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**MITOCHONDRIAL STRESS IN ADVANCED FIBROSIS AND CIRRHOSIS
ASSOCIATED WITH CHRONIC HEPATITIS B, CHRONIC HEPATITIS C OR
NONALCOHOLIC STEATOHEPATITIS**

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Footnotes

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Concept, set-up and design (AM, TA); acquisition and analysis of data (DL, AM, IT, CMB, MR, SN, AS, TA); writing original and final drafts (AM, TA, DL); all authors were involved in critical review of the manuscript and approved the final version of the manuscript.

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Abbreviations

Bp, base pair; CHB, chronic hepatitis B; chronic hepatitis C (CHC); COX4I1, cytochrome c oxidase subunit 4I1; DAMPs, damage associated molecular patterns; HBV, hepatitis B virus; HBV pgRNA, Hepatitis B virus pregenomic RNA; HBx, Hepatitis B virus X protein; HCC, Hepatocellular carcinoma; HRP, horseradish peroxidase; HSPA9, stress-70 protein, mitochondrial (heat shock protein family A (Hsp70) member 9; HSPD1, 60 kDa heat shock protein, mitochondrial (heat shock protein family D (HSPD1) member 1); IL1 β , interleukin 1 beta; IL6, Interleukin 6; LONP1, Lon peptidase 1; MFN1, mitofusin 1; MFN2, mitofusin 2; MnSOD, Manganese superoxide dismutase; MT-ATP8, mitochondrially encoded ATP

synthase membrane subunit 8; MT-CO1, mitochondrially encoded cytochrome c oxidase I; MT-CO2, mitochondrially encoded cytochrome c oxidase II; MT-ND1, mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit; ^{mt}UPR, mitochondrial unfolded protein response; mtDNA, mitochondrial DNA; NASH, non-alcoholic steatohepatitis; nDNA, nuclear DNA; NOS, NO synthase; OPA1, mitochondrial dynamin like GTPase (alias optic atrophy 1); PPARG, peroxisome proliferator-activated receptor gamma; PPARGC1 α , PPARG coactivator 1 alpha; PTEN, phosphatase and tensin homolog; PINK1, PTEN induced kinase 1; PRKN, parkin RBR E3 ubiquitin protein ligase (alias parkin, Parkinson juvenile disease protein 2); RNS, Reactive nitrogen species; ROS, Reactive oxygen species; TFAM, transcription factor A, mitochondrial; TOMM20, translocase of outer mitochondrial membrane 20; TNF α , tumor necrosis factor alpha; UTRN, utrophin (alias DRP1, Dynamin Related Protein1).

ABSTRACT

Background & aims: Hepatitis B virus infection causes oxidative stress and alters mitochondria in experimental models. We postulated that HBV might alter liver mitochondria also in human, and the resulting mitochondrial stress might account for the progression of fibrosis in chronic hepatitis B (CHB).

Approach & results: The study included 146 treatment-naïve CHB mono-infected patients. Patients with CHB and advanced fibrosis or cirrhosis (F3-F4) were compared to patients with no-mild-moderate fibrosis (F0-F2). Patients with CHB were further compared to patients with chronic hepatitis C (CHC) (n=33), nonalcoholic steatohepatitis (NASH) (n=12) and healthy controls (n=24). We detected oxidative damage to mtDNA including mtDNA strand breaks, and identified multiple mtDNA deletions in patients with F3-F4 as compared to patients with F0-F2. Alterations in mitochondrial function, mitochondrial unfolded protein response, biogenesis, mitophagy and liver inflammation were observed in patients with advanced fibrosis or cirrhosis associated with CHB, CHC and NASH.

In vitro, significant increases of the mitochondrial formation of superoxide and peroxynitrite as well as mtDNA damage, nitration of the mitochondrial respiratory chain complexes and impairment of complex I occurred in HepG2 cells [replicating HBV](#) or transiently expressing Hepatitis B virus X protein. mtDNA damage and complex I impairment were prevented with the superoxide scavenging Mito-Tempo or with iNOS specific inhibitor 1400W.

Conclusion: Our results emphasized the importance of mitochondrial oxidative stress, mtDNA damage and associated alterations in mitochondrial function and dynamics in the advanced fibrosis or cirrhosis in CHB and NASH. Mitochondria might be a target in drug development to stop fibrosis progression.

INTRODUCTION

Chronic hepatitis B (CHB) affects around 257 million individuals worldwide, with a risk of advanced fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (1).

HBV chronic infection can be classified into five phases depending on serum ALT levels, Hepatitis B virus e antigen (HBeAg) and HBV viral load : (I) HBeAg-positive chronic infection, (II) HBeAg-positive chronic hepatitis, (III) HBeAg-negative chronic infection, (IV) HBeAg-negative chronic hepatitis and (V) HBsAg-negative phase (2).

In CHB, patients with advanced fibrosis or cirrhosis (Metavir score, F3-F4) should undergo surveillance because they are at risk of liver decompensation and HCC when compared to patients with no-mild-moderate fibrosis (F0-F2) (3–7).

Mitochondria play important roles in inflammation, innate immune and antiviral responses, and both processes require healthy mitochondria (8–13). Mitochondria-derived danger signals promote liver inflammation and directly activate hepatic stellate cells and drive progression of liver fibrosis in mice and in patients with non alcoholic steatohepatitis (NASH) (11–14).

The 16.5-kilo-base-pair mitochondrial DNA (mtDNA) is double stranded circular DNA encoding critical subunits of the mitochondrial respiratory chain (15). Because of the asynchronous mode of replication of heavy and light strands of mtDNA, the region between the origins of replication O_H and O_L is consistent with mutational and instability hot spots region (16–18). This mtDNA segment harbors all of the deletions described in various mitochondrial disorders (17–19).

Unlike chromosomal DNA, mtDNA is more vulnerable to oxidative damage, due to its lack of protective histones and its attachment to the mitochondrial inner membrane, a main source of oxygen radicals in the cell (20–22). Replication of oxidatively damaged mtDNA leads to point mutations and diverse mtDNA deletions which impair mitochondrial respiration in various liver diseases and progressive neurological disorders (19,22,23).

Mitochondrial dysfunction and mitochondrial adaptive and protective mechanisms have been described in diverse chronic liver diseases (9). These mechanisms include mitochondrial biogenesis, mitochondrial fission/fusion, mitochondrial unfolded protein response (^{mt}UPR), mitophagy and induction of mitochondrial antioxidant capacities/pathways, all of which maintain mitochondrial homeostasis and ultimately

cell survival (9). Mitochondrial fusion is mediated by mitofusins 1 and 2 (MFN1, 2), and mitochondrial dynamin like GTPase (OPA1) while mitochondrial fission requires dynamin-related protein 1 (Utrophin, UTRN) (9). Mitochondrial biogenesis maintains mitochondrial mass and is regulated by peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PPARGC1 α) and mitochondrial transcription factor (TFAM). PPARGC1 α and TFAM control the replication and transcription of mtDNA (9). Defective mitochondria are cleared via Parkinson juvenile disease protein 2 (PRKN) and Phosphatase and Tensin-Induced Putative Kinase-1 (PINK1)-mediated mitophagy (9). ^{mt}UPR involves mitochondrial HSPD1/A9 chaperones and LONP1 peptidase, controls the stoichiometric balance between mitochondrial proteins encoded by the nuclear and mitochondrial genomes, ensures mitochondrial quality and proteasis, and senses mitochondrial protein misfolding (9).

The mitochondrial antioxidants include reduced glutathione (GSH)-peroxidases, manganese superoxide dismutase (MnSOD) and peroxiredoxins (9,24). Although mitochondria form large amounts of the superoxide radical anion, MnSOD accelerates its dismutation into hydrogen peroxide, which is detoxified into water by mitochondrial GSH-peroxidases (9,25). By decreasing superoxide steady-state levels, MnSOD limits the formation of reactive nitrogen species (RNS) such as peroxynitrite, a strong oxidant generated by the reaction of superoxide with nitric oxide (NO). MnSOD is inducible by reactive oxygen species (ROS) and cytokines, but it is inactivated by peroxynitrite (9,25,26). Peroxynitrite and peroxynitrite-generated reactive intermediates can nitrate proteins and damage lipids and DNA and have been shown to be involved in mtDNA depletion (25–27). In keeping with the role of inducible nitric oxide synthase (iNOS) in liver lesions, and the ability of MnSOD to decrease peroxynitrite formation, overexpression of MnSOD protected against liver mitochondrial oxidative stress mice (26).

In vitro and *in vivo* studies have shown that hepatitis B virus (HBV) or Hepatitis B virus X protein (HBx) cause oxidative stress and alter mitochondrial dynamics and homeostasis (9,10,13,28–35). HBV-induced oxidative stress might speed up the oxidative damage to mtDNA in patients with CHB. If this hypothesis is correct, one would expect massive oxidative damage to mtDNA in patients with CHB. The resulting mitochondrial dysfunction and associated alterations in the mitochondrial dynamics would favor the progression of fibrosis in these patients. Conversely,

advanced fibrosis and cirrhosis themselves might aggravate HBV-induced mtDNA damage and mitochondrial dysfunction. To verify these hypothesis, patients with CHB were compared to patients with NASH, chronic hepatitis C (CHC) and healthy control livers. We provide here an association of these mitochondrial lesions with advanced fibrosis or cirrhosis in a large cohort of patients with CHB, NASH and CHC. The possible underlying mechanisms of HBV- or HBx-associated mitochondrial oxidative stress, mtDNA damage and mitochondrial dysfunction were further explored in human HepG2 cells replicating HBV or expressing wild-type or mutated HBx protein *in vitro*.

PATIENTS AND METHODS

Patients and Controls

One hundred ninety-one (191) patients and 24 control subjects were enrolled (Table 1, Suppl. Fig. 1). The study included 146 patients with CHB, 12 patients with NASH, 33 patients with chronic hepatitis C (CHC) and livers from 24 non-CHB, non-NASH and non-CHC control subjects (hereafter designed as 'Controls') (Table 1, Suppl. Fig. 1). All patient and controls demographics and laboratory parameters were recorded at the time of the liver biopsy.

Patients with CHB were eligible if they had been HBsAg-positive for at least 6 months before inclusion, and were excluded if they had evidence of alcohol intake >20 g/day, co-infections or NASH. Seventy-seven over the 146 (53%) of the patients with CHB were HBeAg-positive chronic hepatitis (phase II) and 69 (47%) were HBeAg-negative chronic hepatitis (phase IV).

Patients with NASH or with CHC were older than controls and patients with CHB ($p<0.05$) (Table 1). Patients with NASH had also higher BMI ($p<0.001$), blood glucose ($p<0.01$) and triglycerids ($p<0.05$) than other groups. However, all the group patients and controls had comparable ALT and AST, but patients with CHB had lesser γ -GT ($p<0.001$) (Table 1).

Liver biopsy specimens were formalin-fixed and paraffin-embedded. Sections (5 μ m-thick) were stained with H&E, Mallory trichrome, Picrosirius red, and Perls and analyzed by a pathologist blinded to the clinical and laboratory data.

In patients with CHB and CHC, necroinflammation and fibrosis were assessed according to the Metavir scoring system (5). Significant fibrosis was defined as Metavir score F3 or cirrhosis F4. Because patients with advanced fibrosis or cirrhosis (F3-F4) are at risk of HCC and liver decompensation when compared to patients with F0-F2, we stratified our patients in 2 groups regarding fibrosis grade: absent to moderate fibrosis (F0-F2) and advanced fibrosis or cirrhosis (F3-F4) (Tables 1, 2). Necroinflammation was graded as A0 to A3 and steatosis was assessed as the percentage of hepatocytes containing fat droplets (Table 1).

One hundred (100) patients with CHB had F0-F2 and 46 with F3-F4 (Tables 1, Suppl. Fig. 1). Similarly, 21 patients with CHC were with F0-F2 and 12 had F3-F4 (Table 1, Suppl. Fig. 1). All the 12 patients with NASH were with cirrhosis (F4) (Tables 1, Suppl. Fig. 1). No patient had cirrhosis decompensation or HCC. Only 12 (8%) over

the 146 patients with CHB had important steatosis (>33% of fat-engorged hepatocytes).

The 24 control liver specimens were obtained for diagnostic purposes and comprised percutaneous normal liver biopsy specimens obtained from 24 adults with mildly elevated serum alanine aminotransferase activity addressed to Beaujon Hospital (Clichy, France), in whom all causes of liver disease had been ruled out (medication, alcohol, chronic viral hepatitis, autoimmune processes, and metabolic disease) (Table 1). In these control subjects, liver biopsies were performed percutaneously under local anesthesia (36). We selected liver samples on the basis of a histologically normal pattern: no portal or lobular inflammation and/or necrosis; absence of portal, central, or perisinusoidal fibrosis; and no other significant abnormal features (steatosis <5%, no iron overload, no ballooning or liver cell clarification, no cholestasis or bile duct lesion). The 24 controls were all with no or minimal steatosis (<5% of fat containing hepatocytes in 21 and 10% in 3) (Table 1).

Study approval

The study was performed in accordance with the guidelines of the 1975 Declaration of Helsinki and the principles of Good Clinical Practice. All patients gave written consent, and the study was approved by the Ethics Committee of Beaujon Hospital (Département de la Recherche Clinique-Assistance Publique–Hôpitaux de Paris).

Analyses flow chart

Different amounts of DNA, RNAs and/or proteins were obtained from distinct groups of patients and controls depending on the size of liver fragments that remained available after histological studies (Suppl. Fig. 1, Table 2).

In patients with CHB, RNA, DNA and proteins were simultaneously obtained in 42 cases (31 with F0-F2 and 11 with F3-F4), and both DNA and proteins obtained in 13 patients. The remaining 91 patients have only mRNA (64 cases), DNA (25 cases) or proteins (4 cases). mtDNA damage search, mRNAs levels, immunoblotting, and liver ATP content were then assessed in distinct groups of 65, 104, 44, and 30 patients with CHB, respectively (Suppl. Fig. 1).

The 33 patients with CHC had only total RNA, and all the 12 patients with NASH had both RNA, DNA and only 7 among 12 had RNA, DNA and proteins. mtDNA damage

has been reported in CHC and therefore was not searched in the present study (37–39)

Seven (7) of the 24 controls subjects (non-CHB, non-CHC, non-NASH) had RNA, DNA and proteins simultaneously, and 8 had both RNA and DNA while 16 had both DNA and proteins.

mRNAs and/or proteins expression were analyzed for the main genes involved in mitochondrial function (MT-CO1, MT-CO2 and COX4I1 subunits for complex IV; MT-ATP8 subunit for complex V), mitochondrial biogenesis (PPARGC1 α , TFAM), mitochondrial fusion/fission (MFN1/2, OPA-1, UTRN), ^{mt}UPR (HSPD1, HSPA9, LONP1 protease), mitophagy (PINK1, PRKN), oxidative stress (iNOS, MnSOD), and genes for the inflammatory cytokines (TNF α , IL6, IL1 β) (Suppl. Fig. 1) using specific primers (Suppl. Table 2) and/or antibodies (Suppl. Table 3). All these parameters were analyzed in normally distributed groups of patients and controls for age, ALT, steatosis or HBV DNA.

Detection and sequencing of mtDNA deletions

Liver DNA was isolated as previously described (22,23). The instability hot spot mtDNA region (6080 bp) extending from nucleotide (nt) 8167 to 14246 was screened by long polymerase chain reaction (LPCR) for the presence of mtDNA deletions. DNA samples from controls and patients with CHB or NASH were subjected to LPCR with primers A-B (Suppl. Table 2) which concomitantly amplify undeleted and deleted mtDNA in a single reaction. The A and B primers co-amplify a long fragment of 6080-bp from the undeleted mtDNA template and smaller fragment(s) from deleted mtDNA molecules whenever present. The LPCR program included 30 cycles of 95°C for 45 s, 61°C for 10 s, and 68°C for 8 min, and a final extension at 68°C for 7 min. LPCR products were analyzed on 1.5% agarose gels stained with SYBR Safe DNA intercalating agent, and DNA bands were detected by fluorography.

The LPCR products were sequenced to determine the exact sizes and boundaries of the detected mtDNA deletions. MtDNA bands were excised from the gel, placed on the filter of a Spin-X Centrifuge Tube Filter, frozen at -20°C and recovered by centrifugation. One hundred nanograms of the recovered product were then subjected to a single primer PCR sequencing reaction, using BigDye™ Terminator v3.1 Cycle Sequencing Kit and either primer C or D (Suppl. Table 1). After heating to

94°C for 2 min, the reaction was cycled as follows: 25 cycles of 30 s at 94°C, 30 s at 55°C, and 4 min at 60°C. After removal of unbound dye terminators, samples were loaded on an Applied Biosystems 373A sequencer, and run for 12 h on a 6% denaturing acrylamide gel.

Slot blot and Southern blot hybridization

Slot blot was used to quantitate mtDNA and nDNA. Hepatic DNA (100 ng) DNA samples from controls and patients with CHB or NASH was blotted onto a Hybond-N1 nylon membrane and hybridized with a 6.080-kb probe that was generated by long PCR and $\alpha^{32}\text{P}$ -dCTP-labeled by random priming. Membranes were stripped and hybridized with a human C₀t-1 nDNA probe as described previously (40) mtDNA and nDNA were assessed by densitometry analysis of autoradiographs.

The proportions of mtDNA in its supercoiled, circular, and 16569-bp linear forms were analyzed by Southern blot using human mtDNA specific-radiolabeled probe (40). Liver DNA (1.5-3 μg) was loaded on 0.7% agarose gels without DNA intercalating labelling agents. Electrophoresed DNA was transferred to a Hybond-N1 nylon membrane and hybridized with the α -dCTP³²-labelled 6.080-kb human mtDNA probe. The mtDNA forms were quantitated by densitometry of autoradiographs.

Real-time RT-qPCR assessment for liver mRNA levels

Frozen liver fragments from controls and patients with CHB, CHC or NASH were dissolved in 1mL of iced-Trizol and crushed during 60s at 30Hz using Eppendorf-TissusLyser II. Chloroform was added, and the aqueous phase was collected after centrifugation. Total RNAs was precipitated at -20°C overnight using 2-propanol and 1 μg of glycogen RNA grade. RNA was washed in 75% ethanol and the pellet obtained was resuspended in DEPC RNase Free water. Total RNA (0.25-1 μg) treated with DNase I was retro-transcribed using random primers and Superscript II reverse transcriptase kit. mRNA levels were estimated by real-time RT-qPCR using specific primers (Suppl. Table 2) and SYBR Green I Master Mix. GAPDH was used as a reference gene, and the $2^{-\Delta\Delta\text{Cq}}$ method was used to estimate the relative mRNA expression following MIQE guidelines (41).

Liver ATP determination and Western blotting analysis

Frozen liver biopsies from patients with CHB were homogenized in a lysis buffer (either 50 mmol/L Tris-HCl, pH 7.2, 250 mmol/L NaCl, 0.1% NP-40, 2 mmol/L EDTA, 10% glycerol; or 70 mmol/L sucrose, 220 mmol/L Mannitol 2 mmol/L, HEPES-KOH 0,1 mmol/L EDTA II, pH 7.4) containing protease and phosphatase inhibitors cocktail. To measure hepatic ATP levels, 10 protein pools each from 3 patients with CHB (30 µg of liver proteins from each patient) and 4 pools of 3 controls each were constituted and permitted 10 determinations of liver ATP levels in patients with CHB and 4 determinations in the non-CHB controls. Thus, equal amounts of liver proteins from 30 patients were mixed to constitute 5 pools of patients with CHB and F0-F2 and 5 pools with F3-F4. Similarly, 12 controls were pooled into 4 mixes. These 14 liver protein pools were then ground in liquid nitrogen and transferred into 100 µl of ice-cold 1 M perchloric acid. After centrifugation at 4 °C, supernatants were neutralized with 5 M K₂CO₃ and centrifuged again at 4 °C. The pellets were resuspended in phosphate-buffered saline (PBS) containing 0.2N NaOH and used to determine protein content. The supernatant was used to measure ATP with Luminescent ATP Detection Assay Kit on a luminescence plate reader following the manufacturer's recommendations.

For Western blotting, homogenates were centrifuged, and the supernatant was collected. Total protein concentrations were determined by the Bradford reagents. Proteins (25-75 µg) were mixed with Laemmli buffer and subjected to SDS-PAGE. The proteins were blotted on nitrocellulose membranes using a semi-dry transfer system and the transfer buffer (25 mmol/L Tris, 190 mmol/L glycine, 20% ethanol). The membranes were blocked overnight in Tris-buffered saline (TBS) containing 10% BSA 0.05 % tween20 and incubated with specific antibodies (Suppl. Table 3). After 3 washes in TBS-Tween 0.05%, blots were incubated with horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugated secondary antibody.

Statistical analysis

Data are expressed as mean ± SD. Categorical variables are expressed as absolute and relative frequencies. Statistical analyses were performed using Microsoft Excel Office 365 and GraphPad Prism Software version 9.0.1. The Mann-Whitney U-test

was used to assess differences between means. The chi-square (K^2) or the chi-square corrected for continuity ($K c^2$) tests were used to assess differences between frequencies. The Pearson method was used to assess the correlation between numerical variables. Patients with CHB, CHC or NASH were compared to the controls. Patients with F3-F4 were compared to those with F0-F2 within each group of patients and between these groups. n values for each experiment are indicated in figure legends. The significance was setup at $p < 0.05$.

RESULTS

mtDNA deletions in patients with CHB or NASH

mtDNA deletions were looked for by long PCR and characterized by sequencing. In addition of the expected 6080-bp fragment amplified from undeleted mtDNA, the hepatic DNA from patients with CHB and patients with NASH gave amplification products of smaller lengths corresponding to diverse mtDNA deletions (Fig. 1A, B). To determine the exact position of these rearrangements, these mtDNA bands were sequenced (Suppl. Fig. 2).

Four different 5385-bp, 4977-bp, 4881-bp and 2451-bp mtDNA deletions, all flanked by direct repeats were observed in these patients (Suppl. Fig. 2A).

The 4977-bp and 5385-bp deletions start within the gene for subunit 8 of ATP synthase (MT-ATP8) and the deletion 4881-bp starts within the gene for subunit 3 of cytochrome *c* oxidase (MT-CO3, complex IV) (Suppl. Fig. 2A, B). The three deletions then involve the genes for subunit 6 of ATP synthase, MT-CO3, subunits 3, 4L, 4 and 5 of NADH dehydrogenase (complex I) as well as the interspersed genes encoding tRNA-His, tRNA-Gly, tRNA-Arg, tRNA-Ser and tRNA-Lys (Suppl. Fig. 2B).

The 2451-bp deletion starts within subunit 3 of NADH dehydrogenase (ND3) gene and affected the genes for subunits 4, 4L and 5 of complex I, and tRNA-Tyr, tRNAs-Arg, tRNA-His, tRNA-Ser and tRNA-Leu (Suppl. Fig. 2A,B).

The common 4977-bp deletion was observed in 46 (71%) of 65 patients with CHB and in 7 (58%) of the 12 patients with NASH and cirrhosis, but only in 5 (21%) of the 24 controls (Suppl. Table 3). The 2451-bp deletion was detected in 9/19 (47%) patients with F3-F4 fibrosis and in 12/46 (26%) with F0-F2 fibrosis but in none of the patients with NASH nor in the controls. The 4881-bp and 5385-bp deletions were respectively observed in 5 and 2 patients with CHB and F3-F4 fibrosis but in none of the patients with CHB and F0-F2 fibrosis, NASH or in the controls (Suppl. Table 3).

Considering all the four deletions, 56 patients with CHB (86%), but only five of the 24 control subjects (19%) carried at least one mtDNA deletion ($\chi^2=34.68$; $p<0.005$) (Suppl. Table 3). The difference in the prevalence of mtDNA deletions persisted when patients with CHB and F3-F4 were compared to patients with CHB and F0-F2. While eighteen of the 19 (95%) patients with CHB and F3-F4 fibrosis exhibited at least one mtDNA deletion, only thirty of the 46 (65%) patients with CHB and F0-F2 carried a single mtDNA deletion ($\chi^2=20.04$; $p<0.005$), and one patient harbored 2

distinct deletions. Two of the patients with F3-F4 fibrosis had two concomitant deletions and two exhibited three concurrent deletions (Fig. 1C).

Compared to the controls, the prevalence of the 4977-bp deletion was significantly higher in patients with NASH and cirrhosis. While 7 (58%) of the 12 patients with NASH and cirrhosis exhibited the 4977-bp deletion, only five of the 24 controls (19%) carried this common deletion ($\chi^2 = 5.06, p < 0.025$) (Suppl. Table 3).

Similarly, the difference in the prevalence of the 4977-bp deletion persisted when patients with CHB and F3-F4 were compared to patients with NASH and cirrhosis. While eighteen of the 19 (95%) patients with CHB and F3-F4 fibrosis exhibited at least one mtDNA deletion, only seven of the 12 (58%) patients with NASH and cirrhosis carried a single mtDNA deletion ($\chi^2 = 6.93, p < 0.005$) (Suppl. Table 3). This difference persisted even when only patients with CHB and cirrhosis were compared to the patients with NASH and cirrhosis (i.e. patients with CHB and F3 fibrosis excluded from the analysis).

The 5 patients with CHB exhibiting two or three different mtDNA deletions were those with the highest viral loads (HBV DNA: 6.00, 6.6, 7.2, 7.9 and 8.2 log₁₀ IU/mL). All the seven patients with NASH and with the 4977 bp deletion had massive steatosis (5 with 80% and 2 with 70% of fat containing hepatocytes).

mtDNA levels in patients with CHB and patients with NASH

MtDNA levels were analysed by Slot blot. Nuclear DNA (nDNA) levels were not modified when Slot blots were hybridized with the human C₀t-1 nDNA probe (Fig. 1C, D). We thus used the mtDNA/nDNA hybridization ratio to quantitate mtDNA changes (Fig. 1E). The mtDNA/nDNA ratio significantly declined in patients with CHB to 43% of control values ($p < 0.001$) (Fig. 1E). Compared to controls, mtDNA/nDNA ratio significantly decreased by 55% in patients with CHB and F0-F2 ($p < 0.001$) and by 65% in patients with CHB and advanced fibrosis-cirrhosis ($p < 0.001$) (Fig. 1E).

Similarly, mtDNA/nDNA ratio significantly declined to 53% of control values in patients with NASH and cirrhosis ($p < 0.001$) (Fig. 1E). mtDNA/nDNA ratio was also different when patients with CHB and F3-F4 fibrosis were compared to patients with NASH and cirrhosis (35.37 ± 11.55 and 53.41 ± 11.55 , respectively; $p < 0.001$).

Patients with CHB and highest viral loads (HBV DNA >5 log₁₀ IU/mL) all had the lowest mtDNA levels (mtDNA/nDNA ratio <0.3). Similarly, patients with NASH and >66% of steatosis had minimal mtDNA levels (mtDNA/nDNA ratios <0.5).

Quantitative-PCR confirmed mtDNA depletion in patients with CHB (Suppl. Fig. 3A). We looked also for mtDNA strand breaks, topology and changes in the of the main mtDNA forms in patients with CHB and controls (Suppl. Fig. 3B, C) (See 'SUPPLEMENTARY RESULTS' section).

Exacerbated decreases in mtDNA-encoded cytochrome c oxidase subunits 1 (MT-CO1) and 2 (MT-CO2) in CHB, CHC or NASH with advanced fibrosis-cirrhosis as compared to patients with F0-F2

Compared to control livers (n=8), MT-CO1 mRNA decreased significantly by 83% in patients with CHB and F3-F4 (n=21), 70% in patients with CHC and F3-F4 (n=12), and 65% in patients with NASH and cirrhosis (n=12), ($p<0.001$) (Fig 2A). Similarly, MT-CO2 mRNA decreased significantly as compared to controls by 82%, 61% and 59% in patients with advanced fibrosis or cirrhosis and CHB (n=21), CHC (n=12), and NASH (n=12), respectively, ($p<0.001$) (Fig 2C). Although significant, these decreases in MT-CO1 and MT-CO2 mRNAs were to a lesser extend in patients with F0-F2 fibrosis as compared to controls (Fig 2A, C).

MT-CO1 and MT-CO2 mRNAs levels were then compared between F3-F4 and F0-F2 in patients with CHB or CHC. In patients with CHB and F3-F4 (n=21), MT-CO1 mRNA (0.17 ± 0.11 , n=21) and MT-CO2 mRNA (0.18 ± 0.10 , n=21) decreased respectively by 53% and by 56% ($p<0.001$) as compared to patients with CHB and F0-F2 (0.32 ± 0.24 , n=66 and 0.32 ± 0.22 , n=66) (Fig. 2A, B). In patients with CHC, MT-CO1 (F0-F2 n=21, 0.61 ± 0.32 and F3-F4 n=12, 0.30 ± 0.18) and MT-CO2 (F0-F2 n=21, 0.64 ± 0.27 and F3-F4 n=12, 0.39 ± 0.23) mRNAs expression decreased significantly by 50% and 40% ($p<0.001$), respectively, in patients with F3-F4 (n=12) compared to those with F0-F2 (n=21) (Fig. 2A, C).

We also observed a significant decrease ($p<0.001$) in the expression of MT-CO2 mRNA, but not MT-CO1 mRNA, in patients with CHB and F3-F4 when compared to patients with CHC and F3-F4 and a significant decrease in the expression of both MT-CO1, -CO2 ($p<0.05$) when patients with CHB and F3-F4 were compared to patients with NASH and cirrhosis (Fig. 2A, C).

At the protein levels, MT-CO1/ β -Actin ratio significantly decreased in patients with CHB and F0-F2 (n=23; 1.49 ± 1.01 , $p<0.01$) or F3-F4 (n=11; 0.81 ± 0.43 , $p<0.001$) as compared to controls (n=7; 2.56 ± 0.72) (Fig. 2B). Compared to controls (n=7), MT-CO1/ β -Actin and MT-CO2/ β -Actin ratios were significantly decreased in patients with

NASH and cirrhosis (n=7) ($p<0.01$) (1.45 ± 0.62 and $.41\pm0.32$ respectively) (Fig. 2B, D).

Again, the decrease in the MT-CO1/ β -Actin ($p<0.01$) and MT-CO2/ β -Actin ($p<0.001$) ratio was worst in patients with CHB and advanced fibrosis or cirrhosis (n=11, 0.81 ± 0.43 and n=11, 0.55 ± 0.23) than patients with CHB and F0-F2 (n=23, 1.49 ± 1.01 and n=21, 1.07 ± 0.35). MT-CO1/ β -Actin significantly decreased in CHB and F3-F4 as compared to those with NASH and cirrhosis (n=7; 1.45 ± 0.61 , $p<0.05$) (Fig. 2B).

However, nDNA-encoded COX4I1 mRNA levels were unchanged in CHB and CHC whatever the degree of fibrosis, but significantly decreased in patients with NASH and cirrhosis as compared to control subjects ($p<0.001$) (Suppl. Fig. 4A). COX4I1 mRNA also significantly decreased in NASH and cirrhosis when compared to patients with CHB and F3-F4 ($p<0.001$) or with CHC and F3-F4 ($p<0.001$) (Suppl. Fig. 4A).

Altered ^{mt}UPR in patients with CHB, patients with CHC and patients with NASH

The mitochondrial LONP1 peptidase and chaperonins HSPA9 and HSPD1 involved in the ^{mt}UPR (7) were investigated (Fig. 3). LONP1 degrades protein aggregates due to misfolding/disassembling of surplus of mitochondrial proteins, while HSPA9 and HSPD1 allow respiratory chain complexes assembly (7).

Although the expression of the ^{mt}UPR markers was altered in both patients with F0-F2 or with F3-F4 fibrosis, this alteration was more accentuated in patients with advanced fibrosis or cirrhosis associated with CHB or NASH (Fig. 3).

The expression of LONP1 mRNA was induced in patients with CHB and with CHC whatever the degree of fibrosis, but this induction failed in patients with NASH and cirrhosis (Fig. 3A). Compared to controls (n=8), LONP1 mRNA levels significantly increased by 120% in patients with CHB and F0-F2 (n=66; 2.2 ± 0.83 , $p<0.001$), by 65% in patients with CHB and F3-F4 (n=18; 1.65 ± 0.56 , $p<0.05$), by 140% in patients with CHC and F3-F4 (n=11; 2.4 ± 1.03 , $p<0.05$), but significantly decreased in patients with NASH and cirrhosis (n=12; 0.18 ± 0.13 , $p<0.001$) (Fig. 3A). Western blotting confirmed the induction of LONP1 gene at the protein levels in patients with CHB and F0-F2 and to a lesser extent in F3-F4 (controls n=7; 0.23 ± 0.13 vs CHB F0-F2 n=7; 0.81 ± 0.29 , $p<0.01$ and vs CHB F3-F4 n=6, 0.52 ± 0.10 , $p<0.01$) (Fig. 3B).

Contrary to LONP1 mRNA, HSPA9 mRNA levels instead decreased significantly in patients with CHB or with NASH but not in CHC, while HSPD1 mRNA significantly decreased in all these groups as compared to control subjects (Fig. 3C, E).

In patients with CHB, LONP1 mRNAs levels significantly decreased in patients with advanced fibrosis-cirrhosis (F3-F4, n= 20; 1.65 ± 0.56) as compared to those with F0-F2 (n=66; 2.2 ± 0.83 , $p<0.05$) (Fig. 3A). Similarly, LONP1/ β -Actin significantly decreased by 1.3-fold (CHB F3-F4, n=6; 0.52 ± 0.10 vs CHB F0-F2, n=7; 0.82 ± 0.29 $p<0.05$) (Fig. 3B).

Similarly, the relative HSPA9 mRNA expression decreased significantly by 36% in patients with CHB and F3-F4 as compared to patients with CHB and F0-F2 (F3-F4, n=20; 0.37 ± 0.19 and F0-F2, n=66; 0.57 ± 0.23 ; $p<0.001$) (Fig. 3C). This difference was confirmed by Western blotting with a 1.7-fold significant decrease in patients with CHB and F3-F4 (n=10; 0.57 ± 0.33) compared to those with F0-F2 (n=10; 0.98 ± 0.25 , $p<0.01$) (Fig. 3D).

As compared to patients with CHB and F0-F2, HSPD1 mRNAs levels significantly decreased in patients with CHB and advanced fibrosis-cirrhosis (F3-F4, n=20; 0.37 ± 0.17 and F0-F2, n=66; 0.53 ± 0.24 ; $p<0.05$) (Fig. 3E).

These differences in the expression HSPA9 and HSPD1 mRNAs were not observed between F3-F4 and F0-F2 within the group of patients with CHC (Fig. 3C, E).

We finally compared patients with advanced fibrosis or cirrhosis within groups of CHB, CHC and NASH. As compared to patients with CHB and F3-F4 or with CHC and F3-F4, LONP1 and HSPA9 mRNAs significantly decreased in patients with NASH and cirrhosis (n=12; 0.18 ± 0.13 , $p<0.001$ and n=12; 0.30 ± 0.20 , $p<0.001$, respectively) (Fig. 3A, C).

The mitophagy markers were induced in patients with CHB, CHC or NASH but this induction was altered during the fibrosis progression in CHB

The expression of the mitophagy markers was induced in patients with F0-F2 or F3-F4 whatever the etiology, but to a lesser extend in CHB and NASH patients with advanced fibrosis-cirrhosis (Fig. 4). Compared to controls (n=8, 1 ± 0.38), PRKN mRNA increased significantly by 144-fold in patients with F0-F2 (n= 66, 144 ± 54.3 , $p<0.001$) and by 45-fold in patients with F3-F4 (n= 20, 44.7 ± 20.2 , $p<0.001$) in the group of CHB (Fig. 4A). In patients with CHC, PRKN mRNA increased significantly by 35-fold in patients with F0-F2 (n= 18, 35.5 ± 25.1 $p<0.001$) and by 115-fold in patients with F3-F4 (n= 12, 115 ± 64.2 , $p<0.001$) as compared to controls (Fig. 4A). PRKN mRNA increased also significantly by 20-fold in patients NASH and cirrhosis (n=12, 20.3 ± 11.4 $p<0.001$) as compared to controls (Fig. 4A). Compared to controls, the

induction of PRKN protein was confirmed in patients with CHB ($p<0.001$) and in patients with NASH ($p<0.01$) (Fig. 4B).

Similarly, PINK1 mRNA increased significantly by 4-fold in patients with F0-F2 ($n=60$, 4.03 ± 1.12 , $p<0.001$) and by 1.9-fold in patients with F3-F4 ($n=18$, 1.94 ± 0.53 , $p<0.001$) in the group of CHB as compared to controls ($n=8$, 1 ± 0.50) (Fig. 4C). In patients with CHC, PINK1 mRNA increased significantly by 7-fold whatever the fibrosis stage (F0-F2, $n=19$; 7.20 ± 3.25 , $p<0.001$ and F3-F4, $n=12$; 7.11 ± 3.44 , $p<0.001$) as compared to control values (Fig. 4C). PRKN and PINK1 mRNAs increased also significantly by 20- and 1.3-fold, respectively, in patients with NASH and cirrhosis ($n=12$; 20.3 ± 11.4 , $p<0.001$ and $n=12$; 1.32 ± 0.75 , $p<0.001$) as compared to controls (Fig. 4C). The induction of PINK1 protein was confirmed in patients with CHB and F0-F2 ($n=16$; 0.88 ± 0.36 , $p<0.001$) and F3-F4 ($n=7$; 0.71 ± 0.27 , $p<0.01$) (Fig. 4D).

In the group of patients with CHB, PRKN as well as PINK1 mRNAs significantly decreased respectively by 3.2- ($p<0.001$) and by 2.1-fold ($p<0.001$) in patients with F3-F4 as compared to patients with F0-F2 fibrosis (Fig. 4A, C). PRKN protein expression also significantly decreased by 1.3-fold in patients with CHB and F3-F4 as compared to those with F0-F2 ($p<0.05$) (Fig. 4B).

In the group of patients with CHC, a 3.2-fold increase in the PRKN mRNA expression was observed in F3-F4 ($n=12$; 115 ± 64.2) when compared to F0-F2 ($n=19$; 35.5 ± 25.1 , $p<0.001$), while PINK1 mRNA remains unchanged whatever the fibrosis stage (Fig. 4A, C).

When we compared the advanced fibrosis or cirrhosis between these 3 groups, we noticed that PRKN and PINK1 mRNA expression significantly collapsed in patients with NASH and cirrhosis as compared to patients with CHB and F3-F4 ($p<0.001$) or with CHC and F3-F4 ($p<0.001$ and $p<0.01$, respectively) (Fig. 4A, C).

mRNA and/or protein levels for the mitochondrial biogenesis markers PPARGC1 α and TFAM and for the mitochondrial import marker TOMM20 in patients with CHB, CHC or NASH

The mitochondrial transcription factor A (TFAM) is involved in both mtDNA transcription and replication and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PPARGC1 α) is involved in mtDNA replication (7).

Compared to control subjects, both TFAM and PPARGC1 α mRNAs levels significantly dropped in patients with CHB whatever the degree of fibrosis (Fig. 5A, C). TFAM mRNA significantly decreased also in patients with CHC as compared to controls, while PPARGC1 α mRNA levels were unchanged (Fig. 5A, C). Compared to controls, patients with NASH and cirrhosis had significantly higher levels of PPARGC1 α mRNA but lower levels of TFAM mRNA (Fig. 5A, C).

While TFAM and PPARGC1 α mRNAs levels were similar in patients with CHB and F3-F4 (n=20; 0.23 \pm 0.13 and n=20; 0.43 \pm 0.25 respectively) as compared to those F0-F2 (n=60; 0.29 \pm 0.15, and n=60; 0.58 \pm 0.45, respectively), these levels significantly decreased in patients with CHC and F3-F4 (n=20; 0.44 \pm 0.19 and n=20; 0.63 \pm 0.31 respectively) compared to patients with CHC and F0-F2 (n=60; 0.68 \pm 0.29, p <0.05 and n=60; 1.71 \pm 1.19, p <0.01 respectively)(Fig. 5A, C).

Patients with NASH and cirrhosis had significantly higher TFAM (n=12; 0.39 \pm 0.19, p <0.05) and PPARGC1 α (n=12; 2.82 \pm 1.21, p <0.01) mRNAs levels than patients with CHB and advanced fibrosis-cirrhosis (Fig. 5A, C). Patients with NASH had also significant increases in PPARGC1 α (p <0.001) mRNA levels but similar TFAM mRNA levels when compared to patients with CHC and advanced fibrosis-cirrhosis (Fig. 5A, C).

Compared to controls subjects (n=7, 1.59 \pm 0.75), TFAM/ β -actin ratios significantly decreased in patients with CHB and F3-F4 (n=7; 0.74 \pm 0.25, p <0.05) but unchanged in patients with CHB and F0-F2 (n=10; 1.11 \pm 0.37, p =0.26) or in patients with NASH and cirrhosis (n=12; 1.26 \pm 0.39, p =0.62)(Fig. 5B).

TOMM20 is involved in the mitochondrial import machinery (7). TOMM20 protein/ β -actin ratios significantly decreased in patients with CHB and F0-F2 fibrosis, but were not altered in patients with CHB and F3-F4 or in patients with NASH and cirrhosis as compared to controls subjects (Suppl. Fig. 4B).

Altered expression of the mitochondrial fusion marker mitofusin1 and the fission marker OPA1 in patients with CHB and F3-F4

Mitofusin1/ β -Actin and OPA1/ β -Actin ratios were significantly decreased by 1.8 and 1.5-fold in patients with CHB and F3-F4 (n=5; 0.70 ± 0.37 and n=5, 0.71 ± 0.36 , respectively) compared to those with F0-F2 (n=6; 1.31 ± 0.49 , $p < 0.05$ and n=7; 1.09 ± 0.32 , $p < 0.05$ respectively) (Suppl. Fig 4C, D)

Decreased liver ATP levels and mtDNA-encoded subunit 8 of ATP synthase (MT-ATP8) in patients with CHB

Liver ATP levels were 62% of control values in patients with CHB (0.5 ± 0.1 and 0.2 ± 0.1 $\mu\text{mole/mg}$ protein; $p < 0.05$) (Fig. 6A). A significant decrease of liver ATP was observed in patients with CHB and F3-F4 when compared to those with F0-F2 (0.1 ± 0.1 and 0.4 ± 0.2 ; $p < 0.01$) (Fig. 6A). Compared to control livers, the expression of MT-ATP8 protein significantly decreased by 58% ($p < 0.01$) in patients with CHB whatever the degree of fibrosis (Fig. 6B).

Increased iNOS protein but unchanged MnSOD protein expression in patients with CHB and F3-F4 when compared to patients with CHB and F0-F2

Compared to control subjects, inducible NO synthase (iNOS) protein but not manganese superoxide dismutase (MnSOD) significantly increased in patients with CHB (Figure 6C, D). iNOS was also significantly induced in patient with F3-F4 fibrosis compared to patients with F0-F2 fibrosis (Figure 6C) while MnSOD remained unchanged in these two groups (Fig. 6D).

Increased expression of Liver TNF α and IL6 mRNAs in patients with CHB and F3-F4

Liver TNF α mRNAs were 1.72 ± 0.2 and 0.99 ± 0.2 ($p < 0.05$), and liver IL6 mRNAs were 7.82 ± 0.90 and 1.14 ± 0.26 ($p < 0.05$) as assessed in 6 patients with CHB and F3-4 and 10 with F0-F2, respectively (Fig. 6E, F). Liver IL1 β mRNAs were comparable in both groups (1.04 ± 0.40 in F3-F4 fibrosis and 1.24 ± 0.51 in F0-F2).

HBV replication and wild-type HBx or mutated HBx(G124L, I127A) expression in human HepG2 cells *in vitro*

Transfection with pcDNA3.1Flag-HBx expressing wild-type HBx protein, pcDNA3.1Flag-HBx(G124L, I127A) expressing mutated HBx or Payw1.2 expressing

whole HBV genome confirmed the expression of HBx protein (Suppl. Fig. 5A) or HBV pregenomic RNA (HBV pgRNA) (Suppl. Fig. 5B).

Time course of LDH leakage in HepG2 cells replicating HBV or expressing wild-type HBx protein *in vitro*

LDH activity was assessed in the extracellular medium as cell survival marker at 24, 48, 72 and 96 h after transfection (Suppl. Fig. 5C). Medium LDH activity was minimal in non transfected cells, in cells transfected with empty vectors, or in cells transfected for 24 h with pcDNA3.1Flag-HBx or with Payw1.2 vectors (Suppl. Fig. 5C). LDH activity dramatically increased at later time-points indicating that necrotic damage and cell death occurred after longest times of HBx expression or HBV replication. The % of attached cell (survivals) significantly decreased starting at 48 h post-transfection with vectors expressing HBx or HBV. We therefore kept 24 h time-point to further investigate the effects of HBx and HBV on mtDNA damage, mitochondrial oxidative stress, mitochondrial antioxidant system and mitochondrial function.

HBV and HBx protein induced iNOS, decreased MnSOD activity and increased the nitration of tyrosine residues in mitochondrial complexes I, III and V in cultured HepG2 cells *in vitro*

Twenty-four hours post-transfection, both HBV and HBx significantly induced iNOS protein (iNOS/ β -actin ratios were 2.59 ± 0.30 and 4.33 ± 0.26 , respectively) compared to corresponding empty vectors (0.52 ± 0.14 and 1.25 ± 0.35 , respectively, $p < 0.05$) (Suppl. Fig. 6A). Although HBV significantly induced MnSOD protein (Suppl. Fig. 6B), it significantly inactivates MnSOD activity ($p < 0.05$) (Suppl. Fig. 5C). However, HBx did not induce MnSOD protein, rather it decreased residual MnSOD activity (Suppl. Fig. 6C). Cu,ZnSOD activity remained unchanged.

There were no changes in the protein levels of mitochondrial respiratory chain complexes as separated by blue native polyacrylamide gel electrophoresis 24 h after HBV or HBx transfection (Suppl. Fig. 6D). The separated respiratory chain complexes were transferred onto nitrocellulose membranes and revealed with an antibody against 3-nitrotyrosine (Suppl. Fig. 6D). In pGEM or pcDNA3.1 transfected HepG2 cells, 3-nitrotyrosine residues were not detected or less presented in the proteins of complexes I, III and V, but significantly increased after the transfection

with Payw1.2 expressing whole HBV genome or with pcDNA3.1FlagHBx (Suppl. Fig. 6D).

HBV and wild-type HBx but not mutated HBx(G124L, I127A) increased mitochondrial ROS and RNS formation, depleted mtDNA and mitochondrial GSH and decreased complex I activity *in vitro*

HBx has been shown interact with the mitochondrial voltage-dependent anion channel (54), and to bind to the mitochondrial Bcl-2 family members (Bcl-2 and Bcl-xL) to cause the mitochondrial transition pore (MTP) opening and the fall of the mitochondrial membrane potential $\Delta\psi$ (Geng X et al. 2012). HBx containing G124L and I127A substitutions prevented HBx binding to mitochondrial Bcl-2 and Bcl-xL and thus prevented HBx-induced MTP opening and $\Delta\psi$ collapse (Geng X et al. 2012). pcDNA3.1Flag-HBx(G124L, I127A) was therefore used to further explore the possibility that Hbx-induced mitochondrial stress might involve its binding to Bcl-2 and Bcl-xL.

Twenty-four hours after transfection, both HBV and **wild-type** HBx significantly increased the formation of superoxide anion and hydrogen peroxide by purified mitochondria isolated from transfected HepG2 cells (Suppl. Fig 7A, Suppl. Table 4). HBV and **wild type** HBx also significantly increased the peroxynitrite formation by whole HepG2 cells homogenates (Suppl. Table 4).

Consequently, HBV and **wild type** HBx significantly depleted mtDNA at 24 h **post-transfection**. mtDNA/nDNA ratios were 0.78 ± 0.17 in pGEM-transfected HepG2 cells and 0.29 ± 0.12 in HepG2 **replicating** HBV, and were 1.17 ± 0.19 in HepG2 transfected with pcDNA3.1 and 0.49 ± 0.09 in HepG2 cells expressing wild type HBx protein (Suppl. Fig. 7C). However, mtDNA deletions were not detected in cells **replicating** HBV or **expressing** HBx protein up to 72 h posttransfection (Suppl. Fig 7D). Culture medium nitrite/nitrate concentrations were $4.4 \pm 0.6 \mu\text{M}$ and $3.8 \pm 1.2 \mu\text{M}$ after transfection with the corresponding control plasmids, and increased significantly to $8.85 \pm 1.21 \mu\text{M}$ and $7.34 \pm 0.89 \mu\text{M}$ in culture supernatants from HepG2 **replicating** HBV or **expressing wild type** HBx, respectively ($p < 0.05$) (Suppl. Table 4).

Both mitochondrial GSH and total GSH were significantly depleted in HepG2 cells **replicating** HBV or **expressing wild type** HBx. Mitochondrial GSH was 8.84 ± 1.12 and 3.63 ± 0.58 nmol/mg protein in pGEM and payw1.2-HBV transfected HepG2,

respectively ($p < 0.05$) and was 9.24 ± 1.12 and 4.21 ± 1.10 nmol/mg protein in pcDNA3.1 and pcDNA-FlagHBx transfected HepG2, respectively ($p < 0.05$) (Suppl. Table 4).

Finally, plasmids expressing HBV or wild type HBx significantly decreased the complex I activity by 46% and 37%, respectively, as compared to the corresponding empty vectors (Suppl. Table 5).

Importantly, HBx(G124L, I127A) failed to increase mitochondrial ROS formation, failed to deplete mtDNA or mitochondrial GSH and preserved complex I activity compared to wild type HBx (Suppl. Fig. 7A,C; Suppl. Tables 4,5).

Mito-Tempo or 1400W protected against HBx- and HBV-induced mtDNA depletion and against respiratory complex I impairment

To gain further insight into the type and origin of the reactive species involved in mtDNA depletion, we treated transfected cells with Mito-TEMPO (superoxide scavenger) or with 1400W (selective iNOS inhibitor).

Twenty four hours after transfection, Mito-Tempo fully prevented HBx- and HBV-mediated mtDNA depletion, while 1400W partially protected against HBx- and HBV-mediated complex I inactivation in HepG2 cells (Suppl. Table 5).

DISCUSSION

The present study investigated the incidence of mtDNA damage and/or alterations in the mitochondrial function and homeostasis in patients with CHB, CHC and NASH, and in healthy control subjects. We also compared patients with advanced fibrosis or cirrhosis (F3-F4) to patients with minimal-mild-moderate fibrosis (F0-F2). The study explored also *in vitro* the possible mechanisms involved in HBV-induced mitochondrial stress.

Four mtDNA deletions (5385-bp, 4977-bp, 4881-bp and 2451-bp) associated with mtDNA depletion and mtDNA strand breaks were identified in patients with CHB (Fig. 1, Suppl. Figs. 2, 3). Only the 4977-bp deletion associated with mtDNA depletion was detected in NASH patients (Fig. 1B). The expression mtDNA-encoded MT-CO1, MT-CO2 and MT-ATP8 as well as liver ATP levels significantly decreased in patients with CHB when compared to healthy controls (Figs. 2, 6). The expression mtDNA-encoded MT-CO1 and MT-CO2 also decreased in patients with NASH or with CHC as compared to controls (Fig. 2). The expression of the markers for ^{mt}UPR, mitophagy and mitochondrial biogenesis all significantly altered in patients with advanced fibrosis or cirrhosis compared to patients with F0-F2 (Figs. 3-6).

The 4977-bp and 5385-bp but not 4881-bp and 2451-bp have been previously reported. The common 4977-bp mtDNA deletion is associated with normal age (20,21). The 4977-bp and 5385-bp deletions have been previously reported in Wilson disease and in alcoholics with microvesicular steatosis (22,23). The mtDNA 4977-bp deletion has been reported also in non-alcoholic fatty liver diseases (42), in HBV-related HCC (43,44), as well as in a case of CHB patient with toxic myopathy associated with long-term use of oral anti-viral regimens (45). Accumulation of oxidative mtDNA damage (mainly 8-hydroxydeoxyguanine) has been also previously reported in CHC (38).

In patients with CHB, the higher prevalence and the diversity of these mtDNA deletions were associated with advanced fibrosis or cirrhosis (Suppl. Table 3). Fifty-six of the sixty-five (86%) patients with CHB including 30 of 46 (65%) patients with F0-F2 and 18 of 19 (95%) with F3-F4 had either one, two, or three different mtDNA deletions, whereas only 5 of 24 (21%) control subjects had a single mtDNA deletion

(Suppl. Table 3). In patients with NASH and cirrhosis, 7 of the 12 (58%) presented a single 4977-bp mtDNA deletion (Suppl. Table 3).

Subsequently, the expression of mtDNA-encoded MT-CO1 and MT-CO2 was significantly decreased in F3-F4 when compared to F0-F2 (Fig. 2). The expression of these mtDNA-encoded genes might be interpreted as the consequence of mtDNA deletions and lower mtDNA content detected in the present study, but also as consequence of several other mtDNA point mutations including those described in the mtDNA regulatory sequence D-Loop in HBV-infected patients (43,44,46). Point mutations in the D-Loop region might also explain the observed mtDNA depletion observed in our patients as this sequence controls both transcription and replication of mtDNA (15).

The mitophagy markers PRKN and PINK1 as well as ^{mt}UPR markers HSPD1, HSPA9 and LONP1 or biogenesis marker TFAM were all downregulated in patients with F3-F4 when compared to patients with F0-F2 (Figs. 3-5). These mitochondrial lesions were associated with iNOS induction and liver inflammation in patients with CHB and F3-F4 (Fig. 6).

We did not observe any correlation between HBV viral loads and the expression of the studied genes in the global cohort of patients with CHB. No difference in the prevalence of mtDNA deletion or in the expression of the studied genes was observed when HBeAg-positive patients compared to HBeAg-negative patients.

Impaired mitochondrial function leads to liver steatosis, a lesion ascribed as consequence of altered lipids metabolism and mitochondrial β -oxidation of fatty acids (9). NASH is a condition characterized by the presence of fat droplets associated with liver fibrosis, necroinflammation, lipid peroxidation and underlying lipotoxicity (9,47–49). Lipid metabolic changes play a critical role in NASH development (7). The common 4977 bp mtDNA deletion appeared progressively with normal age and has been reported in patients with hepatic steatosis (7, 15-19). Despite their old age, patients with NASH and cirrhosis had only one mtDNA deletion as compared to patients with CHB and F3-F4 (Table 1, Suppl. Table 4). This age-related mtDNA deletion may favor steatosis in patients with NASH. **Conversely**, lipid hydroperoxides may cause mtDNA damage in patients with NASH which in turn might aggravate hepatic steatosis and the development and progression of fibrosis (9,50). All the 7

patients with NASH and mtDNA deletion developed significant hepatic steatosis ($\geq 66\%$ of fat containing hepatocytes in 5 and $>33\%$ - $<66\%$ in 6). Conceivably, subjects with mtDNA deletions (and possibly many other mtDNA mutations) may poorly reoxidize NADH (reduced nicotinamide adenine dinucleotide) in the mitochondria leading to hepatic steatosis as a consequence of impaired oxidation of fatty acids (9). In the present study, mtDNA deletions were associated with decreases in the expression of mtDNA-encoded genes which may block electron flow within respiratory chain complexes, thus causing an excess of reducing equivalents NADH in the liver. Such alterations in the mitochondrial redox state inhibit the oxidized nicotinamide adenine dinucleotide (NAD^+)-dependent fatty acids dehydrogenases involved in the β -oxidation of fatty acids (9). In NASH, the increased hepatic free fatty acids then uncouple respiration from ATP formation (9). Mitochondria-derived ROS oxidize the unsaturated lipids to cause lipid peroxidation. Extensive lipid peroxidation was observed in animal models of steatohepatitis and lipid peroxidation products alter mtDNA and react with mitochondrial proteins, including respiratory chain complexes (9). These effects tend to partially block the transfer of electrons in the respiratory chain. Even when adaptive changes such as mitochondrial biogenesis occur, any block in the flow of electrons along [the respiratory chain](#) may cause the formation of high amounts of ROS (9). Moreover lipid peroxidation and ROS consume antioxidant enzymes and vitamins thus compromising ROS scavenging, causing further lipid peroxidation, increasing mitochondrial damage, and higher ROS formation in NASH (9). Reactive lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) increase hepatic fibrogenesis by two mechanisms. MDA and 4-HNE enhance the hepatic production of transforming growth factor $\beta 1$ (TGF- $\beta 1$), which activates hepatic stellate cells to evolve into collagen secreting myofibroblasts. MDA and 4-HNE also directly enhance collagen production by hepatic stellate cells (9). However, this might not be the case in HBV-infected patients because hepatic steatosis is a relatively uncommon in CHB patients unlike patients with NASH or CHC (46–48 and the present study). Only 12 (8%) over the 146 patients with CHB had significant steatosis ($>33\%$ of fat-engorged hepatocytes) compared to 24% in CHC and 92% in NASH in the present study (Table 1). mtDNA depletion, strand breaks, and the high prevalence of mtDNA deletions and their diversity in patients with CHB as compared to controls or to patients with NASH all suggest that HBV

infection may instead cause massive oxidative damage to liver mtDNA which progressively accumulates in patients with advanced fibrosis. These observations also suggest that mitochondrial dysfunction and altered mitochondrial dynamics would speed-up the progression of CHB-related fibrosis. Indeed, mtDNA damage is a hallmark of oxidative stress (9). Although ROS were not measured in our patients, the mtDNA damage detected in livers from patients with CHB or with NASH and advanced fibrosis suggested that significant oxidative stress rises in these livers. In line with this hypothesis, we demonstrated that transient replication of HBV or expression of HBx protein increased the mitochondrial formation of superoxide anion and peroxynitrite radicals (Suppl. Table 4). Moreover, HBx has been shown to interact with the mitochondrial voltage-dependent anion channel, consistent with its role in decreasing the mitochondrial membrane potential and thus its increasing of in the intracellular levels of ROS (54). HBx interacted also with Bcl-2 and Bcl-xL to induce oxidative stress and cell death (Geng X et al. 2012). HBx containing G124L and I127A mutations prevented HBx binding to Bcl-2 and Bcl-xL and abrogated its role in opening MTP (Geng X et al. 2012). In the present study, mutated HBx(G124L and I127A failed to increase mitochondrial oxidative stress, and failed to cause mtDNA depletion and complex I inactivation (Supple. Fig. 7, Suppl. Tables 4,5). Importantly, we have reported previously that specific protection against hepatocyte mitochondrial dysfunction plays a preventive role in early stages of fibrogenesis and delays its onset in transgenic mice specifically overexpressing Bcl-2 in their hepatocytes (Mitchell et al. 2009). Analyses of mtDNA, respiratory chain complexes, and lipid peroxidation showed that these Bcl-2 transgenic animals were protected against carbon tetrachloride-induced mitochondrial dysfunction and oxidative stress resulting in reduced fibrosis (Mitchell et al. 2009).

HBV and HBx also block electron flow within respiratory chain and depress mitochondrial antioxidant capacities, thus increasing ROS formation within mitochondria (9,10,29,54,55). ROS-mediated mtDNA damage includes single- and double-strand breaks that favor slipped mispairing of repeated sequences during replication (23,40,40,56,57). This may explain why the detected mtDNA deletions were all flanked by direct repeats in these patients (Suppl. Fig. 3 1), consistent with a mechanism of slipped mispairing (56,58). The higher frequency of the 4977-bp deletion in our patients may be explained by the long 13-bp direct repeats in this case favoring misannealing.

In addition to direct ROS attacks, mitochondrial endonucleases could also contribute to mtDNA fragmentation (59). Furthermore, oxidative mtDNA lesions may cause transient formation of abasic sites which have been shown to be potent stimulators of double stranded DNA cleavage by mitochondrial topoisomerase II (59). Alternatively, mtDNA depletion might be caused by mitophagy-mediated clearance of excessively damaged mitochondria (9). [mtDNA degradation occurred to different extent in patients with F3-F4 as compared to those with F0-F2](#) (Fig. 1, [Suppl. Fig. 3](#)) suggesting that mitophagy process might be different in these patients. Accordingly, the expression of the mitophagy markers PINK1 and PRKN significantly dropped in advanced fibrosis [when compared to F0-F2 fibrosis although they were induced in both groups as compared to controls](#) (Figs. 4).

Finally, interferons have been shown to suppress mtDNA gene transcription by activating RNase L contributing to the mtDNA degradation and to a decreased synthesis rate of mitochondrial transcripts (9,24). The RNase L is expressed in mitochondria, where it degrades mtDNA-encoded mRNAs in a translation dependent process (24).

Our data *in vitro* confirmed HBV and HBx protein both inducing mitochondrial superoxide release ([Suppl. Fig. 7A, Suppl. Table 4](#)). Superoxide itself is not only poorly reactive with biological compounds but also it can not cross biological membranes. However, it reacts with NO (formed within mitochondria or elsewhere) to form the highly reactive peroxynitrite (24,26,27). Although the presence of iNOS within mitochondria is debated, the freely diffusible NO formed elsewhere can cross mitochondrial membranes to react with superoxide and form peroxynitrite within the mitochondria (24). Indeed, iNOS is also highly expressed in peroxisomes which are not only essential metabolic organelles with a central role in the synthesis and turnover of complex lipids, but also an important source of ROS, NO and RNS (60–62). Moreover, iNOS inhibited H₂O₂-scavenging peroxisomal catalase leading to high hydrogen peroxide accumulation within this organelles, which may account for further hepatocellular damage through the lipotoxicity of lipid peroxidation byproducts (63). Thus, the combination of the increased mitochondrial superoxide formation ([Suppl. Fig 7A,, Suppl. Table 4](#)) with the concomitant increased iNOS expression wherever it took place ([Fig. 6, Suppl. Fig. 6](#)) [could](#) markedly increase the mitochondrial formation of peroxynitrite in HBV-infected hepatocytes.

Peroxynitrite and/or peroxynitrite-generated reactive intermediates can damage mtDNA (25–27). Overexpression of MnSOD, selective inhibition of iNOS, or peroxynitrite scavengers, all attenuated mtDNA depletion in diverse situations (25–27). iNOS inhibitor 1400W and superoxide scavenger Mito-Tempo protected against HBx- and HBV-induced mtDNA depletion and respiratory complex I impairment (Suppl. Table 5). Taken together, these observations suggest that the reaction of superoxide with NO to form the DNA-damaging peroxynitrite is involved in the mtDNA damage triggered by HBV.

Peroxynitrite also damages proteins, causing nitrotyrosine formation, tyrosine oxidation, and loss of function (24,25). In the present study, HBV as well as HBx increased 3-nitrotyrosine residues in the proteins of mitochondrial complexes I, III and V (Suppl. Fig 6). Such nitration has been shown to decrease respiratory complex I activity (24,25) [to further enhance and sustain mitochondrial ROS formation \(9\)](#).

MnSOD is another target for ROS- and peroxynitrite-mediated inactivation (25). Despite the induction of the MnSOD protein, the MnSOD activity rather decreased in HepG2 cells after transfection with HBV or HBx, whereas Cu,ZnSOD activity remained unchanged (Suppl. Fig. 6). The reduced MnSOD activity in HBV-infected hepatocytes may further increase the intramitochondrial concentrations of superoxide and its reaction with NO to form peroxynitrite.

Thus, several redundant mechanisms may be involved in oxidative stress after HBV infection, including TNF α , respiratory chain impairment, decreased MnSOD activity and induction of iNOS.

To preserve mitochondrial homeostasis, hepatocytes respond by inducing mitochondrial anti-oxidative capacities such as MnSOD, by increasing mitochondrial fission to allow the mitophagy degradation of dysfunctional mitochondria, and by increasing mitochondrial [fusion and](#) biogenesis to restore mtDNA levels and mitochondrial mass (9,64). When these adaptive responses failed, then hepatocytes activate the protective ^{mt}UPR mechanisms through mitochondrial HSPA9 and HSPD1 to allow a correct folding of the mtDNA- and nDNA-encoded mitochondrial proteins (9). The observed mtDNA damage in patients with CHB [and NASH](#) may cause the unbalance between these proteins of two genetic origins and lead to mitochondrial protein aggregates known as toxic for mitochondria (9). The mitochondrial peptidases

LONP1 then degrade the excess of imported mitochondrial proteins encoded by nDNA. Ultimately, hepatocyte might block the mitochondrial import of nDNA-encoded polypeptides and extensively damaged mitochondria cleaned by mitophagy in extreme situations. All of these adaptive responses seem compromised in advanced fibrosis as shown by the decreases in the expression of specific proteins such as LONP1 peptidase and mitochondrial HSPA9 and HSPD1 required for proper ^{mt}UPR, PINK1 and PRKN involved in the mitophagy process (Figs. 3, 4) as well as TOMM20, MFN1 and OPA1 involved respectively in the mitochondrial import, fusion and fission (Suppl. Fig 4). We noted however that mitophagy markers were induced in CHB and F0-F2 but this induction failed in F3-F4 (Fig. 4).

Whatever the etiology, the above discussed mechanisms of oxidative mtDNA damage, mitochondrial dysfunction, and altered mitochondrial dynamics might all favor the development of fibrosis in several ways. *First*, the preexisting (inherited, acquired or age-related) mtDNA deletions might aggravate the effects of HBV in CHB and the effects of lipoperoxidation products in NASH on mitochondrial function, which in turn might accelerate the development and progression of liver fibrosis. Patients with mtDNA deletions in the present study have developed hepatic fibrosis to different extend. Those subjects, however, with multiple mtDNA deletions (and possibly many other mtDNA mutations) may produce more oxygen radicals and poorly eliminate ROS because of the altered mitochondrial antioxidant capacities (9,65,66). This may lead to marked increase in the lipid peroxidation products known to activate hepatic stellate cells (HSCs) leading to collagen accumulation (9). **Patients with NASH and mtDNA deletions and patients with CHC and mitochondrial dysfunction were all significantly older than patients with CHB and controls (Table 1). Unlike in NASH group where patients with mtDNA deletions were significantly older, the age of patients with mtDNA deletions and CHB was not different from the age of all other patients without the deletions in the present study. Similarly, the age of patients with CHB patients and advanced fibrosis was not different from the age of all other patients without mtDNA deletions. Thus, these observations excluded the effect of age on mtDNA in CHB and likely supported direct damaging effects of HBV on mtDNA in advanced fibrosis. *Second*, one can not exclude that steatosis itself likely cause mtDNA damage and alter mitochondrial homeostasis. Steatosis and ROS favor the accumulation of lipid peroxidation products known to cause mtDNA**

damage, which may then favor the development of fibrosis. Lipohydroperoxides lead to mtDNA adducts, single-strand breaks and favors slipped mispairing of repeated sequences during replication of damaged mtDNA molecules resulting in impaired oxidative phosphorylation, even though the percentage of mtDNA affected with these deletions is usually low (20,29,67). This may be the case in NASH and CHC but unlike in CHB. In NASH, massive steatosis associated with liver inflammation and oxidative stress lead to lipid peroxidation products hallmarks (7). However, significant steatosis (thus lipid peroxidation) is a rare histological feature in our patients with CHB (Table 1) and other studies (50–52). *Third*, altered mitophagy together with associated liver necrosis might favor the release of mitochondrial danger signals to induce expression of inflammatory cytokines known to favor the development and progression of liver fibrosis (9,11,68,69). *Progressing work in our laboratory indicated that the plasma mtDNA levels were altered in patients with CHB, and coincided with the increase in the plasma TNF α and IL6 concentrations and liver TNF α and IL6 mRNAs. Reduced mitophagy might thus promote immune and inflammatory responses through the mitochondrial damage-associated molecular patterns (mDAMPs) (9,11,70–72). As such mDAMPs, mtDNA activate Toll-like receptor 9 and NLRP3 inflammasome and leads to the expression and maturation of inflammatory cytokines (9,70–76). Importantly, it has been reported also that mtDNA directly activates HSCs and collagen synthesis in mice fed high fat diet and in patients with NASH (11). Finally, the observed effects of HBV may occur in liver residing other cell types including immune cells. HBV has been shown to cause mitochondrial dysfunction of HBV-specific CD8 T cells leading to their exhaustion and senescence (12,77). In this case, mitochondrial dysfunction indirectly impair the resolution of liver fibrosis due the exhausted CD8 T and thus favors the progression of this lesion. Determining which of these hypotheses is correct clearly requires further studies.*

Although the present study has several strengths (relevant knowledges for the understanding of the pathophysiology and mechanisms of chronic liver diseases), it has also several main limitations: first of all, our inability to simultaneously investigate all of the addressed parameters (RNA, DNA and proteins) and to characterize the intrahepatic HBV biomarkers (cccDNA, 3.5kb mRNAs and the cccDNA transcriptional activity) in all samples due to the limited sizes of available biopsies. Second, the heterogeneity of different cell types (hepatocytes, inflammatory cells, HSCs) has not been addressed. Third, the role of HBV-induced mitochondrial dysfunction in

fibrosis progression need further explorations. To this end, it will be of great interest to treat *in vitro* HSCs with purified mtDNA (as a mDAMP) or to co-culture HBV-infected hepatocytes with HSCs and monitor their differentiation into collagen-secreting myofibroblasts and their expression of profibrogenic factors/markers. Studying mitochondrial stress in HBV-infected liver humanized mouse model treated or not with CCl₄ would allow to explore the role of these mitochondrial lesions in the progression of fibrosis. *Finally*, experiments in other relevant models relying on HBV 'infection' (with wild type and HBx mutant viruses) are needed to confirm whether the mitochondrial alterations observed in our 'transfection system' are induced by HBV replication and/or by other HBV proteins in addition to HBx.

We propose that mtDNA damage resulted in disturbance of mitochondrial homeostasis, function and dynamics in patients with CHB and patients with NASH. Suppressed ^{mt}UPR in combination with altered mitophagy leads to an overall significant mtDNA damage accumulation which subsequently increases mitophagic burden leading to mDAMPs release, and subsequent expression of inflammatory and profibrogenic mediators (Fig. 7). All these mitochondrial alterations likely play important roles in triggering/exacerbating inflammatory and profibrotic responses that promote the development and progression of fibrosis (Fig. 7).

Our observation that HBV- and HBx-induced mtDNA damage and mitochondrial dysfunction is preventable *in vitro* raises the hope that the dire prognosis of advanced fibrosis-cirrhosis could be therapeutically improved or slowed *in vivo*. Further investigations of the modulation of these potential mitochondrial biomarkers in large cohorts of patients with other liver diseases are urgently needed to confirm the feasibility of these markers for therapeutic and diagnosis purposes.

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Table 1. Characteristics of patients with CHB, CHC, NASH and controls.

	CHB	CHC	NASH	Controls
Total Number (n)	146	33	12	24
Gender (M/F)	92/54	14/19	6/6	13/11
Age (years)(mean ± SEM)	41 ± 4	48 ± 7*	52 ± 15*	42 ± 10
BMI (Kg/m ²)(mean ± SEM)	24.5 ± 3.3 ^{§§§}	26.7 ± 4.4 [§]	32.9 ± 7.7	23.1 ± 3.7 ^{§§§}
ALT (IU/L)(mean ± SEM)	111 ± 13	94 ± 41	102 ± 36	74 ± 37
AST (IU/L)(mean ± SEM)	74 ± 14	60 ± 23	81 ± 45	51 ± 28
γ-GT (IU/L)(mean ± SEM)	79 ± 19 [#]	133 ± 124	111 ± 103	198 ± 87*
Total cholesterol (mmol/L) (mean, range)	4.4 (2.9-6.5)	4,7 (3,4-6.9)	4,6 (2.6-6.4)	3,9 (2,7-5,2)
Triglycerides (mmole/L)(mean, range)	1.13 (0.38-3.69)	1.05 (0.47-2.15)	1.98 (0.77-5.5)	1.01 (0.43-1.39)
Glucose (mmole/L) (mean ± SEM)	5.0 ± 0.6 ^{§,&}	5.5 ± 1.1 ^{#,*}	6.0 ± 1.2 ^{#,*}	4.7 ± 0.3
Creatinin (μmol/L) (mean ± range)	0.65 (0.34-1.34)	0.86 ^{#,*} (0.59-1.25)	1.2 ^{#,*,&} (0.8-1.68)	0.66 (0.58-0,82)
Albumin (g/L) (mean ± SEM)	45 ± 7	45 ± 3	47 ± 4	43 ± 3
Platelets (10 ⁹ /L) (mean ± SEM)	174 ± 43	225 ± 48*	186 ± 50	271 ± 69
Virology				
HBeAg positive, n(%)	77(53)	-	-	-
HBsAg (mean, log ₁₀ IU/mL)	3,8	-	-	-
HBV DNA or HCV RNA (log ₁₀ IU/mL) (mean, range)	5.4 (2.2-8.2)	5,9 (5.0-7.1)	-	-
Liver histology findings				
Necroinflammatory activity				
A0 A1 A2 A3	35 68 30 13	2 19 11 1	0 1 3 8	22 1 0 1
Fibrosis stage				
F0-F2 (n)	100	21	0	24
F0 F1 F2	26 40 34	1 12 8	0 0 0	23 1 0
F3-F4 (n)	46	12	12	0
F3 F4	29 17	7 5	0 12	0 0
Steatosis (n)				
≤5% of hepatocytes	118	16	0	21
5%-33% of hepatocytes	16	9	1	3
>33%-66% of hepatocytes	11	6	6	0
≥66% of hepatocytes	1	2	5	0

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, Body Mass Index; HBeAg, Hepatitis B virus e antigen; HBsAg, Hepatitis B virus surface antigen; γ-GT, γ-glutamyl transferase. For ALT level, upper limit of normal is 34 IU/L in women and 45 IU/L in men. For AST level, upper limit of normal is 31 IU/L in women and 35 IU/L in men. For γ-GT level, upper limit of normal is 38 UI/L in women and 55 UI/L in men. [#]Different from controls, *p*<0.05; *Different from CHB, *p*<0.05; [&]Different from CHC, *p*<0.05; [§]Different from NASH, *p*<0.05; ^{§§}Different from NASH, *p*<0.01; ^{§§§}Different from NASH, *p*<0.001; Mann-Whitney U-test.

Table 2. Characteristics of patients with CHB and with mtDNA analysis, mRNAs and/or protein assessment.

	mtDNA Analysis n=65		mRNA Assessment n=104		Protein Assessment n=44	
	F0-F2	F3-F4	F0-F2	F3-F4	F0-F2	F3-F4
Number (n)	46	19	76	28	26	18
Gender M/F (n)	32/14	10/9	61/15	20/8	18/8	10/8
Age (years) (mean ± SEM)	42 ± 2	45 ± 5	40 ± 1	45 ± 2	41 ± 2	47 ± 3
BMI (Kg/m ²) (mean ± SEM)	26.3 ± 2.3	24.5 ± 1.9	25.1 ± 0.6	24.5 ± 0.9	24.9 ± 0.9	23.7 ± 1.4
Serum levels at liver biopsy						
ALT (IU/L) (mean ± SEM)	109 ± 22	112 ± 15	109 ± 15	121 ± 27	101 ± 21	72 ± 18
AST (IU/L) (mean ± SEM)	75 ± 13	83 ± 13	75 ± 9	73 ± 12	82 ± 16	71 ± 19
γ-GT (IU/L) (mean ± SEM)	88 ± 22	80 ± 15	76 ± 20	92 ± 17**	146 ± 53	56 ± 14
Total cholesterol (g/L) (mean ± SEM)	0.8 ± 0.3	1.1 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	0.7 ± 0.1	0.8 ± 0.3
Triglycerides (mmole/L; mean ± SEM)	1.0 ± 0.4	0.9 ± 0.3	1.2 ± 0.1	1.6 ± 0.2	1.7 ± 0.3	2.0 ± 0.8
Glucose (mmole/L) (mean ± SEM)	4.8 ± 0.3	5.0 ± 0.3	4.9 ± 0.2	4.9 ± 0.3	5.4 ± 0.2	4.9 ± 0.2
HBsAg (log ₁₀ IU/mL) (mean ± SEM)	4.3 ± 0.2	5.0 ± 0.5	3.7 ± 0.1	4.6 ± 0.6	4.0 ± 0.2	4.8 ± 0.7
HBV DNA (log ₁₀ IU/mL) (mean ± SD)	4.7 ± 0.3	4.8 ± 0.6	5.5 ± 0.2	5.4 ± 0.4	4.8 ± 0.5	5.6 ± 0.9
Fibrosis stage						
F0	12	0	19	0	5	0
F1	21	0	40	0	14	0
F2	13	0	17	0	7	0
F3	0	10	0	17	0	12
F4	0	9	0	11	0	6

Note: RNA, DNA and proteins were simultaneously obtained in 42 cases (31 with F0-F2 and 11 with F3-F4), and both DNA and proteins obtained in 13 patients. The remaining 91 patients have either DNA, mRNA or proteins. The remaining 91 patients have only mRNA (64 cases), DNA (25 cases) or proteins (4 cases). For ALT level, upper limit of normal is 34 IU/L in women and 45 IU/L in men. For AST level, upper limit of normal is 31 IU/L in women and 35 IU/L in men. For γ-GT level, upper limit of normal is 38 UI/L in women and 55 UI/L in men. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, Body Mass Index; HBeAg, Hepatitis B virus e antigen; HBsAg, Hepatitis B virus surface antigen; γ-GT, γ-glutamyl transferase; SEM, Standard Error of the Mean; SD, Standard Deviation. **Different from F0-F2, $p < 0.01$. Mann-Whitney U-test.

FIGURES LEGENDS:

Figure 1. Liver mtDNA levels, proportions of the three main mtDNA topology forms and mtDNA deletions.

(A, B) Representative electrophoresis gels of long PCR detection of mtDNA deletions in livers from controls, patients with CHB and F0-F2 fibrosis or F3-F4 fibrosis and patients with NASH. In patients with CHB, primers A-B gave 6080-bp product amplified from undelated mtDNA, and 3629-bp, 1199-bp, 1103-bp or 695-bp products from deleted mtDNA (exact sizes were determined from the subsequently characterized 5385-bp, 4977-bp, 4881-bp and 2451 mtDNA deletions in these patients) (A). In patients with NASH, the fragments of 1103-bp corresponding to 4977-bp deletion was obtained (B). (C, D, E) Representative Slot blots (C, D) and mtDNA/nDNA ratios (mean \pm SEM) from 65 patients with CHB (46 with F0-F2 and 19 with F3-F4) and from 12 patients with NASH are expressed as percentages of 24 control values (E). M, 1 Kb DNA Ladder Plus. ###Different from control subjects, $p < 0.001$; *** Different from F0-F2 or from patients with NASH; $p < 0.001$, Mann-Whitney U-test. Bp, base pairs; Ctrl, Controls; CHB, Chronic Hepatitis B; NASH, steatohepatitis, mtDNA, mitochondrial DNA; nDNA, nuclear DNA

Figure 2. Liver mRNA and protein levels for mtDNA-encoded cytochrome c oxidase subunit 1 (MT-CO1) and 2 (MT-CO2).

(A, B) MT-CO1 mRNA and protein levels. MT-CO1 mRNA levels were compared in patients with CHB and F3-F4 (n=21) or F0-F2 (n= 66), patients with CHC and F3-F4 (n=12) or F0-F2 (n=12), patients with NASH and cirrhosis (n=12) and controls (n=8) (A). MT-CO1 and β -Actin protein levels were assessed in patients with CHB and F3-F4 (n=11) or F0-F2 (n=23), patients with NASH and cirrhosis (n=7) and controls (n=7). (B). (C, D) MT-CO2 mRNA and protein levels. MT-CO2 mRNA levels were compared in patients with CHB and F3-F4 (n=21) or F0-F2 (n= 66), patients with CHC and F3-F4 (n=12) or F0-F2 (n=21), patients with NASH and cirrhosis (n=12) and controls (n=8) (C). MT-CO2 and β -Actin protein levels were assessed in patients with CHB and F3-F4 (n=21) or F0-F2 (n=11), patients with NASH and cirrhosis (n=7) and controls (n=7) (D). Representative Western blot gels are shown. Ctrl, Controls. ###, ###Different from control subjects, ### $p < 0.01$, ### $p < 0.001$; *, **, ***Different from F0-F2 or from patients with NASH; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney U-test. U-test. Ctrl, Controls.

Figure 3. Evaluation of the ^{mt}UPR and mitophagy markers. (A) LONP1 mRNA levels were compared in patients with CHB and F3-F4 (n=18) or F0-F2 (n=66), patients with CHC and F3-F4 (n=11) or F0-F2 (n=21), patients with NASH and F4 fibrosis (n=12) and controls (n=8). (B) LONP1 protein levels were compared in patients with CHB and F3-F4 (n=7) or F0-F2 (n=6), patients with NASH and F4 fibrosis (n=7) and controls (n=7). (C) HSPA9 mRNA levels were compared in patients with CHB and F3-F4 (n=20) or F0-F2 fibrosis (n=66), patients with CHC and F3-F4 (n=12) or F0-F2 (n=21), patients with NASH and F4 fibrosis (n=12) and controls (n=8). (D) HSPA9 protein levels were compared in patients with CHB and F3-F4 (n=10) or F0-F2 (n=17), patients with NASH and F4 (n=7) and controls (n=7). (E) HSPD1 mRNA levels were compared in patients with CHB and F3-F4 (n=20) or F0-F2 (n=66), patients with CHC and F3-F4 (n=11) or F0-F2 (n=20), patients with NASH and F4 (n=12) and controls (n=8). Representative Western blots are shown. #, ##, ### Different from controls ($p < 0.05, 0.01, 0.001$); *, **, *** F3-F4 compared to F0-F2 fibrosis within the same etiology or comparison of F3-F4 between etiologies, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney U-test. U-test. Ctrl, Controls.

Figure 4. Expression of PRKN and PINK1 mRNA and proteins. (A) PRKN mRNA levels were compared in patients with CHB and F3-F4 (n=20) or F0-F2 (n=66), patients with CHC and F3-F4 (n=21) or F0-F2 (n=12), patients with NASH and F4 (n=12) and controls (n=8). (B) PRKN protein levels were compared in patients with CHB and F3-F4 (n=7) or F0-F2 (n=12), patients with NASH and F4 (n=7) and controls (n=7). (C) PINK1 mRNA levels were compared in patients with CHB and F3-F4 (n=18) or F0-F2 (n=60), patients with CHC and F3-F4 (n=12) or F0-F2 (n=19), patients with NASH and F4 fibrosis (n=12) and controls (n=8). (D) PINK1 protein levels were compared in patients with CHB and F3-F4 (n=7) or F0-F2 (n=16), patients with NASH and F4 fibrosis (n=7) and controls (n=7). #, ##, ### Different from controls ($p < 0.05, 0.01, 0.001$); *, **, *** F3-F4 compared to F0-F2 fibrosis within the same etiology or comparison of F3-F4 between etiologies, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney U-test. U-test. Ctrl, Controls.

Figure 5. Protein and/or mRNA levels for the mitochondrial biogenesis markers TFAM and PPARGC1 α . (A) TFAM mRNA levels were compared in patients with CHB and F3-F4 (n=20) or F0-F2 fibrosis (n=60), patients with CHC and F3-F4 (n=22) or F0-F2 fibrosis (n=60), patients with NASH and F4 fibrosis (n=12) and controls (n=8). (B) TFAM protein levels were compared in patients with CHB and F3-F4 (n=7) or F0-F2 (n=10), patients with NASH and F4 fibrosis (n=7) and controls (n=7). (C) PPARGC1 α mRNA levels were compared in patients with CHB and F3-F4 (n=20) or F0-F2 (n=60), patients with CHC and F3-F4 (n=12) or F0-F2 (n=21), patients with NASH and F4 fibrosis (n=12) and controls (n=8). #, ##, ### Different from controls ($p < 0.05$, 0.01 , 0.001); *, **, *** F3-F4 compared to F0-F2 fibrosis within the same etiology or comparison of F3-F4 between etiologies, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney U-test. U-test. Ctrl, Controls.

Figure 6. Liver ATP, mtDNA-encoded ATP synthase 8 (MT-ATP8), iNOS and MnSOD protein levels and liver mRNA of TNF α and IL6. (A) ATP levels were measured in 4 pools of 30 μ g liver homogenates each obtained from 3 controls and 10 pools of 30 μ g liver homogenates each obtained from 3 patients with CHB (5 pools of CHB patients with F0-F2 and 5 pools with F3-F4). (B) Western blot analysis for MT-ATP8 subunit. MT-ATP8/ β -Actin protein ratios (mean \pm SEM) for 30 patients with CHB are expressed as percent of the mean of 12 control values. (C, D) Western blot analysis for iNOS and MnSOD proteins and corresponding iNOS/ β -actin and MnSOD/ β -actin ratios from 5 controls, 5 patients with F0-F2 fibrosis and 5 with F3-F4 fibrosis. Relative mRNA expression for TNF α (E) and IL6 (F) were determined by RT-qPCR in 6 patients with CHB and F3-F4 and in 10 patients with F0-F2 fibrosis using specific primers. *Different from controls, $p < 0.05$; **Different from controls, $p < 0.01$; #Different from F0-F2, $p < 0.05$, Mann Whitney U-test. CHB, Chronic Hepatitis B; ATP, Adenosine Triphosphate; MT-ATP8, mitochondrial ATP synthase subunit 8; iNOS, inducible NO synthase; MnSOD, manganese superoxide dismutase; TNF α , tumor necrosis factor alpha; IL6, interleukin 6. Representative gels are shown.

Figure 7. Suggested mechanisms for the role of mtDNA damage and mitochondrial stress in the progression of fibrosis. By depressing liver antioxidant capacities and partially blocking electrons flow within the mitochondrial respiratory chain, HBV proteins or lipoperoxidation products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) increase mitochondrial ROS formation, depress mitochondrial antioxidants and favors hepatic oxidative stress. ROS and these lipotoxic intermediates then cause mtDNA damage. The replication of damaged mtDNA molecules leads to progressive accumulation of mtDNA deletions resulting in profound mitochondrial dysfunction, which further increases ROS production and further mtDNA damage accumulation in a vicious cycle. ROS and mitochondrial damage associated molecular patterns (mDAMPs) also activate the proliferation and dedifferentiation of hepatic stellate cells (HSCs) into myofibroblasts secreting collagens and fibrosis develops. In some patients, the mitochondrial resilience involving adaptive mechanisms (^{mt}UPR, biogenesis, fusion, fission, mitophagy) might maintain or restore mitochondrial homeostasis and limit ROS formation and liver inflammation. The fibrosis in this case stabilizes or regresses. However, in other patients, the altered mitochondrial ^{mt}UPR in combination with mitophagic burden lead to significant accumulation of extensively damaged mitochondria within infected- or fat-engorged hepatocytes. Leakage of mtDNA and other mDAMPs in the cytosol could activate autocrine inflammatory responses. Alternatively, hepatocyte necrosis may lead to mDAMPs release in the extracellular medium leading to an activation of paracrine inflammatory responses in the neighboring hepatocytes, Kupffer cells and resident macrophages. High ROS and inflammatory cytokines in this case could sustain mitochondrial stress which may favor the progression fibrosis.