

Research Paper**The role of Hemochromatosis C282Y and H63D mutations in the development of type 2 diabetes mellitus in Greece**

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ABSTRACT

Several authors have suggested a positive association between diabetes type 2 (DM2) and the C282Y and H63D mutations of the hereditary hemochromatosis gene but others have disputed it. There are also papers reporting an increased iron load in diabetes type 2 and a possible association with the pathogenesis of the disease. We therefore performed a study in 100 type 2 diabetics and 100 age and sex matched controls to assess the possibility that C282Y and H63D mutations constitute a risk factor for DM2 in Greece. We also evaluated the iron load in 500 diabetes type 2 patients and 423 age and sex matched controls. We did not find any differences in the allele frequencies of the above mutations between patients with diabetes type 2 and controls. The allele frequencies were estimated to be 0.0075 for the C282Y and 0.115 for the H63D mutation. Subjects with even one mutation (C282Y or H63D) had higher transferrin saturation compared to those with no such mutations. This seems to apply to both diabetics ($49 \pm 8,6$ vs $44,5 \pm 5,4$, $p < 0,01$) and controls ($49,3 \pm 7,3$ vs $42,6 \pm 3,3$, $p < 0,01$). Patients with DM2 had higher transferrin saturation compared to the general population. These differences were found among men ($n=250$, mean \pm SD $31,8+11$ vs $n=73$, mean \pm SD $29,5+8$, $p=0,05$) as well as among women ($n=250$, mean \pm SD $28,5+10$ vs $n=350$, mean \pm SD $25,5+9,6$, $p=0.001$). The DM2 patients had higher ferritin levels compared to controls. In conclusion, DM2 patients have increased iron load. The C282Y and H63D mutations contribute to increased iron load in both DM2 and controls. There was no difference in the frequency of C282Y and H63D alleles between DM2 and controls in the Hellenic population.

Key words: iron, hereditary hemochromatosis, diabetes, C282Y, H63D.

INTRODUCTION

Hereditary Hemochromatosis (HH) is an autosomal recessive disorder characterized by excessive intestinal

iron absorption that leads to iron overload of parenchymal cells in many organs, including the liver and the pancreas and several mutations of the HFE gene (C282Y and H63D) have been linked to HH¹⁻⁶. It seems that homozygosity for the C282Y mutation of the HFE and compound heterozygosity C282Y/H63D can cause HH, but the prevalence of the above mutations varies significantly in different ethnic groups¹⁻⁶. Several studies have tried to test the hypoth-

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Received 06-12-02, Revised 27-12-02, Accepted 03-01-03

esis that heterozygosity for hereditary hemochromatosis-causing mutations could be a risk factor for the development of diabetes Mellitus type 2 (DM2). There are studies in favor of such an association and others that disprove it⁷⁻¹³. Although the penetrance of the C282Y mutation has recently been reported to be low¹⁴, heterozygosity might affect the iron load of the body¹⁵⁻¹⁶. As expected, Iron overload can negatively affect insulin secretion as well as insulin sensitivity¹⁷⁻¹⁹. There are indications that patients with DM2 are iron overloaded²⁰⁻²² and blood letting in high ferritin type 2 diabetic patients improves insulin sensitivity and beta cell function²³⁻²⁴. The prevalence of hereditary hemochromatosis in patients with type 2 diabetes varies significantly in various populations²⁵⁻²⁶. The aim of our study was to determine the prevalence of the C282Y and H63D mutations in the general Greek population and in patients with type 2 diabetes and to examine if the aforementioned mutations contribute to the iron load of the body in patients with type 2 diabetes in the Hellenic population.

METHODS

Study design

Part 1: 100 unselected patients with type 2 diabetes and 100 non-diabetics matched for age, sex and BMI were genotyped for C282Y and H63D mutations of the HFE gene. Participants were recruited from the diabetes center and the endocrine clinic of the university hospital of Patras.

Part 2: 587 outpatients with type 2 diabetes attending the diabetes center were screened for hemochromatosis by measuring iron and total iron binding capacity (TIBC) in the fasting state. 502 non diabetic outpatients attending the endocrine clinic for goiter served as the control group. Subjects with transferrin saturation more than 38% were genotyped for C282Y and H63D mutations. All participants were tested for hemoglobin, hematocrit, mcv, ferritin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, urea, and creatinine. Inclusion criteria for the diagnosis of diabetes were either fasting plasma glucose concentration greater than 126 mg/dl or plasma glucose concentration greater than 200 mg/dl at 2 hours in an oral glucose tolerance test.

Exclusion criteria for participation in the study were anemia, renal failure or any known inflammatory disease, which might have affected the iron metab-

olism, and alcohol consumption of more than 2-3 glasses of wine daily. Using these criteria, 87 diabetics and 79 controls were excluded from the study. The remaining 500 diabetics and 423 controls were enrolled.

Laboratory methods

Serum iron, total iron binding capacity (TIBC), ferritin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, urea and creatinine were measured with routine hospital laboratory assays from fasting samples. Transferrin saturation index was calculated according to the formula $\text{Iron} \times 100 / \text{TIBC}$.

DNA was extracted from whole blood using Nucleospin Blood QuickPure kit (Machery-Nagel). Oligonucleotides were constructed according to published DNA sequence (GenBank U630319): forward primer 5'-GGAGTTTCGAACCTAAAGACGT-3' and reverse primer 5'-AGGGCTCCCAGATCACAATG-3' for the C282Y mutation, and forward primer 5'-TCAGAGCAGGACCTTGGTCTT-3' and reverse primer 5'-ACTCTGACTCAGCTGCAGCCA-3' for the H63D mutation. 100 ng of genomic DNA was used as template in a PTC-200 thermocycler. PCR was performed in a total volume of 50 µl, using 10 pmol of each appropriate primer, 200 mM of each dNTP, 1.5 mM MgCl₂, 2.5 Units of Taq DNA polymerase and 10 x reaction buffer. After an initial denaturing at 95 °C for 5 min, reactions were subjected to 30 cycles of 45 sec denaturing at 95 °C, 45 sec annealing at 60 °C and 45 sec extension at 72 °C, followed by a final extension at 72 °C for 5 minutes.

The two fragments (one for each mutation) were amplified by polymerase chain reaction (PCR), then purified from the gel and subsequently digested with RsaI (C282Y) and DpnII. (H63D), respectively. Their products were analyzed on 2% agarose gel.

Statistical analysis

SPPSS 10 was used for the statistical analyses. Student's t test and Pearson's χ^2 test were used and p values less than 0.05 were considered significant.

RESULTS

1. Prevalence of C282Y and H63D among patients with type 2 diabetes and controls

In part 1 of the study, the prevalence of the above mutations in the general population and in patients

with DM2 were determined. The two groups were matched for age, sex, and BMI, and were unselected for transferrin saturation. The characteristics of these two groups as well as the frequencies of the two mutations are shown in table 1. The frequencies of the two mutations in diabetes type 2 group did not differ significantly from those in the general population (controls). Pooled data from the two groups were used to calculate the allele frequency for the two mutations. For the C282Y allele, 3 mutations were found out of 400 chromosomes tested and the allele frequency was estimated to be $3/400=0,0075$. For the H63D allele, 46 mutations were found out of 400 chromosomes tested and the allele frequency was estimated to be $46/400=0,115$.

2. Iron indices among type 2 diabetics

In part two of the study, 587 diabetes type 2 patients and 502 controls were screened for transferrin saturation, hemoglobin, hematocrit, MCV, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, blood urea and creatinine. From this pool, 87 diabetics and 79 controls were excluded from the study because they did not meet the inclusion criteria. The remaining 500 diabetics and 423 controls were enrolled. All of them had a repeat measurement of fasting transferrin saturation as well as serum ferritin determination.

The age, sex and BMI as well as the transferring saturation and the serum ferritin values in patients and controls are shown in table 2. Patients and controls were comparable in terms of age and BMI. Among men, the diabetics had higher transferrin saturation ($31,8\pm 11$ vs $29,5\pm 8$, $p<0,05$) compared to controls and their ferritin values were also significantly higher compared to controls (159 ± 80 vs 87 ± 51 , $p<0,001$). Among women, the differences in terms of transferrin saturation were more striking. Women with type 2 diabetes had mean transferrin saturation $28,5\pm 10$ compared to $25,5\pm 9,6$ of the control group, (p value $<0,001$) and their ferritin values were also significantly higher compared to controls (87 ± 60 vs 57 ± 52 , $p<0,01$).

The allocation of people according to transferrin saturation is presented in tables 4 and 5. At every level of transferrin saturation, the percentage of diabetics was higher compared to controls. For both males and females, the percentage of patients with diabetes

Table 1. Genotypes of unselected patients with diabetes type 2 and controls.

Genotype	DM2 n=100	Controls n=100	P value
WT/WT	75	76	0.8
C282Y/WT	1	2	0.5
H63D/WT	24	22	0.7
H63D/C282Y	0	0	NA
H63D/H63D	0	0	NA
C282Y/C282Y	0	0	NA

Table 2. Clinical characteristics and iron indices of males with diabetes type 2 and controls.

	DM2	Controls	P value
n	250	73	
Age (years)	62 ± 11	59 ± 9	NS
BMI (kg/m^2)	$27,6\pm 4$	$25,5\pm 3$	NS
Ferritin (ng/ml)	159 ± 80	87 ± 51	0,001
Iron ($\mu\text{g}/\text{dl}$)	100 ± 33	97 ± 46	0,5
TIBC ($\mu\text{g}/\text{dl}$)	322 ± 66	317 ± 63	0,5
Transferrin saturation index (Ironx100/TIBC)	$31,8\pm 11$	$29,5\pm 8$	0,05

Table 3. Clinical characteristics and iron indices of females with diabetes type 2 and controls.

	DM2	Controls	P value
n	250	350	
Age (age)	63 ± 11	62 ± 11	NS
BMI (kg/m^2)	29 ± 5	27 ± 4	NS
Ferritin (ng/ml)	87 ± 60	57 ± 52	0,01
Iron ($\mu\text{g}/\text{dl}$)	86 ± 30	80 ± 28	0,01
TIBC ($\mu\text{g}/\text{dl}$)	310 ± 62	323 ± 67	0,01
Transferrin saturation index (Ironx100/TIBC)	$28,5\pm 10$	$25,5\pm 9$	0,001

Table 4. Distribution of males with diabetes type 2 and controls at the different levels of transferring saturation.

Transferrin saturation %	DM2 N=250	Controls N=73	P value
38	95 (38)	15 (20,5)	0.006
45	45 (18)	4 (5,4)	0.009
50	23 (9,2)	0	0.007
55	9 (3,6)	0	0.1
60	3 (1,2)	0	0.3

* percentage in parenthesis

Table 5. Distribution of females with diabetes type 2 and controls at the different levels of transferrin saturation

Transferrin saturation %	Group 1 N=250	Group 2 N=350	P value
38	41 (16,4)	36 (10,3)	0.027
45	17 (6,8)	12 (3,4)	0.05
50	7 (2,8)	5 (1,4)	0.2
55	4 (1,6)	1 (0,3)	0.08
60	1 (0,4)	1 (0,28)	0.8

* percentage in parenthesis

type 2 and transferrin saturation more than 38% was significantly greater than that in the controls.

HFE mutations, transferrin saturation and Ferritin values (Table 6)

Subjects with even one mutation (C282Y or H63D) had higher transferrin saturation compared to those with no such mutations. This seems to apply to both diabetics ($49 \pm 8,6$ vs $44,5 \pm 5,4$, $p < 0,01$) and controls ($49,3 \pm 7,3$ vs $42,6 \pm 3,3$ $p < 0,01$). Ferritin was higher in control subjects carrying a mutation compared to those with no such mutation (99 ± 43 vs 68 ± 29 , $p < 0,01$). However, no such difference was detected among diabetics.

Hereditary hemochromatosis among patients with type 2 diabetes

The frequency of the C282Y allele was very low in the Greek population tested (0.0075) and homozygotes (C282Y/C282Y) were not detected in either group. We found 4 compound heterozygotes C282Y/H63D in the diabetics and none in the control group. This difference did not reach statistical significance ($p = 0.06$).

Table 6. Iron indices according to HFE genotype

	At least one mutation	No such mutation	P value
Transferrin saturation % (DM2)	$49 \pm 8,6$	$44,5 \pm 5,4$	0,01
Transferrin saturation % (controls)	$49,3 \pm 7,3$	$42,6 \pm 3,3$	0,01
Ferritin (ng/ml) mean \pm SD (DM2)	165 ± 88	149 ± 119	0,4
Ferritin (ng/ml) mean \pm SD (controls)	99 ± 43	68 ± 29	0,01

DISCUSSION

The allele frequency for the C282Y mutation in the Hellenic population was much lower (0.0075) than the one reported in people of Celtic origin³. Analogous findings to our study were reported from southern Europe⁴. The allele frequency for the H63D mutation (0.115) was comparable to the one in other populations^{3,4}. In our subjects, Homozygotes for hemochromatosis mutations were not detected, possibly because of the low frequency of C282Y mutation in our group. Based on these data, we cannot draw conclusions as to whether hereditary hemochromatosis is more prevalent among patients with diabetes type 2 in our population. Mutations of the transferrin receptor and the ferroportin 1 have recently been reported⁵⁻⁶ in Italian people. These mutations may account for at least a fraction of HH patients in whom the C282Y/C282Y or C282Y/H63D genotypes are not detected. We assume that the Greek population is genetically closer to the southern Italian population in terms of hereditary hemochromatosis causing mutations, and most likely a greater percentage of HH patients carry mutations other than the C282Y and H63D.

We did not detect any differences in the frequencies of C282Y or H63D alleles between type 2 diabetics and controls. This finding does not support the view that these mutations are in linkage disequilibrium for diabetes type 2. The discordance between our findings and those of other studies might be attributed to the different genetic background of the tested subjects.

It seems that the presence of these mutations even in the heterozygous state increases the iron load of the organism.²⁷⁻²⁸ The iron load could render peripheral tissues, and especially the liver, less sensitive to the action of insulin and consequently speed up the progression of the natural course of diabetes.

The mean serum ferritin level was found to be higher in patients with diabetes type 2 in accordance with other studies. This finding could either represent an elevated iron body stores in diabetes type 2 or simply reflect inflammation without true iron load, or else reflect a decreased clearance of glycosylated ferritin. Patients with diabetes type 2 seem to have higher transferrin saturation compared to the general population. This difference is even greater in women. The

increased ferritin level is seen in the group of type 2 diabetes as a whole, as well as in the subgroup of people with transferrin saturation more than 38%. The increased serum ferritin combined with the increased transferrin saturation could represent increased iron load with a potential effect on the hepatocyte and the beta cell of the pancreas. Increased iron load of the liver could lead to resistance to the action of insulin and hyperinsulinemia. There are very few studies focused on this question so that a conclusive answer as to whether diabetics type 2 have increased iron deposition at the hepatocyte²⁹ cannot be offered. In a recent study, we found that the iron load was significantly increased in offspring of type 2 diabetic subjects and was proportional to the insulin resistance index (unpublished observations). In conclusion, the allele frequency for the C282Y mutation was found lower (0.0075) in the Greek population than that reported in people of Celtic origin. The allele frequency for the H63D mutation (0.115) is comparable to other populations. Patients with diabetes type 2 have higher serum ferritin values as well as transferring saturation compared to the general population. The presence of either C282Y or H63D mutations contribute to the iron load of the organism. No difference in the frequency of C282Y and H63D alleles between patients with diabetes type 2 and the general population was detected in Greece.

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