

## Pediatric non-alcoholic steatohepatitis: The first report on the ultrastructure of hepatocyte mitochondria

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### Abstract

**AIM:** To investigate the ultrastructure of abnormal hepatocyte mitochondria, including their cellular and hepatic zonal distribution, in bioptates in pediatric non-alcoholic steatohepatitis (NASH).

**METHODS:** Ultrastructural investigations were conducted on biopsy liver specimens obtained from 10 children (6 boys and 4 girls) aged 2-14 years with previously clinicopathologically diagnosed NASH. The disease was diagnosed if liver biopsy revealed steatosis, inflammation, ballooned hepatocytes, Mallory hyaline, or focal necrosis, varying degrees of fibrosis in the absence of clinical, serological, or histological findings of infectious liver diseases, autoimmune hepatitis, metabolic liver diseases, or celiac disease. For ultrastructural analysis, fresh small liver blocks (1 mm<sup>3</sup> volume) were

fixed in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer. The specimens were postfixed in osmium tetroxide, subsequently dehydrated through a graded series of ethanols and propylene oxide, and embedded in Epon 812. The material was sectioned on a Reichert ultramicrotome to obtain semithin sections, which were stained with methylene blue in sodium borate. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined using an Opton EM 900 transmission electron microscope.

**RESULTS:** Ultrastructural analysis of bioptates obtained from children with non-alcoholic steatohepatitis revealed characteristic repetitive mitochondrial abnormalities within hepatocytes; mainly mitochondrial polymorphisms such as megamitochondria, loss of mitochondrial cristae, and the presence of linear crystalline inclusions within the mitochondrial matrix of an increased electron density. The crystalline inclusions were particularly evident within megamitochondria (MMC), which seemed to be distributed randomly both within the hepatic parenchymal cell and the zones of hepatic lobule, without special variations in abundance. The inclusions appeared as bundles viewed longitudinally, or as an evenly spaced matrix in cross section, and frequently caused mitochondrial deformation. The average diameter of these linear structures was 10 nm and the average space between them 20 nm. Sometimes enlarged intramitochondrial granules were seen in their vicinity. Foamy cytoplasm of hepatocytes was found, resulting from the proliferation of smooth endoplasmic reticulum and glycogen accumulation. The perivascular space of Disse was frequently dilated, and contained transitional hepatic stellate cells, as well as mature and/or newly forming collagen fiber bundles.

**CONCLUSION:** Marked ultrastructural abnormalities observed in hepatocyte mitochondria, especially their polymorphism in the form of MMC and loss of mitochondrial cristae, accompanied by foamy cytoplasm,

clearly indicate a major role of these organelles in the morphogenesis of pediatric NASH. Our findings seem to prove the high effectiveness of electron microscopy in the diagnosis of the disease.

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**Key words:** Pediatric non-alcoholic steatohepatitis; Hepatocyte ultrastructure; Megamitochondria with crystalline inclusions; Foamy cytoplasm of hepatocytes

**Core tip:** Our electron-microscopic analysis of liver biopsies, being the first known investigation into pediatric non-alcoholic steatohepatitis (NASH), revealed characteristic repetitive mitochondrial abnormalities within hepatocytes, mainly mitochondrial polymorphism in the form of megamitochondria, loss of cristae, and the presence of linear crystalline inclusions within the mitochondrial matrix of increased electron density, frequently accompanied by foamy cytoplasm. As the discovery of these morphological indices of hepatocyte injury may be very useful in the diagnosis of NASH in pediatric patients, the ultrastructural analysis of liver biopsy can be recommended as an effective diagnostic method.

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## INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), regarded as a hepatic manifestation of metabolic syndrome, is a growing health problem worldwide both among children and adults. Importantly, the more severe form of this condition, non-alcoholic steatohepatitis (NASH), may progress to fibrosis, as well as cryptogenic cirrhosis and its complications, including hepatocellular carcinoma<sup>[1-6]</sup>. Unfortunately, the pathogenesis of NASH still remains unknown.

The last decade has brought increasing evidence that mitochondrial dysfunction, more specifically respiratory chain deficiency, plays a causative role in the pathophysiology of NASH, whatever its initial cause<sup>[2,7-14]</sup>. Basaranoglu *et al*<sup>[2]</sup> have noted that the pathogenesis of NASH is multifactorial, and that excess intracellular fatty acids, oxidant stress, ATP depletion, and mitochondrial dysfunction are essential for hepatocyte injury in this condition.

Despite clinical symptoms, laboratory data, and imaging findings, the microscopic analysis of liver biopsies still remains the golden standard for the diagnosis of non-alcoholic steatohepatitis<sup>[4,7]</sup>.

Histopathological investigations of liver biopsy specimens from NASH patients have revealed, aside from steatosis accompanied by lobular inflammatory infiltration, evidence of hepatocyte injury in the absence of clinical, serological, or histological findings of infectious liver diseases, autoimmune hepatitis, metabolic liver disease, or celiac disease<sup>[1,4,7,8,15]</sup>. Damage to liver parenchymal cells have been demonstrated in the form of hydropic change (ballooning cell injury), Mallory's hyaline bodies, and focal lytic necrosis causing sinusoidal distortion, and reduced intrasinusoidal volume and microvascular blood flow<sup>[1,3,4,7,8,15-17]</sup>.

It has been reported that in the course of non-alcoholic steatohepatitis, cell types other than hepatocytes are involved, *i.e.*, non-parenchymal hepatic cells: hepatic stellate cells (HSCs), Kupffer cells, sinusoidal endothelial cells, and inflammatory cells and platelets. This leads to deregulation of microvascular blood flow and may result in pericellular/perisinusoidal fibrosis and cirrhosis<sup>[5,6,17-19]</sup>. However, the diagnostic criteria of NASH have not yet been established.

In spite of increasing interest in the morphogenesis of non-alcoholic steatohepatitis, there are only a few reports, mainly limited to adult patients, on the ultrastructure of hepatocytes in this pathology<sup>[20-24]</sup>. We could not find any other publications to compare to the current findings, apart from our Letter to the Editor of the *American Journal of Gastroenterology*<sup>[25]</sup> on the ultrastructure of hepatocyte mitochondria in five children with NASH published 10 years ago.

Thus, the study objective was to analyze the image of abnormal electron microscopic findings observed in the hepatocytes of liver biopsy material collected from a larger group of children with clinicopathologically diagnosed NASH. The search for repetitive specific ultrastructural indices of this disease in liver biopsies would facilitate its early morphological diagnosis, which may result in undertaking targeted therapy.

## MATERIALS AND METHODS

### Patients

This study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Ultrastructural investigations were conducted on liver specimens obtained by needle biopsy from 10 children (6 boys and 4 girls) aged 2-14 years (mean 5 years) with previously clinicopathologically diagnosed NASH. Laboratory tests revealed increased serum alanine transaminase activity, and ultrasound examination showed liver brightness in all study patients. NASH was diagnosed if the liver biopsy revealed steatosis, inflammation, ballooned hepatocytes, Mallory hyaline, focal necrosis, or varying degrees of fibrosis in the absence of clinical, serological, or histological findings of infectious liver diseases (hepatitis B, hepatitis C, cytomegalovirus, or *Toxoplasma gondii*), autoimmune hepatitis, metabolic liver diseases (Wilson's disease, cystic fibrosis, galactosemia, or

alpha 1-antitrypsin deficiency), and celiac disease. Alcohol consumption was excluded in all the children.

The study was carried out in the Department of Medical Pathomorphology, Medical University of Białystok, by an electron microscopist blinded to the clinical information.

### Ultrastructural analysis

For transmission electron microscopic investigations, fresh small tissue blocks (1 mm<sup>3</sup> volume) from the liver biopsies were fixed in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) at room temperature. Subsequently, the specimens were postfixed in 2% osmium tetroxide (OsO<sub>4</sub>) in 0.1 mol/L cacodylate buffer (pH 7.4) for 1 h. The material was then dehydrated through a graded series of ethanols and propylene oxide, embedded in Epon 812, and sectioned on a Reichert ultramicrotome (Reichert Ultracut S) to obtain semithin sections. Next, the sections were stained with 1% methylene blue in 1% sodium borate and preliminarily examined under a light microscope to select Epon blocks. The blocks were then used to prepare ultrathin sections ca. 75 nm thick, which were placed on grids, double stained with uranyl acetate and lead citrate, examined, and photographed *via* an Opton EM 900 transmission electron microscope (Zeiss, Oberkochen, Germany). This processing procedure had been used in our earlier ultrastructural investigations of the liver in children<sup>[18,25,26]</sup>.

## RESULTS

Out of 10 clinicopathologically diagnosed cases of pediatric NASH, nine presented with repetitive mitochondrial abnormalities observed within hepatic parenchymal cells under transmission electron microscope. In 7 patients, the majority of mitochondria were affected, especially within ballooned hepatocytes; in 2 cases the abnormalities were found in a smaller number of hepatic parenchymal cells. The mitochondrial lesions showed no particular differences in quality or topography. They were similarly pronounced in the organelles located both in the vicinity of the cell nucleus, at a certain distance from the nucleus, and on the cell periphery (Figure 1).

The major submicroscopical exponent was the polymorphism of these organelles, with the presence of the so-called megamitochondria, accompanied by variously pronounced defects of the cristae, including their complete loss and increased electron-density of the matrix to its condensation (Figure 1). Frequently, remnants of such cristae, resembling bristles, were found on the periphery of enlarged organelles.

The characteristic feature of the ultrastructure of the altered mitochondria, especially megamitochondria, was the presence of intramitochondrial linear crystalline inclusions within the changed matrix, seen as long parallel strands that often caused deformation of these organelles. They appeared as bundles when viewed longitudinally,

or as an evenly-spaced matrix in cross section (Figure 1). The average diameter of these linear structures was 10 nm and the average space between them 20 nm. Sometimes in their vicinity there were enlarged intramitochondrial dense granules (Figure 1C, E, F).

The megamitochondria with characteristic crystalline inclusions (MMC) seemed to be distributed randomly, both within the hepatic parenchymal cell and the zones of the hepatic lobule, without special variations in abundance. They were found in close vicinity to lipid material (Figure 1C, E, F), and also in hepatocytes with no distinct features of steatosis.

We also observed a wide range of changes in hepatocellular ultrastructure, including the foamy cytoplasmic appearance resulting from smooth endoplasmic reticulum proliferation (Figure 1A, B, D) and glycogen accumulation (Figure 1C, E, F), mainly in the nuclear regions (Figure 1C). Heterogeneous lipofuscin pigment granules of various size and shapes were occasionally found.

The sinusoids with the adherent vascular pole of hepatocytes that showed distinct mitochondrial lesions had markedly swollen endothelial linings (Figure 1A). Kupffer cells in the sinusoidal lumen were considerably activated, sometimes showing features of erythrophagocytosis (their morphology in children with NASH was described in our earlier paper)<sup>[18]</sup>.

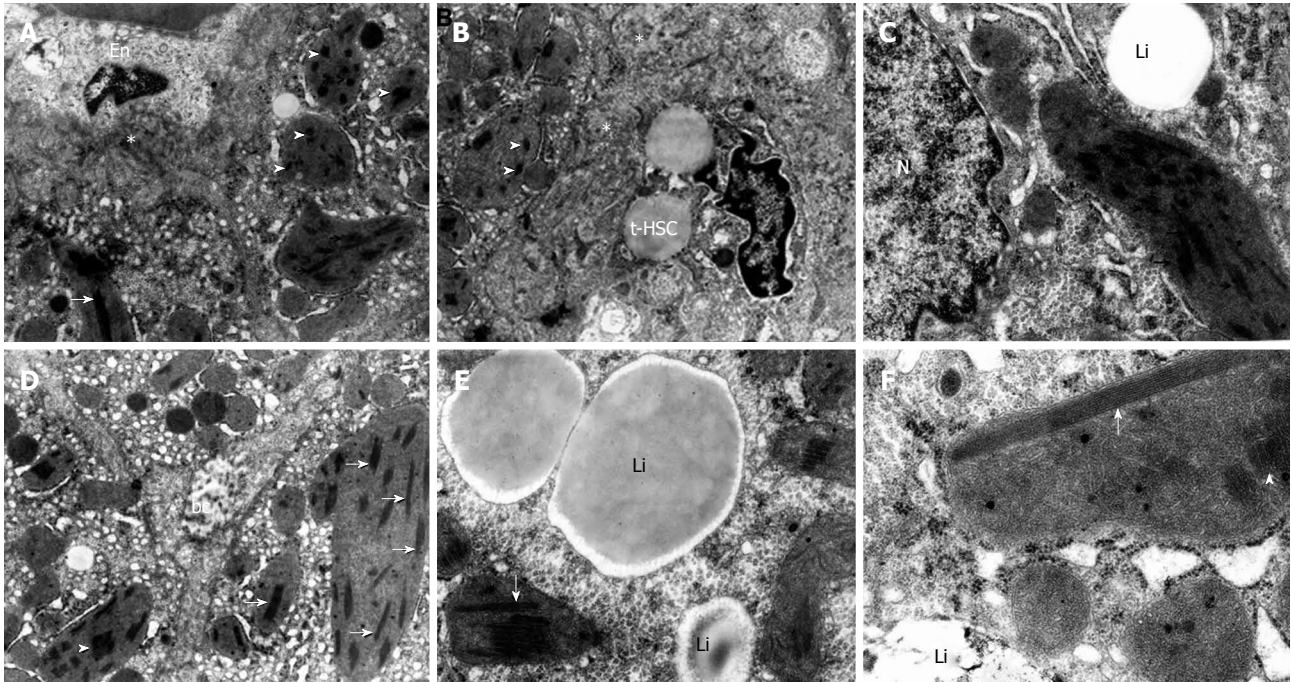
The perivascular space of Disse was frequently dilated and contained transitional (*i.e.*, activated) hepatic stellate cells (t-HSCs) (Figure 1B), deposits of electron-dense material, as well as mature and/or collagen forming fiber bundles (Figure 1A, B).

## DISCUSSION

Although light microscopy is routinely used to evaluate liver biopsy specimens from NASH patients, the ultrastructural investigations that make the diagnosis more profound are conducted in a limited number of cases, especially with respect to children. No reports have been published, apart from those in our center, on the electron-microscopic picture of liver biopsies from pediatric patients with NASH. We focused not only on hepatocytes<sup>[25,27]</sup>, but also on the changes concerning non-parenchymal liver cells, especially Kupffer and liver progenitor/oval cells<sup>[18,26,28]</sup>.

The current ultrastructural observations of hepatocytes obtained from pediatric NASH biopsies were consistent with our preliminary findings presented recently at the 186<sup>th</sup> Falk Symposium<sup>[27]</sup>, as well as in the previously mentioned Letter to the Editor<sup>[25]</sup>. The studies revealed numerous mitochondrial abnormalities similar to those found in adults<sup>[20,24]</sup>, including polymorphisms such as megamitochondria, loss of cristae, increased electron-density of the matrix, and the presence of characteristic linear crystalline inclusions within the matrix. The megamitochondria with crystalline inclusions were seen as parallel strands, and reduced cristae were the major repetitive ultrastructural abnormalities in pediatric NASH cases





**Figure 1** Ultrastructural appearance of polymorphic hepatic mitochondria, with megamitochondria at the foreground, containing linear crystalline inclusions and reduced cristae in bioptates from different pediatric patients with non-alcoholic steatohepatitis. Abnormal mitochondria distributed randomly in topographically varied parts of hepatocytes. A-F: Linear inclusions - cut longitudinally (arrows) and in cross section (arrowheads) in the matrix of moderately or markedly increased electron density in the majority of altered mitochondria, especially elongated and rounded megamitochondria. A, B: Megamitochondria (MMC) located in the vascular pole of hepatocytes; the mitochondrial lesions are accompanied by the proliferation of smooth endoplasmic reticulum; considerably swollen endothelial cell (En) of the sinusoidal vessel; transitional hepatic stellate cells (t-HSC), accumulation of electron-dense material, and collagen formed (asterisk) in the dilated space of Disse (original magnification  $\times 7000$ ); C: The MMC located in the nuclear region of hepatocyte is accompanied by glycogen accumulation; the megamitochondrion shows enlarged intramitochondrial dense granules in the vicinity of linear crystalline inclusions; hepatocyte nucleus (N); lipid material (Li) within hepatocyte cytoplasm (original magnification  $\times 12000$ ); D: MMC distributed randomly within the biliary pole of hepatocytes accompanied by the proliferation of smooth endoplasmic reticulum; bc - biliary canalculus (original magnification  $\times 7000$ ); E, F: MMC located at a certain distance from the nucleus of hepatocyte; mitochondrial abnormalities are accompanied by glycogen accumulation; some MMC show enlarged intramitochondrial dense granules in the vicinity of linear crystalline inclusions; lipid material (Li) (original magnification  $\times 12000$ ,  $\times 20000$ , respectively).

analyzed in the current study. These MMC seemed to be distributed randomly, both within the hepatocytes and the zones of the hepatic lobule.

In addition to mitochondrial alterations, we quite frequently observed foamy cytoplasm of hepatocytes resulting from the proliferation of smooth endoplasmic reticulum and glycogen accumulation, which was also reported by Ahishai *et al*<sup>[20]</sup> in adult patients with NASH. The space of Disse was frequently dilated and contained t-HSCs, which were accompanied by electron-dense material and collagen accumulation. However, the sinusoids were lined with distinctly swollen endothelium and markedly activated Kupffer cells.

The mitochondrial abnormalities showing varying degrees of cristae loss and increased matrix density evidently indicate decreased intra-mitochondrial protein synthesis and respiratory chain dysfunction, which was also emphasized by Ahishali *et al*<sup>[20]</sup> with respect to adult patients with NASH. However, according to Caldwell *et al*<sup>[21]</sup> and Le *et al*<sup>[22]</sup>, the formation of megamitochondria with crystalline inclusions of unknown composition may, in patients with NASH, represent an adaptive process to oxidative stress rather than a secondary injury.

The formation of crystalline inclusions in the mitochondrial matrix has been noted in a variety of other con-

ditions. Similar crystal-like structures have been reported mainly in the early phase of alcoholic steatohepatitis or in copper metabolism disorders such as Wilson's disease<sup>[29,30]</sup>. As already mentioned, they have been recently described in non-alcoholic fatty liver in adult patients, where their presence is correlated indirectly with oxidative stress<sup>[20,24]</sup>, and their absence is noted when cirrhosis develops<sup>[21,24]</sup>. According to Le *et al*<sup>[22]</sup>, as well as in our own observations concerning children, in NASH the MMC seem to be distributed randomly within the hepatic zones and without variation in abundance, regardless of the stage of fibrosis. In contrast, rodent models of NASH do not develop mitochondrial crystalline inclusions. The major electron-microscopic changes in rodents include severe mitochondrial swelling with coexisting proliferation of the smooth endoplasmic reticulum and abnormal granular endoplasmic reticulum<sup>[31]</sup>.

Considering the role of mitochondria in the pathophysiology of NASH, it has been emphasized that hepatic oxidative stress increases the release of lipid peroxidation products, as reflected by increased fatty acid beta-oxidation and cytokines, which together trigger liver lesions<sup>[2,7,9,10,12,13,19,24]</sup>.

In conclusion, this is the first full report on the hepatocyte ultrastructure in pediatric NASH. In our study, the

most significant repetitive electron microscopic alterations in this pathology involved the appearance of randomly distributed megamitochondria within hepatocytes, with characteristic crystalline inclusions seen as parallel strands and reduced mitochondrial cristae, including their complete loss. Additionally, foamy cytoplasm of liver parenchymal cells was found, resulting from the proliferation of smooth endoplasmic reticulum and glycogen accumulation. The submicroscopic changes observed in hepatocytes, especially mitochondrial abnormalities, may play a major role in the morphogenesis of pediatric non-alcoholic steatohepatitis.

We hope that similar reports will appear in other centers to provide comparative material for our current study in children. The discovery of submicroscopic repetitive indices of damage to liver parenchymal cells, specific to this pathology, may have both diagnostic and therapeutic implications for the treatment of fatty liver disease in pediatric patients.

Considering the great usefulness of transmission electron microscopy in the diagnosis of NASH in children, ultrastructural analysis of liver biopsies should be recommended as an effective diagnostic tool, at least in diagnostically difficult cases, despite its high costs and effort.

## COMMENTS

### Background

Despite increasing interest in non-alcoholic steatohepatitis (NASH), there are only a few reports on the hepatocyte ultrastructure in this pathology. This manuscript is the first full original paper known to be conducted on biopsy material in the course of this pathology in children.

### Innovations and breakthroughs

The main objective of the study was ultrastructural analysis of the image of abnormal hepatocyte mitochondria, including their cellular and hepatic zonal distribution in biopsy material in clinicopathologically diagnosed NASH in children. The electron microscopic investigations revealed characteristic mitochondrial structural defects within hepatocytes, mainly mitochondrial polymorphism (megamitochondria), loss of mitochondrial cristae, and the presence of linear crystalline inclusions within the mitochondrial matrix of increased electron density. The crystalline inclusions were particularly evident within megamitochondria. The megamitochondria with characteristic inclusions (MMC) seemed to be distributed randomly, both within the cell and in the hepatic zones, without any special variation in abundance. In addition to mitochondrial alterations, the authors could quite frequently observe foamy cytoplasm of hepatocytes resulting from the proliferation of smooth endoplasmic reticulum and glycogen accumulation.

### Applications

The authors' ultrastructural investigations indicate that pediatric NASH is associated with marked abnormalities in hepatocyte mitochondria, especially their polymorphism in the form of MMC, as well as the loss of mitochondrial cristae accompanied by foamy cytoplasm of hepatocytes. These findings seem to prove the high effectiveness of electron microscopy in the diagnosis of the disease.

### Peer review

This study appears to be properly conducted from a technical view-point. The conclusions of the authors are supported by the data reported.

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