### J. Cell. Mol. Med. Vol 15, No 2, 2011 pp. 445-456

**Mitochondrial DNA depletion and fatal infantile hepatic failure due to mutations in the mitochondrial**

**polymerase** 'Y **(POLG) gene: a combined morphological/enzyme histochemical and immunocytochemical/biochemical**

**and molecular genetic study**

## J. Müller-Höcker a, \*, R. Horvath b, S. Schäfer a, H. Hessel a, W. Müller-Felber c,

***J. Kühr d, W. C. Copeland e, P. Seibel f***

*a Pathologisches Institut der Ludwig-Maximilians-Universität, München, Germany*

*b Friedrich-Baur-Institut, Department of Neurology, Ludwig-Maximilians University, Munich, Germany and Mitochondrial Research Group, Institute for Ageing and Health, Newcastle University, Newcastle upon Tyne, UK*

*c Friedrich-Baur Institut an der Neurologischen Klinik und Poliklinik der Ludwig-Maximilians Universität, München, Germany*

*d Municipal Hospital Karlsruhe, Clinic for Pediatric and Adolescent Medicine, Karlsruhe, Germany*

*e National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA*

*f Molekulare Zellbiologie, Biotechnologisch-Biomedizinisches Zentrum Universität Leipzig, Leipzig, Germany*

### Received: December 1, 2008; Accepted: May 6, 2009

***Abstract***

Combined morphological, immunocytochemical, biochemical and molecular genetic studies were performed on skeletal muscle, heart muscle and liver tissue of a 16-months boy with fatal liver failure. The pathological characterization of the tissues revealed a severe depletion of mtDNA (mitochondrial DNA) that was most pronounced in liver, followed by a less severe, but still significant depletion in skeletal muscle and the heart. The primary cause of the disease was linked to compound heterozygous mutations in the polymerase 'Y (POLG) gene (DNA polymerase 'Y; A467T, K1191N). We present evidence, that compound heterozygous POLG mutations lead to tissue selective impairment of mtDNA replication and thus to a mosaic defect pattern even in the severely affected liver. A variable defect pat- tern was found in liver, muscle and heart tissue as revealed by biochemical, cytochemical, immunocytochemical and in situ hybridiza- tion analysis. Functionally, a severe deficiency of cytochrome-c-oxidase (cox) activity was seen in the liver. Although mtDNA depletion was detected in heart and skeletal muscle, there was no cox deficiency in these tissues. Depletion of mtDNA and microdissection of cox- positive or negative areas correlated with the histological pattern in the liver. Interestingly, the mosaic pattern detected for cox-activity and mtDNA copy number fully aligned with the immunohistologically revealed defect pattern using Pol 'Y, mtSSB- and mtTFA-antibodies, thus substantiating the hypothesis that nuclear encoded proteins located within mitochondria become unstable and are degraded when they are not actively bound to mtDNA. Their disappearance could also aggravate the mtDNA depletion and contribute to the non- homogenous defect pattern.

**Keywords:** depletion of mtDNA • polymerase 'Y • mitochondrial single stranded binding protein(mtSSB) •

mitochondrial transcription factor A (mtTFA) • liver failure • in situ hybridization

*\*Correspondence to: J. MÜLLER-HÖCKER,*

*Institute for Pathology, Ludwig-Maximilians-Universität München, Thalkirchnerstrasse 36, 80337 München, Germany.*

*Tel.: 004989218073611*

*Fax: 0049218073671*

*E-mail:* [*josef.mueller-hoecker@med.uni-muenchen.de*](mailto:josef.mueller-hoecker@med.uni-muenchen.de)

*© 2011 The Authors*

# Introduction

Mitochondrial diseases are a heterogeneous group of disorders caused by mutations in the mitochondrial DNA (mtDNA), but also in nuclear genes [1–3]. These nuclear-derived factors important for replication and transcription of the mtDNA include the human

doi: 10.1111/j.1582-4934.2009.00819.x

*Journal of Cellular and Molecular Medicine © 2011 Foundation for Cellular and Molecular Medicine/Blackwell Publishing Ltd*

mitochondrial transcription factor A (h-mtTFA), DNA poly- merase 'Y, (Pol 'Y) and mitochondrial single stranded binding pro- tein (mtSSB) [4, 5]. The disorders involve mostly skeletal muscle and brain, but other organs may also be affected. In this context, liver failure due to depletion of mtDNA is relatively common [6–11]. Depletion of mtDNA may be caused by mutations in different genes. Mutations in the gene encoding the catalytic subunit of polymerase 'Y [12–14] are associated with depletion of mtDNA and subsequently liver failure and have been described most often in Alpers-Huttenlocher disease [15–19]. However, these mutations were also found in a wide spectrum of other clinical disorders, including autosomally inherited progressive external ophthalmoplegia, sensory-neural neuropathy, ataxia, epilepsy and parkinsonism [20, 21]. Therefore, an interesting question is how other coexisting metabolic alterations might influence the

clinical presentation [22].

In this study, we describe the morphological, cyto-immunocy- tochemical, biochemical and molecular genetic results in a 16-months old child, who died of liver failure due to mutations in the polymerase 'Y (POLG) gene. Our study showed heterogenic tis- sue involvement with a mosaic defect pattern in the liver probably related to concomitant secondary dysfunction of further proteins involved in mtDNA maintenance like mitochondrial single stranded binding protein (mtSSB) and mitochondrial transcription factor A (mtTFA). The results help to explain the occasionally observed recovery from mtDNA depletion and indicate also that a slight increase in the mtDNA copy number might be enough to retain the normal respiratory function, which may open possibilities for ther- apeutic trials in the future.

# Materials and methods

## Case report

*The patient, a boy, was the first child of healthy non-consanguineous/ genetically unrelated parents. There was no history of miscarriage in the family and the history of the family was negative for liver disease.*

*Pregnancy and delivery were normal (birth weight 3920 g, APGAR 6/9/10). Psychomotor development was normal during the first 4 months of life, he started smiling at 2 months and to grasp objects at 4 months of age. At the age of 5 months the patient developed recurrent vomiting and failure to thrive. Liver enzymes at the age of 6 months were: 200 U/L for AST, 107 U/L for ALT and 507 U/L for* 'Y*-glutamyl-transferase, but total bilirubin was normal. In parallel with deterioration of liver function psy- chomotor retardation was noticed and the ability to grasp objects and to roll over was lost. The muscle tone was increased. EEG, as well as MRI and MR-spectroscopy of the brain at the age of 7–8 months were inconspicu- ous. Epileptic episodes have not been observed. Serum lactate was slightly elevated (2.8–3.7 mmol/l). Extensive clinical search did not reveal the eti- ology of the liver disease. There was a normal level of alpha-1-antitrypsine, coeruloplasmin, copper. Serological test for hepatitis A/B/C, CMV, EVB, rubella, toxoplasmosis and HSV1 were negative. Immunological screening did not detect ANAs, ANCAs, ds-ANAs, Gliadin-antibodies. Cystic fibrosis*

*was excluded by iontophoresis. There were no clinical signs of a cardiomy- opathy and sonography of the heart proved unconspicuous. In the following months, gastrointestinal problems recurred because of persisting vomiting and reflux. Ileus due to small bowel obstruction was suspected and parental feeding was necessary.*

*At the age of 15 months AST was still elevated (482 U/L and total biliru- bin had risen to 3.4 mG/dL (conjugated 2 mG/dL).*

*At the age of 16 months liver function rapidly detoriorated (fibrinogen 80 mg/dl, thromboplastin time 15%. AST up to about 1900 U/L, ALT up to 350 U/L total bilirubin 19,1 mG/dL, the conjugated fraction being 15 mG/dL) and ascites developed.*

*He reacted well to acoustic stimuli but was unable to fixate objects and to sit without support. An EEG examination showed diffuse suppression of signals. Finally, intestinal bleeding led to haemorrhagic shock and subse- quent fatal multi-organ failure. An autopsy was not performed, but in addi- tion to biopsy probes of the skeletal muscle and the liver, necropsy probes were taken from the heart. The patient was included in a previous study on the molecular genetic and clinical spectrum of POLG mutations [20].*

*Morphological biochemical and molecular genetic studies were per- formed, the latter being restricted to analysis of mtDNA since the multisys- temic clinical presentation pointed first of all to a mitochondrial disorder.*

## Morphology, cytochemistry and biochemistry

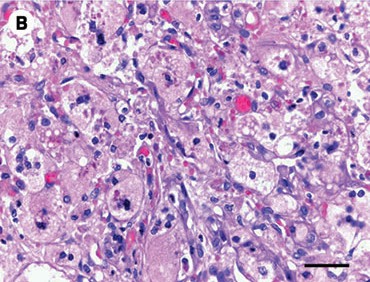
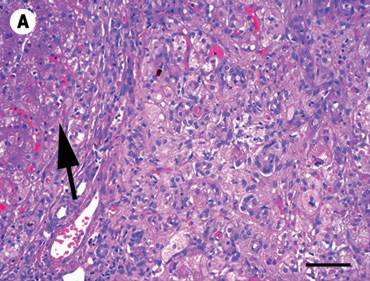
*Liver, skeletal and heart muscle were fixed in 4% buffered formalin and embedded for routine staining procedures. (HE, PAS, van Gieson, Prussian iron stain). For electron microscopy, tissues were fixed in 6.25% glu- taraldehyde/Soerensen phosphate buffer for 2 hrs. After washing in buffered saccharose and osmication (2% distilled water) for 1 hr, tissues were dehydrated in acetone and embedded in Epon (polymerisation at 78*°*C overnight) for the preparation of semi-thin und ultra-thin sections (coun- terstaining using uranyl acetate and lead citrate). Frozen tissue (stored at –80*°*C) was used for light microscopical and ultra-cytochemical detection of cytochrome-c-oxide as previously described [23]. Immuno-cytochem- istry using the ABC Elite kit (DAKO) was performed as previously described for the detection of cytochrome-c-oxidase subunits II-III and Vab [24], mtTFA, DNA-polymerase* 'Y *[25] and mtSSB [26]. Biochemical analysis of the respiratory chain enzymes was performed on homogenates of deep frozen muscle [27]. Measurements were carried out for NADH/CoQ-oxi- doreductase (complex I and II, succinate-oxidoreductase (complex II and III) cytochrome-c-oxidase (complex IV) and citrate synthase.*

## DNA analysis

*mtDNA and nuclear DNA were extracted from skeletal muscle, heart and liver and also from COX negative and COX positive areas and mtDNA copy number and mtDNA/nDNA ratios were determined by real-time PCR using a fluorescent temperature cycler (Light Cycler, Roche Molecular Biochemicals, Mannheim, Germany). The Light Cycler FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals) was used [28, 29]. We amplified the mtDNA ATP6 gene with unlabelled primers between nucleotide positions 8981 and 9061 with the forward primer, 5*'*- ACCAATAGCCCTGGCCGTAC-3*' *and the backward primer 5*'*-GGTG- GCGCTTC-CAATTAGGT-3*'*. For the detection of nDNA we selected exon number 8 of the GAPDH-gene between nucleotide positions 4280–4342, using the forward primer 5*'*-CGGGGCTCTCCAGAACATC-3*' *and the back- ward primer 5*'*-ATGACCTTGCCCACAGCCT-3*'*. The control range was*

### J. Cell. Mol. Med. Vol 15, No 2, 2011

*isolated human placenta mtDNA. The double stranded DNA was purified by Centricon™ 100 Microconcentrators (Amicon, Beverly, MA, USA). The PCR-DNA fragments were labelled by random primed incorporation of digoxigenin-labelled deoxyuridine triphosphate applying the digoxigenin labelling kit of Boehringer Mannheim.*



# Results

## Liver

Light microscopy of the liver (Fig. 1) showed a severe alteration of liver parenchyma with massive ballooning of liver cells that often formed giant cells. The cytoplasm of the altered liver cells had a fine vesicular appearance (Fig. 1B). Often bilirubinostasis was present. The portal tracts were enlarged showing regular pre-existing bile ducts and severe proliferation of bile ductules, containing bile plugs. In the PAS stain no globular diastase resist- ant cytoplasmic inclusions were found. Besides the altered hepa- tocytes, small islands of better-preserved or normal looking hepatocytes were present (Fig. 1A). A stain for iron (Perl-stain) was negative.

#### Fine structure

Most of the hepatocytes were stacked full with slightly enlarged mitochondria. These mitochondria had a floccular granular matrix, loss of matrix granules and a reduced amount of cristae (Fig. 2A, B). Occasionally, mitochondria with tubular cristae formations were also present (Fig. 2C). Deposits of bile and lipid droplets were a constant feature. Corresponding to the light microscopical findings there were also hepatocytes with a normal content of mitochondria and regular cristae (Fig. 2D). The rough endoplas- mic reticulum was inconspicuous.

***Fig. 1*** *Liver, light microscopy. (****A****) Liver displaying a destroyed architecture. Adjacent to the altered liver parenchyma an island with bet- ter structural preservation is seen ( ). (****B****) Higher magnification show- ing a vesicular/granular aspect of the hepatic cytoplasm. (Hematoxilin and eosin). Bar A: 50* µ*M, Bar B: 25* µ*M.*

*determined for skeletal muscle, liver and heart using 30 control muscle DNA samples, 9 control liver DNA samples, and 3 control heart DNA samples.*

*Sequencing of the nuclear encoded deoxyguanosine kinase (DGUOK)*

*[30] and POLG was performed as described [20]. Microdissection of frozen liver sections (30* µ*m) was performed after cytochrome-c-oxidase staining from cytochrome-c-oxidase positive and negative areas by P. A. L.*

*M. (Microlaser Technologies, 82347 Bernried, Germany) for quantitative mtDNA- PCR-analysis.*

## In situ hybridization of mtDNA

*In situ hybridization was performed as previously described [31–34], on formalin fixed paraffin embedded liver tissue as well as on skeletal and heart muscle using specific probes of mtDNA produced by PCR from*

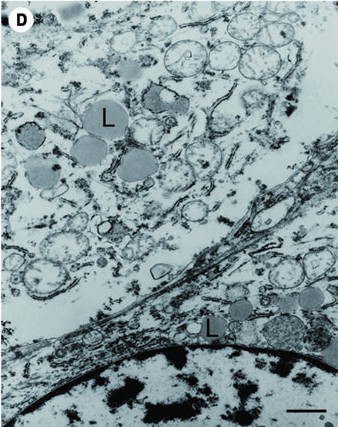
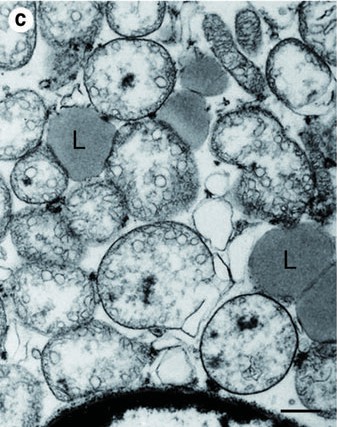
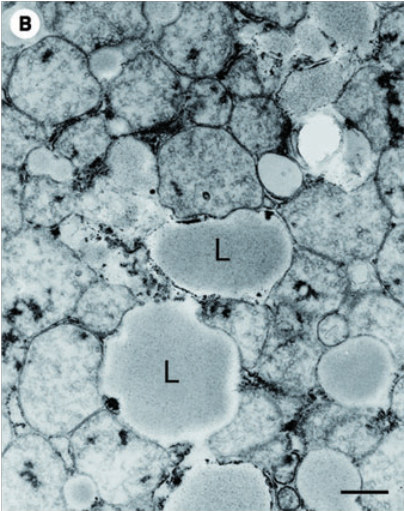
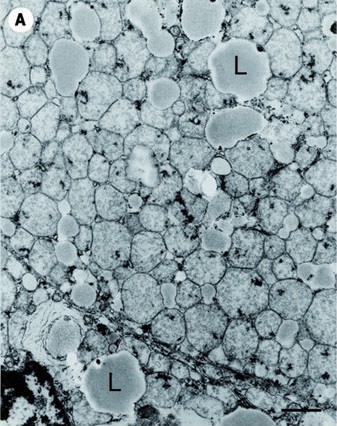
#### Cytochemistry

In most of the hepatocytes cytochrome-c-oxidase (cox-) activity was deficient. (Fig. 3A) However, there were also small islands with preserved activity (Fig. 3B). Succinate dehydrogenase was regularly detectable both in the areas with and without deficiency of cytochrome-c-oxidase (Fig. 3C). At the ultrastructural level occasionally a co-existence of defective and normal reacting mito- chondria could be found (not shown).

#### Immunohistochemistry

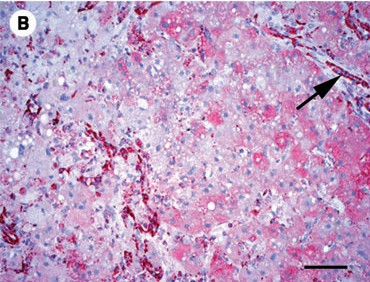
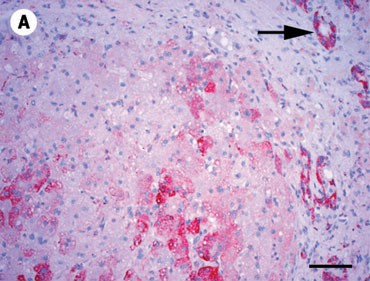
Immunohistochemistry disclosed a severe loss of cytochrome-c- oxidase subunits II/III, Vab, sparing small islands of hepatocytes. In contrast, the bile ducts reacted normally (Fig. 4A, B).

There was also a severe defect of DNA-polymerase 'Y sparing, however, small hepatocytic foci (Fig. 5). Also, mtSSB and mtTFA were partially deficient leading to the coexistence of both defective and normal-reactive hepatocytes (Fig. 6A, B).



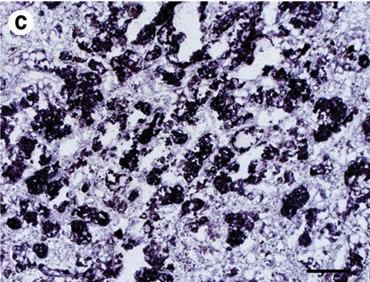
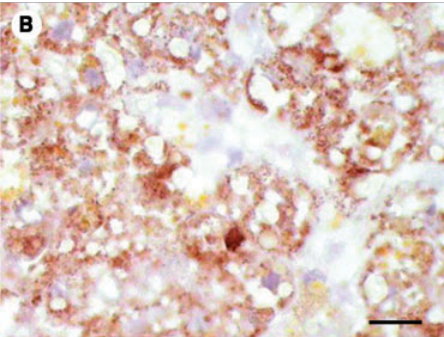
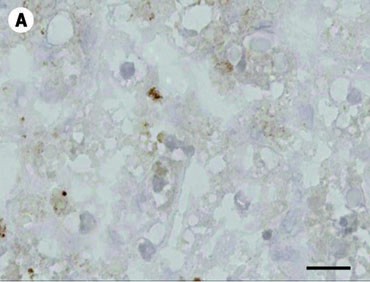
***Fig. 2*** *Ultrastructural changes in the liver. (****A****) The hepatocytes are stacked full of abnormal mitochondria. (****B****) The mitochondria have lost their matrix granules and are mainly devoid of cristae. Only single abortive cristae of tubular type are seen. Between the mitochondria occur lipid droplets (L). (****C****) Hepatocyte full of abnormal mitochondria having cristae of tubular type. (****D****) Normal hepatocyte with regular ultrastructure. The mitochondria show inconspicuous cristae of lamellar type. Lipid droplets are seen (L). Bar A: 1* µ*M, Bar B–D: 0.5* µ*M.*

### J. Cell. Mol. Med. Vol 15, No 2, 2011



***Fig. 4*** *Cytochrome-c-oxidase immunohistochemistry in the liver. (****A****) The subunit II/III lacks in most of the hepatocytes but is retained in the bil- iary ducts ( ). (****B****) Subunit Vab. A mosaic defect pattern is seen with coexisting deficient and reacting hepatocytes. The bile duct epithelium reacts normally ( ). Bar A, B: 50* µ*M.*

#### In situ hybridization of mtDNA

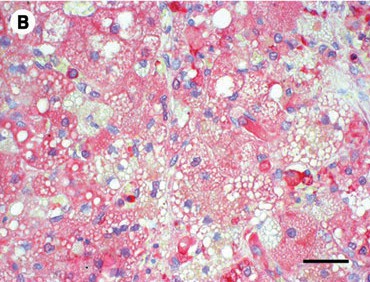
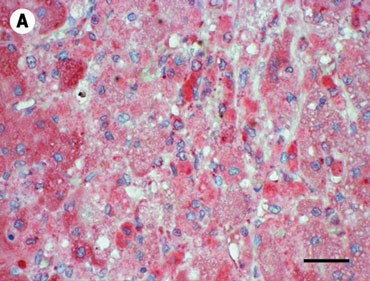


In situ hybridization of mtDNA disclosed a severe reduction of mtDNA in the altered hepatocytes (Fig. 7A, B), but there were multiple small foci of hepatocytes with preserved staining (Fig. 7A, C). No staining defect could be found in the bile duct epithelium (Fig. 7B).

## Heart and skeletal muscle

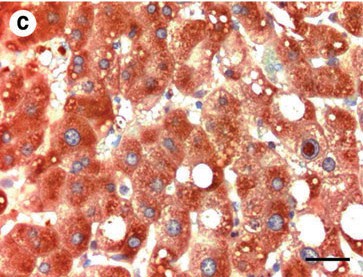
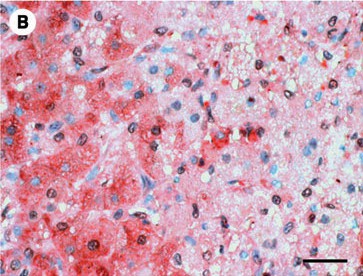
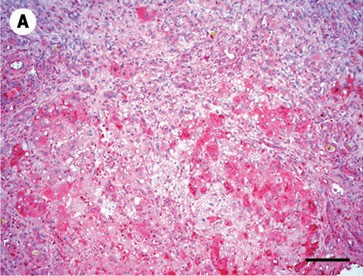
In the heart (Fig. 8) and skeletal muscle single cells/fibres were present with an accumulation of lipids and mitochondria. The mitochondria were enlarged and had irregular cristae of a tubular type. However, no defects of cytochrome-c-oxidase could be detected (Fig. 9). Succinate dehydrogenase was also normal.

***Fig. 3*** *Liver. (****A****) Cytochrome-c-oxidase (cox)-stain, is completely nega- tive in the hepatocytes. (****B****) Cox-stain of hepatocytes with preserved activity. (****C****) SDH-stain for comparison with generally retained activity. Bar A–C: 50* µ*M.*



***Fig. 6*** *Liver. Immunohistochemistry of (****A****) mtSSB, (****B****) mtTFA. Both proteins are focally lacking. Bar A, B: 25* µ*M.*

Furthermore, immunohistochemistry disclosed no defects of cytochrome-c-oxidase subunits II/III, Vab and of DNA- polymerase 'Y in the skeletal muscle. Also no defects were present for mtTFA and mtSSB in the heart. In the skeletal muscle mtTFA and mtSSB could not be demonstrated with confidence, even in controls.



In situ hybridization of mtDNA could not detect a clear reduction

of mtDNA in the heart. In the skeletal muscle the signal intensity was generally weak apparently because of artificial influences.

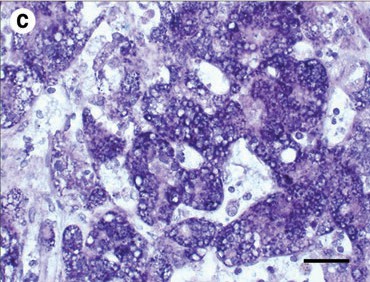
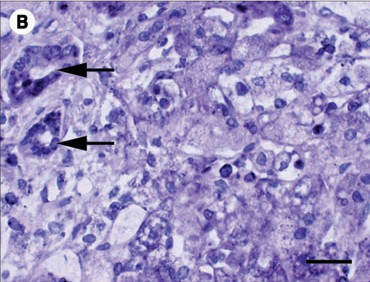
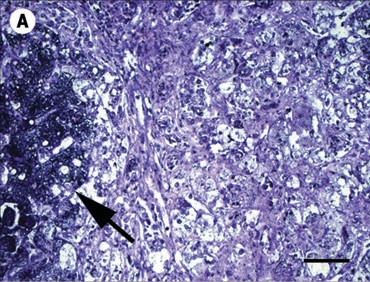
## Biochemistry

***Fig. 5*** *Polymerase* 'Y *immunohistochemistry in the liver. (****A****) Liver with POLG-mutation showing a severe defect. Small islands of hepatocytes have retained some protein. (****B****) Higher magnification to show the coex- istence of defective hepatocytes and hepatocytes with demonstrable polymerase* 'Y *protein. (****C****) In a control liver polymerase* 'Y *is homoge- nously expressed in the hepatocytes. Bar A: 50* µ*M, Bar B, C: 25* µ*M.*

Analysis of the respiratory chain revealed a severely reduced activity of all mtDNA-encoded enzyme complexes (I. III, IV) in the liver (Table 1). In the heart the enzyme activities were below the normal range; however, skeletal muscle showed normal respira- tory chain activities (Table 1).

*J. Cell. Mol. Med. Vol 15, No 2, 2011*

## Molecular genetics/mtDNA-Quantification



A severe depletion of mtDNA was found in the liver and a less severe but still significant depletion was detected in skeletal mus- cle and in heart homogenates (Table 2).

The quantitative analysis of mtDNA in cox-negative and cox- positive hepatocytes revealed a very low copy number of mtDNA in cox-negative and a significantly higher number in cox-positive hepatocytes (Table 2). But also in the cox-positive cells the mtDNA copy number was lowered, measuring only about 24% of the lowest normal value.

Molecular genetic analysis of DNA isolated from blood cells of the index patient revealed two heterozygote missense mutations in the POLG gene, A467T and K1191N. The A467T mutation has been frequently described in patients with Alpers-Huttenlocher syndrome, but also in other POLG phenotypes. The other mutation (K1191N) affects a highly conserved amino acid in the polymerase domain in trans with the A467T mutation [20]. Both parents har- boured one of the two mutations found in the child proving com- pound heterozygosity.

# Discussion

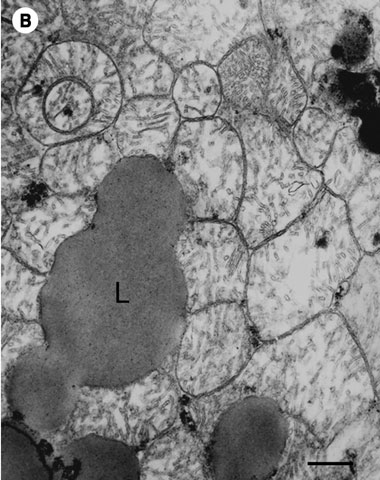
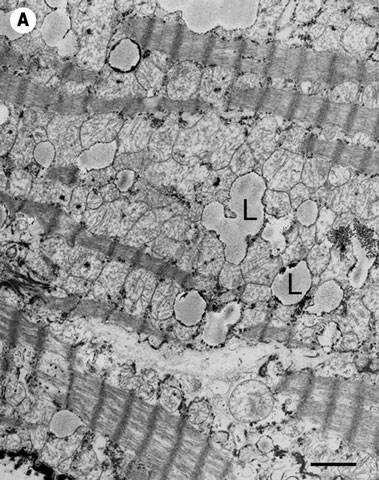
Mitochondrial DNA depletion syndrome is an autosomal recessive disorder affecting either selectively or in combinations various organs, especially the liver, heart, kidneys, skeletal muscle and brain. In contrast to other mitochondrial diseases, severe depletion of mtDNA often affects the liver and can lead to liver failure. [6–11]. Depletion of mtDNA in the liver has also been found in oncocytic hepatocytes [35], associated with defects of the respiratory chain [25] most probably as an effect of cellular ageing [31].

The enzyme responsible for mtDNA replication is Pol 'Y, which consists of a 140 kD catalytic polypeptide (encoded by POLG or POLG1) and two identical 55 kD accessory subunits (encoded by POLG2) that are required for an increased DNA affinity and highly processive DNA synthesis [12, 14, 36, 37]. The majority of muta- tions involved with Pol 'Y have been genetically linked to the POLG gene [15–19] while mutations in POLG2 are rare [38].

Over 150 disease causing mutations have been reported in POLG where the A467T mutation of POLG has been observed to be the most frequent [12, 20, 21] (see also [http://tools.niehs.nih.gov/polg/).](http://tools.niehs.nih.gov/polg/)) The A467T mutation compro- mises the interaction with the accessory subunit and decreases polymerase activity [12]. Patients heterozygous for the A467T mutation, like the patient described here, are usually more severely affected than A467T homozygotes [20, 39]. Patients with compound heterozygous mutations involving A467T display a wide clinical phenotype from PEO to Alpers-Huttenlocher syn- drome, but, in general, mutations in the polymerase domain, such as the K1191N, are associated with Alpers syndrome [40]. The fatal liver failure in our patient was comparable with the liver fail-

***Fig. 7*** *Liver in situ hybrization of mtDNA. (****A****) Severe depletion of mtDNA molecules in the hepatocytes. In an island of hepatocytes mtDNA can be detected ( ) see also* ***C****. (****B****) Higher magnification to show the mtDNA defect in the hepatocytes. The bile duct epithelia react normally ( ).*

*(****C****) Higher magnification. Residual mtDNA can be demonstrated in the cytoplasm of the hepatocytes. Bar A: 50* µ*M, Bar B, C: 25* µ*M.*



***Fig. 8*** *Heart ultrastructure. (****A****) Abnormal cardiomyocyte exhibiting a high content of mitochondria. Higher magnification to show the atypcical mito- chondria with tubular and circular cristae. Original magnification, Bar A: 1* µ*M, Bar B: 0.4* µ*M.*

ure seen in Alpers-Huttenlocher syndrome; however, the lack of epilepsy makes the clinical presentation atypical. Although clini- cally silent the child also had mitochondrial cardiomyopathy. Thus, our studies provide evidence that the heart muscle might also be affected by mutations in POLG.

Furthermore, our study confirms the occurrence of tissue dependent defects [19, 41–44]. Whereas only a mild mitochondrial cytopathy of the heart and skeletal muscle was seen on single cells/ fibre level a severe but nevertheless non-homogenous defect was detected in the liver. The finding of non-homogenous liver involve- ment has been previously reported in three studies [10, 11, 63]. The underlying pathogenic mechanisms were, however, not further elucidated. In the present study, we analyzed the defect pattern by cytochemical and immunocytochemical detection of cytochrome-c- oxidase and for the first time combined the findings with in situ hybridization of mtDNA and additional quantitative PCR analysis of mtDNA on isolated cox-positive and cox-negative liver cells.

In whole liver preparations the mtDNA copy number proved to be 80% reduced (referred to the lowest normal value). In isolated cox-positive liver cells mtDNA was similarly depleted. Cox-nega- tive liver cells, however, exhibited an even more severe depletion. Their mtDNA content was about 9 times lower than that of

cox-positive liver cells. This quantitative analysis correlated well with the results obtained by in situ hybridization of mtDNA. Interestingly, no defect could be localized in the biliary duct cell system. Thus, our findings suggest that even when mtDNA is depleted in liver, the amount of depletion has to reach a critical threshold in single cells in order to express the biochemical defect and this may be variable within single cells. Thereby, similar to the skeletal muscle [45, 46] a mosaic defect pattern may result [63]. In the skeletal muscle 0.01 mtDNA/µm3 has been found sufficient for residual cytochrome c oxidase activity [46]. Therefore, a slight increase in the mtDNA copy number might be enough to traverse the threshold value to regain the normal respiratory function. This observation may open new possibilities for further therapy trials and may explain the occasionally observed reversibility of mtDNA depletion [47–49]. The mechanisms, however, underlying the expression of tissue selectivity and the reversibility remain still unclear [50, 51].

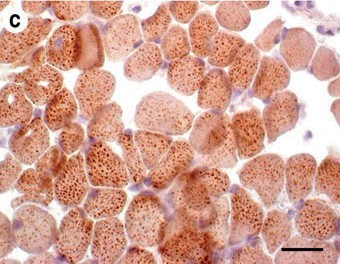
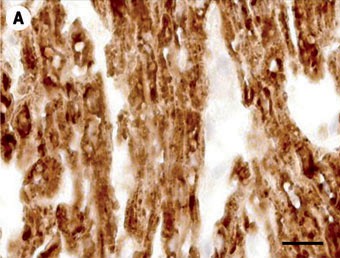
Besides tissue specific isoforms of enzymes enrolled in DNA replication and maintenance, oxidative damage from free radicals might be a causative factor since Pol 'Y is susceptible to oxidative damage in vitro [52, 53]. In the present case, the compound het- erozygous mutations in POLG presented as immunohistochemically

### J. Cell. Mol. Med. Vol 15, No 2, 2011

***Table 1*** *Respiratory chain complex activities*

|  |  |  |  |
| --- | --- | --- | --- |
| ***Tissue*** | ***Skeletal muscle*** | ***Heart*** | ***Liver*** |
| *Complex I U/UCS* | *0.31* | *0.01* | *0.00* |
|  | *(normal 0.17–0.56)* | *(normal 0.09–0.22)* | *(normal 0.5–1.2)* |
| *Complex II* + *III U/UCS* | *0.15* | *0.02* | *0.07* |
|  | *(normal*  *0.08–0.45)* | *(normal*  *0.06–0.1)* | *(normal*  *0.5–0.8)* |
| *Complex IV U/UCS* | *1.3* | *0.51* | *0.12* |
|  | *(normal 1.1–5.0)* | *(normal 0.6–1.3)* | *(normal 1.3–2.6)* |
| *Citrate synthase*  *U/gNCP* | *55* | *246* | *81* |
|  | *(normal 45–100)* | *(normal 250–350)* | *(normal 25–35)* |

severely reduced Pol 'Y protein and a mosaic pattern of cox-staining and mtDNA levels. In a previous case, with DNA depletion caused by a homozygous deoxyguanosine kinase gene (DGUOK) mutation



[54] expression of Pol 'Y was shown to be normal [11].

In our study, we also tested the expression of mitochondrial transcription factor (mtTFA) and of mitochondrial single stranded protein (mtSSB), which both are involved in mtDNA-replication. H-mtTFA is a 25 kD protein, indispensable for transcription and replication of mtDNA [55]. The protein bends and unwinds mtDNA, thus priming transcription and replication [5, 56]. In the liver of the present patient a mosaic defect pattern of both proteins existed. In a previously described case [11] with a hepatic form of mtDNA depletion caused by a homozygous DGUOK gene mutation

[54] mtTFA and mtSSB were also defective, the latter, however only in single abnormal hepatocytes. In a previous study on parathyroids with defects of cytochrome-c-oxidase both mtTFA and polymerase 'Y were regularly expressed [57].

It is generally accepted that h-mtTFA mirrors the mtDNA status of the cell, but it is most likely that reduction of h-mtTFA repre- sents a secondary phenomenon as it is also reduced in cell lines where mtDNA copy number was experimentally reduced [58, 59]. Therefore, it appears that similar as in Saccharomyces cerevisiae

[60] in the liver mitochondrial proteins are soon degraded when they are not properly assembled, e.g. because of reduced binding sites. A similar observation concerns the single strand binding protein (mtSSB). As mtSSB is essential for mtDNA replication and thus the maintenance of the DNA, its amount directly correlates with the mtDNA content [61]. Consequently, it may be assumed that the reduced appearance of mtTFA and mtSSB mirrors the

***Fig. 9*** *Cytochrome-c-oxidase cytochemistry. No defect is seen in the mitochondria of the heart both at light (****A****) and electron microscopy (****B****). Also in the skeletal muscle cytochrome-c-oxidase activity is unaltered*

*(****C****). Bar A, B: 25* µ*M, Bar C: 0.5* µ*M.*

replicative failure of mtDNA. It therefore appears that depletion

represents a dynamic process leading to additional secondary alterations in the mtDNA replicative machinery which might also

***Table 2*** *mtDNA Depletion Test*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| ***Tissue*** | ***Skeletal muscle*** | ***Heart*** | ***Liver*** | ***COX* + *liver cells*** | ***COX-liver cells*** |
| ***mtDNA/nDNA ratio*** | *0.1* | *0.891* | *0.05* | *0.061* | *0.007* |
| ***Normal range*** | *0.25–4.0* | *1–5.0* | *0.25–4.0* |  |  |

aggravate the degree of mtDNA depletion and be a contributory cause of the mosaic defect pattern in the liver.

We also addressed the question of comorbitity with other chronic liver diseases [22]. But there was no evidence for infec- tious liver disease, paucity of intrahepatic bile ducts, fetal hemochromatosis a-1-antitrypsin deficiency or cystic fibrosis as potential contributing factors.

In conclusion, the presented results illustrate that morphologi- cal/cytochemical data and in situ hybridization of mtDNA provide valuable information in diagnosing mtDNA depletion. mtDNA deple- tion in the liver should be suspected on light microscopy when severe ballooning, bilurubinostasis and steatosis and giant hepato- cytes are present. On electron microscopy the most characteristic signs for mtDNA depletion are the accumulation of abnormal mito- chondria with tubular cristae or lack of cristae [11, 62]. Cytochemistry/immunocytochemistry combined with in situ hybridization of mtDNA are especially suited for the detection of mosaic tissue involvement. If biochemical evidence (decreased activity of respiratory chain complexes I, III and IV) in liver also

supports the mitochondrial origin, it can give further support for the depletion, if molecular genetic data are in the border-line range. Our data show that mutations in POLG lead to heterogenous depletion of mtDNA in liver, skeletal and heart and to a mosaic defect pattern even in the severely affected liver. Secondary dysregulation of other proteins involved in mtDNA maintenance like mtTFA and mtSSB probably aggravate the defect and may thereby promote the development of a mosaic defect pattern. The results help to explain the occasionally observed recovery from mtDNA depletion.

# Acknowledgements

*The authors are indebted to Mrs. Diane Raabe and Mrs. Monika Attmanspacher for invaluable help in preparing the manuscript. Funding information: W.C.C. is funded by intramural funds from the National Institutes of Health, NIEHS. This work was in part (P.S.) supported by the Sächsische Ministerium für Wissenschaft und Kunst. RH is supported by the Deutsche Forschungsgemeinschaft HO 2505/2-1.*

# References

1. ***DiMauro S, Schon EA.*** *Mitochondrial res- piratory-chain diseases. N Engl J Med. 2003; 348: 2656–68.*
2. ***Wallace DC.*** *Diseases of the mitochondr- ial DNA. Annu Rev Biochem. 1992; 61: 1175–212.*
3. ***Zeviani M, Di Donato S.*** *Mitochondrial disorders. Brain. 2004; 127: 2153–72.*
4. ***Graziewicz MA, Longley MJ, Copeland WC.*** *DNA polymerase gamma in mito- chondrial DNA replication and repair. Chem Rev. 2006; 106: 383–405.*
5. ***Shadel GS, Clayton DA.*** *Mitochondrial DNA maintenance in vertebrates. Annu Rev Biochem. 1997; 66: 409–35.*
6. ***Bakker HD, Scholte HR, Dingemans KP, et al.*** *Depletion of mitochondrial deoxyri- bonucleic acid in a family with fatal neonatal liver disease. J Pediatr. 1996; 128: 683–7.*
7. ***Ducluzeau PH, Lachaux A, Bouvier R, et al.*** *Depletion of mitochondrial DNA associated with infantile cholestasis and progressive liver fibrosis. J Hepatol. 1999; 30: 149–55.*
8. ***Maaswinkel-Mooij PD, Van den Bogert C, Scholte HR, et al.*** *Depletion of mitochon- drial DNA in the liver of a patient with lactic acidemia and hypoketotic hypo- glycemia. J Pediatr. 1996; 128: 679–83.*
9. ***Mazziotta MR, Ricci E, Bertini E, et al.***

*Fatal infantile liver failure associated with mitochondrial DNA depletion. J Pediatr. 1992; 121: 896–901.*

1. ***Morris AA, Taanman JW, Blake J, et al.*** *Liver failure associated with mitochondrial DNA depletion. J Hepatol. 1998; 28:*

*556–63.*

1. ***Muller-Hocker J, Muntau A, Schafer S, et al.*** *Depletion of mitochondrial DNA in the liver of an infant with neonatal giant cell hepatitis. Hum Pathol. 2002; 33:*

*247–53.*

1. ***Chan SS, Longley MJ, Copeland WC.*** *The common A467T mutation in the human mitochondrial DNA polymerase (POLG) compromises catalytic efficiency and inter- action with the accessory subunit. J Biol Chem. 2005; 280: 31341–6.*
2. ***Copeland WC.*** *Inherited mitochondrial diseases of DNA replication. Annu Rev Med. 2008; 59: 309–24.*
3. ***Longley MJ, Graziewicz MA, Bienstock RJ, et al.*** *Consequences of mutations in human DNA polymerase gamma. Gene. 2005; 354: 125–31.*
4. ***Davidzon G, Mancuso M, Ferraris S, et al.*** *POLG mutations and Alpers syn- drome. Ann Neurol. 2005; 57: 921–3.*
5. ***Ferrari G, Lamantea E, Donati A, et al.*** *Infantile hepatocerebral syndromes asso- ciated with mutations in the mitochondrial DNA polymerase-gammaA. Brain. 2005; 128: 723–31.*
6. ***Naviaux RK, Nguyen KV.*** *POLG mutations associated with Alpers’ syndrome and mitochondrial DNA depletion. Ann Neurol. 2004; 55: 706–12.*
7. ***Naviaux RK, Nyhan WL, Barshop BA, et al.*** *Mitochondrial DNA polymerase gamma deficiency and mtDNA depletion in a child with Alpers’ syndrome. Ann Neurol. 1999; 45: 54–8.*

### J. Cell. Mol. Med. Vol 15, No 2, 2011

1. ***Tesarova M, Mayr JA, Wenchich L, et al.*** *Mitochondrial DNA depletion in Alpers syndrome. Neuropediatrics. 2004; 35:*

*217–23.*

1. ***Horvath R, Hudson G, Ferrari G, et al.*** *Phenotypic spectrum associated with muta- tions of the mitochondrial polymerase gamma gene. Brain. 2006; 129: 1674–84.*
2. ***Hudson G, Chinnery PF.*** *Mitochondrial DNA polymerase-gamma and human disease. Hum Mol Genet. 2006; 15: R244–52.*
3. ***Campbell KM, Arya G, Ryckman FC, et al.*** *High prevalence of alpha-1- antitrypsin heterozygosity in children with chronic liver disease. J Pediatr Gastroenterol Nutr. 2007; 44: 99–103.*
4. ***Muller-Hocker J, Schaefer S.*** *Cytochemistry of cytochrome-c-oxidase at the electron microscopy level. In: Attardi G, Chomyn A, Abelson J, Simon M, edi- tors. Methods in enzymology: mitochondr- ial genetics and biogenesis. New York: Academic Press; 1996. pp. 540–55.*
5. ***Muller-Hocker J, Droste M, Kadenbach B, et al.*** *Fatal mitochondrial myopathy with cytochrome-c-oxidase deficiency and subunit-restricted reduction of enzyme protein in two siblings: an autopsy- immunocytochemical study. Hum Pathol. 1989; 20: 666–72.*
6. ***Muller-Hocker J.*** *Defects of the respira- tory chain in hepatic oncocytes. Virchows Arch. 1998; 432: 349–56.*
7. ***Muller-Hocker J, Schafer S, Li K.*** *Immunocytochemical localization of mito- chondrial single-stranded DNA-binding protein in mitochondria-rich cells of nor- mal and neoplastic human tissue. Appl Immunohistochem Mol Morphol. 2001; 9:*

*276–80.*

1. ***Fischer JC, Ruitenbeek W, Gabreels FJ, et al.*** *A mitochondrial encephalomyopa- thy: the first case with an established defect at the level of coenzyme Q. Eur J Pediatr. 1986; 144: 441–4.*
2. ***Reichenbach J, Schubert R, Horvath R, et al.*** *Fatal neonatal-onset mitochondrial respiratory chain disease with T cell immunodeficiency. Pediatr Res. 2006; 60:*

*321–6.*

1. ***Setzer B, Schlesier M, Walker UA.*** *Effects of didanosine-related depletion of mtDNA in human T lymphocytes. J Infect Dis. 2005; 191: 848–55.*
2. ***Mandel H, Szargel R, Labay V, et al.*** *The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. Nat Genet. 2001; 29:*

*337–41.*

1. ***Muller-Hocker J, Aust D, Rohrbach H, et al.*** *Defects of the respiratory chain in the normal human liver and in cirrhosis during aging. Hepatology. 1997; 26: 709–19.*
2. ***Muller-Hocker J, Seibel P, Schneiderbanger K, et al.*** *Different in situ hybridization patterns of mitochondrial DNA in cytochrome c oxidase-deficient extraocular muscle fibres in the elderly. Virchows Arch A Pathol Anat Histopathol. 1993; 422: 7–15.*
3. ***Muller-Hocker J, Seibel P, Schneiderbanger K, et al.*** *In situ hybridization of mitochondrial DNA in the heart of a patient with Kearns-Sayre syn- drome and dilatative cardiomyopathy. Hum Pathol. 1992; 23: 1431–7.*
4. ***Seibel P, Mell O, Hannemann A, et al.*** *A method for auantitative analysis of detected mitochondrial DNA by PCR in small tissue samples. Meth Mol Cell Biol. 1991; 2: 147–53.*
5. ***Tanji K, Bhagat G, Vu TH, et al.*** *Mitochondrial DNA dysfunction in onco- cytic hepatocytes. Liver Int. 2003; 23:*

*397–403.*

1. ***Graziewicz MA, Longley MJ, Bienstock RJ, et al.*** *Structure-function defects of human mitochondrial DNA polymerase in autosomal dominant progressive external ophthalmoplegia. Nat Struct Mol Biol. 2004; 11: 770–6.*
2. ***Yakubovskaya E, Chen Z, Carrodeguas JA, et al.*** *Functional human mitochondrial DNA polymerase gamma forms a het- erotrimer. J Biol Chem. 2006; 281:*

*374–82.*

1. ***Longley MJ, Clark S, Yu Wai Man C, et al.*** *Mutant POLG2 disrupts DNA poly- merase gamma subunits and causes progressive external ophthalmoplegia. Am J Hum Genet. 2006; 78: 1026–34.*
2. ***Tzoulis C, Engelsen BA, Telstad W, et al.*** *The spectrum of clinical disease caused by the A467T and W748S POLG mutations: a study of 26 cases. Brain. 2006; 129:*

*1685–92.*

1. ***Nguyen KV, Sharief FS, Chan SS, et al.*** *Molecular diagnosis of Alpers syndrome. J Hepatol. 2006; 45: 108–16.*
2. ***Moraes CT, Shanske S, Tritschler HJ, et al.*** *mtDNA depletion with variable tissue expression: a novel genetic abnor- mality in mitochondrial diseases. Am J Hum Genet. 1991; 48: 492–501.*
3. ***Saada A, Shaag A, Elpeleg O.*** *mtDNA depletion myopathy: elucidation of the tis- sue specificity in the mitochondrial thymi- dine kinase (TK2) deficiency. Mol Genet Metab. 2003; 79: 1–5.*
4. ***Santorelli FM, Gagliardi MG, Dionisi-Vici C, et al.*** *Hypertrophic cardiomyopathy and mtDNA depletion. Successful treatment with heart transplantation. Neuromuscul Disord. 2002; 12: 56–9.*
5. ***Wang L, Limongelli A, Vila MR, et al.*** *Molecular insight into mitochondrial DNA depletion syndrome in two patients with novel mutations in the deoxyguanosine kinase and thymidine kinase 2 genes. Mol Genet Metab. 2005; 84: 75–82.*
6. ***Barthelemy C, Ogier de Baulny H, Diaz J, et al.*** *Late-onset mitochondrial DNA depletion: DNA copy number, multiple deletions, and compensation. Ann Neurol. 2001; 49: 607–17.*
7. ***Durham SE, Bonilla E, Samuels DC, et al.*** *Mitochondrial DNA copy number threshold in mtDNA depletion myopathy. Neurology. 2005; 65: 453–5.*
8. ***Bonod-Bidaud C, Chevrollier A, Bourasseau I, et al.*** *Induction of ANT2 gene expression in liver of patients with mitochondrial DNA depletion. Mitochondrion. 2001; 1: 217–24.*
9. ***Ducluzeau PH, Lachaux A, Bouvier R, et al.*** *Progressive reversion of clinical and molecular phenotype in a child with liver mitochondrial DNA depletion. J Hepatol. 2002; 36: 698–703.*
10. ***Vila MR, Segovia-Silvestre T, Gamez J, et al.*** *Reversion of mtDNA depletion in a patient with TK2 deficiency. Neurology. 2003; 60: 1203–5.*
11. ***Elpeleg O.*** *Inherited mitochondrial DNA depletion. Pediatr Res. 2003; 54: 153–9.*
12. ***Elpeleg O, Mandel H, Saada A.*** *Depletion of the other genome-mitochondrial DNA depletion syndromes in humans. J Mol Med. 2002; 80: 389–96.*
13. ***Graziewicz MA, Day BJ, Copeland WC.*** *The mitochondrial DNA polymerase as a target of oxidative damage. Nucleic Acids Res. 2002; 30: 2817–24.*
14. ***Lewis W, Copeland WC, Day BJ.*** *Mitochondrial dna depletion, oxidative stress, and mutation: mechanisms of dys- function from nucleoside reverse transcrip- tase inhibitors. Lab Invest. 2001; 81:*

*777–90.*

1. ***Taanman JW, Kateeb I, Muntau AC, et al.*** *A novel mutation in the deoxyguanosine kinase gene causing depletion of mito- chondrial DNA. Ann Neurol. 2002; 52:*

*237–9.*

1. ***Clayton DA.*** *Replication and transcription of vertebrate mitochondrial DNA. Annu Rev Cell Biol. 1991; 7: 453–78.*
2. ***Fisher RP, Lisowsky T, Parisi MA, et al.***

*DNA wrapping and bending by a*

*mitochondrial high mobility group-like transcriptional activator protein. J Biol Chem. 1992; 267: 3358–67.*

1. ***Muller-Hocker J, Schafer S, Copeland WC, et al.*** *Immunohistochemical detection of human mtDNA polymerase gamma and of human mitochondrial transcription factor A in cytochrome-c- oxidase-deficient oxyphil cells of hyper- functional parathyroids. Virchows Arch. 1998; 433: 529–36.*
2. ***Larsson NG, Oldfors A, Holme E, et al.*** *Low levels of mitochondrial transcription factor A in mitochondrial DNA depletion.*

*Biochem Biophys Res Commun. 1994; 200: 1374–81.*

1. ***Poulton J, Morten K, Freeman- Emmerson C, et al.*** *Deficiency of the human mitochondrial transcription factor h-mtTFA in infantile mitochondrial myopa- thy is associated with mtDNA depletion. Hum Mol Genet. 1994; 3: 1763–9.*
2. ***Black-Schaefer CL, McCourt JD, Poyton RO, et al.*** *Mitochondrial gene expression in Saccharomyces cerevisiae. Proteolysis of nascent chains in isolated yeast mito- chondria optimized for protein synthesis. Biochem J. 1991; 274: 199–205.*
3. ***Schultz RA, Swoap SJ, McDaniel LD, et al.*** *Differential expression of mitochon- drial DNA replication factors in mam- malian tissues. J Biol Chem. 1998; 273: 3447–51.*
4. ***Mandel H, Hartman C, Berkowitz D, et al.*** *The hepatic mitochondrial DNA depletion syndrome: ultrastructural changes in liver biopsies. Hepatology. 2001; 34: 776–84.*
5. ***Roels F, Verloo P, Eyskens F, et al.*** *Mitochondrial mosaics in the liver of 3 infants with mtDNA defects. BMC Clin Pathol. 2009; 9: 4.*