

## Analysis of Mitochondrial DNA Mutation in Pakistani Women with Gestational Diabetes Mellitus

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### Abstract

Gestational diabetes mellitus (GDM), which is considered the most prevalent endocrine condition in pregnancy, has been linked to insulin resistance. Several traditional risk factors for GDM have been confirmed, including lifestyle and environmental factors, while recent studies have focused on the genetic factor, such as mitochondrial DNA (mtDNA) mutation and its association with GDM. Previous research has found a relationship between mutations in the tRNA<sup>Lys</sup> and tRNA<sup>Leu(UUR)</sup> genes and diverse types of diabetes. However, the analysis of mutations in the mitochondrial tRNA<sup>Leu(UUR)</sup> gene and its relationship with GDM patients in Pakistani women is not well investigated. The goal of this study was to investigate whether there is a relationship between the A3243G tRNA<sup>Leu(UUR)</sup> gene mutation and GDM in Pakistani women. We selected 20 GDM pregnant women for this investigation, and DNA was extracted from their saliva. The mitochondrial tRNA<sup>Leu(UUR)</sup> gene was amplified using PCR with specified primers, and 10 samples from different families were sequenced. The present study did not find the A3243G tRNA<sup>Leu(UUR)</sup> mutation in Pakistani GDM women. Further studies are needed for confirmation. (**International Journal of Biomedicine. 2022;12(3):438-443.**)

**Keywords:** gestational diabetes mellitus • mitochondria • tRNA<sup>Leu(UUR)</sup> gene • mutation • PCR

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### Abbreviations

**AQ**, accurate quantification; **GDM**, gestational diabetes mellitus; **T2D**, type 2 diabetes; **mtDNA**, mitochondrial DNA.

### Introduction

Diabetes is a long-term condition marked by inadequate glucose, protein, and fat metabolism. From 135 million in 1995 and 171 million in 2000, the number of diabetics is expected to increase to 300 million in 2025 and 366 million in 2030.<sup>(1)</sup> By 2025, diabetes will be the most common disease in India, China, and the United States. In emerging economies, the number of

diabetics is predicted to grow by 170 percent, from 84 million in 1995 to 228 million in 2025.<sup>(2)</sup> Taiwan, Hong Kong, Singapore, and Mauritius are the countries with the greatest prevalence of newly diagnosed diabetes patients.<sup>(3)</sup>

In pregnant women, GDM manifests in the second and third trimesters and is associated with adverse perinatal outcomes and a high risk of future maternal T2D.<sup>(4)</sup> GDM affects about 7% of all pregnancies, resulting in over 200,000

new cases each year around the world.<sup>(5)</sup> This common and serious pregnancy condition has risks for both the mother and the fetus.

GDM is linked to a variety of adverse maternal outcomes, including gestational hypertension, cesarean section, weight gain, and preeclampsia.<sup>(6,7)</sup> Furthermore, women who develop GDM have a seven-fold increased risk of developing T2D later in life.<sup>(8)</sup> Moreover, their children are more likely to experience fetal and neonatal problems, such as macrosomia, preterm labor, respiratory distress, delivery trauma, neonatal hypoglycemia, and even perinatal mortality.<sup>(9,10)</sup>

Worse, the global burden of this disease is expanding at an alarming rate.<sup>(11)</sup> The negative consequences of gestational diabetes have prompted researchers to dig deeper into the disease's pathogenicity mechanism and functional genes to improve early detection and management. Mutations in mtDNA have recently been linked to a variety of disorders, including diabetes mellitus. Because mitochondria are inherited maternally, the role of mtDNA in disease pathogenesis may play a role in the increased maternal transmission of diabetes in some cases.

Van den Ouweland et al.<sup>(12)</sup> identified a large pedigree in which T2D, in combination with a sensorineural hearing loss, was maternally inherited and associated with the heteroplasmic m.3243A→G mutation in the tRNA<sup>Leu(UUR)</sup> gene, accounting for over 1% of diabetic patients in some populations.<sup>(13-15)</sup> T2D has now been associated with several additional mutations in the tRNA<sup>Leu(UUR)</sup> gene and other mtDNA regions, and there is a mounting body of evidence that mitochondrial genome abnormalities are linked to this condition.<sup>(16)</sup> Furthermore, the most common transition in the tRNA<sup>Leu(UUR)</sup> gene is A3243G, indicating an alteration in Leu-tRNA codon recognition, mitochondrial structure, and amino-acylation.<sup>(17)</sup> About 80% of patients with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) carry this mutation, which causes a variety of symptoms such as cardiomyopathy, deafness, exercise intolerance, and diabetes.<sup>(18)</sup> A study published 2 decades ago described a maternally inherited, adult-onset condition (maternally inherited myopathy and cardiomyopathy, MIMyCa).<sup>(19)</sup> Clinically, it is characterized by a heterogeneous combination of skeletal and cardiac muscle failure, as well as a heteroplasmic point mutation in the tRNA<sup>Leu(UUR)</sup> gene. The A3243G tRNA<sup>Leu(UUR)</sup> mutation is an underlying cause of maternally inherited diabetes and deafness (MIDD) syndrome and MELAS syndrome. The major objective of the present work was to investigate mutations in the tRNA<sup>Leu(UUR)</sup> gene in Pakistani GDM women and compare the amplified sequences to the mitochondrial reference sequence.

## Materials and Methods

The study was approved by the Ethics Committee of the Hazara University (Mansehra, KPK, Pakistan). Written informed consent was obtained from all participants.

We found 20 pregnant ladies from the Ayub Medical Complex's Department of Obstetrics and Gynecology in Abbottabad who were interested in participating in our study. The individuals had to be diagnosed with GDM in the second

or third trimester of pregnancy and be of Pakistani nationality to be included.

### Collection of saliva sample

Before sample collection, the teeth of each patient were brushed, and a 5% sugar solution was given to them. Then a total of 4 ml saliva samples were obtained in 5 ml sterile cups from the 20 GDM patients. The samples were transported to the Molecular Genetics Laboratory at Hazara University(Mansehra), where the samples were stored at -20 °C for further analysis.

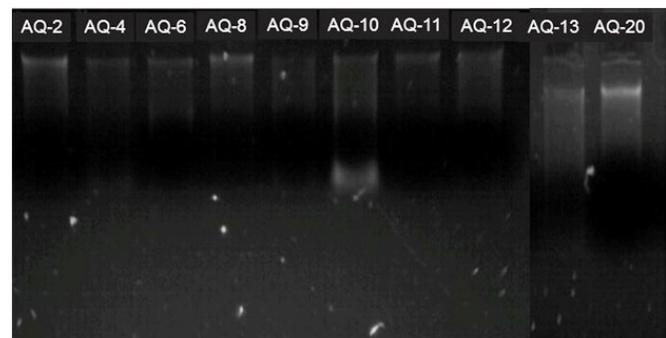
### Molecular analysis

The whole DNA of buccal cavity epithelial cells was extracted, according to Aidar & Line.<sup>(20)</sup> For checking the quality of extracted DNA, agarose gel (0.5g of agarose dissolved in 29.4ml DDH<sub>2</sub>O with 600ul of 50X TAE mixture) with 25ul ethidium bromide was used; then, 5μl of extracted DNA was dissolved with 2μl of loading dye, and the gel was run for 30 minutes at 60V before being photographed under ultraviolet light using the gel documentation system. After that, the DNA was kept at -20°C until it was time to process it.

PCR was used to amplify the desired gene. Initial denaturation was at 95°C for 5 minutes, followed by denaturation at 95°C for 5 minutes, annealing at 50°C for 45 seconds, extension at 72°C for 5 minutes, and final extension at 72°C for 5 minutes; these were the thermal cycling conditions followed for 40 cycles. The final PCR findings were analyzed on a 1% agarose gel. The mixture was then treated with 12ml of ethidium bromide. For cooling, the melted mixture was kept at 25°C. The agarose mixture previously had been put on the gel plate and allowed to solidify. We combined 15ml of PCR product with 2ml of DNA loading blue dye and put it into the agarose gel wells. A 65V was supplied for 25 minutes in an electrophoresis approach until DNA fragments move from left to right. The bands that had been magnified were photographed and inspected under ultraviolet light. To purify the PCR amplification product from an agarose gel, a TIAN gel Midi purification Kit (Cat # DP20902) was used. PCR band including the tRNA<sup>Leu(UUR)</sup> gene was cut with a sterilized surgical blade and kept in labeled Eppendorf tubes until further processing.

## Results

Even though all of the samples were processed for DNA extraction, only 10 of them produced results (Figure 1).

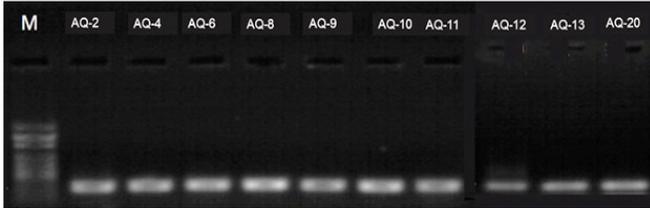


**Fig. 1.** Total genomic DNA extracted from samples.

tRNA<sup>Leu(UUR)</sup> gene amplification

The common primer pair (5'-CAAATTCCTCCC TGTACGAAAGG-3'; 5'-AATGAGGAGTAGGAGTT GGCC-3') was used to amplify the mitochondrial tRNA<sup>Leu(UUR)</sup> gene.

Figure 2 shows the amplification of a 279-base-pair fragment.



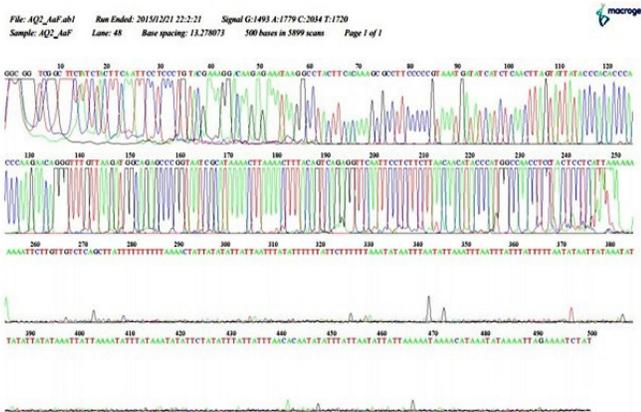
**Fig. 2.** PCR amplification of tRNA<sup>Leu(UUR)</sup> gene. Amplification of a 279 bp fragment. M, molecular size ladder; 1000 bp DNA ladder.

Sequencing analysis

Ten eluted DNA samples were sent to Macrogen Inc., Korea, for sequencing. The resultant sequencing data was compared to the whole mitochondrial sequence rCRS Accession number NC-012920.1. The alignment was checked to see if there was a mutation in the tRNA<sup>Leu(UUR)</sup> gene.

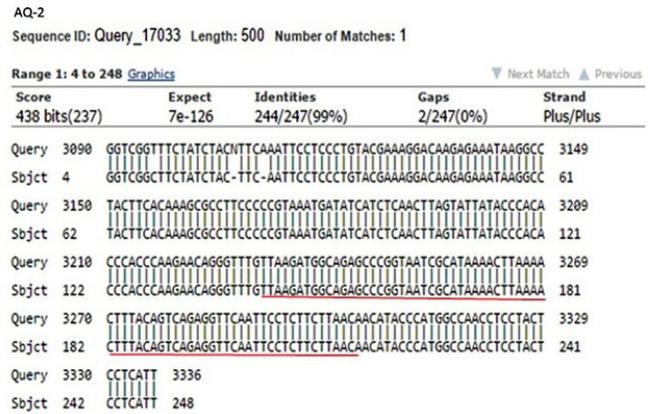
Patients

The Ayub Medical Complex's Department of Obstetrics verified all volunteers. Patients ranged in age from 30 to 39 years (mean age of 34.9 years) and in weight from 50 to 61 kg (mean weight of 55.1 kg), according to records. The patients' histories showed that they had experienced GDM. There were no mutations discovered in any patient's tRNA<sup>Leu(UUR)</sup> gene. Although there was a two-nucleotide mismatch in the alignment when we studied the chromatogram of this sequencing result, we found that it was a technical error rather than an exact miss-match (Figure 3).



**Fig. 3.** Chromatogram of the AQ-2 sample as a sequencing result provided by Macrogen Inc. (South Korea).

The alignment of the patient's AQ-seq data is shown in Figure 4.



**Fig. 4.** The alignment of the patient's AQ-seq data. The Cambridge reference accession number was used to compare the sequence. The highlighted region indicates the tRNA<sup>Leu(UUR)</sup> gene.

**Discussion**

GDM is characterized as glucose intolerance that develops in pregnant women and disappears after birth. Shortly after delivery, glucose homeostasis returns to pre-pregnancy levels. However, afflicted women continue to have a higher chance of acquiring T2D in the future.<sup>(21)</sup> During pregnancy, as gestational age progresses (between 20 and 24 weeks of gestation), the size of the placenta increases. There is a rise in the levels of pregnancy-associated hormones, like estrogen, progesterone, cortisol, and placental lactogen, in the maternal circulation that is accompanied by increasing insulin resistance.<sup>(22)</sup> The fetus' fat and protein supplies are increased as a result of the combination of hyperinsulinemia and hyperglycemia, resulting in macrosomia.<sup>(23)</sup>

GDM causes mitochondrial dysfunction in the fetoplacental unit.<sup>(24)</sup> Mitochondrial diabetes can be a substantial contributor to T2D in some circumstances, while little is known about it.<sup>(25)</sup> As a result of this research, the idea of a genetic cure for diabetes has gained traction. In contrast, lyonization, imprinting, and the prenatal environment could play a role in maternal diabetes.<sup>(21)</sup> Nevertheless, mitochondrial dysfunction is a key pathogenic process in metabolic diseases like diabetes.

In most animals, including humans, although the sperm-derived paternal mitochondria enter the oocyte cytoplasm after fertilization, their mtDNA is never transmitted to the offspring. This pattern of mtDNA inheritance is well known as "maternal inheritance."<sup>(26,27)</sup> The current investigation, which focused on mtDNA alterations in GDM women in Pakistan, did not find the A3243G tRNA<sup>Leu(UUR)</sup> mutation in these patients.

According to genetic studies, T2DM is a multigenic condition in which common mutations interact with environmental variables to trigger the disease.<sup>(28)</sup> Point mutations and deletions in mtDNA have been associated with both GDM and T2DM, and have an impact on transcription and translation.<sup>(29)</sup> Large-scale epidemiological research utilizing unbiased propensity gene polymorphisms could show the in vivo link between candidate genes and complicated disorders.<sup>(30)</sup> One of

the most well-known environmental influences is obesity, which affects the occurrence of any disease in people, especially in women. In terms of age, the link between GDM and obesity has been widely demonstrated.<sup>(30)</sup> Alexandar et al.<sup>(31)</sup> conducted a meta-analysis of mitochondrial mutations in T2DM and GDM patients and found an association of mtDNA mutations T16189C, A12026G, A8296G, and A3243G with T2D. Although mtDNA mutations G15928A, T3394C, T3398C, A8344G, and G3316A showed association with GDM, the authors concluded that these mutations need to be verified in diverse populations. Results of a number of studies suggest that mtDNA mutations may contribute to the development of GDM in some patients.<sup>(32,33)</sup>

The A3243G tRNA<sup>Leu(UUR)</sup> mutation is one of the most common causes of mtDNA-related disorders.<sup>(34)</sup> In a study performed by Gal et al.<sup>(35)</sup> the frequency of the A3243G mtDNA mutation was investigated in patients with maternal sensorineural hearing loss, stroke-like episodes, ataxia, and myopathy with undetermined etiology. The authors screened 631 Hungarian patients between 1999 and 2008 for this mutation. The A3243G substitution was present in 6 patients in heteroplasmic form. The segregation analysis detected 8 further cases. The frequency of the A3243G tRNA<sup>Leu(UUR)</sup> mutation was 2.22% in the investigated patients. Dougherty et al.<sup>(36)</sup> reported a clinically heterogeneous, multigenerational pedigree with the syndrome of MELAS associated with the A3243G tRNA<sup>Leu(UUR)</sup> mutation and found that this mutation is not always associated with the classic MELAS phenotype and that other symptoms (notably cardiac and gastrointestinal abnormalities) should raise the suspicion of a mitochondrial disorder. Schleiffer et al.<sup>(37)</sup> reported an affected German MIDD pedigree with maternal lineage over 3 generations with a positive test for the A3243G tRNA<sup>Leu(UUR)</sup> mutation. The 27-year-old index patient was also diagnosed with chronic pancreatitis with pancreatic calcifications and pancreatic duct dilation on abdominal ultrasound and magnetic resonance cholangio-pancreaticography, although the patient was completely asymptomatic. Debray et al.<sup>(38)</sup> reported on a patient with Kearns-Sayre syndrome and recurrent episodes of acute pancreatitis associated with mitochondrial dysfunction. Toyono et al.<sup>(39)</sup> reported the first case of chronic pancreatitis associated with mitochondrial encephalopathy with the A8344G mtDNA mutation. At the same time, in a study by VERNY et al.,<sup>(21)</sup> none of the 36 unrelated patients with recurrent pancreatitis were carrying the A3243G tRNA<sup>Leu(UUR)</sup> mutation.

Our study has several limitations, such as only saliva samples were collected from patients with GDM, the sample size was small, and samples were collected from one region. Furthermore, in addition to the A3243G tRNA<sup>Leu(UUR)</sup> mutation, other mitochondrial DNA mutations should be examined in GDM patients. The above limitations should be addressed in future studies.

**In conclusion**, GDM patients frequently have mutations in their mtDNA. While the bulk of mtDNA mutations are one-of-a-kind and found only in a small number of people, some are more common than others. The key problem for researchers and physicians is to detect and simulate heteroplasmic mtDNA alterations in vitro, which can be difficult to do. So far, mitochondrial diabetes has been

associated with mtDNA regions involved in mitochondrial translational machinery, chromosome replication, and specific mitochondrial genes encoding important proteins, including NADH-ubiquinone oxidoreductase components. These changes appear to affect the number of copies of mtDNA as well as overall mitochondrial function, according to research conducted in cybrid, cellular, and animal models; they also impair energy production and increase ROS production. These mechanisms are particularly likely to be harmful to certain cell types, such as pancreatic  $\beta$ -cells. By analyzing the genomes of family members, it is now possible to predict the likelihood of disease development in offspring, allowing treatment and disease prevention to begin as soon as possible, thanks to rapid advances in sequencing technologies and a growing body of a thorough understanding of population-specific and unique mtDNA mutations. However, the present study did not find the A3243G tRNA<sup>Leu(UUR)</sup> mutation in Pakistani GDM women. Further studies are needed for confirmation.

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## Data availability statement

The sequences have been uploaded to the National Center for Biotechnology Information using accession numbers: NC-012920.1.

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## Competing Interests

The authors declare that they have no competing interests.

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