

## Lack of association of HFE gene polymorphism with high body iron status in Pakistani patients with type 2 diabetes mellitus

Jibran Sualeh Muhammad,<sup>1</sup> Najmul Islam,<sup>2</sup> Naseema Mehboobali,<sup>3</sup> Khalida Iqbal,<sup>4</sup> Iqbal Azam,<sup>5</sup> Mohammad Perwaiz Iqbal<sup>6</sup>

### Abstract

**Objective:** The Aim of this study was to investigate the relationship of 3 common polymorphisms in the HFE gene (C282Y, H63D and S65C) with high body iron status in a population of Pakistani subjects with type 2 diabetes mellitus (DM) and to explore if there is any novel mutation in HFE gene in a sample of Pakistani subjects with type 2 DM.

**Methods:** In a case-control design, 200 healthy controls and 200 consecutive adult subjects with type 2 DM (both gender; age range of 30-70 years) were enrolled with informed consent. Their serum samples were analyzed for body iron status (ratio of concentration of soluble transferrin receptor to ferritin concentration). DNA from blood was screened for HFE gene polymorphisms via polymerase chain reaction, followed by restriction fragment length polymorphism or via Sanger sequencing to identify any novel mutation(s) in HFE gene.

**Results:** We found that there was lack of any association between HFE polymorphism and body iron status in Pakistani subjects with type 2 DM and healthy controls. H63D was the most common polymorphism found in this population. Single base substitution of G nucleotide instead of C at the codon position 187 in the HFE gene exon 2 was discovered in one subject with DM. There was also a lack of association between D allele (variant allele of H63D) and type 2 DM. A significant relationship was found between CG genotype and abnormal albuminuria in subjects with type 2 DM ( $p = 0.036$ ).

**Conclusion:** In conclusion, HFE gene polymorphism is not associated either with high body iron status or type 2 DM in a hospital based Pakistani population and variant allele of H63D polymorphism appears to be associated with diabetic nephropathy.

**Keywords:** High body iron; HFE gene, Polymorphism, Type 2 diabetes mellitus, Pakistani population, Albuminuria. (JPMA 71: 608; 2021) DOI: <https://doi.org/10.47391/JPMA.563>

### Introduction

HFE gene is located on chromosome 6. Three missense mutations in HFE gene, C282Y, H63D, and S65C have been known to influence body iron status.<sup>1</sup> While being associated with haemochromatosis, their role (especially of HFE C282Y and HFE H63D) in the development of type 2 DM has been investigated in a number of Western populations. Moczulski et al. found high frequency of HFE 282Y allele in Polish subjects with type 2 DM compared to healthy controls and an association of HFE 63D allele with diabetic nephropathy.<sup>2</sup> Colli et al. reported association of HFE H63D polymorphism with high risk of type 2 DM in a Brazilian population.<sup>3</sup> Conversely, no association of HFE mutations with type 2 DM was found in populations belonging to United States and Turkey.<sup>4,5</sup>

A meta-analysis of studies carried out between 1997-2011 on the relationship of HFE polymorphism with the risk of type 2 DM showed no significant association of HFE C282Y with this disease.<sup>6</sup> However, carriers of HFE 63D allele had modestly increased risk of type 2 DM.<sup>7</sup> These reports were indicative of possible variation in the relationship of HFE polymorphism with type 2 DM from one population to another. Since no study had been carried out on South Asian populations to investigate the role of HFE polymorphism with type 2 DM, therefore, the objective of the present study was to find out the frequency distribution of 282Y, 63D, and 65C alleles in Pakistani subjects with type 2 DM and healthy controls and investigate if there is an association between genotypes of HFE C282Y, HFE H63D and HFE S65C polymorphisms with high body iron status and/or type 2 DM.

The possibility of unique mutation(s) in HFE gene (other than the above mentioned 3 polymorphisms) in Pakistani subjects with type 2 DM having an association with high body iron status could not be discounted on the basis of the findings by Pointen et al.<sup>8</sup> Therefore, another objective of the study was to explore the presence of any

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<sup>1</sup>Department of Basic Medical Sciences, College of Medicine, University of Sharjah, Sharjah, United Arab Emirates, <sup>2</sup>Department of Medicine, <sup>3,4,6</sup>Department of Biological and Biomedical Sciences, Medical College, <sup>5</sup>Department of Community Health Sciences, Aga Khan University, Karachi, Pakistan.

**Correspondence:** Mohammad Perwaiz Iqbal. Email: [Perwaiz.iqbal@aku.edu](mailto:Perwaiz.iqbal@aku.edu)

uncommon mutation(s) associated with high body iron status in Pakistani persons with type 2 DM through a small study.

There is some evidence that increased body iron could be associated with diabetic nephropathy, and the progression of this condition could be prevented through iron deficient diet.<sup>9,10</sup> Third objective of this study was to investigate any association between high body iron status and diabetic nephropathy in our study population of patients.

## Methods

This was a case-control study nested into a cohort study intended to investigate an association of high body iron status with the onset of type 2 DM in a hospital based Pakistani population. Purposive sampling technique was used in which subjects fulfilling in the inclusion criteria were included in the study. Prevalence of increased levels of serum ferritin (> 300 ng/ml) in healthy subjects has been found to be 2% in a previous study from our laboratory.<sup>11</sup> Assuming that there would be 6% further increase in the proportion of subjects with serum ferritin levels greater than 300 ng/ml among DM patients, the estimated sample size would be 202 in each group at  $\alpha = 0.05$  and power of 80%.<sup>12</sup> Therefore, we recruited 202 samples from subjects with type 2 DM (30-70 years) and 202 samples from gender and age (within 5 years) matched healthy controls. Two patients and 2 healthy controls were dropped because of some missing information. All 400 participants (200 control and 200 subjects with type 2 DM) from the study were included for the HFE gene polymorphism screening. Out of these, a small nested group of 10 patients and 10 controls, age and gender-matched, were selected randomly for HFE gene sequencing analysis.

Subjects with type 2 DM (144 males and 56 females) with an age between 30-70 years visiting the Endocrinology Clinics of the Aga Khan University Hospital were included. Diagnosis of type 2 DM was confirmed on the basis of clinical examination and biochemical data (fasting blood glucose > 126 mg/dl) as per guidelines of the International Diabetic Federation.<sup>13</sup> Majority of the patients included had the diagnosis of their disease during the last six months. Patients with a history of malabsorption syndrome, tuberculosis, liver diseases, uraemia, or cancer were excluded from the study. Clinical history and relevant biochemical tests were performed for inclusion or exclusion of subjects from the study. The control group (115 males and 85 females) included in this study consisted of normal healthy individuals, matched for age within 5 years. They were recruited from the

personnel of the Aga Khan University and other healthcare institutions in Karachi. Absence of diabetes mellitus and impaired glucose tolerance was confirmed on the basis of clinical examination and biochemical laboratory tests. Subjects with a history of gestational diabetes, polycystic ovary, hypercholesterolaemia, malabsorption syndrome, tuberculosis, uraemia, liver disease or malignancy were excluded from the study. Moreover, pregnant females taking iron supplements or subjects with history of blood transfusion during last six months were also excluded from both control and patient groups.

With prior written informed consent of the patients (cases) and/normal healthy subjects (controls), 10 ml blood was obtained. Five ml was transferred to a tube containing EDTA and the rest was transferred to a plain tube for serum collection. White blood cells (WBCs) were separated from the blood and used for isolation of genomic DNA. Plasma/serum was used for determination of ferritin, soluble transferrin receptor (sTfR), and glucose, using kit methods (Roche Diagnostics Corporation, Indianapolis, IN). Blood serum and plasma were kept frozen in small aliquots at -80°C until analysis. Albumin in urine was determined to assess degree of nephropathy in diabetic patients, while HbA1C was determined in whole blood to monitor the control of diabetes in these patients during the last 3 months.

Genomic DNA was extracted from 5 ml of whole blood using DNA isolation kit for mammalian blood (Roche Diagnostic Corp., Indianapolis, IN). Approximately 100 ng of genomic DNA was subjected to amplification using polymerase chain reaction (PCR) at three regions of the HFE gene carrying mutations C282Y, H63D and S65C followed by restriction fragment length polymorphism (RFLP) using RsaI, BclI, MboI and HinfI.<sup>8,14</sup>

Genomic DNA was subjected to PCR and HFE gene sequencing. Twenty samples (10 with high body iron status and 10 with low body iron status) were randomly selected from patients and healthy control groups. Selection was such that patients group had 5 samples with high body iron status and 5 with low body iron status. Similarly, the healthy control group also had 5 samples with high body iron status and 5 samples with low body iron status. UCSC Genome browser (<https://genome.ucsc.edu/>) was used to obtain the entire genomic sequence of HFE gene. Primers were designed along the intron-exon boundaries of all the six exons of HFE gene by using the software and protocol described previously.<sup>15</sup> All six exons of HFE gene were amplified and samples were sent to Eurofins Scientific., USA (<https://www.eurofins.com/genomic->

services/our-services/custom-dna-sequencing/) for performing Standard Sanger sequencing of our PCR products. PCR was carried out in a 20 µl reaction system with Absolute Master Mix (Moleculon, Auckland, New Zealand) using the primers targeting exon-intron boundaries of HFE gene. Sanger sequencing is the gold standard for sequencing technology that is very flexible for sequencing highly focused sets of genes or regions.

The present study was approved by the Ethical Review Committee of the Aga Khan University, Karachi, Pakistan.

Differences in allele and genotype distributions in the study groups were analysed by Chi-Square test. Two-way ANOVA was used to investigate the relationship of HFE H63D polymorphism with parameters of body iron status. For studying the relationship of HFE H63D polymorphism with glycaemic control in diabetes patients, Pearson's Chi-Square test was used, however, for investigating the relationship of HFE H63D polymorphism with albuminuria in diabetic patients, Fisher Exact test was employed.

Gene sequencing results were aligned with the HFE sequence in National Center of Biotechnology Information (NCBI) database and analysed for unique mutation(s) by comparing the data from patients and control samples. Later, bioinformatics software, polymorphism phenol typing v2 (polyPhen-2) (<http://genetics.bwh.harvard.edu/pph2>), was used to predict the potential pathogenic functions of the missense mutations only, and Mutation Taster (<http://www.mutationtaster.org/>) was used to analyse frame-shift mutations. Polyphen-2 is a tool for prediction of possible impact of an amino acid substitution on the structure and function of a human protein, SIFT is a tool that sorts intolerant from tolerant amino acid substitutions and predicts whether an amino acid substitution in a protein will have a phenotypic effect or not. Mutation Taster is a web-based application to evaluate DNA sequence variants for their disease-causing potential.

## Results

We found that there is no significant difference between the distribution of frequencies of genotypes of 3 HFE gene polymorphisms (C282Y, H63D, S65C) between cases and controls. Since the variant alleles in 2 polymorphisms, C282Y and S65C were in low numbers, further analysis to investigate any association of these genotypes with type 2 DM or body iron status could not be carried out. However, variant allele of H63D polymorphism (63D) was present in reasonable numbers in both subjects with type 2 DM and healthy controls. In order to investigate any relationship of genotypes of H63D with parameters of body iron status, the means of serum ferritin, sTfR and ratio of sTfR to ferritin were compared in subjects with these genotypes using Two-way ANOVA. As shown in Table-1, there appears to be an insignificant ( $p = 0.08$ ) increase in body iron status in subjects with homozygous ancestral genotype (CC) compared to heterozygous CG genotype in both persons with type 2 DM and healthy controls.

In order to investigate whether there is any relationship of H63D genotypes with glycaemic control in subjects with diabetes, the percentages of ancestral CC and heterozygous CG genotypes were compared in groups of patients with good, inadequate and poor glycaemic control on the basis of their HbA1C levels. Pearson's Chi-Square test revealed, no relationship between H63D genotypes and glycaemic control in Pakistani subjects with type 2 DM (Table-2;  $p = 0.24$ ).

In order to find out whether subjects with diabetes in Pakistani population with H63D mutation are more prone to developing diabetic nephropathy, we divided our patient population into 3 groups on the basis of urine albumin concentrations — normoalbuminuria, microalbuminuria and macroalbuminuria. The proportions of ancestral CC genotype of H63D and heterozygous CG genotype of H63D in these three groups were compared using Fisher Exact test. A significant relationship was found between CG genotype and abnormal albuminuria (Table-3;  $p = 0.036$ ).

**Table-1:** Relationship of parameters of body iron with genotypes of H63D polymorphism in HFE gene in diabetic patients and healthy controls (mean±SD).

Parameters*	Diabetic patients (n=200)		Healthy Controls (n=200)		Group	p value*	
	H63D Genotypes		H63D Genotypes			Genotypes	Group* genotypes
	Ancestral CC	Heterozygous CG	Ancestral CC	Heterozygous CG			
Ferritin (ng/ml)	141±126	125±10	70±63	83±66	0.15	0.904	0.249
sTfR (µg/ml)	3.61±2.21	3.81±1.78	3.39±1.57	3.1±1.55	0.32	0.88	0.262
sTfR/ferritin	83±223	109±202	151±303	185±439	0.03	0.087	0.904

\*p values were obtained by using Two-way analysis of variance (Two-way ANOVA)

Note: There were only 5 homozygous variant (GG genotype) in all the samples of this study. Because of this very small number they were not included in the final analysis. Values are mean±SD.

**Table-2:** Frequency distribution of genotypes of H63D polymorphism in HFE gene with respect to levels of HbA1C in subjects with type 2 DM (n=176).

HbA1C* (%)	H63D genotypes		p value**
	Ancestral CC (n=137) n(%)	Heterozygous CG (n=39) n(%)	
<7.0 (Good control)	45(32.8)	10(25.6)	0.24
7.0-9.0 (Inadequate control)	61(44.5)	15(36.5)	
>9.0 (Poor control)	31(22.6)	14(35.9)	

\*Refers to control of serum glucose.

\*\*Percentages were compared using Pearson's Chi-Square test.

**Table-3:** Frequency distribution of genotypes of H63D polymorphism in HFE gene with respect to levels of urinary albumin in subjects with type 2 DM (n=101).

Urine albumin (mg/l)	H63D genotypes		p value**
	Ancestral CC (n=79) n(%)	Heterozygous CG (n=22) n(%)	
Below 30 (normal)	34(43.0)	4(18.2)	0.03
31-300 (microalbuminuria)	37(46.8)	12(54.5)	
Above 300 (macroalbuminuria)	8(10.1)	6(27.3)	

\*Proportions in two groups were compared using Fisher Exact test.

Note: There were 3 samples among patients with homozygous variant GG genotype which were not included in the final analysis for being a very small number.

For exploring any novel mutations, HFE gene sequencing was carried out. In order to optimize experimental conditions, samples from 2 subjects with diabetes (one with high body iron status and one with low body iron status) and 2 control samples (one with high body iron status and another one with low body iron status) were used. HFE gene is located at the short (p) arm of chromosome 6 at position 22.2, from base pairs 26,087, 281 to 26,096,216, and the coding region of HFE gene consists of a total of 6 exons. The final PCR products of 20 samples (10 patients and 10 controls) were sent to Eurofins Scientific for DNA sequencing. After obtaining the DNA sequences, the sequence of each exon from all the samples was manually analysed and checked by the principal author for presence of any novel mutations in the coding region of HFE gene.

Among the 10 subjects' samples with diabetes mellitus, single base substitution of G nucleotide instead of C at the codon position 187 in the HFE gene exon 2 was discovered in one patient. This substitution resulted in a change of single amino acid from Histidine to Aspartic acid at position 63 (c.187C>G; H63D). Furthermore, this change in the amino acid sequence of the HFE protein due to substitution mutation is likely to affect several features of the final HFE protein having an overall pathogenic effect. However, among the 10 healthy control samples no polymorphism was observed.

Therefore, among the 20 samples sequenced in this study, the only polymorphism found was H63D which is the most common HFE gene polymorphism in Pakistani population.

## Discussion

This study revealed that H63D is the common mutation in HFE gene in this population. This was found in a female subject with type 2 DM with high body iron status. Type 2 DM is a common chronic condition, the aetiology of which is multi-factorial and not fully established yet. Several known risk factors exist; many of them are lifestyle-related factors such as being overweight and lack of physical activity. It has been suggested that high body iron status might also play a role in the development of type 2 DM. This hypothesis is originally driven from the fact that the prevalence of type 2 DM is quite high in patients with haemochromatosis, a hereditary disease characterized by iron overload.<sup>16</sup> HFE gene mutations are very common in patients with iron over-load disorders such as hereditary haemochromatosis (HH). A meta-analysis of 449 studies indicated an association of increased body iron stores with the risk for the development of type 2 DM.<sup>7</sup> Two mutations have been reported in HFE gene, which appear to be associated with iron over-load disorders. In up to 10% of alleles in the North European general population, cysteine has been replaced by tyrosine at amino acid 282 and the mutation is termed as C282Y. The second mutation has been observed to be around 13% in the United States and Slovenian populations, resulting in a substitution of aspartic acid in place of histidine at amino acid 63 and is termed as H63D.<sup>17,18</sup> The high correlation of these HFE mutations to the development of iron overload has led many researchers to consider HFE as a model for population-based genetic screening for iron over-load. Results of the current study showed that the allelic frequencies of the C282Y and S65C mutation in the study subjects were very low (0.5% to 2.0%). These observations are similar to the findings of a study on Brazilian population in which the prevalence of these (C282Y and S65C) mutations have been reported to be only 0.29% and 0.87%, respectively.<sup>19</sup> H63D polymorphism, instead, is the most common HFE gene polymorphism found in Pakistani population with percentages of CG genotypes to be 22.1% and 22.5% among healthy subjects and persons with type 2 DM, respectively. The proportion of D allele was slightly higher among persons with type 2 DM compared to healthy subjects, however, the increase was statistically insignificant. Rong et al. in their meta-analysis of 23 studies carried out between 1997-2011, reported a modestly increased odds ratio (OR=1.2) for type 2 DM in persons carrying D allele (variant allele) compared to

persons with ancestral allele.<sup>6</sup> However, Zhang et al. commented that the effect could only be regarded as very small according to the hierarchy for grading credibility of molecular evidence for complex diseases.<sup>19</sup> Our results also showed lack of association between D allele and type 2 DM in Pakistani population. Nevertheless, subjects with type 2 DM with D allele (CG genotype) were found to be more likely to develop albuminuria as compared to those patients with ancestral H allele (CC genotype). This is suggestive that patients harbouring the variant allele are more prone to developing diabetic nephropathy, a complication of type 2 DM. Only about 10-40% of persons with diabetes develop nephropathy and genetic predisposition to developing this complication has also been indicated by Breyer.<sup>20</sup> Two diabetic nephropathy genes - carnosinase (CNDP1) and cell motility 1 (ELM01) have already been reported.<sup>21</sup>

The results of the present study implicating the HFE gene point towards the role iron overload could be playing as a risk factor for diabetic nephropathy. These results corroborated findings by Moczulski et al. who have also shown that being carrier of H63D mutation is a risk factor for nephropathy in subjects with type 2 DM.<sup>2</sup> The mechanism by which overload of iron could be causing diabetic nephropathy has not been unravelled, however, it has been hypothesized that excess iron could be causing tubular damage in diabetic patients.<sup>22</sup> This is further supported by the results of a randomised trial in which a low-iron, carbohydrate-restricted and polyphenol-enriched diet compared to standard protein-restricted diet was shown to slow down the progression of diabetic nephropathy.<sup>23</sup> How H63D mutation could result into iron overload is not clear but it could be related to change in the HFE protein. This mutation involves substitution of a nucleotide at codon position 187 in the HFE gene exon 2 leading to a change in amino acid sequence at the position 63. This would result into an HFE protein lacking a few of its functional domains including one of the alpha-helices and its extracellular domain (Mutation Taster, <http://www.mutationtaster.org/>). Crystallography based studies have demonstrated that histidine at position 63 normally forms a salt bridge with 2 helix in the HFE protein which interacts with transferrin receptor, and this bridge is lost in H63D mutation. This might be leading to altered iron metabolism.<sup>24,25</sup>

In a case control study from Pakistan, it was observed that nephropathy was the most important complication of diabetes affecting approximately 90% of subjects with type 2 diabetes.<sup>26</sup> Our results pertaining to H63D polymorphism in Pakistani diabetic patients could be of value in the management of patients with variant allele.

Early identification of their genotype (with variant allele) would enable them to be more watchful about the management of their disease to obviate any risk of developing nephropathy.

## Conclusion

In conclusion, the percentages of HFE gene polymorphisms — C282Y and S65C were very low in Pakistani population. H63D polymorphism was most commonly found in this population, however it was not found to be associated with high body iron status in either persons with type 2 DM or healthy controls. No unique mutations in HFE gene were found in 20 samples analysed in this study. There is a lack of association between D allele (variant allele of HFE-H63D) and type 2 DM in this population. Nonetheless, subjects with type 2 DM having this variant allele are more likely to develop albuminuria as compared to those patients with ancestral H allele. Thus, patients with variant H63D polymorphism are more prone to develop diabetic nephropathy.

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**Conflict of Interest:** None to declare.

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