, I, J, K, T, U2e, U3, U4, X and W) originated in the Middle East, and it has been suggested that individuals reaching the Middle/Upper Paleolithic after colonization and subsequent settlement 40-45 thousand years ago mixed through Neolithic dispersal or close contact (Richards et al., 2000; Torroni et al., 1993).

Haplogroup H, which constitutes approximately 40% of the current European population and has a very high frequency in almost the entire distribution area, originated from haplogroup HV just before the last glacial maximum. This group, which is about 25-30 thousand years old, originated in western Asia and spread from there to central Asia, Europe and East Africa (Achilli et al., 2004; Roostalu et al., 2007). H2, the subhaplogroup of haplogroup H, originated in Central Asia and Eastern Europe between 10,300 and 13,300 years ago, then diverged into haplogroup H2a between 8,900 and 12,200 years ago, and then into haplogroup H2a2 between 7,300 and 11,000 years ago. H2a2a1, a subgroup of H2a2, is thought to be 5,000-9,100 years old (Behar, Harmant, et al., 2012; Behar, van Oven, et al., 2012).

The T haplogroup is now thought to have diverged from the JT haplogroup in western Asia about 20,500-29,800 years ago, and from the J haplogroup about 29,400-39,100 years ago. The T2 haplogroup, a subgroup of the T haplogroup, diverged about 16,800-21,900 years ago. The J haplogroup first diverged into the J1 subhaplogroup between 21,700 and 32,200 years ago, and the J1b haplogroup, derived from this subgroup, emerged during the LGM between about 12,800 and 19,700 years ago. It is not yet clear how many years ago J1b7, a subbranch of J1b, emerged (Behar, van Oven, et al., 2012).

Previous studies have shown that the majority of present-day European ancestry is derived from three main sources: the Mesolithic hunter-gatherer lineage in Europe, the Neolithic lineage in northwestern Anatolia, which is closely related to the emergence of agriculture in Europe, and the steppe lineage, which is a mixture of Upper Palaeolithic hunter-gatherers in the Caucasus and early farmers in northern Iran (Haak et al., 2015; Hofmanová et al., 2016; Jones et al., 2015; Kılınc et al., 2016; Lazaridis et al., 2016; Mathieson et al., 2015). However, in 2018, Mathieson I et al. showed that 105 of 215 new individuals reported from Paleolithic, Mesolithic, and Eastern European Neolithic contexts are almost exclusively associated with the hunter-gatherer lineage, 98% of the Balkan Neolithic population is associated with the Northwest Anatolian Neolithic, and Greek Neolithic individuals dating to ca. 4000 BC are more closely related to the Upper Palaeolithic hunter-gatherer-related lineage in the Caucasus than are Northwest Anatolian Neolithic and Balkan Neolithic individuals (Mathieson et al., 2018).

Considering the origin of the modern European population and the spread of the most frequent haplogroups H, T, and J from western Asia to Europe, the presence of these haplogroups in three individuals and the dating and location of the Resuloğlu cemetery support the migration movement from Asia to Europe through the Black Sea during the Neolithic. This finding is consistent with the general understanding of population history and migration patterns during

this period. However, since the ancient DNA obtained was in the form of fragments, not all amplicons that were intended to be replicated in all individuals could be replicated. For this reason, we would like to point out that the haplogroups identified are not completely definitive and that haplogroups belonging to sub-branches of the haplogroups obtained in this study can be obtained with the amplicon analyses that can be added to the individuals. As mentioned above, even in this state, the general haplogroup results obtained show a high degree of accuracy considering the history of the geographical region.

In addition, further analysis of the variations found in the individuals revealed the presence of T16189C and C150T mutations in two individuals (M196, M11), which have previously been associated with an increased risk of melanoma and cervical cancer-HPV infection. These findings are in line with studies that have examined diseases that leave traces on skeletal remains to determine the health profile of the population. The studies indicate that the community dealt with various infections and metabolic diseases, and our findings clearly show that some of these diseases are based on genetic mutations and variations (Atamtürk & Duyar, 2009, 2010). The marks of the diseases observed on the skeletal remains of individual M196 are shown below (Figure 3).



Figure 3. The disease marks observed on the skeletal remains of individual M196.

The T16189C variant in the human mitochondrial DNA control region has been associated with various diseases, including endometrial cancer (Liu et al., 2003), as well as several other multifactorial diseases (Khogali et al., 2001, 2001; Mueller et al., 2011, 2011; Weng et al., 2005). The T to C substitution at position 16189 often results in the formation of a continuous poly-C tract between nucleotides 16180 and 16195 within the D-loop region, leading to heteroplasmic length variations of the poly-C tract in different mtDNA molecules (Berger et al., 2011, 2011; Mueller et al., 2011). Lial, et al. (2010) showed that different poly-C variants show differences in the average mtDNA copy number (Liou et al., 2010). Since the 16189 nucleotide is very close to the termination-associated sequence of the D-loop region, it is suggested that the T16189C variant may affect mtDNA replication (Poulton, 2002; Roberti et al., 1998). A case-control study conducted by Ebner et al. in 2011 showed that the T16189C variant has a higher incidence in melanoma patients compared to controls (Ebner et al., 2011).

The C150T variation has been identified in tumor sequences in a number of studies (Chen & Kadlubar, 2004; Yoneyama et al., 2005b, 2005b). The C150T polymorphism was found to increase the risk of cervical cancer in a study conducted by Zhai K et al. in 2011, which examined D-loop sequence variations in Chinese women, including 142 cervical cancer patients and 136 controls, both HPV-positive and HPV-negative. In addition, HPV-positive individuals

were found to be more likely to carry the C150T polymorphism than HPV-negative individuals (Zhai et al., 2011). However, although the C150T variant has been associated with longevity in several previous studies, the mechanism behind this association remains uncertain (MITOMAP, 2009; Santoro et al., 2006, 2006; Zhang et al., 2003).

The general perception is that cancer is a disease of the modern era. However, the oldest evidence of hominin cancer was found in the skeleton of *Australopithecus sediba*, dated to 1.98 million years ago in the Malapa region of South Africa. Cancer cases from ancient times contradict this perception (Lieverse et al., 2014a 2014a; Randolph-Quinney et al., 2016). Certain types of tumors can leave their mark on bone (Bass, 1983). Apart from a case of prostate cancer detected at the protein level in a study by Schultz et al. in 2007, previously reported cases of ancient carcinoma have been identified by morphological observations of the effects of cancer on bones (Lieverse et al., 2014b 2014b; Schultz et al., 2007). Here we report the possible cases of malignancy identified at the molecular level in ancient samples.

This study is one of the first aDNA studies conducted in the region in terms of determining the origins of ancient civilizations and the first detection of malignancy at the molecular level in ancient individuals obtained from the site. It is a groundbreaking study for its encouraging interdisciplinary work in providing supportive data to the fields of archaeology and anthropology.

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Author Contribution

BS, SEM, SY, EA, Investigation, Methodology, Formal analysis; DA, İD, EA, Data curation, Resources, Supervision; BS, SEM, SY, DA, İD, EA, Writing the original draft, Review and editing the draft; EA, Project Administration, Funding acquisition

Data Availability Statement

The data that support the findings of this study are openly available in figshare at <https://doi.org/10.6084/m9.figshare.23733588.v2>

Conflict of Interest Statement

All authors declare that they have no conflicts of interest.

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