Global DNA methylation levels are modulated by mitochondrial DNA variants

**Aim:** In the present study, we investigated whether global DNA methylation levels are affected by mitochondrial DNA (mtDNA) variants, which are known to modulate mitochondrial functions. **Materials & methods:** Global DNA methylation levels were evaluated in peripheral blood DNA collected from adult subjects and *in vitro* using the DNA of cybrid cells harboring mtDNAs of different haplogroups. In these cells, mRNA expression of genes involved in DNA methylation processes, and ATP and reactive oxygen species levels were also analyzed. **Results:** The analysis revealed that methylation levels were higher in the subjects carrying the J haplogroup than in non-J carriers. Consistently, cybrids with J haplogroup mtDNA showed higher methylation levels than other cybrids. Interestingly, we observed overexpression of the MAT1A gene and low ATP and ROS levels in J cybrids. **Conclusion:** Our findings indicate that mtDNA-specific interactions between mitochondria and the nucleus regulate epigenetic changes, possibly by affecting oxidative phosphorylation efficiency.

**KEYWORDS:** ATP, cybrid cell lines, global DNA methylation, MAT1A gene, mitochondrial DNA variability, ROS

Mitochondria hold a central position between energy uptake and energy production. As a consequence, they play a key role in a wide variety of both pathological (e.g., aging, cancer, neurodegenerative diseases and diabetes) and nonpathological (e.g., heat production, reactive oxygen species [ROS] generation, apoptosis and cellular differentiation) traits [1-3]. Over the past two decades, many studies have focused their attention on the influence of variability of mitochondrial DNA (mtDNA), whose genes are responsible for the synthesis of 13 enzyme subunits of the oxidative phosphorylation (OXPHOS) complex, on mitochondrial function and on the above-mentioned traits.

mtDNA variants define specific mtDNA haplotypes. Haplotypes with a common phylogenetic origin are categorized into haplogroups which display a continent-specific distribution. For example, approximately 99% of the mtDNA of the European population falls within nine different haplogroups (H, J, U, X, T, I, K, W and V) as identified by Torroni *et al.* [4]. More recently, sequencing of the complete mtDNA genome has allowed the subdivision of haplogroups into smaller subhaplogroups [5]. The mtDNA haplogroups were initially considered as being neutral and used only for phylogeny analysis and population studies [6-8]. Afterwards, a series of experimental evidence suggested that they are not in fact neutral, and an association between common mtDNA variants and physiological or pathological phenotypes has gradually emerged. The first strong evidence demonstrating such a functional consequence came from the association between the J haplogroup and Leber hereditary optic neuropathy (LHON) [9]. Subsequently, mtDNA variants have been found to affect the quality of aging, sperm motility and the susceptibility to late-onset pathologies, such as neurodegenerative and cardiovascular diseases, diabetes and cancer [10-17]. However, other studies have reported inconsistent results, suggesting the existence of complex interactions among mtDNA genotype, nuclear background and environment [18-22]. It has been widely reported that mtDNA molecules belonging to different haplogroups may differ in the degree of oxidative phosphorylation activity, and in turn, result in different percentages of oxygen consumption, ATP and mitochondrial ROS production or heat generation [23-30]. On the other hand, there is also evidence that different mtDNA haplogroups may maintain similar efficiency in OXPHOS performance through the fine-tuning of ROS production and mitochondrial biogenesis [31,32]. It has also been proposed that the variants which alter OXPHOS coupling efficiency, thus inducing less ATP and more heat production, are more frequent in cold areas where they purportedly confer an advantage, thus suggesting that climatic selection has played a role in shaping the present worldwide distribution of mtDNA variation [33-35].
Functional differences between haplogroups have also emerged from in vitro studies carried out in cybrid cell lines, which were first described by King and Attardi as sharing the same nuclear genome but as having different mitochondrial genomes [36]. These cells are generated by the fusion of mtDNA-depleted cells (Rho0) with enucleated cells harboring particular types of mtDNA molecules [36,37]. In particular, in these cell models, intracellular calcium dynamics, mtDNA copy number, mitochondrial ROS production and the expression levels of several nuclear-encoded genes were demonstrated to be influenced by mtDNA variability [38–44]. For example, differences in mtDNA and mRNA levels, mitochondrial membrane potential, cytochrome oxidase activity, growth capacity and oxygen consumption were observed between cybrids harboring haplogroups H and UK [28]. By contrast, no difference in bioenergetic capacities and coupling efficiencies was identified between H and T cybrids [31,45].

More recently, the importance of the interactions between mitochondrial and nuclear genomes has also emerged from studies on epigenetic changes and, more specifically, on the DNA methylation of cytosines. In fact, the efficiency of mitochondrial energy producing machinery and mitochondrial metabolism can modulate the activity of MAT, the enzyme responsible for SAM synthesis from L-methionine and ATP [46–50]. It follows that dysfunctions in mitochondrial activity may have direct effects on epigenetic markers and, consequently, disrupt gene expression patterns and cellular and organism functioning [51–55]. Recently, Smiraglia et al. reported that the depletion of mtDNA (Rho0 cells) induced changes in DNA methylation of different genes and that these changes were partially reversed by the re-introduction of mtDNA into these cells [54]. As mitochondrial functions strongly rely on proteins that are coded by the nuclear genome, epigenetic changes in the methylation status of nuclear genes, and thus in their expression, may affect mitochondrial function with the onset of a vicious cycle [55]. Although it has not been completely elucidated, this complex interplay between mitochondrial function and epigenetic modifications appears to have important consequences on many traits [48].

On the basis of these observations, we carried out population and in vitro studies to investigate the relationship between epigenetic modifications and mtDNA variability. Global DNA methylation levels were measured in peripheral blood DNA collected from adult subjects and in DNA samples extracted from cybrid cell lines which have identical nuclear DNA but harbor mtDNA molecules of different haplogroups (H, J, U, X and T). In addition, using these cell lines we analyzed the expression profiles of different genes involved in methylation processes as well as ATP and ROS levels, major products of mitochondrial activity and of the communication network between mitochondrial and nuclear genomes.

**Materials & methods**

**Population sample**

A total of 354 (163 men and 191 women) unrelated adult individuals participated in the present study. The Ethics committee of the University of Calabria, Italy approved the recruitment and the use of the information gathered, as well as the use of the biological specimens collected on 9 September 2004. All subjects lived in Calabria (south of Italy) and their origin in the area was ascertained up to the grandparents’ generation. A more detailed sample description can be found elsewhere [56]. Health status was ascertained by medical visit and, at that time, peripheral blood samples were also obtained. Before the interview each subject provided informed consent for her/his phenotypic and genetic data to be used anonymously for genetic studies.

**Cell lines & culture conditions**

143B.TK osteosarcoma and cybrid cell lines were grown, in a water-humidified incubator at 37°C in 5% CO2/95% air, in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, CA, USA) containing 4.5 g/l glucose, 110 µg/ml pyruvate and supplemented with 10% fetal bovine serum (Invitrogen). Rho0 cells, obtained by culturing 143B.TK with 50 ng/ml ethidium bromide, were grown in the above medium supplemented with 50 µg/ml uridine (Sigma, MO, USA).

Cybrid production was carried out as described elsewhere [37,40,42]. Briefly, platelets were isolated through differential centrifugations from blood samples of five donors, previously characterized for their mtDNA haplogroup. 106 Rho0 cells, collected by low-speed centrifugation and resuspended in DMEM, were mixed with an equal number of platelets and the culture medium was eliminated by centrifugation. Cells were resuspended in 100 µl of 42% polyethyleneglycol 1500 (PEG 1500) and seeded in standard DMEM for 48 h, and then in selective uridine-free DMEM supplemented with 10% fetal bovine serum. After 2–3 weeks,
distinct colonies emerged which were isolated by trypsinization in cloning rings and propagated. We routinely assessed the cellular state of the native Rho<sup>0</sup> and cybrid cell lines by carrying out control experiments including proliferation, quantification of mtDNA (quantitative-competitive PCR) and mitochondrial membrane potential (MMP) assays (cytofluorimetric analysis of cells stained with MitoTracker® Green, Invitrogen and tetramethyl rhodamine methylster), which have been fully described elsewhere [40,42].

### DNA samples
Each subject had 6 ml of venous blood drawn. Plasma/sera were used for routine laboratory analyses, while DNA was extracted from blood buffy coats following standard procedures. DNA samples from 143B.TK osteosarcoma, Rho<sup>0</sup> and cybrid cell lines were obtained by phenol/chloroform purification. The DNA concentration and 260/280 absorbance ratio were determined spectrophotometrically.

### Restriction analysis of DNA samples
A total of 100 ng of population sample DNA and DNA extracted from cell lines were incubated separately with 5 U of HpaII and 5 U of MspI restriction endonucleases in a total volume of 30 µl overnight at 37°C and subsequently at 65°C for 20 min to inactivate the two endonucleases.

### Measurement of global DNA methylation levels
Global DNA methylation levels of population sample DNA and DNA extracted from cell lines were estimated using the CpGlobal method [57]. A total of 2 µM of both biotin-11-dCTP (Perkin Elmer, MA, USA) and biotin-11-dGTP (Perkin Elmer) were added to the digested DNA samples in 20 µl final volume containing biotinylation buffer (40 mM Tris-HCl pH 7.5, 20 mM Tris-HCl and 50 mM NaCl) and 2 U of Sequenase<sup>®</sup> (USB Corporation, CA, USA). After incubation at 37°C for 30 min, the samples were incubated on an orbital platform at room temperature overnight in presence of 100 µl of Reacti-Bind<sup>™</sup> DNA Coating Solution (Pierce, IL, USA). The solution was removed through three consecutive washes with Dulbecco’s phosphate buffered saline (Sigma). Samples were then incubated at room temperature for 30 min in presence of 200 µl of the detector block solution (KPL, WA, USA). After the removal of the solution, 150 µl of the detector block solution containing 0.5 µg/ml of horseradish peroxidase (HRP) streptavidin (KPL) were added and the samples were incubated at room temperature for 30 min. The detector block solution was removed through four consecutive washes with biotin wash solution 1x (KPL). After a 2-min incubation at room temperature in presence of 150 µl of LumiGLO® Chemiluminescence substrate (KPL), the chemiluminescence emitted from each sample was quantified using a Lumat LB9507 luminometer (EG&G Bertold, Wildbad, Germany). Each sample was analyzed three independent times in triplicate. In order to determine the possible ‘background effect’ and to calculate the net luminescence for each sample, a control lacking enzyme was also analyzed. The data were calculated as global DNA methylation index (GDMI) by dividing the mean net luminescence values for the HpaII enzyme by the mean net luminescence values for the MspI enzyme. The GDMI values inversely correlate to the global DNA methylation levels, whereby high GDMI values indicated lower methylation level, while low GDMI values indicated hypermethylation.

A subsample (108 samples) of the entire study group was also analyzed by using Sigma’s Imprint® Methylated DNA Quantification Kit according to the manufacturer’s protocol.

### mtDNA analysis
Haplogroup typing was carried out by restriction analyses of mtDNA according to Torroni et al. and De Benedictis et al. [4,10].

### Gene-expression analysis
Expression levels of the following genes were analyzed: DNMT1, DNMT3A, DNMT3B, MAT1A, MAT2B, MBD2 and MBD4. Total RNA was extracted from cells using RNeasy® Mini Kit (Qiagen, Hilden, Germany). The RNA concentration was measured for each sample using a spectrophotometer and purity of the sample evaluated using the 260/280 nm absorbance ratio. RNA samples were treated with DNA-free DNase to remove any residual genomic DNA contamination. Reverse transcription (RT) was carried out using the ImPromII Kit<sup>™</sup> (Promega, WI, USA). A RT mix including 500 ng total RNA and 0.5 µg of oligo-dT primers was preheated at 70°C for 5 min. The reaction was carried out in a 40-µl final volume containing 1x RT buffer, 0.5 mM of each dNTP, 3 mM MgCl<sub>2</sub>, 20 U RNase inhibitor and 5 U reverse transcriptase. The mix was incubated at 25°C for 5 min, then at 37°C for 1 h and successively, at 95°C for 10 min to inactivate the reverse transcriptase. The cDNA obtained was
then used as a template for real-time PCR carried out with the SYBR® Green qPCR Master Mix (Promega) in a StepOne Plus™ machine (Applied Biosystems, CA, USA). Forward and reverse primers were as follows: DNMT1For 5’-AGAACCGTGTCTCATGCTTACA-3’; DNMT1Rev 5’-GGGGCTAGGTGAAGGTCAG-3’; DNMT3AFor 5’-CCGATGCTGCGGACAAGAAT-3’; DNMT3ARev 5’-CCCGTCACTGCCCAAGAC3’; MAT1AFor 5’-CAGTGTCGCAAGACCCGCAT-3’; MAT1ARev 5’-TAGGGCAATGTCGCTTGATGGTG-3’; MAT2BFor 5’-CTGGAGAATTTAGCAAAAGGACGACG-3’; MAT2BRev 5’-GCTCCATTGTTTCCAGGAC-3’; MDB2For 5’-CCCACAACGGAATGAGATGAACGC-3’; MDB2Rev 5’-TGAGACCTTTGGTAGTCTCCAG-3’; MBD4For 5’-CCACCCGTACCTCTAGT-3’; MBD4Rev 5’-CTGACCCCAAACCTGACCAAGA-3’; GAPDHFor 5’-ATGGGGAAGGTGAAGGTCGAC-3’; GAPDHRRev 5’-GGGGTCATTGATGGTG-3’; ENLITEN® ATP stock standard supplied in the kit.

The PCR mixture (10 µl) contained 1 µl of cDNA, 1x GoTaq® qPCR Master Mix, 0.2 µM of each primer and 1x CXR reference dye. The thermal profile used for the reaction included a 2-min heat activation of the enzyme at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s, followed by a melt analysis ramping at 60°C to 95°C. All measurements were taken in the log phase of amplification. Negative controls (in which water instead of cDNA was added) were also run in each plate. StepOne Software V 2.0 was used to analyze data. Gene expression values were normalized to GAPDH gene expression, used as internal control. In addition, the normalized values measured in the 143B.TK cell line were used as reference values (relative quantification) for the other cell lines.

### Measurement of ATP levels

ATP measurement was performed using ENLITEN® ATP assay system bioluminescence detection kit for ATP measurement (Promega), according to the manufacturer’s protocol. Briefly, 1 x 10⁶ 143B.TK, Rho° and cybrid cells were seeded in 6-well plates. In the exponential growth phase, cells were washed with phosphate buffered saline, trypsinized and counted on a hemocytometer with an inverted light microscope. A total of 5 x 10⁶ cells were collected and centrifuged at 400 g for 5 min at 4°C. Then, the intracellular ATP was extracted by resuspending pellets in 100 µl 0.5% trichloroacetic acid. After ATP extraction, the trichloroacetic acid in the samples was neutralized and diluted by adding tris-acetate buffer pH 7.75. Last, the bioluminescence emitted from samples was assessed using a luminometer. For all luminescence assays, blank values were subtracted from measurement values. Concentration values of ATP produced in our cell lines (nmoles of ATP) were obtained by fitting luminescence values to a standard ATP curve, constructed by analyzing series of dilutions ranging from 1 x 10⁻¹¹ to 1 x 10⁻⁶ M of the ATP stock standard supplied in the kit.

### Total ROS/superoxide detection

Intracellular ROS were quantified using two fluorescent dye reagents: oxidative stress detection reagent (green), which reacts directly with a wide range of ROS/reactive nitrogen species (RNS) species; and superoxide detection reagent (orange), which reacts specifically with superoxide (O²⁻) (total ROS/Superoxide Detection Kit, Enzo Life Science, NY, USA).

143B.TK, Rho° and cybrid cells (5 x 10⁵ each) were seeded in 6-well plates. In the exponential growth phase, cells were washed with 1x wash buffer (Enzo Life Science), collected and centrifuged at 400 g for 5 min. Pellets were washed with 1x wash buffer (Enzo Life Science), and the cell suspensions were centrifuged at 400 g for 5 min. The supernatant was discarded and the cell pel- let was resuspended in 500 µl of ROS/superoxide detection mix (Enzo Life Science). The cell suspensions were incubated for 30 min at 37°C in the dark. In all analyses a minimum of 1 x 10⁴ cells per sample were acquired and analyzed with Cell Quest Software (BD Bioscience, CA, USA).

### Statistical analysis

Descriptive statistics for continuous and categorical variables were used to describe the characteristics of the analyzed samples. For continuous variables (GDMI, gene expression levels, ATP extraction, ATP/superoxide quantification) measures of central tendency and dispersion, including mean, median and standard error of the mean were reported. Departures from normality assumption were tested using the Kolmogorov–Smirnov normality test. One-way analysis of variance for multiple comparisons and student’s t-test for pair-wise comparisons were adopted to compare continuous normally distributed variables with respect to the mtDNA haplogroup classification. In case of evidence of non-normality, the corresponding nonparametric Kruskal–Wallis or Mann–Whitney tests (as appropriate) were used.

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Results

Global DNA methylation level analysis in population DNA samples

Global DNA methylation levels were determined as GDMI values by applying the CpGlobal assay developed by Anisowicz et al. to DNA collected from human individuals who were previously analyzed for their mtDNA variability [57]. As a quality control, in each assay we also evaluated the global methylation levels of unmethylated, fully and partially methylated samples of human genomic DNAs. Specifically, in the experimental conditions we adopted, we observed that fully methylated control human DNA was digested by MspI but not by HpaII, fully unmethylated control human DNA was totally digested by both enzymes, whereas the DNA sample obtained by mixing an equal ratio of unmethylated and methylated control human DNA showed an intermediate result. As expected, the GDMI values in the fully methylated, in the mix of methylated and unmethylated, and in the fully unmethylated control DNAs were about 0.1, 0.5 and 1.0, respectively. We maintained that the above results allowed us to validate effectiveness of the experimental conditions [59]. The distribution of the GDMI values in our population sample was not normally distributed as indicated by the Kolmogorov–Smirnov test result (p = 0.007; Supplementary Figure 1, www.futuremedicine.com/doi/suppl/10.2217/epi.11.109). Table 1 reports for each gender the frequency distributions of mtDNA haplogroups with the relevant mean values of age and GDMI in the analyzed sample. The frequency distributions observed in the present study were in agreement with those previously reported for the Calabrian population [10]. We found no significant age and sex difference with respect to the haplogroup classification in the analyzed sample (p = 0.783 and p = 0.534, respectively). In addition, we observed that in both sexes subjects carrying mtDNA belonging to the J haplogroup exhibited lower GDMI values than those carrying mtDNA belonging to non-J haplogroup (p = 0.019 in males; p = 0.002 in females). As GDMI inversely correlates with the global DNA methylation levels, we can infer that the J haplogroup carriers have higher global DNA methylation levels with respect to the non-J haplogroup carriers.

A subset of the whole sample analyzed with Sigma’s Imprint Methylated DNA Quantification Kit gave consistent results (data not shown).

Global DNA methylation level analysis in cybrid cell lines

In order to better clarify the influence of the mtDNA variability on global DNA methylation, we carried out an in vitro study by applying the CpGlobal assay to cybrid cell lines which were constructed by repopulating 143B.TK Rho0 cells with mitochondria derived from platelets of healthy human donors harboring mtDNA of H, J, U, X and T haplogroups. At the beginning of all experiments, these cells were carefully evaluated for proliferation rate, number of copies of mtDNA and MMP as previously reported [40, 42]. We did not observe any significant difference in the above parameters among cybrids and between any of them and the parental 143B.TK cell line (data not shown). As expected, MMP was lower in the Rho0 cells.

Results we obtained indicated that global DNA methylation levels were different among cybrids (Figure 1; p < 0.001), with the J cybrids showing lower GDMI values and, thus, higher methylation levels than each of the other lines (p < 0.05 in all comparisons). Since mtDNA is the sole variant among the cybrid lines, these results demonstrate a correlation between mtDNA variability and global DNA methylation levels, thus confirming the evidence emerging from the population study. Moreover, the comparison between the global methylation levels of the 143B.TK and Rho0 cell lines demonstrated that these levels are also associated with the depletion of mtDNA as Rho0 cells exhibited higher levels with respect to the native line (p = 0.009).

Methylation in cybrids was also tested with Sigma’s Imprint Methylated DNA Quantification Kit and gave consistent results (data not shown).

Gene-expression analysis

Once we determined that the global DNA methylation status was increased in the J cybrid line, quantitative real-time PCR assays were carried out in order to identify which of the following genes, DNMT1, DNMT3A, DNMT3B, MAT1A, MAT2B, MBD2 and MBD4, involved in DNA methylation processes, were responsible for the hypermethylation profile. By
Table 1. Global DNA methylation index values of the population samples according to the mitochondrial DNA haplogroup.

<table>
<thead>
<tr>
<th>Haplogroup</th>
<th>Males</th>
<th></th>
<th></th>
<th>Females</th>
<th></th>
<th></th>
<th>Total</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Rel. Freq.</td>
<td>Age (years)</td>
<td>GDMI</td>
<td>n</td>
<td>Rel. Freq.</td>
<td>Age (years)</td>
<td>GDMI</td>
<td>n</td>
<td>Rel. Freq.</td>
</tr>
<tr>
<td>H</td>
<td>47</td>
<td>0.29±0.04</td>
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<td>0.52±0.03</td>
<td>73</td>
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<tr>
<td>I</td>
<td>5</td>
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<td>88.80±5.03</td>
<td>0.42±0.12</td>
<td>3</td>
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<tr>
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<td>0.39±0.08</td>
<td>15</td>
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<td>86.53±3.95</td>
<td>0.36±0.05</td>
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<tr>
<td>K</td>
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<td>0.11±0.03</td>
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<td>18</td>
<td>0.09±0.02</td>
<td>84.06±3.39</td>
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<tr>
<td>Others</td>
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<tr>
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<td>84.96±0.99</td>
<td>0.52±0.02</td>
<td>191</td>
<td>–</td>
<td>83.96±0.92</td>
<td>0.51±0.01</td>
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</table>

These values, which represent means of three independent triplicate experiments, are also reported by age and gender.

GDMI: Global DNA methylation index; mtDNA: Mitochondrial DNA; n: Absolute frequency of the individuals belonging to the haplogroup class ± standard error of the mean; Rel. Freq.: Relative frequency of the individuals belonging to the haplogroup class ± standard error of the mean.

Comparing the mRNA levels of the above genes among cybrids, a significant difference was observed only for the MAT1A gene (Supplementary Figure 2 & Figure 2; p < 0.001). More specifically, the J cybrids showed higher mRNA levels of the MAT1A gene than each of the non-J cybrids (p < 0.05 in all comparisons). These results suggested that the mtDNA variability may modulate the expression of MAT1A and that the overexpression of this gene might be involved in the hypermethylation status of the J cybrids.

The role of mtDNA in modulating the expression profiles of the above genes was also supported by the comparison of the Rho0 cells with the parental cell line. Indeed, as the J cybrids, the Rho0 cells showed higher MAT1A mRNA levels than the parental cell line (Figure 2; p = 0.011).

Measurement of ATP & ROS levels
To investigate the molecular mechanisms underlying the differences in the MAT1A expression profiles previously reported between J and non-J cybrids, we measured intracellular ATP and ROS levels, major products of mitochondrial activity and regulators of cross signaling between mitochondrial and nuclear genomes. The J cybrids showed lower intracellular ATP (Figure 3a) and lower ROS levels (Figure 3b; p < 0.05 in all comparisons) than each of the non-J cybrids. Interestingly, Rho0 cells exhibited lower ATP levels but higher ROS levels with respect to the parental cell line.

Discussion
The aim of the present study was to investigate whether mtDNA variants could affect global DNA methylation profiles. Indeed, while it is quite clear that these variants are able to regulate several intracellular functions as well as to induce changes in nuclear gene expression, no data has been documented to date about their influence on epigenetic processes. From our population association study and in vitro analyses, it has emerged that the subjects and cybrid cells harboring mtDNA molecules belonging to the J haplogroup have higher global DNA...
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methylation levels than non-J carriers. These data indicate a clear correlation between mtDNA variability and global DNA methylation. The above correlation might be explained by a differential activation of signaling pathways involved in the crosstalk between nucleus and mitochondria, widely described in the literature. These pathways, by acting either directly on energy production efficiency or indirectly by interacting with nuclear genes, could be responsible for qualitative and quantitative differences in living cells in a mtDNA-specific manner [60,61]. What is more, the difference in DNA methylation levels between native and Rho<sup>0</sup> cells we observed are in line with data reported by Smiraglia et al. and provide additional evidence for the above interactions, thus confirming the existence of a retrograde response in human cells [54,62]. Interestingly, it has been repeatedly reported that the variants defining the J haplogroup have functional consequences on several complex traits, including LHON, multiple sclerosis and longevity [10,12,17,63–66]. The data we present here may highlight a novel function for this haplogroup implying that its phenotypic effects may partly be due to its effects on epigenetic changes.

We are aware that the CpGlobal method relies on measurements of luminescence following DNA digestion with HpaII and MspI enzymes, and thus is dependent on their cutting efficiency, and that a large debate exists regarding the technical manipulations leading to cybrid production and its effect on casual chromosomal rearrangements. Conversely, we are confident of the reliability of our results since: a series of control experiments demonstrated the effectiveness of the CpGlobal method [40,42]; the global DNA methylation levels determined by the CpGlobal assay were confirmed by adopting an independent method on a subset of the entire study group; all significant differences between cybrids were replicated three times on independent cybrid clones.

Gene-expression studies reported in this paper also demonstrate that the hypermethylation observed in the J cybrids might be due to the MAT1A gene which is overexpressed in these cells with respect to the other cybrids.

How could the mtDNA sequence-dependent genetic information be responsible for the regulation of the MAT1A gene, and thus, of the hypermethylation in the J cybrids? It is possible that such effects are mediated by the lower ATP levels we observed in the J cybrids than in non-J cybrids.

This hypothesis is also confirmed by observing that Rho<sup>0</sup> cells, which exhibit minimum levels of ATP, have a global methylation pattern similar to that displayed by J cybrids. We retain that the presence/absence of the mtDNA or the variability of its sequence affects the global methylation levels by regulating ATP levels. It is likely that the effect of the ATP on methylation may be threshold-dependent and that the ATP levels of both Rho<sup>0</sup> cells and J cybrids are below this threshold. Further studies are needed to evaluate this hypothesis.

The low ATP levels and the above-mentioned association with different cellular phenotypes of the J haplogroup have been
extensively ascribed to the low OXPHOS efficiency, given that the variants clustered in J haplogroup mainly fall within mitochondrial complex I (ND1 subunit) and complex III (cytochrome b) [24,29,30,67–70]. In line with this hypothesis, oxygen consumption has been reported to be lower in individuals from the J haplogroup with respect to the H haplogroup, as well as the T4216C transition, characterizing the J haplogroup, has an effect on the structural integrity of the ND1 complex and, subsequently, on its activity [24–26]. In addition, Arning et al. reported that haplogroup J variations “partially uncouple OXPHOS” [29]. Here, the demonstration that J cybrids produce lower ATP levels than the non-J cybrids adds further experimental evidence of the uncoupling effect of this haplogroup on oxidative phosphorylation, although some considerations need to be outlined. First, we observed differences in OXPHOS efficiency in cybrids harboring the J and T haplogroups, although they share a common root in the mitochondrial tree, such as the 4216/ND1 and 15542/cytb variants. Second, we observed differences in OXPHOS efficiency between J and non-J cybrids, but not among non-J cybrids, all of them characterized by different clusters of mtDNA variants (Supplementary Table 1). Last, according to previously reports, we did not observe any difference in OXPHOS efficiency between H, ‘tightly coupling OXPHOS’ and U, X and T haplogroups, which harbor variants that are thought to be ‘partially uncoupling’ OXPHOS [29,45]. Scenarios we described induce us to also consider the role of nonsynonymous ‘private’ variants for which a functional role has yet to be determined. These variants are thought to contribute to overall phenotypic variance of each haplogroup through complex interactions with the environment and nuclear background. We retain that, in our study, the role of private variants on the functional effects of the J haplogroup is quite irrelevant. This assumption comes from both the consistence of results we obtained in population and in vitro studies and by the sequence analysis of the whole mtDNA of our J cybrids demonstrating that they do not harbor private variants [40].

By combining our results with published data, we hypothesize that ATP levels affect MAT1A gene transcription. Such effect may be obtained either by chromatin epigenomic remodeling or by modulating the methylation levels of its promoter region, which is known to be regulated by epigenetic changes [71–73]. Alternatively, ATP may regulate the activity of transcription factors involved in MAT1A gene expression. In this regard, it is important to note that factors, such as AP1, CRE-BP, E2F and CAAT, have several motif binding sites located within the MAT1A promoter region, exhibit activity correlated to ATP levels and are also involved in the cross-signaling between nucleus and mitochondria [74,75]. We are currently carrying out experiments which could clarify the involvement of MAT1A in the hypermethylation of the J cybrids and confirm one or the other hypothesis.

With regards to ROS levels, we found conflicting results: low levels of ROS correlated with high DNA methylation in J cybrids and high levels of ROS correlated with high DNA methylation in Rho0 cells. This might suggest that ROS are not predictors of DNA methylation status, although we need to highlight

![Figure 3. Measurement of ATP and reactive oxygen species levels in 143B. TK, Rho0 and cybrid cell lines. (A) ATP levels are reported as nM determined in three independent triplicate experiments with standard errors of the mean. Analysis of variance test p < 0.001; Student’s t-test: 143B.TK vs Rho0 p < 0.001; J vs H p = 0.027; J vs U p = 0.003; J vs X p = 0.04; J vs T p = 0.046. (B) ROS levels are represented as percentage of ROS-staining positive cells. Data represent the means of three independent experiments with standard errors of the mean. Analysis of variance test p < 0.001; Student’s t-test: 143B.TK vs Rho0 p < 0.001; J vs H p = 0.032; J vs U p = 0.028; J vs X p = 0.031; J vs T p = 0.038. ROS: Reactive oxygen species.](image)
that Rho0 cells are in an ‘artificial state’ due to mitochondrial dysfunction caused by mtDNA depletion and to the consequent compensatory mechanisms [76].

Conclusion
On the whole, our data provide evidence that mtDNA variability modulates global DNA methylation levels, possibly via the regulation of OXPHOS efficiency. This may represent an alternative mechanism for the remodeling of gene expression that commonly occurs during lifetime. Conversely, whether the observed epigenomic differences due to mtDNA variability are epiphenomena or part of the causal pathways leading to biological functions is still largely to be determined. Future studies seeking to determine the methylation levels of specific mitochondrial genes will need to clarify this point.

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Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary
• Several published studies report evidence for mitochondrial functions affecting DNA epigenetic changes during a human’s lifetime.
• Variants of mitochondrial DNA (mtDNA) contribute to the interindividual variability in mitochondrial function by a network of signaling between the nucleus and mitochondria.
• The results reported in this study aim to determine whether global DNA methylation levels are affected by mitochondrial DNA variability.
• Population and in vitro results we obtained demonstrate a clear correlation between J mtDNA haplogroup and global DNA methylation levels.
• The above correlation seems to be associated with differences in ATP levels and in MAT1A gene expression.
• This work indicates that mtDNA-specific interactions between mitochondria and the nucleus regulate epigenetic changes, possibly by changes in oxidative phosphorylation efficiency.

References
Papers of special note have been highlighted as:
• of interest
• of considerable interest
• Excellent review of the role played by mitochondria in aging and age-related diseases.
• First evidence demonstrating functional effects of mitochondrial variants.
• First evidence that the mitochondrial variants are associated with a complex trait such as aging.
First experimental demonstration that cells depleted of their own mitochondrial DNA can be repopulated with exogenous mitochondria. The cybrid cell lines so obtained currently represent a powerful tool to investigate the crosstalk between nucleus and mitochondria.

**First evidence that mitochondrial variability influences the expression of nuclear genes.**


Global DNA methylation levels are modulated by mitochondrial DNA variants


** Important review of the influence of mitochondrial functions on epigenetic changes.


Global DNA methylation levels are modulated by mitochondrial DNA variants.