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Variations at the H-strand replication origins of mitochondrial DNA and mitochondrial DNA content in the blood of type 2 diabetes patients

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ABSTRACT

Mitochondrial DNA (mtDNA) sequence variation in the segment of the D-loop region encompassing the initiation sites for replication and transcription was analyzed in the blood of 277 Italian type 2 diabetes patients and 277 Italian healthy subjects. Compared with the Cambridge Reference Sequence, diabetic patients show a slightly higher propensity to accumulate base changes in this region, with respect to controls, although no significant association can be established between any of the detected changes and the diabetic condition. Subjects, patients and controls, harbouring base changes at the replication origins (positions 57 and 151) and at position 58 were analyzed for mtDNA content. The mtDNA content increased three–four times only in the diabetic patients bearing the m.151C>T transition, whereas in those bearing the m.58T>C change the mtDNA content doubled, independently of the affiliation haplogroup. This result suggests that the m.151C>T transition and, to a lower extent, the m.58T>C might confer to the blood cells of diabetic patients the capability of increasing their mtDNA content, whereas the same transitions have no effect on control subjects.

1. Introduction

Diabetes is a collection of diseases characterized by chronic hyperglycaemia. It can be classified into two main groups, insulin-dependent diabetes mellitus or type 1, at early onset, and non insulin-dependent diabetes mellitus or type 2, at late onset. While type 1 diabetes is associated with the autoimmune destruction of pancreatic β cells, thus producing insulin deficiency [1,2], type 2 diabetes, which is the most prevalent form of disease, is due to a multiplicity of environmental and genetic factors [3,4]. One particular form of type 2 diabetes is the so-called mitochondrial diabetes [5], characterized by a maternal transmission together with a bilateral hearing defect in most of the affected patients. In the large majority of cases the mitochondrial diabetes is associated with a m.3243A>G mutation in mtDNA. Also associated with mitochondrial diabetes is a deletion of 10.4 kb [6]. Following these observations many other base changes were identified in diabetic patients; however, the role of such base changes in the development of the disease is still controversial [7–16].

The sequential accumulation of new mtDNA mutations along radiating maternal lineages has given rise to haplogroups which tend to be restricted to specific geographic areas and/or ethnic groups. Each haplogroup constitutes a branch or a sub-branch in the worldwide mtDNA phylogeny and is characterized by a distinguishing combination of mutations [17]. The preferential association of a clinical disorder with a specific haplogroup might suggest that one or more of the haplogroup-specific sequence changes might play a role in the disease expression [18,19]. However, no evidence of an association between type 2 diabetes and the major European mtDNA haplogroups has been up to now reported [20,21].

In this study the mtDNA sequence variation of a segment of the D-loop region, never analyzed until now, namely that containing the initiation sites for mtDNA replication and transcription has been determined in the total blood of 277 type 2 diabetes patients and 277 healthy subjects. Furthermore, the mtDNA relative content in a subset of diabetic patients and in one of controls has been measured to verify the eventual different influence of some specific changes on this relevant parameter. Also the relationship with the haplogroups affiliation has been studied.

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2. Materials and methods

2.1. Subjects

A total of 277 Italian patients with type 2 diabetes (the diagnosis was made according to the American Diabetes Association criteria), 141 men and 136 women, mean age 63 ± 8 years, were recruited from the same Italian region (Marche) by the Diabetology Unit, INRCA Ancona, Italy. 277 Italian subjects in good health (without diabetic familiarity), 103 men and 174 women, mean age 58 ± 11 years, were enrolled as a control group. Detailed medical history of all diabetic patients and controls was recorded. In all subjects antropometric determinations were performed as well as the following routine laboratory screening including levels of: fasting plasma glucose, blood ureic nitrogen, creatinine, lipid profile, reactive protein C, fibrinogen and counts of red blood cells, white blood cells and platelets. All patients gave their written informed consent before they enrolled in the study, which was approved by the local Ethics Committees.

2.2. Isolation and amplification of DNA

Total DNA was prepared from peripheral blood using Quiamp DNA Blood Maxi Kit (Qiagen) and stored at −20 °C. A part of the D-loop region was amplified by PCR from total DNA with primers 16.4 For (16495–16514) -0.6 Rev (609–590). Nucleotide numbering is according to the Cambridge Reference Sequence (CRS) [22]. The reaction was performed as reported in [23]. The ability of the primer pair to amplify nucleus-embedded mtDNA pseudogenes was investigated performing the PCR with 400 ng of total DNA extracted from human osteosarcoma-derived mtDNA-deficient cell lines (143 B ρ^0). Control experiments on DNA extracted from blood and 143 B ρ^+ cells were also performed.

2.3. Sequencing of the D-loop region

To facilitate the direct sequencing of PCR-amplified products, both used primers were tagged with M13 sequence as reported in [23]. PCR products were purified by using a Quiaquick PCR-purification Kit (QUIAGEN) and sequenced by MWG Biotech facilities. All sequences were analyzed with Chromas program and compared with the CRS by means of BLAST program.

2.4. Quantification of the mtDNA/nDNA ratio by real-time PCR

The mtDNA content was measured by real-time PCR using an ABI Prism 7000 real-time PCR (Applied Biosystems). The mtDNA quantity was referred to the nuclear DNA (nDNA) amount by simultaneous measurement of the nDNA. The primers for the mtDNA were 3485-ND1-For (5′-CCCTAAAACCCGCCACATCT-3′) and 3553-ND1-Rev (5′-TAGAAGAGCGATGGTGAGAGCTAA-3′) and were used together with the mtDNA probe (5′FAM-CCATCACCCTCTACATCACCGGCC-TAMRA3′)

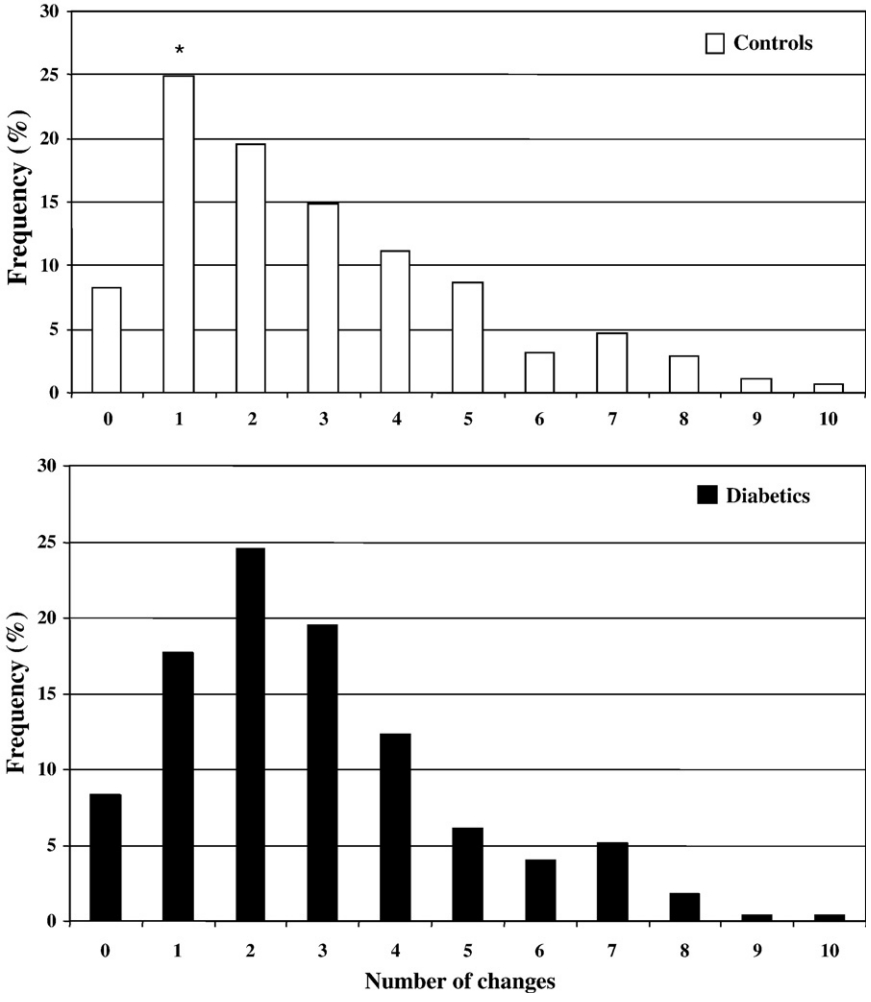

Fig. 1. Frequencies of control subjects and diabetic patients bearing the reported numbers of sequence changes in the analyzed segment of the D-loop region. Each frequency was the ratio between the number of subjects harbouring a specific amount of changes and the total of all analyzed subjects. A statistically significant difference was found between the frequency of diabetic patients harbouring only one mutation and that of controls (*$p<0.05$).

made for nt 3506–3529. The primers for the 28S rRNA were 28S-7358-For (5′-TTAAGGTAGCCAAATGCCTCG-3′) and 28S-7460-Rev (5′-CCTTGGCTGTGGTTTCGCT-3′) and the 28S rRNA probe was (5′FAM-TGAACGAGATTCCCACTGTCCCTACCTACTATTC-TAMRA3′) made for nt 7408–7440. The method has been validated by evaluating the equal reaction efficiency of the two amplicons. Each sample was analyzed in triplicate and in 4–7 different experiments. The PCR was performed separately for the target ND1 gene and the reference 28S rRNA gene. The PCR mixture contained the specific primers (200 nM), specific TaqMan probes (100 nM), ~50–100 ng DNA, 12.5 μl TaqMan Universal PCR MasterMix (Applied Biosystems, California, USA). Amplification conditions were 50 °C for 2 min to remove carry over by UNG activity, 95 °C for 10 min to inactivate UNG enzyme and activate Taq Polymerase and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The difference in threshold cycle values (ΔCt, namely $Ct_{ND1} - Ct_{28SrRNA}$) was used as a measure of the relative abundance of the mitochondrial genome. In particular, the mtDNA/nDNA ratio is reported as $2^{-\Delta Ct}$. To compare the mtDNA amount among diabetic patients and controls, the control class was taken as a reference (calibrator) and the number of fold increase of mtDNA content in diabetics compared to controls was calculated by the following equation: $R = 2^{-\Delta Ct(\text{diabetics})} / 2^{-\Delta Ct(\text{controls})}$. The standard deviation of the quotient was calculated as reported in User Bulletin # 2 Applied Biosystems. Data were submitted to *t*-test to assess significance of the differences observed among groups.

2.5. Statistics

Statistical analysis was carried out by using SPSS Base 11.5 software. Student's *t*-test and chi square test were used. Statistical significance was set at $p<0.05$.

3. Results

3.1. Identification of sequence variants in the mtDNA D-loop region

To verify whether specific sequence changes in the segment of the mtDNA D-loop region, harbouring the initiation sites for replication and transcription, were associated to type 2 diabetes, we determined the sequence of part of the D-loop and of the $tRNA^{Phe}$ gene, from position 16495 to position 609, in the total blood of 277 diabetic patients and 277 controls, all living in a specific Italian region, namely Marche. In order to exclude coamplification of nuclear mtDNA pseudogenes, amplification experiments were carried out on DNA extracted from mtDNA-deficient human osteosarcoma-derived cell lines (143 B ρ^0) obtaining no PCR products. Control PCR experiments on DNA extracted from blood and 143 B ρ^+ cells were also performed.

The comparison of the obtained mtDNA sequences with the CRS [22] shows a variable number of sequence changes (from 0 up to 10) both in healthy subjects and in diabetic patients. All found sequence

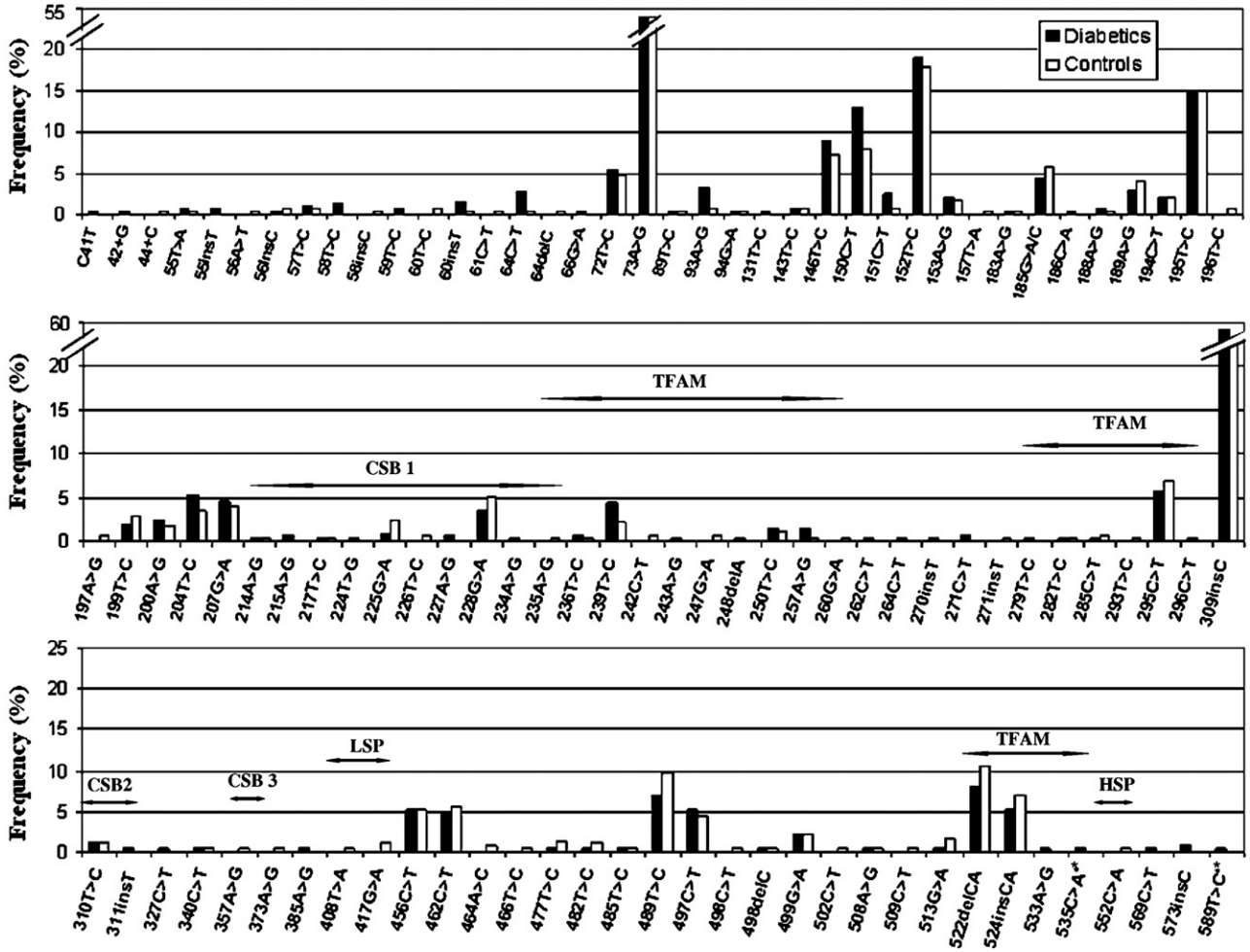

Fig. 2. Frequencies of control subjects and diabetic patients bearing each of the specific base changes found in the analyzed segment of the D-loop region. Each frequency was the ratio between the number of subjects harbouring the specific change and the total of all analyzed subjects. The double-headed arrows indicate the localization of Conserved Sequence Box 1–3 (CSB1-3), H- and L-strand promoters (HSP and LSP) and TFAM binding sites. Positions of regulatory sequences are from MITOMAP (http://www.mitomap.org), and from Ghivizzani et al. [24]. Base changes, found in two diabetic patients, not previously reported in mtDNA databases are indicated with an asterisk.

changes are homoplasmic. Diabetic patients have a slightly higher propensity to accumulate changes in the analyzed segment of mtDNA control region; in fact the number of subjects harbouring a single change is significantly higher in control subjects ($n=69$) than in diabetic patients ($n=49$) ($\chi^2=3.9$; $p<0.05$), whereas the frequencies of subjects harbouring two or three changes are higher in diabetics than in controls. These last differences do not reach, however, a statistical significance (Fig. 1).

Fig. 2 shows the frequencies of control subjects and diabetic patients bearing each of the specific base changes found in the analyzed segment of the D-loop region. These base changes affected 107 positions out of 685 examined; 29 of them concerned exclusively diabetic patients, 26 of them control subjects and the remaining both types of individuals. Two base changes (m.535C>A and m.589T>C), only found in diabetic patients, were never reported previously in databases. The position of the sequence variations in relationship with that of sites critical for the regulation of mtDNA metabolism (replication origins, Conserved Sequence Box 1–3 (CSB1-3), H- and L-strand promoters (HSP and LSP) and TFAM binding sites) was analyzed. No association was verified between the disease and any of the reported mtDNA base changes in this group of Italian diabetic patients. Interestingly, we observed sequence changes at the two H-strand replication origins, that is at positions 57 and 151 [25] in 3/277 control subjects and in 6/277 diabetic patients.

3.2. Determination of the mtDNA content in controls and diabetic patients containing base changes at the H strand replication origins or nearby

Since all analyzed patients had a normal peripheral blood cell number, similar to that of control subjects, a comparison between the two groups was possible and we tested whether sequence variations at the mtDNA replication origins or nearby were associated with differences in the mtDNA content. We determined, by real-time PCR, the mtDNA content relative to nuclear DNA (nDNA) in the total blood of the three control subjects and of the six diabetic patients harbouring changes at the two H-strand replication origins (positions 57 and 151) as well as of 11 controls and 12 diabetic patients not harbouring such changes. Moreover, the relative content of mtDNA was measured also in a subset of three diabetic patients harbouring a base change nearby the replication origin 57, namely at position 58, which is exclusively present in these three diabetic patients (see Fig. 2). With the exception of D1, D2 and D3 subjects, all other individuals analyzed for the mtDNA level were from different pedigrees.

The sequence changes and the relative mtDNA content together with the age, the sex, the haplogroup affiliation, the amount of platelets and the glycated haemoglobin (HbA1c) content of each analyzed individual are reported in Table 1. No correlation between mtDNA content and age for diabetic patients and controls was found

Table 1
mtDNA content and other relevant parameters in subsets of diabetic patients and controls bearing or not bearing changes at the replication origins or nearby

Sample	Sex	Age	Haplogroup	Platelets (10^3/μl)	HbA1c (%)	Control region sequence changes	mtDNA/nDNA
C1	M	60	H15	191	–	55, **57**	1.10 ± 0.15
C2	M	64	K	180	–	56T, **57**, 58 + C, 73, **151**	1.40 ± 0.15
C3	F	59	T2b	233	5.5	73, **151**, 152, 309 + C	1.27 ± 0.42
Mean							1.26 ± 0.32
D1	M	67	T2b	189	7.0	55 + T, **57**, 59, 73, **151**, 152, 309 + 2C	4.20 ± 0.42
D2	F	61	T2b	141	6.6	55 + T, **57**, 59, 73, **151**, 152, 309 + 2C	3.50 ± 0.90
D3	F	71	T2b	190	7.4	55 + T, **57**, 59, 73, **151**, 152, 309 + 2C	3.40 ± 0.80
Mean							3.80 ± 0.76*
D4	M	70	T2b	154	6.8	73,150, **151**, 309 + C, 573 + 2C	3.35 ± 0.49
D5	F	73	N1b	270	7.7	73, **151**, 152, 200, 309 + C	3.90 ± 0.94
D6	M	77	N1b	132	11.5	73, **151**, 152, 200, 309 + C	3.36 ± 0.75
Mean							3.54 ± 0.64*
D7	M	69	R0a	153	5.5	**58**, 60 + T, 64, 309 + C	1.95 ± 0.06
D8	M	66	R0a	216	8.6	**58**, 60 + T, 64, 309 + C	2.00 ± 0.27
D9	F	52	R0a	215	8.2	**58**, 60 + T, 64, 309 + C	2.50 ± 0.51
Mean							2.16 ± 0.41*
C4	M	58	H	227	5.9	309 + 2C	0.97 ± 0.15
C5	M	69	H5	236	5.5	152, 456	1.00 ± 0.10
C6	F	72	H5	124	6.6	146, 456	1.03 ± 0.10
C7	M	61	H	166	5.4	146, 195	0.97 ± 0.15
C8	M	78	H	275	6.4	–	1.00 ± 0.10
C9	F	55	T2b	384	6.1	73	0.97 ± 0.23
C10	F	39	T2b	199	5.6	73	0.81 ± 0.33
C11	F	62	T2b	220	5.7	73	0.92 ± 0.23
C12	F	46	T2b	236	6.5	73	1.27 ± 0.57
C13	M	84	N1b	272	5.5	73, 152, 200, 309 + C,522delCA	1.49 ± 0.37
C14	F	55	N1b	205	5.7	73, 152, 338, 522delCA	1.25 ± 0.12
Mean							1.05 ± 0.29
D10	F	57	T1	195	8.0	73, 152, 309 + C, 456	0.99 ± 0.21
D11	F	57	R0a	207	7.4	60 + T, 64, 72G	0.80 ± 0.05
D12	M	61	H1	209	6.7	195	1.25 ± 0.31
D13	M	62	H5	176	7.8	204, 456	0.96 ± 0.18
D14	F	67	I	239	6.5	73, 199, 204, 250	0.71 ± 0.13
D15	M	58	T2b	162	6.4	73	1.80 ± 0.14
D16	F	69	T2b	346	8.2	73, 309 + C	1.15 ± 0.21
D17	M	57	T2b	256	8.6	73	1.10 ± 0.10
D18	F	68	T2	284	8.0	73, 146,309 + C, 522delCA	0.91 ± 0.20
D19	F	66	T2	291	7.3	73, 309 + C	1.77 ± 0.30
D20	M	52	T2	203	9.0	73, 146, 309 + C	1.07 ± 0.40
D21	M	55	T2	187	7.1	73, 146,309 + C, 522delCA	1.85 ± 0.63
Mean							1.19 ± 0.39

* $p<0.001$ = statistically significant difference between the sample mean value and the mean value of control class (C4–C14). Indicated in bold are the sequence changes at the replication origins and a specific change among the nearby ones in the subjects analyzed for mtDNA content. HbA1c (%) = percentage of glycated haemoglobin.

in our analyzed subjects. The mtDNA content of the subset of the three healthy individuals (C1–C3) bearing the m.57T>C and/or the m.151C>T transitions was unchanged when compared with that of the 11 age-matched controls (C4–C14) not bearing base changes at the replication origins. The subsets of the six diabetic patients constituted by three patients (D1–D3) presenting the m.57T>C and the m.151C>T and three patients (D4–D6) with the m.151C>T change alone showed a three–four-fold increase of mtDNA content compared with age-matched controls (C4–C14) and diabetic patients (D10–D21) without the above-reported base changes. The transition at position 57 does not seem to influence the mtDNA content since the increase of mtDNA content was the same in D1–D3 (bearing the m. 57T>C transition) and in D4–D6 patients (not bearing the m. 57T>C transition). As far as the three patients D7–D9 with the m.58T>C change, their content of mtDNA was only doubled compared with age-matched controls (C4–C14) and diabetic patients (D10–D21) without this change. No relationship was found between the mtDNA content and the haplogroup to which the analyzed subjects belong; in fact, patients D1–D6, exhibiting the highest contents of mtDNA, belong to the same haplogroups (T2b and N1b) of the control C3, bearing the m.151C>T transition, of the D15–D17 diabetic patients and of the C9–C14 control subjects not bearing any of the above reported base changes and all having the same mtDNA content.

4. Discussion

The present study about type 2 diabetes and mtDNA sequence variation in the segment of D-loop region containing the transcription and replication origins deals with a large size of sampled individuals (277 diabetics and 277 controls), highly homogeneous as geographical and population origin. They have all been recruited, in fact, in the same Marche region of the Italian peninsula. The analyzed segment of mtDNA has never been studied until now. The results about the base changes in this mtDNA segment show that diabetic individuals have a slightly higher propensity to accumulate mutations in their mtDNA. Consistent conclusions were obtained by Sherrat et al. [26] who analyzed the sequence of another segment of the D-loop region in a smaller number of individuals. However, no association was verified between the disease and any of the reported mtDNA base changes in this group of Italian diabetic patients as other authors did analyzing different regions of mtDNA and groups of subjects with distinct geographical origins [14,16]. Our analysis of the pattern of nucleotide substitutions in control and diabetic individuals shows that the most frequent substitutions are A–G and T–C (about 65% of the total mutations), while C–T and G–A account only for 30%. This result supports the previously reported hypothesis [27] that the majority of mtDNA mutations derive from errors of the gamma-DNA polymerase activity due to the uneven composition of the mitochondrial deoxynucleotide pool. The data reported here do not confirm those by Kamiya and Aoki [28] who, by studying a small population (six diabetics and six controls), found an incidence of somatic transversion mutations significantly higher in diabetic patients than in controls.

A relevant result of this study concerns the mtDNA content in a subset of diabetic patients harbouring the m.151C>T and/or the m.57T>C or the m.58T>C transitions (9/277 patients). In fact, only these diabetic patients (D1–D9, see Table 1) present a statistically significant increase of the mtDNA content in blood with respect to a similar number (12/277) of other patients not harbouring the same base changes and featuring an unchanged mtDNA content (D10–D21, see Table 1). The mtDNA content in the blood of the diabetic patients D1–D9 is remarkably increased also with respect to control subjects with or without these changes (C1–C3 and C4–C14, respectively, see Table 1). Such data strongly suggest only in diabetic patients some kind of association between the presence of one of these transitions (the m.151C>T or the m.58T>C) and the increase of mtDNA content. The highest increase (three–four times) of mtDNA content was found in those patients having the m.151C>T transition (D1–D6). In the diabetic patients analyzed here the increase of mtDNA content did not appear to be associated with a specific haplogroup.

According to the asymmetric model of mtDNA replication [29] and to Fish et al. [25], as for the human mtDNA synthesis, the major replication origin of mtDNA is position 57 which is mainly responsible for mtDNA maintenance under steady-state conditions, whereas position 151 may be more important in the replication of mtDNA during the recovery after mtDNA depletion. In this study the m.57T>C transition does not influence the mtDNA content, whereas the m.151C>T transition and, to a lower extent, the m.58T>C transition seem to confer to blood cells of diabetic patients the capability of increasing their mtDNA content, probably as an adaptation to the disease-related oxidative stress [30,31]. Many published reports associate an increase of mtDNA copy number to an oxidative stress situation: in aging or/and smoking cigarettes individuals [32], in cultured human lung fibroblasts exposed to H_2O_2 [33], in human leucocytes from different age groups [34]. Using cell lines carrying different common mouse mtDNA haplotypes in an identical nuclear background, Moreno-Loshuertos et al. [35] showed that different amounts of ROS are associated to different mtDNA variants and that cells producing more ROS have higher mtDNA copy numbers as a consequence of compensatory mechanisms triggered by ROS.

The detailed mechanism which regulates mtDNA copy number in animal cells remains unclear [36,37]. The diabetes-associated oxidative stress in subjects carrying the here described mtDNA base changes might constitute another experimental model to dissect the problem.

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