A Mitochondrial DNA Variant Elevates the Risk of Gallstone Disease by Altering Mitochondrial Function

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SUMMARY

Mitochondrial DNA 827A>G induces aberrant mitochondrial function and abnormal cholesterol transport through the transporter ABCG5/8 (ATP binding cassette subfamily G member 5/8) via activation of the AMPK signaling pathway, which increases the formation of gallstones.

BACKGROUND AND aims: Gallstone disease (cholelithiasis) is a cholesterol-related metabolic disorders with strong familial predisposition. Mitochondrial DNA (mtDNA) variants accumulated during human evolution are associated with some metabolic disorders related to modified mitochondrial function. The mechanistic links between mtDNA variants and gallstone formation need further exploration.

METHODS: In this study, we explored the possible associations of mtDNA variants with gallstone disease by comparing 104 probands and 300 controls in a Chinese population. We constructed corresponding cybrids using trans-mitochondrial technology to investigate the underlying mechanisms of these associations. Mitochondrial respiratory chain complex activity and function and cholesterol metabolism were assessed in the trans-mitochondrial cell models.

RESULTS: Here, we found a significant association of mtDNA 827A>G with an increased risk of familial gallstone disease in a Chinese population (odds ratio [OR]: 4.5, 95% confidence interval [CI]: 1.2–4.0). Compared with 827A cybrids (haplogroups B4a and B4c), 827G cybrids (haplogroups B4b and B4d) had impaired mitochondrial respiratory chain complex activity and function and activated JNK and AMPK signaling pathways. Additionally, the 827G cybrids showed disturbances in cholesterol transport and accelerated development of gallstones. Specifically, cholesterol transport through the transporter ABCG5/8 was increased via activation of the AMPK signaling pathway in 827G cybrids.

CONCLUSIONS: Our findings reveal that mtDNA 827A>G induces aberrant mitochondrial function and abnormal cholesterol transport, resulting in increased occurrence of gallstones. The results provide an important biological basis for the clinical
diagnosis and prevention of gallstone disease in the future. (Cell Mol Gastroenterol Hepatol 2021;11:1211–1226; https://doi.org/10.1016/j.cjcmgh.2020.11.015)

**Keywords:** mtDNA 827A>G; Gallstone Disease; Mitochondrial Function; Cholesterol Transport; Chinese Population.

Gallstone disease (cholelithiasis) is a common disease that is related to abnormal metabolism of lipids. Most gallstones are caused by supersaturation of cholesterol in the bile that leads to cholesterol crystallization and stone nidus formation, and <10% of stones are black and brown pigment stones. The etiology of gallstone disease is multifactorial; age, sex, pregnancy, diet (macronutrients, alcohol, and coffee), and other factors are involved. Moreover, the significant familial predisposition and ethnic differences in prevalence of this disease indicate the potential influences of genetic factors. A recent analysis of U.S. populations suggested that genetic factors are responsible for at least 30% of gallstone disease cases, and the highest prevalence of gallstones is observed in Native Americans (>70% among women).

Previous genome-wide association studies have identified a variant of the hepatobiliary cholesterol transporter ATP binding cassette subfamily G member 8 (ABCG8) as the most frequent genetic risk factor in gallstone disease patients. Another study has demonstrated that the lithogenic genes ATP binding cassette subfamily G member 5 (ABCG5) and ABCG8, as well as UGT1A1 (UDP glucuronosyltransferase family member A1), may cause gallstone formation. Moreover, rare mutations in ATP binding cassette subfamily B member 4 (ABCB4), ATP binding cassette subfamily B member 11 (ABCB11), CFTR (cystic fibrosis transmembrane conductance regulator), and CYP7A1 (cytochrome P450 family 7 subfamily A member 1) may promote gallstone formation by altering bile composition. Interestingly, gallstone formation is frequent in patients with diabetes. One group has clarified that insulin resistance elevates biliary cholesterol secretion by upregulating ABCG5 and ABCG8 via dysregulation of the transcription factor FOXO1 (forkhead box protein 01) in hepatocytes. In addition, activation of the nuclear receptor LXR (liver X receptor) can enhance biliary cholesterol secretion by increasing ABCG5 and ABCG8 levels in hepatocytes. Collectively, these findings reveal that nuclear variants indeed contribute to gallstone formation to some extent.

Mitochondria play important roles in the metabolism of glucose and lipids by conducting oxidative phosphorylation (OXPHOS) and producing adenosine triphosphate (ATP). Thus, mitochondrial DNA (mtDNA) abnormalities can influence the production of ATP and in turn contribute to the etiologies of common metabolic diseases. Type 2 diabetes is a classic metabolic disease that has been linked to mtDNA variants, such as 3310C>T, 3394T>C and 3243A>G. Furthermore, mtDNA variants have been identified as being responsible for many metabolic defects, including hypertension and dyslipidemia. Notably, mitochondrial function has been found to be associated with beta-oxidation and OXPHOS in fat and steroid metabolism. In addition, observations of maternal bias in the maternal transmission of gallstone disease have suggested that mtDNA variants are associated with familial gallstone disease. Therefore, we hypothesized that mtDNA variants may contribute to the occurrence of gallstones, which are manifestations of lipid metabolic abnormalities.

In this study, we explored the possible associations between mtDNA variants and gallstone disease by comparing 104 probands and 300 controls in a Chinese population. The results showed a strong association of gallstone disease with mtDNA haplogroup B4b’d’e’. A variant defining haplogroup B4b’d’e’, 827A>G, in mitochondrial 12S ribosomal RNA (rRNA) emerged as the most likely candidate responsible for the observed association. Thus, we investigated the pathological mechanism of gallstone disease associated with 827A>G by using transmitochondrial technology in this study. Mitochondrial respiratory chain complex activity and function, and cholesterol metabolism were assessed in the transmitochondrial cell models. Our findings revealed that mtDNA 827A>G induced aberrant mitochondrial function and abnormal cholesterol transport, resulting in increased occurrence of gallstones.

**Results**

**Associations of mtDNA Variants With Gallstone Disease**

We first investigated the relationships between mtDNA variants and gallstone disease in a Chinese population. DNA samples from 104 unrelated probands (mean age 44.9 years; range, 19–81 years) with confirmed gallstone disease (cholelithiasis) and a total of 300 unrelated individuals (mean age 64 years; range, 50–86 years) were collected from the Greater Shanghai Area. The average total cholesterol and triglyceride concentrations of the patients were 4.41 (normal range, 2.8–5.17) mmol/L and 1.67 (normal range, 0.56–1.7) mmol/L, respectively. We sequenced the mtDNA HVSI regions of the 104 patients and 300 controls, and the results showed that 2 variants, 16217C (P = 0.041) and 16136C (P = 0.007), showed significantly higher frequencies in the cases than in the controls. Interestingly, both variants were mutations defining B4 haplogroup (ie, 16217C defined B4, and 16136C defined B4b1). The P values for the chi-square test and Fisher’s exact test of the
associations between gallstone disease and the closely related polymorphisms are presented in Table 1. A 9-bp deletion at 8281–8289, the diagnostic marker of haplogroup B4, showed no association between the cases (n = 23 of 104) and the controls (n = 50 of 300) (P > 0.05). In addition, the prevalence of B5, the sister group of B4, was not significantly different between the cases (n = 4 of 104) and controls (n = 12 of 300), further supporting the significance of haplogroup B4. Moreover, the mitochondrial whole genome results showed the variant 827A>G in mitochondrial 12S rRNA was the candidate most likely responsible for gallstone disease (Table 2).

Next, we explored the frequency differences of the mutations defining the subhaplogroups of B4 between cases and controls to identify the subhaplogroups most associated with gallstone disease. In the phylogenetic tree (Figure 1A) and median-joining network (Figure 1B) of haplogroup B4, we found that haplogroup B4b'/d'e' showed a higher odds ratio (4.428) and more significant P value (1.2 × 10⁻⁴) (n = 15 of 104 patients and n = 11 of 300 controls) than the other haplogroups in B4 (B4, B4a, B4b1, and B4d), indicating that B4b'/d'e' was the haplogroup most likely associated with gallstone disease and that the variant harbored in this haplogroup might contribute to gallstone disease.

During the evolutionary history of modern humans, haplogroup B4 might have originated in East Asia approximately 40,000 years ago. B4b'/d'e' arose from B4 with the mutations 827A>G (a variant in 12S rRNA) and 15535C>T (a synonymous mutation in CytB). Thus, we hypothesized that 827A>G might have a considerable effect on the etiology of gallstone disease. B2, a subhaplogroup of B4b'/d'e', was a founder haplogroup and expanded in the

| Table 1. Association of mtDNA Haplogroups B4b'/d'e' with Gallstone Disease |
|-----------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| mtDNA Mutation and Haplogroup | mtDNA Mutation and Haplogroup | mtDNA Mutation and Haplogroup | mtDNA Mutation and Haplogroup | mtDNA Mutation and Haplogroup | mtDNA Mutation and Haplogroup | mtDNA Mutation and Haplogroup |
| 16217C | 16261 | 827G | 499A | 13942G | 16136C | 6413C |
| Cases (n = 104) | 20 | 5 | 15 | 11 | 4 | 11 | 8 |
| % | 19.23 | 4.81 | 14.42 | 10.58 | 3.85 | 10.58 | 7.69 |
| Controls (n = 300) | 34 | 15 | 11 | 11 | 0 | 11 | 3 |
| % | 11.33 | 5.00 | 3.67 | 3.67 | 0 | 3.67 | 1.00 |
| P (χ²) | 0.041 | 0.938 | 1.2 × 10⁻⁴ | 7.5 × 10⁻³ | 6.4 × 10⁻⁴ | 7.5 × 10⁻³ | 2.3 × 10⁻⁴ |
| P (FET) | 0.046 | <0.001 | 3.5 × 10⁻⁴ | 0.012 | 0.004 | 0.012 | 1.3 × 10⁻³ |
| OR | 1.863 | 0.960 | 4.28 | 3.107 | / | 3.107 | 8.25 |
| 95% CI | 1.02–3.38 | 0.34–2.71 | 2.08–9.44 | 1.35–7.13 | / | 1.35–7.13 | 2.68–25.3 |

**NOTE.** Probabilities of observed associations were calculated based on Pearson chi-square test, FET, and OR. CI, confidence interval; FET, Fisher’s exact test; OR, odds ratio.

| Table 2. Analysis of Whole Mitochondrial Genome of the 4 B4b'/d'e' Individuals |
|-----------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Position | CRS | P13 | P46 | P74 | P89 | Region | AA Change |
| 827 | A | G | G | G | G | 12S rRNA | — |
| 4820 | G | A | A | G | G | ND2 | None |
| 5292 | T | T | T | T | C | ND2 | Ser > Arg |
| 6023 | G | A | A | G | G | COXI | None |
| 6216 | T | C | C | T | T | COXI | None |
| 6413 | T | C | C | T | T | COXI | None |
| 13590 | G | A | A | A | G | ND5 | None |
| 13801 | A | A | A | A | G | ND5 | Thr > Ala |
| 13942 | A | A | A | A | G | ND5 | Thr > Ala |
| 14587 | A | A | A | G | A | ND6 | None |
| 15315 | C | C | C | T | T | Cytb | Ala > Val |
| 15535 | C | T | T | T | T | Cytb | None |
| 15930 | G | G | G | G | A | tRNA-Thr | — |

AA, amino acid; CRS, Cambridge Reference Sequence; rRNA, ribosomal RNA; tRNA, transfer RNA.

*Presents the site is mutant.
Americas after the Last Glacial Maximum (approximately 20,000 years ago). Thus, the frequencies of 827A>G B4 carriers were higher in Native Americans (14%–44%) than in East Asians (2%–8%) (Figure 1C; Supplementary Table 1). Interestingly, we found that the prevalence of gallstone disease was also significantly higher in Native Americans (14%–35%) than in East Asians (3%–12%) ($P = .002$, t test), suggesting the possible role of B4b'd'e'j in gallstone disease.

In addition, conservation analysis of vertebrates showed that 827A>G was generally conserved among all the species tested (Figure 1D), indicating that this adenosine might be
important in maintaining 12S rRNA function in different species and that mutations at this site are subject to purifying selection. We further predicted the RNA structure of 12S rRNA with or without 827A>G using the RNAfold program implemented in the Vienna RNA Package 2.0 (Figure 1E). The results indicated that the 827A>G variant in 12S rRNA might change the nearby loop structure.

In summary, we found that the 12S rRNA variant 827A>G harbored in haplogroup B4b’d’e] might affect mitochondrial function and in turn affect the prevalence of gallstone disease.

The 827G Cybrids Exhibited Lower Respiratory Chain Complex Activity Than the 827A Cybrids

To analyze the effect of the 827A>G variant on the regulation of the mitochondrial respiratory chain complex, we constructed 2 groups of sister branch haplogroup cell models: 827A cybrids (B4a/B4c haplogroups) (n = 6), and 827G cybrids (B4b/B4d haplogroups) (n = 6) according to the mtDNA phylogenetic tree. First, to determine whether 827A>G affects mitochondrial respiratory chain complex activity, we evaluated the transcript levels of 12S rRNA and mtDNA-encoded OXPHOS genes in the 827A and 827G cybrids. The results illustrated that the RNA levels of 12S rRNA, ND4/4L, ND5, ND6, CO3, ATP6, and ATP8 were significantly higher in the 827A cybrids than in the 827G cybrids (Figure 2A and C). In addition, mitochondrial ribosomal small subunit transcript levels were lower in the 827G cybrids than in the 827A cybrids, consistent with the results of the transcriptomic gene expression analysis of all 30 mitochondrial ribosomal small subunits (Figure 2A and B). Moreover, nuclear DNA (nDNA)-encoding OXPHOS gene expression tended to be lower in the 827G cybrids than in the 827A cybrids (Figure 2D). However, the mtDNA copy number did not significantly differ in either cybrids or peripheral blood mononuclear cell samples between the 827A (n = 206) and 827G (n = 82) genotypes (Figure 2E and F).

We further determined the mtDNA-encoding and nDNA-encoding protein expression levels in the 827A and 827G cybrids. The results revealed that the expression levels of most of the proteins, especially the ND5 subunit, were lower in the 827G cybrids than in the 827A cybrids, which may have impaired the assembly of OXPHOS complex I (Figure 2G and H). This finding confirms that 12S rRNA plays important roles in the processes of mtDNA transcription and translation. Finally, we examined the activity of the 4 respiratory chain complexes, and the results showed that complex I activity relative to citrate synthase activity was significantly higher in the 827A cybrids than in the 827G cybrids (Figure 2I).

Collectively, these results indicated that the 827G cybrids had lower respiratory chain complex activity than the 827A cybrids.

Compared With the 827A Cybrids, the 827G Cybrids Presented Diminished Mitochondrial Function

To corroborate the effect of the 827A>G variant on the mitochondrial respiratory chain complex, we detected OXPHOS function in the 827A and 827G cybrids. The results showed that the total ATP content, basal mitochondrial respiration, and OXPHOS-driven mitochondrial respiration were significantly lower in the 827G cybrids than in the 827A cybrids (Figure 3A and B). However, reactive oxygen species (ROS) generation was increased by 50% in the 827G cybrids compared with 827A cybrids (Figure 3C), which may have further activated mitochondrial quality control protein expression and mitochondrial-nuclear signaling pathways. Thus, compared with the 827A cybrids, the 827G cybrids presented diminished mitochondrial OXPHOS function.

The Mitochondrial Protein Quality Control Response and Retrograde Signaling Pathways Were Activated in the 827G Cybrids

We further investigated whether the ROS increases caused by mitochondrial dysfunction activated the expression of mitochondrial quality control proteins. The results showed that the levels of CLPP and LONP1 were significantly higher in the 827G cybrids than in the 827A cybrids, suggesting that the 827G cybrids experienced ROS stress. Notably, the mitophagy-related protein PINK1 tended to be downregulated in the 827G cybrids compared with the 827A cybrids (Figure 4A and B). Consistent with this result, the messenger RNA (mRNA) expression levels of the mitophagy-associated genes p62 and LC3 also tended to be
Figure 2. The 827G cybrids exhibited lower respiratory chain complex activity than the 827A cybrids. (A) Mitochondrial 12S rRNA and ribosomal protein small subunit–related gene transcript levels in the 827A (n = 6) and 827G (n = 6) cybrids. The relative mitochondrial ribosomal protein transcript levels in the 827G cybrids were normalized to the levels in the 827A cybrids. (B) Heatmaps of all the mitochondrial ribosomal protein small subunit–related gene levels between the 827A (n = 6) and 827G (n = 6) cybrids. The gradual color change from red to green represents the expression change from upregulation to downregulation. (C) Mitochondrial RNA levels in the 827A (n = 6) and 827G (n = 6) cybrids. The relative mitochondrial RNA levels in the 827G cybrids were normalized to the levels in the 827A cybrids. ND4/4 L, ND4, and ND4L. (D) Heatmaps of OXPHOS nDNA-encoded gene expression between the 827A (n = 6) and 827G (n = 6) cybrids. (E) MtDNA copy number levels of the 827A (n = 6) and 827G (n = 6) cybrids. The relative mtDNA copy number levels in the 827G cybrids were normalized to the levels in the 827A cybrids. (F) MtDNA copy number levels of the peripheral blood mononuclear cell samples of the 827A (n = 206) and 827G (n = 82) genotypes. The relative mtDNA copy number levels for the 827G genotype were normalized to the levels for the 827A genotype. (D) Immunoblot analysis of the levels of OXPHOS mtDNA-encoded and nDNA-encoded proteins in whole-cell extracts of the 827A (n = 6) and 827G (n = 6) cybrids. TOMM20 and ACTIN were used as loading controls for the mtDNA-encoded and nDNA-encoded proteins, respectively. (E) Quantified signal intensities of the OXPHOS mtDNA-encoded protein bands/TOMM20 bands and nDNA-encoded protein bands/ACTIN bands. The levels of proteins in the 827G cybrids were normalized to those in the 827A cybrids (the mean value for cybrids 827A was set to 1). (F) The enzymatic activity levels of mitochondrial complexes I, II, III, and IV and of citrate synthase were measured in mitochondria isolated from 827A (n = 6) and 827G (n = 6) cybrids. The enzymatic activity levels in the 827G cybrids were normalized to those in the 827A cybrids. The data are presented as the mean ± SD from at least 3 independent tests per experiment. *P < .05; **P < .01.
lower in the 827G cybrids than in the 827A cybrids (Figure 4C). These findings suggest that deficient mitophagy in the 827G cybrids hampered the cybrids to cope with stress.

As altered mitochondrial ROS generation has been found to be related to modification of many mitochondria-to-nucleus retrograde signaling pathways, we examined several major signaling pathways in the 827A and 827G cybrids. The results indicated that the JNK and AMPK pathways, which are related to OXPHOS gene expression and catabolism, respectively, were activated in the 827G cybrids (Figure 4D and E). In addition, the nuclear receptor RXRα, which mediates the biological effects of OXPHOS gene activation, was significantly downregulated in the 827G cybrids compared with the 827A cybrids (Figure 4F and G). Thus, the results suggested that OXPHOS complex capacity was decreased and that catabolism was increased for energy compensation in the 827G cybrids.

Abnormal Lipid Metabolism and Cholesterol Transport Processes Occurred in the 827G Cybrids

We further investigated whether abnormal lipid metabolism occurred in the 827G cybrids and found that the mRNA expression levels of fatty acid metabolism–related genes (CPT1A, CPT1B, VLCAD, ACOX1, and PPARα) were significantly downregulated in the 827G cybrids compared with the 827A cybrids (Figure 5A). In addition, the protein levels of CPT1A and PPARα were significantly decreased in the 827G cybrids compared with the 827A cybrids (Figure 5B and C). The results indicated that long-chain fatty acid transport and fatty acid β-oxidation processes were aberrant in the 827G cybrids.

Additionally, we examined the gene expression levels of the main enzymes in the metabolic processes of glycolysis and the tricarboxylic acid (TCA) cycle. The results illustrated that the gene expression of the key glycolysis enzymes PFKL and LDHA and the key TCA cycle enzymes PDHA, IDH, SDHA, FH, and MDH1 were evidently downregulated in the 827G cybrids compared with 827A cybrids. Downregulation of glycolysis in the 827G cybrids might have reduced the levels of the substrate for TCA and cholesterol synthesis, acetyl coenzyme A, thus hampering the TCA cycle and decreasing ATP generation. In addition, the levels of acetyl coenzyme A used for cholesterol synthesis might have decreased due to excessive accumulation of cholesterol in gallstones (Figure 5D and E). However, cholesterol synthesis showed no significant difference between the 827A and 827G cybrids (Figure 5F). The protein level and enzymatic activity of the
Figure 4. The mitochondrial protein quality control response and retrograde signaling pathways were activated in the 827G cybrids. (A) Representative Western blot of the mitochondrial quality control proteins PINK1, CLPP, LONP1, AFG3L2, HSP60, GRP75, and VDAC in whole-cell extracts from the 827A (n = 6) and 827G (n = 6) cybrids. ACTIN was used as a loading control. (B) Quantified signal intensities of all the target protein bands/ACTIN bands. The levels of proteins in the 827G cybrids were normalized to those in the 827A cybrids (the mean value for the 827A cybrids was set to 1). (C) Mitophagy associated gene expression in the 827A (n = 6) and 827G (n = 6) cybrids. The relative gene expression in the 827G cybrids was normalized to the expression in the 827A cybrids. (D) Representative Western blot of the relative phosphorylation of JNK, P38, ERK, AKT, and AMPK in whole-cell extracts from the 827A (n = 6) and 827G (n = 6) cybrids. ACTIN was used as a loading control. The levels of proteins in the 827G cybrids were normalized to those in the 827A cybrids. (E) Quantified signal intensities of all the target protein bands/ACTIN bands. The levels of proteins in the 827G cybrids were normalized to those in the 827A cybrids (the mean value for the 827A cybrids was set to 1). (F) Representative Western blot of the mitochondrial retrograde signaling mediators NRF1 and RXRα in whole-cell extracts from the 827A (n = 6) and 827G (n = 6) cybrids. ACTIN was used as a loading control. (G) Quantified signal intensities of the NRF1 and RXRα protein bands/ACTIN bands. The levels of proteins in the 827G cybrids were normalized to those in the 827A cybrids (the mean value for 827A cybrids was set to 1). The data are presented as the mean ± SD from at least 3 independent tests per experiment. *P < .05; **P < .01; ***P < .001.
rate-limiting enzyme of cholesterol synthesis, HMGCR, also did not differ between the 827A and 827G cybrids (Figure 5G–I). As expected, the total cellular cholesterol levels were similar between the 827A and 827G cybrids (Figure 5J–L).

Next, we focused on the transport processes in the 827A cybrids and 827G cybrids. The expression of the transporter ABCA1, which mediates cholesterol efflux to peripheral blood, was downregulated in the 827G cybrids compared with the 827A cybrids; however, the expression of the transporter ABCG5/8, which mediates cholesterol efflux to bile and the gallbladder, was upregulated in the 827G cybrids compared with the 827A cybrids (Figure 5M and N).

Because evidence has shown that hypoxic conditions contribute to the formation of gallstones, 28 we further evaluated whether hypoxia promoted the formation of gallstones in 827G cybrids. The cybrids were cultured in 1% O2 for 36 hours, and the results showed that the levels of the transporter ABCG5/8 increased dramatically in the 827G cybrids compared with the 827A cybrids, partly dependent on the HIF1α pathway (Figure 5M and N).

Taken together, our results showed that abnormal cholesterol transport, but not cholesterol synthesis, promoted gallstone formation in the 827G cybrids.

**Discussion**

In this study, we demonstrated a strong association of the mtDNA haplogroups B4b’d’e’j with gallstone disease in a Chinese population. Furthermore, mtDNA 827A>G, a highly conserved site in mitochondrial 12S rRNA defining the B4b’d’e’j haplogroups, induced aberrant mitochondrial function and elevated the occurrence of gallstones by inducing abnormal cholesterol transport through ABCG5/8 via activation of the AMPK signaling pathway.

Mitochondrial 12S rRNA plays an important role in the synthesis of mtDNA-encoded proteins, which may contribute to mitochondrial OXPHOS function. 29 The human mitochondrial ribosomal small subunit comprises mitochondrial 12S rRNA and 30 ribosomal proteins encoded by nDNA in eukaryotic cells. 30 Variants in the mitochondrial 12S rRNA genes, especially 827A>G, have been found to be associated with various diseases, such as cardiomyopathy, 31 nonsyndromic and aminoglycoside-induced hearing loss, 32,33 and age-related hearing loss in Chinese individuals. 24 However, the underlying mechanisms remain unknown and need further exploration.

The 143B osteosarcoma ρ0 cell line was the first mtDNA-deleted cell model successfully constructed 45 and has been widely used to study human evolution-related topics, such as mtDNA haplogroup function in East Asia 46 and high-altitude population adaptability. 37 In addition, many diseases, including diabetes, 38 osteoarthritis, 39 and hepatocellular carcinoma, 40 have been studied in this cell model. Therefore, we chose the 143B osteosarcoma ρ0 cell line in combination with 827A- and 827G-related haplogroups plasma for our research.

Notably, the mitochondrial complex I ND5 subunit, which plays a pivotal role in complex I assembly and activity, was decreased dramatically in the 827G cybrids. 41 Complex I is the largest complex (approximately 1000 kD) of the 5 OXPHOS complexes and occupies the initial position in the respiratory electron transport chain (I–III–IV); thus, decreased complex I levels obviously affect mitochondrial function. 42,43 Consistent with these findings, we found decreased respiration chain function and increased ROS levels along with downregulated complex I activity in the 827G cybrids compared with the 827A cybrids. These changes further activated mitochondrial-nuclear signaling pathways, including the JNK and AMPK signaling pathways. JNK signaling can downregulate RXRα transcript levels and OXPHOS complex biogenesis, which further aggravates mitochondrial OXPHOS and lipid metabolism dysfunction. 44 On the other hand, evidence has shown that lipid metabolism dysfunction induced PPARα downregulation, which may inhibit the expression of PPARα/RXRα. 45,46 Moreover, AMPK activation may increase the expression of the transporter ABCG5/8 to increase the risk of cholesterol entering the bile and gallbladder. 47 As shown in Figure 6, we similarly found that the 827G cybrids showed decreased PPARα and RXRα expression, which may have inhibited cholesterol efflux into peripheral blood by downregulating the expression of the transporter ABCA1, while promoting cholesterol efflux into the bile and gallbladder by increasing the expression of transporter ABCG5/8.

As previously described, hypoxia is a risk factor for the formation of gallstones. One study has demonstrated that adipocyte HIF2α reduces atherosclerosis by promoting ceramide catabolism and thus increasing hepatic cholesterol elimination to the gallbladder. 40 Additionally, activation of the HIF1α subunit pathway in steatotic liver contributes to the formation of gallstones. 20 Consistent with previous studies, our study indicated that the levels of ABCG5/8 were dramatically higher in the 827G cybrids compared than in the 827A cybrids and that this difference was partly dependent on the HIF1α pathway. Notably, such increases in ABCG5/8 levels may increase the risk of gallstones by reinforcing activation of AMPK signaling under hypoxia. 40,50 Interestingly, in our gallstone cases, the blood lipid levels did not exceed the upper limit. These findings provide a possible explanation for why mtDNA 827A>G preferentially increases the risk of cholesterol gallstones rather than atherosclerosis.

Accumulating evidence has shown that the prevalence of gallstones among Native Americans is the highest in the world. 7,63 Interestingly, a high frequency of haplogroup B, almost all of which are haplogroup B2 (1 of the branches of haplogroup B4b), has also been observed in Native Americans. 52,53 For example, the Pima Indian, the most commonly studied Amerindian tribe in the context of gallbladder diseases, has the highest prevalence of gallstone and the highest frequency of haplogroup B2 among Native Americans. 54,55 It has also been shown that the prevalence of gallbladder diseases increases in Chilean populations with increasing level of Native Americans admixture as measured by analysis of mtDNA and other genetic markers. 56 It seems that the presence of mtDNA haplogroup B4b’d’e’j is responsible for the ethnic differences in gallstone and even
other metabolic disorders, in America. Interestingly, the frequencies of 827A>G B4 carriers in Africa, Europe, and South and West Asia were found to be generally low in the current study, but the prevalence of gallstone disease was also considerable (4%–23%) (Figure 1C), indicating that genetic risks other than the mtDNA variant 827A>G or environmental factors, such as lifestyles, are also important in gallstone disease. Therefore, our study, to some extent,
The lipid metabolism and cholesterol transport processes were abnormal in the 827G cybrids. (A) Lipid metabolism process–associated gene expression in the 827A (n = 6) and 827G (n = 6) cybrids. Relative gene expression in the 827G cybrids was normalized to the 827A cybrids. (B) Immunoblotting analysis of the levels of CPT1A, ACOX1, and PPARα in whole-cell extracts from the 827A (n = 6) and 827G (n = 6) cybrids. GAPDH was used as a loading control. (C) Quantified signal intensities of all the target protein bands/ACTIN bands. The protein levels in the 827G cybrids were normalized to those in the 827A cybrids (the mean value for cybrids 827A was set to 1). (D) Glycolysis process–associated gene expression in the 827A (n = 6) and 827G (n = 6) cybrids. The relative gene expression in the 827G cybrids was normalized to the expression in the 827A cybrids. (E) TCA process–associated gene expression in the 827A (n = 6) and 827G (n = 6) cybrids. The relative gene expression in the 827G cybrids was normalized to the expression in the 827A cybrids. (F) Cholesterol synthesis process–associated gene expression in the 827A (n = 6) and 827G (n = 6) cybrids. The relative gene expression in the 827G cybrids was normalized to the expression in the 827A cybrids. (G) Representative Western blot of the cholesterol synthesis rate-limiting enzyme HMGCR in whole-cell extracts from the 827A (n = 6) and 827G (n = 6) cybrids. GAPDH was used as a loading control. (H) Quantiﬁed signal intensities of HMGCR protein bands/GAPDH bands. The levels of proteins in the 827G cybrids were normalized to those in the 827A cybrids (the mean value for 827A cybrids was set to 1). (I) HMGCR enzymatic activity detection in the 827A (n = 6) and 827G (n = 6) cybrids. The relative activity in the 827G cybrids was normalized to the activity in the 827A cybrids. (J) Whole-cell cholesterol content in the 827A (n = 6) and 827G (n = 6) cybrids. The relative content in the 827G cybrids was normalized to the content in the 827A cybrids. (K) Cholesterol efflux into the cultured medium of the 827A (n = 6) and 827G (n = 6) cybrids. The relative efflux for the 827G cybrids was normalized to the efflux for the 827A cybrids. (L) Total whole-cell and medium cholesterol content for the 827A (n = 6) and 827G (n = 6) cybrids. The relative content in the 827G cybrids was normalized to the content in the 827A cybrids. (M) Representative Western blot of ABCA1, ABCG5, ABCG8, CYP7A1, HIF1α, and HIF2α in whole-cell extracts from the 827A (n = 6) and 827G (n = 6) cybrids under 21% O2, and 1% O2, respectively. ACTIN was used as a loading control. The protein levels in the 827G cybrids were normalized to those in the 827A cybrids. (N) Quantified signal intensities of all the target protein bands/ACTIN bands. The levels of proteins in the 827G cybrids were normalized to those in the 827A cybrids (the mean value for cybrids 827A was set to 1). The data are presented as the mean ± SD for at least 3 independent tests per experiment. *P < .05; **P < .01; ***P < .001.
explained the etiology underlying the susceptibility to gallstone disease in Native Americans.

In summary, our study demonstrates a potential link between mtDNA 827A>G and gallstone disease. We have revealed that mtDNA 827A>G induces aberrant mitochondrial function and abnormal cholesterol transport, resulting in increased occurrence of gallstones. Our findings provide a significant biological basis for the clinical diagnosis and prevention of gallstone disease in the future.

Materials and Methods

Study Participants

Blood samples of 104 cases and 300 controls were collected in the Greater Shanghai Area with informed consent. The protocol of the study was approved by the Ethical Committee of the Chinese National Human Genome Center in Shanghai. The presence (cases) or absence (controls) of gallstone disease (cholelithiasis) was diagnosed by ultrasound and/or cholecystectomy. The mean age of onset in the patients was 44.9 (range, 19–81) years, and all controls were above 50 years of age (mean age 64.0 years; range, 50–86 years). The proportions of men among the cases and controls were 46.8% and 54.0%, respectively.

DNA samples from the blood of 206 827A (B4a/c)- and 82 827G (B4b’d’e’j’)- genotype participants and all platelet samples from healthy individuals used to construct the transmitochondrial cybrids model were obtained from the Taizhou Institute of Health Sciences, Fudan University. The study was approved by the Human Ethics Committee of Fudan University.

mtDNA Sequencing, Genotyping, Median-Joining Network Analysis, Conservation Analysis, and RNA Structure Analysis

Genomic DNA from peripheral blood was extracted using a standard SDS lysis protocol for genotyping. HVSI and other sites (8281–8289 deletion, 827A>G, 499G>A, 13942A>G, 6023G>A, and 6413T>C) in the coding region were genotyped by sequencing or polymerase chain reaction–restriction fragment length polymorphism assay. For complete sequence analysis of certain samples, Sanger sequencing was performed using 24 previously reported pairs of mtDNA primers. The single nucleotide polymorphisms (SNPs) in each participant were identified by comparing the obtained sequences with the revised Cambridge Reference Sequence by using Codon Code Aligner 3.0.1 (Codon Code Corporation, Centerville, VA). The mtDNA haplogroup was assigned by comparing the target SNPs with the SNPs of the most up-to-date Chinese mtDNA haplogroup tree. A median-joining phylogeny of these individuals was reconstructed using NETWORK software (Fluxus Technology Ltd, Colchester, Essex). Conservation analysis of 827 nucleotides was performed with UGENE software (NCIT UNIPRO, LLC, Novosibirsk, Russia). The RNA structure analysis of 12S rRNA with 827A or 827G was performed using the RNAfold program in the Vienna RNA Package 2.0.

Cell Line Generation and Culture Conditions

143B ρ0 human osteosarcoma cells lacking mtDNA were cultured in high-glucose Dulbecco’s modified Eagle medium (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (Thermo Fisher Scientific), 100-µg/mL pyruvate, and 50-µg/mL uridine. Transmitochondrial cybrids were formed by fusing 143B ρ0 cells and platelets from healthy individuals with different haplogroups, as described previously. In this study, 12 cybrids distributed in an mtDNA tree were constructed, including 6 B4a/B4c-haplogroup cybrids (named 827A cybrids) and 6 B4b/B4d-haplogroup cybrids (named 827G cybrids). All plasma samples were collected from Taizhou. The cybrids were cultured in high-glucose DMEM containing 10% fetal bovine serum at 37°C in an atmosphere with 5% CO2. Pathogenic mtDNA mutations and cross-contamination during single-clone selection were ruled out through Sanger sequencing of the whole mitochondrial genome in all cybrid cells during culture (Supplementary Table 2).

Analyses of mtDNA Content, Mitochondrial RNA, Mitochondrial Ribosome Subunits, Mitophagy, and Metabolism-Associated Gene Expression

Both mtDNA content and the mRNA levels of 13 mtDNA-encoded OXPHOS subunits were determined using the 2−ΔΔCT method as previously described. Briefly, genomic DNA and total RNA were extracted using standard protocols, and the total RNA was then treated with DNase and reverse-transcribed using 6 random primers (Takara, Dalian, China). Quantitative real-time polymerase chain reaction was performed using primers targeted to mtDNA, mitochondrial RNA, a subset of mitochondrial ribosome subunits, mitophagy-related genes, metabolism-related genes, and nuclear housekeeping genes on a QuantStudio 7 Flex Real-Time polymerase chain reaction system (Thermo Fisher Scientific) by using SYBR Green qPCR Mastermix (Takara). All primers used in these analyses are listed in Supplementary Table 3.

Mitochondria Isolation and Respiratory Chain Complex Enzymatic Activity Assay

Mitochondria from cultured cybrids were isolated as previously described. The enzymatic activity of 4 respiratory chain complexes was measured in the mitochondria of cybrids as previously described. The respiratory chain complex enzymatic activity in each case was normalized against that of citrate synthase, a mitochondrial matrix marker enzyme.

Immunoblotting and Antibodies

Proteins were extracted using RIPA lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Thirty micrograms of protein was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride...
Figure 7. Immunoblotting validation analysis of the differentially expressed proteins. (A) Representative Western blot of proteins ND5, CLPP, LONP1, CPT1A, PPARα, AMPKα, P-AMPKα, JNK1/2, P-JNK1/2, and RXRα in whole-cell extracts from the 827A (n = 6) and 827G (n = 6) cybrids. ACTIN was used as a loading control. The protein levels in the 827G cybrids were normalized to those in the 827A cybrids. (M) Representative Western blot of ABCA1, ABCG5, and ABCG8 in whole-cell extracts from the 827A (n = 6) and 827G (n = 6) cybrids under 21% O₂ and 1% O₂, respectively. ACTIN was used as a loading control. The protein levels in the 827G cybrids were normalized to those in the 827A cybrids. *P < .05; **P < .01; ***P < .001.
membranes. The proteins were blotted with antibodies at a dilution of 1:1000, as listed in Supplementary Table 4, and detected with ECL reagents (Bio-Rad, Hercules, CA). The validation data of the differentially expressed proteins was presented in Figure 7.

ATP, ROS, Oxygen Consumption, and Cholesterol Measurements

ATP content and ROS levels were determined using an ATP measurement kit and Mitosox (Thermo Fisher Scientific), respectively, as previously described. Endogenous oxygen consumption in intact cells was determined using an Oxgraph-2k (Oroboros, Innsbruck, Austria) as described previously. After fragmenting the mRNA, first-strand complementary DNA was synthesized and then sequenced using a NovaSeq 6000 platform (Illumina, San Diego, CA) as described previously. To obtain high-quality reads, reads containing adaptors, reads containing adapter sequences, and poly-N and low-quality reads were removed from the raw data. The reference genome and gene model annotation files were downloaded directly from genome websites. The reference genome was built using STAR, and paired-end high-quality reads were aligned to the reference genome. Differential expression analysis was performed using the DESeq2 R package. DESeq2 provides statistical routines for determining differential expression from digital gene expression data by using a model based on the negative binomial distribution. A heatmap of gene expression between six 827A cybrids and six 827G cybrids was created.

Statistical Analysis

The associations of mtDNA variants with gallstone disease were investigated with both the chi-square test and Fisher’s exact test. The odds ratio and 95% confidence interval were calculated to evaluate the level of risk. The data are presented as the mean ± SD from 3 independent experiments. Means were compared using independent Student’s t tests using SPSS 21.0 software (IBM, Armonk, NY, USA), and \( P < .05 \) was considered to indicate significance.

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**Supplementary Table 1.** The Frequency of Mitochondrial Haplogroup B4b'd'e'j and the Prevalence of Gallstone Disease in Global Populations

| Population             | B4b'd'e'j Haplogroups (%) | Gallstone Disease (%) | References |
|------------------------|---------------------------|-----------------------|------------|
| Mapuche Indians, Chile | 36.84                     | 35.2                  | 1,2        |
| Peru                   | 43.88                     | 14.3                  | 3,4        |
| Argentinian            | 10.35                     | 20.5                  | 5,6        |
| Mexican American       | 13.54                     | 17.8                  | 7,8        |
| Puerto Rican           | 6.54                      | 9.5                   | 9,10       |
| Mexican                | 26.45                     | 14.1                  | 11,12      |
| Taiwan, China          | 3.19                      | 6.26                  | 13-16      |
| Sichuan, China         | 2.93                      | 10.7                  | 16,17      |
| Shanghai, China        | 4.18                      | 5.84                  | 16,18,19   |
| Zhejiang, China        | 2.98                      | 8.8                   | 16,20      |
| Jiangsu, China         | 4.24                      | 4.21                  | 16,21      |
| Xinjiang, China        | 7.79                      | 11.64                 | 16,22      |
| Japan                  | 3.15                      | 3.2                   | 10,23      |
| Korea                  | 3.45                      | 4.85                  | 24,25      |
| Thailand               | 2.11                      | 3.1                   | 26-28      |
| Italy                  | 0                         | 13.9                  | 10,29      |
| British                | 0                         | 23.6                  | 10,30      |
| Ghana                  | 0                         | 5.9                   | 31,32      |
| Tunisia                | 0                         | 4                     | 32,33      |
| Sudan                  | 0                         | 5.2                   | 27,32      |
| India                  | 0                         | 6.12                  | 10,34      |
| Iran                   | 0                         | 4.7                   | 35,36      |
| Germany                | 0                         | 21.2                  | 37,38      |
### Supplementary Table 2. Analysis of Whole Mitochondrial Genome of the 12 Cybrids

| Position | Gene              | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|-------------------|-----------|----------|-----------|----------------|
| 73       | D-loop            | A         | G        | no        | polymorphic site |
| 152      | D-loop            | T         | C        | no        | polymorphic site |
| 193      | D-loop            | A         | G        | no        | polymorphic site |
| 263      | D-loop            | A         | G        | no        | polymorphic site |
| 373      | D-loop            | A         | G        | no        | polymorphic site |
| 709      | 12S rRNA          | G         | A        | no        | polymorphic site |
| 750      | 12S rRNA          | A         | G        | no        | polymorphic site |
| 1438     | 12S rRNA          | A         | G        | no        | polymorphic site |
| 2010     | 16S rRNA          | T         | C        | no        | polymorphic site |
| 2706     | 16S rRNA          | A         | G        | no        | polymorphic site |
| 4769     | 16S rRNA          | A         | G        | no        | polymorphic site |
| 5201     | ND2               | T         | C        | no        | polymorphic site |
| 5465     | ND2               | T         | C        | no        | polymorphic site |
| 7028     | CO1               | C         | T        | no        | polymorphic site |
| 8860     | ATPase6           | A         | G        | Thr > Ala | polymorphic site |
| 9123     | ATPase6           | G         | A        | no        | polymorphic site |
| 10289    | ND3               | A         | G        | no        | polymorphic site |
| 11719    | ND4               | G         | A        | no        | polymorphic site |
| 13269    | ND5               | A         | G        | no        | polymorphic site |
| 14751    | Cytb              | C         | T        | Thr > Ile | polymorphic site |
| 14766    | Cytb              | C         | T        | Thr > Ile | polymorphic site |
| 15326    | Cytb              | A         | G        | Thr > Ala | polymorphic site |
| 16182    | D-loop            | A         | C        | no        | polymorphic site |
| 16183    | D-loop            | A         | C        | no        | polymorphic site |
| 16189    | D-loop            | T         | C        | no        | polymorphic site |
| 16217    | D-loop            | T         | C        | no        | polymorphic site |
| 16261    | D-loop            | C         | T        | no        | polymorphic site |
| 16299    | D-loop            | A         | G        | no        | polymorphic site |
| 16519    | D-loop            | T         | C        | no        | polymorphic site |

#### Analysis of whole mitochondrial genome-1-B4a4

| 73       | D-loop            | A         | G        | no        | polymorphic site |
| 146      | D-loop            | T         | C        | no        | polymorphic site |
| 263      | D-loop            | A         | G        | no        | polymorphic site |
| 709      | 12S rRNA          | G         | A        | no        | polymorphic site |
| 750      | 12S rRNA          | A         | G        | no        | polymorphic site |
| 1438     | 12S rRNA          | A         | G        | no        | polymorphic site |
| 2706     | 16S rRNA          | A         | G        | no        | polymorphic site |
| 4769     | ND2               | A         | G        | no        | polymorphic site |
| 5465     | ND2               | T         | C        | no        | polymorphic site |
| 6080     | CO1               | A         | T        | no        | polymorphic site |
| 7028     | CO1               | C         | T        | no        | polymorphic site |
| 7052     | CO1               | A         | G        | no        | polymorphic site |
| 7271     | CO1               | A         | G        | no        | polymorphic site |
| 8860     | ATPase6           | A         | G        | Thr > Ala | polymorphic site |
| 9123     | ATPase6           | G         | A        | no        | polymorphic site |
| 9822     | CO3               | C         | A        | Leu > Ile | polymorphic site |
| 10238    | ND3               | T         | C        | no        | polymorphic site |
| 11719    | ND4               | G         | A        | no        | polymorphic site |
| 14766    | Cytb              | C         | T        | Thr > Ile | polymorphic site |
| Position | Gene       | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|------------|-----------|----------|-----------|----------------|
| 15326    | Cytb       | A         | G        | Thr > Ala | polymorphic site |
| 15661    | Cytb       | C         | T        | no        | polymorphic site |
| 16167    | D-loop     | C         | T        | no        | polymorphic site |
| 16182    | D-loop     | A         | C        | no        | polymorphic site |
| 16183    | D-loop     | A         | C        | no        | polymorphic site |
| 16189    | D-loop     | T         | C        | no        | polymorphic site |
| 16217    | D-loop     | T         | C        | no        | polymorphic site |
| 16261    | D-loop     | C         | T        | no        | polymorphic site |
| 16317    | D-loop     | A         | T        | no        | polymorphic site |
| 16519    | D-loop     | T         | C        | no        | polymorphic site |

Analysis of whole mitochondrial genome-3-B4a

| Position | Gene       | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|------------|-----------|----------|-----------|----------------|
| 73       | D-loop     | A         | G        | no        | polymorphic site |
| 263      | D-loop     | A         | G        | no        | polymorphic site |
| 310      | D-loop     | T         | C        | no        | polymorphic site |
| 316      | D-loop     | G         | C        | no        | polymorphic site |
| 750      | 12S rRNA   | A         | G        | no        | polymorphic site |
| 1438     | 12S rRNA   | A         | G        | no        | polymorphic site |
| 2706     | 16S rRNA   | A         | G        | no        | polymorphic site |
| 4769     | ND2        | A         | G        | no        | polymorphic site |
| 5465     | ND2        | T         | C        | no        | polymorphic site |
| 6386     | CO1        | C         | T        | no        | polymorphic site |
| 7028     | CO1        | C         | T        | no        | polymorphic site |
| 8860     | ATPase6    | A         | G        | Thr > Ala| polymorphic site |
| 9123     | ATPase6    | G         | A        | no        | polymorphic site |
| 11227    | ND4        | C         | T        | no        | polymorphic site |
| 11719    | ND4        | G         | A        | no        | polymorphic site |
| 13781    | ND5        | T         | C        | Ile > Thr| polymorphic site |
| 14053    | ND5        | A         | G        | Thr > Ala| polymorphic site |
| 14766    | Cytb       | C         | T        | Thr > Ile| polymorphic site |
| 15326    | Cytb       | A         | G        | Thr > Ala| polymorphic site |
| 16182    | D-loop     | A         | C        | no        | polymorphic site |
| 16183    | D-loop     | A         | C        | no        | polymorphic site |
| 16189    | D-loop     | T         | C        | no        | polymorphic site |
| 16217    | D-loop     | T         | C        | no        | polymorphic site |
| 16261    | D-loop     | C         | T        | no        | polymorphic site |
| 16299    | D-loop     | A         | G        | no        | polymorphic site |
| 16355    | D-loop     | C         | T        | no        | polymorphic site |
| 16390    | D-loop     | G         | A        | no        | polymorphic site |
| 16519    | D-loop     | T         | C        | no        | polymorphic site |

Analysis of whole mitochondrial genome-4-B4b1a3

| Position | Gene       | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|------------|-----------|----------|-----------|----------------|
| 73       | D-loop     | A         | G        | no        | polymorphic site |
| 207      | D-loop     | G         | A        | no        | polymorphic site |
| 263      | D-loop     | A         | G        | no        | polymorphic site |
| 408      | D-loop     | T         | A        | no        | polymorphic site |
| 499      | D-loop     | G         | A        | no        | polymorphic site |
| 750      | 12S rRNA   | A         | G        | no        | polymorphic site |
| 827      | 12S rRNA   | A         | G        | no        | polymorphic site |
| 1438     | 12S rRNA   | A         | G        | no        | polymorphic site |
| 2706     | 16S rRNA   | A         | G        | no        | polymorphic site |
| 4769     | ND2        | A         | G        | no        | polymorphic site |
| Position | Gene     | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|----------|-----------|----------|-----------|----------------|
| 4820     | ND2      | G         | A        | no        | polymorphic site |
| 6023     | COI      | G         | A        | no        | polymorphic site |
| 6413     | COI      | T         | C        | no        | polymorphic site |
| 7028     | COI      | C         | T        | no        | polymorphic site |
| 8466     | ATPase8  | A         | G        | no polymorphic site |
| 8860     | ATPase6  | A         | G        | no polymorphic site |
| 9055     | ATPase6  | G         | A        | Ala > Thr | polymorphic site |
| 9338     | COIII    | A         | T        | no        | polymorphic site |
| 9615     | COIII    | T         | C        | no        | polymorphic site |
| 9966     | COIII    | G         | A        | Val > Ile | polymorphic site |
| 11719    | ND4      | G         | A        | no        | polymorphic site |
| 13590    | ND5      | G         | A        | no        | polymorphic site |
| 14766    | Cytb     | C         | T        | Thr > Ile | polymorphic site |
| 15326    | Cytb     | A         | G        | no polymorphic site |
| 15535    | Cytb     | C         | T        | no        | polymorphic site |
| 16136    | D-loop   | T         | C        | no        | polymorphic site |
| 16183    | D-loop   | A         | C        | no        | polymorphic site |
| 16189    | D-loop   | T         | C        | no        | polymorphic site |
| 16519    | D-loop   | T         | C        | no        | polymorphic site |

Analysis of whole mitochondrial genome-5-B4b1c1

| Position | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|-----------|----------|-----------|----------------|
| 7        | D-loop    | A         | G         | no            | polymorphic site |
| 263      | D-loop    | A         | G         | no            | polymorphic site |
| 499      | D-loop    | G         | A         | no            | polymorphic site |
| 750      | 12S rRNA  | A         | G         | no            | polymorphic site |
| 827      | 12S rRNA  | A         | G         | no            | polymorphic site |
| 1438     | 12S rRNA  | A         | G         | no            | polymorphic site |
| 1717     | 16S rRNA  | T         | C         | no            | polymorphic site |
| 2706     | 16S rRNA  | A         | G         | no            | polymorphic site |
| 3918     | ND1       | G         | A         | no            | polymorphic site |
| 4769     | ND2       | A         | G         | no            | polymorphic site |
| 4820     | ND2       | G         | A         | no            | polymorphic site |
| 7028     | COI       | C         | T         | no            | polymorphic site |
| 7521     | tRNA Asp  | G         | A         | no            | polymorphic site |
| 8860     | ATPase6   | A         | G         | Thr > Ala     | polymorphic site |
| 9101     | ATPase6   | T         | G         | Ile > Thr    | polymorphic site |
| 9861     | COIII     | T         | C         | Phe > Leu    | polymorphic site |
| 11239    | ND4       | A         | G         | no            | polymorphic site |
| 11719    | ND4       | G         | A         | no            | polymorphic site |
| 11914    | ND4       | G         | A         | no            | polymorphic site |
| 13590    | ND5       | G         | A         | no            | polymorphic site |
| 14587    | ND6       | A         | G         | no            | polymorphic site |
| 14766    | Cytb      | C         | T         | Thr > Ile    | polymorphic site |
| 15326    | Cytb      | A         | G         | Thr > Ala    | polymorphic site |
| 15535    | Cytb      | C         | T         | no            | polymorphic site |
| 16136    | D-loop    | T         | C         | no            | polymorphic site |
| 16183    | D-loop    | A         | C         | no            | polymorphic site |
| 16189    | D-loop    | T         | C         | no            | polymorphic site |
| 16217    | D-loop    | T         | C         | no            | polymorphic site |
| 16218    | D-loop    | C         | T         | no            | polymorphic site |
| 16519    | D-loop    | T         | C         | no            | polymorphic site |
## Supplementary Table 2. Continued

| Position | Gene               | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|--------------------|-----------|----------|-----------|----------------|
| **Analysis of whole mitochondrial genome-6-B4b1c** |
| 73       | D-loop             | A         | G        | no        | polymorphic site |
| 263      | D-loop             | A         | G        | no        | polymorphic site |
| 309      | D-loop             | C         | T        | no        | polymorphic site |
| 310      | D-loop             | T         | C        | no        | polymorphic site |
| 499      | D-loop             | G         | A        | no        | polymorphic site |
| 750      | 12S rRNA           | A         | G        | no        | polymorphic site |
| 827      | 12S rRNA           | A         | G        | no        | polymorphic site |
| 1438     | 12S rRNA           | A         | G        | no        | polymorphic site |
| 2706     | 16S rRNA           | A         | G        | no        | polymorphic site |
| 4769     | ND2                | A         | G        | no        | polymorphic site |
| 4820     | ND2                | G         | A        | no        | polymorphic site |
| 7028     | COI                | C         | T        | no        | polymorphic site |
| 7080     | COI                | T         | C        | Phe > Leu| polymorphic site |
| 8343     | tRNA Lys           | A         | G        | no        | polymorphic site |
| 8860     | ATPase6            | A         | G        | Thr > Ala| polymorphic site |
| 11719    | ND4                | G         | A        | no        | polymorphic site |
| 13401    | ND5                | T         | C        | no        | polymorphic site |
| 13590    | ND5                | G         | A        | no        | polymorphic site |
| 14587    | ND6                | A         | G        | no        | polymorphic site |
| 14766    | Cytb               | C         | T        | Thr > Ile| polymorphic site |
| 15535    | Cytb               | C         | T        | no        | polymorphic site |
| 16136    | D-loop             | T         | C        | no        | polymorphic site |
| 16182    | D-loop             | A         | C        | no        | polymorphic site |
| 16183    | D-loop             | A         | C        | no        | polymorphic site |
| 16189    | D-loop             | T         | C        | no        | polymorphic site |
| 16217    | D-loop             | T         | C        | no        | polymorphic site |
| 16218    | D-loop             | C         | T        | no        | polymorphic site |
| 16519    | D-loop             | T         | C        | no        | polymorphic site |
| **Analysis of whole mitochondrial genome-7-B4c1b2c** |
| 73       | D-loop             | A         | G        | no        | polymorphic site |
| 150      | D-loop             | C         | A        | no        | polymorphic site |
| 263      | D-loop             | A         | G        | no        | polymorphic site |
| 709      | 12S rRNA           | G         | A        | no        | polymorphic site |
| 750      | 12S rRNA           | A         | G        | no        | polymorphic site |
| 1119     | 12S rRNA           | T         | C        | no        | polymorphic site |
| 1438     | 12S rRNA           | A         | G        | no        | polymorphic site |
| 2706     | 16S rRNA           | A         | G        | no        | polymorphic site |
| 3394     | ND1                | T         | C        | Tyr > His| polymorphic site |
| 3435     | ND1                | C         | T        | no        | polymorphic site |
| 3497     | ND1                | C         | T        | Ala > Val| polymorphic site |
| 3571     | ND1                | C         | T        | Leu > Phe| polymorphic site |
| 4173     | ND1                | A         | G        | no        | polymorphic site |
| 4769     | ND2                | A         | G        | no        | polymorphic site |
| 7028     | COI                | C         | T        | no        | polymorphic site |
| 8860     | ATPase6            | A         | G        | Thr > Ala| polymorphic site |
| 9123     | ATPase6            | G         | A        | no        | polymorphic site |
| 11440    | ND4                | G         | A        | no        | polymorphic site |
| 11719    | ND4                | G         | A        | no        | polymorphic site |
| 14766    | Cytb               | C         | T        | Thr > Ile| polymorphic site |
| Position | Gene     | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|----------|-----------|----------|-----------|----------------|
| 15326    | Cytb     | A         | G        | Thr > Ala | polymorphic site |
| 15346    | Cytb     | G         | A        | no        | polymorphic site |
| 16129    | D-loop   | G         | A        | no        | polymorphic site |
| 16140    | D-loop   | T         | C        | no        | polymorphic site |
| 16166    | D-loop   | A         | G        | no        | polymorphic site |
| 16183    | D-loop   | A         | C        | no        | polymorphic site |
| 16189    | D-loop   | T         | C        | no        | polymorphic site |
| 16217    | D-loop   | T         | C        | no        | polymorphic site |
| 16274    | D-loop   | G         | A        | no        | polymorphic site |
| 16335    | D-loop   | A         | G        | no        | polymorphic site |
| 16519    | D-loop   | T         | C        | no        | polymorphic site |
| 73       | D-loop   | A         | G        | no        | polymorphic site |
| 150      | D-loop   | C         | A        | no        | polymorphic site |
| 195      | D-loop   | T         | C        | no        | polymorphic site |
| 263      | D-loop   | A         | G        | no        | polymorphic site |
| 709      | 12S rRNA | G         | A        | no        | polymorphic site |
| 750      | 12S rRNA | A         | G        | no        | polymorphic site |
| 1119     | 12S rRNA | T         | C        | no        | polymorphic site |
| 1438     | 12S rRNA | A         | G        | no        | polymorphic site |
| 2706     | 16S rRNA | A         | G        | no        | polymorphic site |
| 3434     | ND1      | A         | G        | Tyr > Cys | polymorphic site |
| 3497     | ND1      | C         | T        | Ala > Val | polymorphic site |
| 3571     | ND1      | C         | T        | Leu > Phe | polymorphic site |
| 4769     | ND2      | A         | G        | no        | polymorphic site |
| 7028     | COI      | C         | T        | no        | polymorphic site |
| 7175     | COI      | T         | C        | no        | polymorphic site |
| 7888     | COII     | C         | T        | no        | polymorphic site |
| 8200     | COII     | T         | C        | no        | polymorphic site |
| 8860     | ATPase6  | A         | G        | Thr > Ala | polymorphic site |
| 10325    | COIII    | G         | A        | no        | polymorphic site |
| 11719    | ND4      | G         | A        | no        | polymorphic site |
| 13928    | ND5      | G         | C        | Ser > Thr | polymorphic site |
| 14766    | Cytb     | C         | T        | Thr > Ile | polymorphic site |
| 15326    | Cytb     | A         | G        | Thr > Ala | polymorphic site |
| 15346    | Cytb     | G         | A        | no        | polymorphic site |
| 16140    | D-loop   | T         | C        | no        | polymorphic site |
| 16182    | D-loop   | A         | C        | no        | polymorphic site |
| 16183    | D-loop   | A         | C        | no        | polymorphic site |
| 16189    | D-loop   | T         | C        | no        | polymorphic site |
| 16217    | D-loop   | T         | C        | no        | polymorphic site |
| 16223    | D-loop   | C         | T        | no        | polymorphic site |
| 16274    | D-loop   | G         | A        | no        | polymorphic site |
| 16305    | D-loop   | A         | T        | no        | polymorphic site |
| 16519    | D-loop   | T         | C        | no        | polymorphic site |

Analysis of whole mitochondrial genome-9-B4c1b2c

| Position | Gene     | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|----------|-----------|----------|-----------|----------------|
| 73       | D-loop   | A         | G        | no        | polymorphic site |
| 150      | D-loop   | C         | A        | no        | polymorphic site |
| 263      | D-loop   | A         | G        | no        | polymorphic site |
| 709      | 12S rRNA | G         | A        | no        | polymorphic site |
| 750      | 12S rRNA | A         | G        | no        | polymorphic site |
| Position | Gene       | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|------------|-----------|----------|-----------|----------------|
| 1119     | 12S rRNA   | T         | C        | no        | polymorphic site |
| 1438     | 12S rRNA   | A         | G        | no        | polymorphic site |
| 1534     | 12S rRNA   | C         | T        | no        | polymorphic site |
| 2706     | 16S rRNA   | A         | G        | no        | polymorphic site |
| 3435     | ND1        | C         | T        | no        | polymorphic site |
| 3497     | ND1        | C         | T        | Ala > Val| polymorphic site |
| 3571     | ND1        | C         | T        | Leu > Phe| polymorphic site |
| 4769     | ND2        | A         | G        | no        | polymorphic site |
| 7028     | COI        | C         | T        | no        | polymorphic site |
| 8860     | ATPase6    | A         | G        | Thr > Ala| polymorphic site |
| 9128     | ATPase6    | T         | C        | Ile > Thr| polymorphic site |
| 9575     | COIII      | G         | A        | no        | polymorphic site |
| 10493    | ND4L       | T         | C        | no        | polymorphic site |
| 11440    | ND4        | G         | A        | no        | polymorphic site |
| 11719    | ND4        | G         | A        | no        | polymorphic site |
| 12026    | ND4        | A         | G        | Ile > Val| polymorphic site |
| 14766    | Cytb       | C         | T        | Thr > Ile| polymorphic site |
| 15326    | Cytb       | A         | G        | Thr > Ala| polymorphic site |
| 15346    | Cytb       | G         | A        | no        | polymorphic site |
| 16136    | D-loop     | T         | C        | no        | polymorphic site |
| 16140    | D-loop     | T         | C        | no        | polymorphic site |
| 16183    | D-loop     | A         | C        | no        | polymorphic site |
| 16189    | D-loop     | T         | C        | no        | polymorphic site |
| 16217    | D-loop     | T         | C        | no        | polymorphic site |
| 16249    | D-loop     | T         | C        | no        | polymorphic site |
| 16274    | D-loop     | G         | A        | no        | polymorphic site |
| 16291    | D-loop     | C         | T        | no        | polymorphic site |
| 16335    | D-loop     | A         | G        | no        | polymorphic site |
| 16519    | D-loop     | T         | C        | no        | polymorphic site |

Analysis of whole mitochondrial genome-10-B4d1a

| Position | Gene       | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|------------|-----------|----------|-----------|----------------|
| 73       | D-loop     | A         | G        | no        | polymorphic site |
| 263      | D-loop     | A         | G        | no        | polymorphic site |
| 272      | D-loop     | A         | G        | no        | polymorphic site |
| 750      | 12S rRNA   | A         | G        | no        | polymorphic site |
| 827      | 12S rRNA   | A         | G        | no        | polymorphic site |
| 1438     | 12S rRNA   | A         | G        | no        | polymorphic site |
| 2706     | 16S rRNA   | A         | G        | no        | polymorphic site |
| 4769     | ND2        | A         | G        | no        | polymorphic site |
| 7028     | COI        | C         | T        | no        | polymorphic site |
| 8860     | ATPase6    | A         | G        | Thr > Ala| polymorphic site |
| 11719    | ND4        | G         | A        | no        | polymorphic site |
| 11914    | ND4        | G         | A        | no        | polymorphic site |
| 12612    | ND5        | A         | G        | no        | polymorphic site |
| 12732    | ND5        | T         | C        | no        | polymorphic site |
| 13942    | ND5        | A         | G        | Thr > Ala| polymorphic site |
| 14034    | ND5        | T         | C        | no        | polymorphic site |
| 14766    | Cytb       | C         | T        | Thr > Ile| polymorphic site |
| 15038    | Cytb       | A         | G        | Ile > Val| polymorphic site |
| 15326    | Cytb       | A         | G        | Thr > Ala| polymorphic site |
| 15535    | Cytb       | C         | T        | no        | polymorphic site |
## Supplementary Table 2. Continued

| Position | Gene       | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|------------|-----------|----------|-----------|----------------|
| 15930    | tRNA Thr   | G         | A        | no polymorphic site |
| 16182    | D-loop     | A         | C        | no polymorphic site |
| 16183    | D-loop     | A         | C        | no polymorphic site |
| 16189    | D-loop     | T         | C        | no polymorphic site |
| 16193    | D-loop     | T         | C        | no polymorphic site |
| 16221    | D-loop     | T         | C        | no polymorphic site |
| 16523    | D-loop     | T         | C        | no polymorphic site |

### Analysis of whole mitochondrial genome-11-B4d1a

| Position | Gene       | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|------------|-----------|----------|-----------|----------------|
| 73       | D-loop     | A         | G        | no polymorphic site |
| 146      | D-loop     | T         | C        | no polymorphic site |
| 263      | D-loop     | A         | G        | no polymorphic site |
| 750      | 12S rRNA   | A         | G        | no polymorphic site |
| 827      | 12S rRNA   | A         | G        | no polymorphic site |
| 959      | 12S rRNA   | C         | T        | no polymorphic site |
| 1438     | 12S rRNA   | A         | G        | no polymorphic site |
| 2706     | 16S rRNA   | A         | G        | no polymorphic site |
| 4769     | ND2        | A         | G        | no polymorphic site |
| 7028     | COI        | C         | T        | no polymorphic site |
| 8860     | ATPase6    | A         | G        | Thr > Ala polymorphic site |
| 11719    | ND4        | G         | A        | no polymorphic site |
| 11914    | ND4        | G         | A        | no polymorphic site |
| 12732    | ND5        | T         | C        | no polymorphic site |
| 13942    | ND5        | A         | G        | Thr > Ala polymorphic site |
| 14766    | Cytb       | C         | T        | Thr > Ile polymorphic site |
| 15038    | Cytb       | A         | G        | Ile > Val polymorphic site |
| 15326    | Cytb       | A         | G        | Thr > Ala polymorphic site |
| 15535    | Cytb       | C         | T        | no polymorphic site |
| 16182    | D-loop     | A         | C        | no polymorphic site |
| 16183    | D-loop     | A         | C        | no polymorphic site |
| 16189    | D-loop     | T         | C        | no polymorphic site |
| 16193    | D-loop     | T         | C        | no polymorphic site |
| 16519    | D-loop     | T         | C        | no polymorphic site |
| 16221    | D-loop     | T         | C        | no polymorphic site |
| 16523    | D-loop     | T         | C        | no polymorphic site |

### Analysis of whole mitochondrial genome-12-B4d1a

| Position | Gene       | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|------------|-----------|----------|-----------|----------------|
| 73       | D-loop     | A         | G        | no polymorphic site |
| 146      | D-loop     | T         | C        | no polymorphic site |
| 263      | D-loop     | A         | G        | no polymorphic site |
| 750      | 12S rRNA   | A         | G        | no polymorphic site |
| 827      | 12S rRNA   | A         | G        | no polymorphic site |
| 959      | 12S rRNA   | C         | T        | no polymorphic site |
| 1438     | 12S rRNA   | A         | G        | no polymorphic site |
| 2706     | 16S rRNA   | A         | G        | no polymorphic site |
| 4769     | ND2        | A         | G        | no polymorphic site |
| 7028     | COI        | C         | T        | no polymorphic site |
| 8860     | ATPase6    | A         | G        | Thr > Ala polymorphic site |
| 11719    | ND4        | G         | A        | no polymorphic site |
| 11914    | ND4        | G         | A        | no polymorphic site |
| 12732    | ND5        | T         | C        | no polymorphic site |
| 13942    | ND5        | A         | G        | Thr > Ala polymorphic site |
| Position | Gene | rCRS Base | Mutation | AA Change | mtDNA Database |
|----------|------|-----------|----------|-----------|----------------|
| 14766    | Cytb | C         | T        | Thr > Ile | polymorphic site |
| 15038    | Cytb | A         | G        | Ile > Val | polymorphic site |
| 15326    | Cytb | A         | G        | Thr > Ala | polymorphic site |
| 15535    | Cytb | C         | T        | no        | polymorphic site |
| 16182    | D-loop | A   | C        | no        | polymorphic site |
| 16183    | D-loop | A   | C        | no        | polymorphic site |
| 16189    | D-loop | T   | C        | no        | polymorphic site |
| 16519    | D-loop | T   | C        | no        | polymorphic site |

AA, amino acid; rCRS: revised Cambridge Reference Sequence; mtDNA, mitochondrial DNA; rRNA, ribosomal RNA.

*MITOMAP, mtDB, mtSNP, and PhyloTree mt.
Supplementary Table 3. Primers for the Determination of mtDNA Content, mtRNA, Fatty Acid Oxidative, Mitochondrial Translation, Mitophagy, Tricarboxylic Acid, and Cholesterol Synthesis

| Primer Name                      | Sequence (5'-3') |
|----------------------------------|------------------|
| Primers for mtDNA copy number determination |                   |
| mt-F3212 / mt-R3319            | CACCCAAGAACAGGGTTTGT |
| 18SF / 18SR                     | TGGCCATGGGTATGTTTAA |
|                                 | TAGAGGGACAGTGCGGTTTC |
|                                 | CGCTGAGCCAGTCAGTTG  |
| Primers for mtRNA determination  |                   |
| mt-ND1F3310-nt-ND1R3440        | CCCATGGCCAACTCTCTACTCTC |
|                                 | AGCCGTCAGGGCCCTACAAGG |
| mt-ND2F4614-nt-ND2R4677        | AACCTCTGTCACACAGAGCAG |
|                                 | GGTATATGGATCGGGTTGCT |
| mt-ND3F10168-nt-ND3R10376      | ACGAGTGGCGCTTCCACCTC |
|                                 | TCACCTCATAGGCCAGACTCTAGG |
| mt-ND4 (L) F10621-nt-ND4 (L) R10694 | CCCACTTCCTTCTAGGCAATTT |
|                                 | TAGGCCCCACGCTGCTTTG |
| mt-ND5F13350-nt-ND5R13426      | TGCTCCGGGCTCATTATC |
|                                 | TGAAGTGGCTCTATTTCCTTGCATATCT |
| mt-ND6F14243-nt-ND6R14439      | GCCCCCGACCACTAGATCTCCTC |
|                                 | CCTGAGCAGGTTGGGCTAGG |
| mt-CO1F6168-nt-CO1R6587        | GCCCGCGAGATCGGCTTTCG |
|                                 | GCCGCTCCCTTCTCCGGGGTCG |
| mt-CO2F7972-nt-CO2R8157        | ACCAGGGACACCTCGACTCCTC |
|                                 | ACCCCCGGTCGTTGAGCCGTT |
| mt-CO3F9555-nt-CO3R9935        | CCCCAACAGGGCTACACCCCGC |
|                                 | ATGCCAGTATCAGGGGCCGCG |
| mt-CYBF15263-nt-CYBR15326      | CCCACCCCTCACAATGCGTTTAATAGA |
|                                 | TTATGAGCAGGGCAACTGATT |
| mt-ATP6F8848-nt-ATP8R8910      | GCCAGGCTGACCTCTTCG |
|                                 | GAGTGGGCTAGGCAAAGTATTTT |
| mt-ATP8F8939-nt-ATP8R8472      | CCCACCCATAATTACCCCCACT |
|                                 | GTGTTAGGTTGTTAGTTTTGTTATATTTATAG |
| 18SF/18SR                      |                   |
| Primers for the determination of fatty acid oxidative genes expression |                   |
| CPT1AF / CPT1AR                 | ATGCGGTACCTCCCTGAAGATG |
|                                 | GTGGCGACAGCTACCTCTG |
| CPT1BF / CPT1BR                 | CCTGCTACTAGGCAACTGCTA |
|                                 | AGAAGTGGCCCAATGAGGGA |
| VLCADF / VLCADR                 | TAGGAAGGCAGGCAACAGCCT |
|                                 | CACAGTGGCAAACTCCTCAGA |
| MCADF / MCADR                   | AGAACCTGGAGACGGCTCAGAT |
|                                 | GGATCTGAGTACGAAGCTG |
| SCADF / SCADR                   | CGGCCAGTTTACACACCTACTC |
|                                 | GCAATGAGAAAACACTCCTTTC |
| ACOX1F / ACOX1R                 | GCCGCATCAGTGAAGGAACTC |
|                                 | AGGTGAAGGCGCTTCAGTCAG |
| ACLYF / ACLYR                   | GCCCTGCTCTATGAGCAGAACAT |
|                                 | GTCCAGATGTTGCTACTCCTT |
| PPARaF / PPARaR                 | TCGCGAGGATGATTCTGTGAG |
|                                 | GACCAACAGTAAATGCAAGG |
| PGC1aF / PGC1aR                 | CCAAGGAGTGGCGCTCTGTTCA |
|                                 | CGGTGTCTGATGTTGCTGAGT |
| 18SF / 18SR                     |                   |
| Primers for the determination of mitochondrial translation genes expression |                   |
| 12S rRNAF / 12S rRNAR           |                   |
|                                 | AATAGGTTCCTCAGCCCT |
|                                 | TGAGGTTTCCCCGTTGAGT |
| Primer Name          | Sequence (5’-3’)                                      |
|---------------------|------------------------------------------------------|
| MRPS11F / MRPS11R   | CCTTTGCTCTTGTCAGACAGA                                |
|                     | GCCCTTACCAACATCGCGATG                                 |
| MRPS16F / MRPS16R   | GGTGCCCTAACCTAGACAGA                                  |
|                     | CCGTTTCTGTCAGTCTTCA                                   |
| MRPS6F / MRPS6R     | GCGCTCTTTATAGAGATCTGTGAG                             |
|                     | GGACAGGTCTCCACAGATGCT                                 |
| MRPS22F / MRPS22R   | CTACGCAAAGCTCTGTGAGAG                                 |
|                     | CAAACATGCTGTCCTGCTATAAC                              |
| MRPS27F / MRPS27R   | CGAGGAAACAGACAGTCAGAAG                                |
|                     | ATGTCTCTGTCCTACAGAGTG                                 |
| 18SF / 18SR         | TAGAGGAGACGATGGGCTTC                                  |
|                     | CGCTGAGCCAGATGCT                                     |
| Primers for the determination of mitophagy genes expression |                                                     |
| LC3F / LC3R         | GCTACAAGGAGGTGAGACGCT                                |
|                     | CTGGTTACACGAGAGAAGAG                                  |
| P62F / P62R         | TGGTAGCGTCCTGCGAGGAG                                  |
|                     | AGTGTCTCTGTTTTCACCTCCG                                |
| 18SF / 18SR         | TAGAGGAGACGATGGGCTTC                                  |
|                     | CGCTGAGCCAGATGCT                                     |
| Primers for the determination of glycolysis genes expression |                                                     |
| SLC2A1F / SLC2A1R   | TGCGAGCCTCTCCAACATGGAC                                |
|                     | CAGAACCAGACAGACATGGAC                                 |
| HK2F / HK2R         | GATGGTGACTTGTGATGGTGTTGC                             |
|                     | CCTCCATGAGCAGCCATGCT                                 |
| GPIF / GPIR         | CTGGTAGACGGCAGAGATGTA                                 |
|                     | TCCGTGATGCTTGCCTGCTGT                                |
| PFKMF / PFKMR       | GCTTTACTAGCTAGTCAGACCC                                |
|                     | CCAAATCTACAGATGCAGAAA                                |
| PFKLF / PFKLR       | AAGAAGTAACTGTCAGACG                                  |
|                     | CGGAGGATTTCTCCACAAATGGAC                              |
| PFKPF / PFKPR       | AGGAGGATCTGCTCCAGTGTA                                |
|                     | ATCGCTCTGTCACATCCTGAG                                 |
| PKMF / PKMR         | ATGGCTGACACATTCTGCGGAC                                |
|                     | CTTCAACGTCTCCACTGATCG                                 |
| LDHAF / LDHAR       | GGAATCTCAACATGGACAGC                                  |
|                     | AGACGCTTTGCTCCCTGCTGT                                |
| LDHBF / LDHBR       | GGAACAAATTTGTAGTGCTGT                                 |
|                     | AAGGCTCCATGCTGCAAGATCCA                              |
| Primers for the determination of TCA genes expression |                                                     |
| PDHA1F / PDHA1R     | GAGATGTTGACAGGACAAATCGTTCG                          |
|                     | TCATGTTGAGTATGAGTATGTTG                               |
| ACO1F / ACO1R       | TCCCTAGTTGATGTGGTACAG                                 |
|                     | TGCGTCAAGCAGACAGGAC                                   |
| ACO2F / ACO2R       | CAATCGTCACCTCAGAAGC                                  |
|                     | GTCTGTGGTGATGAGG                                     |
| IDH1F / IDH1R       | CTATGATGAGTCAGTCCAGTG                                |
|                     | CTTGCTCTTCTACTGTCCTGGC                               |
| IDH2F / IDH2R       | AGATGAGCAGTGACTGTCAGG                                |
|                     | CTGGATGAGGATGACTGGAAG                                 |
| IDH3AF / IDH3AR     | TCATGTTGACACCAAGTGGCAA                                |
|                     | TGCGTCAAGCCTCAGGCAA                                  |
| IDH3BF / IDH3BR     | TGCACTGAGSAGAGAAGTGGCA                               |
|                     | TGAGGCGATTCATAGGGAGG                                 |
| IDH3GF / IDH3GR     | CGAATGTGGTTAAGAGTGGCA                                |
|                     | TGGTGGAGCGGAGGAGGGAAG                                |
| OGDHF / OGDHR       | GAGGCTCTGACTGACTGACTGCA                              |
|                     | TACATGAGGCGCGTGAGGAC                                 |
### Supplementary Table 3. Continued

| Primer Name          | Sequence (5'-3')                                      |
|----------------------|-------------------------------------------------------|
| SUCLA2F / SUCLA2R    | GCAAGAAGCTGGTGCTCCGGTT                                  |
|                      | CCACCAGCTAAACCTGTGCCCT                                  |
| SDHAF / SDHAR        | GAGATGTGTTGTCATTCATCCAT                                  |
|                      | GCTGTCTGAAATGCGCCAGCA                                  |
| FHF / FHR            | CCGCTGAAGTTAAACAGGATGTATG                                 |
|                      | ATCCAGTCTGCCCATTACAGGAG                                  |
| MDH1F / MDH1R        | GGTGTCTCTTGAAGGACTGCAAG                                  |
|                      | CATCCAGGTTTTTGAAGGACACG                                  |
| MDH2F / MDH2R        | CTGGACATCGTCAAGACACCAA                                  |
|                      | GGATGATGTTTTCCACTACAG                                  |
| CSF / CSR            | CACAGGGTATCAGCGCAACCAA                                  |
|                      | CCAATACCAGCTGCCCTTCTG                                  |

Primers for the determination of cholesterol synthesis genes expression

| Primer Name          | Sequence (5'-3')                                      |
|----------------------|-------------------------------------------------------|
| CYP51AF / CYP51AR    | CTCTTACCATGGTGCTGCTTT                                  |
|                      | CTTGAGACTGTCTGCCCCTTG                                  |
| FDFT1F / FDFT1R      | TGTGACCTCTGAACAGGAGTAGG                                  |
|                      | GCCCATAGAGTTGGCAGGTCT                                 |
| DHCR24F / DHCR24R    | CAGGAGAACACTTGGCTGGAAG                                  |
|                      | CCACATGCTTTAAAGGACACG                                  |
| DHCR7F / DHCR7R      | TCCACAGCGCATGGACCAATGC                                  |
|                      | CGAAAGTGCTGAGTCATGACATG                                 |
| HSD17B7F / HSD17B7R  | GCTGTAGGACTTTCAAGAGGAGTG                                 |
|                      | GCACTGCGAGATGCTCCAGA                                  |
| NSDHLF / NSDHLR      | CAGTTTTCTCAGTGCTGACACC                                  |
|                      | ACGCCCTCAAGAATGACACTG                                  |
| HMGCRF / HMGCRR      | GACGTGAACTATGCTGAGTAGG                                  |
|                      | GGTATCTGTGTTCCAGACACTAAGG                               |
| HMGCS1F / HMGCS1R    | AAGTCACACAAAGATGCTACACCG                               |
|                      | TCAGCGAAGACATCTGCGTCAACA                                |

mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA.
| Antibodies          | Source          |
|---------------------|-----------------|
| Anti-ND1            | Proteintech     |
| Anti-ND2            | Proteintech     |
| Anti-ND3            | Abcam           |
| Anti-ND5            | Proteintech     |
| Anti-CYTB           | Proteintech     |
| Anti-CO1            | Abcam           |
| Anti-CO2            | Proteintech     |
| Anti-ATP6           | Proteintech     |
| Anti-ATP8           | Proteintech     |
| Anti-TOMM20         | Proteintech     |
| Anti-GRIM19         | Abcam           |
| Anti-SDHA           | Abcam           |
| Anti-CORE2          | Abcam           |
| Anti-ATP5           | Abcam           |
| Anti-GAPDH          | Proteintech     |
| Anti-PINK1          | Proteintech     |
| Anti-CLPP           | Proteintech     |
| Anti-LONP1          | Proteintech     |
| Anti-AFG3L2         | Proteintech     |
| Anti-HSP60          | Proteintech     |
| Anti-GRP75          | Proteintech     |
| Anti-ACTIN          | Proteintech     |
| Anti-RXRA           | Abcam           |
| Anti-NRF1           | Abcam           |
| Anti-JNK1/2         | Cell Signaling Technology |
| Anti-P-JNK1/2       | Cell Signaling Technology |
| Anti-p38            | Cell Signaling Technology |
| Anti-phospho-p38 (Thr389) | Cell Signaling Technology |
| Anti-ERK1/2         | Cell Signaling Technology |
| Anti-phospho-ERK (Thr202/Tyr204) | Cell Signaling Technology |
| Anti-AKT1           | Cell Signaling Technology |
| Anti-P-AKT (Thr473) | Cell Signaling Technology |
| Anti-AMPKα          | Cell Signaling Technology |
| Anti-P-AMPKα         | Cell Signaling Technology |
| Anti-CPT1A          | Abcam           |
| Anti-ACOX1          | Abcam           |
| Anti-PPARα          | Abcam           |
| Anti-ABCA1          | Proteintech     |
| Anti-ABCG5          | Proteintech     |
| Anti-ABCG8          | Proteintech     |
| Anti-CYP7A1         | Proteintech     |
| Anti-HIF1α          | Proteintech     |
| Anti-HIF2α          | Proteintech     |
| Anti-HMGCR          | Proteintech     |
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