ORIGINAL ARTICLE

Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease

Carlos Jose Pirola,[1] Tomas Fernández Gianotti,[1] Adriana Laura Burgueño,[1] Manuel Rey-Funes,[2] Cesar Fabian Loidl,[2] Pablo Mallardi,[3] Julio San Martino,[3] Gustavo Osvaldo Castaño,[4,5] Silvia Sookoian[4,5,6]

► Additional materials are published online only. To view these files please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2012-302962).

[1]Department of Molecular Genetics and Biology of the Complex Diseases, Institute of Medical Research A Lanari-IDIM, University of Buenos Aires-National Council of Scientific and Technological Research (CONICET), Ciudad Autónoma de Buenos Aires, Argentina
[2]Institute of Cellular Biology and Neuroscience "Prof. E. De Robertis", School of Medicine, University of Buenos Aires-National Council of Scientific and Technological Research (CONICET), Ciudad Autónoma de Buenos Aires, Argentina
[3]Department of Pathology, Hospital Diego Thompson, San Martin, Buenos Aires, Argentina
[4]Liver Unit, Medicine and Surgery Department, Hospital Abel Zubizarreta. Ciudad Autónoma de Buenos Aires, Argentina
[5]Research Council in Health, Ciudad Autónoma de Buenos Aires, Argentina
[6]Department of Clinical and Molecular Hepatology, Institute of Medical Research A Lanari-IDIM, University of Buenos Aires-National Council of Scientific and Technological Research (CONICET), Ciudad Autónoma de Buenos Aires, Argentina

Correspondence to
Dr Carlos Jose Pirola and Dr Silvia Sookoian, Instituto de Investigaciones Médicas IDIM-CONICET, Av. Combatiente de Malvinas 3150. (C1427ARO) Buenos Aires, Argentina; pirola.carlos@lanari.fmed.uba.ar; sookoian.silvia@lanari.fmed.uba.ar

Revised 26 June 2012
Accepted 14 July 2012

ABSTRACT

Objective & design Nonalcoholic fatty liver disease (NAFLD) is a clinical condition that refers to progressive histological changes ranging from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH). We evaluated the status of cytosine methylation (5mC) of liver mitochondrial DNA (mtDNA) in selected regions of the mtDNA genome, such as D-loop control region, and mitochondrially encoded NADH dehydrogenase 6 (*MT-ND6)* and cytochrome C oxidase I (*MT-CO1*), to contrast the hypothesis that epigenetic modifications play a role in the phenotypic switching from SS to NASH.

Methods We studied liver biopsies obtained from patients with NAFLD in a case-control design; 45 patients and 18 near-normal liver-histology subjects.

Results *MT-ND6* methylation was higher in the liver of NASH than SS patients ($p<0.04$) and *MT-ND6* methylated DNA/unmethylated DNA ratio was significantly associated with NAFLD activity score ($p<0.02$). Liver *MT-ND6* mRNA expression was significantly decreased in NASH patients (0.26 ± 0.30) versus SS (0.74 ± 0.48), $p<0.003$, and the protein level was also diminished. The status of liver *MT-ND6* methylation in NASH group was inversely correlated with the level of regular physical activity ($R=-0.54$, $p<0.02$). Hepatic methylation levels of D-Loop and *MT-CO1* were not associated with the disease severity. DNA (cytosine-5) methyltransferase 1 was significantly upregulated in NASH patients ($p<0.002$). Ultrastructural evaluation showed that NASH is associated with mitochondrial defects and peroxisome proliferation.

Conclusion Hepatic methylation and transcriptional activity of the *MT-ND6* are associated with the histological severity of NAFLD. Epigenetic changes of mtDNA are potentially reversible by interventional programs, as physical activity could modulate the methylation status of *MT-ND6.*

Significance of this study

What is already known on this subject?

- The role of mitochondria and mitochondrial DNA (mtDNA) in metabolic-syndrome related phenotypes has long been recognised.
- The molecular mechanisms associated with nonalcoholic fatty liver disease (NAFLD) progression are still unknown, but the evidence strongly suggests that mitochondrial dysfunction might be critically involved in the pathogenesis of NAFLD progression.

What are the new findings?

- We answered the question whether mtDNA methylation plays a role in the molecular changes that mediate the transition from one state without liver injury (simple steatosis) to a more aggressive disease (nonalcoholic steatohepatitis); the status of 5mC of human mtDNA in the liver tissue was never explored before.
- We observed that mitochondrially encoded NADH dehydrogenase 6 (*MT-ND6*) is highly methylated in the liver of NASH patients but not in patients with simple steatosis, and the status of methylation significantly impacts on the *MT-ND6* transcriptional regulation, an effect probably dependent on the enhanced expression of the mitochondrial-targeted isoform of the DNA (cytosine-5) methyl transferase 1 (*DNMT1*).
- Liver *MT-ND6* transcriptional activity and protein expression were significantly decreased in nonalcoholic steatohepatitis, suggesting that the expression of this mitochondrial gene, which is regulated by an epigenetic modification, may play an important role in the pathogenesis of the disease progression.
- Our results also show that the status of *MT-ND6* cytosine methylation is potentially inducible by environmental factors, such as life style intervention.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a clinical condition that refers to potentially progressive histological changes ranging from fatty liver alone (simple steatosis, SS) to nonalcoholic steatohepatitis (NASH), a disease stage characterised by liver cell injury, a mixed inflammatory lobular infiltrate and variable fibrosis.[1] Clinical data do not necessarily parallel the magnitude of the histological lesions and the histological stages are hardly distinguished without confirmation by a liver biopsy. Epidemiological data reveal that patients with SS usually have a benign prognosis in contrast to patients with NASH, who can progress to cirrhosis and, eventually, to hepatocellular

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- Mitochondrial epigenetics is a novel mechanism to understand the pathobiology of complex diseases such as NAFLD and other diseases with a mitochondrial dysfunction involvement.
- Lifestyle changes such as regular physical activity might modulate the status of hepatic DNA methylation of *MT-ND6*.
- Because of the inherent plasticity of epigenetic modifications, either physiologically or pathologically produced, the epigenetic regulation of liver *MT-ND6* in NASH patients might have therapeutic potential.

carcinoma.[2] Remarkably, evidence from clinical trials showed that hepatic steatosis and lobular inflammation are potentially reversible[3]; however, the molecular mechanisms by which the histological lesions could be reverted are still unknown.

In addition, although the understanding of the mechanisms related with the disease progression remains a major challenge, some evidence exists on the molecular pathways that might contribute to the phenotypic switching from one state without liver injury (SS) to a more aggressive disease (NASH). For instance, hepatocyte apoptosis, lipotoxicity, and oxidative stress[4–6] are plausible molecular mediators of cellular injury, inflammatory recruitment, and fibrogenesis. Interestingly, the above-mentioned molecular pathways converge into a common point: mitochondrial dysfunction, which critically determines the activity of the oxidative phosphorylation cascade, and is associated with early proapoptotic events, defects in fatty acid oxidation, and impaired insulin signalling.

Earlier evidence indicated that whereas insulin resistance, increased fatty acid β oxidation, and hepatic oxidative stress are present in both SS and NASH, only NASH is associated with mitochondrial structural and molecular defects.[7] In addition, patients with NASH have a significantly decreased activity of mitochondria respiratory chain complexes.[8] Therefore, the evidence strongly suggests that mitochondrial dysfunction is involved in the pathogenesis of NAFLD progression. Although some evidence from animal studies is emerging,[9] the molecular mechanisms leading to liver mitochondrial dysfunction in human NASH are still unknown.

Defects of the mitochondrial genome are widely recognised as responsible of mitochondrial dysfunction as mitochondrial DNA (mtDNA) critically controls the mitochondrial gene expression machinery.[10] Until very recently, mutations and deletions of mtDNA were the only mechanisms to explain changes in the transcriptional mitochondrial profile. Recent evidence showed that an isoform of DNA methyltransferase 1 (DNMT1) is targeted to mtDNA and appears to be responsible for mtDNA methylation of cytosine at the carbon-5 position (5mC), in CpG dinucleotides, which in turn regulates mitochondrial function and gene transcription.[11] Hence, the presence of 5mC in human mtDNA led us to question whether this epigenetic modification might play a role in the molecular changes that explain why NASH is progressive and SS is not. Therefore, in this study, we evaluated the status of cytosine modifications of liver mtDNA in selected regions of the mtDNA genome, such as the displacement loop (D-loop), the mitochondrially encoded NADH dehydrogenase 6 (*MT-ND6)*, and cytochrome C oxidase I (*MT-CO1 or COX1*), because a previous report showed that methylation of mtDNA is nonrandom,[12] and Shock *et al* showed that the CpG-dependent interaction of DNMT1 with the mitochondrial genome occurs in specific points, which significantly affects the mitochondrial transcriptional machinery.[11]

PATIENTS AND METHODS

Patients

To explore the role of liver mtDNA methylation in the modulation of the histological disease severity, we studied liver biopsies obtained from 45 unrelated adult NAFLD patients, including 23 patients with SS and 22 patients with NASH. Patients were considered for inclusion if they had histopathological evidence of fatty liver disease, either SS or NASH, on liver biopsy performed within the study period.

Moreover, to understand to what extent liver mtDNA methylation might modulate metabolic syndrome (MS)-associated phenotypes, the livers of 18 subjects without MS (Non-MS-near normal liver histology) were included in this study, in a case-control design. Subjects without MS were selected from patients attending the Liver Unit, whose age and sex matched the NAFLD patients; liver specimens were obtained by percutaneous liver biopsy, and the reason for performing a liver biopsy in these subjects was based on the presence of persistently mildly elevated serum liver enzymes activity. In all the Non-MS-near normal liver histology subjects, all causes of common liver disease were ruled out, and they were included in the study if they did not have histological evidence of fatty change; the histological diagnosis of control livers was minimal changes or mild cholestasis. Surgical liver biopsies were avoided because significant changes in gene expression were previously reported in the frame of surgical stress.[13]

The case participants and the Non-MS-near normal liver histology subjects were selected during the same study period from the same population of patients attending the Liver Unit, and all of them share the same demographic characteristics (occupation, educational level, place of residence, and ethnicity).

More details can be found in the online supplementary material.

All the investigations performed in this study were conducted in accordance with the guidelines of the 1975 Declaration of Helsinki. Written consent from individuals was obtained in accordance with the procedures approved by the Ethical Committee of our institution.

Physical, anthropometric, and biochemical evaluation

Health examinations included anthropometric measurements, a questionnaire on health-related behaviours and biochemical determinations as previously described.[14] Details about physical, anthropometric and biochemical evaluation are shown in table 1. Regular physical activity was defined as all forms of activity, such as walking or cycling for everyday journeys, active play, work-related activity, active recreation such as working out in a gym, dancing, or competitive sport and the overall amount of activity was expressed in hours / week; there was no specific exercise intervention, and data about regular physical activity was surveyed at baseline by the time of liver biopsy.

Liver biopsy and histopathological evaluation

The degree of steatosis was assessed according to the system developed by Kleiner *et al* based on the percentage of hepatocytes containing macrovesicular fat droplets.[15] NASH[1] and NAFLD Activity Score (NAS)[15] were defined as previously

Table 1 Clinical and biochemical characteristics of the whole population according to disease status

Variables	NON-MS-near normal liver histology subjects	SS patients	NASH patients	p Value* p<	p Value† p<	p Value‡ p<
Number of subjects	18	23	22	—	—	—
Female/male, %	10/8	14/9	12/10	NS	NS	NS
Age, years	48.3±8.7	51.6±10.5	48±9.2	NS	NS	NS
Smoking habit, cigarettes/day	0.7±2.6	3.1±5.9	0.5±1.5	NS	NS	NS
Physical activity, h/week	1.4±2.8	0.8±1.5	0.5±0.9	NS	NS	NS
BMI, kg/m^2	25.3±4.1	31.6±5	31.2±6	0.0001	NS	0.001
Waist circumference, cm	94.2±12	106.9±8.2	102.6±16.5	0.001	NS	0.01
Waist/hip ratio	0.9±0.1	1.0±0.1	0.9±0.1	NS	NS	NS
SABP, mm Hg	117.8±18.6	124.4±14	124.7±11.0	NS	NS	NS
DABP, mm Hg	70±11.2	76.7±13.0	80±8.4	NS	NS	0.04
Fasting plasma glucose, mmol/l	4.81±0.56	5.69±0.89	5.62±0.67	0.001	NS	0.001
Fasting plasma insulin, pmol/l	41.0±10.4	97.2±81.3	97.9±68.3	0.01	NS	0.002
HOMA-IR index	1.24±0.8	3.4±0.5	3.6±0.5	0.001	NS	0.002
Total cholesterol, mmol/l	5.02±0.98	5.21±0.91	5.73±1.42	NS	NS	NS
HDL-cholesterol, mmol/l	1.77±0.41	1.22±0.34	1.37±0.34	0.01	NS	0.02
LDL-cholesterol, mmol/l	2.71±0.93	3.24±1.01	3.53±1.29	NS	NS	NS
Triglycerides, mmol/l	1.51±1.17	1.93±0.62	2.43±1.50	0.03	NS	0.04
Uric acid, mmol/l	226±101	315±89	345±107	NS	NS	0.05
ALT, U/L	81.3±76	68.6±40	59.9±41	NS	NS	NS
AST, U/L	51.9±38	38.4±15	37.9±25	NS	NS	NS
GGT, U/L	104.5±114	71.1±49	73.3±42	NS	NS	NS
AP, U/L	238.2±194	215.4±104	178.4±71	NS	NS	NS
Histological features						
Degree of steatosis, %	—	35±22.1	62.6±20.6	—	0.001	—
Lobular inflammation (0—3)	—	0.4±0.5	1.3±0.9	—	0.003	—
Portal inflammation (0—2)	—	0.5±0.5	1.1±0.4	—	0.001	—
Hepatocellular ballooning (0—2)	—	0	1.2±0.4	—	0.0000001	—
Fibrosis stage	—	0	1.1±1.1	—	0.01	—
NAS score	—	2.3±1.1	5.9±1.8	—	0.00001	—

Results are expressed as mean±SD. All measurements are in SI units.
*Indicates comparisons between SS versus Non-MS-near normal liver subjects.
†Stands for comparisons between NASH versus Simple steatosis.
‡Stands for comparisons between NASH versus Non-MS-near normal liver. p Value stands for statistical significance using Mann—Whitney U test, except for female/male proportion that p value stands for statistical significance using χ^2 test.
ALT and AST, serum alanine and aspartate aminotransferase; AP, alkaline phosphatase; BMI, body mass index; DABP, diastolic arterial blood pressure; GGT, gamma-glutamyl-transferase; HOMA-IR, homeostatic model assessment-insulin resistance; MS, metabolic syndrome; NS, non significant; NASH, nonalcoholic steatohepatitis; SABP, systolic arterial blood pressure; SS, simple steatosis.

reported; a NAS threshold of five was used for further comparisons with variables of interest.[15] More details can be found in the online supplementary material.

Liver immunohistochemistry

Immunostaining was performed on liver specimens of NAFLD patients previously included in paraffin. Details about immunostaining can be found in the online supplementary material.

Electron microscopy (EM) of the liver tissues

Electron microscopy studies were performed on liver specimens by immersion of a small sample (1 mm) in 2.5% glutaraldehyde and 0.1 M phosphate buffer (pH 7.4); sections were mounted in copper grids, counterstained with lead citrate, and observed in a transmission electronic microscope (Zeiss E.M. 10C) by experienced electron microscopists (RFM and CFL).

Bisulfite treatment of DNA and methylation-specific PCR

Briefly, this technique is based on the bisulfite treatment of genomic DNA, thereby converting all the unmethylated cytosines to uracils, while conserving the methylated cytosines, using the EZ DNA Methylation Kit, according to the manufacturer's protocol (Zymo Research Corporation, Orange, CA, USA). The level of methylated DNA is expressed as the ratio of the estimated amount for methylated DNA to the unmethylated DNA levels, calculated for each sample using the fluorescence threshold cycle (Ct) values for a previously estimated efficiency.[16] More details can be found in the online supplementary material.

RNA preparation and real-time RT-PCR for quantitative assessment of mRNA expression

The primer sequences are shown in table 2; more details can be found in the online supplementary material.

Statistical analysis

The quantitative data were expressed as mean±SD, unless otherwise indicated. Because a significant variance difference was observed between the groups for most of the variables and as the distribution was significantly skewed in most cases, we chose to be conservative and assessed the differences between the groups by using the non-parametric Mann—Whitney *U*-or Kruskal—Wallis tests. For multiple regression analysis or ANCOVA for adjusting for covariables, such as HOMA, as the methylated DNA/unmethylated DNA ratio and HOMA and were not normally distributed, we used log-transformation of these variables. Correlation between the two variables was performed by Spearman's rank correlation test or Pearson's

Table 2 PCR primers used for methylation-specific PCR (MS-PCR) and quantification of mRNA abundance of target genes

	The primers sequences for methylation-specific PCR	
Gene	Forward primer 5′ →3′	Reverse primer 5′ →3′
MT-ND6-M	TTTCGTATTAATAGGATTTTTTCGA	AATTATCTTTAAATATACTACAACGAT
MT-ND6-U	TTTTGTATTAATAGGATTTTTTTGA	ATAATTATCTTTAAATATACTACAACAAT
MT-CO1-M	GGAATATTATATTTATTATTCGGCGT	ACTAATCAATTACCAAAACCTCCG
MT-CO1-U	TGGAATATTATATTTATTATTTGGTGT	CTAATCAATTACCAAAACCTCCAAT
D-loop-M	TAGGAATTAAAGATAGATATTGCGA	ACTCTCCATACATTTAATATTTTCGTC
D-loop-U	GGTAGGAATTAAAGATAGATATTGTGA	ACTCTCCATACATTTAATATTTTCATC
	The primer sequences for mRNA gene expression	
Gene	Forward primer 5′ →3′	Reverse primer 5′ →3′
MT-ND6	AATAATTTATGAAGGAGAGG	CAAACAATGTTCAACCAGTA
MT-CO1	ACAGACCGCAACCTCAACAC	AGCCTGGTAGGATAAGAATA
MTRNR2	ACTAACCCCTATACCTTCTG	TTCCCACTATTTTGCTACAT
DNMT1	CATGGCCGGCTCCGTTCCAT	AGCTGTCTCTTTCCAAATCTTTGA
B-actin	CTG GCA CCC AGC ACA ATG AAG	AAA GGG TGT AAC GCA ACT AAG

M, methylated-specific primers; U, unmethylated-specific primers; *MT-ND6*, mitochondrially encoded NADH dehydrogenase 6; *MT-CO1*, mitochondrially encoded cytochrome c oxidase I (*COX1*); *D-Loop*, displacement loop, *MTRNR2*:http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=full_report&list_uids=4550 mitochondrially encoded 16S RNA. *DNMT1*: DNA (cytosine-5-)-methyltransferase 1.

correlation test, after log-transformation of the variables. To perform these analyses, we used the CSS/Statistica program package, V. 6.0 (StatSoft, Tulsa, OK, USA).

RESULTS

Mitochondrially encoded NADH dehydrogenase 6 is highly methylated in the liver of NASH patients

Our first approach was to explore the association between the hepatic 5mC of the D-loop, *MT-ND6* and *MT-CO1* (sequence details can be found in the Online only material) and the histological disease severity. Thus, we compared the levels of 5mC of the DNA of the mitochondrial genes in the liver of patients with SS versus patients with NASH. Interestingly, we observed that liver *MT-ND6* methylated DNA/unmethylated DNA ratio was significantly associated with NASH ($p<0.04$, even after adjusting by HOMA-IR; figure 1A), showing that 28.4% of alleles were methylated in NASH versus 20.6% in SS. Likewise, liver **MT-ND6** methylation levels were significantly associated with the NAS score (figure 1A), showing that the ratio of **MT-ND6** methylated DNA/unmethylated DNA levels is higher in patients with a NAS score above 5.

In addition, by grouping the dependant variable according to the severity of liver fibrosis as absent or mild (F0-F1) and moderate or severe (F2-F3), we observed a significant association with the status of liver *MT-ND6* methylation, showing that patients with more advanced fibrosis had higher levels of liver *MT-ND6* methylation (figure 1A). Despite the degree of steatosis was significantly higher in the NASH group compared with SS

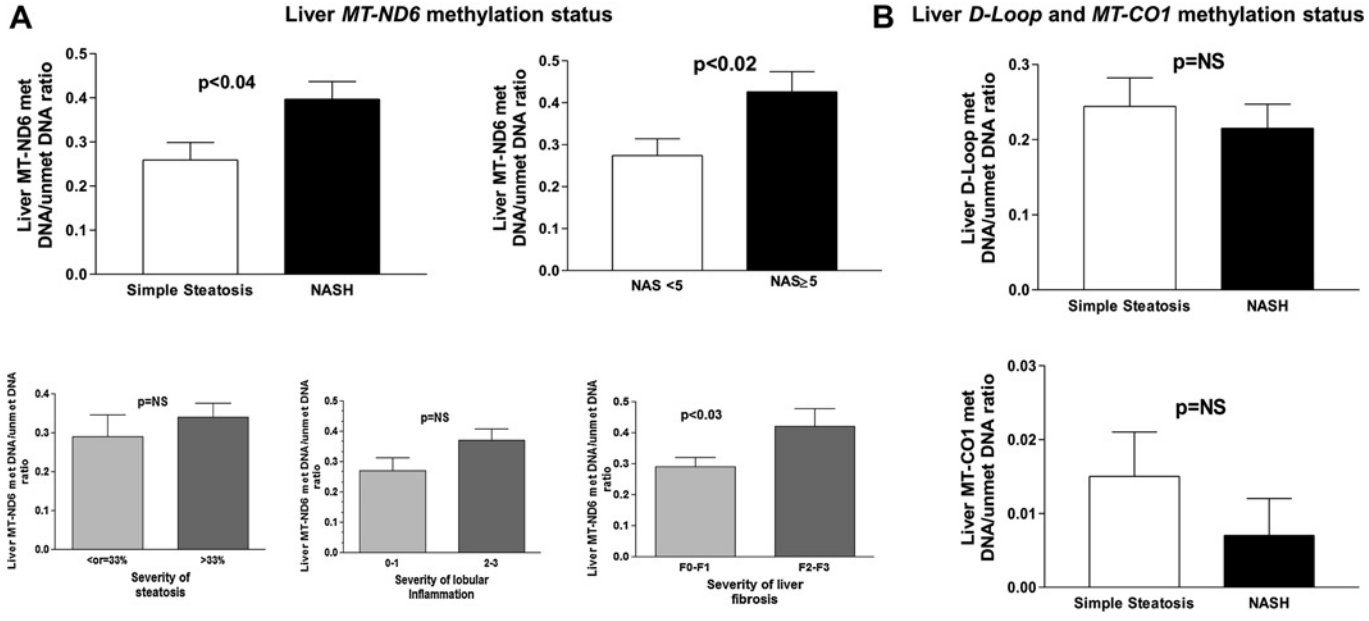

Figure 1 Status of DNA cytosine methylation of mitochondrially encoded NADH dehydrogenase 6 (*MT-ND6*), cytochrome C oxidase I (*MT-CO1*), and mitochondrial *D-Loop* in the liver of patients with nonalcoholic fatty liver disease according to histological disease severity (nonalcoholic steatohepatitis (NASH) vs simple steatosis (SS)) Each bar represents mean±SE values. In each sample, the level of methylated DNA is expressed as the ratio of the estimated amount for methylated DNA to the unmethylated DNA levels. The severity of fatty liver infiltration was graded as mild (< or =33%) moderate or severe (>33%); liver fibrosis was graded as absent or mild (F0-F1) and moderate or severe (F2-F3), as described in the Methods Section.

patients, liver *MT-ND6* methylation status was not different among patients with mild (degree < or =33%) and patients with moderate or severe (>33%) fatty liver infiltration (figure 1A), suggesting that the observed effect was not related to the extent of steatosis. Likewise, lobular inflammation was not associated with liver *MT-ND6* methylation status (figure 1A).

On the contrary, the histological disease severity was not associated either with the ratio of D-Loop (19.6% of methylated alleles in SS vs 17.7% in NASH) or *MT-CO1* methylated DNA/unmethylated DNA levels (1.5% of methylated alleles in SS vs 0.7% in NASH; figure 1B). Then, *MT-CO1* was mostly unmethylated in the liver of NAFLD patients as well as in the Non-MS subjects, which is consistent with what was previously found for this particular gene in another system.[11] There was a preferential cytosine methylation of the *MT-ND6* over the D-Loop region in NASH with respect to SS patients (ratio: 1.01±0.65 vs 1.77±0.86, p<0.009) demonstrating that these mitochondrial genes were differently affected by changes in cytosine methylation of mtDNA.

In fact, the ratio of *MT-ND6* methylated/methylated D-Loop levels was significantly higher in patients with moderate or severe fibrosis (1.83±0.72) in comparison with patients with absent or mild (1.23±0.85), p<0.05.

We further evaluated whether the status of hepatic 5mC of the targeted mitochondrial genes was associated with metabolic stressors such as peripheral insulin resistance (measured by HOMA-IR), hyperglycaemia, hypertriglyceridaemia, and hypercholesterolaemia; for this purpose, we included liver samples of the Non-MS-near normal liver histology group in the analysis (table 1). Surprisingly, we observed a significant inverse correlation between the hepatic *MT-CO1* methylated DNA/unmethylated DNA ratio and high-density lipoprotein (HDL) cholesterol levels ($R=-0.42$, p<0.004), and a significant association between *MT-CO1* methylated DNA/unmethylated DNA ($R=0.34$, p<0.01) and BMI.

Lifestyle changes such as regular physical activity might modulate the status of hepatic DNA methylation of *MT-ND6*

Recent evidence suggests that regular physical activity is able to modulate hepatic fat content,[17–19] and potentially influences NAFLD severity.[20] Exercise also involves changes and adaptation of oxidative phosphorylation (OXPHOS) in skeletal muscle by increasing the amount of mitochondrial proteins and direct stimulation of OXPHOS complexes.[21] Thus, we explored in the whole population (patients and Non-MS-near normal liver histology subjects, $n=64$) whether regular exercise could have an impact on the levels of 5mC in the selected mitochondrial genes, and we observed that *MT-ND6* methylated DNA/unmethylated DNA ratio was inversely correlated with the level of physical activity measured in h/week (Pearson's correlation coefficient $R=-0.30$, p<0.04). The analysis was in addition restricted to the NASH group, and interestingly, we observed and even strong inverse correlation (Pearson's correlation coefficient $R=-0.54$, p<0.02). Nevertheless, carefully controlled lifestyle intervention programs will better define the impact of epigenetic changes on human NAFLD progression.

Functional assessment of the impact of *MT-ND6* methylation on NASH pathobiology: 1-Liver *MT-ND6* transcription and protein levels are significantly reduced in patients with NASH

To further evaluate whether the transcriptional activity of liver *MT-ND6* is associated with NAFLD severity, we measured the mRNA relative abundance in the liver of patients with NASH and SS. Interestingly, we observed that the liver of NASH patients shows significantly reduced *MT-ND6* mRNA levels in comparison with SS (figure 2), and NAS score was significantly (p<0.007) and negatively correlated (Spearman $R=-0.50$) with *MT-ND6* mRNA levels in the whole population of patients with NAFLD. In addition, liver abundance of *MT-ND6* mRNA was significantly reduced in patients with a more severe histological disease (fibrosis moderate or severe F2-3), figure 2, suggesting that the transcriptional activity of the *MT-ND6* is strongly associated with the histological severity of NAFLD.

Furthermore, liver expression pattern of MT-ND6 protein was evaluated according to the histological disease severity of NAFLD, and we observed lower levels of liver MT-ND6 expression in patients with NASH in comparison with those showing SS (figure 2). Overall, positive staining for MT-ND6 was easily identified in hepatocyte cytoplasma showing a pattern, primarily but not exclusively restricted to regions surrounding lipid droplets (figure 2), showing that DNA methylation of *MT-ND6* is accompanied by a decrease in its protein level.

In addition, we evaluated the levels of *MT-CO1* mRNA in the liver of NAFLD patients and non-significant differences were observed between the two groups (figure 2).

Then, the ratio *MT-ND6* mRNA/*MT-CO1* mRNA ratio was significantly diminished in NASH versus SS patients (0.49±0.69 vs 1.12±0.45, p<0.002) demonstrating that these mitochondrial genes were differently affected by changes in cytosine methylation of mtDNA in relation with disease severity. Accordingly, this *MT-ND6* mRNA/*MT-CO1* mRNA ratio was inversely correlated with NAS score (Spearman $R=-0.60$, p<0.0006).

2-Hepatic expression of the *DNMT1* is significantly higher in the liver of NASH patients: a mechanistic explanation

DNMT1 is regarded as the maintenance DNA methyltransferase because of its critical role in the establishment and regulation of tissue-specific patterns of methylated cytosine residues. In addition, DNMT1 is the only member of the DNA methyltransferase family found to have an isoform targeted to the mitochondria.[11] In this study, we examined the liver transcriptional activity of the *DNMT1* isoform among NAFLD patients in order to answer the question whether its expression is higher in patients suffering from NASH. We were able to demonstrate that the *DNMT1* mRNA/ β-actin mRNA ratio was significantly higher in the liver of NASH patients (4.6±8.0) compared with SS (0.0004±0.002), mean ±SD ×1000, p<0.002.

NASH is associated with changes in mitochondrial morphology and peroxisome proliferation

Mitochondrial morphology is not only critical for maintaining optimal mitochondrial function but also crucially linked to energy metabolism and OXPHOS function.[22 23] Hence, we examined sections of the liver tissue by EM and, notably, in the hepatocytes of NASH patients, we found that mitochondrial morphology was considerably altered and characterised by structural defects, such as mitochondria with loss of their inner membrane and deep infoldings, known as cristae and also loss of the typical dense mitochondrial granules, and larger mitochondria (figure 3). In addition to the defects in mitochondrial morphology, NASH patients showed increased peroxisome proliferation (figure 3).

These defects were observed in the majority of cells displayed. Supplementary figure 1 shows liver mitochondria in close physical association with large lipid droplets, a finding that supports the localisation of the immunoreactivity product of MT-ND6 described above. The online supplementary figure 2

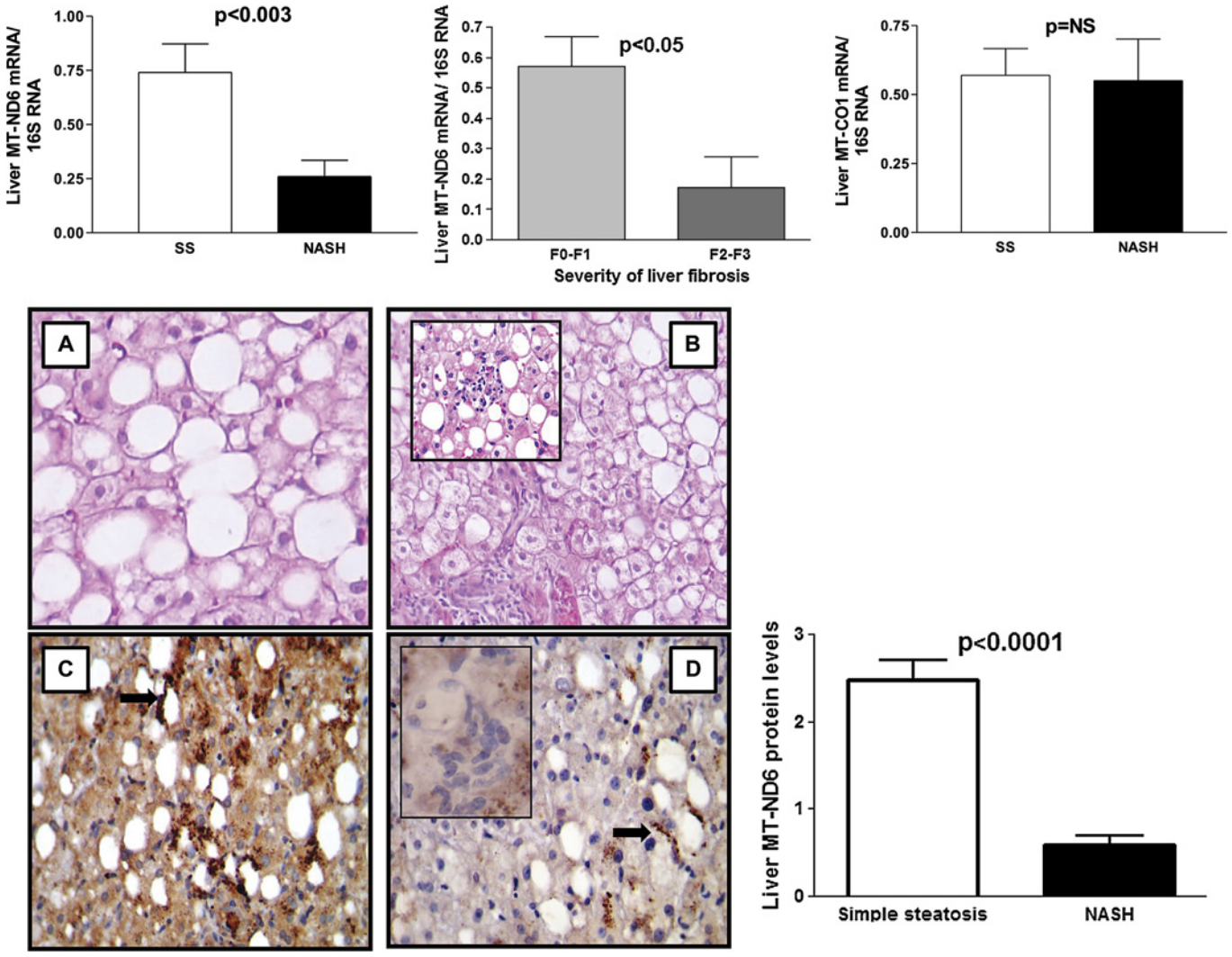

Figure 2 Liver abundance of *MT-ND6* and *MT-CO1* mRNA evaluated by quantitative real-time PCR in patients with nonalcoholic fatty liver disease according to the histological disease severity (nonalcoholic steatohepatitis (NASH) vs simple steatosis (SS)) Each bar represents the mean ± SD values. In each sample, the abundance of target genes was normalised to the amount of mitochondrially encoded 16S RNA to carry out comparisons between the groups. Liver fibrosis was graded as absent or mild (F0-F1) and moderate or severe (F2-F3) as described in the Methods Section. Representative liver expression pattern of MT-ND6 protein and its semiquantitative evaluation by immunohistochemistry in patients with NAFLD MT-ND6 immunoreactivity was examined by light microscopy on liver sections. A and C show a representative figure of a patient with SS, and B and D show a representative figure of a patient with NASH (inset shows lobular inflammation). A and B show specimens stained with H&E; C and D show MT-ND6 immunostaining (counterstaining was with haematoxylin). Hepatocytes of SS patients show intense granular staining in comparison with weak staining of NASH. Overall, the staining was particularly localised surrounding the lipid droplets (black arrows), regardless the NAFLD histological severity. Original magnifications: 400×.

shows a representative electron micrograph of liver mitochondria isolated from a patient with near normal liver histology.

DISCUSSION

In this study, we asked whether epigenetic modifications of mtDNA in the liver tissue play a role in the histological disease severity of NAFLD. Actually, 5mC of mtDNA seems to be a critical and potentially reversible mechanism of mitochondrial transcriptional control, but its role in the pathogenesis of human diseases has never been studied before.

We provide, to our knowledge, the first evidence that methylation of *MT-ND6* in the liver is higher in NASH than in SS patients. In addition, liver *MT-ND6* transcriptional activity and protein expression were significantly decreased in NASH with respect to SS patients, suggesting that the expression of this mitochondrial gene, which is regulated by an epigenetic modification, may play an important role in the pathogenesis of the disease progression. Moreover, we observed higher levels of *DNMT1* mRNA in the liver of NASH patients. On the contrary, we observed that liver methylation levels of *MT-CO1* and D-Loop were unrelated to the severity of NAFLD, with hepatic *MT-CO1* almost completely unmethylated.

Why could the regulation of hepatic *MT-ND6* be relevant in the pathogenesis of NAFLD progression?

Mutational analysis and in vivo studies showed that of the 13 proteins encoded by the mtDNA, only MT-ND6 (encoded by the light strand of the mtDNA) is an essential component of complex I.[24] Therefore, maintaining adequate levels of MT-ND6 is critical for the proper assembly of complex I,[25] which is the largest enzyme complex of the OXPHOS system and defects of which result in mitochondrial dysfunction.[26 27]

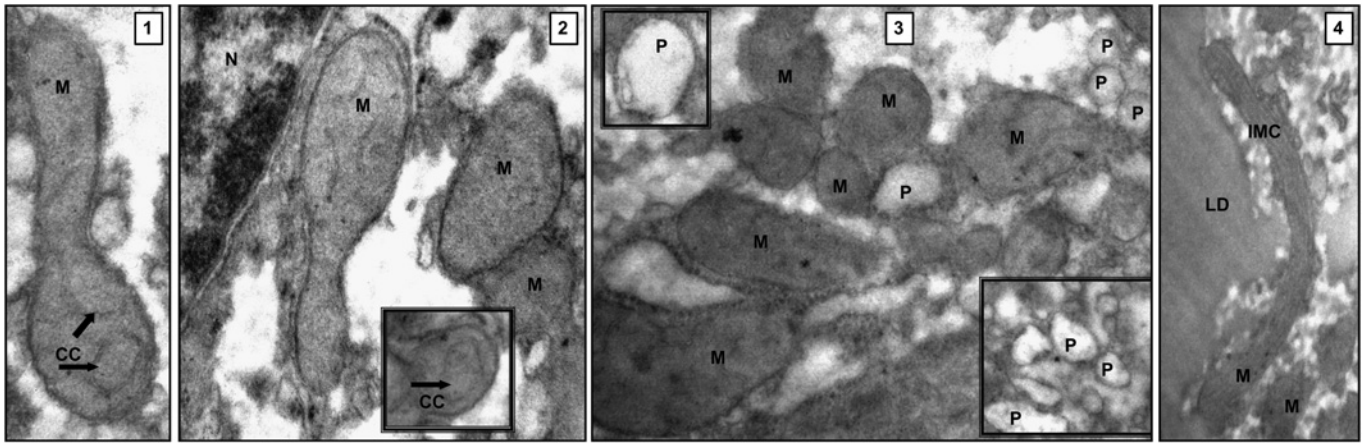

Figure 3 Ultrastructural changes in mitochondrial morphology and peroxisome proliferation observed in the hepatocytes of nonalcoholic steatohepatitis (NASH) patients. Representative electron micrograph of liver mitochondria isolated from a NASH patient showing altered morphology characterised by changes in organelle shape (panels 1, 2, 3), loss of mitochondrial infoldings and replacement by circular cristae (CC) (panels 1,2), mitochondria with condensed (opaque) matrix (panel 3), marked peroxisome proliferation in the hepatocytes' cytoplasm (inset, panel 3), and intramitochondrial crystals (IMC) in longitudinal form (4). M: mitochondria; N: nuclei; P: peroxisome. Magnification ×25.000/40.000.

Immunohistochemical staining for the MT-ND6 subunit has proven to be useful in identifying partial complex I disassembly[28]; hence, this observation reinforces our findings about significantly decreased hepatic protein expression of MT-ND6 in NASH patients might have functional implications.

Thus, the biological evidence mentioned earlier strongly suggest that the regulation of *MT-ND6* transcription, either by a direct effect or by modulation of the complex I activity significantly affects mitochondrial physiology and a number of key mitochondrial functions that could be involved in NAFLD progression.

The ultrastructural changes observed in the liver mitochondrion of NASH patients support our hypothesis about the potential association between disruption of the transcriptional activity of *MT-ND6* and mitochondrial dysfunction because morphological changes such as loss of mitochondrial cristae and changes in mitochondrial shape and size are often associated with OXPHOS defects and decreased mitochondrial protein expression.[29] Remarkably, loss of mitochondrial infoldings was previously observed by Sanyal A *et al*[7] in NASH patients but not in SS patients, and other mitochondrial disturbances, such as intramitochondrial crystals (figure 3), seem to be characteristics of NASH.[30]

Furthermore, we observed increased peroxisome proliferation in NASH patients, a finding reported to be a response to mitochondrial dysfunction.[31]

We might wonder about what factors could influence liver mtDNA methylation of selected genes in the context of NAFLD. Actually, in this study, we cannot show or demonstrate a cause-effect relation because the human mtDNMT1 isoform was very recently discovered.[11] and there is scarce data about its regulation. However, the evidence suggests that this locus is strongly influenced by oxidative stress,[11] a common finding in NASH. It is therefore possible that a some liver molecular events, such as lipotoxicity, oxidative stress, and local hypoxia, could influence the behaviour of the epigenetic modifications of the liver mitochondrial genome. Taken together, our data invite us to speculate that methylation and decreased transcription of liver *MT-ND6* participates in the molecular events associated with the histological progression of human NAFLD.

Remarkably, our results also showed that the status of *MT-ND6* cytosine methylation is potentially modified by environmental factors, such as physical activity, an attainable lifestyle intervention. This unexpected finding leads us to question whether the epigenetic regulation of liver *MT-ND6* in NASH patients might have any therapeutic potential. Because of the inherent plasticity of epigenetic modifications, either physiologically or pathologically produced, we are optimistic that therapeutic approaches and quick interventions are able to modify the natural history of NAFLD. We might also hypothesise that the reversion of liver inflammation by vitamin E[3] could be associated with modulation of the mitochondrial transcriptional activity as α tocopherol is able to restore the mitochondrial respiratory enzymatic activity.[32] This reasoning may also be applicable to other vitamins such as folic acid, which participates in the methyl donor metabolism that improves hyperhomocysteinaemia, a well-known cardiovascular risk factor.[33]

Finally, in this study, we also explored the impact of epigenetic modifications of liver mtDNA on the MS-associated traits. Analysing methylation levels of the target genes in the liver of Non-MS subjects, in comparison with NAFLD patients, we observed that the hepatic *MT-CO1* methylation status was significantly associated with the subjects' BMI and plasma HDL cholesterol levels. These findings, despite being unexpected, raise interesting questions about the role of hepatic mtDNA methylation on the pathogenesis of intermediate phenotypes associated with the MS, and suggest that fatty liver is not an innocent bystander comorbidity but seems to play a critical role in the systemic abnormalities associated with atherosclerosis.[34–36]

In conclusion, our study shows that epigenetic control and transcriptional activity of mitochondrially-encoded mitochondrial genes, such as *MT-ND6*, might be involved in the pathogenesis of NAFLD progression. Although the cross-sectional nature of our study does not allow us to prove the impact of changes in mtDNA methylation on the reversibility of the histological lesions, some clues about the putative molecular events that could modulate the severity of the disease are suggested. In addition, our study supports the notion of the complex nature of the NAFLD pathogenesis, which requires a dynamic balance between genetic and environmental factors that potentially operate at the epigenetic level in order to modulate its natural history. Liver mitochondria and, in turn, the dysfunction of the OXPHOS system might participate in the phenotypic switching from SS to NASH.

Contributors CJP and SS designed the study, analysed and interpreted the data, and prepared and wrote the manuscript. TFG and AB performed molecular experiments. SS and GC collected samples and performed liver biopsies. MRF and CFL performed electron microscopy studies, and PM and JSM performed immunohistochemistry.

Funding Supported in part by Grants UBACYT CM04 (Universidad de Buenos Aires), PICT 2008-1521 and 2010-0441 (Agencia Nacional de Promoción Científica y Tecnológica). SS, TFG, ALB, CFL and CJP belong to Consejo Nacional de Investigaciones Científicas (CONICET). SS and GC belong to Consejo de Investigación en Salud del Gobierno de la Ciudad Autonóma de Bs. As.

Competing interests None.

Ethics approval This study was approved by the Ethical Committee of our institution where patients were recruited (Hospital Abel Zubizarreta. Ciudad Autónoma de Buenos Aires, Argentina).

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Brunt EM,** Janney CG, Di Bisceglie AM, *et al*. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* 1999;**94**:2467—74.
2. **Starley BQ,** Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology* 2010;**51**:1820—32.
3. **Sanyal AJ,** Chalasani N, Kowdley KV, *et al*. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010;**362**:1675—85.
4. **Fuchs M,** Sanyal AJ. Lipotoxicity in NASH. *J Hepatol* 2012;**56**:291—3.
5. **Cheung O,** Sanyal AJ. Recent advances in nonalcoholic fatty liver disease. *Curr Opin Gastroenterol* 2010;**26**:202—8.
6. **Browning JD,** Horton JD. Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest* 2004;**114**:147—52.
7. **Sanyal AJ,** Campbell-Sargent C, Mirshahi F, *et al*. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001;**120**:1183—92.
8. **Perez-Carreras M,** Del HP, Martin MA, *et al*. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* 2003;**38**:999—1007.
9. **Carabelli J,** Burgueno AL, Rosselli MS, *et al*. High fat diet-induced liver steatosis promotes an increase in liver mitochondrial biogenesis in response to hypoxia. *J Cell Mol Med* 2011;**15**:1329—38.
10. **Scarpulla RC.** Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiol Rev* 2008;**88**:611—38.
11. **Shock LS,** Thakkar PV, Peterson EJ, *et al*. DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in mammalian mitochondria. *Proc Natl Acad Sci U S A* 2011;**108**:3630—5.
12. **Pollack Y,** Kasir J, Shemer R, *et al*. Methylation pattern of mouse mitochondrial DNA. *Nucleic Acids Res* 1984;**12**:4811—24.
13. **Asselah T,** Bieche I, Laurendeau I, *et al*. Significant gene expression differences in histologically "normal" liver biopsies: implications for control tissue. *Hepatology* 2008;**48**:953—62.
14. **Sookoian S,** Rosselli MS, Gemma C, *et al*. Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: impact of liver methylation of the peroxisome proliferator-activated receptor gamma coactivator 1alpha promoter. *Hepatology* 2010;**52**:1992—2000.
15. **Kleiner DE,** Brunt EM, Van NM, *et al*. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;**41**:1313—21.
16. **Gemma C,** Sookoian S, Alvarinas J, *et al*. Maternal pregestational BMI is associated with methylation of the PPARGC1A promoter in newborns. *Obes (Silver Spring)* 2009;**17**:1032—9.
17. **Hallsworth K,** Fattakhova G, Hollingsworth KG, *et al*. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. *Gut* 2011;**60**:1278—83.
18. **Johnson NA,** George J. Fitness versus fatness: moving beyond weight loss in nonalcoholic fatty liver disease. *Hepatology* 2010;**52**:370—81.
19. **Musso G,** Gambino R, Cassader M, *et al*. A meta-analysis of randomized trials for the treatment of nonalcoholic fatty liver disease. *Hepatology* 2010;**52**:79—104.
20. **Thoma C,** Day CP, Trenell MI. Lifestyle interventions for the treatment of non-alcoholic fatty liver disease in adults: a systematic review. *J Hepatol* 2012;**56**:255—66.
21. **Korzeniewski B.** Regulation of oxidative phosphorylation in different muscles and various experimental conditions. *Biochem J* 2003;**375**:799—804.
22. **Alirol E,** Martinou JC. Mitochondria and cancer: is there a morphological connection? *Oncogene* 2006;**25**:4706—16.
23. **Chen H,** Chomyn A, Chan DC. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *J Biol Chem* 2005;**280**:26185—92.
24. **Bai Y,** Attardi G. The mtDNA-encoded ND6 subunit of mitochondrial NADH dehydrogenase is essential for the assembly of the membrane arm and the respiratory function of the enzyme. *EMBO J* 1998;**17**:4848—58.
25. **Kruse SE,** Watt WC, Marcinek DJ, *et al*. Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. *Cell Metab* 2008;**7**:312—20.
26. **Smeitink J,** van den Heuvel L, DiMauro S. The genetics and pathology of oxidative phosphorylation. *Nat Rev Genet* 2001;**2**:342—52.
27. **Valentino ML,** Barboni P, Ghelli A, *et al*. The ND1 gene of complex I is a mutational hot spot for Leber's hereditary optic neuropathy. *Ann Neurol* 2004;**56**:631—41.
28. **Gasparre G,** Romeo G, Rugolo M, *et al*. Learning from oncocytic tumors: why choose inefficient mitochondria? *Biochim Biophys Acta* 2011;**1807**:633—42.
29. **Koopman WJ,** Verkaart S, Visch HJ, *et al*. Human NADH:ubiquinone oxidoreductase deficiency: radical changes in mitochondrial morphology? *Am J Physiol Cell Physiol* 2007;**293**:C22—9.
30. **Caldwell SH,** de Freitas LA, Park SH, *et al*. Intramitochondrial crystalline inclusions in nonalcoholic steatohepatitis. *Hepatology* 2009;**49**:1888—95.
31. **Butow RA,** Avadhani NG. Mitochondrial signaling: the retrograde response. *Mol Cell* 2004;**14**:1—15.
32. **Fosslien E.** Mitochondrial medicine—molecular pathology of defective oxidative phosphorylation. *Ann Clin Lab Sci* 2001;**31**:25—67.
33. **Herrmann W.** Significance of hyperhomocysteinemia. *Clin Lab* 2006;**52**:367—74.
34. **Sookoian S,** Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. *J Hepatol* 2008;**49**:600—7.
35. **Sookoian S,** Castano GO, Burgueno AL, *et al*. Circulating levels and hepatic expression of molecular mediators of atherosclerosis in nonalcoholic fatty liver disease. *Atherosclerosis* 2010;**209**:585—91.
36. **Sookoian S,** Gianotti TF, Rosselli MS, *et al*. Liver transcriptional profile of atherosclerosis-related genes in human nonalcoholic fatty liver disease. *Atherosclerosis* 2011;**218**:378—85.