Reduced Mitochondrial DNA Content and Heterozygous Nuclear Gene Mutations in Patients With Acute Liver Failure

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ABSTRACT

Objectives: Historically, mitochondrial disorders have been associated with predominantly multisystem or neurological symptoms. If present, hepatic complications were thought to be a late feature. Recently, mutations in at least 4 nuclear genes have been identified in infants presenting with rapidly progressive hepatic failure, which may be precipitated by infection or drugs. We aimed to determine whether hepatic mitochondrial DNA (mtDNA) depletion is associated with apparently isolated hepatic failure in individuals with acute liver failure (ALF) of known or unknown etiologies undergoing liver transplant (LT). In addition, we wished to establish whether there was an excess of mutations in gene known to cause hepatic mtDNA depletion.

Methods: Using previously established methods, we demonstrated that end-stage liver disease from known causes did not lead to hepatic mtDNA depletion.

Results: Using thresholds derived from receiver-operator curve analysis, 66% of cases with ALF had probable or definite mtDNA depletion, including 34% with definite mtDNA depletion. There was a small but significant increase in the proportion of patients undergoing LT for ALF with heterozygous mutations known to lead to mtDNA depletion and hepatic failure compared with controls ($P = 0.001$).

Conclusions: Liver disease severe enough to require LT does not cause secondary mtDNA depletion; however, the majority of patients undergoing LT for ALF had reduced mtDNA content, which fell within the range seen in patients with classic mtDNA depletion. A subset of patients with ALF has mutations in genes known to lead to mtDNA depletion and hepatic failure. Together, these results suggest defective mtDNA maintenance is associated with ALF.

Key Words: *DGUOK*, mitochondrial DNA depletion, next-generation sequencing, *POLG*, valproate

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Until recently, hepatic complications were thought to be a late feature of mitochondrial disorders, which are generally recognized as multisystem diseases presenting predominantly with neurological and muscular dysfunction (1); however, patients with mitochondrial disorders can present with isolated hepatic failure; indeed, patients may even present with apparent viral hepatitis (2–5).

Mitochondrial disorders may be caused by defects in nuclear or mitochondrial genes. A subset of these defects leads to mitochondrial DNA (mtDNA) depletion (a reduction in cellular mtDNA content). These are usually caused by molecular defects in the nuclear genes responsible for mtDNA biogenesis, and the maintenance of mtDNA integrity and deoxynucleotide pools (6). Mutations in 9 nuclear genes are known to cause mtDNA depletion (2,3,6–19).

Mutations in 4 of these nuclear genes have been identified in infants presenting with rapidly progressive hepatic failure: deoxyguanosine kinase (*DGUOK*), a gene coding for mitochondrial inner membrane protein (*MPV17)*, DNA polymerase γ (*POLG*), and *TWINKLE*, a DNA helicase (*C10orf2)* (2–5,17,20–24). Patients with these mutations have presented with hepatic failure with (11,12,17,20–22,24–26) or without (2–5,24) a neurological component. Anecdotal evidence from published cases has suggested a link between primary mtDNA depletion and both drug- and viral-induced acute hepatic failure (3,5,27). In addition, 3 other genes have been shown to result in a reduced mtDNA content in the liver: ribonucleotide reductase M2 B *(RRM2B),* succinate-coenzyme A ligase ADP-forming β subunit (*SUCLA2*), and thymidine phosphorylase (*TP*) without clear evidence of liver dysfunction in reported cases (28).

Presently, it is unclear how frequently mtDNA depletion is associated with isolated hepatic failure. The determination of mtDNA depletion requires the primarily affected organ to be assayed (3,28); therefore, in the case of hepatic disorders, liver tissue is required. We have previously validated an assay using real-time quantitative polymerase chain reaction to evaluate mtDNA content relative to haploid nuclear DNA in muscle and liver specimens, thus establishing normal ranges for liver tissue (28). Because previous data have demonstrated mitochondrial dysfunction results from bile acid accumulation (29), it was first necessary to determine whether hepatic disease, particularly of a cholestatic nature from known etiologies, severe enough to require liver transplant (LT), affects mtDNA content. If such liver dysfunction does not lead to secondary mtDNA

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depletion, then hepatic mtDNA content could be used as a tool to evaluate the association between clinically isolated hepatic failure in individuals with acute liver failure (ALF) and disordered mtDNA maintenance.

A recent systematic study demonstrated an association between heterozygous mutations in *POLG* and drug-induced hepatic injury following exposure to sodium valproate (30). We therefore also set out to establish the prevalence of mutations in nuclear encoded genes associated with hepatic mtDNA depletion in patients with ALF.

METHODS

Liver Specimens/DNA Samples

DNA from unrelated healthy subjects was obtained from excess tissue from 65 LT donors, provided by the National Institutes of Health–sponsored Liver Tissue Cell Distribution System for Minneapolis, MN and Pittsburgh, PA (healthy controls) (Table 1). Although delay in freezing, tissue mishandling, or length of time in storage has been demonstrated to not affect copy number (28), all liver tissue used from the Liver Tissue Cell Distribution System was flash frozen within 30 minutes of harvesting. An additional 205 DNA samples from unrelated healthy subjects were obtained through a separate institutional review board–approved protocol, and were used to evaluate the population frequency of detected mutations.

DNA from liver specimens from 18 patients with molecularly proven mtDNA depletion syndromes (mtDNA depletion homozygous or compound heterozygous for deleterious mutations in *DGUOK, MPV17, POLG,* or *TWINKLE*) was selected from those submitted to the Mitochondrial Diagnostic Laboratory at Baylor College of Medicine or the Genetics Center, Children's Hospital of Wisconsin (mtDNA depletion controls).

Individuals with end-stage liver disease requiring LT, as a result of clearly defined, nonmitochondrial etiology according to the Liver Tissue Cell Distribution System registry, were identified to evaluate whether end-stage liver disease leads to secondary hepatic mtDNA depletion. These LT cases (LT controls; n = 28) comprised 12 patients with extrahepatic biliary atresia, 6 with progressive familial intrahepatic cholestasis type 1 (PFIC-1), 5 with Wilson disease, 3 with tyrosinemia type 1, and 2 with Alagille syndrome.

TABLE 1. Source of liver specimens/DNA samples

	n
Previously healthy LT donors (healthy controls)	65
Individuals with known mtDNA depletion (mtDNA depletion controls)	18
Patients with liver disease of known etiology requiring LT (LT controls)	28
Extrahepatic biliary atresia	12
PFIC-1	6
Wilson disease	5
Tyrosinemia type 1	3
Alagille syndrome	2
Healthy individuals* (population controls)	205
Patients with FHF undergoing LT (FHF test group)	45
Viral-induced FHF	10
Drug-induced FHF	17†
FHF resulting from unknown cause	18

FHF = fulminant hepatic failure; LT = liver transplant; PFIC-1 = progressive familial intrahepatic cholestasis type 1.

* DNA was obtained from liver samples except in this group of individuals in whom DNA was obtained from blood samples.

† One sample was too degraded to extract DNA, and in a further sample the DNA was of insufficient quality to perform sequencing.

DNA samples were also obtained from the liver tissue of 45 LT patients following ALF because of "unknown," "drug-induced," or "viral" etiology (ALF test), via the Liver Tissue Cell Distribution System.

Where appropriate, studies were performed with the consent of the patient or his or her legal guardian. All studies were performed according to protocols approved by the institutional review board of Children's Hospital of Wisconsin and in accordance with the Declaration of Helsinki.

Hepatic mtDNA Content Analysis

DNA was extracted from control and ALF test specimens and mtDNA content was determined as described previously (28,31). To allow for appropriate statistical comparison, the normally distributed cycle threshold difference (ΔCt) was used.

mtDNA content in healthy controls (n = 65) and LT controls (n = 28) was compared with the mtDNA depletion controls to determine whether they could be distinguished from one another based on mtDNA content alone. Thresholds for defining probable and definite depletion were determined using a receiver operator curve analysis (supplemental figure, *http://links.lww.com/MPG/A236* (Receiver-operator curve comparing cycle threshold in patients with proven mitochondrial [mtDNA] depletion [mtDNA depletion controls] vs healthy controls and patients with hepatic failure [LT controls]. Based on these data the following thresholds were defined: probable mtDNA depletion [100% sensitivity, 96% specificity at 8.19 cycle difference] and definite mtDNA depletion [100% specificity, 84% sensitivity at 7.47 cycle difference]). These thresholds were then applied to the ALF test group.

Molecular Genetic Analysis

ALF test samples underwent the following molecular genetic analyses to evaluate whether they had pathologic variants in 7 genes that have been associated with reduced hepatic mtDNA content (28): bidirectional dideoxy (Sanger) sequencing of coding exons for *POLG, MPV17, RRM2B,* succinate-coenzyme A ligase ADP-forming β subunit (*SUCLA2*), *DGUOK,* thymidine phosphorylase (*TP*), and *TWINKLE* (28); in addition, they were subject to long-range PCR with 454 sequencing using the titanium upgrade (Roche, South San Francisco, CA), as described previously (4). In addition, 270 controls (65 healthy controls and 205 population controls) were also subject to Sanger dideoxy sequence-based evaluation of all exons in which a variant was found in the ALF test samples. The 65 healthy control samples were also subject to screening by long-range PCR and subsequent 454 sequencing. A pathogenic mutation was defined according to the American College of Medical Genetics' criteria. For *POLG*, it was defined as pathogenic if it was annotated as such in the POLG database (*tools.niehs.nih.gov/polg*) for the other genes, we defined a mutation as a variant published as pathogenic in the Human Gene Mutation Database (Biobase) with a population frequency in dbSNP (*www.ncbi.nlm.nih.gov/SNP*) and the exome variant server (*evs.gs.washington.edu*) of <5% that after review of the primary literature, the authors agreed would meet criteria for clinical reporting.

RESULTS

Hepatic mtDNA Content

Data from 63 of 65 healthy controls and 15 of 18 mtDNA depletion controls have been published (28). These control samples showed a stable mtDNA content across age, sex, and stated ethnicity (28).

The mtDNA content measured by cycle threshold difference (ΔCt) in samples from patients undergoing LT because of end-stage liver disease of nonmitochondrial etiology (LT controls) was not statistically different from the normal range (ie, the range defined by the healthy control group; Fig. 1); however, the mtDNA content for several samples from patients with tyrosinemia type 1, PFIC-1, Wilson disease, or Alagille syndrome was lower than that in the healthy control sample with the lowest mtDNA content encroaching the range seen in patients with mutations in *DGUOK*. As expected, the mtDNA content for liver samples from patients with confirmed mtDNA depletion syndromes designated as the mtDNA depletion group had marked mtDNA depletion, with an mtDNA content <25% of the mean for healthy controls.

Using a receiver-operated curve analysis, we compared mtDNA content in the mtDNA depletion controls with healthy controls and LT controls; the area under the curve was 0.9932. Using these data, we were able to establish the threshold for probable mtDNA depletion (100% sensitivity, 96% specificity) at 8.19 cycles different, and definite mtDNA depletion (100% specificity, 84% sensitivity) at 7.47 cycles different (supplemental figure, *http://links.lww.com/MPG/A236*). Individual samples were reviewed in light of the thresholds, demonstrating that 2 patients with PFIC-1, 2 with Alagille syndrome, and 1 with tyrosinemia type 1 were classified as having probable mtDNA depletion.

Hepatic mtDNA Depletion in Patients With ALF

DNA was obtained from 44 of the 45 samples in the ALF test group; 1 sample from a patient with drug-induced ALF was too degraded to extract DNA. Hepatic mtDNA content was significantly lower in patients undergoing LT for ALF (ALF test) compared with healthy controls plus LT controls (mean 7.86, $P = 3.94 \times 10^{-25}$). Furthermore, mtDNA content remained significantly lower in the ALF test group than controls when the ALF test group was split into subgroups defined by the cause of ALF: ''unknown'' mean 7.51, *t* test $P = 4.79 \times 10^{-24}$; ''drug induced'' mean 8.61, *t* test $P = 4.79 \times 10^{-7}$, ''viral'' mean 7.32, *t* test $P = 1.58 \times 10^{-15}$.

Applying the probable and definite thresholds for mtDNA depletion derived from the receiver-operator curve analysis to the ALF test group, 29 of 44 (66%) patients had probable or definite mtDNA depletion, with 15 of 44 (34%) of these falling into the definite mtDNA depletion category (Fig. 2, Table 2). Interestingly, a lower proportion of patients with drug-induced liver failure exhibited mtDNA depletion than patients with ALF because of unknown or viral etiologies. Regardless of the age cutoff used, there was no difference in the proportion of samples from pediatric patients and adult patients with mtDNA depletion.

Genetic Analysis in Patients With ALF

Genetic analysis was possible for 43 of 45 samples in the ALF test group; 1 sample was too degraded to extract DNA and DNA was of insufficient quality to analyze in another sample. We detected 7 (16%) pathogenic mutations in hepatic mtDNA depletion genes in the 43 ALF test samples and 7 (3%) in the 270 controls (healthy controls + population controls; Fisher exact test $P = 0.001$), when using a conservative definition of pathogenic mutations (ie, only those mutations presently predicted to be pathogenic).

When considering *DGUOK*, a higher mutation rate was found in the ALF test samples than controls (Fisher exact test $P = 0.014$). In the ALF test group, 3 (7%) samples had the p.Q170R mutation (20) and 1 (2%) had the p.E165 V mutation (3), whereas 4 (1%) of the controls had mutations, all p.Q170R.

A higher rate of *POLG* mutations was also found in the ALF test samples than in the controls (Fisher exact test $P = 0.036$). In the ALF test group, 3 (7%) samples had mutations, 1 had the p.E1143G variant, 1 had the p.A467T variant, and 1 had both the p.T251I and p.P587L variants (which are frequently reported on the same chromosome and are considered 1 mutation (26)), and in the control group, 3 (1%) had mutations, all having both the p.T251I and p.P587L variants.

No pathogenic mutations were detected in *TP*, *RRM2B*, *MPV17*, or *SUCLA2* in either group, and no mutations in *TWINKLE* that are presently considered pathogenic were detected.

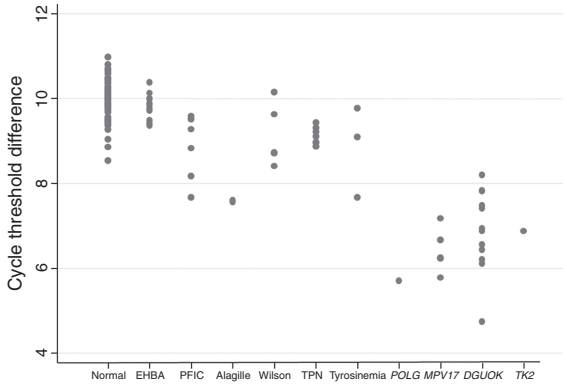

FIGURE 1. Mitochondrial DNA content in hepatic failure. mtDNA depletion was not seen in patients with hepatic failure of nonmitochondrial etiology, although a small reduction in mtDNA was seen in patients with progressive familial intrahepatic cholestasis type 1 (PFIC-1), Alagille syndrome (Alagille), Wilson disease (Wilson), and tyrosinemia type 1 (Tyrosinemia). Conversely, liver tissue from patients with 2 mutations in *POLG*, *MPV17*, *DGUOK*, and *TK2* show marked mtDNA depletion, with mtDNA content <25% of the mean value for normal controls. *DGUOK*= deoxyguanosine kinase; EMBA = extrahepatic biliary atresia; TPN = total parenteral nutrition–induced cholestasis.

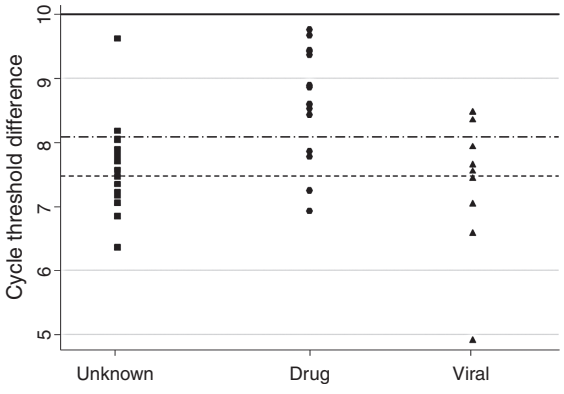

FIGURE 2. Mitochondrial DNA content in test patients with acute liver failure caused by unknown, drug-induced, or viral etiology. Thresholds for probable mtDNA depletion (100% sensitivity, 96% specificity at 8.19 cycle difference: upper dashed line) and definite mtDNA depletion (100% specificity, 84% sensitivity at 7.47 cycle difference: lower dashed line) were defined using receiver-operator curve analysis. Mean cycle difference for normal liver tissue was 10 (based on healthy control data, solid line), and 8.6 is 2 standard deviations below the mean.

TABLE 2. Demographics and mitochondrial copy number in liver obtained from individuals with acute liver failure are detailed with mutations detected in *DGUOK* and *POLG*

Category	Agent	Race	Age, y	Sex	dCT	*POLG*	*DGUOK*
Unknown		C	12	M	6.35	c.3199T>A (p.S1067T)	c.494A>T (p.E165V)
Unknown		C	3.5	M	6.84		
Unknown		H	5	M	7.05		
Unknown		C	7	M	7.16		
Unknown		U	5		7.20		
Unknown		U	13	F	7.21		
Unknown		U	2.2		7.34		
Unknown		A	38	M	7.46		
Unknown		U	16	F	7.56	c.3428A>AG (p.E1143G)	
Unknown		C	7	M	7.70	c.1760C>T (p.P587PL) c.752C>T (p.T251I)	
Unknown		A	42	F	7.74		
Unknown		U	2.2	M	7.74		c.509A>G (p.Q170R)
Unknown		C	42	F	7.82		
Unknown		U	55	F	7.85		
Unknown		C	24	F	7.88		
Unknown		H	17	M	8.03		
Unknown		C	62	M	8.04		
Unknown		N	5.5	M	8.17		
Unknown		U	60		9.61		c.509A>G (p.Q170R)
Drug	Acetaminophen	C	5.6	F	*		
Drug	Isoniazid	U	9.5	M	6.92		
Drug	Valproic acid	C	2.9	F	7.24	c.1399G>A (p.A467T)	
Drug	Herbal infusion	A	38	F	7.77		
Drug	Acetaminophen	C	48	F	7.85		
Drug	Acetaminophen	C	34	M	8.42		
Drug	Acetaminophen	U	35	F	8.52		
Drug	Carbon terachloride	C	31	F	8.58		
Drug	Acetaminophen	C	32	F	8.59		
Drug	Isoniazid	C	53	M	8.85		c.509A>G (p.Q170R)
Drug	Acetaminophen	N	39	F	8.88		
Drug	Valproic acid	C	16	F	9.36		
Drug	Acetaminophen	U	15	M	9.41		
Drug	Acetaminophen	C	29	F	9.43		
Drug	Acetaminophen	C	60	F	9.66	*	
Drug	Acetaminophen	C	41	F	9.75		
Viral	ND	B	26	F	4.91		
Viral	Hepatitis A	N	14	M	6.58		
Viral	ND	C	39	M	7.04		
Viral	Hepatitis A	U	26	F	7.44		
Viral	ND	C	29	F	7.55		
Viral	Hepatitis A	C	1.8	M	7.65		
Viral	Influenza	C	1.9	M	7.93		
Viral	Hepatitis A	C	24	F	8.35		
Viral	ND	A	39	M	8.47		
Viral	ND	A	39	M	8.47		

A = African American; B = Asian/Pacific Islander; C = white non-Hispanic; dCT = change in cycle threshold; H = white Hispanic; N = Native American; ND = not documented; U = unknown.
* Insufficient quantity or quality of DNA.

DISCUSSION

The diagnosis of mtDNA depletion as a cause of liver failure is clinically challenging, with patients often presenting with no other features to suggest a mitochondrial etiology (2,3,20,32–35). Hepatic mtDNA depletion may go unrecognized in many cases, or may only come to light when a subsequent affected child is born (3).

The results of this study demonstrate that the presence of hepatic disease severe enough to require LT does not, by itself, lead to secondary mtDNA depletion (LT control group; Fig. 1), including hepatic diseases known to reduce mitochondrial function such as bile accumulation as a result of cholestasis caused by biliary atresia (29). The reduced mtDNA content in the 2 patients with Alagille syndrome in the LT control group was a surprising finding. The underlying mechanism behind this reduction in mtDNA content may be related to the pivotal role of *NOTCH/MYC* signaling in mitochondrial biogenesis (36). Thus one may postulate that these cases with *JAG1* mutations may have reduced *NOTCH* signaling,

thus reduced mtDNA replication, suggesting an alternate genetic etiology leading to a reduction in hepatic mtDNA content. Further evaluation in a larger cohort of patients with Alagille syndrome is required before firm conclusions can be drawn.

Definite mtDNA depletion was demonstrated in 34% (15/44) of ALF test patients, and 66% (29/44) were identified as having probable or definite mtDNA depletion, based on mtDNA content. Definite/probable mtDNA depletion was identified in patients irrespective of the underlying cause of ALF. This demonstrates that there is a strong association between hepatic mtDNA depletion and ALF in cases in which there appears to have been an environmental trigger, that is, drug-induced and viral ALF; however, this study was not designed to establish causality. Importantly, mtDNA depletion was found in both adults and children with ALF, and there was no significant association between mtDNA depletion and age.

Heterozygous mutation ''carriers'' were found at a significantly higher rate in patients undergoing LT for ALF than in the control group ($P = 0.001$), most notably *DGUOK*. The status of the p.Q170R variant remains controversial because it is more frequent in the general population than other variants in *DGUOK* (3), and is more frequent than would be expected given the published frequency of the severe hepatocerebral phenotype; however, this mutation has been demonstrated to cause infantile-onset hepatocerebral mtDNA depletion in one infant, in trans with a null mutation (20) and more recently been shown to cause later-onset myopathy in a cohort of patients (37). This glutamine is found within the highly constrained α helix domain of the protein (GERP 5.67, Phastcons 1.0 at the variant nucleotide). Furthermore, the p.Q170R variant in *DGUOK* is at a highly conserved position, with all homologous proteins having a glutamine and none having an arginine. In silico prediction, by POLYPHEN 2 (38), predicts that this change is possibly damaging; however, the effects of the p.Q170R variant in *DGUOK* in isolation have not been determined by enzymatic testing and, to date, no studies on individuals homozygous for this variant have been published. For the healthy controls, we have no data on ethnicity.

We also saw a higher rate of carriers of pathogenic *POLG* mutations in patients undergoing LT for ALF. This is consistent with recent data describing a higher rate of heterozygous *POLG* mutations in patients with sodium valproate–induced liver toxicity than in ethnically matched controls (odds ratio 23.6, 95% confidence interval 8.4–65.8) (30). Our data suggest that *POLG* mutations may be a general risk factor for liver disease and not specific to drug-induced liver injury.

Although population stratification could account for the differences, the majority of controls were from Wisconsin and would be expected to be mostly white. Because the common pathogenic mutations in *DGUOK* and *POLG* show increased frequency in those of northern European ancestry, if there is stratification it would be expected to favor the null hypothesis.

Given that a causal gene can only be identified in 20% of patients with multisystemic mtDNA depletion (39), the failure to detect pathogenic variants in the majority of our cohort with reduced mtDNA content is not surprising. Rather, it suggests that presently unrecognized genes may lead to reduction in hepatic mtDNA content, predisposing to liver dysfunction. Consequently, further work is required to identify such mutations and elucidate the mechanisms leading to reduced mtDNA content. Given the costs associated with the Sanger methods used in this study, there is a potential need for larger-scale, genome-wide sequencing to evaluate the pathophysiology and predisposition toward mtDNA depletion and ALF.

Our results, in combination with recent data (27,30), further suggest that carriers of mutations in genes associated with mtDNA depletion, specifically *POLG* and *DGUOK,* are at increased risk for liver failure. Therefore, the prescription of hepatotoxic drugs, in particular, isoniazid and sodium valproate, should be considered carefully in individuals carrying, or who are suspected of carrying, mutations in mtDNA depletion genes. These data suggest that being a carrier for mutations, even without depletion, may be a potential mechanism underlying ''idiosyncratic'' drug reactions in a subset of cases. Furthermore, in conjunction with previously published data (5,40), our data suggest a more aggressive role for vaccines against hepatotropic viruses in those with hepatic mtDNA depletion. Given the relatively low population incidence of mtDNA depletion and the expense of testing, there is presently insufficient evidence to recommend sequence-based testing for these genetic disorders before initiating potentially hepatotoxic therapy in patients; however, these recommendations may change as the cost of sequencing falls.

In summary, end-stage liver disease severe enough to require an LT does not cause secondary mtDNA depletion. The majority of individuals in this study undergoing LT for ALF had reduced hepatic mtDNA content, which fell within the range seen in patients with genetically caused mtDNA depletion. A subset of ALF cases has single variants in genes known to lead to mtDNA depletion and hepatic failure. Together, these results suggest an important role for mtDNA replication and maintenance in ALF.

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