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Úloha lipidů v patogenezi jaterních onemocnění

The role of lipids in the pathogenesis of liver diseases

Disertační práce/Doctoral thesis

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Abstrakt

V předkládané práci jsem se zaměřil na studium úlohy lipidů v patogenezi jaterních onemocnění, konkrétně cholestázy a nealkoholického postižení jater při steatóze (NAFLD). Hlavním cílem bylo objasnit změny metabolismu gangliosidů v játrech u různých typů cholestázy a popsat význam hemoxygenázy-1 (HMOX1) a přidruženého oxidačního stresu. Druhým cílem bylo popsání účinků n-3 polynenasycených mastných kyselin (n-3 PUFA) na patogenezi NAFLD u myšího modelu a u pacientů s metabolickým syndromem a NAFLD.

Naše výsledky naznačují, že zvýšení biosyntézy gangliosidů a jejich redistribuce může představovat obecný obranný mechanismus hepatocytů při cholestáze (jak indukované estrogeny, tak i obstrukčního typu). Tyto změny úzce souvisejí s oxidačním stresem a mohou chránit hepatocyty před škodlivým účinkem nahromaděných žlučových kyselin. Nedostatečná aktivita HMOX1 a následný oxidační stres potencují patologické změny jaterního parenchymu a vedou ke tkáňově specifické modulaci syntézy a redistribuci gangliosidů (*in vivo* a *in vitro*). Naproti tomu má aktivace HMOX1 opačný účinek a může působit hepatoprotektivně. Dokázali jsme, že pozorované změny mohou být částečně zprostředkovány modulací aktivity proteinkinázy C.

Výsledky druhé části práce prokazují příznivé účinky suplementace n-3 PUFA na patogenezi NAFLD. *In vivo* experimenty na myším modelu odhalily příznivé účinky n-3 PUFA na histopatologické změny, sérové markery jaterního poškození a složení lipidů v plazmě i játrech. Ve dvojitě zaslepené, randomizované, placebem kontrolované studii jsme u pacientů s NAFLD zaznamenali významné snížení aktivity γ-glutamyl transferázy v séru po 12-ti měsících podávání n-3 PUFA, stejně jako změny plazmatického lipidomu. Z dosažených výsledků usuzujeme, že n-3 PUFA mohou představovat potenciální agens v prevenci a léčbě tohoto stále častěji se vyskytujícího onemocnění.

Klíčová slova: gangliosidy, hemoxygenáza, cholestáza, játra, n-3 PUFA, NAFLD.

Abstract

In this thesis I have focused on the role of lipids in the pathogenesis of liver diseases, specifically on cholestasis and non-alcoholic fatty liver disease (NAFLD). The first major aim was to clarify the changes in liver ganglioside metabolism in various types of cholestasis and to elucidate the role of heme oxygenase-1 (HMOX1) and associated oxidative stress. The second objective was to determine the effects of n-3 polyunsaturated fatty acids (n-3 PUFA) administration on NAFLD development in a rodent dietary model of NAFLD and in patients with metabolic syndrome and NAFLD.

Our results suggest that increased ganglioside biosynthesis and their re-distribution might represent a general protective mechanism of hepatocytes under cholestatic conditions (both estrogen-induced and obstructive aetiology). These changes are closely related to oxidative stress and might protect hepatocytes against deleterious effect of accumulated bile acids. The lack of HMOX1 activity and subsequent oxidative stress potentiate pathological changes in the liver and resulted in tissue-specific modulation of synthesis and re-distribution of gangliosides (*in vivo* and *in vitro*). Contrary to it, HMOX1 activation has an opposite effect and may represent a general hepatoprotective mechanism. We have proven that observed changes might be, at least partly, mediated through modulation of protein kinase C activity.

Secondly, we have demonstrated beneficial effects of n-3 PUFA supplementation on NAFLD development. *In vivo* experiments revealed the favourable effects of n-3 PUFA on histopathological changes, serum markers of liver damage, fatty acid compound of plasma and liver and show anti-inflammatory properties. In our double blind randomised placebo-controlled study, we observed significant decrease in γ-glutamyl transferase serum activity after 12 months of n-3 PUFA administration as well as changes in plasma lipidome. Based on our results, we conclude that n-3 PUFA could represent a potential promising way in prevention and treatment of this increasingly common disorder.

Key words: cholestasis, gangliosides, heme oxygenase, liver, n-3 PUFA, NAFLD.

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1. INTRODUCTION

This thesis is focused on the role of lipids in development of liver injury including cholestatic liver diseases and non-alcoholic fatty liver disease (NAFLD). The liver plays pivotal role in energy and lipid metabolism. Many lipid compounds including cholesterol, triacylglycerols (TAG) or lipoproteins are synthesized in the liver and alteration in their metabolism might contribute to development of chronic liver disorders. On the contrary, accumulation of excess fat in the liver tissue predispose to a pro-inflammatory state and subsequent development of fibrosis, which is considered a crucial factor in liver-related mortality.

During cholestasis, elevated concentrations of bile acids (BA) and other bile components are responsible for hepatocyte damage. Among others they affect the plasma membrane, which consists (dominantly) of three major classes of lipids: cholesterol, (glyco)phospholipids and sphingolipids. Last named complex lipids – sphingolipids – are key players in the resistance of plasma membrane against the detergent action of bile.

Moreover, disorders in lipid metabolism are crucial determinants for development of NAFLD considered the most frequent chronic liver disease of the present. The most important mechanism in lipid-mediated injury is dysregulation of lipid influx and outflux with conversion of inert species into potentially toxic mediators (Neuschwander-Tetri, 2010). The second factor in lipid-mediated injury is the activation of hepatic stellate cells (HSC; so-called *Ito cells* or lipocytes) (Wobser *et al.*, 2009) which are responsible for fibrogenesis (Kocabayoglu and Friedman, 2013). Equally principal factor is the type of digested lipids such as trans fats and cholesterol. They may be relevant for disease development, including inflammation, liver injury, and fibrosis (Mari *et al.*, 2006), (Kohli *et al.*, 2010).

1.1 LIPIDS

Lipids are molecules soluble in nonpolar solvents with unique physical-chemical properties. Despite their general hydrophobic character, some lipids possess amphipathic properties representing a key feature in their role as fundamental elements of cellular and organelle membranes. Lipids might be divided into several categories (Fahy *et al.*, 2009) and have broad spectrum of functions including: structural (membranes), storage of energy, signalling and others.

Fatty acids

Fatty acids (FA), or fatty acid residues consist of hydrocarbon chain, usually in the range from four to 24 carbons long, that terminates with a carboxyl group. The hydrocarbon chain can be either saturated or unsaturated and might be connected to functional groups including oxygen, nitrogen, sulphur and/or halogen. In unsaturated FA there is a possibility of either *cis* or *trans* isomerism. FA are synthesized by chain-elongation of an acetyl-CoA basis through the action of FA synthases and encompass a wide spectrum of metabolic functions. They are essential for energy storage, energy production, cell proliferation, metabolic homeostasis and for regulation of inflammatory processes. Bioactive FA are fundamental for cell signalling pathways as well as intermediates in the biosynthesis and structure of many complex lipids (e.g. phospholipids).

Glycerolipids

Glycerolipids are a group of compounds consisting of mono-, di-, and tri-substituted glycerols typically by different FA. Triacylglycerols (TAG) – FA esters of glycerol – represent the most abundant lipid group in mammals. Their biosynthesis starts at carbon 2 of the glycerol backbone when monoacylglycerols and diacylglycerols are created (Coleman and Lee, 2004).

Their leading role is energy store and releasing of glycerol and FA mainly from adipose tissue is the beginning step in utilizing fat.

Role of simple lipids in the pathogenesis of liver diseases - fatty acids as a major source of lipotoxicity

Several types of simple lipids including FA and glycerolipids from diet and visceral adipose tissue are delivered to the liver by the portal vein. There is extensive body of evidence indicating that excessive free FA influx into the liver cause not only steatosis, but is associated also with chronic inflammation, organelle dysfunction (ER-stress, mitochondria damage, oxidative stress), cell injury and/or cell death. This dysregulation of a lipid environment and/or intracellular lipid composition leading to an accumulation of harmful lipids is called *lipotoxicity* (Schaffer, 2003). Recent data suggest that total amount of triglycerides stored in hepatocytes is not the major determinant of lipotoxicity (Listenberger *et al.*, 2003), (McClain *et al.*, 2007). On the contrary, dietary FA and other specific lipid compounds (cholesterol, lysophosphatidylcholine, ceramides) are the key elements in lipotoxicity-induced liver damage as well as insulin resistance (Alkhouri *et al.*, 2009), (Mattace Raso *et al.*, 2013), (Marra and Svegliati-Baroni, 2018). Accumulated lipids are primarily neutral lipids stored in dynamic organelles called lipid droplets (lipid bodies, adiposomes) with own lifecycle within the cell. These droplets provide storage of energy accumulated during periods of energy surplus and their abnormalities participate in liver disease development (Gluchowski *et al.*, 2017). Nearly two-thirds of accumulated fats come from free FA delivered to the liver (Donnelly *et al.*, 2005), (Lambert *et al.*, 2014). The origin of free FA is especially fatty tissue (dysfunctional and insulin-resistant adipocytes) (Barrows and Parks, 2006), which serves also as a source of many hormones, cytokines (interleukins, TNFα and others), chemokines and adipokines (including growth factors such as IGF-1,

TGFβ or VEGF). All these factors together with impaired FA metabolism contribute to the development and progression of many liver diseases, especially progression of NAFLD to NASH with subsequent fibrosis (Polyzos *et al.*, 2010). Many authors conclude that an activation of lipotoxic pathways is considered a pivotal event in the pathogenesis of NAFLD and NASH including: 1) an increased supply of free FA in increased lipolysis (subcutaneous/visceral fat) and/or increased dietary fat intake; 2) reduced FA oxidation; 3) increased *de novo* lipogenesis in the liver; 4) reduced VLDL secretion from the liver (Fabbrini *et al.*, 2008), (Lambert *et al.*, 2014).

Phospholipids

Phospholipids are ubiquitous lipid molecules responsible for creating lipid bilayer, the key components of all cell membranes. The phospholipid molecule usually consists of two hydrophobic FA tails and a hydrophilic phosphate group. They are regarded derivatives of phosphatidic acids classified in various distinct classes with many physiological functions. In human, they are most abundant in neural tissue. Glycerophospholipids, the glycerol-based phospholipids, are the main component of biological membranes. The most important glycerophospholipids are phosphatidylcholine (known as lecithin), phosphatidylethanolamine and phosphatidylserine. They are also cleaved to membranes and form second messengers including diacylglycerol, inositol trisphosphate or phosphatidylinositol 4,5-bisphosphate. Cardiolipin is a type of diphosphatidylglycerol found predominantly in the inner mitochondrial membrane where it is fundamental for proper function of many enzymes of mitochondrial energy metabolism.

Sphingolipids

Sphingolipids constitute a very heterogeneous and diverse group of lipid macromolecules, which counts hundreds of distinct species derived from their structural diversity. Their basal building block is long-chain sphingoid base – sphingosine (Carter *et al.*, 1947). Ceramide is the basal sphingolipid with a fatty acid attached via an amine bond in the C2 position. More complex sphingolipids are created by attaching various groups in the C1 position of ceramide. Combinations within long-chain sphingoid base, FA and head group variants are clarifying the bottomless amount of sphingolipid species. The most important groups of sphingolipids are phosphosphingolipids (sphingomyelins) and glycosphingolipids (cerebrosides, gangliosides and globosides) (Merrill, 2011).

The major function of sphingolipids is the formation of mechanically stable and chemically resistant outer leaflet of the plasma membranes by their ability to create microdomains called lipid rafts. Simple sphingolipids such as sphingosine-1-phosphate or ceramide are important bioactive mediators involved in differentiation, proliferation, inflammation, apoptosis and/or senescence (Hannun and Obeid, 2008), (Bartke and Hannun, 2009). Complex sphingolipids (glycosphingolipids) have also specific functions such as cell signalling, cell-cell interaction and recognition, which explain their predominant occurrence in the central nervous system (Yu *et al.*, 2012), (Olsen and Faergeman, 2017). Moreover, gangliosides, a subgroup of glycosphingolipids, are present in other cell types and have many functions under physiological as well as pathological conditions.

1.2 GANGLIOSIDES

1.2.1 The structure of gangliosides

Glycosphingolipids (GSLs) are amphipathic molecules composed of a hydrophilic carbohydrate chain and a hydrophobic ceramide moiety that contains a sphingosine and a fatty acid residue (Hakomori, 1990). GSLs containing one or more sialic acid (N-acetylneuraminic

acid or N-glycolylneuraminic acid) residues in the carbohydrate chain are gangliosides; sialic acid differentiates gangliosides from neutral GSLs and sulfatides. Gangliosides are ubiquitously distributed in all vertebrates tissues, most abundantly occurred in the nervous system (Yu *et al.*, 2009). Diversity and heterogeneity of the structure, content, sequence, and connections in their oligosaccharide chains are characteristic features of gangliosides. So far, more than 180 gangliosides with distinct carbohydrate structure have been described in vertebrates (Yu *et al.*, 2012, Yu *et al.*, 2011). This complexity is increased multiply when heterogeneity in the lipophilic components is taken into account.

Gangliosides had been discovered by the German biochemist Ernst Klenk (1896 – 1971), who gave them the name "gangliosides". The detailed structure of a gangliosides was elucidated in 1963 (Kuhn and Wiegandt, 1963). In 1962, Svennerholm proposed a nomenclature of brain gangliosides (Svennerholm, 1963), (Svennerholm, 1964). Based on the order of the carbohydrate residues, GSLs are classified into series, including gala-, neogala-, ganglio-, globo-, isoglobo-, lacto-, neolacto-, lactoganglio-, and muco- series. Due to variability in the structure, two nomenclature systems of gangliosides were created: the short-hand nomenclature according to Svennerholm (based on the migration of gangliosides in chromatography (Svennerholm, 1963)) (see Tab. 1); and the more comprehensive IUPAC system (Chester, 1998). Both nomenclatures specify the glycan part of gangliosides. According to Svennerholm, the names of gangliosides contain information about the series ("G" = ganglio, "L" = lacto) and the amount of sialic acids ("A" = 0, "M" =1, "D" = 2, "T" = 3, "Q" = 4, "P" = 5) (Kolter, 2012).

The lipid portion of gangliosides shared with all sphingolipids is called ceramide (Fig. 1). It is formed by long-chain amino alcohol, 2-amino-1,3-dihydroxy-octadec-4-ene with trivial name sphingosine (Karlsson, 1970), connected to a FA by an amide linkage. Heterogeneity of gangliosides is not only found within the above-mentioned carbohydrate

structure	abbreviation
Neu5Acα2-3Galβ1Cer	GM4
Neu5Acα2,3Galβ1,4Glcβ1Cer	GM3
GalNAcβ1,4(Neu5Acα2,3)Galβ1,4Glcβ1Cer	GM2
Galβ1,3GalNAcβ1,4(Neu5Acα2,3)Galβ1,4Glcβ1Cer	GM1a
Neu5Acα2,3Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1Cer	GM1b
Neu5Acα2,8Neu5Acα2,3Galβ1,4Glcβ1Cer	GD3
GalNAcβ1,4(Neu5Acα2,8Neu5Acα2,3)Galβ1,4Glcβ1Cer	GD2
Neu5Acα2,3Galβ1,3GalNAcβ1,4(Neu5Acα2,3)Galβ1,4Glcβ1Cer	GD1a
Galβ1,3GalNAcβ1,4(Neu5Acα2,8Neu5Acα2,3)Galβ1,4Glcβ1Cer	GD1b
Neu5Acα2,8Neu5Acα2,3Galβ1,3GalNAcβ1,4(Neu5Acα2,3)Galβ1,4Glcβ1Cer	GT1a
Neu5Acα2,3Galβ1,3GalNAcβ1,4(Neu5Acα2,8Neu5Acα2,3)Galβ1,4Glcβ1Cer	GT1b
Galβ1,3GalNAcβ1,4(Neu5Acα2,8Neu5Acα2,8Neu5Acα2,3)Galβ1,4Glcβ1Cer	GT1c
Neu5Acα2,8Neu5Acα2,3Galβ1,3GalNAcβ1,4(Neu5Acα2,8Neu5Acα2,3)Galβ1,4GlcβCer	GQ1b

Table 1. *Ganglioside nomenclature according to Svennerholm (Svennerholm, 1963).*

portion but also within the ceramide structure in different sphingoid bases, respectively: sphinganine, sphingosine, and phytosphingosine of different chain lengths. (Pruett *et al.*, 2008). C18- and C20-sphingosine are the most common sphingoid bases of gangliosides in humans (Kolter, 2012). The functional implications of the diversity in the lipid part are largely unknown.

1.2.2 The biosynthesis of gangliosides

GSL, as well as gangliosides, are primarily synthesized in the endoplasmic reticulum (ER) and further modified in the Golgi apparatus. *De novo* biosynthesis of gangliosides can be suppressed by salvage processes, in which all molecules are recycled (Tettamanti *et al.*, 2003).

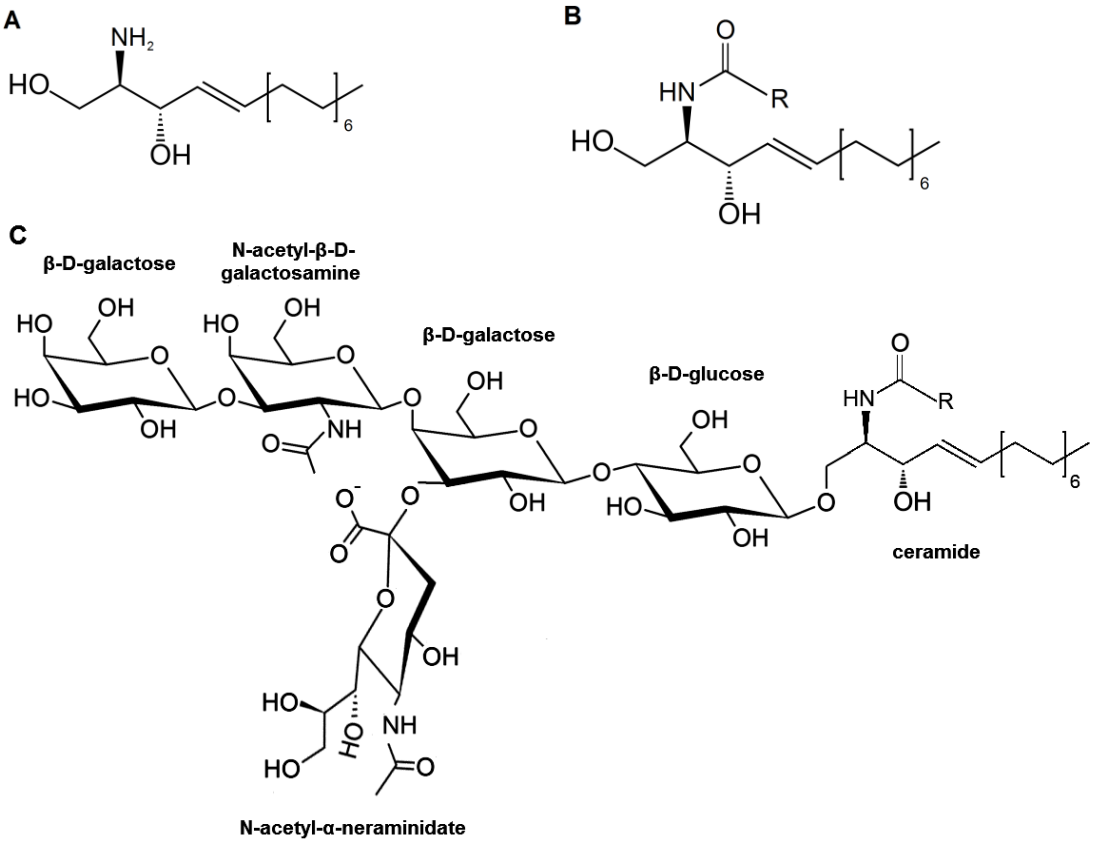

Figure 1. *Structure of sphingosine (A), ceramide (B) and GM1 ganglioside (C).*

Biosynthesis of gangliosides begins with the generation of ceramide. This process takes places in the cytoplasmic leaflet of the ER membrane (Mandon *et al.*, 1992), (van Meer and Hoetzl, 2010). Condensation of L-serine and a coenzyme A-activated FA is catalyzed by the pyridoxal phosphate-dependent serine palmitoyltransferase (EC 2.3.1.50) (Braun and Snell, 1968), (Ikushiro and Hayashi, 2011). The next step is the reduction of 3-ketosphinganine to sphinganine by 3-ketosphinganine reductase (EC 1.1.1.102), and subsequent acylation of sphinganine to dihydroceramides of variant chain lengths (sphingosine N-acyltransferase; 2.3.1.24) (Mandon *et al.*, 1991), (Mullen *et al.*, 2012). Dihydroceramides are dehydrogenated by the dihydroceramide desaturase des1 (EC 1.14.-.-) with the formation of ceramide (Geeraert *et al.*, 1997), or hydroxylated to phytoceramides by des2 (Fabrias *et al.*, 2012).

The synthesis of GSL continues by the membrane-bound glycosyltransferases in the Golgi apparatus. The major regulator of ceramide flux into the cell is CERT – ceramide transfer protein (Hanada, 2010). Ceramide, a precursor of sphingomyelin and all GSL, is sequentially coupled by monosaccharide units. Glycosidation is associated to exocytosis through the Golgi to the plasma membrane (Daniotti and Iglesias-Bartolome, 2011). The glycosylation sequence is precise, and is based on the specificity, compartmentalization and topology of the participating enzymes and its transport speed is based on rate of bulk vesicle flow (Young *et al.*, 1992).

Ceramide is a subject of various metabolic pathways. In the biosynthesis of gangliosides, ceramide is catalytically converted to glucosylceramide (GlcCer) through UDP-glucose:ceramide glucosyltransferase (Glc-T; EC 2.4.1.80) (Paul *et al.*, 1996) on the cytoplasmic face of Golgi (Coste *et al.*, 1986) and subsequently, after translocation across the membrane on the luminal side of Golgi (Lannert *et al.*, 1994), is converted to lactosylceramide (LacCer) through UDP-galactose:glucosylceramide galactosyltransferase (Gal-T1; EC 2.4.1.274), which transfers a galactose residue from UDP-Gal to GlcCer (Senn *et al.*, 1983). Formed LacCer is a general precursor of most glycolipids.

According to the amount of sialic acids coupled to the "inner" galactosyl residue, gangliosides of the ganglio-series are classified into four groups: *0*-, *a*-, *b*-, and *c-series*; which only *a*- and *b*- described in mammalian cells. The derivatives from LacCer are produced by the action of specific enzymes catalysing formation of complex gangliosides GM3, GD3, and GT3. These gangliosides are potentially converted to more complex gangliosides of the *0*-, *a*-, *b*- and *c*-series by sequential glycosylations catalysed by specific glycosyl- and sialyltransferases (Fig. 2) (Basu, 1991), (Furukawa, 2007), (Audry *et al.*, 2011). Their glycan chains are oriented extracytoplasmatically. Transferases catalysing the first step in the biosynthesis of gangliosides show strong specificity towards their glycolipid substrates

and therefore the precursors (LacCer, GM3, GD3, and GT3) regulate the amount of *0*-, *a*-, *b*-, and *c*-series of gangliosides.

The subsequent steps in the biosynthetic pathway represented by glycosyl- and sialyltransferases transfer the carbohydrates to acceptors from all series. It is known that these enzymes form specialized functional complexes (Giraudo and Maccioni, 2003) and might be excluded from membrane domains of the Golgi apparatus, known as the GSL-enriched microdomains (GEM) (Crespo *et al.*, 2004). Thus coupled enzymes reflect the functional state of the current cell (Uliana *et al.*, 2006) and ganglioside profile vary between different cell type/development phase. Gangliosides leave the Golgi via the luminal surface of the transport vesicles after synthesis (Tettamanti and Riboni, 1993), (Sandhoff and van Echten, 1994).

Biosynthesis of gangliosides is regulated by many factors at the transcriptional and post-transcriptional, translational and post-translational levels as well as by epigenetic modifications (Suzuki *et al.*, 2011). The key factor seems to be the transcriptional regulation of transferase genes (Yu *et al.*, 2004). But other factors play important role: spatial neighbourhood to other transferases, topological organization or kinetic parameters (Sandhoff and Harzer, 2013). As mentioned above, all these factors are influenced also by formation of functional complexes (Roseman, 1970) and are still intensively studied (Giraudo *et al.*, 2001), (Giraudo and Maccioni, 2003), (Spessott *et al.*, 2012). An additional level of regulation of ganglioside expression has been suggested - the plasma membrane-associated enzymes – sialidase called Neu3, and others with ß-hexosaminidase, ß-glucosidase and ß-galactosidase activities. All these enzymes are able to remodel the plasma membrane gangliosides (Sonnino *et al.*, 2010). In summary, due to high degree of complexity of glycolipid pattern, a paradigm of combinatorial biosynthesis was supposed - complex ganglioside are generated by so called “combinatorial biosynthetic pathways” (Kolter *et al.*, 2002), (Kolter, 2012).

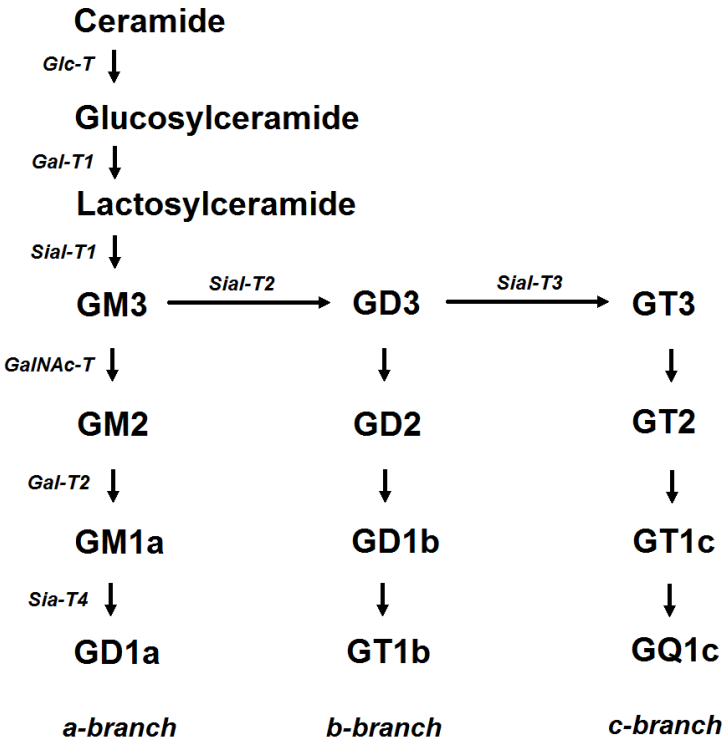

Figure 2. *Scheme of ganglioside biosynthesis.*

Transfer steps for synthesis of a-, b-, and c-series of gangliosides are shown. The relative amount of GM3, GD3 and GT3 represent the entry substrate for the biosynthesis of complex-type gangliosides. Further synthesis is regulated by transferases that catalyse the addition of various sugar residues to different ganglioside acceptor. These enzymes are identical for all branches. Ganglioside names are abbreviated according to Svennerholm.

Abbreviations: Glc-T, UDP-Glc: ceramide glucosyltransferase; Gal-T1, UDP-Gal: glucosylceramide galactosyltransferase; Sial-T1, CMP-NeuAc: lactosylceramide sialyltransferase; Sial-T2, CMP-NeuAc: GM3 sialyltransferase; Sial-T3, CMP-NeuAc: GD3 sialyltransferase; GalNAc-T, UDP-GalNAc: GM3/GD3/GT3 N-acetylgalactosaminyltransferase; Gal-T2, UDP-Gal: GM2/GD2/GT2 galactosyltransferase; Sial-T4, CMP-NeuAc: GM1/GD1b/GT1c sialyltransferase.

1.2.3 Degradation of gangliosides

The degradation of gangliosides takes place in endosomes and lysosomes and starts after the receptor-mediated endocytosis of the certain part of the plasma membrane. This process requires a combination of appropriate conditions – pH, presence of matching glycosidase and suitable composition of the ganglioside-containing membrane (Kolter and Sandhoff, 2010). Two membranes (perimeter and luminal) are present in lysosomes with different structure and function (Furst and Sandhoff, 1992), but only the intralysosomal (luminal) one is the site of lipid degradation. Degradation of gangliosides begins with the activity of above mentioned water-soluble glycosidases, which sequentially cleave off the terminal monosaccharide units of GSL in the presence of other factors – typically for GSL with short oligosaccharide chain – (pro)saposins, activating proteins, and transporting proteins (Schulze *et al.*, 2009).

This process is sequential and defects in this pathway – in participating enzymes and other proteins – are related to inherited diseases called lysosomal storage diseases. They can be classified according to the accumulated lipid substance – gangliosidoses, sphingolipidoses, mucopolysaccharidoses etc. For details of pathogenesis and therapy please see reviews (Xu *et al.*, 2010). (Schultz *et al.*, 2011), (Sandhoff and Harzer, 2013).

1.2.4 Functions of gangliosides

Gangliosides are ubiquitously found in all tissues but predominantly in the nervous system (Yu *et al.*, 2009), where their occurrence in grey matter is about 5-fold higher than in white matter. The total content of gangliosides seems to depend mainly on the proportions of grey and white matter. In adult human brain regions, the values range from 2 to 14 μg lipid-bound sialic acid/mg protein (Kracun *et al.*, 1984). In the brain, ganglioside expression is connected to the cell proliferation, synaptogenesis, neurogenesis, and synaptic transmission (Rahmann, 1995), (Wang, 2009).

In cells, gangliosides are primarily concentrated in the outer layer of the plasmatic membrane (Hakomori, 2003). Due to high heterogeneity of the oligosaccharide part should allow specific interactions between the plasma membrane surface and many proteins (Regina Todeschini and Hakomori, 2008). In general, functional roles of gangliosides can be divided into two groups: a) interactions outside the cell (by many authors called "trans" interactions), and b) within the same membrane ("cis" interactions). The glycan part of ganglioside which participates in sugar code of cell surface is for "trans" interactions. Due to these properties, gangliosides have irreplaceable role in cell-cell recognition and membrane protein regulation (Schnaar, 2004), (Lopez and Schnaar, 2009). These carbohydrate-to-carbohydrate interactions or interactions with lectins and adhesins are the basis of cell recognition (and membrane organization as well as intracellular signalling pathways) (Hakomori, 2004), (Bucior and Burger, 2004). Gangliosides work also as receptors for toxins and microbial pathogens which use sugar-binding proteins to recognize and bind to host glycoconjugates with high affinity (Imberty and Varrot, 2008). The best known example is the GM1 as the receptor for cholera toxin produced by *Vibrio cholerae*. Cholera toxin is made up of six protein subunits – a single A-subunit and 5 copies of the B-subunit making a pentamer. The B-subunit ring of cholera toxin binds GM1 ganglioside on the surface cells (predominantly in lipid rafts). This binding is highly specific and widely used in imunohistochemical techniques. Once bound, the entire toxin (A- and B-subunit) is endocytosed and A-subunit is responsible for toxic effects leading to well-known life-threatening dehydration (Sanchez and Holmgren, 2011).

"Cis" interactions are linked to membrane domains and gangliosides influence many pathways in this manner. Experimental data suggest that gangliosides are considered crucial molecules responsible for the rigidity of cell membranes (Pascher, 1976), (Harris *et al.*, 1978), (Pascher *et al.*, 1992). Gangliosides have unique physicochemical properties (geometry of the hydrophilic headgroups, hydrogen-bond network, carbohydrate–water linkage e.g.) and have

significant impact on membrane domain formation and organization (Sonnino *et al.*, 2006). Existence of domains with special composition and physical-chemical properties different from the surrounding membrane is supported by many authors. These domains – often called as caveolae, lipid rafts or detergent resistant microdomains (DRM, due to their inability of some detergents to solubilize them) – are involved in cell-cell recognition and adhesion, signal transduction, and play a pivotal role in stabilization and formation of plasma membrane (Pike, 2006), (Brown, 2006), (Lingwood and Simons, 2010), (Simons and Sampaio, 2011). The size and lifetime of rafts are not yet completely understood, but it is believed that gangliosides are encased into these domains together with cholesterol, sphingomyelin and GPI-anchored proteins (Cantu *et al.*, 2011). Gangliosides, respectively sialic and oligosialic acids are able to influence also membrane potentials (surface charge) and pH at the membrane surface (Salazar *et al.*, 2004), (Janas and Janas, 2011).

1.3 PATHOGENESIS OF CHOLESTASIS

Cholestasis is an impairment of bile secretion and/or flow, followed by a lack of bile in the intestine, and the accumulation of potentially toxic bile acids (BA) in the liver and systemic circulation (Paumgartner, 2006). This may originate either from a functional defect in bile production in the hepatocytes (hepatocellular cholestasis) or from an impairment in bile secretion and flow at the level of biliary tree (ductular/ductal cholestasis) (Trauner *et al.*, 1999). The production of bile is one of the most important functions of the liver. Many endogenous and exogenous substances are eliminated from the body by hepatocyte uptake, metabolized to less toxic and more water-soluble compounds, and excreted into the bile. Bile removes endogenous (toxic) compounds e.g. excess cholesterol, bilirubin and by-products of metabolism of various xenobiotics from the body. It also delivers bile salts to the small

intestine where they participate in the digestion and absorption of ingested lipids (Boyer, 2013).

1.3.1 Bile acid metabolism, transport and functions

BA are steroid acids with many physiological functions predominantly in the liver, biliary tree, and intestine. They are formed in the liver from cholesterol and their synthesis is a complex, multienzyme process. Uncharged and insoluble molecule of cholesterol is transformed to a bile acid molecule. This molecule, when ionized, is membrane dissolving, water-soluble detergent with amphipathic properties. This formation comprises enzymatic reactions localized in cytosol, microsomes, mitochondria, and peroxisomes. Two pathways are recognized – the classical and alternative one. In humans, the classical BA synthetic pathway is predominant, with only 10% of BA being produced via the alternative pathway (Axelson *et al.*, 2000); for details see review (Kullak-Ublick *et al.*, 2004). In the first step, two primary BA are synthesized – cholic and chenodeoxycholic acids. After their synthesis, BA are conjugated mostly with glycine or taurine. This conjugation makes them impermeable to cell membranes (in physiological pH), and allows high concentrations of BA in bile and intestine. (Monte *et al.*, 2009), (Hofmann, 1999a). The transmembrane transport of BA requires specialized membrane transporters in hepatocytes, cholangiocytes and enterocytes (Kullak-Ublick *et al.*, 2004). Under normal physiological conditions, monovalent BA are excreted into bile via the bile salt export pump (BSEP, *ABCB11*), an adenosine triphosphate (ATP)-binding cassette (ABC) transporter localized in the canalicular (apical) part of hepatocyte membrane (Meier and Stieger, 2002). Divalent (glucuronidated and sulphated) bile salts are transported by canalicular multidrug resistance-associated protein-2 (MRP2, *ABCC2*) (Akita *et al.*, 2001). MRP2 mediates also export of multiple organic anions including conjugated bilirubin, for details see review (Alrefai and Gill, 2007).

After excretion by canalicular (apical) part of hepatocyte membrane into bile canaliculus, primary bile is modified by cholangiocytes during its passage through the biliary tree. Major role in this modification plays the Na^+ dependent bile salt transporter (ASBT *SLC10A2*) (Lazaridis *et al.*, 1997), (Benedetti *et al.*, 1997). This first step of absorption of bile salts is called "cholehepatic shunt pathway" which has the regulatory effect of BA on cellular signalling mechanisms (Kullak-Ublick *et al.*, 2004).

The crucial role in BA homeostasis plays their reabsorption in terminal ileum. BA are returned to the liver and repeatedly secreted into bile. This continuous cycle of secretion, absorption, and resecretion is called the enterohepatic circulation of BA (Hofmann, 1976). Reabsorption is a major determinant of the BA pool size and tightly regulates the expression of key synthesizing enzymes CYP9A1 and CYP27 (Xu *et al.*, 2000). The reabsorption involves uptake into ileal epithelium via ileal bile acid transporter ASBT (*SLC10A2*), the shift to the basolateral membrane by intracellular ileal BA-binding protein (I-BABP) (Kramer *et al.*, 2001), and efflux by MRP3 (*ABCC3*), and organic solute transporter α and β (OST; *SLC51A and B*) into portal blood (Rost *et al.*, 2002). BA circulate tightly bound to albumin (Roda *et al.*, 1982) and lipoproteins (Wolkoff and Cohen, 2003). The final step – the uptake of BA into hepatocytes by sinusoidal (basolateral) part of hepatocyte membrane is predominantly mediated by Na^+ - taurocholate co-transporting polypeptide (NTCP, *SLC10A1*) (Hagenbuch and Meier, 1994), (Kullak-Ublick *et al.*, 2004). Next to the sodium-dependent uptake system is Na^+-independent hepatic uptake by the organic anion transporting polypeptides (OATPs) (Tirona and Kim, 2002) , in humans predominantly OATP-C (*SLC21A6*) and OATP8 (*SLC21A8*). (Hagenbuch and Meier, 2003).

In pathological conditions during cholestasis, BA are transported to the blood across the sinusoidal (basolateral) membrane of hepatocytes. This efflux is mediated by other

members of multidrug resistance-associated proteins, namely MRP3 (*ABBC3*) and MRP4 (*ABCC4*) (Trauner and Boyer, 2003) (Geier *et al.*, 2007).

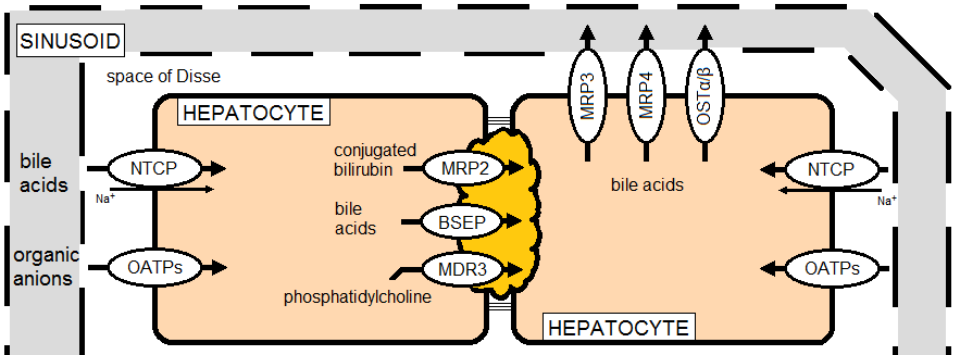

Figure 3. *Scheme of transport of bile acids and bilirubin in the hepatocyte*

From the sinusoidal blood, bile acids are transported by NTCP. BSEP facilitate their transport to the bile canaliculus at the apical (canalicular) membrane of hepatocytes.

Bilirubin is transported to the hepatocyte by OATP, then is conjugated in ER, and subsequently secreted into bile by MRP2. In addition, even under physiologic conditions, a fraction of bilirubin conjugates is secreted by MRP3 across the sinusoidal membrane into blood.

So called "shifting" or "hopping" of many compounds (as well as bile acids) by other transporters (OATPs predominantly) from periportal to centrizonal hepatocytes may serve as a protection of the periportal hepatocytes.

Many physiological functions of BA are well established. Best known is their participation in the digestion to help emulsification and absorption of dietary fats and liposoluble vitamins. Forming mixed micelles accelerate lipid absorption. The second role is the elimination of cholesterol from the body by its converting into BA and by micellar solubilization of cholesterol in bile, ultimately leading to elimination via faecal route. Additional functions of BA involve intestinal calcium uptake (Sanyal *et al.*, 1994), modulation of pancreatic enzyme secretion and cholecystokinin release (Koop *et al.*, 1996) and eliminating substances that cannot be efficiently excreted in urine because they are insoluble, or protein bound. Well known is also the antimicrobial action in the bowel that exerting differential antimicrobial activity against several strains and against fungi and preventing bacterial over-growth in the small intestine (Begley *et al.*, 2005). One of the most important roles of BA is their negative feedback regulation of cholesterol biosynthesis. In the last few decades, the role of BA as signalling molecules with many paracrine and endocrine functions has become evident. The specific nuclear receptor "farnesoid X receptor" (Parks *et al.*, 1999), (Wang *et al.*, 1999) and their membrane receptor TGR5 (Kawamata *et al.*, 2003) (Houten *et al.*, 2006) are responsible for regulation of their own hepatic synthesis and hepatic and intestinal transport (Chiang, 2002). BA are assumed in triggering the adaptive response to insults to the liver (cholestasis eg.). There are proofs that BA play an important role in energy-related metabolism, resp. in hepatic glucose homeostasis (Ma *et al.*, 2006).

BA homeostasis as well as other hepatobiliary mechanisms is controlled and regulated by nuclear receptors and their target genes, creating a complex regulatory network. Best known is farnesoid X receptor (FXR) which play crucial role in BA biosynthesis and transport (Matsubara *et al.*, 2013). FXR can induce expression of bile salt export pump (Bsep) and prevents BA uptake into hepatocyte by down-regulating of Na+/taurocholate co-transporting polypeptide (Ntcp) (Cai and Boyer, 2006). The synthesis of BA is influenced by FXR due to

changes in expression of small heterodimer partner (Shp) and fibroblast growth factor (Fgf15) which lead to transcriptional repression of genes involved in BA metabolism (Denson *et al.*, 2001), (Kong *et al.*, 2012). FXR plays role also in anti-bacterial defence in intestine, liver regeneration, and hepatocarcinogenesis (Wang *et al.*, 2008). Therefore, FXR regulates synthesis and transport of BA and protects against BA-induced hepatotoxicity (Meng *et al.*, 2015).

1.3.2 Disturbances in BA metabolism and BA-induced cell injury – role of oxidative stress and bile salt resistant microdomains:

Disorders in BA metabolism leading to cholestasis can be caused by many reasons: disturbances in synthesis or defect in conjugation, defective membrane transport in the hepatocyte or ileal enterocyte, disorders in transport between organs, and/or disturbances in bacterial degradation during enterohepatic circulation (Hofmann, 1999b).

A major consequence of cholestasis is the development of severe liver injury due to rapid accumulation of BA within hepatocytes (Gujral *et al.*, 2003). Accumulated BA in the plasma and the liver alter the expression of various genes involved in phospholipid and cholesterol homeostasis resulting in inflammation, cell death (Rodrigues and Steer, 2000) and liver injury mainly through inducing the oxidative stress (Sokol *et al.*, 2001), (Fuentes-Broto *et al.*, 2009). The molecular mechanisms behind cholestatic liver injury have been intensively studied, but are still not well understood (Woolbright and Jaeschke, 2012). In hepatocytes, BA operate as inflammagens and directly activate the signalling pathways leading to upregulation of production of proinflammatory mediators (Allen *et al.*, 2011). Recent data support the hypothesis that liver injury associated with cholestasis may not occur through direct BA-induced apoptosis, but may mainly involve inflammatory cell-mediated liver cell necrosis (Woolbright and Jaeschke, 2012). Some authors also point to importance of the disruption of

phospholipid/sphingolipid homeostasis in the pathogenesis of cholestasis (Matsubara *et al.*, 2011).

1.3.3 Cholestatic syndromes – obstructive cholestasis as the major cause of cholestasis

Cholestasis can be classified as intra- and extrahepatic. Cholestatic syndromes are known due to their overlap - many of them encompass the injury of intra- and/or extrahepatic bile ducts (*intra- / extrahepatic cholestasis*). Mechanical or *obstructive cholestasis* is the most common cause of jaundice characterized by an absence of bile in the intestine. Two causes of a blockade to the free bile flow in bile duct are most frequent - lithiasis and tumours. With disease progression, alterations in biochemical cholestatic markers and clinical signs of cholestasis are typically present – jaundice, pruritus, abdominal pain, cholangitis etc. In contrast, intrahepatic cholestasis is caused by parenchymal injuries of various aetiology. Other types of cholestasis are rare – autoimmune liver diseases (primary sclerosing cholangitis, primary biliary cholangitis), or extrahepatic biliary atresia. Severe infections cause systemic as well as intrahepatic increase in production of proinflammatory cytokines which can result in so-called sepsis-induced cholestasis (Chand and Sanyal, 2007). In general, the inflammation worsens the development of cholestasis by impairment in bile flow due to decreased aquaporin channels and increased nitric oxide production (Bhogal and Sanyal, 2013). Many reviews are focused on cholestasis of all these origins (Lee and Boyer, 2000), (Li and Crawford, 2004), (Boyer, 2007), (Wagner *et al.*, 2009).

Cholestasis is characterized by histopathological profound changes: dilated bile ducts (inspissated bile), portal tract oedema, bile ductular proliferation, neutrophilic infiltration with biliary piecemeal necrosis which includes cholate stasis (with *feathery degeneration*), ductular reaction, and fibrogenesis. Sometimes, the discrepancy between morphological and clinical cholestasis can be seen.

1.3.4 Intrahepatic cholestasis of pregnancy and estrogen-related cholestasis

Also known as obstetric cholestasis, intrahepatic cholestasis of pregnancy (ICP) is liver disorder in pregnancy that is most commonly seen in the third trimester and it is the second most common cause of jaundice in pregnant women (after viral hepatitis) (Abedin *et al.*, 1999). Twin pregnancies have a higher incidence of cholestasis than single pregnancies (Gonzalez *et al.*, 1989). ICP is characterized by a pruritus and elevated concentrations of maternal serum BA. Although most affected pregnant women have a mild form of the disease, ICP can be associated with spontaneous preterm childbirth, meconium staining, and intrapartum fetal distress, as well as low birth weight (Fisk and Storey, 1988), (Arrese and Reyes, 2006), (Heinonen and Kirkinen, 1999), (Wong *et al.*, 2008).

For a long time, estrogens have been known to cause ICP in susceptible women during pregnancy, during hormone replacement therapy, or after administration of oral contraceptives (Schreiber and Simon, 1983), (Yamamoto *et al.*, 2006). The detailed etiology is still unknown, but hypersensitivity to estrogens and estrogen-sulfated metabolites is considered to be the major factor as well as the heterozygous non-sense mutation of the multidrug-resistance-associated protein-3 (MDR3) gene (Jacquemin *et al.*, 1999). Estrogens are known to induce cholestasis in animal models (Lee and Boyer, 2000), (Stieger *et al.*, 2000). The pathophysiology is multifactorial and includes a) disorders in bile flow due to decreased sinusoidal uptake (downregulation of basolateral transporters NTCP, *SCL10A1*; and OATPs family) and reduced canalicular transport of BA (reduction of apical transporters MRP2, *ABCC2*; and BSEP, *ABCB11*), and b) factors which are more or less independent on BA. Almost 40 years ago, decreased fluidity of the cytoplasmic membrane in erythrocytes of patients with intrahepatic cholestasis (Balistreri *et al.*, 1981), as well as in the livers of mice with intrahepatic cholestasis (Boelsterli *et al.*, 1983), was described. Changes in the

membrane cholesterol and sphingomyelin contents were at least partially responsible for these changes (Smith and Gordon, 1988). Amphipathic BA could harm plasma membranes and induce structural and/or functional impairment of the hepatocyte membrane through their detergent action on lipid portion of plasma membranes. Another mechanism for the development of cholestatic liver injury arises from ultrastructural changes such as increased tight junctions permeability (Elias *et al.*, 1983), altered cell polarity, disruption of cell-to-cell junctions, cytoskeletal changes and membrane fluidity (Trauner *et al.*, 1999). It is known that above mentioned lipid rafts, tightly packed in a liquid ordered state, (Munro, 2003), (Rajendran and Simons, 2005) are needed to protect the membranes against the detergent action of BA (Guyot and Stieger, 2011).

The possible relationship between decreased membrane fluidity and changes in the content and localization of GSL in intrahepatic cholestasis was recently investigated (Jirkovska *et al.*, 2007). Based on this study, the redistribution of gangliosides on the sinusoidal membrane of the hepatocyte seems to be a protective mechanism of hepatocytes against the harmful effects of BA accumulated during ethinylestradiol (EE)-induced cholestasis. A significant increase of total lipid sialic acid - the hallmark of gangliosides content - together with a high increase of gangliosides synthetized in the so-called *b*-biosynthetic pathway was found in EE-induced cholestasis in rats (Majer *et al.*, 2007). These results suggest that changes in the localization and content of gangliosides may serve as a protective mechanism against cholestatic liver injury.

1.4 HEME CATABOLIC PATHWAY AND HEME OXYGENASE

Heme oxygenase (HMOX; EC 1.14.99.3) catalyzes the rate-limiting step in the oxidative degradation of heme, converting it into biliverdin IXα, which is subsequently metabolized to bilirubin IXα by biliverdin reductase (EC 1.3.1.24) (Singleton and Laster, 1965), (Tenhunen

et al., 1970), carbon monoxide, and iron (Fig. 4) (Tenhunen *et al.*, 1968), (Tenhunen *et al.*, 1969), (Yoshida and Kikuchi, 1978). This reaction proceeds through three successive oxygenation reactions (Kikuchi *et al.*, 2005), (Matsui *et al.*, 2010). Each step of oxidation requires reducing equivalents from NAPDH: cytochrome p450 reductase (EC 1.6.2.4) (Noguchi *et al.*, 1979), (Sugishima *et al.*, 2014). To date, two distinct HMOX isoforms are described – HMOX1 and HMOX2 (Maines *et al.*, 1986), (Trakshel *et al.*, 1986), which are coded by two different genes, but their amino acid sequences share about 40% similarity (Muller *et al.*, 1987), (McCoubrey *et al.*, 1992). HMOX1 is the inducible form known as heat shock protein (HSP), due to its molecular weight 32 kDa also called HSP32 (Keyse and Tyrrell, 1989), compared to 36 kDa for HMOX-2 (Trakshel *et al.*, 1991). HMOX1 activity is highest in the spleen due to erythrocyte turnover. (Ewing and Maines, 1992). HMOX2 is expressed constitutively and under physiological condition is the major isoform found in mammalian tissues including spleen, testes, brain, liver, kidney and lung particularly (Maines, 1988).

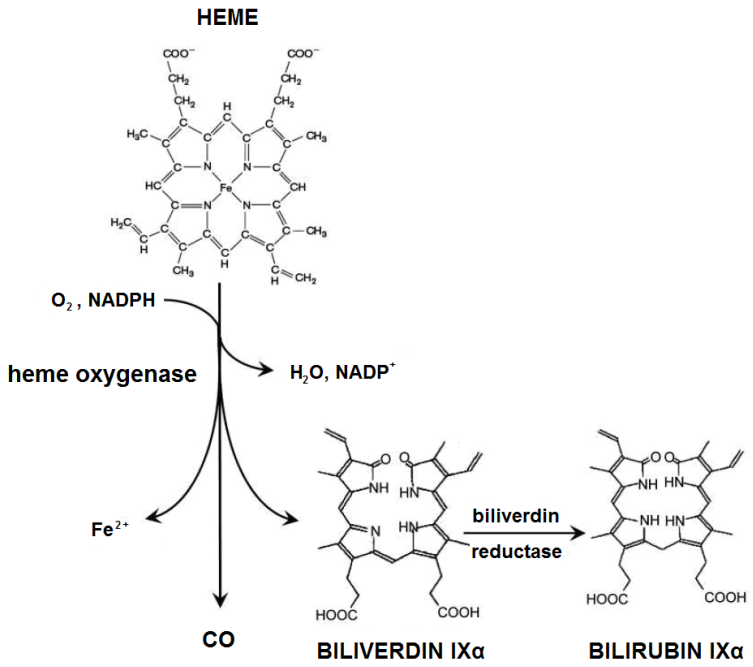

Figure 4. *Heme catabolic pathway.*

1.4.1 Regulation of expression of HMOX1

The major transcriptional regulator of *HMOX1* gene is the nuclear factor erythroid 2-related factor-2 (Nrf2), which recognizes and binds to the StRE/ARE motif (stress-responsive element/antioxidant element) (Alam *et al.*, 1999). Other factors also influence the transcription - Keap1 (Kelch-like ECH associated protein), which acts as an anchor for Nfr2 (Itoh *et al.*, 2003), and Bach1 (BTB and CNC homologue 1). Bach1 has been identified as a negative transcription factor, competing with Nrf2 for binding site at the StRE (Sun *et al.*, 2002). In the regulation of *HMOX1* gene expression, microRNAs are also involved. These small non-coding RNAs decrease mRNA stability and modulate the expression of upstream regulatory factors (Nrf2/Keap1 and Bach1) (Cheng *et al.*, 2013), (Pulkkinen *et al.*, 2011). Expression of *HMOX1* gene could be affected by microsatellite polymorphisms (which may result in the impaired transcriptional regulation) (Kramer *et al.*, 2013). Activity of HMOX1 is upregulated by its substrate, heme (Porteri *et al.*, 2009), (George *et al.*, 2013), and downregulated by metaloporphyrins (Ndisang *et al.*, 2010), (Zhong *et al.*, 2010), (Muchova *et al.*, 2011). In conclusion, all above mentioned elements confirmed the complexity of HMOX1 regulation and many studies suggest associations between these elements and protection and/or risk of many pathological conditions/diseases (Ryter and Choi, 2016).

Localization of HMOX

HMOX1 is localized in the smooth ER and other intracellular membranes, including microsomes, inner mitochondrial membrane (Converso *et al.*, 2006), and also to the plasma membrane caveolae, so called DRM (Kim *et al.*, 2004). There is also evidence that caveolin-1 interacts with and modulates HMOX activity and can act as a natural competitive inhibitor of HMOX1 with heme (Taira *et al.*, 2011).

1.4.2 HMOX function

HMOX operates as a "protective" enzyme possessing anti-inflammatory, anti-apoptotic and anti-proliferative actions, which are mediated predominantly by one or more active end-products of HMOX-catalysed degradation. HMOX could restore homeostasis in many situations and is speculated that HMOX, resp. HMOX1 functions as a "therapeutic funnel" (Otterbein *et al.*, 2003a), (Bach, 2005). In general, HMOX plays a key role in the cellular and tissue defences against oxidative stress (Poss and Tonegawa, 1997b). Genetic deficiency of HMOX1 in humans as well as knockout animals (*Hmox*$^{-/-}$) confirm the importance of HMOX1 in physiological processes (Yachie *et al.*, 1999), (Poss and Tonegawa, 1997a). The studies with *Hmox*$^{-/-}$ mice have demonstrated the tissue protective effects of HMOX1 in models of cardiac, pulmonary, skin, and other diseases (Ryter and Choi, 2016).

The anti-inflammatory HMOX functions are mediated primarily by generation of its biologically active end-products, especially bilirubin and carbon monoxide (CO). Bilirubin, discovered by Rudolf Virchow in 1847 (referred as haematoidin), represents a major bile pigment. About 80% comes from degradation of erythrocyte haemoglobin, remaining 20% from other origin (other heme proteins degradation, inefficient erythropoiesis) (London *et al.*, 1950), (Berk *et al.*, 1969). For many years, bilirubin was considered only a waste product of heme catabolism with no particular physiological function. In plasma, unconjugated bilirubin is bound to albumin. The uptake from plasma to the liver is mediated via passive transmembrane diffusion as well as active transport. Bilirubin is excreted from the organism after its conjugation by bilirubin UDP-glucuronosyltransferase (UGTA1, EC 2.4.1.17) (Schmid, 1956), (Schmid, 1957), (Gorski and Kasper, 1977) in hepatocytes, making it water-soluble molecule which might be transported across apical membrane of hepatocyte to bile via ATP-dependent multidrug resistance-associated protein-2 (MRP2, *ABCC2*) (Paulusma *et al.*,

1996), (Buchler *et al.*, 1996). MRP2 is expressed predominantly in the apical (canalicular) membrane of hepatocyte, and, to a lesser extent, in the small bowel, kidney, brain, and placenta (Cherrington *et al.*, 2002). Recent studies have revealed that a fraction of bilirubin conjugates is primarily secreted by MRP3 into the blood, from where is re-uptaken by sinusoidal membrane-bound organic anion transporting polypeptide (OATP1B1 and OATP1B3). This sinusoidal liver-to-blood cycle mediates shifting of bilirubin from periportal to centrizonal hepatocytes (van de Steeg *et al.*, 2010), (Iusuf *et al.*, 2012a), represents an enterohepatic circulation of bilirubin, and contributes to efficient hepatic detoxification (Iusuf *et al.*, 2012b), (van de Steeg *et al.*, 2012).

Antioxidant properties of bilirubin were confirmed by many authors, for the first time in late eighties by prof. Stocker *et al.* (Stocker *et al.*, 1987), (Dennery *et al.*, 1995). Many studies have found that bilirubin has a lot of biochemical and biological actions. Up to date, there is a strong body of evidence confirming that bilirubin and biliverdin reductase form a complex network with important physiological consequences and provide a complex antioxidant system (Vitek and Schwertner, 2007), (O'Brien *et al.*, 2015).

CO, one of the end-products of heme catabolic pathway, is known to be toxic at high concentrations due to its combination with haemoglobin to produce carbonylhaemoglobin. However, low concentrations of CO are essential for the regulation of many biological systems (Verma *et al.*, 1993), (Bilban *et al.*, 2008). CO is fast signalling molecule – thanks to its ability to diffuse through membranes – and affects several intracellular signalling pathways including guanylate cyclase activation (Ingi *et al.*, 1996), mitogen-activated protein kinases (MAPKs) (Otterbein *et al.*, 2000), and/or modulation of nitric oxide synthase (Zuckerbraun *et al.*, 2003). CO exhibits biological significance by its vasoregulatory, anti-inflammatory, anti-apoptotic and anti-proliferative effects (Morita *et al.*, 1997), (Otterbein *et al.*, 2003b). CO inhibits LPS-inducible proinflammatory cytokine production by macrophages. Inhibits Toll-

like receptor trafficking to the lipid rafts during LPS stimulation, through a mechanism involving the downregulation of NADPH:oxidase-dependent reactive oxygen species (ROS) production (Nakahira *et al.*, 2006). CO has also anti-apoptotic effects, due to the inhibition of TNF-alpha-initiated apoptosis (Petrache *et al.*, 2000), modulation of MAPK, and the upregulation of NF-κB-dependent antiapoptotic genes (Brouard *et al.*, 2000), (Brouard *et al.*, 2002). In endothelial cell, CO can inhibit the initiation of the extrinsic apoptotic pathway through by downregulation of ERK1/2-dependent ROS production (Wang *et al.*, 2007). Recent studies believe that CO might represent a novel therapeutic modality. The recently developed CO-releasing molecules, so called CO-RMs may provide new way of pharmacological delivery of CO (Motterlini *et al.*, 2005).

1.4.3 HMOX activity and liver diseases

HMOX1 activity affects many physiological functions and plays key role in hepatic fat accumulation, fibrogenesis, and oxidative injury (Poss and Tonegawa, 1997b). The activation of inducible isoenzyme HMOX1 has been shown to protect the liver/hepatocytes from toxic, inflammatory, and oxidative insults. Contrary to it, lack of HMOX1 activity is associated with increased vulnerability to oxidative stress due to free radical formation and oxidative stress-related cytotoxicity. It´s main causes are reduced antioxidant bilirubin and vasoactive carbon monoxide formation, disruption of iron homeostasis, and accumulation of pro-oxidative heme (Fraser *et al.*, 2011). Under basal conditions, HMOX1 is expressed mainly in Kupffer cells and at low concentrations in hepatocytes (Abshagen *et al.*, 2008). The up-regulation of HMOX1 is rapid not only in Kupffer cells, but also in hepatocytes in response to natural stimuli or many pathological conditions – hypoxia, ischemic/reperfusion injury, alcoholic liver disease, non-alcoholic fatty liver disease or viral hepatitis B and C (Jarvelainen *et al.*, 2000), (Li Volti *et al.*, 2008), (Mandal *et al.*, 2010), (Sass *et al.*, 2012).

In the cholestatic liver disease, it has been shown that BA act as potent inhibitors of HMOX1 activity and expression (Muchova *et al.*, 2011). The decreased HMOX1 activity in the cholestatic liver causes relative depletion of intracellular bilirubin, a potent endogenous antioxidant (Vitek *et al.*, 2002). The relative lack of this intracellular antioxidant together with high levels of pro-oxidative BA participates in the development of oxidative stress-mediated liver injury. In addition, Muchova *et al.* reported the anticholestatic effect of HMOX1 induction (Muchova *et al.*, 2015).

There is also evidence that HMOX1 interacts with caveolin-1, an important component of caveolae or so-called DRM or rafts (Kim *et al.*, 2004). Caveolin-1 modulates the activity of HMOX1 and can act as a natural competitive inhibitor of HMOX1 with heme (Taira *et al.*, 2011). Despite the knowledge that gangliosides modulate ROS formation in human leukocytes (Gavella *et al.*, 2010), neuronal cell (Avrova *et al.*, 1998), and there are in close relationship to HMOX1 in caveolae, only few reports discussing the possible role of HMOX1 or oxidative stress in ganglioside metabolism are present. Moreover, there are only few data exploring the role of HMOX in the pathogenesis of the cholestatic liver disease, the connection between HMOX and ganglioside metabolism during cholestasis or possible underlying mechanisms are completely unknown.

1.5 NON-ALCOHOLIC FATTY LIVER DISEASE

1.5.1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is an increasingly diagnosed condition associated with obesity, hyperlipoproteinaemia, insulin resistance and metabolic syndrome first described by Ludwig *et al.* in 1980 (Ludwig *et al.*, 1980). Definition of NAFLD comprise (a) evidence of hepatic steatosis, either by imaging or by histology and (b) lack of secondary causes of hepatic fat accumulation (such as significant alcohol consumption, hereditary disorders etc.) (Chalasani *et al.*, 2018). NAFLD encompasses wide spectrum of potentially progressive liver disease that comprises of mere hepatic steatosis, hepatic necroinflammation (non-alcoholic steatohepatitis, NASH), which often leads to liver fibrosis and subsequent progress to cirrhosis with high risk of complications development – portal hypertension, hepatocellular carcinoma and liver failure (Fig. 5) (Angulo, 2002), (Schuppan and Schattenberg, 2013). Due to a dramatic increase of risk factors for NAFLD, that are obesity, metabolic syndrome and insulin resistance (diabetes mellitus type 2), NAFLD has become the most frequent chronic liver disease in Western/developed countries (Loomba and Sanyal, 2013). The prevalence of NAFLD seems to be underreported and varies widely from 20 to 30% in adult population (Vernon *et al.*, 2011), (Fazel *et al.*, 2016), (Younossi *et al.*, 2016), (Bellentani, 2017). The development of NAFLD is not uniform, and only a small portion of patients (approximately 2-3%) progress to end-stage liver disease (White *et al.*, 2012).

1.5.2 Pathophysiology of NAFLD

The basic pathophysiological mechanism common for metabolic syndrome and NAFLD is the imbalance between increased energy intake (mainly due to consumption of foods characterized by a high energy density) and decreased energy expenditure (mainly due to sedentary life style) (Zelber-Sagi *et al.*, 2011) leading to excessive fat accumulation in the

adipose tissue and other organs (liver, pancreas, muscles, omentum). The severity of steatosis correlated with progression to NASH in humans (Wanless and Lentz, 1990) and many recent reports suggest that accumulation of lipids in the liver, and especially disturbances in the composition of hepatic FA, might play a causal role in the development of NASH (Day and James, 1998), (Araya *et al.*, 2004), (Allard *et al.*, 2008), (Gentile and Pagliassotti, 2008). Fat tissue acts as an endocrine organ producing free FA, adipokines (including TNF-α, IL-6, leptin, resistin, visfatin) and other compounds like retinol-binding protein-4 (Tilg, 2010). These metabolites are together with many epigenetic and genetic factors affecting the liver and together contributing to NASH development and progression of liver inflammation and fibrosis independent of insulin resistance and hepatic steatosis. Only small portion of patients with steatosis progress to NASH (Schuppan and Schattenberg, 2013), characterized by typical histopathological features: steatosis, ballooning and mixed acute and chronic lobular inflammation with zone 3 perisinusoidal fibrosis (Brunt *et al.*, 1999). Factors leading to this progression probably include products of an intermediary metabolism of FA, oxidative stress due to increased oxidation of FA, cytokines produced both in the fat tissue and the liver, and bacterial toxins from gut. Predisposition to progression is also strongly influenced by genetic heritability (Dongiovanni *et al.*, 2013), (Loomba *et al.*, 2015). Two common genetic variants increasing liver fat content have been identified by genome wide association studies (Romeo *et al.*, 2008), specifically the I148M variant of the *Patatin-like phospholipase domaincontaining-3* gene (PNPLA3) and the E167K variant of *Transmembrane 6 superfamily member 2 gene* (TM6SF2) (Kozlitina *et al.*, 2014). Both PNPLA3 and TM6SF2 variants were found to increase the susceptibility to NAFLD development (Valenti *et al.*, 2010), (Liu *et al.*, 2014), (Dongiovanni *et al.*, 2015) The PNPLA3 protein has hydrolase (lipase) activity towards triglycerides in hepatocytes and retinyl esters in hepatic stellate cells, while TM6SF2 is involved in hepatic very-low density lipoproteins (VLDL) secretion and has a regulatory

functions in liver fat metabolism (Kozlitina *et al.*, 2014), (Holmen *et al.*, 2014), (Pirola and Sookoian, 2015). Recently, a genome wide association study identified the single nucleotide polymorphism (rs641738 C>T) in the Membrane bound O-acyltransferase domain containing 7/Transmembrane Channel-Like 4 (MBOAT7/TMC4) locus that has been shown to predispose to cirrhosis development in alcohol abusers. The *MBOAT7* polymorphism has been also reported as another disease modifier in NAFLD (Mancina *et al.*, 2016), (Luukkonen *et al.*, 2016).

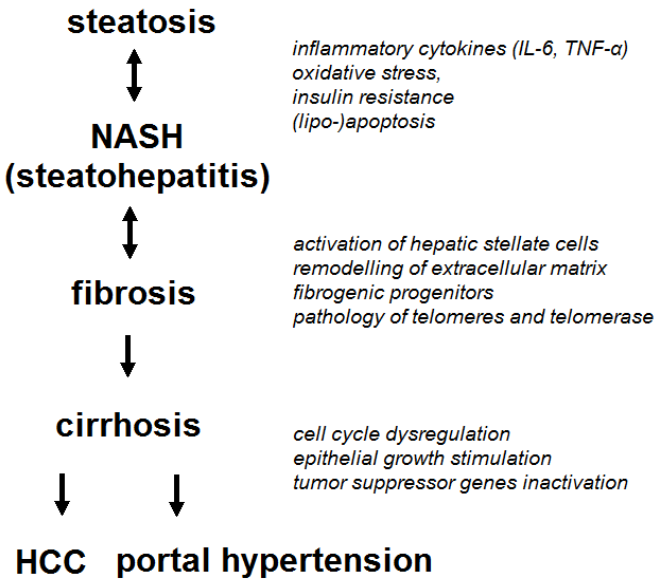

Figure 5. *NAFLD development*

NAFLD encompasses wide spectrum of potentially progressive liver disease that comprises of mere hepatic steatosis, hepatic necroinflammation (non-alcoholic steatohepatitis, NASH), which often leads to liver fibrosis and subsequent progress to cirrhosis with high risk of complications development – portal hypertension, hepatocellular carcinoma and liver failure. Major risk factors for NAFLD development are sedentary lifestyle, inappropriate (Western type) diet, and genetic predisposition. Excess of free fatty acids with insulin resistance lead to a liver injury through lipotoxicity and oxidative stress, resulting in necroinflammatory changes (necroapoptosis) and subsequent fibrogenesis.

1.5.3 Role of lipids in NAFLD development

Despite these findings, the detailed molecular mechanisms responsible for progression from simple steatosis to NASH have not been fully elucidated. Due to complexity of NAFLD pathogenesis, recently a "multiple parallel-hit theory" has been proposed to explain the mechanism of development and progression of NAFLD (Buzzetti *et al.*, 2016). Lipid accumulation is considered the most important mechanism in liver damage and altered repair and regeneration. Few years ago, steatosis, the earliest and most prevalent stage of NAFLD, was referred as the "first hit" (Day and James, 1998) and predispose liver tissue to the inflammation, fibrosis and cellular death characteristic for NASH. It was believed that accumulation of TAG predisposes to further liver damage in NASH pathogenesis. Recent data provide convincing evidence that FA and their metabolites are the true lipotoxic agents (Neuschwander-Tetri, 2010) and TAG accumulation does not cause insulin resistance or cellular injury in the liver. Lipids accumulated in hepatocytes are derived from circulating FA (from diet and adipose tissue) and *de novo* lipogenesis and their accumulation lead to overproduction of ROS, ER-stress and lipotoxicity (Sanyal *et al.*, 2001), (Fu *et al.*, 2011). Under physiological conditions, FA are transported to mitochondria for ß-oxidation or esterified for either excretion in VLDL or storage as lipid droplets. Hepatic lipid metabolism is regulated by many factors, intracellular signalling pathways (Savage and Semple, 2010), and various nuclear receptors (PPARα, PPARγ, FXR) (Knight *et al.*, 2005), (Schadinger *et al.*, 2005), (Shen *et al.*, 2011). Insulin also plays an important role in the regulation of FA metabolism – *de novo* lipogenesis is regulated by SREBP-1c, and Akt-regulated production of VLDLs. FA uptake (FA transport proteins) is regulated by signalling via PPARα as well as by hormonal regulation via insulin and leptin (Wierzbicki *et al.*, 2009), (Ge *et al.*, 2010). During fasting state, lysosomal pathway called "autophagy" recycles dispensable cellular parts into energy sources and represents important way in regulation of hepatic lipid homeostasis (Finn

and Dice, 2006), (Czaja, 2010). Animal studies shown that autophagy represents a key process in hepatic lipolysis and lipid droplet degradation (Singh *et al.*, 2009), (Shibata *et al.*, 2009) and is altered in NAFLD (Jung *et al.*, 2010).

Another source of ROS overproduction caused by hepatic lipid accumulation is decreased hepatic blood flow by direct compression and systemic hypercatecholemia which are typical for NAFLD. FA increased production of TNFα, hepatic TNF receptor activation (Crespo *et al.*, 2001) induces SREBP-1c overexpression, which promotes hepatic lipogenesis and lipid accumulation (Endo *et al.*, 2007). Kupffer cells activation, mitochondrial stress and development of hepatic inflammation and apoptosis are logical consequence of ROS accumulation (Sato, 2007). Serum free FA levels correlate with hepatocyte apoptosis and interfere with nuclear receptors, which might additionally influence the extent of liver injury and pro-oxidative stress (Nolan and Larter, 2009). The predominant form of hepatocellular injury in NAFLD is apoptosis. Its activity correlates with disease severity and might be monitored by cytokeratin-18 fragments in serum, which is a surrogate marker of NAFLD progression (Feldstein *et al.*, 2009). In conclusion, serum FA levels have been identified as supposed cause of lipotoxicity (Bechmann *et al.*, 2012) and correlate with severity of NAFLD.

FA-mediated lipotoxicity seems to be crucial factor for development of NASH due to overwhelming of the capacity of the liver to store and export FA (in the form of VLDL), typically due to increased influx from the periphery or hepatic *de novo* lipogenesis (Cohen *et al.*, 2011), (Trauner *et al.*, 2010). This concept highlights the role adipose tissue and enzymes involved in TAG hydrolysis (adipocyte TAG lipase; hormone-sensitive lipase; monoglycerol lipase) which catalyse the TAG breakdown (Haemmerle *et al.*, 2006), (Zechner *et al.*, 2012).

There are also other factors contributed in the pathogenesis of NAFLD, NASH respectively. FXR and its signalization influences the metabolism of glucose and lipids in the

liver (Kim *et al.*, 2016). BA, endogenous FXR and TGR5 plasma membrane bound G-coupled receptor ligands, could activate several signalling pathways to regulate biological processes - liver gluconeogenesis, lipogenesis, metabolism of cholesterol, and even inflammation (Vitek and Haluzik, 2016). BA also exert protective effects via FXR-dependent action by the suppression of hepatic fatty acid and TAG gene expression.

Choline is essential for *de novo* synthesis of phosphatidylcholine, required for VLDL packaging (TAG export) (Vance, 2008). Methionine, an essential amino acid, is affecting the protein synthesis and its deficiency predispose to the development of oxidative stress and inflammation (Caballero *et al.*, 2010). Based on this knowledge, methionine- and choline-deficient diet (or high fat methionine- and choline-deficient diet) is a widely used experimental dietary model of NASH (Jha *et al.*, 2014), (Hebbard and George, 2011). Western style diet-induced obesity causes severe microbial dysbiosis which directly impacts hepatic metabolism. (Henao-Mejia *et al.*, 2012). The action of metabolites produced by microbiome, and their actions on hepatic metabolism is called “gut-liver axis”. There are many signals suggesting that modification of microbiome might be a potential therapeutic approach combating the NAFLD (Boursier *et al.*, 2016), (Panasevich *et al.*, 2017).

1.5.4 NAFLD and NASH diagnosis

The majority of NAFLD patients have simple steatosis and do not have NASH, which has a significant liver-related adverse outcome (to cirrhosis and mortality). Liver biopsy together with non-invasive scoring systems and imaging techniques represent the most useful methods for the diagnosis of all NAFLD stages. The precise diagnosis of NASH still requires a liver biopsy that is suboptimal (Younossi *et al.*, 2018).

The role of ultrasound as a screening modality to identify the presence of abnormal liver morphology is undisputable. Despite its high sensitivity in detection of simple liver

steatosis, there is a growing need to quantify the liver steatosis and monitor the treatment efficacy. Best accuracy in quantifying of steatosis has magnetic resonance (MR) spectroscopy (Zhang *et al.*, 2018). In the last few years new methods have been developed to detect liver fibrosis. Most data have MR elastography (Huwart *et al.*, 2008), transient elastography (TE), followed by shear wave elastography (SWE) and acoustic radiation force impulse (ARFI) (Dietrich and Cantisani, 2014). TE (Fibroscan®) is the only non-imaging method, while ARFI and SWE are implanted in ultrasound device. The transient elastography with a special tool called Controlled Attenuation Parameter (CAP) is a useful for quantifying the liver steatosis (Sasso *et al.*, 2012), (Myers *et al.*, 2012), (Mikolasevic *et al.*, 2016).

For assessment of NASH as well as liver fibrosis, the liver biopsy has long been the only method for accurate diagnosis. The NAFLD activity score (NAS) based on histological findings (steatosis, inflammation, and ballooning) is widely used in the quantification of disease activity (NAS > 5 is associated with NASH diagnosis) (Brunt *et al.*, 1999). Together with fibrosis stage are key features in liver biopsy evaluation in NAFLD patients (Kleiner *et al.*, 2005).

Due to many potential problems with liver biopsy procedure as well as during its assessment (Machado and Cortez-Pinto, 2013), many non-invasive methods have been developed to predict NASH, or to quantify liver fibrosis. Many of these scores are listed below (see Discussion, Chapter 4.2.) and all of them together with new imaging techniques have a strong data background. Their specificity and sensitivity in NASH and/or liver fibrosis detection indicates that they might be at least as good as liver biopsy (Lurie *et al.*, 2015), (Chalasani *et al.*, 2018). Most of them are cheaper, safer, well tolerated, and good acceptable to the patient than liver biopsy, because they can be also repeated as often as necessary. Thanks to these modalities, we can screen the presence of advanced liver fibrosis in a risk

population (diabetes mellitus, metabolic syndrome etc.) and exclude patients with low-risk NAFLD in whom liver biopsy is not needed.

1.5.5 NAFLD treatment

Despite intensive effort, no established pharmacological treatment of NAFLD has been approved. Together with increased consumption of Western diet rich in processed sugars (fructose, sucrose) and fat, which has been directly implicated in the recent increase in the prevalence of NAFLD (Oddy *et al.*, 2013), there is an imperative need for development of new effective treatment strategies. The lifestyle risk factors for NAFLD development and progression to NASH are inappropriate diet, sedentary behaviour, physical inactivity, and lack of exercise. Up to date, the first line treatment of NAFLD and NASH patients should be lifestyle change, including dietary habits modification and physical activity increase leading to weight reduction (Chalasani *et al.*, 2012). Based on recent findings, the question is no longer whether lifestyle modification represents effective treatment of NAFLD/NASH, the question is now how to implement these methods as therapy in everyday practice (Romero-Gomez *et al.*, 2017). Other treatment strategies for NAFLD are focused on associated metabolic conditions such as diabetes, dyslipidaemia or pro-oxidative status of liver parenchyma. Pharmacological interventions to treat obesity-associated diseases require multiple agents and are often associated with adverse side effects (Beaton, 2012). Vitamin E and pioglitazone have been shown to benefit selected patients with biopsy-proven NASH. Other agents (statins or metformin) are not indicated for direct treatment of NASH. New treatment options are being developed and many studies are in advanced research phase (Fiorucci *et al.*, 2018). There is lack of long-time prospective investigations describing the effect of reduction sedentary behaviour, increasing physical activity and/or diet modification on NAFLD incidence or development. Few recent studies have been mainly relatively short,

lasting in the main between 6 and 12 months (Zhang *et al.*, 2017). One of the most important studies showing histologic regression of NASH/fibrosis during lifestyle change is not controlled (Vilar-Gomez *et al.*, 2015); many other studies are very small, or based only on the evaluation of changes in liver enzymes, without any imaging technique. Further randomised, well controlled trials are needed to find out complex and effective treatment option for NAFLD patients.

2. AIMS

The aim of this thesis was to elucidate the role lipids in the pathogenesis of selected liver diseases, focusing on cholestatic and non-alcoholic fatty liver disease. Specifically, our aims were:

1. to define optimal conditions for the ganglioside detection using cholera toxin B subunit *(reported in "Histochemical detection of GM1 ganglioside using cholera toxin B subunit. Evaluation of critical factors optimal for in situ detection with special emphasis to acetone pre-extraction" (Petr et al., 2010)).*

2. to assess changes in the composition and localization of gangliosides within hepatocytes in experimental EE-induced cholestasis following HMOX1 induction *(reported in "The Effect of Heme Oxygenase on Ganglioside Redistribution Within Hepatocytes in Experimental Estrogen-Induced Cholestasis" (Petr et al., 2014)).*

3. to clarify the changes in liver ganglioside metabolism in obstructive cholestasis and to verify the described effects of HMOX1 modulation on both cholestatic liver injury and on ganglioside metabolism *(reported in "Changes in Liver Ganglioside Metabolism in Obstructive Cholestasis - the Role of Oxidative Stress" (Smid et al., 2016))*

4. to assess the role of *Hmox1* knockout and associated oxidative stress on ganglioside metabolism and to identify the possible underlying mechanisms *(reported in "Heme oxygenase-1 may affect cell signalling via modulation of ganglioside composition" (Smid et al., 2018)).*

5. to clarify the relevance of serum non-invasive parameters and scoring systems in the staging of liver fibrosis and NASH in patients with NAFLD *(reported in "Use of Non-Invasive Parameters of Non-Alcoholic Steatohepatitis and Liver Fibrosis in Daily Practice - An Exploratory Case-Control Study" (Dvorak et al., 2014)).*

6. to determine the effects of n-3 PUFA administration on NAFLD/NASH development in a rodent high fat methionine-choline deficient (HFMCD) dietary model *(reported in manuscript "The effects of n-3 polyunsaturated fatty acids in a rodent nutritional model of non-alcoholic steatohepatitis").*

7. to determine the effects of n-3 PUFA administration on NAFLD development in NAFLD patients with metabolic syndrome *(reported in manuscript "Prague NAFLD Study: Non-alcoholic Fatty Liver Disease and n-3 Polyunsaturated Fatty Acids in Patients with Metabolic Syndrome").*

3. PUBLICATIONS RELATED TO THIS THESIS

3.1

Petr T, Šmíd V, Šmídová J, Hůlková H, Jirkovská M, Elleder M, Muchová L, Vítek L, Šmíd F. Histochemical detection of GM1 ganglioside using cholera toxin-B subunit. Evaluation of critical factors optimal for in situ detection with special emphasis to acetone pre-extraction. *European Journal of Histochemistry*. 2010; May 12;54(2):e23.

3.2

Petr T, Šmíd V, Kučerová V, Váňová K, Leníček M, Vítek L, Šmíd F, Muchová L. The effect of heme oxygenase on ganglioside redistribution within hepatocytes in experimental estrogen-induced cholestasis. *Physiological Research*. 2014; 63(3), 359-367.

3.3

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3.4

Šmíd V, Šuk J, Kachamakova-Trojanowska N, Jašprová J, Valášková P, Jozkowicz A, Dulak J, Šmíd F, Vítek L, Muchová L. Heme Oxygenase-1 May Affect Cell Signalling via Modulation of Ganglioside Composition. *Oxid Med Cell Longev*, 2018; 3845027.

3.5

Dvořák K, Stříteský J, Petrtýl J, Vítek L, Šroubková R, Leníček M, Šmíd V, Haluzík M, Brůha R. Use of non-invasive parameters of non-alcoholic steatohepatitis and liver fibrosis in daily practice--an exploratory case-control study. *PLoS One*, 2014; 9, e111551.

3.6

Šmíd V, Dvořák K, Hůrková K, Strnad H, Rubert J, Stříteský J, Staňková B, Zachariášová M, Hajšlová J, Brůha R, Vítek L. The effects of n-3 polyunsaturated fatty acids in a rodent nutritional model of non-alcoholic steatohepatitis. *Manuscript*.

3.7.

Šmíd V, Dvořák K, Šedivý P, Kosek V, Drobný M, Hajšlová J, Hájek M, Vítek L, Brůha R. Prague NAFLD Study: Non-alcoholic Fatty Liver Disease and n-3 Polyunsaturated Fatty Acids in Patients with Metabolic Syndrome. *Manuscript*.

Histochemical detection of GM1 ganglioside using cholera toxin-B subunit. Evaluation of critical factors optimal for *in situ* detection with special emphasis to acetone pre-extraction

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Abstract

A comparison of histochemical detection of GM1 ganglioside in cryostat sections using cholera toxin B-subunit after fixation with 4% formaldehyde and dry acetone gave tissue-dependent results. In the liver no pre-treatment showed detectable differences related to GM1 reaction products, while studies in the brain showed the superiority of acetone pre-extraction (followed by formaldehyde), which yielded sharper images compared with the diffuse, blurred staining pattern associated with formaldehyde. Therefore, the aim of our study was to define the optimal conditions for the GM1 detection using cholera toxin B-subunit.

Ganglioside extractability with acetone, the ever neglected topic, was tested comparing anhydrous acetone with acetone containing admixture of water. TLC analysis of acetone extractable GM1 ganglioside from liver sections did not exceed 2% of the total GM1 ganglioside content using anhydrous acetone at -20°C, and 4% at room temperature. The loss increased to 30.5% using 9:1 acetone/water. Similarly, photometric analysis of lipid sialic acid, extracted from dried liver homogenates with anhydrous acetone, showed the loss of gangliosides into acetone 3.0±0.3% only. The loss from dried brain homogenate was 9.5±1.1%.

Thus, anhydrous conditions (dry tissue samples and anhydrous acetone) are crucial factors for optimal *in situ* ganglioside detection using acetone pre-treatment. This ensures effective physical fixation, especially in tissues rich in polar lipids (precipitation, prevention of *in situ* diffusion), and removal of cholesterol, which can act as a hydrophobic blocking barrier.

Introduction

Gangliosides are charged glycosphingolipids (GSLs) containing one or more sialic acid residues in their carbohydrate chains. They are highly enriched in the outer leaflet of the plasma membrane and most of their functions are associated with this location. In contrast to glycerolipids, GSLs are more rigid molecules, and therefore protect cells against harmful environmental factors by keeping the outer leaflet of the plasma membrane mechanically stable and chemically resistant.[1-3] Carbohydrate moieties of gangliosides function as surface antigens, receptors for bacteria, bacterial toxins, viruses, and other bioactive molecules. Quantities and types change during development, differentiation, tumorigenesis, cell adhesion, and signal transduction.[4] *In situ* detection of GSLs in tissue sections is important for reliable information on their subcellular distribution. GM1 ganglioside is used as the representative member of the GSL family. The detection of GM1 is based on its strong binding affinity with cholera toxin B-subunit, which is highly sensitive for GM1, thus detecting this predominantly among all other gangliosides.[5-7] It can occur an additional staining due to binding of the other gangliosides, mainly GD1b, but its resolution from GM1 in histochemical detection is not possible. TLC analysis of gangliosides with resorcinol and cholera toxin staining should bring attention to unusual samples with high ratio of other gangliosides to GM1. Comparison of GM1 detection with cholera toxin and with anti-GM1 monoclonal antibody was referred by Kotani *et al.*[8]

GM1 detection is also widely used for detection of glycolipid-enriched membrane micro domains, called rafts. The fluctuations in cell surface glycolipid signaling molecules, at ultramicroscopic level, has been demonstrated in connection with fluorescence-topographic imaging at nano scale optical microscopy.[9]

The use of proper fixation technique is generally considered crucial for immunohistological detection of GSLs.[10] Acetone fixation of cryostat sections is recommended and preferred to formaldehyde, as it provides better accessibility of antibodies or bacterial ligands to GSLs.[10,11] For this reason, acetone fixation has been used in several studies.[8,12,13]

The first report on the effect of water in acetone on extraction of polar lipids was published by an author of our group.[14] In this report, it was demonstrated that the water content of acetone used during the extraction procedure, and even the water content of the treated samples, affects the degree of polar lipid extraction.

Since then, acetone fixation has been used in several studies.[8,12,13] However, these studies did not mention whether water was excluded from the acetone step, as even whether the water content of the studied sample might contribute to significant extraction of highly polar lipids.[15] The aim of the present study was to define the optimal conditions for the detection of GM1 using cholera toxin B-subunit by comparing formaldehyde fixation, acetone pre-extraction or a combination of both (acetone + formaldehyde sequence) in tissues with different polar lipid content; in this study liver and brain were used as representative tissues with significantly different polar lipid content.

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Key words: fixation, GM1 ganglioside, cholera toxin, anhydrous acetone, 4% formaldehyde.

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Materials and Methods

Chemicals

Paraformaldehyde, cholera toxin B-subunit biotin-labeled (ChT-B-biotin), streptavidin-peroxidase-polymer, albumin, biotin, and diaminobenzidine (DAB)-tetrahydrochloride tablets were supplied by Sigma (St Louis, MO, USA); avidin was obtained from Fluka (Buchs, Switzerland); cholera toxin B-subunit conjugated with peroxidase (ChT-B-Px) came from List laboratories (USA) and DEAE Sephadex was supplied by GE (Healthcare, UK). All other chemicals were purchased locally from Penta (Czech Republic).

Experimental animals

Female Wistar rats (Anlab, Prague, Czech

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Republic) were housed under controlled temperature and a natural light-dark cycle. Liver and brain samples were taken from the Wistar rats and snap frozen.

All aspects of the study met the accepted criteria for the experimental use of laboratory animals and all protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University, Prague, Czech Republic.

Preparation of fixatives

Formaldehyde: 4% formaldehyde was freshly prepared by depolymerization of paraformaldehyde.

Dry acetone: 1000 mL of acetone was dried over 100-150 g of anhydrous calcium chloride in a closed glass bottle (with occasional mixing) for several days, then decanted and distilled under elimination of air moisture. The obtained dry acetone was stored in tightly closed bottles.

Quantification of GM1 ganglioside extraction with acetone from liver sections

Twelve 6 μm cryostat sections from normal rat liver were cut and placed on the same microscopic slide. One slide, containing 12 sections, represented one sample for TLC densitometry. The samples (slides) used in each extraction were in triplicates.

The loss of GM1 ganglioside during acetone fixation was studied by comparison of the following extractions:

1) The samples (slides) were extracted directly with chloroform-methanol-water (C-M-W) 10:10:1 v/v/v with shaking on an orbital shaker at room temperature (25°C) for 10 min. These samples were used to measure the total quantity of GM1 in the tissue sections.

2) Other samples were extracted with i) dry acetone at room temperature for 15 min, ii) dry acetone at -20°C for 15 min, and iii) a cold acetone-water 9:1 v/v mixture, at -20°C for 15 min.

The samples from the acetone extraction were re-extracted with C-M-W 10:10:1 v/v/v by shaking at room temperature for 10 min.

All extracts were evaporated under a stream of nitrogen, dissolved in chloroform-methanol 1:1 v/v, and then applied onto HPTLC aluminium sheets of silica gel (Merck; Darmstadt, Germany). Chromatograms from the experiments were developed in a solvent mixture C:M:0.2% aqueous $CaCl_2$ (50:45:11). After drying, the silica gel layer was impregnated with 0.1% polyisobutylmethacrylate in cyclohexane. Non-specific binding was blocked using 1% BSA in PBS. Thereafter, the biotin-labeled cholera toxin B-subunit (diluted 1:300) was bound to the GM1 ganglioside at room temperature for 30 min. After extensive washing, streptavidin-peroxidase-polymer (diluted 1:400) was used for ultra-sensitive binding to biotin and amplification of the peroxidase enzyme signal. After washing, a blue reaction product was formed by reaction of the peroxidase with a solution of 1-chloronaphtol and H_2O_2 in a citrate phosphate buffer (pH=7.2).

Densitometry of the chromatogram in reflectance mode at 580 nm (CAMAG TLC Scanner II, Switzerland) was used for evaluation of the percentage distribution of GM1, in both the acetone and C-M-W extracts.

Quantification of acetone extraction of gangliosides from dried liver and brain homogenates

A very fine-grained homogenates were prepared from 1 g of liver and 0.5 g of brain, dried using a two-day lyophilization and then rapidly transferred into desiccator (to eliminate condensation of air moisture on the cold samples). One group of samples was extracted with C-M-W (4:8:3) repeated extractions (3x) with shaking (extracts A, n=6). The collected extracts represented the total gangliosides in the sample. The second group was extracted with dry acetone at room temperature for 15 min with shaking (extracts B, n=6) and afterwards re-extracted (3x) with C-M-W (4:8:3) using the same extraction technique described for extract A (extracts C, n=6).

Gangliosides from the extracts were isolated with ion exchange chromatography (DEAE Sephadex), base treatment, dialysis and silica gel column chromatography according to the procedure described by Ledeen *et al.*[16] Total gangliosides (total lipid sialic acid) in the extracts were determined using the resorcinol-HCl method.[17]

Histochemical detection of GM1 ganglioside in rat liver sections

Cryostat liver sections 6 μm thick were dried overnight at room temperature, and fixed with freshly prepared 4% formaldehyde at room temperature for 5 min. The parallel sections were fixed in dry acetone at -20°C for 2 or 15 min, then briefly transferred into a desiccator in order to eliminate air moisture condensation on the cold microscopic slides and thereby dilution of the acetone with condensed water. The influence of temperature on GM1 extraction from liver sections was studied by comparing fixation with dry acetone at -20°C for 2 or 15 min and fixation at room temperature for 2 or 15 min. The effect of the water content in the acetone fixative was studied in a further experiment comparing fixation using dry acetone with an acetone-water mixture (9:1 v/v), both at -20°C for 15 min. After acetone fixation, liver sections were dried in desiccator at room temperature and then put into PBS.

Histochemical detection of GM1 ganglioside was performed with ChT-B-biotin, according to the procedure described by Jirkovská *et al.*[18] In brief, the endogenous peroxidase activity was blocked by incubation in PBS, supplemented by 1% H_2O_2 and 0.1% sodium azide. Endogenous biotin was blocked using a blocking kit (DakoCytomation, Denmark). In order to block non-specific binding, sections were treated with 3% BSA in PBS for 15 min. Sections were then incubated with ChT-B-biotin, diluted 1:300 with PBS plus 3% BSA, for 16.5 h at 8°C. After washing with PBS, the sections were incubated with streptavidin-peroxidase-polymer diluted 1:400 with PBS at room temperature for 60 min. Peroxidase activity was visualized using DAB tetrahydrochloride in darkness for 20 min.

In each series two control tests were included. First, ChT-B-biotin was omitted in a negative test; second, fixed sections were extracted with a C:M (1:1 v/v) and C-M-W 1:1:0.3 v/v/v mixtures at room temperature, both for 15 min with shaking before detection of GM1. In order to obtain comparable results, corresponding areas were selected on parallel liver sections and photographed under constant exposure conditions.

Densitometric analysis of GM1 ganglioside in tissue sections

Liver sections obtained from 23 Wistar rats were analyzed. In each animal, the optical density of GM1 staining in formaldehyde-fixed samples represented 100% and the density of GM1 staining after acetone fixation was expressed as a proportion.

The mean optical density of the GM1 ganglioside reaction product in liver parenchyma was measured using an Olympus Cue 2 densitometric program at 70 areas in each section, at a magnification of 500x. Areas of sections containing either large vessels or technical artifacts (disruptions, etc.) were excluded from measurements.

Comparison of histochemical detection of GM1 ganglioside in rat liver and brain sections

Parallel cryostat sections from liver tissues were treated with the following fixatives: 1) 4% formaldehyde; 2) anhydrous acetone. Sections from brain tissue were fixed with: 1) formaldehyde; 2) Baker's solution (formaldehyde containing calcium chloride); 3) acetone followed by formaldehyde (A+F); and 4) acetone followed by Baker's solution (A+B). Acetone followed by formaldehyde gave better-preserved morphology than acetone alone. After acetone fixation, liver and brain sections were dried in a desiccator, put into formaldehyde or Baker's

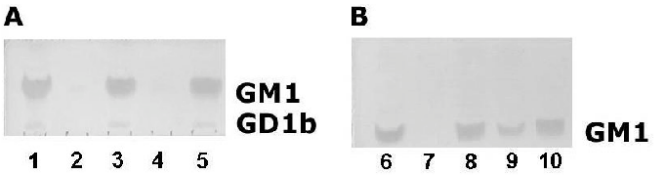

Figure 1. TLC of gangliosides extracted under various conditions. (A) Effect of temperature on dry acetone extractability of GM1 ganglioside. (1) direct extraction with C-M-W; (2) extract with dry acetone at -20°C; (3): C-M-W extract after previous extraction with dry acetone at -20°C; (4): acetone extract at room temperature; (5): C-M-W extract after previous extraction with dry acetone at room temperature. Note: GM1 ganglioside detected with ChTB (GD1b ganglioside band was also detected with much lower sensitivity). GM1 content in all acetone extracts was below the detection level of very sensitive cholera toxin staining. (B) Comparison of GM1 extraction with dry acetone and with aqueous acetone. (6) Direct extraction with C-M-W; (7) extract with dry cold acetone at -20°C. (8): C-M-W extract after previous extraction with dry acetone at -20°C; (9) extracts with mixture acetone-water 9:1 shows significant loss of GM1; (10) C-M-W extracts after previous extraction with acetone-water mixture.

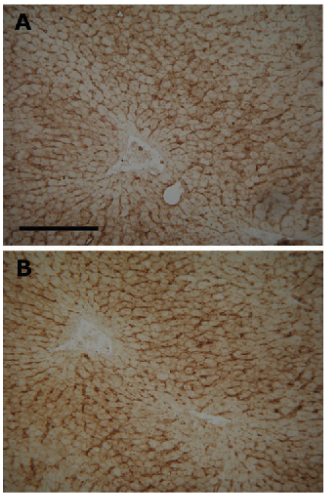

Figure 2. Histochemical detection of GM1 ganglioside in liver cryostat sections after various fixation conditions. Fixation with (A) dry cold acetone for 15 min at -20°C and with (B) 4% formaldehyde for 5 minutes at room temperature gave comparable results. Rat liver cryostat sections stained with cholera toxin B-subunit. Bar 200 µm.

solution and then into PBS. Histochemical detection of GM1 ganglioside was performed using cholera toxin B-subunit labeled with peroxidase (ChT-B-Px) diluted 1:500 with PBS plus 3% BSA and incubated for 16.5 h at 8°C. Cell nuclei were counterstained with hematoxylin. Other steps were applied according to the previously mentioned staining protocol (*see above*), except for blocking of biotin.

Unmasking treatment (cholesterol removal) with methyl-β-cyclodextrin (MCD) was performed on brain sections only. 12 mM MCD in PBS was used for 30 min at room temperature and for 45 min at 37°C. A 20 mM MCD concentration was used for 30 min at room temperature. After MCD treatment, sections were briefly washed in PBS and then fixed with formaldehyde.

Determination of cholesterol

Cholesterol was determined in the lipid extracts from both rat liver and brain samples using gas chromatography, with isotope dilution mass spectrometry,[19] using D7-cholesterol as the internal standard.

Statistical analysis

SigmaStat software (Jandel Scientific) was used for statistical analyses. Data are presented as the median and IQ range, and assessed using the Mann Whitney rank sum test. A $P<0.05$ was considered as statistically significant.

Results

Effect of different fixation procedures on GM1 ganglioside extraction from the liver sections

The loss of GM1 ganglioside due to the fixation of dry cryostat liver sections with dry acetone was studied and compared with those samples extracted with chloroform-methanol-water 10:10:1 v/v/v (taken as 100% of GM1). First, the effect of the temperature of dry acetone was studied at -20°C and 25°C; the sections extracted with acetone were re-extracted with C-M-W 10:10:1. The results of the TLC of gangliosides in extracts stained with the cholera toxin B-subunit are shown in Figure 1A. A similar procedure was used to demonstrate the effect of water content in acetone on the extraction of gangliosides (Figure 1B).

Both chromatograms were evaluated using densitometry. Figures 1A and 1B illustrate that the GM1 content from dry acetone extracts was not detectable, even when the highly sensitive cholera toxin method was used (Figure 1). The greatest part of GM1 persisted after acetone fixation and extraction; and it was extracted by the C-M-W re-extraction. Therefore, the percentage of GM1 in the acetone extracts was counted as the difference between the mean density of GM1 from samples extracted directly with C-M-W 10:10:1 v/v/v (taken as 100% of GM1) minus the mean density detected in C-M-W re-extracts. GM1 content in C-M-W re-extracts was 98.1% after cold acetone, and 96.2% after acetone at 25°C (i.e., the loss of GM1 was 1.9% with dry acetone at -20°C and 3.8% at 25°C, respectively). On the other hand, the acetone:water mixture (9:1) yielded a remarkable 30.5% loss of GM1 (Figure 1B, line 9). The almost ten times higher extraction of GM1 in acetone:water (9:1) extract demonstrates that the water content in acetone has a significant effect on GM1 extraction.

Effect of fixation procedures studied on liver and brain dried homogenates

The semi-quantitative results obtained from the densitometry of chromatograms were supported by a quantitative determination (photometric resorcinol-HCl method) of the loss of total gangliosides from dried liver and brain homogenate into acetone.

The mean value of the amount of total gangliosides extracted with C-M-W (4:8:3) from dried liver and brain homogenate (extract A) was taken as 100% of total gangliosides. The loss of gangliosides from liver into acetone at room temperature (extract B) was 3.0±0.3%) and from brain 9.5±1.1% and in the C-M-W re-extract of liver (extract C) remained 97.0±0.3%), and in re-extracts from brain about 90.5%.

Histochemical detection of GM1 ganglioside in liver cryostat sections under various fixation conditions

Comparable GM1 staining intensity and pattern were observed in liver cryostat sections after both formaldehyde and anhydrous acetone fixations (Figure 2). For dry acetone, the effects of fixation temperature and time on

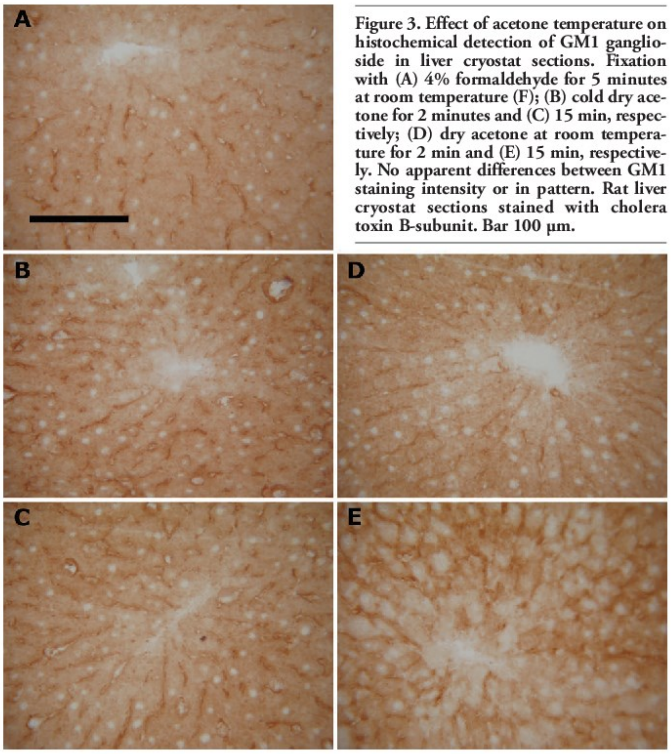

Figure 3. Effect of acetone temperature on histochemical detection of GM1 ganglioside in liver cryostat sections. Fixation with (A) 4% formaldehyde for 5 minutes at room temperature (F); (B) cold dry acetone for 2 minutes and (C) 15 min, respectively; (D) dry acetone at room temperature for 2 min and (E) 15 min, respectively. No apparent differences between GM1 staining intensity or in pattern. Rat liver cryostat sections stained with cholera toxin B-subunit. Bar 100 µm.

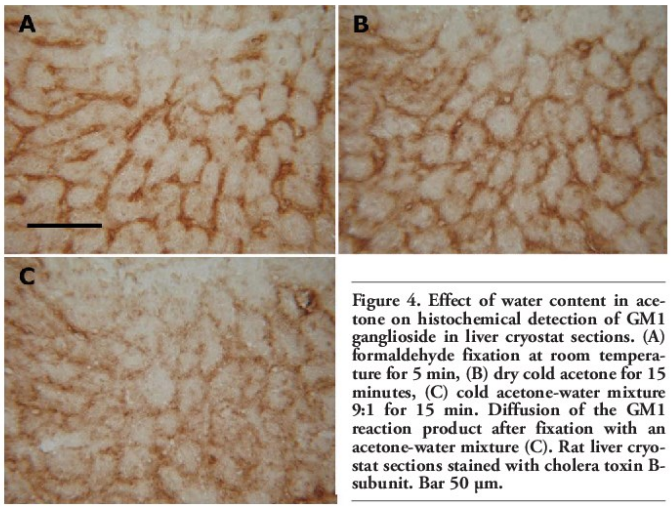

Figure 4. Effect of water content in acetone on histochemical detection of GM1 ganglioside in liver cryostat sections. (A) formaldehyde fixation at room temperature for 5 min, (B) dry cold acetone for 15 minutes, (C) cold acetone-water mixture 9:1 for 15 min. Diffusion of the GM1 reaction product after fixation with an acetone-water mixture (C). Rat liver cryostat sections stained with cholera toxin B-subunit. Bar 50 µm.

GM1 staining were also examined. Liver sections fixed with dry acetone at -20°C or 25°C, both for 2 and 15 min, were compared with those fixed with formaldehyde at room temperature for 5 min. No apparent difference in the staining intensity and/or location of the reaction product was observed in corresponding areas of parallel sections (Figure 3). On the contrary, fixation with an acetone-water mixture caused apparent diffusion of the GM1 reaction product (Figure 4).

Densitometric analysis of GM1 staining in liver sections

Densitometry was used for evaluation, in order to obtain objective data regarding the optical density of GM1 staining in tissue sections. The differences in the optical density of GM1 staining in liver tissue from 23 normal animals after formaldehyde and anhydrous acetone fixation were not statistically significant (*data not shown*). Finally, neither formaldehyde nor dry acetone fixation caused diffusion of the reaction product in the liver sections.

Comparison of histochemical detection of GM1 ganglioside in liver and brain cryostat sections after different fixations

Unlike the liver samples, where formaldehyde and acetone fixations of cryostat sections gave minimal differences, different pretreatment of brain sections led to remarkably different GM1 staining patterns. Cryostat brain sections fixed with formaldehyde (F) displayed a diffuse GM1 staining pattern in the neuropil of cortical and subcortical grey matter, without focal accentuation of the staining (Figure 5 A,C). The slight and diffuse positivity seen in the white matter probably corresponds to myelin. Baker's solution (B) used instead of formaldehyde (F) had no apparent effect on the diffuse character of GM1 staining. After fixation with anhydrous acetone followed by formaldehyde (A+F), overall GM1 staining was less intensive but the staining pattern became much more distinct, revealing the presence of definable subcellular structures (Figure 5B,D). The finely granular GM1 staining changed for coarsely granular with the anhydrous acetone plus Baker's solution (A+B) sequence (*not shown*).

Treatment of brain sections with MCD led to decreased GM1 staining intensity against formaldehyde fixation (Figure 5 G,H). However, the sharp, focally accented staining pattern was less expressed than after acetone (A+F) as demonstrated (Figure 5 H,I).

In the liver, the distinct, finely granular

GM1 staining of hepatocyte membranes at both sinusoidal and canalicular poles did not display any substantial difference in sections fixed either in formaldehyde or in anhydrous acetone (Figure 5 E,F).

Discussion

In general, there are two major issues regarding the problem of optimal GM1 and ganglioside *in situ* detection: i) reliability of acetone pre-treatment, which should ensure effective retention of gangliosides *in situ*, in other words acetone pre-treatment should not lead to a significant loss of the lipids; and ii) evaluation of all factors responsible for optimal *in situ* demonstration.

As far as the reliability of acetone pre-treatment is concerned, the quantitative analysis of GM1 ganglioside in extracts of liver sections showed a negligible loss. Likewise, densitometric evaluation of the reaction product of histochemical GM1 detection in liver sections carried out in formaldehyde fixed sections and sections pretreated with acetone did not show statistically significant differences. Loss of gangliosides by acetone extraction of dried liver homogenate at room temperature was negligible (3.0±0.3%). An increased loss of gangliosides in brain up to 9.5±1.1% corresponds to the less intensive GM1 staining in brain cryostat sections.

It might be possible to see an analogy with the conclusions of Ilangumaran and Hoessli,[20] who proposed that in rafts, the GSL-rich core of the microdomains persists depletion of cholesterol with methyl-β-cyclodextrin (MCD), while the cholesterol-rich annulus surrounding the core, containing as well some GM1, seems to be partially released by MCD. It might be that acetone similarly extracts some GM1 from cholesterol-rich areas. Further extensive studies will be necessary to support this hypothesis.

Our study also confirmed the risk of pre-treatment with acetone; extraction must be carried out with anhydrous acetone; this observation was previously described.[14] It should be stressed that anhydrous acetone and dry cryostat sections are both essential for *in situ* preservation of gangliosides. Breaking of any of these conditions leads to ganglioside dislocation *in situ* or directly to their extraction extra situ. This might explain the loss of gangliosides described by Schwarz and Futerman,[15] who found an approximate 56% reduction of gangliosides after acetone fixation and a 46% reduction after formaldehyde-acetone fixation of cultivated hippocampal neurons.

Acetone extraction is recommended for better *in situ* detection of gangliosides in many studies.[8,12]

The enhanced staining intensity after acetone pre-treatment is explained by the improvement of permeabilization.[21] Glycosphingolipid globotriaosylceramide was more accessible to verotoxin in kidney sections after acetone pre-treatment; an effect ascribed to the removal of exceeding cholesterol. Similar effects were seen after methyl-β-cyclodextrin.[13,22] Schwarz and Futerman[15] also demonstrated brighter labeling of gangliosides in cultivated hippocampal neurons after combined fixation with formaldehyde and acetone, despite significant gangliosides removal dur-

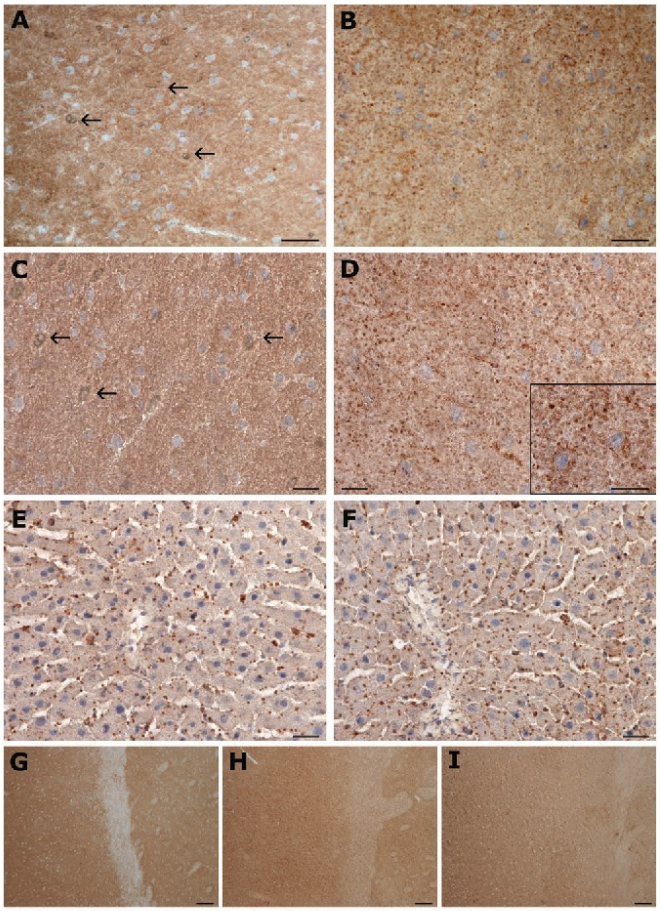

Figure 5. Compared effect of different fixatives on histochemical detection of GM1 ganglioside in rat liver and brain. Cryostat sections stained with cholera toxin B-subunit. (A, B) Brain cortical grey matter; low power field microphotographs. (A) Diffuse positivity in the neuropil after formaldehyde fixation and (B) focally accented staining after anhydrous acetone + formaldehyde (A+F) combination. Bars 50 µm. (C, D) Brain cortical grey matter; high power field microphotographs. (C) Diffuse staining in the neuropil after formaldehyde fixation. Note also a migration of unstained myelin lipids forming myelin figures (A,C); some of them are marked by arrows. (D) Granular staining after acetone fixation (A+F) suggesting localization of a reactive product to the cell membranes (for a detail see insert in D). Bars 25 µm. (E, F) High power field pictures of the liver sections fixed with (E) formaldehyde or (F) acetone + formaldehyde (A+F). Finely granular staining on the cell membranes of hepatocytes is uninfluenced by the type of fixation. Bars 25 µm. Cell nuclei were counterstained with hematoxylin. (G,H,I) survey of brain cortex; pre-treatment of cryostat sections with (G) formaldehyde, (H) MCD + formaldehyde and (I) anhydrous acetone + formaldehyde. Slight gradual (G → I) decrease in overall staining intensity and increase in sharpness and condensation of the staining pattern. Bars 100 µm.

ing pre-treatment (*see also above*).

The finely granular GM1 positivity seen in acetone (A+F) fixed sections could represent the combined effect of unmasking, due to cholesterol extraction and precipitation resulting from the low solubility of GSLs in acetone (difference from MCD). The increased granularity and contrast seen when the combination of anhydrous acetone plus Baker's solution (A+B) was used for fixation, could be explained by the well known aggregation of gangliosides in presence of Ca^{2+} ions.[23,24]

Our results in brain samples showed better outcomes *in situ* detection of GM1 after acetone pre-extraction of dry cryostat sections (Figure 5A-D) which also inhibited the considerable diffusion of myelin lipids into the mounting medium in acetone unextracted sections.

This suggests that the benefits of acetone, as a process of physical fixation, are related to precipitation of polar lipids generally (inhibition of their local diffusion), and to removal of the possible masking effect of cholesterol.

However, we should be aware that cryostat section technique is inherently influenced by thawing and subsequent drying of the sections that might influence *in situ* localization of polar compounds, including gangliosides. The current study offers the optimal way to cope with this problem.

Conclusions

We present for the first time evidence that both pre-treatments (formaldehyde and acetone) provide good information on GM1 localization at the light microscopy level in liver sections. In the brain, the quality of GM1 detection was found to be higher in acetone pretreated sections; optimal conditions vary based on the lipid composition of the tissue. The benefits of acetone pre-treatment are linked to the use of anhydrous conditions.

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The Effect of Heme Oxygenase on Ganglioside Redistribution Within Hepatocytes in Experimental Estrogen-Induced Cholestasis

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Summary

Cholestasis is characterized by the elevation of serum total bile acids (TBA), which leads to the production of both free radicals and oxidative stress. Although they do not share the same mechanisms, membrane glycosphingolipids (GSL) and the antioxidant enzyme heme oxygenase-1 (HMOX1) both act against the pro-oxidative effect of TBA. The aim of the study was to assess the role of HMOX on GSL redistribution and composition within hepatocytes in the rat model of estrogen-induced cholestasis. Compared to the controls, an increase of total gangliosides in the liver homogenates of the cholestatic group (P=0.001) was detected; further, it paralleled along with the activation of their biosynthetic b-branch pathway (P<0.01). These effects were partially prevented by HMOX activation. Cholestasis was accompanied by a redistribution of GM1 ganglioside from the cytoplasm to the sinusoids; while HMOX activation led to the retention of GM1 in the cytoplasm (P=0.014). Our study shows that estrogen-induced cholestasis is followed by changes in the synthesis and/or distribution of GSL. These changes are not only triggered by the detergent power of accumulated TBA, but also by their pro-oxidant action. Increases in the antioxidant defenses might represent an important supportive therapeutic measure for patients with cholestatic liver disease.

Key words

GM1 ganglioside • Heme oxygenase • Oxidative stress • Intrahepatic cholestasis • 17α-ethinylestradiol

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Introduction

Estrogen-induced cholestasis is a pathological condition characterized by impaired bile flow and the accumulation of bile acids in the plasma of susceptible women, either after estrogen administration or in pregnancy (Vore 1987). Accumulated bile acids in the plasma and the livers of cholestatic individuals can cause liver injury through mechanisms including oxidative stress and structural and/or functional damage of the hepatocyte membrane, due to the detergent properties of bile acids (Kullak-Ublick and Meier 2000, Sokol *et al.* 2001, Roma *et al.* 2008, Fuentes-Broto *et al.* 2009).

Heme oxygenase (HMOX) is a rate-limiting enzyme in heme catabolism, which has antioxidative, anti-inflammatory, and cytoprotective properties. The beneficial effects of HMOX are mainly produced through its bioactive products – bilirubin and carbon monoxide (CO) (Vitek and Schwertner 2007, Muchova *et al.* 2011). CO is an important signaling molecule involved in bile secretion, as well as bile canaliculi contractility and liver perfusion; while bilirubin acts as a strong antioxidant, and can protect the liver directly against the oxidative stress triggered by bile acids (Reyes and Simon 1993, Muchova *et al.* 2011).

Gangliosides are glycosphingolipids (GSL), assembled from a lipophilic ceramide portion and

structurally variable hydrophilic oligosaccharide portion containing N-acetylneuraminic acid. Two main biosynthetic pathways of the gangliosides have been described in mammalian cells: 1] a-pathway comprising GM3, GM2, GM1, and GD1a gangliosides; and 2] b-pathway with GD3, GD2, GD1b, and GT1b gangliosides (Kolter *et al.* 2002).

Gangliosides, as well as other GSL, are highly concentrated in the outer layer of the plasmatic membrane. Furthermore, gangliosides, thanks to their unique physical and chemical properties, are considered crucial molecules responsible for the rigidity of cell membranes (Pascher 1976, Harris *et al.* 1978, Pascher *et al.* 1992), as well as contributing to the protection against oxidative stress (Gavella *et al.* 2010).

More than 30 years ago, a decreased fluidity of the liver plasma membrane was observed in cholestasis that had been induced by ethinylestradiol (EE) (Balistreri *et al.* 1981, Smith and Gordon 1988); however, the possible consequences, resulting from the decreased fluidity of the cholestatic membrane, an increase in b-pathway gangliosides, and the redistribution of GM1 ganglioside from the cytoplasm to the sinusoidal membrane of hepatocytes have only recently been described, in 2007 (Jirkovska *et al.* 2007, Majer *et al.* 2007). This supports the hypothesis that not only the increased synthesis of gangliosides, but also their redistribution in a situation of limited biosynthesis, may serve as a protective mechanism against the strong detergent effects of the bile acids accumulated during cholestasis.

The aim of the present study was to assess the potential role of HMOX on GSL redistribution and composition within hepatocytes in the rat model of ethinylestradiol-induced cholestasis.

Materials and Methods

Chemicals

Paraformaldehyde, cholera toxin B-subunit biotin-labeled (ChT-B-biotin), streptavidin-peroxidase polymer, albumin, biotin, 17α-ethinylestradiol, hemin, N-acetylneuraminic acid, ammonium acetate, PAP pen for immunostaining, monosialoganglioside GM1 from bovine brain, 1,2-propanediol, diaminobenzidine (DAB)-tetrahydrochloride tablets, and RNAlater were all supplied by Sigma Aldrich (St. Louis, MO, USA); avidin and N,N-dimethylformamide were obtained from Fluka (Buchs, Switzerland); the DEAE Sephadex was supplied by GE Healthcare (Little Chalfont, UK). High Performance Silica Plates, resorcinol, and silica gel 60 came from Merck (Darmstadt, Germany); the Faramount Mounting Medium was received from Dako (Glostrup, Denmark); the Total RNA Purification Kit was from Norgen Biotek Corporation (Thorold, Canada); the High Capacity cDNA Reverse Transcription Kit and TaqMan Gene Expression Master Mix were from Applied Biosystems (Foster City, USA). All other chemicals were purchased from Penta (Prague, Czech Republic).

Experimental animals

Female Wistar rats were obtained from Anlab (Prague, Czech Republic), and housed under a controlled temperature and with a natural light-dark cycle. The animals had free access to water and food throughout the experiment. They were forced to fast overnight before the experiment.

Cholestasis was induced by subcutaneous injections of EE (5 mg/kg) diluted in 1,2-propanediol, and applied daily for 18 days (E group). The control groups (C) only received the 1,2-propanediol. Activation of HMOX1 (aE group) was achieved by intraperitoneal administration of heme (15 μmol/kg in 4 doses on days 0, 5, 10, and 15). The heme was dissolved in 0.1 M NaOH, stabilized by albumin solution (1.5 mmol/l heme: 0.15 mmol/l BSA), and then adjusted by 0.1 M HCl to a final pH of 7.4. The minimum number of animals per group was n=6.

After intramuscular anesthesia with ketamine (90 mg/kg) and xylazine (10 mg/kg), the animals were sacrificed, and blood was then obtained from the inferior vena cava for biochemical analyses. Their livers were weighed and then immediately processed for histochemical analysis, isolation of gangliosides and RNA, as well as the determination of HMOX activity.

All aspects of the study met all of the accepted criteria for the experimental use of laboratory animals; all protocols were approved by the Animal Research Committee of the First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic.

Determination of serum markers of liver injury and HMOX activity

The following serum markers of cholestasis and hepatocellular injury were evaluated: total bile acids (TBA), total bilirubin (Bili-T); the activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). TBA levels were

determined spectrophotometrically using a Bile Acids kit (Trinity Biotech, Jamestown, NY, USA), all of the other markers were determined on an automatic analyzer (Modular analyzer, Roche Diagnostics GmbH, Mannheim, Germany). HMOX activity was measured by gas chromatography, as previously described (Vreman and Stevenson 1988, 2001), and calculated as pmol CO/h/mg fresh weight.

Peroxyl radical scavenging capacity

The peroxyl radical scavenging capacity of the rat serum was detected fluorometrically as the proportion of chain-breaking antioxidant consumption present in the serum, relative to that of Trolox (a reference and calibration antioxidant compound), as previously described (Iuliano *et al.* 2000).

Analysis of liver gangliosides

The chloroform-methanol extraction of glycolipids from the liver tissue was performed according to Ledeen *et al.* (1973), with minor modifications by Ueno *et al.* (1978). The extract was purified on a silica gel column, again following the method of Leeden *et al.* (1973). An additional purification of the samples using a Folch partition (Folch *et al.* 1957) was necessary for the removal of heme in the aE samples. This substance has a similar mobility in thin layer chromatography (TLC) as gangliosides, and might interfere with the densitometric measurement. Part of the extract was used for the determination of the total sialic acid (total gangliosides) by the photometric method with resorcinol reagent (Svennerholm 1957, Takki-Luukkainen and Miettinen 1959), the other part was used for TLC quantification of the content of the major gangliosides. GM1 ganglioside, with a known concentration, was applied onto each HPTLC plate in order to correct for any differences caused by the intensity of spraying with the resorcinol HCl reagent. The densitometric measurement was evaluated using CATs3 software (CAMAG, Switzerland).

Determination of glycosyltransferases expression

Total liver RNA was isolated from samples stored in RNAlater tissue storage reagent (Sigma Aldrich, St. Louis, USA) using a Total RNA Purification kit (Norgen Biotek Corporation, Canada); and cDNA was generated using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA). Real-time PCR was performed with a TaqMan® Gene Expression Assay Kit (Applied Biosystems, Foster City, USA) for sialyltransferase II (*St8Sia1*, Rn00563093_m1) and galactosyltransferase II (*B4Galt2*, Rn01417399_m1). Data were normalized to β-actin (*Actb*, Rn00667869_m1) and expressed as fold change from the control levels.

Histochemical detection of GM1 ganglioside in rat liver

Microscopic distribution of GM1 ganglioside (the representative of GSL) was studied in the liver tissue sections by histochemical detections, based on the strong binding of the cholera toxin B-subunit to GM1. Blocks of liver tissue were collected from each animal, frozen, and cryostat sections (6 μm thick) were prepared from all blocks. Each animal was represented by six sections.

Before histochemical detection, the slides with sections were fixed with anhydrous acetone and 4 % formaldehyde, as described by Petr *et al.* (2010). GM1 ganglioside was detected by a histochemical reaction with ChT-B-biotin (diluted 1:500 in PBS with 3 % BSA), followed by incubation with streptavidin-peroxidase polymer, diluted 1:400 in PBS. Peroxidase activity was visualized using diaminobenzidine tetrahydrochloride.

Image analysis of the liver sections

Selected areas from each slide were photographed with an objective magnification of 40X (NA=500). Photographs were taken under constant conditions. The entire area was systematically inspected, and every sixth to seventh screen was photographed using the stratified random sampling method (Hamilton 1995). Lumens of large vessels and artificially damaged areas were excluded from the analysis. The sinusoidal membrane of hepatocytes (sin) and adjacent areas of the cytoplasm (cyt) were traced and subsequently measured. The optical density of the GM1 reaction product was determined using a computer image analysis program ACC 6.0 (SOFO, Czech Republic).

Statistical analysis

Normally distributed data are presented as the mean ± SD, and were analyzed using Student's t-test; skewed data are expressed as the median and 95 % CI, and were analyzed by the Mann-Whitney Rank Sum test. Kruskal Wallis ANOVA with post hoc analysis was used for multiple comparisons. Differences with $P<0.05$ were considered statistically significant. The analyses were performed using STATISTICA CZ software, v. 10.

Results

HMOX activation increases serum antioxidant capacity of cholestatic animals

As expected, EE exposure resulted in significant increases of serum Bili-T (1.0±0.3 vs. 3.2±2.0 μmol/l, P=0.005), TBA (5.5±5.3 vs. 115.2±71.3 μmol/l, P=0.005), and ALP (1.8±0.4 vs. 3.2±0.9 μkat/l, P=0.008), compared to the corresponding controls. Administration of heme, a specific inducer of HMOX1, substantially increased liver HMOX activity in the EE-treated group (261.2±129.1 vs. 434.9±143.7 pmol CO/h/mg FW, P=0.031).

To investigate whether HMOX induction can increase the total antioxidant status of experimental animals, we measured the serum antioxidant capacity of cholestatic rats exposed to heme. Indeed, the heme administration increased the peroxyl radical scavenging capacity of the sera of EE-treated animals by 68 % (P=0.016, Fig. 1).

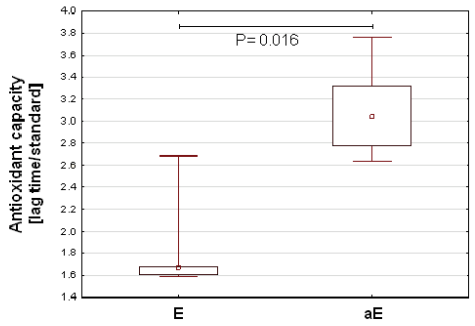

Fig. 1. The effect of HMOX induction on serum antioxidant capacity of estrogen-exposed animals. Data are expressed as a ratio between the lag time of the serum to that of the standard (Trolox), presented as median, 25-75 % (boxes) and 5-95 % (whiskers). E, estrogen-exposed animals; aE, estrogen-exposed animals treated with heme

HMOX activation prevents an increase of total ganglioside content in the cholestatic liver

Total liver gangliosides (measured as total sialic acid content) were significantly elevated in the cholestatic samples, compared to control group C [40.3 (27.4-49.9) vs. 17.0 (8.9-26.5) nmol/g, P=0.001]. A much smaller and non-significant increase in the total liver ganglioside content has been observed in the cholestatic samples treated with heme [17.0 (8.9-26.5) vs. 31.9 (16.9-36.8) nmol/g, P>0.05] (Fig. 2).

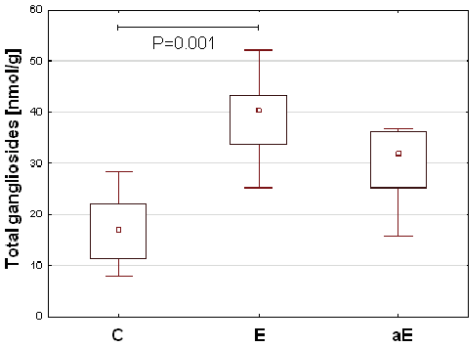

Fig. 2. The effect of HMOX induction on total liver gangliosides of cholestatic animals. Liver content of total gangliosides, expressed as median, 25-75 % (boxes) and 5-95 % (whiskers). C, control animals; E, estrogen-exposed animals; aE, estrogen-exposed animals treated with heme

Activation of b-terminal branch of ganglioside synthesis in cholestasis is prevented by HMOX

In this set of experiments, we measured changes in the concentration of individual liver gangliosides after induction of cholestasis and HMOX activation.

No changes in the concentration of gangliosides of the a-biosynthetic pathway (GM1 and GD1a) were observed. However, a significant activation of the terminal part of the b-branch gangliosides (represented by a two-fold increase of GD1b and GT1b, P=0.005 and P<0.001, respectively) was detected in the cholestatic animals. Interestingly, this activation was partially prevented by HMOX activation, only when mild increases in GD1b and GT1b were observed (Fig. 3).

To determine the rates of activation of the a- and b-branch of ganglioside biosynthetic pathways, we studied the relative expressions of galactosyltransferase II (unspecific GM1-synthase) and sialyltransferase II (specific GD3-synthase) in liver homogenates. Compared to controls, no significant changes were observed in the expression of GM1-synthase in the livers of cholestatic animals (98±27 % and 113±43 % in E and aE, respectively, P>0.05). On the other hand, the expression of GD3-synthase, showing an activation of the b-biosynthetic branch, was markedly increased in the cholestatic samples (230±77 % of controls, P=0.04). This increase was not as pronounced when HMOX had been activated in the cholestatic animals (increased only to 186±84 %, P>0.05) (Fig. 3).

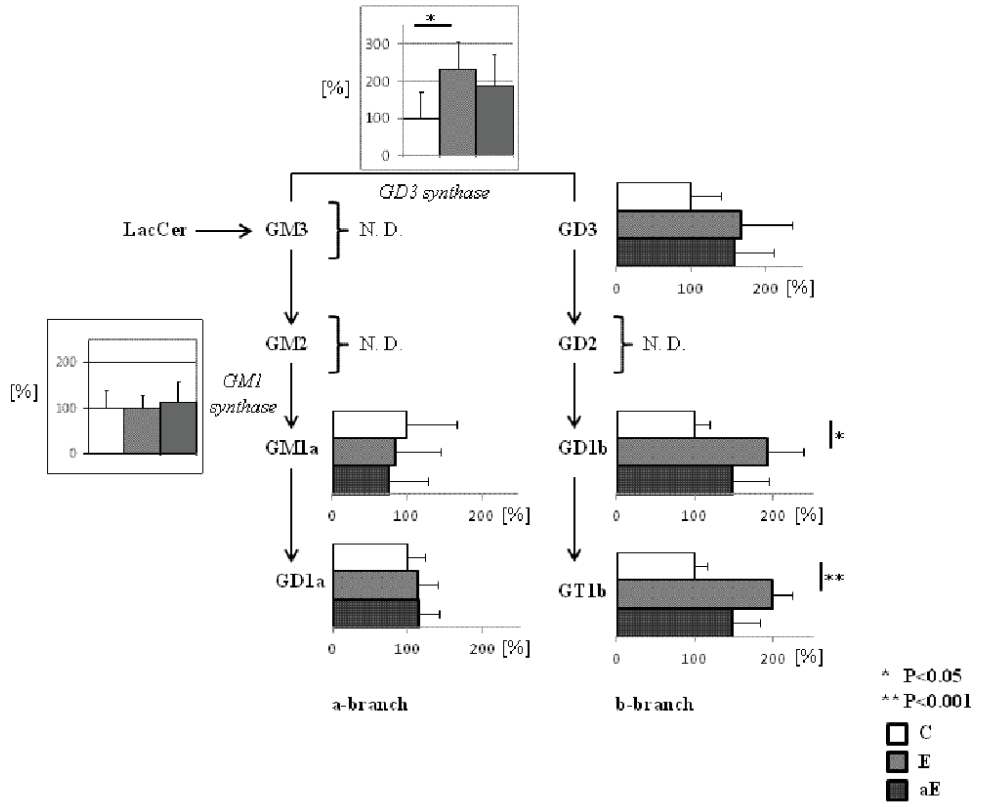

Fig. 3. Induction of ganglioside biosynthesis in cholestatic hepatocytes. Relative liver content of individual gangliosides of a- and b-biosynthetic pathways, and relative expressions of key enzymes in ganglioside synthesis. ND, not detected; LacCer, lactosylceramide (precursor of ganglioside synthesis); C, control animals; E, estrogen-exposed animals; aE, estrogen-exposed animals treated with heme

HMOX induction affects the GM1 distribution in the cholestatic hepatocyte

To study the changes in the distribution of glycolipids within the liver cell after cholestatic liver injury and HMOX activation, we measured the histochemical localization of GM1 ganglioside (the representative of GSL) in the liver sections.

When compared to the controls, cholestasis was accompanied by a substantial shift of GM1 ganglioside from the cytoplasm to the sinusoidal membrane. Pre-treatment with heme completely abolished this effect; GM1 staining increased in the cytoplasm, but was substantially reduced in membranes (Fig. 4).

This observation was quantified by the image analysis of GM1 in the subsinusoidal compartment of the cytoplasm (cyt) and the sinusoidal membranes (sin) of hepatocytes. Based on this analysis, the sin/cyt ratio was calculated, indicating the relative distribution of glycolipids within the liver cell. The results clearly show the shift of GM1 ganglioside from the cytoplasm to membranes in cholestatic samples (sin/cyt ratio 1.51±0.19 vs. 1.71±0.10, P=0.65); while the shift was reversed by HMOX induction (sin/cyt ratio 1.71±0.10 vs. 1.31±0.04, P=0.014) (Fig. 5).

Discussion

The present study shows that in estrogen-induced cholestasis, it is not only the detergent properties, but also the pro-oxidant properties of the accumulated bile acids that are responsible for increased synthesis and membrane distribution of gangliosides. If the antioxidative response is augmented, the cholestatic pattern of the liver gangliosides is partially reversed.

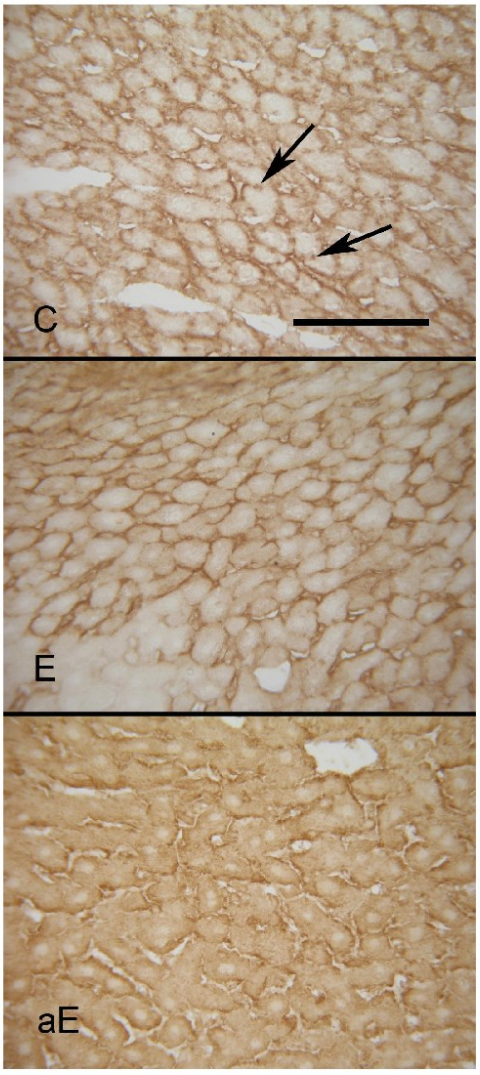

Fig. 4. The effect of HMOX1 induction on GM1 localization in the cholestatic liver. GM1 in the liver sections was detected using ChT-B-biotin with streptavidin-peroxidase polymer, and visualized using diaminobenzidine tetrahydrochloride (brown color). The apparent shift of GM1 positivity from sinusoidal and canalicular membrane (arrowheads) in control group (**C**) to sinusoidal membrane, only in the cholestatic group (**E**); or to the cytoplasm in the cholestatic group with activated HMOX1 (**aE**) can be observed. Bar = 100 µm. C, control animals; E, estrogen-exposed animals; aE, estrogen-exposed animals treated with heme

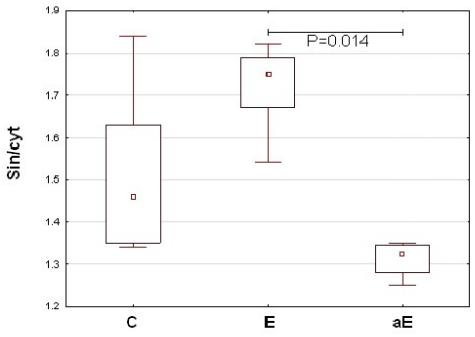

Fig. 5. The effect of HMOX1 induction on redistribution of GM1 ganglioside within the liver cells. Image analysis of the intensity of GM1 staining in the subsinusoidal compartment of cytoplasm (cyt) and sinusoidal membranes (sin) of hepatocytes, expressed as sin/cyt ratio. C, control animals; E, estrogen-exposed animals; aE, estrogen-exposed animals treated with heme

To investigate whether oxidative stress, increased by accumulated bile acids in estrogen-induced cholestasis is responsible for changes in liver ganglioside metabolism, we used an experimental model with activation of the antioxidant enzyme HMOX. We found that administration of the HMOX substrate, heme, to EE-treated animals leads to a significant increase in the serum antioxidant capacity. As we have recently shown (Zelenka *et al.* 2012), activation of HMOX is associated, *via* formation of its bioactive products, with strong cytoprotective and antioxidant actions, counteracting the pro-oxidative effect of accumulated bile acids in cholestasis (Muchova *et al.* 2011).

In agreement with data published earlier by our group (Majer *et al.* 2007), we observed an increase in ganglioside synthesis (measured as sialic acid content) and the activation of b-branch ganglioside biosynthesis in cholestatic livers. Gangliosides, due to their physical chemical properties, protect cells against harmful extrinsic factors by forming detergent-resistant and rigid domains in the outer leaflet of the plasma membrane (Kanfer and Hakomori 1983, Hakomori 2003). Thus, the increase in total ganglioside content, and particularly the b-series of gangliosides (containing more sialic acid molecules) in cholestatic animals might be a logical response to this insult. Interestingly, antagonizing the pro-oxidative actions of bile acids by HMOX activation in cholestatic animals tended to reverse this increase towards values closer to those of the controls. Considering reports emphasizing the antioxidant properties of gangliosides (Avrova *et al.* 1998, Gavella *et al.* 2007), and the effect of GM1 ganglioside on membrane fluidity in primary rat hepatocytes (Sergent *et al.* 2005), we speculate that oxidative stress might be an

important factor in regulating ganglioside biosynthesis and membrane stabilization during estrogen-induced cholestasis.

Cholestasis is not only characterized by an increase in ganglioside biosynthesis, but also by their shift from the cytoplasm to the plasma membrane (Jirkovska *et al.* 2007). In accord with this data, we observed the highest membrane GM1 ganglioside content in cholestatic livers. On the other hand, in estrogen-treated animals with HMOX induction, gangliosides did not follow the cholestatic pattern, and a significant drop in membrane ganglioside content was observed.

There are some limitations of our study. First, we can only speculate on the role of GSL redistribution within the membrane of hepatocyte. GSL are part of dynamic membrane rafts having not only protective properties against detergent effects of bile acids but also important signaling functions. This topic needs to be addressed in future studies. Secondly, to clarify the exact role of HMOX on GSL metabolism, further studies with HMOX knock-out animals should be performed.

We conclude that estrogen-induced cholestasis is followed by changes in the synthesis and/or distribution of gangliosides within the hepatocyte. These changes are not only triggered by the detergent properties of highly concentrated bile acids, but also by oxidative stress, and thus might represent a general mechanism of hepatoprotection. Therefore, enhancement of the antioxidative defense mechanisms by HMOX induction might represent an important supportive therapeutic measure under cholestatic liver conditions.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

GSL, glycosphingolipids; HMOX, heme oxygenase; GM1, II^3NeuAc-$GgOse_4$Cer; GM2, II^3NeuAc-$GgOse_3$Cer; GM3, II^3NeuAc-LacCer; GD1a, IV^3NeuAc,II^3NeuAc-$GgOse_4$Cer; GD3, $II^3(NeuAc)_2$-LacCer; GD2, $II^3(NeuAc)_2$-$GgOse_3$Cer; GD1b, $II^3(NeuAc)_2$-$GgOse_4$Cer; GT1b, IV^3NeuAc,$II^3(NeuAc)_2$-$GgOse_4$Cer; LacCer, lactosylceramide; EE, 17α-ethinylestradiol; ChT-B-biotin, cholera toxin B-subunit biotin-labeled; TBA, total bile acids; Bili-T, total bilirubin; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase

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Original Article

Changes in Liver Ganglioside Metabolism in Obstructive Cholestasis – the Role of Oxidative Stress

(cholestasis / gangliosides / haem oxygenase / liver)

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Abstract. Bile acids have been implicated in cholestatic liver damage, primarily due to their detergent effect on membranes and induction of oxidative stress. Gangliosides can counteract these harmful effects by increasing the rigidity of the cytoplasmic membrane. Induction of haem oxygenase (HMOX) has been shown to protect the liver from increased oxidative stress. The aim of this study was to determine the changes in the synthesis and distribution of liver gangliosides following bile duct ligation (BDL), and to assess the effects of HMOX both on cholestatic liver injury and ganglioside metabolism. Compared to controls, BDL resulted in a significant increase in total as well as complex gangliosides and mRNA expression of corresponding glycosyltransferases ST3GalV, ST8SiaI and B3GalTIV. A marked shift of GM1 ganglioside from the intracellular compartment to the cytoplasmic membrane was observed following BDL. Induction of oxidative stress by HMOX inhibition resulted in a further increase of these changes, while HMOX induction prevented this effect. Compared to BDL alone, HMOX inhibition in combination with BDL significantly increased the amount of bile infarcts, while HMOX activation decreased ductular proliferation. We have demonstrated that cholestasis is accompanied by significant changes in the distribution and synthesis of liver gangliosides. HMOX induction results in attenuation of the cholestatic pattern of liver gangliosides, while HMOX inhibition leads to the opposite effect.

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Abbreviations: aBDL – BDL with HMOX activation, aC – control with HMOX activation, ALP – alkaline phosphatase, ALT – alanine aminotransferase, AST – aspartate aminotransferase, B3GalTIV – UDP-Gal:βGlcNAc β 1,3-galactosyltransferase, B4GalNTI – β-1,4-N-acetyl-galactosaminyl transferase 1, BA – bile acids, BDL – bile duct ligation, BSA – bovine serum albumin, C – control, cAMP, cyclic adenosine monophosphate, CO – carbon monoxide, CTB – cholera toxin B-subunit, EDTA – ethylenediamine tetraacetic acid, EE – 17α-ethinyl oestradiol, FW – fresh weight, GalTI – UDP-Gal:βGlcNAc β 1,4-galactosyltransferase, GlcT – UDP-glucose ceramide glucosyltransferase, GSL – glycosphingolipids, HMOX – haem oxygenase, iBDL – BDL with HMOX inhibition, iC – control with HMOX inhibition, PBS – phosphate-buffered saline, ROS – reactive oxygen species, SnMP – tin-mesoporphyrin, ST3GalV – ST3 β-galactoside α-2,3-sialyltransferase 5, ST8SiaI – ST8 α-N-acetyl-neuraminide α-2,8-sialyltransferase 1, TBA – total bile acids. GSL are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (Chester, 1998)

Introduction

Cholestasis is an impairment of bile secretion and/or flow, followed by a lack of bile in the intestine and accumulation of potentially toxic bile acids (BA) in the liver and systemic circulation (Paumgartner, 2006). A major consequence of cholestasis is development of severe liver injury due to rapid accumulation of BA within the hepatocytes (Gujral et al., 2003). It is generally assumed that the exposure of hepatocytes to high concentrations of potentially toxic BA is primarily responsible for cholestatic liver injury (Perez and Briz, 2009). The molecular mechanisms behind early liver injury associated with cholestasis have been extensively studied, but are still not well understood (Woolbright and Jaeschke, 2012). BA act as an inflammation agent, directly activating the signalling pathways in hepatocytes that regulate production of proinflammatory mediators (Allen et al., 2011). Recent data support the hypothesis that cholestatic liver injury may not occur through direct BA-induced apoptosis, but may mainly involve inflammatory cell-

mediated liver cell necrosis (Woolbright and Jaeschke, 2012).

Equally, amphipathic BA could disrupt cell membranes and cause structural and/or functional damage of the hepatocyte membrane through their detergent action on lipid components (Billington et al., 1980). Another mechanism for the development of cholestatic liver injury arises from ultrastructural changes such as altered cell polarity, disruption of cell-to-cell junctions, cytoskeletal changes, and membrane fluidity (Trauner et al., 1999).

More than 30 years ago, decreased fluidity of the cytoplasmic membrane in erythrocytes of patients with intrahepatic cholestasis (Balistreri et al., 1981), as well as in the livers of mice with intrahepatic cholestasis (Boelsterli et al., 1983), was described. Changes in the membrane cholesterol and sphingomyelin contents were at least partially responsible for these changes (Smith and Gordon, 1988).

It is known that cholesterol-(glyco)sphingolipid (GSL) complexes, tightly packed in a liquid ordered state (Munro, 2003; Rajendran and Simons, 2005), called lipid rafts, are needed to protect the membranes against the detergent effect/action of BA (Guyot and Stieger, 2011).

Gangliosides, forming a major part of GSL, are assembled from a lipophilic ceramide portion plus a structurally variable hydrophilic oligosaccharide portion containing N-acetylneuraminic acid. They are highly concentrated in the outer layer of the plasmatic membrane, and thanks to their unique physical and chemical properties, they are considered crucial molecules responsible for the rigidity of plasmatic membranes (Pascher, 1976; Pascher et al., 1992).

The possible relationship between decreased membrane fluidity and changes in the content and localization of GSL in intrahepatic cholestasis was recently investigated by our group (Jirkovska et al., 2007). Based on this study, the redistribution of gangliosides on the sinusoidal membrane of the hepatocyte seems to be a protective mechanism of hepatocytes against the harmful effects of BA accumulated during ethinyl oestradiol (EE)-induced cholestasis. A significant increase of total lipid sialic acid – the hallmark of ganglioside content – together with a high increase of gangliosides synthesized in the so-called *b*-biosynthetic pathway (Fig. 1) was found in EE-induced cholestasis in rats (Majer et al., 2007). These results suggest that changes in the localization and content of gangliosides may serve as a protective mechanism against cholestatic liver injury.

Moreover, our recent data also indicate that the changes and redistribution of gangliosides during experimental EE-induced cholestasis are attributable to high concentrations of accumulated BA (Jirkovska et al., 2007), as well as increased oxidative stress (Petr et al., 2014) in the EE-induced cholestasis model. One of the important anti-oxidant and anti-inflammatory factors is haem oxygenase (HMOX; E.C. 1:14:99:3). HMOX catalyses the rate-limiting step in the oxidative degradation of haem, converting it into biliverdin IXα and subsequently to bilirubin IXα by biliverdin reductase (E.C. 1:3:1:24), carbon monoxide, and iron. HMOX plays a key role in the cellular and tissue defences against oxidative stress (Poss and Tonegawa, 1997). Up-regulation of its inducible isoenzyme HMOX-1 has been shown to protect the liver from toxic, inflammatory, and oxidative insults. Recently, we have shown that BA are potent inhibitors of HMOX activity and expression (Muchova et al., 2011). The decreased HMOX activity in the cholestatic liver causes relative depletion of intracellular bilirubin, a potent endogenous antioxidant (Vitek et al., 2002). The relative lack of this intracellular antioxidant together with high levels of pro-oxidative BA participates in the pathogenesis of oxidative stress-mediated liver injury. In addition, we reported the anticholestatic effect of HMOX induction (Muchova et al., 2015) as well as restoration of the changes in the synthesis and distribution of gangliosides in EE-induced cholestasis (Petr et al., 2014).

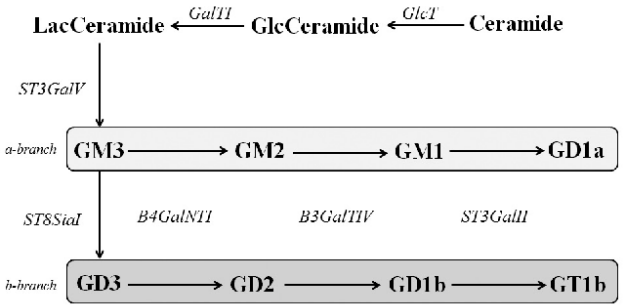

Fig. 1. Scheme of *de novo* biosynthesis of the oligosaccharide moieties of gangliosides
GlcT – UDP-glucose ceramide glucosyltransferase, *GalTI* – UDP-Gal:βGlcNAc β 1,4-galactosyltransferase, *ST3GalV* – ST3 β-galactoside α-2,3-sialyltransferase 5, *ST8SiaI* – ST8 α-N-acetyl-neuraminide α-2,8-sialyltransferase 1, *B4GalNTI* – β-1,4-N-acetyl-galactosaminyl transferase 1, *B3GalTIV* – UDP-Gal:βGlcNAc β 1,3-galactosyltransferase, *ST3GalII* – ST3 β-galactoside α-2,3-sialyltransferase 2.

The aim of the present study was to determine changes in the synthesis and distribution of liver gangliosides following bile duct ligation (BDL), and to assess the effect of the modulation of HMOX activity both on cholestatic liver injury and on ganglioside metabolism.

Material and Methods

Materials

Paraformaldehyde, hemin, biotin, bovine serum albumin (BSA) and diaminobenzidine tetrahydrochloride tablets were supplied by Sigma (St. Louis, MO); avidin was obtained from Fluka (Buchs, Switzerland); the cholera toxin B-subunit (CTB) peroxidase conjugated came from List Biological Laboratories (Campbell, CA). The tin-mesoporfyrin was supplied by Frontier Scientific (Logan, UT). All other chemicals were purchased locally from Penta (Prague, Czech Republic). The TaqMan® Gene Expression Master Mix, High-Capacity RNA-to-cDNA Kit, the TaqMan® Gene Expression Assay kit for rat genes were obtained from Life Technologies (Carlsbad, CA). The QIAshredder kit and RNEasy Plus MiniKit were supplied by Qiagen (Valencia, CA).

Animals and treatments

Female Wistar rats (Anlab, Prague, Czech Republic) were housed under controlled temperature and a natural light-dark cycle. The animals had free access to food and water throughout the experiments, and were fasted overnight prior to the experiment initiation. All aspects of the study met the accepted criteria for the experimental use of laboratory animals, and all protocols were approved by the Animal Research Committee of the First Faculty of Medicine, Charles University in Prague, Czech Republic.

Cholestasis induction

Rats intraperitoneally anaesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) underwent ligation or a sham operation. Cholestasis was induced in three groups of rats (N = 8 per group) by microsurgical ligation of segmental bile ducts and resection of the extrahepatic biliary tract ("BDL") (Aller et al., 2004). Biliary trees were exposed through midline abdominal incision. The control group of rats (N = 6) was sham-operated (controls, "C").

HMOX modulation

HMOX was inhibited by a bolus of tin-mesoporphyrin (SnMP) 15 μM/kg administered intraperitoneally on day 1 ("iBDL", BDL with HMOX inhibition; "iC", control with HMOX inhibition) (George et al., 2013; Porteri et al., 2009). Induction of HMOX was performed by a bolus of hemin 30 μM/kg given intraperitoneally on day 0 and day 3 ("aBDL", BDL with HMOX activation; "aC", control with HMOX activation), (Ndisang et al., 2010; Zhong et al., 2010; Muchova et al., 2015).

Tissue preparation

After five days, the inferior vena cava was cannulated through laparotomy, and blood samples were collected (5 ml), transferred to EDTA-containing tubes, mixed, and placed on ice. An aliquot was centrifuged to separate out the plasma. The livers were then harvested and weighed. Pieces of liver tissue were appropriately processed for further biochemical and histochemical analyses (see below). For quantitative histochemical analysis of GM1 ganglioside, the liver specimens were collected using a systematic uniform random sampling method (Hamilton, 1995).

For RNA analysis, 100 mg of tissue was immediately placed in 1.5 ml microfuge tubes containing RNAlater (Qiagen). The tubes were stored at –20 °C until total RNA isolation.

For HMOX activity, HMOX protein, and lipid peroxidation measurements, 100–150 mg of tissue was diluted 1 : 9 (by weight) in 0.1 M potassium phosphate buffer (pH = 7.4), diced, and sonicated with an ultrasonic cell disruptor (Model XL2000, Misonics, Farmingdale, NY). Sonicates were kept on ice and assayed for HMOX activity within 1 hr, or frozen in liquid nitrogen and stored at -80 °C until analysis of the HMOX protein.

Analysis of plasma markers of cholestasis

In order to determine the degree of cholestasis and liver injury, the following plasma levels were assessed: total bile acids (TBA), total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). TBA were determined spectrophotometrically using a Bile Acids Kit (Trinity Biotech, Jamestown, NY), while all other markers were quantified in an automatic analyser (Model 717, Hitachi, Tokyo).

HMOX activity measurement

Twenty μl of 10% liver sonicate (2 mg fresh weight [FW]) was incubated for 15 min at 37 °C in carbon monoxide (CO)-free septum-sealed vials containing 20 μl of 150 μM methemalbumin and 20 μl of 4.5 mM NADPH as previously described (Vreman et al., 1999). Blank reaction vials contained 0.1 M phosphate buffer (pH = 7.4) in place of NADPH. Reactions were terminated by adding 5 μl of 30% (w/v) sulphosalicylic acid. The amount of CO generated by the reaction and released into the vial headspace was quantitated by gas chromatography with a reduction gas analyser (Trace Analytical, Menlo Park, CA). HMOX activity was calculated as pmol CO/hr/mg FW.

Isolation and TLC analysis of liver gangliosides

Gangliosides were isolated using the procedure previously described (Majer et al., 2007) and finally purified on a small silica gel column (Yu and Ledeen, 1972). Gangliosides were separated in a solvent system (chloroform/methanol/0.2% aqueous $CaCl_2$, 55/45/10, v/v/v), and detected with resorcinol-HCl reagent. Densitometry

was performed according to Majer et al. (2007). Part of the extract was used for determination of the total sialic acid (total gangliosides) by the photometric method with resorcinol reagent (Svennerholm, 1957).

Light microscopy

Small tissue blocks (about 1 cm^3) were fixed in 4% paraformaldehyde followed by the standard procedure for paraffin embedding. Serial sections were cut and stained with haematoxylin and eosin. Each slide was viewed using standard light microscopy.

GM1 histochemistry

GM1 was determined using a modified procedure according to Jirkovska et al. (2007). In brief, 4% formaldehyde was freshly prepared by depolymerization of paraformaldehyde (pH = 7.2). Frozen 6 µm thin sections were first fixed in dry cold acetone (-20 °C) for 15 min, and then in 4% freshly prepared paraformaldehyde for 5 min. Endogenous peroxidase activity was blocked by incubation for 15 min in phosphate-buffered saline (PBS) supplemented with 1% H_2O_2 and 0.1% sodium azide. Endogenous biotin was blocked by means of a DakoCytomation blocking kit (DakoCytomation, Denmark). In order to block nonspecific binding, sections were treated with 3% BSA in PBS for 15 min. GM1 ganglioside in liver sections was detected using CTB biotin labelled (Sigma), diluted 1 : 300 in PBS, plus 3% BSA at 8 °C for 16.5 h, followed with streptavidin-peroxidase polymer at room temperature for 1 h. Peroxidase activity was visualized with diaminobenzidine tetrahydrochloride for 20 min in the dark. Sections were mounted in DAKO S3025 (Dako North America, Inc., Carpinteria, CA). Two negative control tests were performed for each group. First, CTB was omitted in immunohistochemical staining. Second, fixed sections were extracted with chloroform:methanol 2 : 1 at room temperature for 30 min, followed by standard immunohistochemical staining.

Quantitative study of the distribution of GM1 ganglioside in the hepatic lobule

Six liver specimens were used for the experiment. One section of each specimen was used for GM1 ganglioside detection with CTB. In each section, four hepatic lobules with a clearly discernible central vein were selected. In each lobule, one measuring frame in lobular zone II (approximately) was selected for the analysis. The haematoxylin and eosin counterstaining was omitted.

The images of whole sections were photographed at the objective magnification of 40× (NA = 0.12) and stored. The quantity of the reaction product was determined as the mean optical brightness/density of the marked area using the ACC 6.0 image analysis program (SOFO, Brno, Czech Republic). Two different ways were used for quantification. First, the whole section was marked and its mean optical brightness was evaluated. Second, areas of liver parenchyma in the intermediate (zone II) of the hepatic lobules were marked, and their mean optical brightness was determined separately.

Densitometric analysis of GM1 ganglioside in sinusoidal membrane and adjacent cytoplasm areas

Six liver specimens were used from each animal. One section from each specimen was used for GM1 ganglioside detection as described above. In each section, four hepatic lobules with a clearly discernible central vein were selected. In each lobule, one measuring frame in the central lobular zone III and one measuring frame in the corresponding peripheral lobular zone I were selected for analysis. In each frame, 15 areas of sinusoidal surface and 15 areas of adjacent hepatocyte cytoplasm were selected by the stratified random sampling method (Hamilton, 1995) and marked out. The reaction product was quantified as the mean optical density of the analysed areas (determined by the densitometric program ACC 6.0, SOFO) at objective magnification of 40× (NA = 0.7). The ratios of densities measured in the sinusoidal membrane and subsinusoidal intracellular compartment were measured and compared (sin/int).

Quantitative real-time PCR

The liver samples were stored frozen at –80 °C in RNAlater (Sigma Aldrich, St. Louis, MO), and total RNA was isolated using a Qiagen RNAeasy plus kit and QIA shredder (Qiagen). A High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) was used to generate cDNA. Quantitative real-time PCR was performed using a TaqMan® Gene Expression Assay Kit (Life Technologies for the following genes: *GlcT* (Rn00582480_m1), *GalTI* (Rn00581985_m1), *ST3GalV* (Rn01420866_m1), *ST8SiaI* (Rn00563093_m1), *B4GalNTI* (Rn00575768_m1), *B3GalTIV* (Rn01429268_s1), and β-actin endogenous control kit (Rn00667869_m1), all provided by Life Technologies. The data were normalized to β-actin and expressed as percent of control levels.

Statistical analysis

Normally distributed data are presented as the mean ± SD and analysed by the Student's *t*-test. Medians (25–75%) and the Mann-Whitney U test or Kruskall-Wallis test were used in skewed data. Differences with $P < 0.05$ were considered significant.

Results

Induction of cholestasis by BDL

As expected, BDL for five days resulted in a significant increase in plasma cholestatic markers, alkaline phosphatase (ALP) activity, as well as BA and bilirubin concentrations (Table 1). Modulation of HMOX activity had no effect on plasma BA levels, while HMOX activation by hemin led to a significant decrease in ALP activ-

Table 1. The effect of BDL on cholestatic markers, and liver and body weight

Group	**Body weight** [g]	**Liver weight** [g]	**TBA** [μmol/l]	**Bilirubin** [μmol/l]	**ALP** [μkat/l]	**HMOX activity** [pmol CO /hr/mg FW]
C (N = 6)	235.5 ± 9.0	10.4 ± 0.7	17.3 ± 9.2	1.4 ± 0.6	2.0 ± 0.3	327.7 ± 48.0
BDL (N = 8)	224.7 ± 16.0	14.5 ± 1.8*	372.0 ± 132.3*	320.5 ± 74.0*	4.4 ± 0.3*	199.4 ± 37.0*
iBDL (N = 8)	209.3 ± 11.0	13.3 ± 1.9	612.6 ± 164.1*†	215.4 ± 47.0*†	3.5 ± 0.9*	59.3 ± 7.0*†
aBDL (N = 8)	218.9 ± 26.0	15.5* ± 1.7	638.4 ± 188.2*†	291.3 ± 38.1*	3.3 ± 0.5*†	640.6 ± 123.0*†

Cholestatic markers, and liver and body weight in Wistar rats 5 days after surgery. C – control, CO – carbon monoxide, FW – fresh weight. * – $P < 0.05$ vs. C; † – $P < 0.05$ vs. BDL

ity (BDL vs. aBDL, $P < 0.05$). Generally, plasma bilirubin levels were substantially affected by its decreased elimination in cholestasis as well as by the changes in its production following induction/inhibition of HMOX. Accordingly, we observed a significant increase in plasma bilirubin levels in all cholestatic groups compared to controls. However, HMOX inhibition in the iBDL group resulted in a significantly lower plasma bilirubin increase compared to the BDL and aBDL groups (Table 1). Compared to controls, BDL, iBDL and aBDL also led to a significant increase in AST activity, a marker of liver injury (data not shown).

The effect of HMOX modulators on enzyme activity was verified by gas chromatography (Table 1). As expected, inhibition of HMOX activity was achieved in the iBDL group, while its increase was detected following HMOX activation in the aBDL group. A significant decrease in HMOX activity was observed in the BDL group compared to controls.

BDL had no effect on the total body weight of the experimental animals. A 15% drop in total body weight was only observed in the iBDL group compared to the controls (Table 1). By contrast, significant increases in liver weight were recorded in all BDL animals compared to the controls.

Modulation of HMOX activity affects ductular proliferation and biliary infarction in obstructive cholestasis

BDL led to typical morphological features of obstructive jaundice in the liver tissue. The histopathological changes included: portal tract expansion, ductular proliferation (mainly in periportal areas), and interlobular bile duct elongation with dilated lumina and irregular epithelium. The surrounding portal tract tissue was oedematous and infiltrated by nonaggressive inflammatory infiltrate, predominantly with neutrophils. Signs of hepatocellular degeneration were observed – feathery degeneration with flocculent cytoplasm and ballooning with swollen hepatocytes. Variable amounts of intracellular bile pigments and focal signs of necrosis with increased hepatocyte regeneration were observed in all BDL rats. Giant cell transformation with coalescence of hepatocytes, multiple nuclei, and free-floating canaliculi were present.

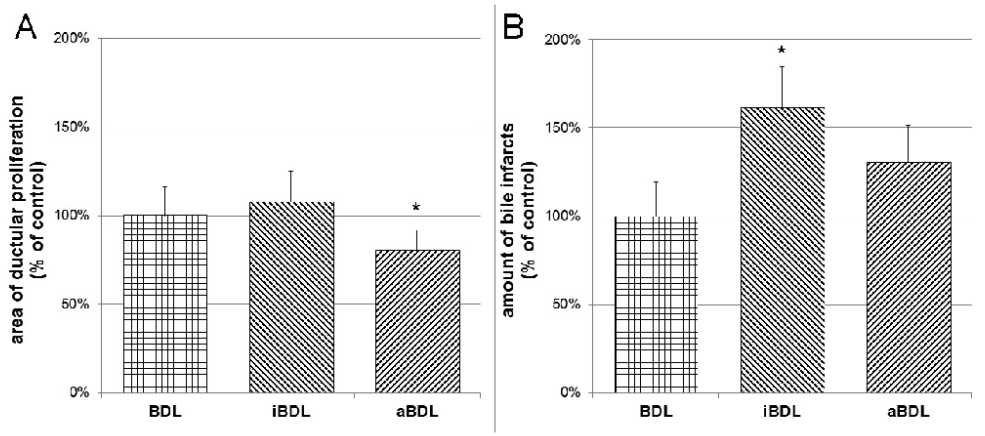

Fig. 2. Area of liver ductular proliferation and number of bile infarcts

A. Area of ductular proliferation in the liver sections of BDL animals. **B**. Number of bile infarcts in liver sections of BDL animals.

Liver sections stained with haematoxylin and eosin were analysed using a systematic uniform random sampling method. Results are expressed as % of controls. * – $P < 0.05$ vs. BDL

HMOX activation resulted in a significant decrease in ductular proliferation (to 83 ± 9 %, $P < 0.05$), while HMOX inhibition had no effect on ductular proliferation compared to BDL animals (Fig. 2A).

BDL rats developed a typical biliary type of liver cell hepatocyte necrosis with typical bile infarcts. The average number of bile infarcts in BDL rats was 0.43 infarcts per liver section. Inhibition of HMOX significantly increased the amount of bile infarcts compared to BDL controls (0.43 ± 0.11 vs. 0.71 ± 0.09 infarcts per liver section, $P < 0.005$). In contrast, no effect was observed upon HMOX activation (0.43 ± 0.11 vs. 0.57 ± 0.1 infarcts per section, $P > 0.05$; Fig. 2B).

BDL and HMOX modulation leads to changes in ganglioside content and spectra

Total lipid sialic acid concentration, a marker of total ganglioside content, was significantly higher in the livers of BDL animals compared to controls (100 ± 20 % vs. 153 ± 15 %, $P < 0.001$). Inhibition of HMOX activity by SnMP resulted in a further increase in total lipid sialic acid content (298 ± 22 %, $P < 0.001$). Unfortunately, due to haem interference, we were not able to measure the sialic acid content in the livers of hemin-treated animals (Table 2).

Next, we analysed the hepatic ganglioside spectra of both *a*- and *b*- biosynthetic pathways (Fig. 3A, 3B and Table 3). While the predominant ganglioside was determined to be GM3 in the control livers, GD1a and GD1b were the major gangliosides in the cholestatic livers. Interestingly, HMOX inhibition led to an increase of GM3 ganglioside, while HMOX activation resulted in significant decreases in the liver content of the *b-series* of gangliosides GD1b and GT1b compared to the BDL group (Table 3). Furthermore, obstructive cholestasis was accompanied by a significant increase in the liver ganglioside content, as determined both by thin layer chromatography (Fig. 3A, 3B) and spectrophotometrically as total sialic acid concentration (Table 2). This increase was most pronounced following HMOX inhibition, whereas a tendency to decrease was observed following HMOX activation (Fig. 3A). BDL led to a significant increase in the total amount of di- and trisialogangliosides. In contrast, activation of HMOX led to a significant decrease in these complex gangliosides (Fig. 3B).

Changes in the mRNA expression of the key enzymes of ganglioside metabolism during BDL

To elucidate the biochemical basis of the observed differences in the ganglioside content spectra, we measured the expression of key enzymes involved in ganglioside synthesis – *GlcT*, *GalTI*, *ST3GalV*, *ST8SiaI*, *B4GalNTI* and *B3GalTIV*.

BDL led to a significant increase in *ST3GalV* mRNA expression compared to controls (197 ± 30 %, $P < 0.05$), (Fig. 4C), and was even more pronounced in iBDL.

Inhibition of HMOX also resulted in a significant increase in *GalTI* (186 ± 22 %, $P < 0.001$), *ST3GalV,* (371 ± 105 %, $P < 0.001$), *ST8SiaI* (185 ± 44 %, $P < 0.05$), and *B3GalTIV* (150 ± 25 %, $P < 0.05$) mRNA expression compared to the control. The effect of HMOX inhibition in the iBDL group observed as overexpression of *ST3GalV* and *ST8SiaI* was also significant compared to the BDL group (Fig. 4). These results are consistent with the results of TLC ganglioside analysis.

HMOX induction resulted in a significant decrease of *B3GalTIV* expression (68 ± 11 %, $P < 0.05$; Fig. 4F). Furthermore, a significant drop in *ST3GalV* mRNA expression was observed in the aBDL group when compared to BDL alone.

Table 2. The effect of BDL on total sialic acid concentrations in the liver tissue

Group	**C** (N = 6)	**BDL** (N = 8)	**iC** (N = 6)	**iBDL** (N = 8)	**aC** (N = 6)	**aBDL** (N = 8)
Total sialic acid [nmol/g liver]	**17.3** ± 3.5	**26.5*** ± 3.9	**29.3*** ± 1.6	**51.6***† ± 6.2	**N.D.**	**N.D.**

Total sialic acid (total gangliosides) concentrations in the liver tissue of Wistar rats 5 days after surgery was measured by the spectrophotometric method with resorcinol reagent. BDL as well as HMOX inhibition led to a significant increase in total sialic acid content. C – control, N.D. – not determined due to haem interference. * – $P < 0.05$ vs. C; † – $P < 0.05$ vs. BDL

Table 3. The effect of BDL on the total amount of gangliosides of the a- and b-branches in the liver tissue

Group	***a-Branch*** [nmol/g liver]			***b-Branch*** [nmol/g liver]		
	GM3	**GM1**	**GD1a**	**GD3**	**GD1b**	**GT1b**
C (N = 6)	2.5 ± 0.9	1.01 ± 0.30	2.01 ± 1.40	1.43 ± 0.50	1.04 ± 0.40	0.62 ± 0.20
BDL (N = 8)	2.62 ± 1.10	1.60 ± 0.40*	5.1 ± 1.3*	1.42 ± 0.30	2.86 ± 0.70*	1.49 ± 0.40*
iBDL (N = 8)	3.69 ± 0.70*†	1.91 ± 0.40*	5.89 ± 1.50*	1.85 ± 0.50*	1.81 ± 0.30*	0.91 ± 0.50
aBDL (N = 8)	3.45 ± 1.20	1.83 ± 0.30*	4.31 ± 0.90*	1.49 ± 0.50	1.47 ± 0.40†	0.92 ± 0.20†

Isolated gangliosides were separated in a solvent system and detected with resorcinol-HCl reagent (nmol/g) with subsequent densitometric analysis. BDL led to a significant increase of terminal gangliosides of the *a*- (GD1a and GM1) as well as *b*-branch (GD1b and GT1b).
C – control, * – $P < 0.05$ vs. C; † – $P < 0.05$ vs. BDL

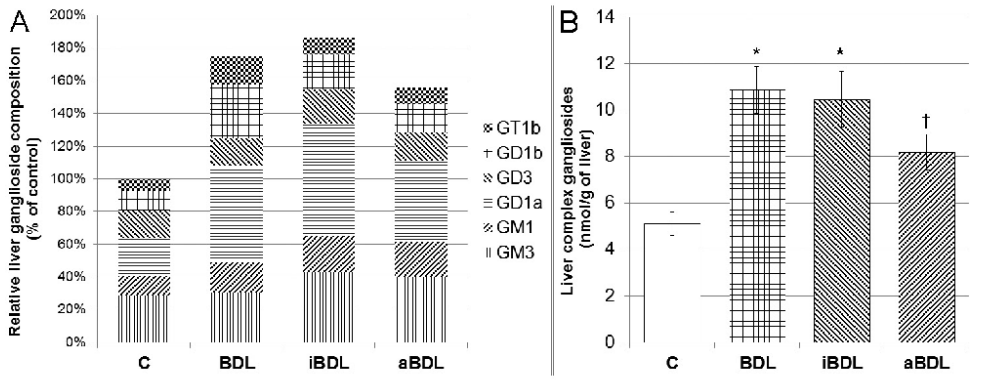

Fig. 3. Effect of BDL on the ganglioside content in the liver
A. Relative amount of gangliosides of the *a*- and *b*-branches in the liver tissue of the control and BDL animals. Isolated gangliosides were separated in a solvent system and detected with resorcinol-HCl reagent, with subsequent densitometric analysis. Results are expressed as % of controls. **B**. Total amount of di- and trisialogangliosides (GD3, GD1, GD1b and GT1b) in the liver tissue of BDL animals.
C – control, * – $P < 0.05$ vs. C, † – $P < 0.05$ vs. BDL

The shift of GM1 ganglioside to the cytoplasmic membrane during cholestasis is affected by HMOX modulation

Under physiological conditions, the GM1 ganglioside is distributed in both the sinusoidal and canalicular hepatocyte membranes, with strong intracellular localization in all lobular zones in the liver sections of the control animals. The same pattern was observed in the control livers with HMOX modulation (iC and aC), indicating no effect of HMOX modulation on ganglioside distribution within the liver cell under physiological conditions.

However, a marked shift of GM1 positivity from the intracellular compartment to the hepatocyte membrane (predominantly sinusoidal) was observed after BDL ($P < 0.01$, Figs. 5 and 6).

Interestingly, HMOX inhibition in BDL animals resulted in an even more pronounced shift of GM1 to the sinusoidal membrane (iBDL vs. BDL; $P < 0.05$, Fig. 5), whereas no shift of GM1 from the intracellular compartment was observed following HMOX induction in BDL rats, resulting in a similar immunohistological pattern as in the control group (Figs. 5 and 6).

Discussion

In the present study, we showed that cholestasis induced by BDL is accompanied by significant changes both in the distribution and synthesis of the liver gangliosides. Moreover, simultaneous induction of the hepatoprotective and anti-cholestatic HMOX enzyme resulted in attenuation of the cholestatic pattern of liver gangliosides.

Even though gangliosides are considered key structural as well as functional parts of the lipid bilayer, and changes in their chemical composition and cellular concentration might have deleterious consequences (d'Azzo et al., 2006), their pathophysiological significance in the liver tissue needs to be elucidated (Sanchez et al., 2000). Altered ganglioside patterns have been reported in cirrhosis and hepatocellular carcinoma in human livers (Tanno et al., 1988) as well as in biliary cirrhosis in the rat (Senn et al., 1991). A different distribution of ganglioside synthases in hepatocytes, Kupffer cells, and sinusoidal endothelial cells was observed in the rat (Senn et al., 1990). Moreover, our group has demonstrated an altered ganglioside pattern in the livers of rats with EE-induced cholestasis (Jirkovska et al., 2007), and its improvement following HMOX induction by haem (Petr et al., 2014; Muchova et al., 2015).

BDL in rats is used as a model of severe obstructive cholestasis in humans. This disease is characterized by interruption of bile flow, followed by a rapid increase in the plasma levels of BA, conjugated bilirubin, and ALP activity.

As expected, BDL was followed by a severe biochemical as well as histological cholestatic pattern. In accordance with our recent data (Muchova et al., 2011), we observed a decrease in liver HMOX activity as a consequence of the inhibitory effect of high plasma and liver concentrations of BA. In contrast to the study on intrahepatic cholestasis (Muchova et al., 2015), HMOX induction with hemin did not lead to improvement in the plasma cholestatic markers. This might be explained by the different mechanisms of the intrahepatic and obstructive cholestasis. While the primary cause of intrahepatic cholestasis is impaired bile flow due to the changes in hepatocyte transporter expression (Trauner et al., 1997), which might be restored by the effect of haem, the biliary tree obstruction is primarily due to a mechanical obstacle followed by secondary changes in the expression of liver transporters.

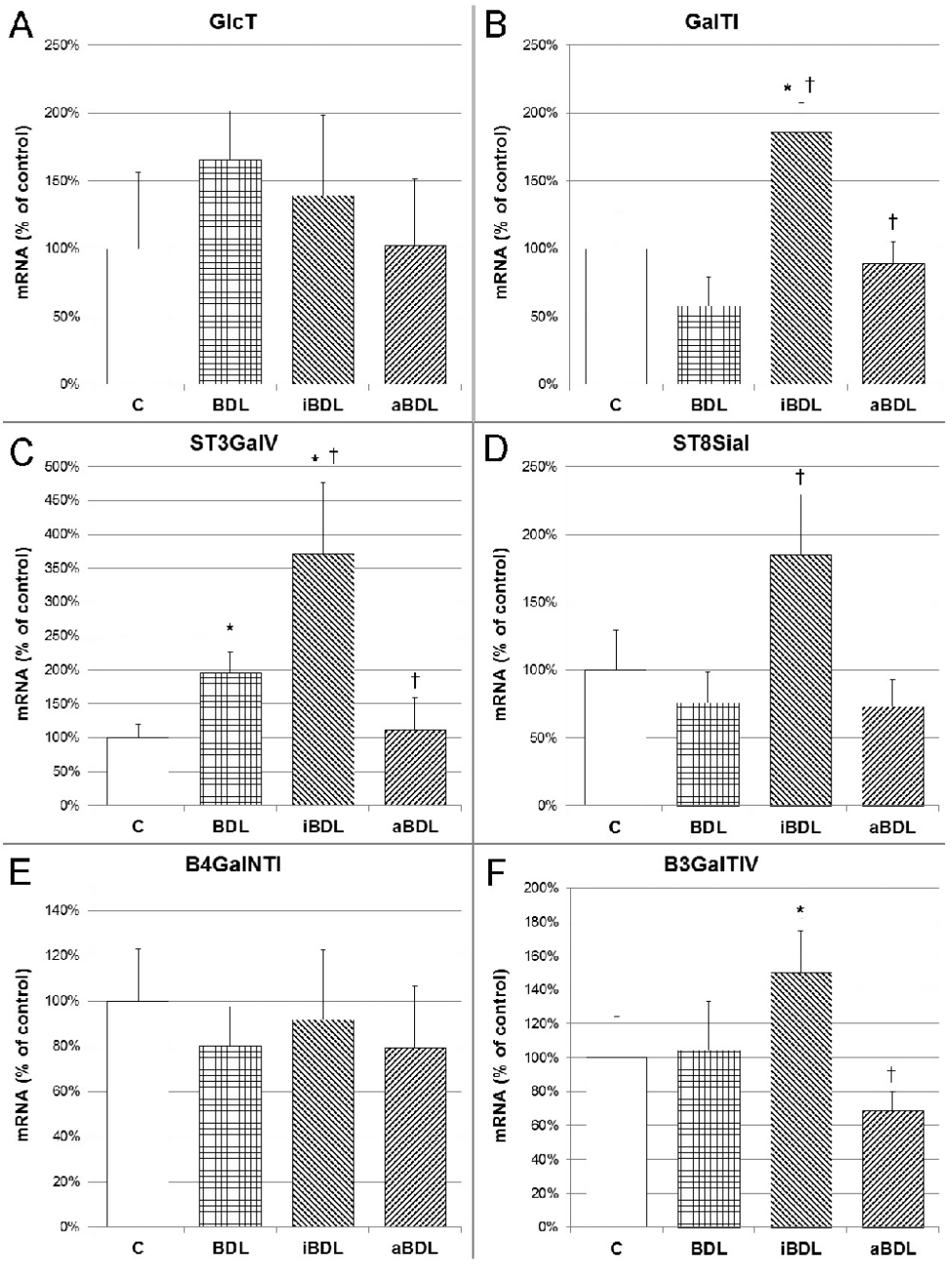

Fig. 4. Effect of BDL on mRNA expression of key enzymes in ganglioside synthesis in the liver
The relative mRNA expression of key glycosyltransferases in ganglioside synthesis was measured in the liver tissue. Results are expressed as % of controls. C – control, * – $P < 0.05$ vs. C, † – $P < 0.05$ vs. BDL

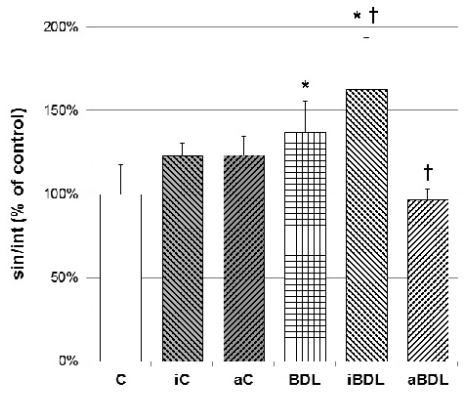

Fig. 5. Distribution of GM1 ganglioside in cholestatic livers Image analysis of the intensity of GM1 ganglioside staining in the subsinusoidal part of the intracellular compartment (int) and sinusoidal membranes (sin) of hepatocytes, expressed as the sin/int ratio. Results are expressed as % of controls. C – control, * – $P < 0.05$ vs. C, † – $P < 0.05$ vs. BDL

A significant decrease in ductular proliferation, the hallmark of obstructive cholestasis, was observed in the liver sections of BDL animals with HMOX induction. Cholangiocytes are mitotically dormant in the normal liver, but start to proliferate upon cholangiopathies including BDL (Glaser et al., 2000, Munshi et al., 2011). Two main signalling pathways are involved in cholangiocyte proliferation, the inositol 1,4,5-trisphosphate/Ca^{2+} signalling pathway and cAMP (Alpini et al., 1998). Interestingly, both of these pathways were found to be regulated by gangliosides in different cell culture models (Ravichandra and Joshi, 1999; Kanda et al., 2001).

Moreover, in our study, a significant increase in complex gangliosides (containing at least two sialic acid residues linked to lactosylceramide) was detected in the liver of BDL animals, as well as those with HMOX inhibition compared to controls. In contrast, HMOX activation resulted in a decrease in liver complex gangliosides when compared with BDL without HMOX modulation. Importantly, a completely different role has been attributed to complex versus monosialyl gangliosides in cellular proliferation and differentiation. While monosialyl gangliosides have an anti-proliferative effect, complex gangliosides enhance cell growth and proliferation (Furukawa et al., 2012), indicating that gangliosides might possess an important regulatory role in ductular proliferation in the cholestatic liver. The proliferation of ductules is a logical response of the liver to BA accumulation, which augment the hepatic bile clearance capacity. However, in the case of obstructive cholestasis, these changes are ineffective (the cause of biliary obstruction lies in the common bile duct) and as a consequence, it could have a destructive effect on the liver parenchyma by replacing the functional hepatocytes by ineffective tissue. In this case, decreasing oxidative stress by hemin administration in cholestasis has a hepatoprotective effect.

The effect of BDL and HMOX modulation on ganglioside synthesis was further supported by determination of mRNA expression of the key enzymes of the ganglioside biosynthesis pathway. BDL animals showed increased *ST3GalV* expression, corresponding to the overall increased ganglioside production from lactosylceramide. HMOX inhibition resulted in a further increase in *ST3GalV,* with simultaneous elevations in *ST8SiaI* and *B3GalTIV,* representing activation of the *b*-branch and terminal gangliosides, respectively. On the other hand, HMOX activation returned the expression of all enzymes to control values. These findings correspond well with the liver ganglioside content as measured by total sialic acid concentrations in the liver, as well as ganglioside spectra in control and cholestatic livers with/without modulation of HMOX.

The accumulation of BA inside hepatocytes is the major cause of cholestatic liver damage (Kullak-Ublick and Meier, 2000), including structural and functional injuries of hepatocyte membranes (Roma et al., 2008), cell death, and activation of inflammatory and fibrogenic signalling pathways (Maher and Friedman, 1993; Muchova et al., 2011). BA may disrupt cell membranes through their detergent action on lipid components and promote generation of ROS, which in turn oxidatively modify lipids, proteins, and nucleic acids; eventually causing hepatocyte necrosis and apoptosis (Perez and Briz, 2009). Recently, it was reported that the canalicular part of the hepatocyte membrane contains detergent-resistant microdomains – lipid rafts – enriched in GSL, including gangliosides, sphingomyelin, and cholesterol, protecting this part of the hepatocyte membrane against the detergent action of BA (Nourissat et al., 2008). There is no doubt that these rafts also exist on the sinusoidal part of the hepatocyte membrane (Zegers and Hoekstra, 1998). Gangliosides as molecules with a high melting point, rigid structure, and highly stable sialic acids are key raft components, responsible for their physical properties.

Recently, Jirkovska et al. (2007) described redistribution of gangliosides within hepatocytes in EE-induced cholestasis. Similarly, in the present study we have shown the shift of the GM1 ganglioside from the intracellular compartment to the sinusoidal membrane of the hepatocyte in cholestatic animals, supporting the concept of a protective effect of gangliosides against the deleterious effect of high plasma levels of BA. Interestingly, this shift was even more pronounced in BDL with HMOX inhibition, while HMOX induction prevented GM1 trafficking to membranes. A similar effect of HMOX induction on ganglioside redistribution was observed by our group (Petr et al., 2014) in the model of EE-induced cholestasis related to reduction of oxidative stress.

We conclude that obstructive cholestasis is accompanied by an increase in the content and synthesis of liver

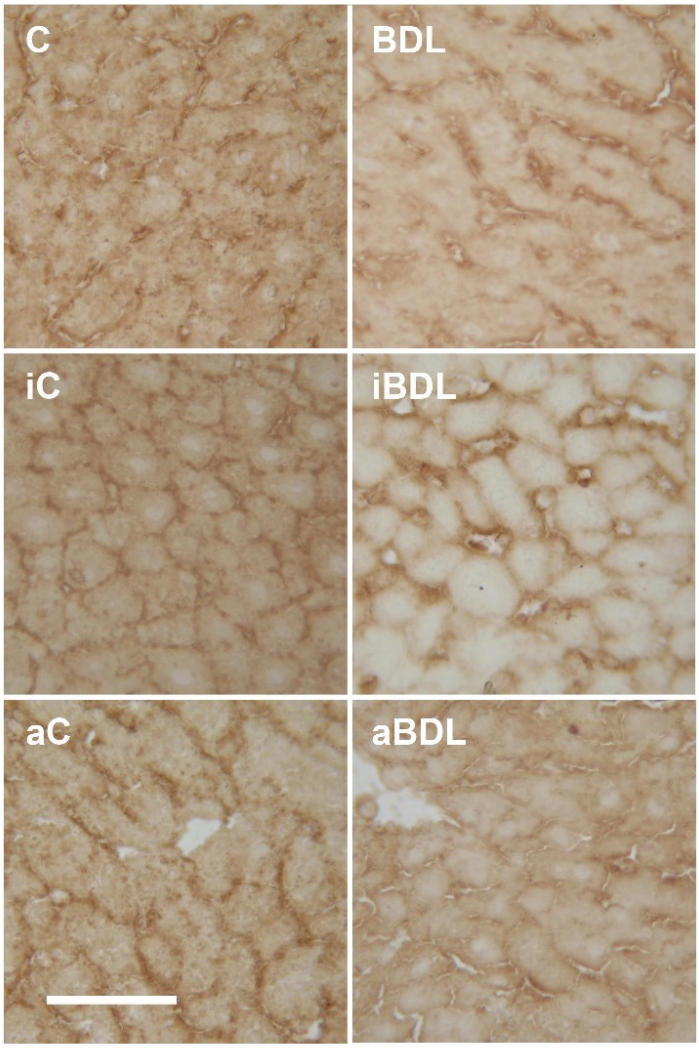

Fig. 6. Localization of GM1 ganglioside in cholestatic livers
In the liver sections, GM1 ganglioside was detected using the cholera toxin B-subunit with streptavidin-peroxidase polymer. Diaminobenzidine tetrahydrochloride (brown colour) was used for visualization. A significant shift of GM1 positivity from the cytoplasm to the sinusoidal membranes was observed after BDL. HMOX inhibition in iBDL animals resulted in an even more pronounced shift of GM1 from the cytoplasm to the sinusoidal membrane, whereas HMOX activation in the aBDL group had the opposite effect. Modulation of HMOX alone in iC and aC had no effect on GM1 localization. C – control. Bar represents 50 μm.

gangliosides followed by their shift into the sinusoidal membranes. This mechanism can protect hepatocytes against the deleterious effect of high systemic concentrations of BA, and also activate proliferation of biliary epithelia. Moreover, the inhibition of antioxidant and hepatoprotective enzyme HMOX in BDL animals further potentiates these changes, while its activation has the opposite effect, indicating the important role of BA-induced oxidative stress in the liver ganglioside metabolism. These results might have potential therapeutic implications, since many drugs and natural products exhibit HMOX-inducing activities (Bach, 2005), and thus might serve as hepatoprotectants.

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Research Article

Heme Oxygenase-1 May Affect Cell Signalling via Modulation of Ganglioside Composition

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Heme oxygenase 1 (Hmox1), a ubiquitous enzyme degrading heme to carbon monoxide, iron, and biliverdin, is one of the cytoprotective enzymes induced in response to a variety of stimuli, including cellular oxidative stress. Gangliosides, sialic acid-containing glycosphingolipids expressed in all cells, are involved in cell recognition, signalling, and membrane stabilization. Their expression is often altered under many pathological and physiological conditions including cell death, proliferation, and differentiation. The aim of this study was to assess the possible role of Hmox1 in ganglioside metabolism in relation to oxidative stress. The content of liver and brain gangliosides, their cellular distribution, and mRNA as well as protein expression of key glycosyltransferases were determined in *Hmox1* knockout mice as well as their wild-type littermates. To elucidate the possible underlying mechanisms between Hmox1 and ganglioside metabolism, hepatoblastoma HepG2 and neuroblastoma SH-SY5Y cell lines were used for *in vitro* experiments. Mice lacking *Hmox1* exhibited a significant increase in concentrations of liver and brain gangliosides and in mRNA expression of the key enzymes of ganglioside metabolism. A marked shift of GM1 ganglioside from the subsinusoidal part of the intracellular compartment into sinusoidal membranes of hepatocytes was shown in *Hmox1* knockout mice. Induction of oxidative stress by chenodeoxycholic acid *in vitro* resulted in a significant increase in GM3, GM2, and GD1a gangliosides in SH-SY5Y cells and GM3 and GM2 in the HepG2 cell line. These changes were abolished with administration of bilirubin, a potent antioxidant agent. These observations were closely related to oxidative stress-mediated changes in sialyltransferase expression regulated at least partially through the protein kinase C pathway. We conclude that oxidative stress is an important factor modulating synthesis and distribution of gangliosides *in vivo* and *in vitro* which might affect ganglioside signalling in higher organisms.

1. Introduction

Heme oxygenase 1 (Hmox1) is a highly inducible antioxidant and cytoprotective enzyme in the heme catabolic pathway generating equimolar amounts of iron, carbon monoxide, and biliverdin which is immediately reduced to bilirubin [1]. Hmox1 activity—also due to the effect of its bioactive products—affects pathophysiology of many neurologic, cardiovascular, and pulmonary diseases [2–4]. In the liver, Hmox1 plays an important role in hepatic fat accumulation, fibrogenesis, ischemia-reperfusion, and oxidative injury [5]. Moreover, upon *Hmox1* knockout, the cells and/or animals become more vulnerable to oxidative stress. Free radical formation as well as oxidative stress-

associated cytotoxicity are increased in *Hmox1* knockouts due to reduced antioxidant bilirubin and vasoactive carbon monoxide formation, disruption of iron homeostasis, and accumulation of prooxidative heme [6]. Due to iron accumulation, liver is one of the tissues most affected by an increased oxidative stress in *Hmox1* knockout mice and increased lipid peroxidation, fibrosis, and hepatic injury have been described in these animals [5]. Furthermore, an increase in some key cytoprotective genes such as NAD(P)H dehydrogenase quinone 1 and glutathione S-transferase P1 and marked decrease in peroxyl radical scavenging activity have been described in *Hmox1* knockouts even under basal (unstimulated) conditions [7]. Bilirubin per se is considered a potent endogenous antioxidant protecting against diseases associated with oxidative stress [8] and counteracting harmful effects of various prooxidants including hydrophobic bile acids (BA) on cells and tissues [9]. In fact, both bilirubin and BA are accumulated in plasma and tissues during cholestasis and while BA are responsible for increased lipid peroxidation and oxidative liver damage, bilirubin has a protective effect [10].

Gangliosides are ubiquitously found in all tissues, but most abundantly in the nervous system [11]. They substantially influence the organization of the membrane and the function of specific membrane-associated proteins due to lipid-lipid and lipid-protein lateral interactions [12]. In the brain, ganglioside expression correlates with neurogenesis, synaptogenesis, synaptic transmission, and cell proliferation [13, 14].

It is known that gangliosides form so called caveolae or "detergent resistant microdomains" (DRM), which are crucial elements for cell-cell recognition, adhesion, and especially membrane stabilization [15, 16]. There is also evidence that caveolin-1, an important component of caveolae, interacts with Hmox1, modulates its activity, and can act as a natural competitive inhibitor of Hmox1 with heme [17]. Moreover, gangliosides have been found to inhibit hydroxyl radical formation *in vitro* [18] and also modulate ROS formation in human leukocytes [19] and neuronal cells [20].

Despite the close relationship of gangliosides and Hmox1 in DRM, there are only few reports discussing the possible role of Hmox1 or oxidative stress in ganglioside metabolism [21, 22]. The aim of this study was to assess the role of *Hmox1* knockout and associated oxidative stress on ganglioside metabolism and to identify the possible underlying mechanisms.

2. Materials and Methods

2.1. Materials. Paraformaldehyde, biotin, bovine serum albumin (BSA), phorbol 12-myristate 13-acetate (protein kinase C (PKC) activator), Ro 31-0432 (PKC inhibitor), chenodeoxycholic acid (CDCA), diaminobenzidine tetrahydrochloride tablets, NADPH, and sulfosalicylic acid were supplied by Sigma-Aldrich (St. Louis, MO, USA); avidin was obtained from Fluka (Buchs, Switzerland), the cholera toxin B subunit (CTB) peroxidase conjugated came from List Biological Laboratories (CA, USA), and the HPTLC silica-gel plates came from Merck (Darmstadt, Germany). Cell plates were supplied by Corning (NY, USA). The TaqMan® Gene Expression Master Mix, High-Capacity RNA-to-cDNA Kit, and the TaqMan Gene Expression Assay kit for mouse and human genes were obtained from Life Technologies (Carlsbad, CA, USA). The QIAshredder kit and RNeasy Plus Mini Kit were supplied by Qiagen (USA). All other chemicals were purchased locally from Penta (Prague, Czech Republic).

2.2. Animals. $Hmox1^{-/-}$ ($n = 9$; KO—knockout) mice and $Hmox1^{+/+}$ ($n = 6$; Wt—wild type) littermates (C57Bl/6xFVB, 8-week-old males) were used for all the experiments. Breeding heterozygote pairs of *Hmox1*-deficient mice were initially kindly provided by Anupam Agarwal, University of Alabama (Birmingham, AL). The $Hmox1^{-/-}$ strain poorly breeds on pure C57/Bl6 background (5.1% of expected $Hmox1^{-/-}$ pups) and therefore is maintained on mixed C57/Bl6 × FVB background (20.1% of expected $Hmox^{-/-}$ pups, when $Hmox^{-/-}$ males are crossed with $Hmox^{+/-}$ females) [23]. All $Hmox1^{+/+}$ controls were C57/Bl6xFVB littermates from the same breeders used to obtain $Hmox1^{-/-}$ mice. They had free access to food and water and were kept in individually ventilated cages with a 12:12 day/night cycle, under a specific pathogen-free regime. All aspects of the study met the accepted criteria of experimental use of laboratory animals, and all protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University, Prague, Czech Republic, and by the 1st Local Ethics Committee for Animal Research, Krakow, Poland.

2.3. Tissue Preparation. Mice were intraperitoneally anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) and sacrificed by cervical dislocation at day 5. The inferior vena cava was cannulated through laparotomy, and blood samples were collected, transferred to EDTA-containing tubes, mixed, and placed on ice. An aliquot was centrifuged to separate out the plasma. The livers and brains were then harvested and weighed. Pieces of liver tissues were appropriately processed for further biochemical and histochemical analyses (see below). For quantitative histochemical analysis of GM1 ganglioside, the liver specimens were collected using a systematic uniform random sampling method [24].

For the RNA analysis, 100 mg of tissue was immediately placed in 1.5 mL microcentrifuge tubes containing RNAlater (Qiagen, Valencia, CA, USA). The tubes were stored at −80°C until total RNA isolation.

2.4. Extraction and TLC Densitometry of Liver and Brain Gangliosides. The chloroform-methanol extraction of gangliosides from the liver tissue was used—the procedure previously described by Majer et al. [25]—and gangliosides were finally purified on a small silica gel column [26]. Brain gangliosides were isolated as described previously [27, 28]. All ganglioside samples were separated in a solvent system (chloroform/methanol/0.2% aqueous $CaCl_2$, 55/45/10, *v*/*v*/*v*) and detected with resorcinol-HCl reagent. The densitometry was performed according to Majer et al. [25] using a CATS3 Software, CAMAG (Switzerland).

GSL are abbreviated according to recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [29]: glycosyltransferases: *GlcT*, UDP-glucose ceramide

glucosyltransferase; *GalTI*, UDP-Gal:betaGlcNAc beta-1,4-galactosyltransferase; *ST3GalV (GM3 synthase)*, ST3 beta-galactoside alpha-2,3-sialyltransferase 5; *ST8SiaI (GD3 synthase)*, ST8 alpha-*N*-acetylneuraminide alpha-2,8-sialyltransferase 1; *B4GalNTI (GM2/GD2 synthase)*, beta-1,4-*N*-acetyl-galactosaminyltransferase 1; and *B3GalTIV (GM1 synthase)*, UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase.

2.5. GM1 Histochemistry. GM1 was determined using a modified procedure according to Jirkovská et al. [30]. In brief, 4% formaldehyde was freshly prepared by depolymerization of paraformaldehyde (pH = 7.2). Frozen 6 μm thin sections were first fixed in dry cold acetone (−20°C) for 15 min and then in 4% freshly prepared paraformaldehyde for 5 min. Endogenous peroxidase activity was blocked by incubation for 15 min in phosphate-buffered saline (PBS) supplemented by 1% H_2O_2 and 0.1% sodium azide. Endogenous biotin was blocked by means of a DakoCytomation blocking kit (DakoCytomation, Denmark). In order to block nonspecific binding, sections were treated with 3% BSA in PBS for 15 min. GM1 ganglioside was detected in liver sections using CTB biotin labelled (Sigma, USA), diluted 1:300 in PBS, plus 3% BSA at 8°C for 16.5 h, followed with streptavidin-peroxidase polymer at room temperature for 1 h. Peroxidase activity was visualized with diaminobenzidine tetrahydrochloride for 20 min in the dark. Sections were mounted in mounting medium Dako S3025 (Dako North America, CA, USA).

Two negative control tests were performed for each group. First, CTB was omitted in immunohistochemical staining. Second, fixed sections were extracted with chloroform : methanol 2:1 at room temperature for 30 minutes followed by standard immunohistochemical staining.

2.6. Densitometric Analysis of GM1 Ganglioside in Sinusoidal Membrane and Adjacent Cytoplasm Areas. Six liver specimens were used from each animal. One section from each specimen was used for GM1 ganglioside detection with CTB as described above. From each section, four hepatic lobules with a clearly discernible central vein were selected. In each lobule, one measuring frame in the central lobular zone III and one measuring frame in the corresponding peripheral lobular zone I were selected for analysis. In each frame, 15 areas of sinusoidal surface and 15 areas of adjacent hepatocyte cytoplasm were selected by the stratified random sampling method [24] and marked out. The reaction product was quantified as the mean optical density of the analyzed areas (determined by the densitometric program ACC 6.0, SOFO, Czech Republic) at objective magnification of 40x (NA = 0.7). The ratios of densities measured in the sinusoidal membrane and subsinusoidal intracellular compartment were measured and compared together (sin/int).

2.7. Cell Culture Experiments. Human neuroblastoma cell line SH-SY5Y (ATCC, Manassas, VA, USA) was cultured in the Minimum Essential Medium Eagle (MEM) and Ham's F-12 medium (1:1, *v*/*v*) with 15% of fetal bovine serum and human hepatoblastoma cell line HepG2 (ATCC, Manassas, VA, USA) in MEM with 10% of fetal bovine serum in a humidified atmosphere (containing 5% CO_2 and 37°C). Authentication of used cell lines was confirmed by an independent laboratory using a method based on an accredited short tandem repeat analysis.

Cells were seeded onto 6-well plates (Corning, NY, USA) at a concentration of 50,000 cells per 1 cm^2 and treated with CDCA and bilirubin for 4 h. SH-SY5Y cells were also treated with PKC activator (phorbol 12-myristate 13-acetate) or PKC inhibitor (Ro 31-0432) for 4 h. After the incubation period, cells were harvested into the lysis buffer and stored at −80°C for further experiments.

2.8. Measurement of Intracellular ROS Production. ROS production was determined using a fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA, Life Technologies, USA). Briefly, SH-SY5Y cells were grown in 12-well plates to 80% confluence. Cells were then incubated with CDCA and/or antioxidant (bilirubin) for 24 h. After the incubation, the cells were washed twice with PBS and loaded with 10 μM CM-H2DCFDA at 37°C for 30 min in the dark, then washed with PBS to remove excess dye. Fluorescence was measured using 485 nm excitation and 540 nm emission wavelengths in microplate reader (Synergy HTX, BioTek, USA). Cells were then lysed with Cell Lysis Buffer (Cell Signaling Technology, USA), and protein concentration was measured using DC Protein Assay (Bio-Rad, USA) according to the manufacturer's instruction. Data were normalized to protein content and expressed as % of controls.

2.9. Lipid Peroxidation. Lipid peroxidation was measured according to the method by Vreman et al. [31]. Twenty microliters of 10% liver or brain sonicates in 0.1 M phosphate buffer, pH = 7.4, were incubated at 37°C with 100 μM ascorbate (80 μL) and 6 μM Fe^{2+} (0.5 μL) in a septum-sealed vial. Butylated hydroxytoluene (10 μM) was added to the blank reaction. The reaction was terminated by adding 2 μL of 60% sulfosalicylic acid. CO produced into the vial headspace was quantified by gas chromatography with a reduction gas analyzer (Peak Laboratories LLC, Mountain View, CA, USA). The amount of CO produced served as an index of lipid peroxidation, was measured as picomoles of CO per hour per milligram of fresh tissue, and was expressed as % of control.

2.10. Western Blotting. Cells grown to 60% confluency were lysed using RIPA buffer supplemented with phosphatase and protease inhibitors (Protease Inhibitor Mix G and Phosphatase Inhibitor Mix I, Serva, Heidelberg, Germany). Samples were separated by SDS-PAGE on 12% polyacrylamide gel and then transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking in Tween-PBS with 5% BSA (Sigma-Aldrich, St. Louis, MO, USA) for at least 1.5 h, membranes were incubated with GM3 synthase and GM2/GD2 synthase antibody (1:2000; Santa Cruz sc-365329 and sc-376505, Dallas, TX, USA), or β-actin (1:2000; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After washing, membranes were incubated with anti-mouse m-IgGκ BP-HRP (Santa Cruz

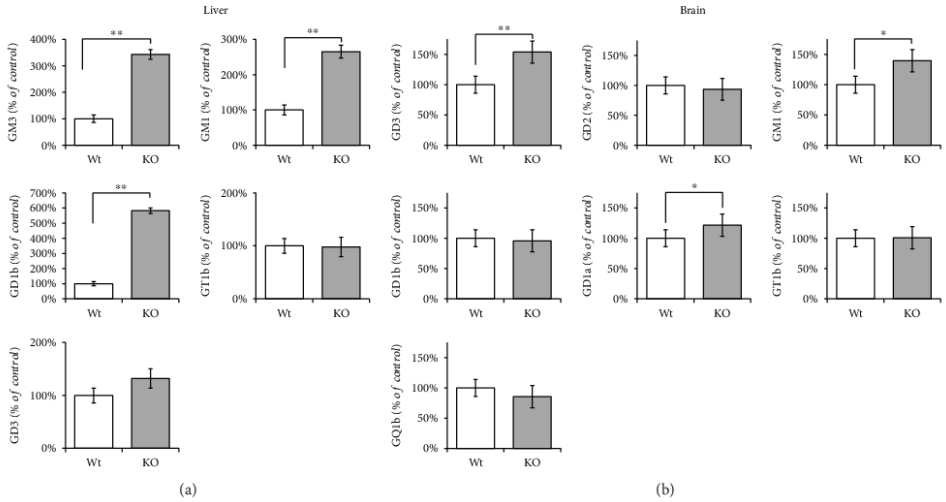

Figure 1: The impact of *Hmox1* knockout on ganglioside composition in mouse (a) liver and (b) brain. Isolated hepatic (a) and brain (b) gangliosides were separated in a solvent system and analyzed by a densitometric method after TLC separation and detection using resorcinol-HCl reagent. Values are expressed as % of control and represent mean SD. Wt: wild-type ($n = 6$); KO: *Hmox1* knockout ($n = 9$). $^{*}P < 0.05$ and $^{**}P < 0.01$.

sc-516102, Dallas, TX, USA) for 1 h. Immunocomplexes on the membranes were visualized with ECL Western Blotting Detection Reagents (Cell Signaling Technology) using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

2.11. Quantitative Real-Time PCR. The liver samples were stored frozen at −80°C in RNAlater (Sigma-Aldrich, St. Louis, USA), and total RNA was isolated using a Qiagen RNAeasy Plus kit and QIAshredder (Qiagen, USA). Cell culture samples were stored in lysis buffer at −80°C, and total RNA was isolated using PerfectPure RNA Cell Kit (5Prime, USA). A High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) was used to generate cDNA. Quantitative real-time PCR was performed using TaqMan® Gene Expression Assay Kit (Life Technologies, Carlsbad, CA, USA) for the following genes: *GlcT* (Hs00234293_m1), *GalTI* (Hs00191135_m1), GM3 synthase (*St3GalV*, Mm00488237_m1, and Hs01105379_m1), GD3 synthase (*STSia8*, Mm00456915_m1, and Hs00268157_m1), GM2/GD2 synthase (*B4GalNT1*, Mm00484653_m1, and Hs01110791_g1), and GM1 synthase (*B3GalT4*, Mm00546324_s1, and Hs00534104_s1), all provided by Life Technologies (Carlsbad, CA, USA). The data were normalized to HPRT and expressed as percent of control levels.

2.12. Statistical Analysis. Normally distributed data are presented as mean ± SD and analyzed by the Student *t*-test. The Mann–Whitney *U* test or Kruskal-Wallis test were used in skewed data. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. The Impact of Hmox1 Knockout on the Liver and Brain Ganglioside Content. To investigate the role of *Hmox1* knockout on the ganglioside pattern, we measured changes in ganglioside composition in the liver as well as the brain, the tissue with the highest glycolipid content *in vivo*. As the ganglioside spectra differ within specific tissues, only major gangliosides and representatives of two main biosynthetic pathways, *a*- and *b*-series, were determined.

In the liver, mice lacking *Hmox1* exhibited marked increases in the concentrations of individual gangliosides. Specifically, *Hmox1* knockout led to a significant increase in GM3 (343 ± 76%, $P < 0.001$) and GM1 (265 ± 62%, $P < 0.001$) representing *a*-series, and GD1b (582 ± 176%, $P < 0.001$) from *b*-series of gangliosides (Figure 1(a)).

In the brain, the most abundant ganglioside was GD1a in both wild-type as well as knockout animals. Together with GM1, GD1a content was significantly higher (GD1a 122% vs. Wt, $P < 0.05$; GM1 140% vs. Wt, $P < 0.05$) in *Hmox1* knockout mice as compared to wild types. The other two major brain gangliosides (GM3, GT1b) stayed unchanged after *Hmox1* knockout. The amount of minor GD3 ganglioside was also significantly increased (154% vs. Wt, $P < 0.01$) (Figure 1(b)). The scheme of de novo biosynthesis of the oligosaccharide moieties of gangliosides is illustrated in Figure 2.

To confirm the level of oxidative stress in *Hmox1* knockouts, we measured the extent of lipid peroxidation in liver and brain tissue homogenates. Importantly, liver lipid peroxidation was increased in *Hmox1* knockout mice as compared to controls (155% ± 51% $Hmox^{-/-}$, $n = 7$, vs. 100% ± 46%

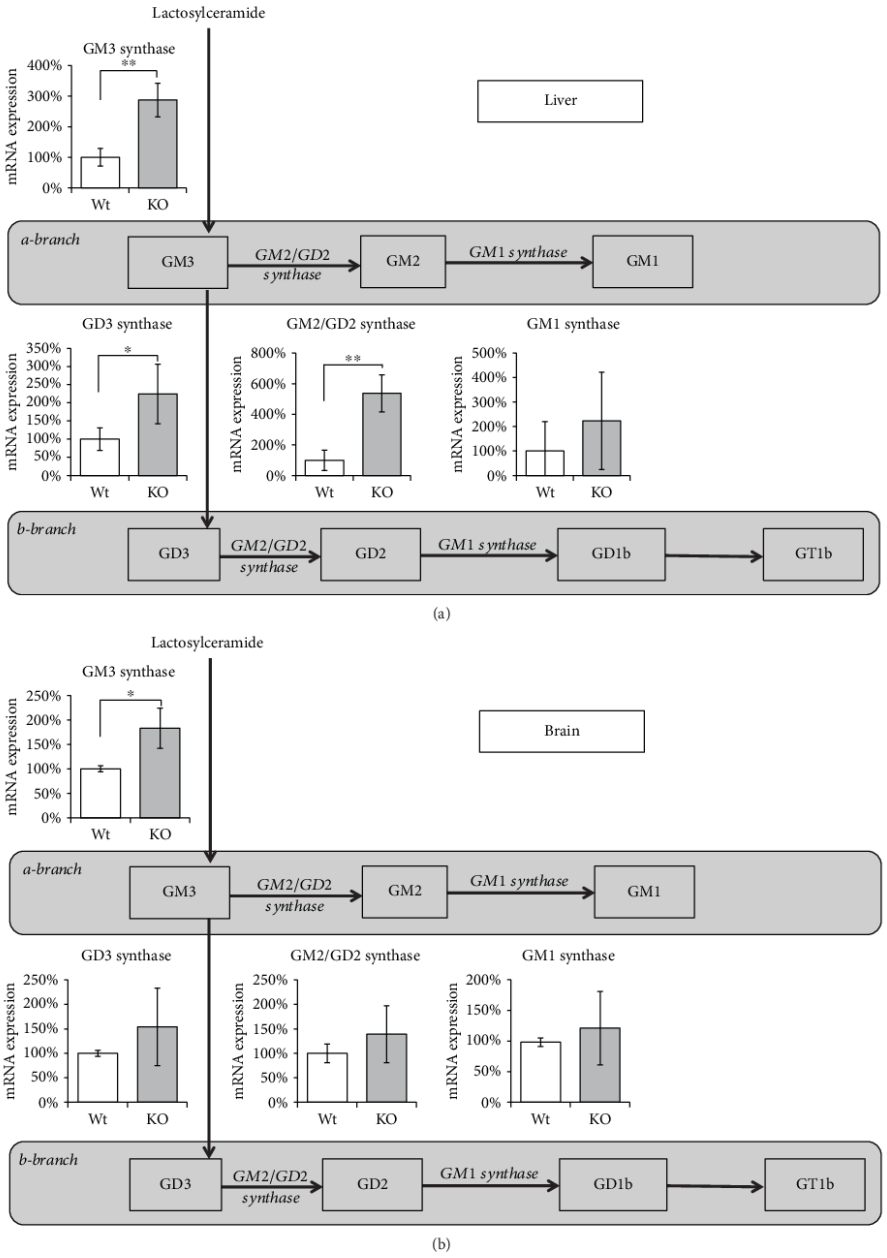

Figure 2: *Hmox1* knockout leads to changes in sialyltransferase expression in the liver and brain. Relative mRNA expression of the key enzymes in ganglioside synthesis was measured in the liver (a) and brain (b) tissues of wild-type (Wt) and *Hmox1* knockout (KO) animals. Values are expressed as % of control. Wt: wild-type ($n = 6$); KO: *Hmox1* knockout ($n = 9$). $^{*}P < 0.01$; $^{**}P < 0.001$.

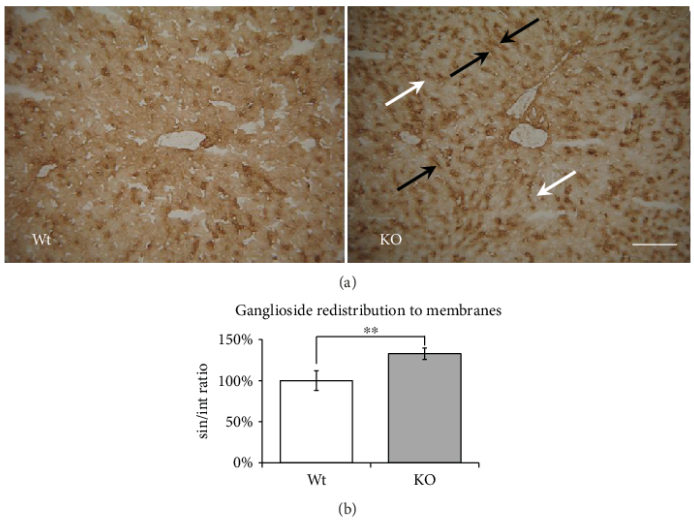

FIGURE 3: The effect of *Hmox1* knockout on distribution/localization of GM1 ganglioside in the liver. (a) Immunohistochemical detection of GM1 ganglioside. In the liver sections, GM1 ganglioside was detected using the cholera toxin B subunit with streptavidin-peroxidase polymer. Diaminobenzidine tetrahydrochloride (brown colour) was used for visualization. The shift of GM1 ganglioside expression from intracellular localization (white arrows) into sinusoidal membranes (black arrows) was observed in *Hmox1* knockout animals. (b) Quantification of GM1 staining in the liver. Image analysis of the distribution of GM1 ganglioside staining in the subsinusoidal part of the intracellular compartment (int) and sinusoidal membranes (sin) of hepatocytes, expressed as the sin/int ratio relative to control (Wt). The reaction product was quantified as the mean optical density of the analyzed areas at objective magnification of 40x (NA = 0.7). Bar represents 100 μm. Wt: wild-type ($n = 6$); KO: *Hmox1* knockout ($n = 9$). $^{**}P < 0.01$.

$Hmox^{+/+}$, $n = 6$, $P = 0.032$). No significant increase was observed in a brain tissue (115% ± 46% $Hmox^{-/-}$, $n = 7$, vs. 100% ± 45% $Hmox^{+/+}$, $n = 6$, $P = 0.311$).

3.2. Hmox1 Knockout Results in Changes in the Expression of Sialyltransferases. Relative mRNA expression of the key sialyltransferases was determined to elucidate the activation rate of *a*- and *b*-series of a ganglioside biosynthetic pathway in mouse liver and brain homogenates. *Hmox1* knockout led to a significant increase in mRNA expression of GM3 synthase (*ST3GalV*) (287 ± 55%, $P < 0.001$; 183 ± 41%, $P < 0.01$) in both liver and brain, and GD3 synthase (*St8SiaI*) (224 ± 89%, $P < 0.01$), the key step in an activation of *b*-biosynthetic branch in the liver. *Hmox1* knockout caused also significant activation of GM2/GD2 synthase (*B4GalNTI*) (538 ± 121%, $P < 0.001$) in the liver. Expression of GM1 synthase (*B3GalTIV*) stayed unchanged in both liver and brain (Figure 2).

3.3. Hmox1 Knockout Leads to a Marked Shift of Gangliosides to the Hepatocyte Membrane. To study possible changes in distribution of gangliosides within mouse hepatocytes, histochemical localization of GM1, the representative of gangliosides, was determined in the liver sections. In control liver specimens, GM1 was detected in both sinusoidal and canalicular membranes, as well as in the intracytoplasmic compartment. In *Hmox1* knockout animals, we observed a pronounced shift in GM1 ganglioside expression from intracellular localization into sinusoidal membranes (Figure 3(a)). To quantify this redistribution pattern of GM1, we measured the GM1 expression under high microscopic magnification expressed as sin/int ratio (GM1 staining in the sinusoidal membrane/subsinusoidal intracellular compartment) (133 ± 7%, $P < 0.01$, Figure 3(b)).

3.4. Ganglioside Pattern in Neuroblastoma Cells (SH-SY5Y) Is Affected by Oxidative Stress. To find out whether changes in the ganglioside pattern might be affected by an increased oxidative stress associated with *Hmox1* knockout, we investigated the regulation of glycosphingolipid (GSL) synthesis using SH-SY5Y neuroblastoma cells rich in gangliosides. CDCA, a potent inducer of ROS production accumulating in the liver during cholestasis, was used to increase oxidative stress *in vitro*, while addition of bilirubin, a potent antioxidant and a product of the Hmox pathway, had an opposite effect (Figure 4).

Administration of CDCA (80 μM) resulted in a significant increase in the major gangliosides GD1a (141%, $P < 0.01$), GM3 (170%, $P < 0.01$), and GM2 (130%, $P < 0.05$) in SH-SY5Y neuroblastoma cells (Figure 5(a)) and GM3 (233%, $P < 0.01$) and GM2 (251%, $P < 0.05$) in hepatoblastoma HepG2 cells (Figure 5(b)). Interestingly, coadministration of bilirubin (CDCA/bilirubin), a potent antioxidant, resulted in normalization of the ganglioside pattern in both cell lines (Figure 5).

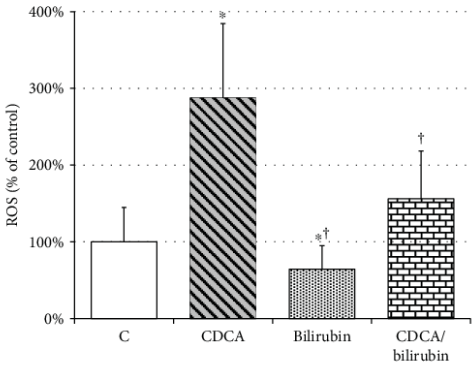

FIGURE 4: The ameliorating effect of bilirubin on CDCA-induced oxidative stress in SH-SY5Y cells. SH-SY5Y cells were incubated with CDCA (80 μM), bilirubin (1 μM), or both (CDCA/bilirubin) for 24 h. ROS production was measured using fluorescent CM-H2DCFDA probe. Values are expressed as % of controls. C: control; CDCA: chenodeoxycholic acid (80 μM); bilirubin: 1 μM bilirubin; CDCA/bilirubin: CDCA (80 μM) + bilirubin (1 μM). $^{*}P < 0.05$ vs. C; $^{\dagger}P < 0.05$ vs. CDCA.

3.5. Oxidative Stress-Mediated Changes in Sialyltransferase (ST3GalV) Expression Are Regulated through the Protein Kinase C Pathway. To elucidate whether oxidative stress induced by CDCA affects the expression of GM3 synthase (*ST3GalV*), a key enzyme in ganglioside metabolism, *in vitro*, SH-SY5Y as well as HepG2 cell lines were incubated with CDCA and/or bilirubin for 4 h. Significant increases in GM3 synthase mRNA expression were observed upon CDCA treatment while cotreatment with bilirubin abolished this effect in both neuroblastoma (Figure 6(a)) and hepatic (Figure 6(c)) cell lines. The results were confirmed by the detection of GM3 synthase protein expression in the SH-SY5Y cell line (Figure 6(b)).

To investigate the possible role of the PKC pathway on oxidative stress-mediated changes in ganglioside expression, we measured the effect of PKC induction/inhibition ($PKC^{+/-}$) on the mRNA expression of *GM3 synthase* (*ST3GalV*), in the SH-SY5Y cell line. PKC activators induced the mRNA expression of *ST3GalV*. On the other hand, PKC inhibitors significantly decreased *ST3GalV* mRNA expression. Importantly, cotreatment of CDCA with PKC inhibitor completely abolished the stimulatory effect of CDCA on *ST3GalV* mRNA (Figure 6(d)). Successful PKC activation and/or inhibition was proven by determination of mRNA expression of PKC alpha, PKC beta, and PKC epsilon (Figure 6(e)).

4. Discussion

Gangliosides play a crucial role in signal transduction pathways, regulating many different cell functions such as proliferation, differentiation, adhesion, and cell death [32, 33]. They are responsible for the rigidity of a plasmatic membrane [34] and participate in a protection against oxidative stress [19, 21]. However, the significance of changes in ganglioside metabolism under oxidative stress remains to be elucidated. To address this issue, we have studied the consequences of the antioxidant enzyme Hmox1 deficiency for ganglioside metabolism in mouse tissues. Unlike in the brain, we found significantly increased lipid peroxidation in the liver of *Hmox1* knockout animals which is in accordance with the published data showing increased lipid peroxidation and hepatic injury mostly due to iron accumulation in the liver tissue [6]. Our results indicate that oxidative stress plays an important role in ganglioside synthesis resulting in changes in their spectra and cellular distribution.

Gangliosides are ubiquitously found in tissues and body fluid with the most abundant expression in the nervous system [35]. The expression levels of gangliosides undergo dramatic changes during various physiological and pathological conditions including cell death, proliferation, differentiation, development, and oncogenesis [36–38] as well as neurological diseases [39, 40]. These effects are largely attributed to the changes in expression levels of ganglioside synthases (glycosyltransferases) [41, 42]. In our previous experiments on rats, we observed the shift in liver ganglioside synthesis towards more complex ones in various types of cholestatic liver diseases [25, 30]. These changes were associated with the accumulation of detergent and prooxidative bile acids as well as with the increase in oxidative stress in these animals [21, 22].

In the present study, *Hmox1* knockout resulting in the absence of an important antioxidant enzyme in experimental mice was accompanied by significant increases in the brain GM1, GD1a, and GD3 and liver GM1, GM3, and GD1b gangliosides. The tissue specificity of these changes might be explained by the different ganglioside composition of the liver and brain tissues. While GM3 is the main ganglioside in the liver, GD1a is the most abundant in the adult brain [38, 43], where GD1a, GM1, GD1b, GT1b, and GD3 belong to most important glycosphingolipids [44]. Several reports suggest gangliosides to possess antioxidant properties, but very little is known about the function of gangliosides in the liver and therefore most data relates to the nervous tissue [11]. Among these, GM1 ganglioside has neuroprotective functions. Micelles containing GM1 inhibited iron-catalysed hydroxyl radical formation *in vitro* [18], GM1 decreased ROS formation in rat brain synaptosomes [45], or protected cells against H_2O_2-induced oxidative damage [46] while GD1b was able to inhibit lipid peroxidation in human sperm cells [47]. On the other hand, some gangliosides might enhance ROS formation and contribute to the cell death. Sohn et al. [48] found GM3, but not GD3 or GT1b, to mediate oxidative toxicity induced by glutamate in immortalized mouse neuronal HT22 cells. GD3 was described to interact with mitochondria and generate ROS [49, 50], and there is strong evidence for involvement of GD3 in autophagosome formation [51, 52]. GD3 is also considered a key player in apoptosis by Fas, ceramide, and amyloid-β [53, 54]. These findings suggest an important role of gangliosides in processes involved in oxidative stress regulation which might explain their compensatory upregulation in the prooxidative environment of *Hmox1* knockout.

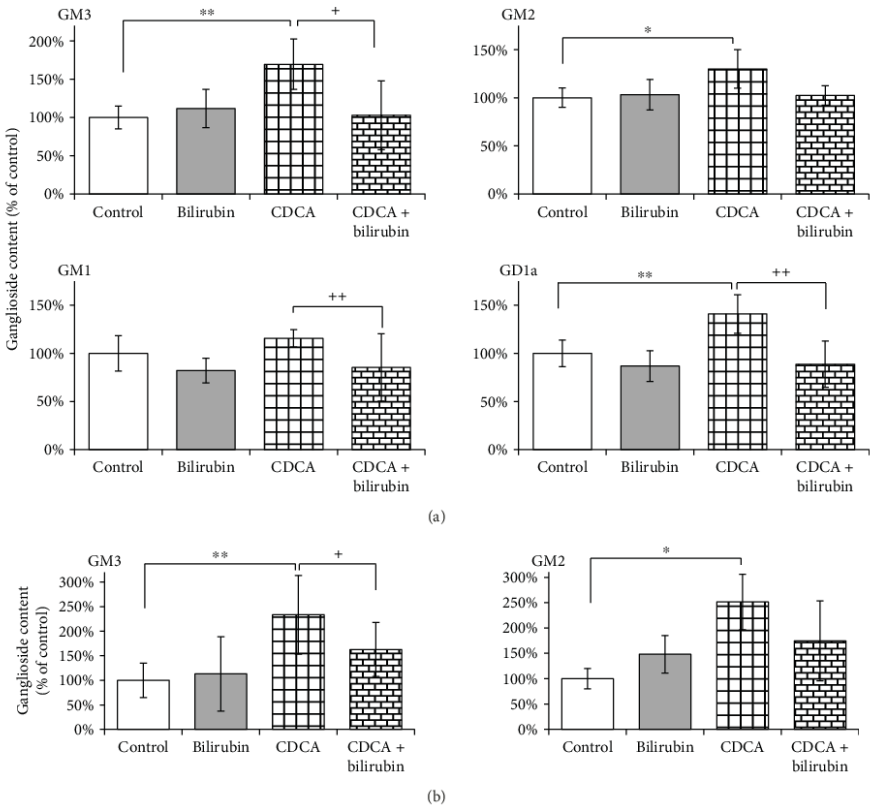

FIGURE 5: The ameliorating effect of bilirubin on CDCA-induced change in ganglioside content in SH-SY5Y cells (a) and HepG2 cells (b). Relative content of individual gangliosides was evaluated using extraction and TLC densitometry after incubation with CDCA or CDCA/bilirubin for 4 in (a) SH-SY5Y cells and (b) HepG2 cells. Values are expressed as % of controls. Bilirubin (1 μM); CDCA: chenodeoxycholic acid (80 μM); CDCA/bilirubin: CDCA (80 μM) + bilirubin (1 μM). $^{*}P < 0.05$ vs. C; $^{**}P < 0.01$ vs. C; $^{+}P < 0.05$ vs. CDCA; $^{++}P < 0.01$ vs. CDCA.

The effect of oxidative stress on changes in ganglioside synthesis was further supported by determination of glycosyltransferase mRNA expressions in mouse liver and brain homogenates. The key regulatory enzymes in the synthesis of nearly all gangliosides, *GM3* and *GD3 synthases*, as well as *GalNAcT*, were found to be significantly increased in *Hmox1* knockouts while GM1 synthase expression stayed unchanged. These data correspond with the observed increases in liver gangliosides and are in accordance with our previous observations on liver glycosyltransferase expression in experimental cholestasis in rats [22]. The increase in liver GM1 ganglioside content in *Hmox1* knockouts allows speculating that expression of *GM1 synthase* is redundant in wild-type animals and is capable of maintaining the induction of the *GalNAcT* product. Interestingly, only GM3 synthase has been found to be significantly upregulated in the brain suggesting the tissue-specific regulation of various sialyltransferases. Moreover, different extents of oxidative stress in particular tissues might affect the final sialyltransferase expression.

Furthermore, in our earlier reports, we described not only an increase in ganglioside synthesis but also their shift into the sinusoidal membranes of hepatocytes upon oxidative stress induced by bile acids [21]. This mechanism could protect hepatocytes against detergent and prooxidant effects of bile acids. A very similar effect was observed in the present study. We have used a selective histochemical approach based on the high binding affinity of *Cholera toxin* B subunit to GM1 ganglioside [6], the representative of the complex gangliosides. A significant shift of GM1 gangliosides from intracellular localization to the membrane compartment was found in *Hmox1* knockout which is also associated with prooxidative condition.

To investigate the mechanism of oxidative stress-mediated changes in ganglioside metabolism, we used the *in vitro* model of the SH-SY5Y neuroblastoma cell line rich

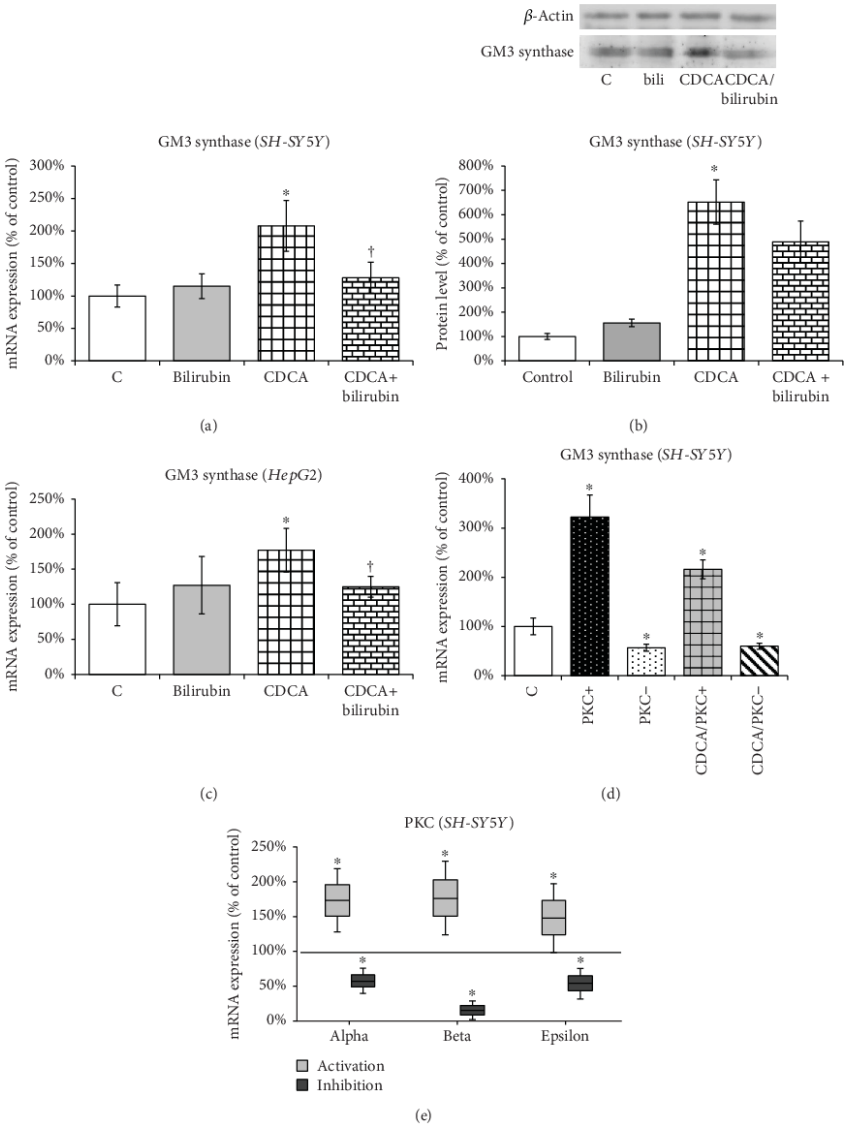

FIGURE 6: The opposite effects of CDCA and bilirubin on regulation of GM3 synthase expression in SH-SY5Y and HepG2 cells. (a) Relative GM3 synthase (ST3GalV) mRNA expression was determined in control cells (C), after 4 h incubation with chenodeoxycholic acid (CDCA) and/or bilirubin in SH-SY5Y cells. (b) Relative GM3 synthase (ST3GalV) protein expression by Western blot was determined in control cells (C), after 4 h incubation with chenodeoxycholic acid (CDCA) and/or bilirubin in SH-SY5Y cells. (c) Relative GM3 synthase (ST3GalV) mRNA expression was determined in control cells (C), after 4 h incubation with chenodeoxycholic acid (CDCA) and/or bilirubin in HepG2 cells. (d) PKC activity was modulated by incubating SH-SY5Y cells with PKC activator (PKC^+) or PKC inhibitor (PKC^-) or their combination with CDCA ($CDCA/PKC^+$, $CDCA/PKC^-$) for 4 h. (e) PKC activation and/or inhibition was proven by determination of mRNA expression vs. control (100% line) of PKC alpha, PKC beta, and PKC epsilon in SH-SY5Y cells. Values are expressed as % of controls. C: control; PKC^+: PKC activation by phorbol 12-myristate 13-acetate (5 μM); PKC^-: PKC inhibition by Ro 31-0432 (5 μM); CDCA: chenodeoxycholic acid (80 μM); CDCA/bilirubin: CDCA (80 μM) + bilirubin (1 μM). $^*P < 0.05$ vs. C; $^\dagger P < 0.05$ vs. CDCA.

in glycosphingolipids and, for the comparison, the human hepatoblastoma HepG2 cell line. Interestingly, the HepG2 cell line was found to be very poor in ganglioside content and completely lacking GD3 synthase. Exposure of SH-SY5Y to oxidative stress induced by chenodeoxycholic acid [55] resulted in a significant increase in all major gangliosides of this cell line—GD1a, GM3, and GM2—while addition of a potent antioxidant, bilirubin [56], resulted in normalization of the ganglioside content. Importantly, the same pattern was observed in the HepG2 cell line in GM3 and GM2 gangliosides. These results are in accordance with our earlier observations [10] that bilirubin may counteract a prooxidative effect of BA on hepatocytes in the model of obstructive cholestasis in rats. Furthermore, accumulation of hydrophobic BA in the brain and their possible involvement in hepatic encephalopathy associated with cholestatic liver diseases has been reported [57]. BA can act as cell signalling effectors through binding and activating receptors on both the cell membrane and nucleus. BA signalling encompasses both direct (FXR, TGR5) and indirect (FGF19, GLP-1) pathways. The role of BA in extrahepatic diseases is becoming more important, and increasing amount of reports suggests that BA might play an important role in neurological function and diseases [58, 59].

To elucidate the mechanism of oxidative stress-induced changes of ganglioside metabolism, we focused on regulation of the main enzyme in complex ganglioside synthesis, GM3 synthase (*ST3GalV*).

PKC appeared to be a logical candidate regulating the expression of GM3 synthase. Hydrophobic bile acids are considered potent inducers of PKC while antioxidants inhibit PKC activity [60, 61]. For more than 30 years, it has been known that ganglioside metabolism is in tight connection to PKC activity [62–64], and the action of glycosyltransferases is controlled through posttranslational modification. Glycosyltransferase activities have been demonstrated to be significantly modulated by the action of PKC [65]. Another study suggested the role of PKC as an activator of GM3 synthase (*ST3GalV*) [66]. Our *in vitro* data support this hypothesis. While PKC activators and oxidative stress induced the expression of *ST3GalV*, PKC inhibitors as well as antioxidants completely abolished this effect.

There are some limitations of our study. First, we were primarily interested in the shift of GSL to the cytoplasmic membrane; however, more studies are needed to assess whether subcellular localization and trafficking of gangliosides are affected as well. Second, in histochemical analyses, we used GM1 as a GSL representative but further studies with individual gangliosides are needed to confirm that the shift of GM1 from the intracellular compartment to the cytoplasmic membrane is a general reaction to loss of Hmox1 action. Finally, the PKC pathway is an important but probably not the only pathway regulating GSL metabolism affected by oxidative stress.

5. Conclusions

We conclude that oxidative stress is an important factor modulating synthesis and distribution of gangliosides *in vivo* and *in vitro*. Knockout of *Hmox1*, an important antioxidant enzyme, results in tissue-specific increases in main gangliosides together with changes in mRNA expression of key enzymes of ganglioside synthesis. We demonstrate that these changes might be, at least partially, mediated through modulation of the PKC pathway.

Data Availability

The raw data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Use of Non-Invasive Parameters of Non-Alcoholic Steatohepatitis and Liver Fibrosis in Daily Practice - An Exploratory Case-Control Study

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Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of a metabolic syndrome. To date, liver biopsy has been the gold standard used to differentiate between simple steatosis and steatohepatitis/fibrosis. Our aim was to compare the relevance of serum non-invasive parameters and scoring systems in the staging of liver fibrosis and non-alcoholic steatohepatitis (NASH) in patients with NAFLD.

Methods and Findings: A total of 112 consecutive patients diagnosed with NAFLD were included. A liver biopsy was performed on 56 patients. The Kleiner score was used for the staging and grading of the histology. Non-invasive parameters for fibrosis (hyaluronic acid; AST/ALT; fibrosis scoring indexes OELF, ELF, BARD score, APRI, NAFLD fibrosis score); and inflammation (M30 and M65 cytokeratin-18 fragments) were measured and calculated. The same analyses were performed in 56 patients diagnosed with NAFLD, who were not indicated for liver biopsy. Based on the liver histology, NASH was diagnosed in 38 patients; simple steatosis in 18 patients. A cut-off value of 750 U/L of serum M65 discriminated patients with and without NASH with a 80% sensitivity and 82% specificity (95% CI:57–95). Fibrosis stage F0–F2 was present in 39 patients; F3–F4 in 17 patients. Serum concentrations of hyaluronic acid were higher in patients with advanced fibrosis ($p < 0.01$); a cut-off value of 25 μg/l discriminated patients with F3–F4 with a 90% sensitivity and 84% specificity from those with F0–F2 (95% CI:59–99). When applying the non-invasive criteria to those patients without a liver biopsy, NASH could only be diagnosed in 16%; however, advanced fibrosis could be diagnosed in 35% of them.

Conclusions: In patients with NAFLD, non-invasive serum parameters with a high accuracy can differentiate those patients with NASH and/or advanced fibrosis from those with simple steatosis. A substantial portion of those patients not indicated for liver biopsy might have undiagnosed advanced fibrosis.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) represents the hepatic manifestation of a metabolic syndrome, a disease affecting a substantial portion of modern populations [1]. NAFLD comprises a spectrum of conditions from simple steatosis, through non-alcoholic steatohepatitis (NASH), ending with liver cirrhosis. With the epidemic increase in the incidence of obesity, NAFLD has become a serious issue for the foreseeable future.

Whereas simple liver steatosis is regarded as a benign condition, NASH can be a progressive liver disease leading to fibrosis and ultimately to cirrhosis. A liver biopsy has long been the only method for NASH diagnosis and for the staging of liver fibrosis. However, there are several drawbacks associated with this procedure. A liver biopsy is an invasive diagnostic method which is distressing to patients. Although it is generally safe, there is some unavoidable risk of major complications (1−3% of patients) or even fatal ones (0.01% of patients) [2]. Other potential problems include sampling heterogenity [3,4] and the subjectivity and intra/interobserver variability.

Several non-invasive tools and methods have been developed to predict NASH, or to quantify liver fibrosis without having to resort to a liver biopsy. They are either based on an assessment of single

Table 1. Comparison of clinical and laboratory parameters between NAFLD patients and control subjects.

Parameter	NAFLD (n = 112)	Controls (n = 14)	p-value
Age (years)	48.9±14,9	43.9±9	ns
Gender (M/F)	79/33	8/6	ns
Serum glucose fasting (mmol/l)	5.98±1.8	4.7±0.4	<0.001
IL2 (ng/l)	7.35±22.7	10.33±28.3	ns
IL6 (ng/l)	18.37±34.4	6.21±6.7	ns
TNFα (ng/l)	11.84±12	12.08±8	ns
M30 (U/l)	379±375	122±49	<0.001
M65 (U/l)	884±675	301±79	<0.001
hsCRP (mg/l)	26.95±27	15.5±13.5	ns
Leptin (mg/l)	10.82±7.3	9.97±8.7	ns
Adiponectin (mg/l)	6.72±5.2	10.88±5.5	0.002
Insulin (mIU/l))	16.09±11.4	7.59±4.1	<0.001
HA (μg/l)	49.5±103	20.9±15	ns

IL 2: interleukin 2, IL 6: interleukin 6, TNFα: tumor necrosis factor alpha, M30, M65: fragments of cytokeratin-18, hsCRP: high sensitive C-reactive protein, HA: hyaluronic acid, ns: non-significant. The results are given as mean ±standard deviation.
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substances, or on the calculation of specific scores. Only a minority of these have been externally validated on large numbers of patients. From the large spectrum of biomarkers tested, cytokeratin-18 fragments have shown the most consistent results for differentiating NASH from steatosis [5].

In addition to the necessity to differentiate NASH from simple steatosis, another necessary goal is the non-invasive assessment of liver fibrosis. For the individual patient, the stage of fibrosis is probably the most important prognostic factor. Moreover, early recognition of cirrhosis enables regular screening for the development of portal hypertension and hepatocellular carcinoma. Many serum tests and scores have been developed, with some of them only available commercially. There are simple tests based on routinely available biochemical markers such as: AST/ALT ratio [6]; BARD score (**B**MI, **A**ST to ALT **R**atio, **D**iabetes mellitus) [7]; NAFLD Fibrosis Score [8]; APRI score (AST to Platelet Ratio Index); and FIB-4 score (based on age, AST/ALT activities and platelet count) [9]. Other scoring systems require special laboratory analyses such as the determination of serum hyaluronic acid (HA) [10], aminoterminal peptide of pro-collagen III (PIIINP), and the tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) levels required for both the OELF (**O**riginal **E**uropean **L**iver **F**ibrosis) [11] and ELF (**E**nhanced **L**iver **F**ibrosis) panels [12].

The aim of our prospective study was to compare the relevance of serum hyaluronic acid levels and other non-invasive scoring systems (APRI, AST/ALT ratio, FIB 4, BARD, NAFLD fibrosis score, ELF, OELF) [8] in the distinguishing of NASH, as well as the staging of liver fibrosis in a group of consecutive NAFLD patients.

Patients and Methods

Patients

A total of 112 patients with NAFLD were included in the prospective study. The patient population was recruited from all consecutive patients referred to the 4th Department of Internal Medicine of the General University Hospital in Prague between 2010 and 2013, and in whom a diagnosis of NAFLD had been confirmed. The clinical and laboratory data were either collected from the time of liver biopsy or from the time of confirmation of NAFLD in those patients not indicated for liver biopsy. As inclusion in the study did not involve any especially invasive examination (only blood sampling coupled with the regular blood check), after careful explanation, no patients declined inclusion. The indication for liver biopsy depends solely on the clinical situation and the patient's decision, and was not the determinant factor for inclusion in the study (see paragraph "Liver biopsy").

The diagnosis of NAFLD was based on liver histology in 56 patients with an available liver biopsy, and on clinical and laboratory parameters in the remainder of the patients. These clinical and laboratory parameters included: 1) elevated levels of ALT, AST, GGT; the presence of steatosis, fibrosis, or cirrhosis on abdominal ultrasound, and 2) a clinical situation compatible with NAFLD (i.e. the presence of metabolic syndrome or certain components of this syndrome), and exclusion of another etiologies of liver disease [13]. Viral hepatitis, drug induced liver disease, autoimmune liver disease, biliary diseases, and inherited metabolic diseases were excluded by specific laboratory and radiologic examinations, as well as the patient history. Alcohol abuse was excluded by the patient's history, short questionnaire, stable GGT activities, and use of either serum carboxyl deficient transferin and/or urine ethyl-glucuronide levels. The presence of an uncontrolled tumor was also an exclusion criterion. The control group for the comparison of the laboratory parameters, inflammatory cytokines, and total antioxidative capacity consisted of 14 healthy individuals. The control population was recruited from the staff of the University hospital in Prague. These individuals were healthy subjects without a history of liver disease or other chronic diseases, and were age- and gender-matched to the patient population. These individuals had normal liver function tests and the same battery of laboratory tests as those patients with NAFLD had that had been used to exclude any liver disease. The study was carried out in full accordance with the Helsinki Declaration, and was approved by the Ethics Committee of the General University Hospital and First Medical Faculty, Charles University in Prague. All subjects had given prior written informed consent.

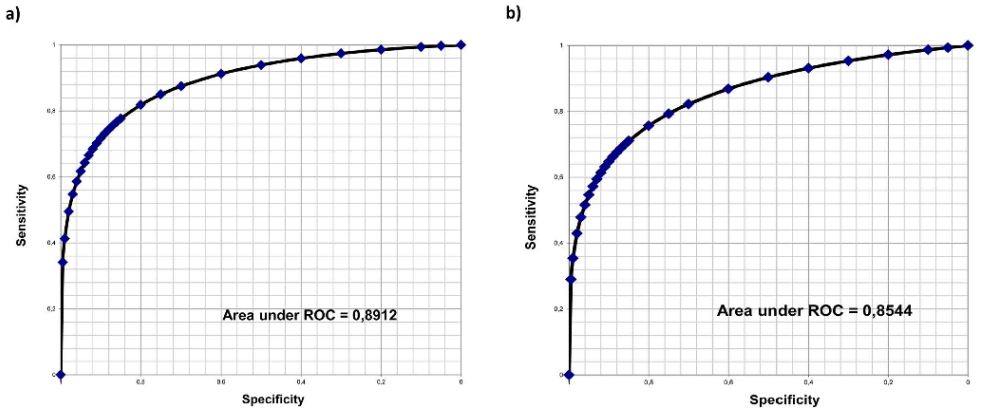

Figure 1. Predictive discrimination of hepatocyte death biomarkers, as determined by ROC plot analysis. The ROC analyses indicate the threshold for M65 (a) and M30 (b) for the best compromise sensitivity/specificity to predict NASH (n = 38 patients) versus steatosis (n = 18 patients) in a group of patients with NAFLD. ROC – receiver operating characteristic curve.
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Table 2. Clinical and laboratory data in patients with NAFLD, and the presence or absence of histological signs of NASH.

Parameter	NASH+ n = 38	NASH- n = 18	p-value
Age (years)	46.4±15	43.6±16	ns
BMI (kg/m^2)	31.2±3.01	28.9±4.47	ns
GGT (μkat/l)	5.34±4.7	2.3±2.9	0.004
ALT (μkat/l)	2.25±1.6	1.54±1.3	ns
AST (μkat/l)	1.28±0.7	0.79±0.4	0.003
Triglycerides (mmol/l)	3.08±3.4	1.46±0.6	0.011
Histological stage of fibrosis (Kleiner score)	2.64±0.9	1.3±1.4;	0.002
PIIINP (pg/ml)	539±138	618±147	ns
TIMP-1 (ng/ml)	91.6±12.9	69.5±17	0.004
IL2 (ng/l)	2.42±6.9	10.57±34	ns
IL6 (ng/l)	13.86±24.7	18.7±38.2	ns
TNFα (ng/l)	10.27±7.2	10.51±6.4	ns
M30 (U/l)	516±394	181±85	0.001
M65 (U/l)	1333±804	836±437	0.014
hsCRP (mg/l)	22.48±18.7	19.38±22.6	ns
Leptin (mg/l)	9.8±6.6	10.65±6.9	ns
Adiponectin (mg/l)	7.53±9.1	6.75±4.4	ns
Insulin (mIU/l)	23.99±20.1	14.04±8.4	ns
HA (μg/l)	69.9±112	20.3±14.1	0.057
AST/ALT ratio	0.65±0.2	0.72±0.4	ns
APRI	0.86±0.5	0.65±0.5	ns
FIB 4	1.52±0.7	1.86±2.4	ns
NAFLD fibrosis score	−1.73±1.4	−2.31±3	ns
BARD score	1.46±1.1	1.31±1.3	ns

BMI: body mass index, GGT: γ-Glutamyltransferase, PIIINP: aminoterminal peptide of pro-collagen III, TIMP-1: tissue inhibitor of matrix metalloproteinase 1, IL 2: interleukin 2, IL 6: interleukin 6, TNFα: tumor necrosis factor alpha, M30, M65: fragments of cytokeratin-18, hsCRP: high sensitive C-reactive protein, HA: hyaluronic acid, ns: non-significant. The results are given as mean ±standard deviation.
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Table 3. Sensitivity and specificity of different parameters for differentiation between the patients with and without histological sings of NASH.

Parameter	Cut-off value	Sensitivity	Specificity	95% CI
M30 (U/l)	211	0.79	0.76	0.56–0.93
	234	0.75	0.81	0.50–0.92
M65 (U/l)	790	0.78	0.85	0.48–0.93
	750	0.80	0.82	0.57–0.95
AST (μkat/l)	0.6	0.71	0.55	0.54–0.88
ALT (μkat/l)	1.02	0.71	0.60	0.52–0.85
GGT (μkat/l)	1.66	0.57	0.50	0.28–0.83

M30, M65: fragments of cytokeratin-18, GGT: γ-Glutamyltransferase, CI: confidence interval.
doi:10.1371/journal.pone.0111551.t003

Non-invasive liver fibrosis scoring systems

The scoring systems examined for the presence of liver fibrosis were calculated on the basis of following formulae: APRI was calculated as AST (IU/L/upper AST limit/platelet count ($\times 10^9$/L) × 100 [14]; the FIB-4 score [9] according to the formula: age × AST (IU/L/platelet count ($\times 10^9$/L) × ALT (IU/l); the NAFLD fibrosis score [8] according to the formula: −1.675+0.037 × age (yrs) +0.094 × BMI (kg/m2) +1.13 × impaired glucose tolerance or diabetes (yes = 1, no = 0) +0.099 × the AST/ALT ratio −0.013 × platelet count ($\times 10^9$/L) −0.66 × albumin (g/dl); and the BARD score (BMI, AST/ALT Ratio, Diabetes) as the sum of the following three values: BMI>28 = 1 point, AST/ALT >0.8 = 2 points, diabetes = 1 point [7]. The OELF score was calculated using the algorithm: −6.38 − (ln(age) × 0.14)+(ln(HA) × 0.616)+(ln(PIIINP) × 0.586)+(ln(TIMP-1) × 0.472) [11]; whereas, the ELF score was calculated by using the algorithm: −7.412+ (ln(HA) × 0.681)+(ln(PIIINP) × 0.775)+(ln(TIMP-1) × 0.494) [12]. The scoring systems were applied to: 1) the group of 56 patients with a liver biopsy in order to evaluate their sensitivity and specificity for distinguishing NASH and advanced fibrosis; and 2) the group of 56 patients, without a clinically clear indication to have a liver biopsy, in order to evaluate the incidence of NASH and advanced fibrosis in these patients.

Liver biopsy

A liver biopsy was available in 56 of the patients. In 43 patients, it was conducted by the percutaneous method with a Menghini needle [15], and in another 13 patients by the transjugular method. The indications for transjugular biopsy were obesity, thrombocytopenia, suspicion of liver cirrhosis, and the need for a hepatic venous pressure gradient measurement [16]. The biopsy samples were routinely stained and then read by a single pathologist (JS) who was blind to the clinical and laboratory data. The stage of liver fibrosis was scored based on the Kleiner *et al.* modification [17] of the Brunt *et al.* proposition [18,19]: stage 0 - no fibrosis, stage 1 - perisinusoidal or portal fibrosis, stage 2 - perisinusoidal and portal/periportal fibrosis, stage 3 - septal or bridging fibrosis, stage 4 - cirrhosis. The indication for liver biopsy reflected usual clinical practice, in particular clinical suspicion of NASH or significant fibrosis. The reasons for not performing a liver biopsy were either the patients rejection of this invasive procedure, or a clinical and laboratory picture of simple steatosis (normal or slightly elevated ALT, typical ultrasound sings).

Laboratory methods

The biochemical parameters were measured by routine laboratory techniques. Highly sensitive CRP (hs-CRP) was measured by immunonephelometry (Behring Nephelometer II), and inflammatory cytokines (IL-2, IL-6, and TNF-α) by Luminex technology (using multiplex kit from EMD Millipore, Darmstadt, Germany). Adiponectin, leptin, and insulin were measured by the ELISA technique (Roche Diagnostics, Indianapolis, IN, USA). Serum HA was measured using a method of latex agglutination (Hyaluronic acid LT, Latex Agglutination Method, Wako Chemicals GmbH. Germany). M30 and M65 levels were measured by commercially available ELISA tests (PEVIVA AB, Sweden). Blood for laboratory examination was collected on the same day as the biopsy was performed.

Statistical methods

For sample size determination, we used an estimation based on the AUROC evaluation for single parameters. To reject the null hypothesis, AUC = 0.50 on the 0.05 significance level with the test power at 80%, the sample size is n = 16 in each group.

The results are presented as the mean values with the standard deviation. Either a two-sample t-test or the Mann-Whitney rank test for non-Gaussian distributed variables were used to estimate the intergroup differences. The correlations between different parameters were evaluated by Pearson resp. Spearman correlation coefficient and linear regression. The Chi-square test, resp. Yates's corrected chi-square test was used for frequency table analyses. All tests were two-sided with $p<0.05$, considered statistically significant. Receiver operating characteristic curve analysis was used to assess the utility of different non-invasive parameters in the discrimination between those patients with/without significant fibrosis or with/without steatohepatitis. The statistical analyses were performed using BMDP Statistical Software (Release 8.1.) and MedCalc software.

Results

Characteristics of Patients

NAFLD was diagnosed in 112 patients −79 males and 33 females. Both gender groups exhibited no differences in both the anthropometric, biochemical parameters, and/or the fibrosis stage (data not shown). The male patients had significantly lower ages compared to the females (46.1±14.5 vs 55.6±14.3 years, p = 0.002).

The clinical and laboratory parameters of all patients with NAFLD as well as the controls are given in Table 1. As expected,

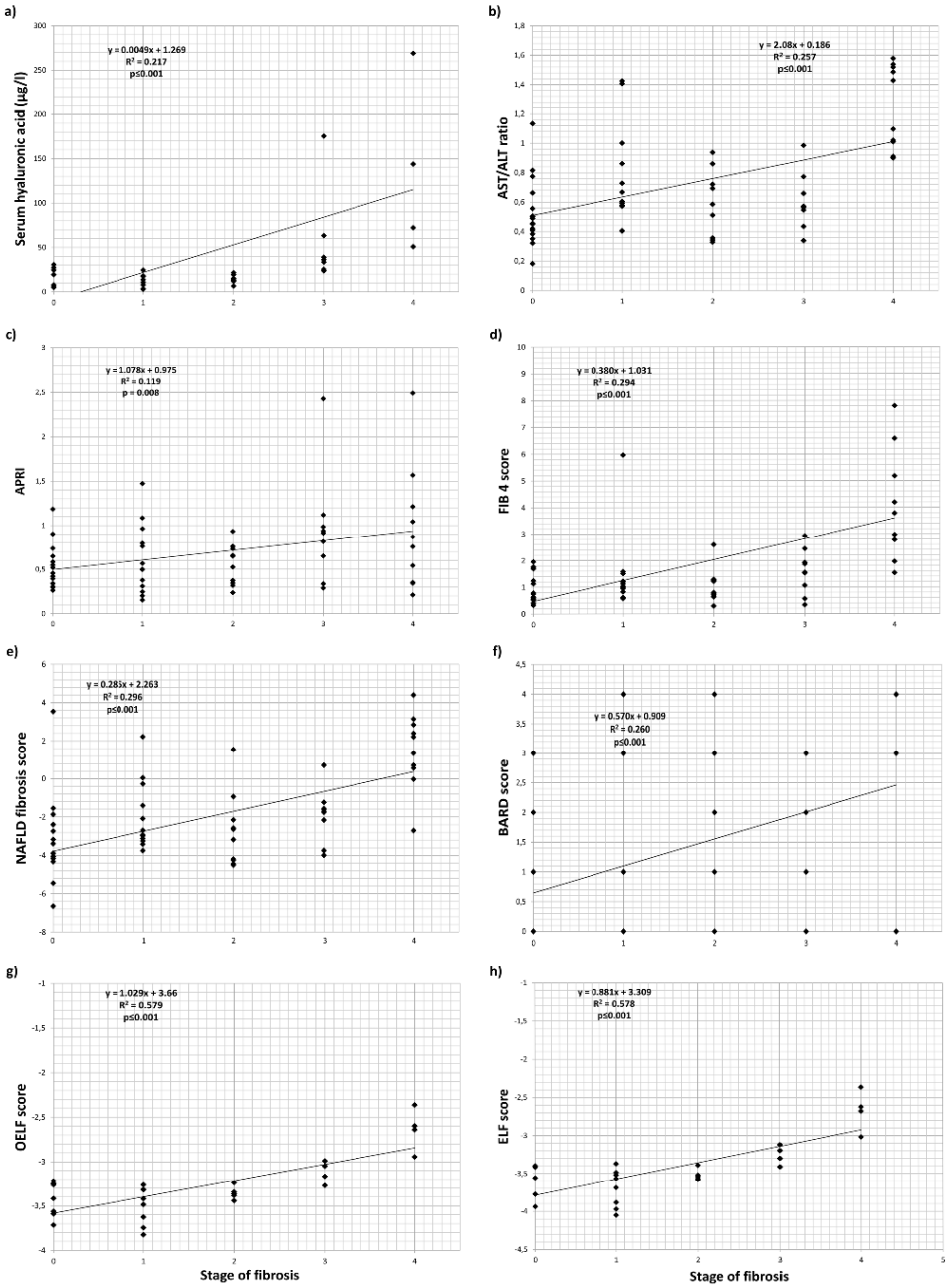
a)
y = 0.0049x + 1.269
R² = 0.217
p≤0.001
Serum hyaluronic acid (µg/l)
b)
y = 2.08x + 0.186
R² = 0.257
p≤0.001
AST/ALT ratio
c)
y = 1.078x + 0.975
R² = 0.119
p = 0.008
APRI
d)
y = 0.380x + 1.031
R² = 0.294
p≤0.001
FIB 4 score
e)
y = 0.285x + 2.263
R² = 0.296
p≤0.001
NAFLD fibrosis score
f)
y = 0.570x + 0.909
R² = 0.260
p≤0.001
BARD score
g)
y = 1.029x + 3.66
R² = 0.579
p≤0.001
OELF score
Stage of fibrosis
h)
y = 0.881x + 3.309
R² = 0.578
p≤0.001
ELF score
Stage of fibrosis

Figure 2. The relationship between serum biomarkers or non-invasive scores and stage of liver fibrosis in 56 patients with biopsy-confirmed NAFLD evaluated by linear regression: Hyaluronic acid (a), AST/ALT ratio (b), APRI score (c), FIB-4 score (d), NAFLD fibrosis score (e), BARD score (f), OELF score (g), and ELF score (h). The non-invasive scores were calculated by formulas given in "Patients and Methods". The fibrosis stage was evaluated by the Kleiner modification of the Brunt proposition as described in "Patients and Methods".
doi:10.1371/journal.pone.0111551.g002

compared to NAFLD/NASH patients, significantly lower serum glucose, adiponectin, insulin levels, and cytokeratin-18 fragments M30 and M65 levels were observed in healthy controls. On the other hand, no differences in the serum levels of inflammatory markers were detected between both of the examined groups. A liver biopsy was performed in 56 patients.

Differentiation of NASH from simple steatosis

Histological signs of NASH were present in 38 patients, with simple steatosis in the remaining 18 patients. Clinical and laboratory data of these two subgroups are presented in Table 2. In brief, patients with NASH had significantly higher serum levels of GGT, AST, and triglycerides. Patients with NASH had more advanced fibrosis, compared to patients with simple steatosis (mean fibrosis stage 2.64±0.9 vs. 1.3±1.4; $p = 0.002$); nevertheless, individuals with F3 and F4 fibrosis were also between those patients without NASH (there were 3 patients with cirrhosis). Serum concentrations of cytokeratin-18 fragments M30 and M65 were significantly higher in patients with NASH, compared to patients with simple steatosis. The sensitivity and specificity of cytokeratin-18 fragments for discrimination between patients with and without NASH were calculated and ROC plotted. The most significant parameter for this discrimination was serum concentration of M65 (sensitivity 80%, specificity 82%, with a cut-off value of 750 U/L). The AUROC for M30 was 0.85; for M65 it was 0.89 (Figure 1a, b; Table 3). No other parameter had a similar sensitivity and specificity (Table 3).

Differentiation of liver fibrosis

The entire group was subdivided into the histological stage of fibrosis (see Table S1). Fibrosis stage F0–F2 was present in 39 patients; F3–F4 in 17 patients. Patients with cirrhosis (F4) were significantly older, compared to other patients ($p<0.01$). The stage of liver fibrosis unambiguously correlated with the serum concentration of HA ($p<0.001$; value <75 µg/l excluded the presence of F3/4 fibrosis, Table 4). Similarly, the stage of liver fibrosis correlated with the various fibrosis indexes (AST/ALT ratio, $p<0.001$; APRI, $p<0.01$; NAFLD fibrosis score, $p<0.001$; FIB 4 score, $p<0.001$; BARD score, $p<0.001$) (see Figure 2 a–h, Table 4). The stage of liver fibrosis did not correlate with the serum markers of inflammatory reaction (hsCRP, IL2, IL6, $p>0.05$, Table 4). Congruently, the stage of liver fibrosis did not correlate with the parameters of hepatocyte apoptosis or necrosis (fragments M30 and M65 of cytokeratin-18).

To discriminate between significant liver fibrosis (F3+F4) and mild to moderate or no fibrosis (F0–F2), the sensitivity and specificity were calculated for the different parameters. The most significant parameter for this discrimination was serum HA concentration (sensitivity 80%, specificity 91%, with a cut-off value of 30 µg/l, resp. 90% and 84% with a cut-off value 25 µg/l; AUROC 0.94; Figure 3 a–d, Table 5); OELF score (sensitivity 92%, specificity 93% with a cut-off value of −3.24; AUROC 0.93); and ELF score (sensitivity 90%, specificity 97% with a cut-off value of −3.37; AUROC 0.97). Other parameters did not reach statistical significance (AUROC values were as follows: AST/ALT ratio: 0.73; APRI: 0.70; NAFLD fibrosis score: 0.81; FIB-4:0.83; and BARD score: 0.77 (Table 5).

Comparison of patients with and without liver biopsy

To assess the applicability of the examined non-invasive markers, clinical and laboratory parameters in patients with a liver biopsy and those without a biopsy were compared (Table 6). Based on the non-invasive parameter (M65, cut-off value 750 U/l), patients indicated for a liver biopsy would more frequently have

Table 4. Correlation between stage of fibrosis and clinical parameters, laboratory parameters, and fibrosis indexes.

Parameter	r	p-value
Age (years)	0.589	<0.001
Thrombocytes (*10^9/l)	−0.353	<0.01
Albumin (g/l)	−0.320	<0.05
BMI (kg/m^2)	0.293	<0.05
TIMP-1 (ng/ml)	0.590	<0.01
IL6 (ng/l)	0.331	<0.05
Insulin (mIU/l)	0.366	<0.05
HA (µg/l)	0.486	<0.001
AST/ALT ratio	0.501	<0.001
APRI	0.345	<0.01
FIB4	0.544	<0.001
NAFLD fibrosis score	0.540	<0.001
BARD score	0.510	<0.001
OELF score	0.761	<0.001
ELF score	0.760	<0.001

BMI: body mass index, TIMP-1: tissue inhibitor of matrix metalloproteinase 1, IL 6: interleukin 6, HA: hyaluronic acid.
doi:10.1371/journal.pone.0111551.t004

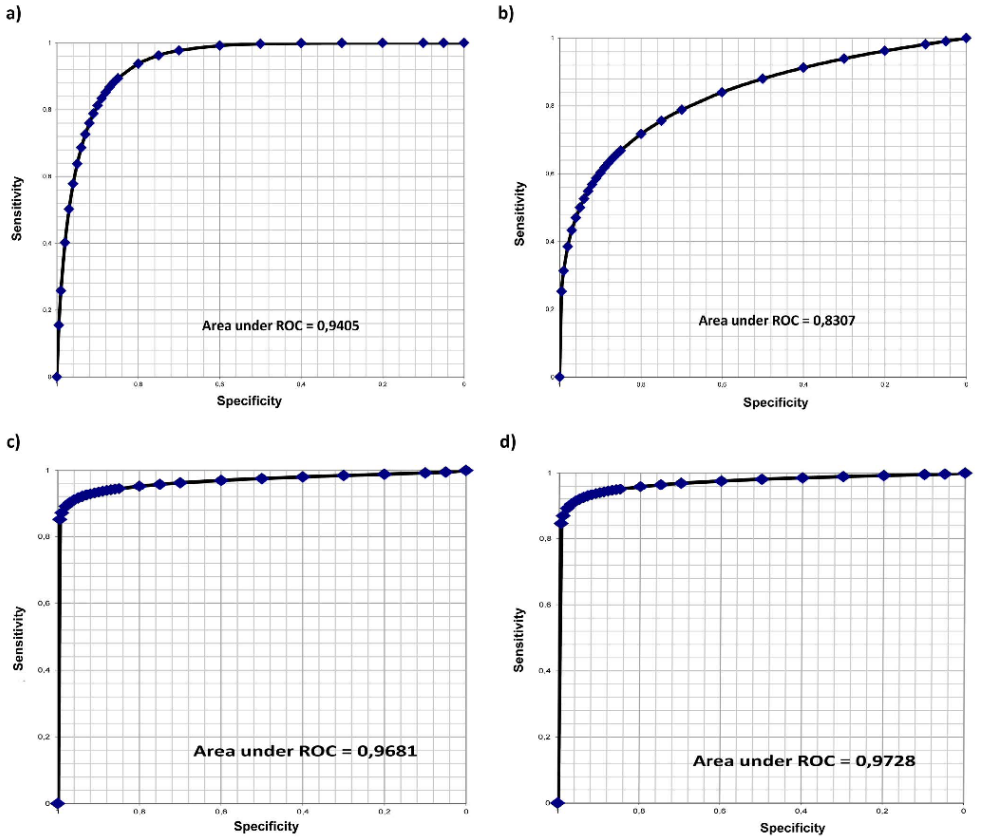

Figure 3. Predictive discrimination of non-invasive fibrosis parameters and scores, as determined by ROC plot analysis. The ROC analyses indicate the threshold for serum hyaluronic acid (a), FIB-4 score (b), OELF (c), and ELF (d) for the best compromise sensitivity/specificity to predict significant fibrosis (F3+F4; n = 17 patients) versus mild or no fibrosis (F0–F2; n = 39 patients) in patients with NAFLD. ROC – receiver operating characteristic curve.
doi:10.1371/journal.pone.0111551.g003

steatohepatitis than those not indicated for liver biopsy (68% vs. 16%, p = <0.001). Regarding liver fibrosis, based on a HA cut-off value of 30 μg/l, significant fibrosis would have a similar proportion of patients not indicated for liver biopsy, compared to those in whom fibrosis was verified histologically (35% vs. 29%, ns).

Discussion

There are many reasons to support the use of non-invasive parameters in the diagnoses of NASH/fibrosis instead of an invasive liver biopsy. Apart from the general trend to use non-invasive diagnostic techniques, it is important to note that there is no specific treatment of NASH to be offered to the patients after a confirmation of such a diagnosis by liver biopsy. Even if some treatment could be implemented in the future, the use of a liver biopsy for repeated evaluation of treatment efficacy is inconceivable [20]. Moreover, the diagnostic value of a liver biopsy is frequently overestimated, being burdened with both intra- and inter-observer variability, with an accuracy below 90% [3].

The main challenge in the use of non-invasive methods for NASH and fibrosis detection is their level of applicability and implementation into daily medical practice. Even so, clinicians are faced with various pitfalls. First, some non-invasive methods, and the most specific and sensitive scores, rely on parameters which are not routinely examined and are costly (such as TIMP-1 or PIIINP for OELF and ELF scores) [11,12]. Secondly, most of the scores and parameters have largely been investigated in cross-sectional studies [8], and their utility in monitoring of the disease's natural history in specific clinical situations (such as screening for fibrosis) is unknown. Additionally, while in the original papers (with definitions of ELF [11] and OELF [12] scores) the formulas gave results in the negative range (or around zero); subsequent papers changed the formula, additionally giving the constant +10 [21,22].

Table 5. Sensitivity and specificity of different parameters for differentiation between those patients with significant fibrosis (F3,4) and those with none to moderate fibrosis (F0–F2).

Parameter	Cut-off value	Sensitivity	Specificity	95% CI
HA (µg/l)	25	0.90	0.84	0.59–0.99
	30	0.80	0.91	0.44–0.96
FIB-4 score	1.24	0.78	0.72	0.59–0.92
	1.51	0.73	0.78	0.50–0.87
NAFLD fibrosis score	−2.16	0.78	0.70	0.54–0.92
APRI	0.65	0.64	0.66	0.40–0.79
AST/ALT ratio	0.67	0.67	0.67	0.44–0.82
OELF score	−3.24	0.92	0.93	0.58–0.99
ELF score	−3.37	0.9	0.97	0.51–0.99
	−3.39	0.93	0.93	0.51–0.99

HA: hyaluronic acid, CI: confidence interval.
doi:10.1371/journal.pone.0111551.t005

This fact emphasizes the need for specifying a precise formula in all papers calculating the OELF/ELF score in order to enable a correct reading of the results.

There are two aspects in the evaluation of steatosis/steatohepatitis in patients with NASH. Although the steatosis could be diagnosed by imaging techniques such as ultrasound, its sensitivity is not high enough [23] to discriminate between simple steatosis and NASH; and a liver biopsy is currently required in routine clinical practice. Various biomarkers or scoring systems have been reported to be useful in the detection of NASH. Emerging data suggests that hepatocyte apoptosis, a highly organized and genetically controlled form of cell death, may play an important

Table 6. Clinical and laboratory data in patients with and without liver biopsy.

Parameter	biopsy +	biopsy −	p-value
Age	44.1±15	52.5±14	**0.007**
BMI	29.6±4.3	30.9±3.3	0.193
GGT (µkat/l)	3±3.5	1.55±1.4	**0.012**
ALT (µkat/l)	1.63±1.3	0.99±0.6	**0.044**
AST (µkat/l)	0.89±0.5	0.65±0.3	**0.030**
Triglycerides (mmol/l)	1.8±1.7	1.8±0.9	0.774
albumin (g/l)	46.1±23.3	46.1±4.9	0.99
Thrombocytes (*10^9/l)	194±66	242±68	**<0.001**
IL2 (ng/l)	10.46±28.8	2.87±5.6	0.445
IL6 (ng/l)	24±42.2	10.1±14.5	0.310
TNFα (ng/l)	11.4±8.5	12.5±16	0.712
M30 (U/l)	433±373	336±375	**0.021**
M65 (U/l)	1091±742	544±346	**<0.001**
hsCRP (mg/l)	20.9±20.6	32.4±32.6	0.368
Leptin (mg/l)	10.9±7.3	10.8±7.4	0.839
Adiponectin (mg/l)	7.1±6.5	6.3±3.6	0.798
Insulin (mIU/l)	18.3±14.6	14.1±7.1	0.250
HA (µg/l)	57.8±131	37.6±36.4	0.298
AST/ALT	0.72±0.36	0.73±0.25	0.293
APRI	0.67±0.47	0.43±0.35	**<0.001**
FIB 4	1.83±2.1	1.26±0.85	0.575
NAFLD fibrosis score	−2.02±2.8	−2.71±2.2	0.196
BARD score	1.43±1.3	1.5±1.4	0.789

Biopsy +: patients with liver biopsy, biopsy −: patients without liver biopsy, BMI: body mass index, GGT: γ-Glutamyltransferase, IL 2: interleukin 2, IL 6: interleukin 6, TNFα: tumor necrosis factor alpha, M30, M65: fragments of cytokeratin-18, hsCRP: high sensitive C-reactive protein, HA: hyaluronic acid. The results are given as mean ±standard deviation.
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role in liver injury and disease progression in NAFLD [24]. An increase in hepatocyte cell death by apoptosis is typically present in patients with NASH, but is absent in those with simple steatosis [25]. The most promising non-invasive parameter of NASH seems to be the examination of circulating levels of cytokeratin-18, a biomarker of hepatocyte necrosis and apoptosis [26]. Among the different fragments of cykokeratine-18, M30 (a marker of apoptosis), and M65 (a marker of cell necrosis) have been widely investigated [27–29]. Although the AASLD guidelines published in 2012 [13] do not recommend the use of cytokeratin-18 in routine clinical practice, a recent meta-analysis on more than 800 patients in 10 studies [30] concluded that both cytokeratin-18 fragments and total cytokeratin-18 have a clinically meaningful benefit in the non-invasive diagnosing of NASH. In this meta-analysis, the area under the ROC curve for cytokeratin-18 fragments in the identification of NASH was 0.845, with 77% sensitivity and 71% specificity. The authors concluded that CK-18 fragments may be a useful biomarker for screening rather than for identifying NASH. Another recent meta-analysis evaluating the use of serum/plasma cytokeratin-18 fragments described a pooled sensitivity of 66% and specificity of 82% in the diagnoses of NASH [31]. In our study, the relevance of cytokeratin-18 fragments was even higher - the AUROC for discrimination of NASH from simple steatosis in our patients was 0.89 for M65, and 0.85 for M30. Also, the sensitivity and specificity of cytokeratin-18 fragments in the discrimination between simple steatosis and NASH was higher (75% sensitivity and 81% specificity for M30; 80% sensitivity and 82% sensitivity for M65). Based on our results, the assessment of M65, with a cut-off value 750 U/l, is the best simple non-invasive method with which to diagnose NASH. Our results also support the suggestion for the use of M30/M65 in the screening of NASH, as the differences between patients with NASH and controls were statistically significant.

Another important finding in this study was that a single biochemical parameter (HA serum concentration) was able to discriminate between NAFLD patients with or without significant liver fibrosis. HA cut-off values of 20−30 μg/g were similar to the results of other authors [32]; however, our sensitivity and specificity numbers were higher. Using this parameter, acceptable false positive and false negative results were recorded.

Similar results regarding fibrosis detection were recorded with the OELF and ELF scores. However, it remains questionable if specific and costly laboratory examinations used for OELF and ELF (PIINP and TIMP-1) can be transferred into routine daily medical practice. The other scores that were calculated in our study correlated closely to the stage of fibrosis; however, the discrimination values did not reach a satisfactory accuracy, having had low sensitivity and specificity (data not shown). Surprisingly, this was also the case with the NAFLD fibrosis score, which is endorsed by current AASLD guidelines [13] as a screening test to exclude low-risk individuals with fibrosis. The NAFLD fibrosis score [8] has been validated to discriminate between various stages of biopsy-verified NAFLD vs. advanced liver fibrosis to identify patients at the highest risk for progression to end-stage liver disease. In our study, the values of the NAFLD fibrosis score differed between patients with different fibrosis stages, but without having statistical significance. We speculate this was due to our specific population (or to other factors).

There are also novel approaches to build-relevant panels for non-invasive fibrosis assessment; for instance, the use of non-linear machine learning techniques [33]. These systems allow for estimating the variable importance, and therefore can be used to further improve precision performance. Using a "machine learning" technique, combining different parameters of fibrosis and cell death, Sowa JP et al. [33] were able, with adequate accuracy, to retrospectively differentiate even those patients with a fibrosis score of 1 or 2. However, this observation should be evaluated in prospective studies.

Another interesting observation concerns the high percentage of possible advanced fibrosis in patients not indicated for liver biopsy. In our study, liver biopsies confirmed NASH in 38 patients (68% of all patients indicated for liver biopsy). Based on a M65 threshold value of 750 U/l, NASH was probably present in 65% of patients (in 4 cases there would have been a false negative diagnosis, and in 2 cases a false positive diagnosis). To the contrary, in patients not indicated for liver biopsy (based on the M65 threshold value mentioned above) NASH would be diagnosed in only 16% (!!) of patients. Significant fibrosis was described from biopsies in 17 patients (30% of all patients indicated for liver biopsy). When using the simple non-invasive test (HA, threshold of 30 μg/l) significant fibrosis would be considered in 29% of patients (in 3 cases a false negative, in 4 cases a false positive). In patients not indicated for liver biopsy, the same percentage as in the biopsy group (35%) fulfilled the criteria for significant fibrosis. This data could indicate that the basic clinical and laboratory parameters used in routine practice, when considering the indication to liver biopsy, could select for patients having steatohepatitis; however, a remarkable proportion of those patients with liver fibrosis might elude a proper diagnosis. Our observation documents the fact that diagnosis of significant fibrosis, when based on routine liver function tests and standard ultrasonography, is not appropriate.

One of the limitations of our study appears to be the fact that not all patients had a liver biopsy performed. Nevertheless, the group of patients without a liver biopsy, which had the same battery of biochemical and clinical examination as those patients with a biopsy available, is interesting for many reasons. First, worldwide, hepatologists face this problem in their daily practice. This is due to the lack of strict criteria existing for an indication for a liver biopsy, as well as not all patients indicated for liver biopsy being willing to undergo such an invasive procedure. Selection of our patients was not based on randomization, but rather depended on the clinical situation. As the application of non-invasive criteria for NAFLD/NASH/fibrosis diagnosis to these patients showed, the selection directed most of the patients with NASH toward a liver biopsy. Among patients without a biopsy, only 15% patients presented with a M65 value corresponding to NASH. By contrast, when considering the non-invasive diagnosis of fibrosis, an even higher percentage of patients without biopsy (compared to those with biopsy) had values corresponding to significant fibrosis (35%, resp. 29%). This emphasizes the need to follow and accurately examine those patients with suspected NAFLD but not indicated for a liver biopsy. Paradoxically, the fact that biopsy was not performed in all of our patients and that the selection of this group was affected by factors common in routine clinical practice is one of the most interesting findings in our study!

In conclusion, our study supports the use of non-invasive parameters such as HA, OELF and ELF scores, or cytokeratin-18 fragments in diagnosing of NASH/fibrosis in those patients with NAFLD. It should be emphasized that a high percentage of patients not undergoing a liver biopsy (for a variety of reasons) might have significant fibrosis.

Supporting Information

Table S1 Clinical and laboratory data in patients with different stages of liver fibrosis.
(DOC)

Author Contributions

Conceived and designed the experiments: KD JP LV VS RB. Performed the experiments: KD JS JP RS VS RB. Analyzed the data: KD JS LV ML VS RB. Contributed reagents/materials/analysis tools: JS JP LV ML VS MH. Contributed to the writing of the manuscript: KD JS JP LV RS ML VS MH RB.

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The effects of n-3 polyunsaturated fatty acids in a rodent nutritional model of non-alcoholic steatohepatitis

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Abstract

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) probably represent the most common chronic liver diseases in western countries with a substantial risk of liver cirrhosis and high cardiovascular morbidity and mortality.

The aim of the study was to determine the effects of administration of n-3 polyunsaturated fatty acids (n-3 PUFA) in the high fat methionine-choline deficient (MCD) dietary model of NASH and to assess the role of n-3 PUFA in development and pathogenesis of NASH.

Male C57BL/6 mice were fed for 6 weeks either with MCD or with chow. There were 4 groups of animals. Two groups fed with MCD and two control groups, both either with or without n-3 PUFA supplementation. Detailed liver histology and serum biochemistry were determined. Lipidomic analysis was performed by using ultra high-performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS). Large chromatographic and spectral data sets generated by metabolic fingerprinting were used to assess the differences between groups. BeadChip analysis was used to profile and identify the differences in liver mRNA transcriptomes.

Feeding with MCD (group M) resulted in histopathological changes of NASH and these changes were ameliorated after n-3 PUFA administration (group MP). n-3 PUFA decreased cholesterol levels ($P < 0.001$), ALT ($P<0.01$) and AST levels ($P < 0.01$). MP developed less pro-inflammatory cytokine profile ($P < 0.01$) than controls. Administration of n-3 PUFA led to lower serum concentrations of saturated and monounsaturated free FA and to higher serum concentrations of polyunsaturated FA in MP. Total serum lipid content and intensities of TAG was significantly lower in MP compared to M ($P < 0.001$).

In the liver, MCD (M) significantly increased amount of TAG compared to C ($P < 0.001$). n-3 PUFA significantly decreased the content of TAG in MP compared to M ($P < 0.001$). In M group the most frequent fractions of TAG were C50, C52, C54 and C56. n-3 PUFA (MP) decreased concetrations of fractions C50, C52 and C54. Contrary to it, n-3 PUFA (MP) singnificantly increased fractions C58 and C60. Only after n-3 PUFA administration (MP) C62 fraction appeared, which was not detected in M. n-3 PUFA administration influenced also the number of double bonds in acyl chains of TAG. After n-3 PUFA administration TAG with more double bonds

were found in MP compared to M. In the liver, groups without n-3 PUFA (control and M) had the highest intensity of arachidonic acid, diets with n-3 PUFA (CP, MP) contained higher amount of docosahexaenoic acid and eicosapentaenoic acid.

Detailed BeadChip analysis revealed differences in liver mRNA transcriptomes after MCD as well as n-3 PUFA administration. 4707 gene transcripts were significantly changed ($P < 0.05$) between MP and M liver specimens and 5021 between M and C groups. n-3 PUFA administration influenced e.g. expression of mRNA of enzymes of cholesterol and fatty acid biosynthesis pathways and led to significant decrease in mRNA expression of pro-inflammatory cytokines (IL-2 ($P < 0.05$); IL-6 ($P < 0.01$); TNF-alpha ($P < 0.05$; MP vs. M).

We conclude that n-3 PUFA may play an important role in the pathophysiology of NASH. In summary, n-3 PUFA have favorable effects on histopathological changes, serum markers of liver damage, FA and TAG compound. n-3 PUFA change the metabolomic profile of liver tissue. We expect that n-3 PUFA may represent a promising way in prevention and treatment of NASH, which should be further studied in clinical trials.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is an increasingly diagnosed condition associated with obesity, hyperlipoproteinaemia, insulin resistance and metabolic syndrome. With epidemic of obesity and diabetes mellitus it is regarded as a problem with increasing importance for the future [1]. NAFLD affects 20% - 30% of the current westernalized world population [2], [3] and encompasses a wide spectrum of liver disease, ranging from simple steatosis (non-alcoholic fatty liver) to non-alcoholic steatohepatitis (NASH). NASH is accompanied by inflammation and hepatocyte injury (ballooning), with or without fibrosis [4] which can progress to cirrhosis with a high risk of liver failure and hepatocellular carcinoma [5]. The course of NAFLD is highly variable, and only a minority of patients (2-3%) progress to end-stage liver disease [6].

The pathogenic mechanism involved in the development of fatty liver remains unclear. It is known that severity of steatosis is well correlated with progression to NASH in humans [7] and several recent reports suggest that accumulation of lipids in the liver, and especially disturbances in the composition of hepatic fatty acids, might play a causal role in the development of NASH [7], [8], [9], [10], [11].

Intricacies of the molecular and cellular mechanisms responsible for progression from simple steatosis to NASH have not been fully elucidated. It has been proposed that steatosis, the earliest and most prevalent stage of NAFLD, often referred to as the "first hit" that in turn lead to the inflammation, fibrosis and cellular death characteristic of NASH [11]. Recently, a "multiple parallel-hit" theory has been proposed to explain the development and progression of NAFLD [12]. This hypothesis encompasses multiple insults such as mitochondrial dysfunction, endotoxemia, insulin resistance, gut microbiota synergistically act together leading to immune system activation and subsequent hepatic injury and cell death.

Excessive accumulation of lipid substrates in the liver has serious adverse effects on cell functions [13] and especially saturated free fatty acids are directly hepatotoxic, perhaps by mediating an endoplasmic reticulum stress response and inducing hepatocyte apoptosis referred to as lipoapoptosis or lipotoxicity [14]. Recent studies suggest that excess fat accumulation in the liver

associated with depletion of n-3 polyunsaturated fatty acids and disturbances in the ratio of saturated/unsaturated fatty acids may play a causal role in the pathophysiology of NASH [15].

Oxidative stress is another important and central mechanism in the progression towards NASH. It brings damage to cells, hereby triggering a hepatic inflammatory response, which can eventually lead to apoptosis [16].

Since the liver plays a key role in lipid metabolism, dietary fats and their oxidized metabolites may not only influence the pathogenesis of liver diseases [17], [18], [19] but may also prevent and/or reverse disease manifestations [20].

Despite high prevalence of NAFLD, there is no established pharmacological treatment and weight reduction and lifestyle modification with increased physical activity stay the only effective therapeutic measures [4], . Together with increased consumption of Western diet rich in processed sugars (fructose, sucrose) and fat, which has been directly implicated in the recent increase in the prevalence of NAFLD [21], there is an imperative need for development of new effective treatment strategies. Pharmacological interventions to treat obesity-associated diseases require multiple agents and are often associated with adverse side effects [22].

It has been reported that n-3 polyunsaturated fatty acids (n-3 PUFA) are able to limit triglyceride deposition in the liver [23], [24], ameliorate hepatic steatosis and insulin resistance [25], [26], whereas a diet deficient in n-3 PUFA with a high n-6/n-3 ratio could induce fatty liver [18] and chronic diseases including NAFLD [27]. The n-6 and n-3 PUFA are functionally and metabolically distinct and are not interconvertible [28]. In the Western type of diet, the level of n-6 PUFA is higher than the n-3 PUFA, which resulted in a shift of n-6/n-3 ration from traditional 2:1 to >10:1 [29]. Hypolipidemic effects of n-3 PUFA depend on complex metabolic and gene expression changes, resulting in suppression of hepatic lipogenesis and increased fatty acid oxidation [30]. n-3 PUFA show anti-inflammatory action in rodents [26], [31] and in humans [32], [33] and improve dyslipidemia [34]. Metabolic actions of n-3 PUFA are determined by tissue production of eicosanoids and other lipid mediators [35], induction of adiponectin [36] and modulation of expression by master transcriptional regulators [37].

The aim of our study was to investigate the impact of substituting n-3 PUFA on the development of NAFLD using well-established model of NAFLD/NASH – methionine- choline-deficient diet [38] and to elucidate the mechanisms involved in the antisteatotic action of n-3 PUFA.

Materials and Methods

Chemicals

Paraformaldehyde, hematoxylin-eosin, RNA-later, $MgSO_4$ p.a. (purity ≥ 99%) were supplied by Sigma-Aldrich (St. Luis, MO, USA). Deionized water was obtained from a Milli-Q® Integral system supplied by Merck (Darmstadt, Germany), HPLC-grade cyclohexane, acetonitrile, formic acid, and ammonium formate (purity ≥ 99%) and. NaCl p.a. (purity ≥ 99%) was supplied by Penta (Chrudim, Czech Republic). Total RNA Purification Kit from Norgen Biotek Corporation (Canada); High Capacity cDNA Reverse Transcription Kit and TaqMan Gene Expression Master Mix from Applied Biosystems (USA). All other chemicals were purchased locally from Penta (Czech Republic).

Animals and experimental design

Male C57BL/6 mice (30 g, age of 3 months), obtained from Anlab (Prague, Czech Republic), were housed under controlled temperature and a natural 12:12 light-dark cycle. All aspects of the study met the accepted criteria of experimental use of laboratory animals and all protocols were approved by the Animal Research Committee of the 1st Faculty of Medicine, Charles University, Prague, Czech Republic.

NASH induction

Adult male mice were divided into 4 groups. Two groups of animals representing NASH model were fed with high fat methionine/choline deficient (MCD) diet ssniff R/M (Soest, Germany) for 6 weeks, each group received either n-3 polyunsaturated fatty acids (n-3 PUFA) (MP) or saline (M) for the whole period of experiment. NASH control groups were fed chow containing n-3 PUFA (CP) or saline (C) in the same dosage as the MCD-fed animals.

n-3 PUFA administration

n-3 PUFA were obtained from Farmax, Czech Republic, each animal received daily either 0.1 ml of a mixture of docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) (56.25mg EPA + 42.5mg DHA) via orogastric tube or saline.

General tissue preparation

Mice were anesthetized with intramuscular application of ketamine (100 mg/kg) and xylazine (16 mg/kg). Peritoneal cavity was opened, and blood was taken from inferior vena cava. Liver was removed, weighted and cut to samples (formol, liquid nitrogen, RNA later).

Serum biochemistry

Serum biochemical markers (total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total cholesterol and triacylglycerols (TAG) were determined by standard assays using an automatic analyzer (Modular analyzer, Roche Diagnostics GmbH, Mannheim, Germany).

Liver histology

Two small liver tissue blocks (about 1 cm^3) were fixed in 4% paraformaldehyde followed by a standard procedure for paraffin embedding. Serial sections 5-7 μm thick were cut a stained with haematoxylin and eosin. Each slide was viewed using standard light microscopy. Histological assessment was performed by an experienced liver histopathologist according to Kleiner [39].

In representative section from each tissue block was determined its area, established NAS score by Kleiner (steatosis, ballooning, intralobular inflammation). The ratio of micro- / macrovesicular steatosis was obtained and was searched for: dissolving hepatocytes, portal and periportal inflammatory infiltrates, Mallory-Denk bodies, and signs of fibrosis.

Adipokines ELISA kit

The concentrations of adiponectin and leptin in mice sera were measured with sandwich enzyme immunoassays with the spectrophotometric detection at 450 nm (ELISA kits, Uscn Life Science Inc., Wuhan, China).

Serum fatty acids composition

Total serum fatty acids were extracted from 50 mL serum as described [40], using dichloromethane instead of chloroform[41]. Ten mg of nonadecanoic acid methyl ester (NuCheck Prep, USA) was

added to each sample before extraction, as an internal standard. Samples were transmethylated to fatty-esther methyl esthers (FAME) by incubation in 1 M sodium methoxide in dry methanol for 20 min at 80°C. The reaction mixture was then cooled, acidified with 98% sulphuric acid and incubated for 1 hour at room temperature to methylate free acids. Lipid methyl esters were extracted with hexane, and the hexane extracts were subsequently dried under a nitrogen flow. Next, the residue was dissolved in 100 µl of n-heptane and stored at -20°C under nitrogen until analyzed. All reactions were performed under nitrogen atmosphere. GC was performed with a Trace-GC gas chromatograph combined with AS 2000 autosampler (Thermo-Finnigan, USA), equipped with a capillary split/splitless injector and a flame ionization detector. Analyses of FAME were performed on a fused-silica capillary column coated with chemically bond stationary phase CP-Sil 88 CB (100 m, 0.32 mm I.D.). The oven temperature was programmed as follows: from 80°C to 260°C at 2°C/min, then to 280°C at 10°C/min, where it was maintained for 45 min. The injector and detector temperatures were set at 250°C and 270°C, respectively. Hydrogen carrier gas was maintained at a head pressure of 70 kPa and total flow of 44 ml/min, with a split ratio of 1:35. Integration software Clarity version 2.4.1.57 (Data Apex Ltd. Prague, Czech Rep.) was used for data acquisition and handling.

Lipidomic analysis

Liver fatty acid composition - sample preparation

0.4 g of liver tissue was homogenized in 4 mL of cyclohexane by Ultraturrax laboratory homogenizer (1 min). Afterwards, 8 mL aqueous acetonitrile (1:1, *v/v*), 1.6 g $MgSO_4$ and 0.2 g of NaCl were added, subsequently the sample was shaken (1 min) and centrifuged (5 min, 10,000 rpm). Three layers were clearly separated. While the upper phase (cyclohexane) and middle one (acetonitrile) were stored in freezer (-18°C) for the follow up instrumental analyses, by contrast the bottom phase (aqueous) was discarded.

Liver fatty acid composition - analytical method

Ultra-High Performance Liquid Chromatography coupled to High Resolution Mass Spectrometry (UHPLC-HRMS) was used for analyses of mouse liver tissue samples. The instrumentation platform employed for analyses of both acetonitrile and cyclohexane extracts consisted of Dionex UltiMate

3000 UHPLC system (Thermo Fisher Scientific, Waltham, USA) coupled to quadrupole-time-of-flight AB SCIEX TripleTOF® 5600 mass spectrometer (AB SCIEX, Concord, ON, Canada).

The ion source for mass spectrometric measurements was a Duo Spray™ with separated ESI ion source and atmospheric-pressure chemical ionization (APCI). In the positive ESI mode the source parameters were as follows: capillary voltage: +4500 V; nebulizing gas pressure: 60 psi; drying gas pressure: 50 psi; temperature: 450°C; and declustering potential: 80 V. The capillary voltage in negative ESI was -4000 V, other source settings were the same as for ESI+.

The data obtained by full mass scan and Information Dependent Acquisition (IDA) method were employed to collect MS and MS/MS spectra. The method consisted of a survey TOF MS experiment ranging from m/z 100 to 1200, and in parallel, Product Ion (PI) spectra for the eight most intensive ions of the survey spectra throughout the chromatographic run. Dynamic Background Subtraction was activated to acquire PI spectra of real eluted compounds, avoiding background ions. PI spectra were collected for ions ranged from m/z 100 to 1200 with the quadrupol extraction window of 1 Da. The former selected precursor ion was excluded for 3 s (mass tolerance of 30 mDa) and totally excluded after three occurrences. The PI spectra were recorded with collision energy of 35 V and collision energy spread of ±15 V. In this way, both low and high energy fragment ions were present in a single spectrum. The total cycle time of TOF MS and IDA methods took 0.55 s.

An automatic m/z calibration was performed by the calibration delivery system (CDS) every 15 samples using positive or negative APCI calibration solution (AB SCIEX, Concord, ON, Canada) according to the batch polarity. Each set of samples in each polarity was preceded by blank control. At the end, the same MS approach was carried out by ESI- mode. The achieved resolving power was >31,000 (m/z 321.0192) full width at half maximum (FWHM) in both polarities. The PI spectra were measured in high sensitivity mode, which provides half resolving power.

Instrument control and data acquisition were carried out with the Analyst 1.6 TF software (AB Sciex, Concord, ON, Canada) and the qualitative analysis was performed using PeakView 2.0 (AB Sciex, Concord, ON, Canada) equipped with Formula Finder. The in-batch sequence of the samples was random to avoid any possible time dependent changes during UHPLC–HRMS analysis, which would result in false clustering.

Liver fatty acid composition - data processing

Molecular formula estimation, structural elucidation and subsequent tentative identification of compounds were performed based on MS and MS/MS accurate mass spectra using PeakView software. PeakView software equipped with FormulaFinder was able to calculate formulas according to MS, isotopic pattern and MS/MS ranking, reflecting differences between theoretical and measured m/z values for both parent and fragments ions, and the match of experimental and theoretical isotope pattern in terms of spacing and relative intensities.

Quantitative real-time PCR

The liver samples were stored frozen at -80°C in RNAlater (Sigma Aldrich, St. Louis, USA), and total RNA was isolated using a Qiagen RNAeasy plus kit and QIA shredder (Qiagen, USA). Cell culture samples were stored in lysis buffer at -80°C, and total RNA was isolated using PerfectPure RNA Cell Kit (5Prime, USA). A High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) was used to generate cDNA. Quantitative real-time PCR was performed using TaqMan® Gene Expression Assay Kit (Life Technologies, Carlsbad, CA, USA) for examined genes, all provided by Life Technologies (Carlsbad, CA, USA). The data were normalized to HPRT and expressed as percent of control levels

Statistical analysis

Normally distributed data are presented as mean ± SD and analyzed by the Student t-test. The Mann-Whitney U test or Kruskall-Wallis test were used in non-normally distributed data. Differences with $P < 0.05$ were considered significant.

Results

n-3 PUFA reduce body and liver weight gain

Administration of MCD resulted in increase of animal weight (M, 34.8 g ± 3.2 vs. C, 31.6 ± 1.1 g; $P<0.05$) as well as liver weight (M, 1.76 ± 0.17 g vs. C, 1.39 ± 0.09 g; $P<0.001$). Co-administration of MCD with n-3 PUFA (MP) contrary resulted in significant decrease of animal and liver weight compared to MCD (animal: MP, 31.0 ± 0.83 g vs. M, 34.8. ± 3.2 g $P<0.01$; liver weight: MP 1.37 ±

0.08 g vs. M, 1.76 ± 0.17 g, P<0.001). n-3 PUFA administration itself did not change the animal (CP, 31.8 ± 1.1 g vs. C, 31.6 ± 1.1 g, P=0.74) or liver weight (CP, 1.51 ± 0.12 vs. C 1.39 ± 0.09 g; P=0.22); see Fig. 1A and B.

Administration of MCD led to deterioration of liver injury

MCD administration for 6 weeks resulted in severe liver injury as evidence by significant elevation of serum ALT activity in M group compared to control (M, 1.29 ± 0.42 µkat/l vs. C, 0.64 ± 0.20, P<0.01) as well as ALP (M, 0.95 ± 0.15 µkat/l vs. C, 0.73 ± 0.07, P<0.01). Supplementation of n-3 PUFA completely abolish the effect of MCD on ALT activity (MP, 0.73 ± 0.37 µkat/l vs. M, 1.29 ± 0.42, P<0.01); see Fig. 2. Compared to control, AST and bilirubin stayed unchanged after all interventions.

n-3 PUFA reduced serum levels of cholesterol, triglycerides and had specific effects on fatty acid composition

Serum triacylglycerols (TAG) levels were significantly decreased after n-3 PUFA supplementation (CP, 0.86 ± 0.16 mmol/l vs. C, 1.08 ± 0.07, P<0,05; MP, 0.59 ± 0.16 mmol/l vs. M, 0.86 ± 0.24, P<0.05), while alone MCD administration had no effect (M, 0.86 ± 0.24 mmol/l vs. C, 1.08 ± 0.07, P=0.073); see Fig. 2B.

Serum cholesterol concentrations were 1.7-fold increased after MCD administration compared to chow (M, 3.28 ± 0.62 mmol/l vs. C, 2.02 ± 0.12; P<0.001). n-3 PUFA co-administration leads to a significant decrease of serum cholesterol compared to MCD (MP, 2.36 ± 0.29 mmol/l vs. M, 3.28 ± 0.62 mmol/l; P<0.001) as well as in CP compared to control (CP, 1.49 ± 0.15 mmol/l vs. C, 2.02 ± 0.12, P<0.0001); see Fig. 2C)

Detailed plasma fatty acids (FA) composition (Fig. 3) was obtained as described above. The total FA concentration was significantly lower in groups treated with n-3 PUFA (CP, MP) compared to controls and MCD respectively (see Fig. 4A). Saturated FA and monosaturated FA were higher in groups C and M compared to groups treated with n-3 PUFA. Serum levels of DHA and EPA are shown in Fig. 4B and 4C.

n-6/n-3 ratio was unchanged in C and M groups, while n-3 PUFA supplementation tended to decrease it. The n-6/n-3 ratio is shown in Fig. 5.

n-3 PUFA significantly decreased liver lipid content in MCD

Total liver lipid was obtained by method described above (see "Methods"). In NASH groups (M, MP) total liver lipid content was significantly higher (M, 13.97 ± 3.3 g of lipid/100 g liver tissue vs. C, 3.08 ± 0.35, P<0.001, MP, 8.29 ± 3.6 g of lipid/100g liver tissue vs. CP, 3.08 ± 0.6, P<0.001 respectively), see Fig. 6. n-3 PUFA reduced liver total lipid content in MP (MP, 8.29 ± 3.6 g of lipid/100 g liver tissue vs. M, 13.97 ± 3.3, P<0.01) and had no effect in chow group (C vs. CP), see Fig. 6.

Liver histology

Control groups (C, CP) are characterized by the absence of steatosis and ballooning degeneration. In both control groups are in the majority of animals small focal intralobular inflammatory infiltrates (C 5/6; CP 4/6), in the vast majority participations of neutrophil granulocytes. Neither porto-lobular inflammatory infiltrates nor fibrosis occur.

Histology of livers from experimental groups M and MP shows both steatosis and more frequent intralobular hepatocyte apoptosis. Steatosis (its proportion of the area of liver section) presents significant focal heterogeneity occurring deposits of panlobular steatosis, but often areas without steatosis (Fig. 7).

Dominantly zonal periportal macrovesicular steatosis (M, 11 from 11 animals; MP, 9/9) was observed. Panlobular steatosis was found in 9 animals from 11 in M group and in 3/9 in MP group. From M group, 8 animals showed microvesicular steatosis. Degree of steatosis was significantly higher in M group compared to MP (M, 69.4 ± 12 % vs. MP. 46.8 ± 9%, P<0.01), see Fig. 8A. Intralobular inflammatory infiltrates were observed in all groups fed by MCD (M, MP) as well as termination of hepatocytes (lipogranulomas). Apoptosis was rarely observed in M and MP groups. For details see Fig. 8. NAS Score was significantly higher in M compared to control, n-3 PUFA administration lead to non-significant decrease of NAS Score (M vs. MP).

Differential metabolic effects on adipokines by n-3 PUFA

Plasma levels of leptin were significantly increased by MCD intervention (C, 10.7 ± 4.1 ng/l vs. M, 33.8 ± 9.8, P<0.001). Administration of n-3 PUFA completely normalized leptin levels (MP, 12.58 ± 5.4ng/l vs. M, 33.8 ± 9.8; P<0.001). Surprisingly, n-3 PUFA decreased leptin levels as well as in control group (CP, 4.9 ± 1.8 vs. C 10.7 4.1, P<0.01); see Fig. 9A.

Adiponectin levels were increased by all interventions, with the M and MP being highest (C, 21.7 ± 2.0 ng/l vs. M, 28.3 ± 4.6, P<0.01; M vs. MP, 34.2 ± 3.3 P<0.01); see Fig. 9B.

Examination of lipids in liver tissue by UHPLC-HRMS

Both cyclohexane and acetonitrile extracts of livers were analyzed by UHPLC-HRMS technique described above. While neutral TAG were expected to be contained mainly in nonpolar solvent represented by cyclohexane, more polar lipids such as phospholipids were assumed to be transferred mainly into acetonitrile, i.e. into more polar solvent. Lipidomic analysis of the TAG, phospholipids and cholestyr esters fractions revealed significant changes in the FA profiles in response to all the interventions.

MCD administration resulted in changes in TAG spectra

MCD significantly increased TAG content in mouse liver tissue (groups M, MP) compared to control groups (C, CP). In contrast, n-3 PUFA in the diet decreased the intensity of TAG (see Fig. 10). Figure 11 shows absolute sum of TAG in all experimental groups.

It should be noted, that livers from mice fed by MCD, contained TAG with higher m/z values, in other words TAG containing FA with longer carbon chains. Higher content of TAG with longer carbon chains (higher number of carbons) C58, C60, C62 and C64 were found in liver of mouse fed with MCD diet with n-3 PUFA (group MP) in comparison with group M. The increase was 156% for C58, 288% for C60, 2362% for C62. C64 was found exclusively in MP. On contrary, TAG with shorter carbon chains – C46, C48, C50 – were more abundant in C and CP (see Fig. 12).

In addition, when n-3 PUFA was also added the number of double bonds in FA present in TAG was increased. Figure 11 shows mass spectra of TAG and clearly documents these trends.

n-3 PUFA influenced the phospholipid content of the liver tissue

Lysophospholipids and phospholipids, more polar lipids, were detected in both positive and negative polarities (ionisation modes). The most abundant phospholipids were phosphatidylcholines and phosphatidylethanolamines, even tought phosphatidylglycerols and phosphatidylinositols were also present. The highest content of phospholipids was found in M, by contrast the lowest amount of

phospholipids was found in C. Administration of n-3 PUFA increased the phospholipid content in chow groups and decreased in MCD groups.

Most significant differences were found in lysophospholipids. Lowest amount was present in group C. In MP, n-3 PUFA significantly decreased amount of lysophospholipids. Apparently, positive ionization mode did not provide a relevant information, because of the fact phospholipids are usually much better identified using negative ionization mode. For this reason, livers of mouse fed by different diets were also explored using negative ionization mode.

The most abundant phospholipids found in negative ionization mode were phosphatidylethanolamines and phosphatidylinositols, however phosphatidylglycerols and phosphatidylcholines were also identified. The highest content of phospholipids was found in group M, by contrast the lowest amount of phospholipids was found in group C . On the other hand, n-3 PUFA increased the phospholipid content in CP compared to C and decreased in MP compared to M. This is the same behivour as was already seen for phospholipids using positive ionization mode, but in this case more pronounced.

Free FA compostion in the liver tissue was determined by administration of n-3 PUFA

The differences in free FA in the liver tissue were mainly related to the administration of n-3 PUFA. In groups without n-3 PUFA (C, M) the most abundant FA was arachidonic acid (C 20:4). Groups with n-3 PUFA contained highest amount of docosahexaenoic acid (C22:6) and eicosapentaenoic acid (C20:5).

Chemometrics - PCA-DA model provided much better separation of all groups

The analysis of lipidomics data separated dietary interventions into four distinct groups according the presence of n-3 PUFA (principal component analysis); see Fig. 13. As expected, EPA and DHA increased in the both acetonitrile and cyclohexane extracts upon n-3 PUFA intervention. Principal component analysis identified species containing DHA and EPA as the most discriminating analytes.

Figure 14 presents average intensities with relative standard deviations of cholesteryl esters. Cholesteryl EPA (m/z 688.60276) and DHA (m/z 714.61851) were detected as ammonium adducts in positive ionization mode. Both esters were present in significantly higher amounts in liver tissue of

mice fed by MCD containing n-3 PUFA. Cholesteryl palmitate (m/z 669.58161) and stearate (m/z 697.61211) were detected as formate adducts in negative ionization mode.

n-3 administration resulted in complex down-regulation of hepatic genes encoding pro-inflammatory cytokines

Hepatic gene expression encoding key cytokines revealed significant changes. The expression of mRNA of genes encoding pro-inflammatory cytokines - interleukin 2, 6 and TNF-alpha were at least 2-3-fold increased after MCD administration: interleukin 2 (M, 259% vs. C, 100%, $P<0.001$), interleukin 6 (M, 2051% vs. C, $P<0.001$) and TNF-alpha (M, 292% vs. C, $P<0.001$). mRNA expressions of these cytokines were markedly reduced (normalized) after n-3 PUFA administration: IL-2 (MP, 124% vs. M, 259%, $P<0.001$), IL-6 (MP, 929% vs. M 2051%, $P<0.001$) and TNF-alpha (MP, 143% vs. M, 292%), see Fig. 15. Contrary to pro-inflammatory cytokines, mRNA expression of interleukin 10 was unchanged.

Discussion

Feeding with MCD diet resulted in histopathological changes typical for NAFLD/NASH and these changes were significantly ameliorated by simultaneous n-3 PUFA supplementation. n-3 PUFA administration significantly decreased plasma cholesterol levels and ALT as well as AST activity levels. Moreover, n-3 PUFA administration led to lower serum concentrations of saturated and monounsaturated free FA and to higher serum concentrations of polyunsaturated FA (mainly EPA and DHA).

Detailed (liver and serum) lipidomic analysis was performed by using UHPLC-HRMS. Total serum lipid content and intensities of TAG were significantly lower after n-3 PUFA administration. In the liver, mice fed only with MCD diet had the highest intensity of arachidonic acid. Contrary to it, livers of mice fed with MCD diet together with n-3 PUFA contained higher amount of EPA and DHA together with higher content of TAGs with longer carbon chains in comparison to MCD diet only. In the liver, real-time PCR analysis revealed significant decrease of mRNA expression of pro-inflammatory cytokines (IL-2, IL-6, TNF-α) after n-3 PUFA supplementation compared to MCD diet.

In summary, n-3 PUFA have favourable effects on histopathological changes, serum markers of liver damage, fatty acid and TAG compound and show anti-inflammatory properties. We suppose that n-3 PUFA may represent a promising way in prevention and treatment of NAFLD, this increasingly common disorder.

Acknowledgments

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Figures

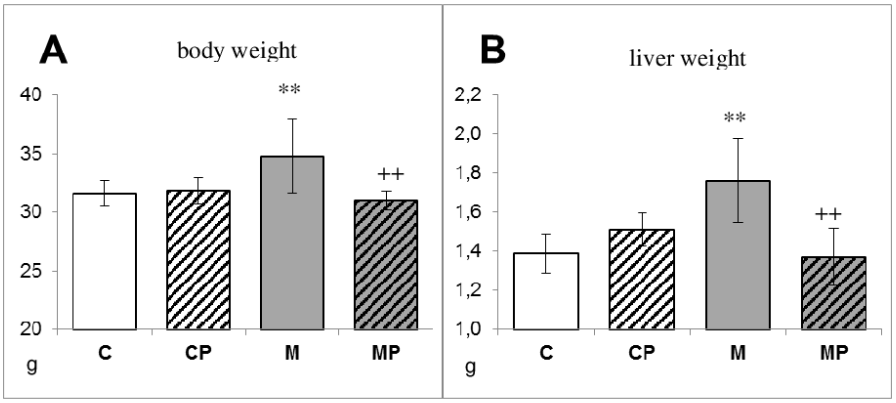

Figure 1. n-3 PUFA reduce body and liver weight gain

Total body weight (A) and liver weight (B) were assessed in the ad libitum fed state.Total body weight (A) after 6 weeks of experiment. Administration of MCD resulted in increase of animal weight as well as liver weight. Co-administration of MCD with n-3 PUFA (MP) resulted in significant decrease of animal and liver weight compared to MCD (to a control level).

*** P<0.01 vs. C; ++ P<0.01 vs. M*

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

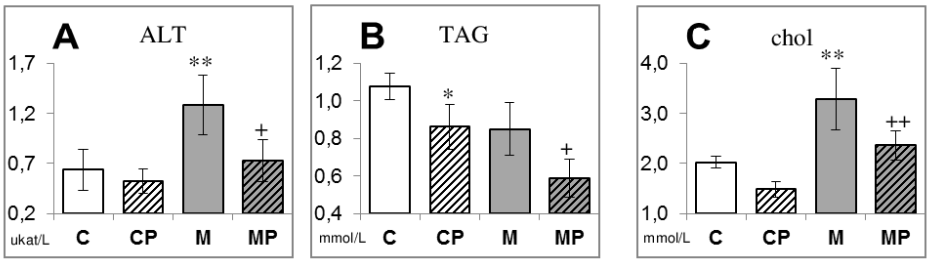

Figure 2. n-3 PUFA reduced serum markers of liver injury and decreased TAG and cholesterol levels in serum

Activity of ALT (A), levels of triacylglycerols (B) and cholesterol (C) were determined at the end of study. MCD administration for 6 weeks resulted in severe liver injury as evidence by significant elevation of serum ALT activity in M group compared to control as well as ALP and cholesterol levels. Supplementation of n-3 PUFA completely abolish the effect of MCD on ALT activity, TAG and cholesterol levels.

** P<0.05 vs. C; ** P<0.01 vs. C; + P<0.05 vs. M; ++ P<0.01 vs. M*

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

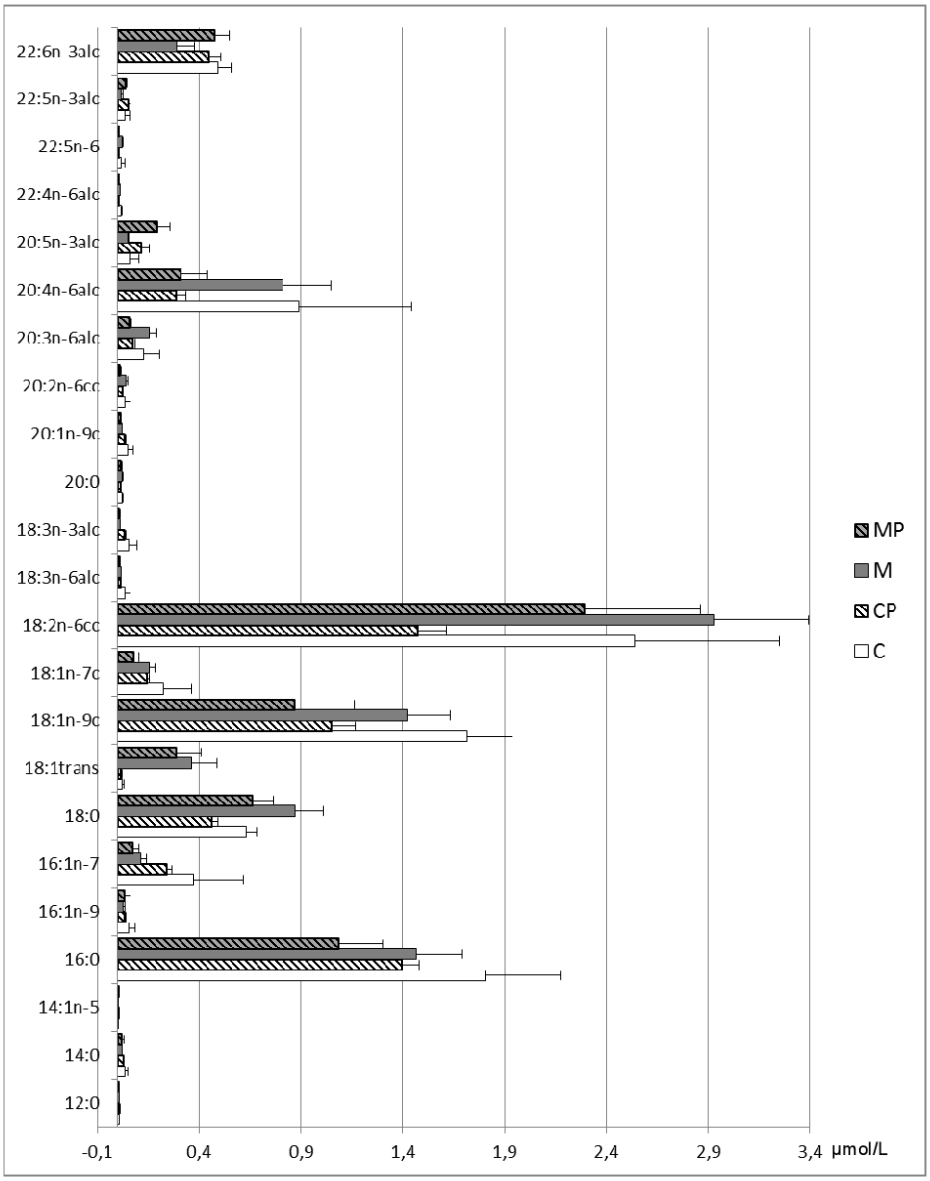

Figure 3. Detailed analysis of serum FA concentrations

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

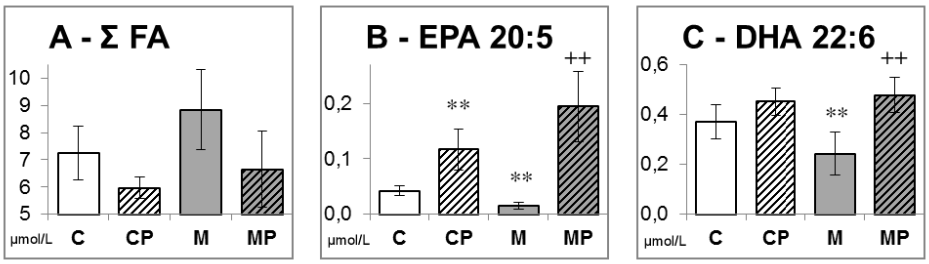

Figure 4. The total FA concentration was significantly lower in groups treated with n-3 PUFA (CP, MP) compared to controls and MCD respectively

Saturated FA and monosaturated FA were higher in groups C and M compared to groups treated with n-3 PUFA. Serum levels of DHA and EPA are shown in B and C.

A – Serum total FA concentration; B – serum concentration of eicosapentaenoic acid; C – serum concentration of docosahexaenoic acid.

*** P<0.01 vs. C; M; ++ P<0.01 vs. M*

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

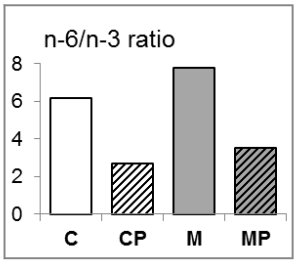

Figure 5. Serum n-6/n-3 ratio.

n-6/n-3 ratio was unchanged in C and M groups, while n-3 PUFA supplementation tended to decrease it.

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

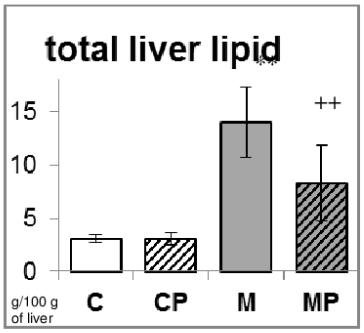

Figure 6. Total liver lipid content

In NASH groups (M, MP) total liver lipid content was significantly higher compared to controls. n-3 PUFA reduced liver total lipid content in MP and had no effect in chow group.

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

*** P<0.01 vs. C; ++ P<0.01 vs. M*

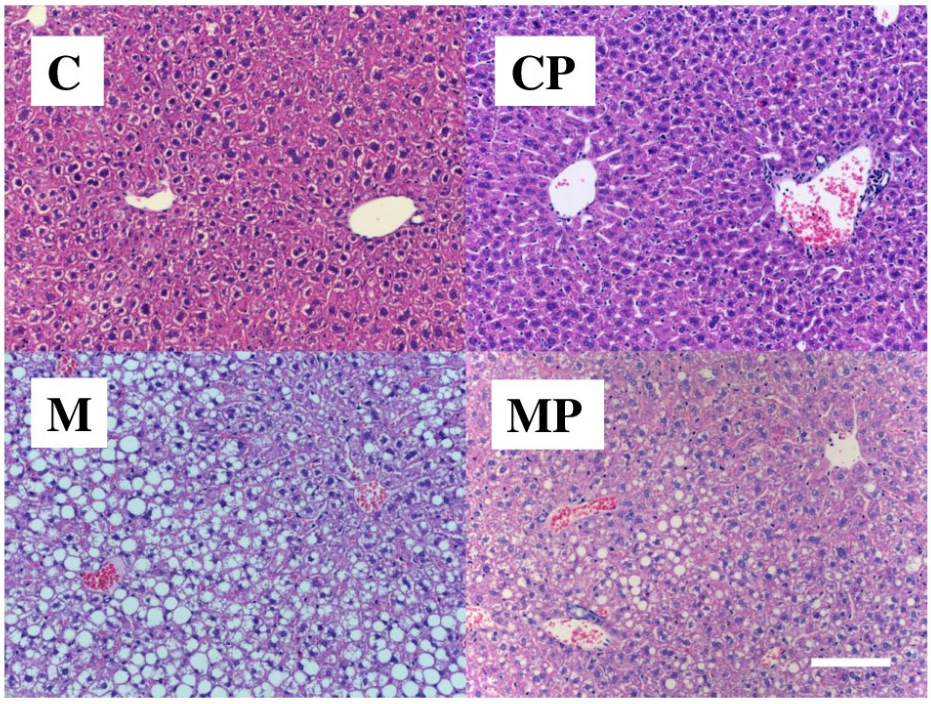

Figure 7. n-3 PUFA supplementation resulted in improvement of liver histology

In group M dominantly periportal macrovesicular steatosis was observed. Panlobular steatosis was found in 9 animals from 11 in M group. Degree of steatosis was significantly higher in M group compared to MP. Experimental groups M and MP shows more frequent intralobular hepatocyte apoptosis. Control groups (C, CP) are characterized by the absence of steatosis and ballooning degeneration.

C, control; CP, control + n-3 PUFA; M, MCD; M, MCD + n-3 PUFA

The bar represents 100 μm.

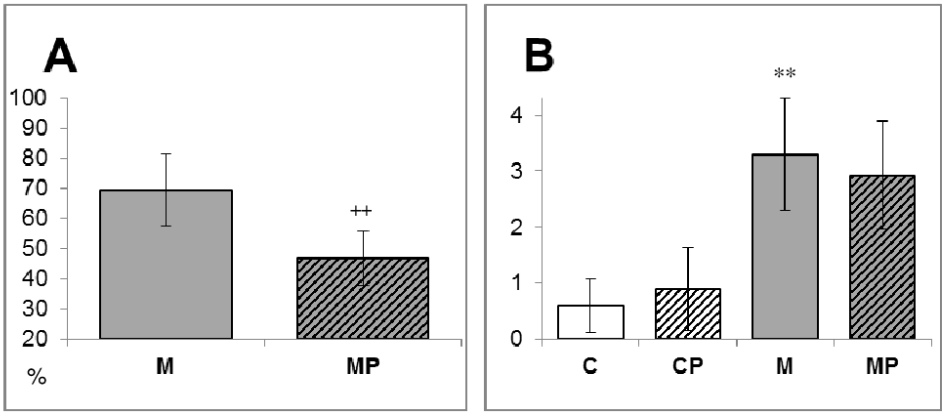

Figure 8. Degree of steatosis and NAS (NASH Acitivity Score)

In MCD groups, degree of steatosis was significantly lower after n-3 PUFA supplementation (MP group compared to M). NAS Score was significantly higher in M compared to control, n-3 PUFA administration lead to non-significant decrease of NAS Score (M vs. MP).

A – area of steatosis. B – NAS Score.

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

*** P<0.01 vs. C; ++ P<0.01 vs. M*

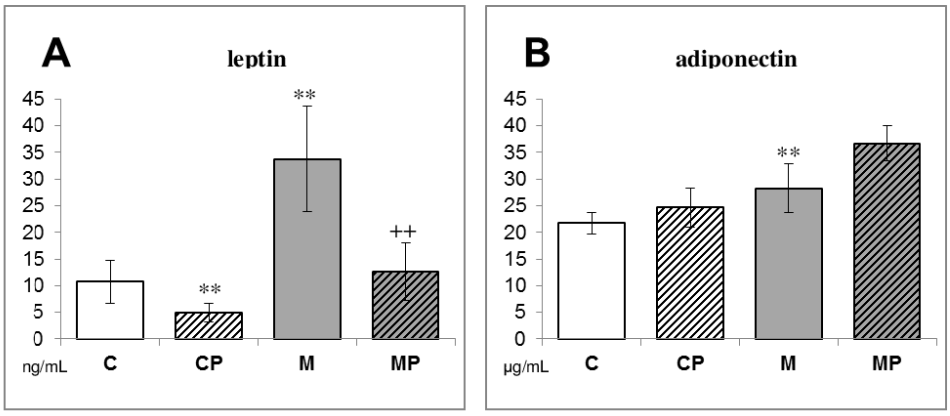

Figure 9. Metabolic effects on adipokines by n-3 PUFA

Plasma levels of leptin were significantly increased by MCD intervention. Administration of n-3 PUFA completely normalized leptin levels. Adiponectin levels were increased by all interventions, with the M and MP being highest.

A – serum leptin levels. B – serum adiponectin levels

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

*** P<0.01 vs. C; ++ P<0.01 vs. M*

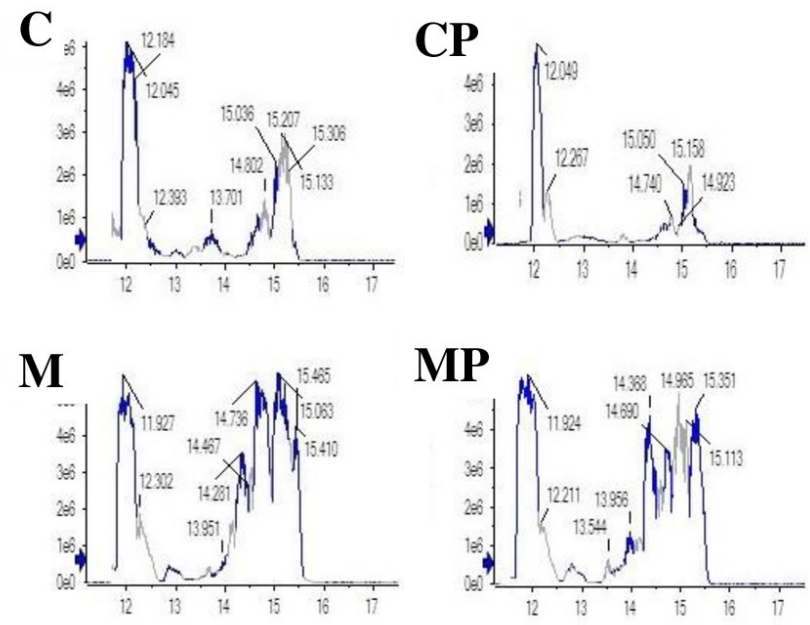

Figure 10. MCD administration resulted in changes in TAG spectra

MCD diet resulted in significant increase of TAG content in the liver tissue. Administration of n-3 PUFA in the diet decreased the TAG content in the liver.

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

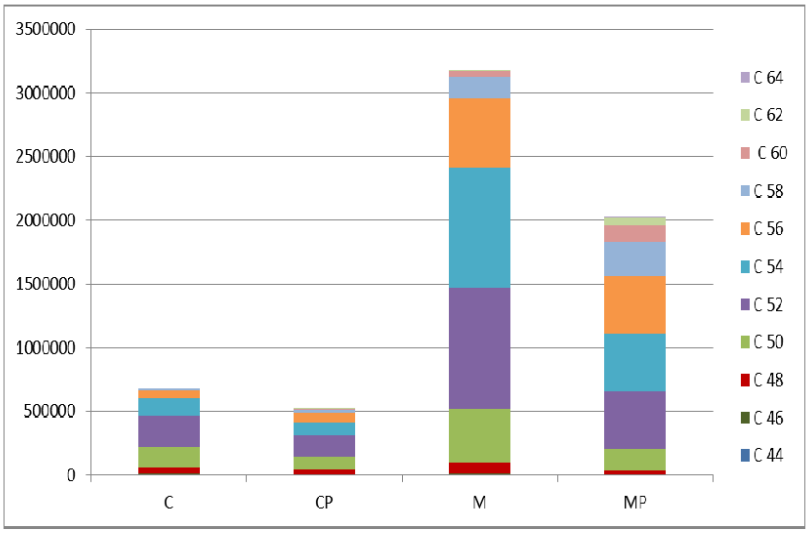

Figure 11. Absolute sum of TAG in all experimental groups

The codes Cx indicate the total number of carbons (x) in fatty acids bond to respective triacylgylcerol.

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

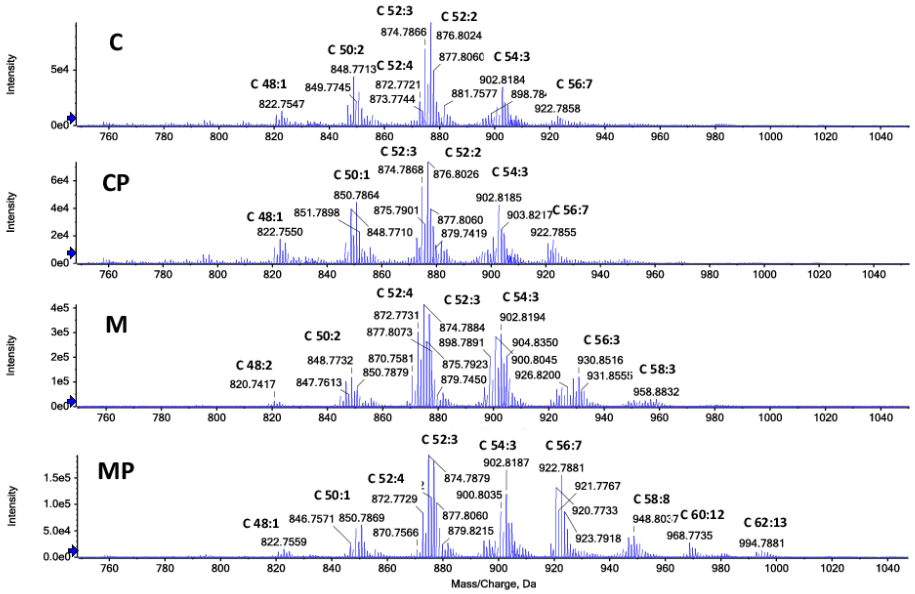

Figure 12. Mass spectra of TAG in liver tissue

Livers from mice fed by MCD, contained TAG with higher m/z values, in other words TAG containing FA with longer carbon chains. Higher content of TAG with longer carbon chains (higher number of carbons) C58, C60, C62 and C64 were found in liver of mouse fed with MCD diet with n-3 PUFA (group MP) in comparison with group M.

The codes C x:y indicate the total number of carbons (x) and number of double bonds (y) in fatty acids bond to respective triacylglycerol.

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

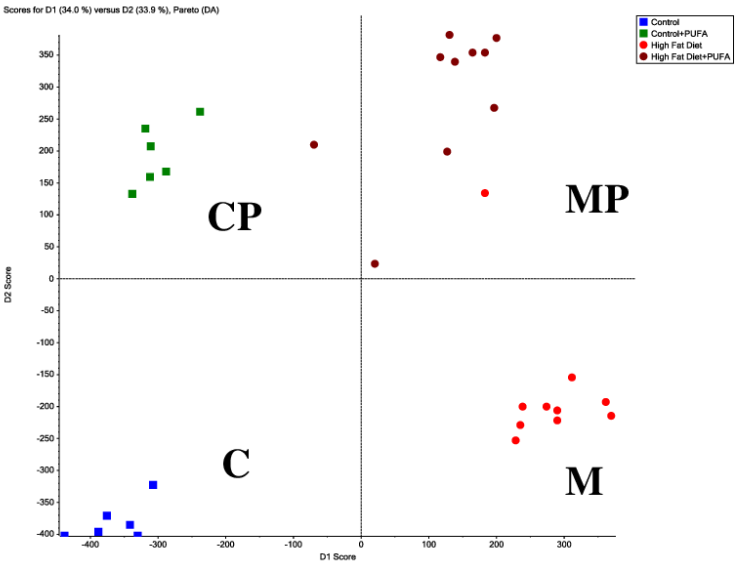

Figure 13. Principal component analysis of the lipidomics data

The analysis of lipidomics data separated dietary interventions into four distinct groups according the presence of n-3 PUFA. As expected, EPA and DHA increased in the both acetonitrile and cyclohexane extracts upon n-3 PUFA intervention. Principal component analysis identified species containing DHA and EPA as the most discriminating analytes.

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

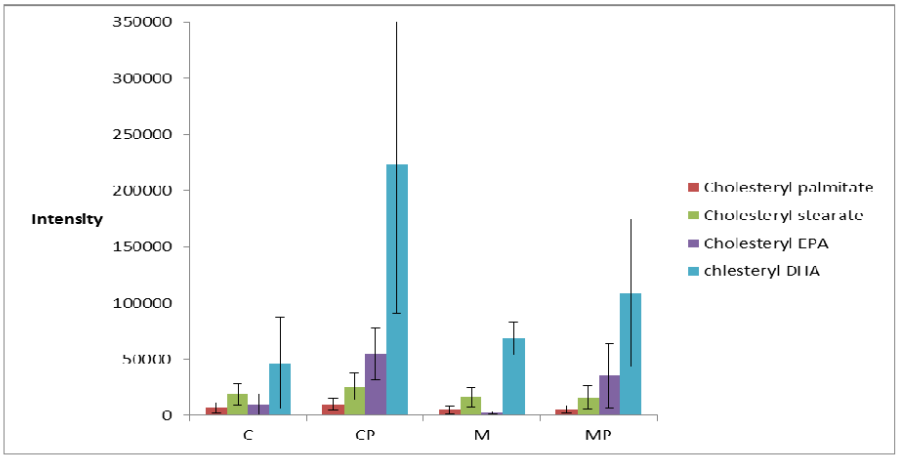

Figure 14. Average intensities of cholesteryl ester ions in mice liver tissue cyclohexane extracts

Cholesteryl EPA (m/z 688.60276) and DHA (m/z 714.61851) were detected as ammonium adducts in positive ionization mode. Both esters were present in significantly higher amounts in liver tissue of mice fed by MCD containing n-3 PUFA.

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

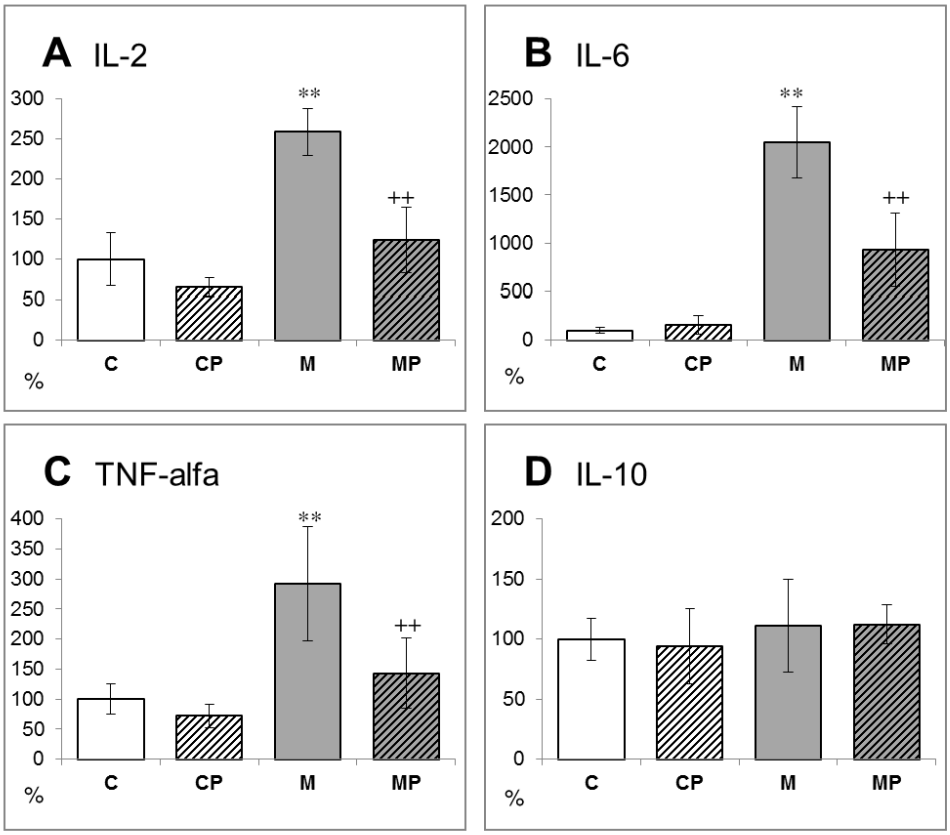

Figure 15. n-3 administration resulted in complex down-regulation of genes encoding pro-inflammatory cytokines in the liver

The expression of mRNA of genes encoding pro-inflammatory cytokines (interleukin 2, 6 and TNF-alpha) were at least 2-3-fold increased after MCD administration. mRNA expressions of these cytokines were markedly reduced (normalized) by n-3 PUFA administration. Contrary to pro-inflammatory cytokines, mRNA expression of interleukin 10 was unchanged. Data presented as

C, control; CP, control + n-3 PUFA; M, MCD; MP, MCD + n-3 PUFA

*** P<0.01 vs. C; ++ P<0.01 vs. M*

Abbreviations

APCI – atmospheric-pressure chemical ionization

ALT – alanine aminotransferase

AST – aspartate aminotransferase

EC – esters of cholesterol

EPA – eicosapentaenoic acid

FAME – fatty-esther methyl esthers

DHA – docosahexaenoic acid

FA – fatty acids

FW – fresh weight

IDA – information dependent acquisition

IL – interleukin

MCD – high fat methionine-choline deficient diet

n-3 PUFA – n-3 polyunsaturated fatty acids

NAFLD – non-alcoholic fatty liver disease

NASH – non-alcoholic steatohepatitis

PI – product ion

TAG – triacylglycerols

TNF – tumor necrosis factor

UHPLC-HRMS – ultra high-performance liquid chromatography coupled to high resolution mass spectrometry

experimental groups:

C - control; CP - control + n-3 PUFA; M - high fat MCD; MP - high fat MCD + n-3 PUFA

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Prague NAFLD Study: Nonalcoholic Fatty Liver Disease and n-3 Polyunsaturated Fatty Acids in Patients with Metabolic Syndrome

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Key words: NAFLD, NASH, n-3 PUFA

Abstract

Non-alcoholic fatty liver disease (NAFLD) represents the most common chronic liver disease in western countries with a global prevalence of 25% in adults. There is no established pharmacological treatment and weight reduction and lifestyle modification with increased physical activity stay the only effective therapeutic measures, but they are difficult to achieve and sustain. It has been reported that n-3 polyunsaturated fatty acids (n-3 PUFA) are able to ameliorate hepatic steatosis and insulin resistance, whereas a diet deficient in n-3 PUFA with a high n-6/n-3 ratio could induce fatty liver. Up to date published papers using n-3 PUFA have yielded contradictory results. The aim of the study was to assess the effects of administration of n-3 PUFA in development of NAFLD in patients during one-year follow-up.

We have examined 60 patients with metabolic syndrome and NAFLD in different stage of disease. Patients were randomized into two groups: 30 used n-3 PUFA in daily dose 1.8 g of eicosapentaenoic acid and 1.36 g of docosahexaenoic acid in four divided doses; 30 patients used placebo in the same scheme. During one-year follow-up were patients periodically examined - anthropometry (weight, waist circumference, BMI), biochemistry (liver enzymes, glucose metabolism etc.), blood count, abdominal ultrasound, liver stiffness measurement using ARFI®, nuclear magnetic resonance spectroscopy (at the start and end of follow-up). After one-year follow-up results were evaluated and statistically processed.

We observed significant decrease in GGT serum activity after 12 months of n-3 PUFA administration, the total amount of liver fat measured using magnetic resonance spectroscopy remained unchanged. We conclude that n-3 PUFA could represent a potential agent in preventing the development of NAFLD in patients with metabolic syndrome.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of potentially progressive liver disease that comprises of simple steatosis, non-alcoholic steatohepatitis (NASH), variable degree of fibrosis and, ultimately, cirrhosis. NAFLD is strongly associated with type 2 diabetes (T2DM) and abdominal obesity and is recognized as the hepatic manifestation of the metabolic syndrome [1]. NAFLD has emerged a leading cause of chronic liver diseases worldwide, as its incidence in adult population (17-46 %) parallels the epidemics of obesity and type 2 diabetes [2] and is becoming one of the most frequent causes of cirrhosis and liver transplantation worldwide [3]. Moreover, its prevalence is expected to increase in the future as a consequence of the increasing adoption of an unhealthy diet and sedentary lifestyle [4]. Unlike to NASH (which develops approximately in 20-30% of patients with NAFLD) simple steatosis has been considered a benign condition in spite of fibrosis progression. Nevertheless the presence of NAFLD is associated with the risk of cardiovascular morbidity and different cancers irrespective to the type of liver lesion [5], [6].

The diagnosis of NAFLD/NASH has been based on liver biopsy [7]. Liver biopsy is not acceptable for wide spread use due to invasivity, therefore non-invasive methods have recently been implemented in the diagnosis of NAFLD. While a diagnosis of steatosis can be made by quantification of liver fat content using ^{1}H magnetic resonance spectroscopy (^{1}H MRS) and fibrosis/cirrhosis could be detected with high accuracy using elastography or serum biomarkers, the precise diagnosis of NASH still requires liver biopsy.

Irrespective of high frequency of NAFLD in population and its consequences for overall mortality, the treatment of NAFLD/NASH is subject of many controversies. The first line treatment of NAFLD (and NASH) should be lifestyle change, including dietary habits modification and physical activity increase leading to weight reduction. However, the percentage of patients targeting long-term lifestyle modification is low, underlining the search for pharmacologic therapy of NAFLD. Despite intensive effort and increasing number of clinical trials, no pharmacological treatment of NAFLD has been approved till now. The only available pharmacotherapy is vitamin E and pioglitazone, whose effect was described at relatively small cohort, and the use is a subject of some controversies.

A growing interest has been directed to the potential favorable effect of n-3 polyunsaturated fatty acids (n-3 PUFA) on NAFLD in the last years. n-3 PUFA supplementation results in the improvement of biochemical aspects of NAFLD as well as amelioration of liver steatosis in some studies, while in other studies the fat liver content was not changed by n-3 PUFA administration. The authors of 2 large and recent metaanalyses [8], [9] concluded that n-3 PUFA may have favorable effect both on liver fat content and liver enzymes in patients with NAFLD, but more randomized studies are required to confirm their effect.

The aim of our study was to assess the effect of 12 month n-3 PUFA treatment on biochemical parameters and liver fat content in patients with NAFLD compare to placebo.

Methods

Study design and patients

The study was a single-center, double-blind, randomized trial of 12 months of n-3 PUFA versus placebo in patients with NAFLD and metabolic syndrome (*www.clinicalTrials.gov* registration number NCT02647294). The primary end point was to test whether administration of n-3 PUFA over a period of 12 months leads to decrease in liver fat percentage (measured by ^{1}H MRS) and no progression of liver fibrosis (determined by liver stiffness measurement) compare to placebo. Secondary end points evaluated improvement of biochemical parameters, surrogate markers of NAFLD and tolerability of medication in active treatment compare to placebo group.

The study was carried out in full accordance with the Helsinki Declaration of 1975, as revised in 1983, and was approved by the Institutional Ethics Committee. Informed consent was obtained from all subjects before entering the study.

Inclusion and exclusion criteria

Patients were recruited from outpatient departments of 4th Department of Internal Medicine (hepatogastroenterology, diabetic and metabolic special centers). They were consecutive patients diagnosed with NAFLD and metabolic syndrome referred to Hepatology unit. The diagnosis of NAFLD was based on clinical and laboratory parameters. These clinical and laboratory parameters included 1.) evidence of hepatic steatosis, either by imaging (in presented study diagnosed by ultrasound) or histology, and 2.) lack of secondary causes of hepatic fat accumulation [3]. Other etiologies of liver disease like viral hepatitis, drug-induced liver disease, autoimmune liver disease, biliary diseases, and inherited metabolic diseases were excluded by specific laboratory and radiologic examinations, as well as by the patient history. Significant alcohol abuse (160 g/week in women and 210 g/week in men) was ruled out by the patient´s history, short questionnaire, stable GGT activities documented in patient´s documentation, and the use of either serum carbohydrate-deficient transferrin and/or urine ethyl-glucuronide levels if required. The presence of any malignancy was also an exclusion criterion. Metabolic syndrome was diagnosed on the basis of diagnostic criteria stated by International Societies Consensus in 2009 [10].

n-3 PUFA administration

Patients with metabolic syndrome and NAFLD were randomly allocated for the treatment with n-3 PUFA (3.6 g/day) or placebo for 12 months. The study medication was supplied by SVUS Pharma a.s. Hradec Kralove, Czech Republic (all expenses of the study including the manufacturing of study medication were funded by the Czech health research council). One tablet with active substance contained 450 mg n-3 PUFA (the main components: eicosapentaenoic acid (EPA) 215 mg and docosahexaenoic acid (DHA) 155 mg). The dosage was 2 tablets four times a day. Placebo capsules with the same dosage contained 450 mg of soya oil and had the same appearance as capsules with active substance. Patients, physicians, nurses, and technicians performing imaging techniques (^{1}H MRS) were masked to treatment assignment throughout the study. Adverse events were recorded on all follow-up visits.

Clinical, anthropometric and biochemical measurements

Clinical, anthropometric and laboratory parameters were recorded at the baseline, at the end of the study and in month 3, 6 and 9 during the study. Biochemical analyses were performed on automatic analyzers (Modular Analyzer; Roche Diagnostics GmbH, Mannheim, Germany) using standard laboratory assays and included examination of triacylglycerols, cholesterol, HDL-cholesterol, LDL-cholesterol, glucose, insulin, serum bilirubin, AST, ALT, ALP, GGT, albumin and total protein. Fatty liver index was calculated as described in the literature [11].

Ultrasonography and magnetic resonance technique

Ultrasonography with liver stiffness measurement (ARFI®, Siemens, Germany) was performed at the baseline, at month 6 and at the end of study. The ^{1}H MRS for visceral and liver fat determination was performed at the baseline and at the end of study. All ^{1}H MRS examinations were performed on a 3T MR scanner (Magnetom-Trio, Siemens, Germany) as described previously [12].

Plasma lipidomics using UHPLC-HRMS/MS

Samples for lipidomic analysis were prepared by plasma protein precipitation by 2-propanol. After centrifugation of precipitated samples, 350 µL of the supernatant was collected and stored in 1.5 mL vial for analysis. The quality control sample was prepared by transferring of 20 µL of supernatant from every sample into a 15 mL centrifugation tube.

For the lipidomic analysis, U-HPLC (Infinity 1290, Agilent) coupled to a high-resolution mass spectrometer with a hyphenated quadrupole time-of-flight mass analyzer (6560 Ion Mobility Q-TOF LC/MS; Agilent) with the Agilent Jet Stream (AJS) electrospray (ESI) source were employed.

An Acquity BEH C18 (1.7 µm, 2.1 mm x 150 mm (Waters, USA)) was used for chromatographic separation. The chromatographic system used with ESI+ detection was: A – 10 mM ammonium formate and 0.1 % formic acid in acetonitrile:water (60:40, v/v); B – 10 mM ammonium formate and 0.1 % formic acid in 2-propanol:acetonitrile (90:10, v/v). For chromatographic separation of plasma detected in ESI- mode, following mobile phases were used: A - 10 mM ammonium acetate and 0.1% acetic acid in acetonitrile:water (60:40; B - 10 mM ammonium acetate and 0.1% acetic acid in 2-propanol:acetonitrile (90:10, v/v). The flow rate was constant at 0.300 mL · min^{-1}. The column

temperature was maintained at 60 °C, the injection volume was increased to 10 µL. The autosampler was kept at 10 °C.

Before injection, the samples were randomized in MS Excel. The QC sample was injected every 10 samples.

The mass analyzer was operated in ESI+ mode in following conditions: Gas temperature 180 °C, Drying Gas 12 L/min, Nebulizer pressure 40 psig, Sheath gas temperature 350 °C, Sheath gas flow 11 l/min, capillary voltage 3000 V, Nozzle voltage 250 V, fragmentor voltage 380 V, octopole radiofrequency voltage 750 V. Data were acquired over the m/z range of 50 – 1700 at the rate of 2 spectra/s. The m/z range was autocorrected on reference masses 121.0509 and 922.0098.

The mass analyzer was operated in ESI- mode in following conditions: Gas temperature 180 °C, Drying Gas 12 L/min, Nebulizer pressure 45 psig, Sheath gas temperature 350 °C, Sheath gas flow 11 L/min, capillary voltage 3500 V, Nozzle voltage 250 V, fragmentor voltage 350 V, octopole radiofrequency voltage 250 V. Data were acquired over the m/z range of 50 – 1700 at the rate of 2 spectra/s. The m/z range was autocorrected on reference masses 119.0363 and 980.0164.

The data was processed LipidMatch [13] suite which uses MZmine 2 for feature extraction and an R script for lipid identification. A custom-built R script based on MetaboAnalystR package was used to filter out features based on their univariate statistics. Statistically insignificant compounds were filtered out if they did not meet the criteria of ANOVA p-value of <0.01 and max. fold change among the groups >3. These data were then loaded by SIMCA, where statistical models were built. When building PLS-DA and OPLS-DA models in SIMCA logarithmic transformation and pareto scaling was used to ensure higher significance of low abundant compounds Fragmentation spectra of the significant compounds were compared to those present in METLIN and LIPIDMAPS databases and their identities confirmed.

Statistical analysis

The results are presented as mean values with standard deviation. Either a two-sample t-test or the Mann-Whitney rank test for non-Gaussian distributed variables was used to estimate intergroup

differences. The correlations between different parameters were evaluated by calculation of Pearson or Spearman correlation coefficients and linear regression analyses. All tests were two-sided, with $P < 0.05$ considered as statistically significant. The statistical analyses were performed using BDMP Statistical Software version PC90 (Cork Technology Park, Ireland) and Statistica CZ v.12 (StatSoft, Prague, Czech Republic).

Results

Baseline characteristics

We have examined patients with metabolic syndrome and NAFLD in different stage of disease – steatosis or NASH had 55 participants, liver cirrhosis had 5 patients. Of the 60 patients enrolled in the study were 45 men and 15 women, the detailed characteristics of participants shows Table 1. There was no significant difference in any of anthropometric or biochemical parameters among the monitored groups (n-3 PUFA versus placebo) at the beginning of the study. No serious adverse events attributed to n-3 PUFA medication occurred during the follow-up.

n-3 PUFA administration led to significant decrease in GGT activity

After one-year follow-up, no changes in anthropometric data (weight, waist circumference, or BMI) were observed in the patients enrolled in the study. Yearlong n-3 PUFA treatment resulted in a significant decrease in GGT activity (n-3 PUFA group 2.27 ± 2.53 vs. 1.43 ± 1.55 ukat/L, $P = 0.0397$), without any change in the placebo group (2.11 ± 3.13 vs. 2.03 ± 2.8 ukat/L, $P = 0.064$); Figure 1. All other observed biochemical parameters remained unchanged in both groups (Table 2). During the follow-up liver elastography measured using ARFI® did not change in either group (Figure 2).

We have determined selected noninvasive parameters of non-alcoholic steatohepatits and liver fibrosis - APRI Score, FIB-4 Score and NAFLD Fibrosis Score. There was no significant difference in any of these parameters among at baseline and end of the study.

MRI determination of liver and abdominal fat showed no differences

^{1}H MRS data were obtained for 58 patients, two patients were unable to undergo the examination because of claustrophobia. Percentage of fat determined by magnetic resonance imaging showed no significant differences between placebo and n-3 PUFA group at the beginning of the study (Table 3). After n-3 PUFA supplementation there was only reduction of liver fat detected by ^{1}H MRS, but below the level of significance.

Detailed lipidomic analysis using UHPLC-HRMS/MS

Detailed lipidomic analysis using UHPLC-HRMS/MS revealed higher intensity of n-3 PUFA in TAG and PC in PUFA treated group. Supervised analysis showed 87 significant features – 75 significant features for samples after n-3 PUFA treatment and 12 significant features for 0. and placebo samples (Fig. 4). PCA analysis showed significant shift of samples with higher intensity of features containing n-3 PUFA (Fig. 5 and Fig. 6).

Discussion

Of the 60 patients enrolled in the study, there was no significant difference in any of monitored parameters among the groups (n-3 PUFA versus placebo) at the beginning of the study. No side/adverse effects were recorded during the study. After one-year follow-up, no changes in anthropometric data (weight, waist circumference, or BMI) were observed in the patients enrolled in the study. These results are in accordance with the objective/meaning of our study, where the goal was not to intervene any habits of patients, but only add n-3 PUFA supplementation. Most of the published papers concerned to simple n-3 PUFA supplementation without scheduled physical activity have comparable results [9]. On the other hand, there was a significant decrease in GGT activity in the n-3 PUFA group without any change in the placebo group. Elevated activity of GGT is typical feature of NAFLD patients and has positive correlation with mortality in type 2 diabetes [14]. It is also

associated with subclinical myocardial injury [15], [16] and predict all-cause cardiovascular and liver mortality [17], [14]. The reduction of GGT activity may represent important prognostic factor for patients with NAFLD. Other observed biochemical parameters remained unchanged in both groups. During the follow-up liver elastography did not change in either group. These results are in accordance with other published studies with n-3 PUFA [9] and we can assume that one-year follow-up is a too short time period for the development or regression of liver fibrosis (and only small portion of patients had significant liver fibrosis).

^{1}H MRS as a non-invasive gold-standard technique for assessing liver fat content [18], [19], [12] was performed at the start and end of follow-up. After n-3 PUFA administration the percentage of fat in hepatic tissue measured by ^{1}H MRS in both groups stayed unchanged (respectively was lower in n-3 PUFA group without significance). Possible explanations of this result is recent knowledge, that liver fat restriction during n-3 PUFA administration was shown especially when combined with dietary restrictions [20], [21]. Also a higher percentage of DHA in relation to EPA seems to be favourable [22], [23] and evaluation of patient´s compliance is needed especially in those subjects with metabolic syndrome.

Despite these facts, we conclude that n-3 PUFA could represent a potential agent in preventing the development of NAFLD in patients with metabolic syndrome and may play a causal role in the pathophysiology of NAFLD/NASH. We observed significant decrease in GGT serum activity after 12 months of n-3 PUFA administration and they seem to be effective especially when combined with dietary restriction and proper dose and treatment duration.

Acknowledgment

This work was supported by grants: AZV 15-28745A a SVV 260370/2018.

Tables and Figures

Variables	placebo	n-3 PUFA	*P* value
Age [yrs]	51.8 ± 12.02	52.07 ± 12.37)	N.S.
Sex, M/F	21/9	24/6	
Weight [kg]	97.7 ± 13.82	96.16 ± 16.74	N.S.
BMI [kg/m2]	32.36 ± 4.58	30.09 ± 3.90	N.S.
Waist circumference [cm]	109.17 ± 9.93	107.5 ± 10.31	N.S.
ALT [µkat/L]	0.99 ± 0.40	0.98 ± 0.51	N.S.
AST [µkat/L]	0.70 ± 0.24	0.69 ± 0.25	N.S.
GGT [µkat/L]	2.11 ± 3.13	2.27 ± 2.53	N.S.
Diabetes [%]	12 / 40%	14 / 47%	N.S.
HbA1c [mmol/L]	37.67 ± 8.72	41.81 ± 9.08	N.S.
Plasma TAG [mmol/L]	1.90 ± 0.88	2.08 ± 1.59	N.S.
Plasma cholesterol [mmol/L]	4.86 ± 0.96	5.06 ± 1.17	N.S.
LDL-cholesterol [mmol/L]	2.62 ± 0.73	2.91 ± 1.14	N.S.
HDL-cholesterol [mmol/L]	1.35 ± 0.34	1.32 ± 0.34	N.S.
MRS liver fat [%]	13.24 ± 0.27	13.02. ± 0.38	N.S.
Volume internal abdomen fat [mL]	14951.7 ± 2931	14712.0 ± 2528	N.S.
APRI Score	0.509 ± 0.25	0.469 ± 0.23	N.S:
FIB-4 Score	1.567 ± 0.91	1.360 ± 0.66	N.S.
NAFLD Fibrosis Score	-1.192 ± 1.68	-1.628 ± 1.22	N.S.

Table 1. Randomization of placebo and n-3 PUFA group – the baseline values

Baseline measurements showed no significant difference in key parameters. Variables are expresses as mean (SD).

Abbreviations: M, male; F, female; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase, Hb1Ac, glycosylated haemoglobin; TAG, triacylglycerol; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

	placebo		n-3 PUFA	
	start	**end**	**start**	**end**
Weight [kg]	97.7 ± 13.8	99.2 ± 15.3	96.5 ± 16.3	98.5 ± 25.1
BMI [kg/m2]	32.4 ± 4.6		30.0 ± 3.9	
Waist circumference [cm]	109.2 ± 9.9	109.7 ± 10.1	106.6 ± 8.8	105.3 ± 9.0
ALT [µkat/L]	0.99 ± 0.40	0.94 ± 0.47	0.98 ± 0.51	0.94 ± 0.42
AST [µkat/L]	0.70 ± 0.24	0.66 ± 0.21	0.69 ± 0.25	0.66 ± 0.21
GGT [µkat/L]	2.11 ± 3.13	2.03 ± 2.80	**2.27 ± 2.53**	**1.43 ± 1.55 ***
Diabetes [%]	12 / 40%		14 / 47%	
HbA1c [mmol/L]	37.67 ± 8.72	41.07 ± 10.75	41.81 ± 9.08	45.79 ± 13.33
Plasma TAG [mmol/L]	1.90 ± 0.88	1.86 ± 0.77	2.08 ± 1.59	2.03 ± 1.49
Plasma cholesterol [mmol/L]	4.86 ± 0.96	4.78 ± 0.83	5.06 ± 1.17	5.21 ± 1.04
LDL-cholesterol [mmol/L]	2.62 ± 0.73	2.58 ± 0.83	2.91 ± 1.14	3.14 ± 1.13
HDL-cholesterol [mmol/L]	1.35 ± 0.34	1.36 ± 0 37	1.32 ± 0.34	1.21 ± 0.33
APRI Score	0.509 ± 0.25	0.455 ±0.22	0.469 ± 0.23	0.465 ±0.23
FIB-4 Score	1.567 ± 0.91	1.409 ±0.76	1.360 ± 0.66	1.365 ±0.65
NAFLD Fibrosis Score	-1.192 ± 1.68	-1.384 ± 1.58	-1.628 ± 1.22	-1.612 ±1.10

Table 2. Biochemistry results after 12 months of n-3 PUFA administration.

After one-year follow-up, anthropometric and biochemical data were collected and analysed. There was no significant difference in any of anthropometric parameters among the monitored groups (n-3 PUFA versus placebo) after n-3 PUFA administration.

** P<0.05; Variables are expressed as mean (SD).*

Abbreviations: M, male; F, female; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase, Hb1Ac, glycosylated haemoglobin; TAG, triacylglycerol; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

	placebo		n-3 PUFA	
	start	**end**	**start**	**end**
Liver fat MRI [%]	9.14 ± 0.66	9.29 ± 0.5	9.14 ± 0.58	8.74 ± 0.57
Liver fat MRS [%]	13.24 ± 0.27	13.40 ± 0.23	13.02. ± 0.38	12.32 ± 0.25
Volume external [mL]	21238.9 ± 3578	23007.4 ±4301	22701.8 ± 4265	21342.8 ± 3023
Volume internal abdomen [mL]	14951.7 ± 2931	14883.1 ± 3149	14712.0 ± 2528	14884.1 ± 2559
Volume external fat [mL]	7750.1 ± 3028.1	8124.3 ± 3322	6526.9 ± 2742	6458.8 ± 1941
Volume abdominal (resp. subfascial) fat [mL]	10128.5 ± 3092	10627.2 ± 2879	9598.6 ± 2278	10386.3 ± 2197
omega FA abdominal [%]	2.92 ± 0.99	4.25 ± 5.6	3.55 ± 1.14	4.03 ±1.5

Table 3. ***^{1}H MRI data after 12 months of n-3 PUFA administration.***

The ^{1}H MRS on a 3T MR scanner for visceral and liver fat determination was performed at the baseline and at the end of study. After 12 months of n-3 PUFA administration no significant changes were observed, the reduction of liver fat content after n-3 PUFA supplementation was below the level of significance.

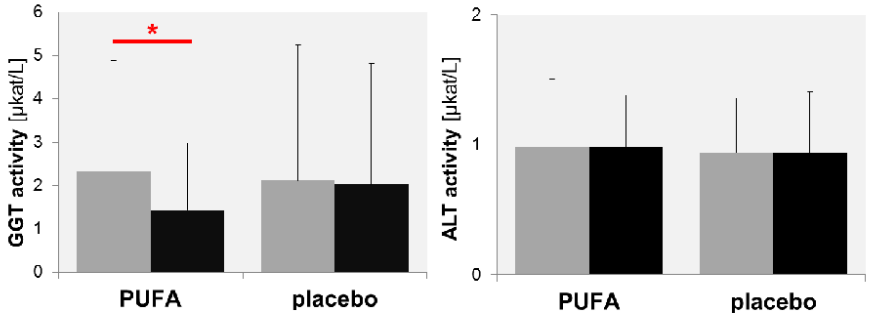

Figure 1. The comparison of GGT and ALT activity at baseline and end of the study.

Biochemical analyses were performed using standard laboratory assays. Activity of GGT significantly decreased after one year of n-3 PUFA administration, without any change in the placebo group. Other biochemical parameters stayed unchanged.

** P<0.05*

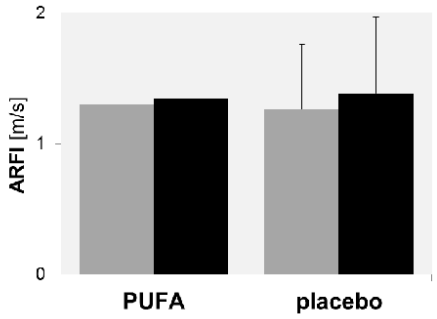

Figure 2. Liver stiffness measurement at baseline and end of the study.

Ultrasonography with liver stiffness measurement using ARFI® was performed at the baseline, at month 6 and at the end of study. No changes were observed in both groups when compared at baseline and end of the study.

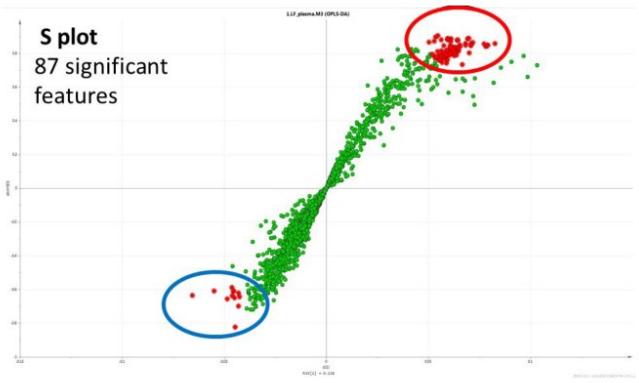

Figure 4. Supervised analysis of UHPLC-HRMS/MS data

UHPLC-HRMS/MS analysis revealed 87 significant features (lipids) – 75 significant features (red circle) for samples after n-3 PUFA treatment and 12 significant features for time 0 and placebo samples (blue circle).

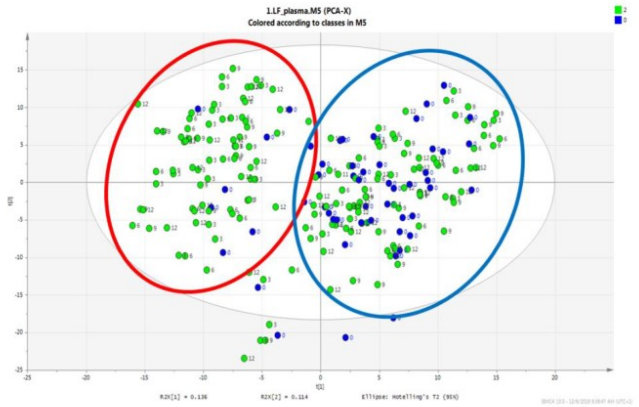

Figure 5. Detailed lipidomic analysis using UHPLC-HRMS/MS divided n-3 PUFA treated and placebo samples (based on intensity of n-3 PUFA in TAG)

Using UHPLC-HRMS/MS method and following PCA analysis samples were divided into two groups: right (blue) cluster represents most of time 0 and placebo samples; left (red) cluster samples after n-3 PUFA treatment (based on intensity of features (lipids) containing n-3 PUFA).

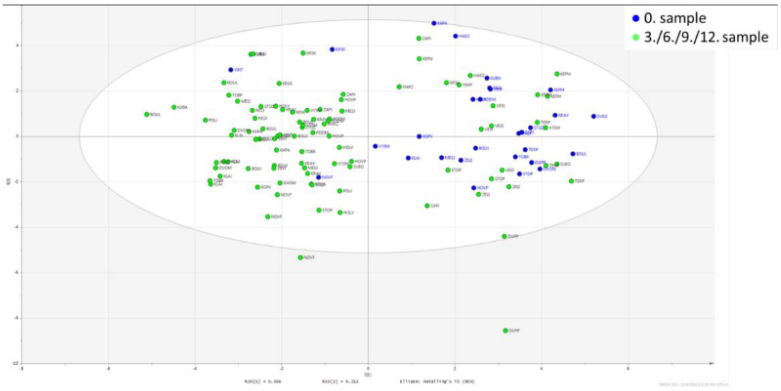

Figure 6. PCA analysis of 87 significant features in n-3 PUFA treated patients

Treatment with n-3 PUFA resulted into a shift from right cluster (blue dost = time 0) to left cluster – cluster with higher intensity of features (lipids) containing n-3 PUFA.

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4. DISCUSSION

4.1. Gangliosides and cholestatic liver disease

In the presented studies, we have demonstrated the importance of lipids in the pathogenesis of selected liver diseases, particularly cholestasis and NAFLD.

At first, in the paper entitled "***Histochemical detection of GM1 ganglioside using cholera toxin-B subunit. Evaluation of critical factors optimal for in situ detection with special emphasis to acetone pre-extraction***" we focused on the use of proper fixation technique of tissue sections, a crucial factor for immunohistological detection of gangliosides – one of major classes of complex lipids (Schwarz and Futerman, 1997). Detection of GSL is important to obtain information on their (sub)cellular localization and tissue distribution. Due to strong affinity with cholera toxin B-subunit, GM1 ganglioside is widely used for detection of gangliosides as well as so-called rafts or DRM (Chen *et al.*, 2009). The optimal conditions for detection of GM1 ganglioside using cholera toxin B-subunit by comparing various types of pre-extraction and fixation techniques (formaldehyde fixation, acetone pre-extraction, or a combination of both) have never been defined before. Second crucial factor in GSL detection is polar lipid content of the tissue. There are signs that acetone fixation compared to formaldehyde provides better accessibility of bacterial ligands or antibodies to GSL.

Acetone extraction is recommended for better *in situ* detection by many authors (Schwarz and Futerman, 1996), (Schwarz and Futerman, 2000) partly also due to improvement of permeabilization by cholesterol removal and so is widely used by many authors (Kotani *et al.*, 1993), (Kotani *et al.*, 1994), (Chark *et al.*, 2004). There are two important issues which most of the published papers did not mention: i) if the water was excluded from the acetone during fixation; ii) the extent of water content in the studied sample, which might influence the extraction of polar lipids. We used liver and brain as representative tissues with different content of polar lipids The first report about the effect of

water in acetone on extraction of polar lipids was published by Elleder and Lojda in 1971 (Elleder and Lojda, 1971). In our study, ganglioside extractability with acetone was tested by comparing anhydrous acetone with acetone containing admixture of water using TLC and photometric analysis. We have confirmed that acetone pre-treatment must be carried out with anhydrous acetone. Our results also bring for the first-time the evidence that both pre-treatments (acetone and formaldehyde) give good and comparable information on GM1 localization at the light microscopy level in the liver tissue.

In the brain sections, acetone pre-treatment seems to be better for detection of GM1 ganglioside, but optimal conditions may vary based on a lipid composition of the tissue. Very important factor seems to be also thawing and subsequent drying of the sections. In conclusion, *in situ* preservation of gangliosides (in tissue sections) has two key conditions: anhydrous acetone and dry cryostat sections.

Acquired knowledge about methodological aspects of GSL fixation was used in our further research focused on the role of gangliosides in the pathogenesis of cholestatic liver disease. Almost 40 years ago, a decreased fluidity of the liver plasma membrane was observed during cholestasis (Balistreri *et al.*, 1981), (Smith and Gordon, 1988), when importance of gangliosides in membrane stabilization during oxidative stress of hepatocytes was proven repeatedly (Sergent *et al.*, 2005), (Nourissat *et al.*, 2008). Interestingly, few recent papers support the hypothesis that gangliosides may play and important role in pathogenesis of estrogen-induced cholestasis and protect hepatocytes against detergent effect of bile acids accumulated during this pathological condition (Jirkovska *et al.*, 2007), (Majer *et al.*, 2007), (Guyot and Stieger, 2011). In plasma membranes, gangliosides form DRM (or so-called rafts or caveolae) and participate in cell‑cell recognition, adhesion, proliferation, differentiation and signal transduction (Hakomori, 2003), (Pike, 2006), (Lingwood and Simons, 2010).

Moreover, gangliosides have been found to modulate ROS formation in human leukocytes (Gavella *et al.*, 2010) and neuronal cells (Avrova *et al.*, 1998), and also inhibit hydroxyl radical formation *in vitro* (Gavella *et al.*, 2007). There is a convincing evidence that caveolin-1, an important component of caveolae, interacts with HMOX1, highly induced protein in diseases arising as a result of inflammatory and pro-oxidative insults (Kim *et al.*, 2004). Gangliosides modulate its activity and can act as a natural competitive inhibitor of HMOX1 with heme (Taira *et al.*, 2011). Despite the close topographic relationship of gangliosides and HMOX1 in DRM (Patel and Insel, 2009), there are missing data discussing this topic.

In our next study, we used estrogen-induced model of cholestatic liver injury to answer the question whether modulation of HMOX1 pathway could influence ganglioside metabolism within hepatocytes. BA accumulated during cholestasis are responsible for increased oxidative stress which might lead to a hepatocyte injury and liver damage (Perez and Briz, 2009). On the other hand, activation of HMOX1 by its substrate, heme, is associated with strong antioxidant and cytoprotective actions via formation of its bioactive products and leads to a significant increase in the serum antioxidant capacity (Reyes and Simon, 1993) (Muchova *et al.*, 2011), (Zelenka *et al.*, 2012). Well known role of HMOX in defending against oxidative injury, regulation of inflammation (Vitek *et al.*, 2002), and its close topological relationship to gangliosides in rafts/DRM (Kim *et al.*, 2004), (Patel and Insel, 2009) provide a theoretical basis for our hypothesis that there might exist a close relationship between HMOX and GSL metabolism. The results are recorded in the paper entitled "***The Effect of Heme Oxygenase on Ganglioside Redistribution within Hepatocytes in Experimental Estrogen-Induced Cholestasis***" *(Petr et al., 2014)*. In accordance to our previous results on the protective action of gangliosides in cholestatic liver injury (Jirkovska *et al.*, 2007), (Majer *et al.*, 2007), we found an induction of ganglioside biosynthesis, as demonstrated by observed increase in total sialic acid content, and the activation of *b-branch*

of ganglioside biosynthetic pathway in cholestatic livers. These changes might be a logical response to stressful conditions during cholestasis.

HMOX1 activation counteracted the pro-oxidative action of BA and affected ganglioside metabolism. Induction of HMOX1 activity by heme significantly increased the peroxyl radical scavenging capacity of sera of cholestatic animals. HMOX1 activation also prevented an increase of total ganglioside content, and an activation of *b-branch* of ganglioside synthesis in cholestatic liver. Moreover, while cholestasis led to a significant shift of GM1 ganglioside from cytoplasm to the sinusoidal membrane, these changes were completely reversed following HMOX1 activation.

While changes in ganglioside content and distribution in cholestatic livers were described previously, we have shown that oxidative stress is a crucial factor triggering this adaptive response. These results are in accordance with recent data supporting the hypothesis that cholestatic liver injury may not occur through direct BA-induced apoptosis, but may mainly involve inflammatory cell-mediated liver cell necrosis (Woolbright and Jaeschke, 2012). Despite the fact that BA act as an inflammation agent and directly activate the signalling pathways regulating production of proinflammatory mediators (Allen *et al.*, 2011), an enhancement of the antioxidative defence mechanisms by HMOX1 induction might be a principal feature regulating ganglioside biosynthesis and membrane stabilization during cholestasis and represents a supportive therapeutic measure.

To investigate whether these changes are specific for estrogen-induced cholestasis or whether they represent more general mechanism, we aimed to assess the changes in liver ganglioside metabolism (synthesis and redistribution) during obstructive cholestasis and to describe the effects of HMOX1 activity modulation (activation and inhibition) on both cholestatic liver injury and ganglioside metabolism. The results of this study are reported in

the paper "***Changes in Liver Ganglioside Metabolism in Obstructive Cholestasis - the Role of Oxidative Stress***" (Smid *et al.*, 2016).

Modulation of HMOX1 activity was achieved using HMOX1 activator heme (Ndisang *et al.*, 2010), (Zhong *et al.*, 2010), (Muchova *et al.*, 2015), or inhibitor tin mesoporphyrin. (Porteri *et al.*, 2009), (George *et al.*, 2013). Extrahepatic cholestasis was induced by microsurgical bile duct ligation (BDL) (Aller *et al.*, 2004), which led to severe cholestasis with typical biochemical pattern and morphological features of obstructive cholestasis in the liver tissue (ductular proliferation, bile infarcts).

In BDL animals, the activation of HMOX1 resulted in a significant decrease in ductular proliferation, the typical process following cholangiopathies and one of major pathological hallmarks of obstructive cholestasis. The neoformation of ductules is a logical response of the liver to BA accumulation following BDL, which increases the hepatic bile clearance capacity. However, in the case of obstructive cholestasis, these changes are ineffective because the location of biliary obstruction lies in the common bile duct. The proliferation of ductules has a destructive effect on the liver parenchyma by replacing the functional hepatocytes by ineffective tissue and can lead to the development of biliary cirrhosis (Munshi *et al.*, 2011). Activation of HMOX1 leading to oxidative stress reduction and subsequent decrease in ductular proliferation has a hepatoprotective effect. Ductular proliferation of cholangiocytes is regulated through two main signalling pathways: the inositol 1,4,5-trisphosphate/Ca^{2+} signalling pathway and cAMP (Alpini *et al.*, 1998), (Glaser *et al.*, 2000), (Munshi *et al.*, 2011). Interestingly, both pathways were found to be regulated by gangliosides in different cell culture models (Ravichandra and Joshi, 1999), (Kanda *et al.*, 2001). Contrary to it, HMOX1 inhibition and associated pro-oxidative state resulted in significant increase of amount of bile infarcts (typical biliary hepatocyte necrosis), and non-significant increase of ductular proliferation.

Changes in the synthesis and distribution of liver gangliosides following BDL were determined. Compared to controls, BDL resulted in a significant induction of ganglioside biosynthesis as demonstrated by observed increase in content of gangliosides (increase of GM1, GD1a, GD1b, GT1b), synthesis (*ST3GalV*) and redistribution of gangliosides. A similar effect compared to these results was seen in EE- induced cholestasis (Jirkovska *et al.*, 2007), (Majer *et al.*, 2007), (Petr *et al.*, 2014). After HMOX1 activation, a significant decrease in complex gangliosides was detected. While monosialyl gangliosides have an anti-proliferative effect, complex gangliosides enhance proliferation and cell growth (Furukawa *et al.*, 2012), suggesting that gangliosides might possess an important regulatory role in ductular proliferation in cholestatic liver. In the liver of BDL animals with HMOX1 inhibition, a significant increase in content and synthesis of complex gangliosides was observed (compared to control animals). We have also shown that HMOX1 inhibition significantly pronounced the shift of GM1 ganglioside from the intracellular compartment to the sinusoidal membrane of the hepatocyte in cholestatic animals. Contrary to it, HMOX1 activation prevented GM1 trafficking to membranes. An analogous effect of HMOX1 induction on ganglioside redistribution was observed by our previous study (Petr *et al.*, 2014) in the model of EE-induced cholestasis related to reduction of oxidative stress. The results of mRNA expression of the key enzymes of the ganglioside biosynthesis pathway supported above described changes in ganglioside metabolism induced by BDL and HMOX1 modulation.

To conclude, we have described changes in the ganglioside pattern during obstructive cholestasis, particularly in synthesis, distribution and localization of gangliosides protecting hepatocytes against deleterious effect of accumulated BA. We have observed the shift in liver ganglioside synthesis towards more complex gangliosides in various types of cholestatic liver injury. Loss of HMOX1 activity and subsequent pro-oxidative state of the liver tissue potentiate these changes. Contrary to it, HMOX1 activation has an opposite effect: decreases

ductular proliferation (reduction of biliary epithelia proliferation) and lowers BDL-induced ganglioside synthesis and redistribution. Thus, these changes appear to be a general mechanism independent on aetiology of a cholestatic liver disease and our findings support the concept of a general hepatoprotective mechanism of gangliosides against the harmful action of BA-induced oxidative stress.

The apparent relationship between HMOX1 and GSL metabolism prompted us to analyse GSL pattern in *Hmox1* knockout animals and identify possible underlying mechanisms. Results are presented in manuscript entitled **"*Heme oxygenase-1 may affect cell signalling via modulation of ganglioside composition*"** (Smid *et al.*, 2018).

We have measured changes in ganglioside composition in *Hmox1* knockout mice liver as well as brain, the tissue with the highest glycolipid content *in vivo*. *Hmox1* knockout led to a marked increase in concentrations of individual gangliosides in both the liver and brain, together with significant changes in the expression of mRNA of sialyltransferases. In accordance to our previous observations in rats with modulated HMOX1 activity (Smid *et al.*, 2016), *Hmox1* knockout led to a marked shift of GM1 ganglioside to the hepatocyte membrane. These findings support the importance of ganglioside metabolism in oxidative stress regulation.

To find out the underlying mechanism, we investigated regulation of ganglioside biosynthesis using hepatoblastoma HepG2 and neuroblastoma SH-SY5Y cells rich in gangliosides. Inducer of ROS, chenodeoxycholic acid (CDCA) (Fuentes-Broto *et al.*, 2009) accumulated during cholestasis, as well as bilirubin, a potent antioxidant and product of HMOX pathway (Vitek and Ostrow, 2009), (Zelenka *et al.*, 2012), were used for *in vitro* experiments. Administration of CDCA resulted in a significant increase in major gangliosides of these cell lines, while addition of a potent antioxidant – bilirubin resulted in normalization

of ganglioside pattern. These observations correspond with data published by Muchova *et al.* (Muchova *et al.*, 2011) that bilirubin may counteract pro-oxidative effect of BA on hepatocytes in the model of obstructive cholestasis in rats. The effect of oxidative stress on changes in ganglioside metabolism was further supported by determination of mRNA expressions of key glycosyltransferases. In particular, we focused on regulation of the main step in ganglioside biosynthetic pathway – GM3 synthase (*ST3GalV*). There is a convincing evidence that ganglioside metabolism is in tight connection to protein kinase C (PKC) activity (Kreutter *et al.*, 1987), (Xia *et al.*, 1989), (Aguilera *et al.*, 1993) including also the action of glycosyltransferases (Yu and Bieberich, 2001). PKC appeared to be a logical candidate regulating the expression of GM3 synthase and our *in vitro* data strongly support this hypothesis. Induction of oxidative stress and PKC activators induced the mRNA expression of *ST3GalV*, antioxidants and PKC inhibitors completely abolished this effect.

Based on our results, we conclude that oxidative stress is an important factor modulating synthesis and distribution of gangliosides *in vivo* and *in vitro*. Lack of a key antioxidant enzyme HMOX1 and subsequent oxidative stress resulted in tissue-specific changes in ganglioside metabolism as demonstrated in *Hmox1* knockout mice model. We have also demonstrated that these processes might be, at least partly, mediated through modulation of PKC activity.

4.2. Lipid dysregulation and NAFLD development – the role of n-3 PUFA

Second part of this thesis is focused on the pathogenesis of NAFLD which represents the most common chronic liver disease in western countries (Marchesini *et al.*, 2003) and it´s development is tightly connected to dysregulation of lipid metabolism. NAFLD is considered as the hepatic manifestation of a metabolic syndrome and hepatic steatosis is a major feature associated with NAFLD with prevalence of 25 – 75% in patients with obesity and type 2 diabetes mellitus in Czech population (Dvorak *et al.*, 2015). Whereas mere hepatic steatosis is regarded as a benign condition, NASH can lead to fibrosis and ultimately to liver cirrhosis with a substantial risk of development of portal hypertension and/or hepatocellular carcinoma (Chalasani *et al.*, 2018). For a long time, the liver biopsy has been the only approach for diagnosis of NASH and for the staging of liver fibrosis. This invasive procedure has many limitations – sampling heterogeneity, variability during analysis (intra-/inter-observer variability) (Ratziu *et al.*, 2005) (Merriman *et al.*, 2006) and possible complications. Although liver biopsy is generally safe method, there is some unavoidable risk of major complication (1 – 3% of patients) or even fatal ones (0.01% of patients) (Bravo *et al.*, 2001). The use of liver biopsy for repeated evaluation of treatment efficacy is also inconceivable (Sumida *et al.*, 2014). Many non-invasive scoring systems have been developed to predict NASH, or to quantify liver fibrosis, but validation on large number of patients is lacking. In our prospective study we have compared the relevance of serum hyaluronic acid levels and other non-invasive scoring systems in the distinguishing of NASH, as well as the staging of liver fibrosis in 112 patients with NAFLD. The results are reported in paper ***"Use of Non-Invasive Parameters of Non-Alcoholic Steatohepatitis and Liver Fibrosis in Daily Practice - An Exploratory Case-Control Study" (Dvorak et al., 2014).***

We have used two groups of tests: 1) first based on routinely available biochemical markers: AST/ALT ratio (Shimada *et al.*, 2002); BARD score (BMI, AST to ALT Ratio,

Diabetes mellitus) (Harrison *et al.*, 2008); NAFLD Fibrosis Score (Angulo *et al.*, 2007); APRI score (AST to Platelet Ratio Index); and FIB-4 score (based on age, AST/ALT activities and platelet count) (Vallet-Pichard *et al.*, 2007), and 2) scoring systems requiring special laboratory analyses such as the determination of serum hyaluronic acid (HA) (Suzuki *et al.*, 2005), aminoterminal peptide of pro-collagen III (PIIINP), and the tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) levels required for both the OELF (Original European Liver Fibrosis) (Rosenberg *et al.*, 2004) and ELF (Enhanced Liver Fibrosis) panels (Guha *et al.*, 2008). These tests were applied to the group of 56 patients with a liver biopsy in order to evaluate their sensitivity and specificity for distinguishing NASH and advanced fibrosis, and to the group of 56 patients without a clinically clear indication to have a liver biopsy in order to evaluate the incidence of NASH and advanced fibrosis.

The trend of using non-invasive parameters in the diagnosis of NASH/fibrosis instead of invasive liver biopsy has many reasons as mentioned above. The most promising non-invasive parameter seems to be the cytokeratin-18, the marker of hepatocyte necrosis (fragment M65) and apoptosis (fragment M30) (Yilmaz *et al.*, 2007), (Wieckowska *et al.*, 2006) (Feldstein *et al.*, 2009) as demonstrated by meta-analysis (800 patients, 10 studies) (Chen *et al.*, 2014). In our study, the relevance of cytokeratin-18 fragments was even higher. Also, the specificity and sensitivity in the discrimination between simple steatosis and NASH was higher. Based on our results, the assessment of M65, with a cut-off value 750 U/l, is the best simple non-invasive method with which to diagnose NASH. The differences between patients with NASH and controls were statistically significant using M30/M65 and we suggest that using of M30/M65 in NASH screening seems to be subsustained.

Other non-invasive parameters pose an accurate diagnostic tool in patients with clearly defined NAFLD. For example, simple biochemical parameter HA was able to distinguish

between NAFLD patients with or without significant liver fibrosis. OELF and ELF scores provide comparable results regarding fibrosis detection.

Next noteworthy result is the finding, that a substantial portion of those NAFLD patients not indicated for liver biopsy might have undiagnosed advanced fibrosis. In the group of patients not indicated for liver biopsy, the same percentage as in the biopsy group (35%) fulfilled the criteria for significant fibrosis based on HA levels.

We conclude that our results support the effort to use non-invasive parameters in diagnosis of NASH/fibrosis in daily practice. For an individual patient, the stage of NAFLD (or the presence of NASH and/or fibrosis) is probably the most important prognostic factor. Only early recognition of patients with NASH and/or advanced fibrosis from those with simple steatosis enables the arrangement of appropriate follow-up and screening for further serious complications.

The development of NAFLD is connected to dysregulation of lipid metabolism when especially FA play a pivotal role. There is convincing evidence indicating the importance of both quantitative and qualitative (saturated *vs.* unsaturated) changes in dietary FA as crucial mechanism in NAFLD progression both *in vitro* and *in vivo* (Wang *et al.*, 2006), (Ricchi *et al.*, 2009), (Juarez-Hernandez *et al.*, 2016). In the last years growing interest has been focused on the potential role of n-3 PUFA on NAFLD development. Results of studies with n-3 PUFA supplementation in animal as well as clinical studies of NAFLD are inconclusive despite many papers had shown various positive effects of n-3 PUFA on NAFLD development (de Castro and Calder, 2018), (Scorletti and Byrne, 2018). The aim of our two studies was to investigate the impact of substituting n-3 PUFA on the development of NAFLD/NASH in patients as well as in murine model of NASH using high fat methionine- and choline-deficient (HFMCD) diet (Anstee and Goldin, 2006). The results of murine study are presented in

manuscript entitled "***The effects of n-3 polyunsaturated fatty acids in a rodent nutritional model of non-alcoholic steatohepatitis***".

Feeding with HFMCD diet resulted in histopathological changes typical for NAFLD/NASH and these changes were significantly ameliorated by simultaneous n-3 PUFA supplementation. n-3 PUFA administration significantly decreased plasma cholesterol levels and ALT as well as AST activity levels. Moreover, n-3 PUFA administration led to lower serum concentrations of saturated and monounsaturated free FA and to higher serum concentrations of polyunsaturated FA (mainly EPA and DHA).

Detailed (liver and serum) lipidomic analysis was performed by using ultra high-performance liquid chromatography coupled to high resolution mass spectrometry (UHPLC-HRMS). Total serum lipid content and intensities of TAG were significantly lower after n-3 PUFA administration. In the liver, mice fed only with HFMCD diet had the highest intensity of arachidonic acid. Contrary to it, livers of mice fed with HFMCD diet together with n-3 PUFA contained higher amount of EPA and DHA together with higher content of TAGs with longer carbon chains in comparison to HFMCD diet only. n-3 PUFA normalize the n-6/n-3 ratio and reduce the availability of FA for the synthesis of TAG in the liver. These changes lead to above-mentioned reduced plasma cholesterol and TAG levels, decreased proinflammatory status of the liver as demonstrated by real-time PCR analysis. We have observed significant decrease of mRNA expression of pro-inflammatory cytokines (IL-2, IL-6, TNF-α) in the liver after n-3 PUFA supplementation compared to HFMCD diet.

The n-6 and n-3 PUFA are functionally and metabolically distinct and are not interconvertible (Schmitz and Ecker, 2008). In the Western type of diet, the level of n-6 PUFA is higher than the n-3 PUFA, which resulted in a shift of n-6/n-3 ratio from traditional 2-4:1 to >10:1 (Kang, 2005). Hypolipidemic effects of n-3 PUFA depend on complex metabolic and gene expression changes, resulting in suppression of hepatic lipogenesis and

increased FA oxidation (Ferramosca *et al.*, 2012). It has been reported that n-3 PUFA are able to limit fat deposition in the liver (Xin *et al.*, 2008), (Di Minno *et al.*, 2012), ameliorate hepatic steatosis and insulin resistance (Sekiya *et al.*, 2003), (Kuda *et al.*, 2009), whereas a diet deficient in n-3 PUFA with a high n-6/n-3 ratio could potentiate inflammatory processes, induce fatty liver (El-Badry *et al.*, 2007) and chronic diseases including NAFLD (Simopoulos, 2008). n-3 PUFA show anti-inflammatory action in rodents (Kuda *et al.*, 2009), (Rossmeisl *et al.*, 2012) and in humans (Roche and Gibney, 2000), (Itariu *et al.*, 2012) and improve dyslipidaemia (Nettleton and Katz, 2005). Our results support these hypotheses. Metabolic actions of n-3 PUFA are complex and are determined by tissue production of eicosanoids and other lipid mediators (Flachs *et al.*, 2009), induction of adiponectin, and modulation of expression by master transcriptional regulators (Sanderson *et al.*, 2008). Compared to n-6 and n-9 PUFA, eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) are most potent inhibitors of Toll-like receptor activation. There is no doubt that disbalance in dietary consumption of n-6/n-3 PUFA is detrimental to human health and lead to immune responses towards a proinflammatory profile.

In summary, n-3 PUFA have favourable effects on histopathological changes, serum markers of liver damage, FA and TAG compound and show anti-inflammatory properties. We suppose that n-3 PUFA may represent a promising way in prevention and treatment of NAFLD, this increasingly common disorder.

Considering these findings, our next aim was to assess the effects of n-3 PUFA administration in development of NAFLD in patients during one-year follow-up. A double-blind randomised placebo-controlled study with n-3 PUFA supplementation was done and results are presented in manuscript entitled *"**Prague NAFLD Study: Nonalcoholic Fatty Liver Disease and n-3 Polyunsaturated Fatty Acids in Patients with Metabolic Syndrome**"*.

Sixty patients with metabolic syndrome and NAFLD in different stage of disease (steatosis/NASH (n=55) / liver cirrhosis (n=5)) were enrolled in the study. Patients were randomized into two groups, first used n-3 PUFA (mixture of EPA and DHA in four divided doses); second group used placebo in the same scheme. During one-year follow-up were patients periodically examined - anthropometry, biochemistry, blood count, abdominal ultrasound, liver stiffness measurement using ARFI®, and nuclear magnetic resonance spectroscopy (MRS) at the start and end of follow-up. After one-year follow-up results were evaluated and statistically processed.

Of the 60 patients enrolled in the study were 45 men and 15 women, the mean age was 51.9 ± 12.2 years, the weight was 97.1 ± 15.2 kg and the mean BMI was 31.25 ± 4.25. There was no significant difference in any of these parameters among the monitored groups (n-3 PUFA versus placebo) at the beginning of the study. Similarly, between both groups there was no significant difference in other key parameters - ALT, GGT, or the percentage of fat in the liver tissue determined by MRS. No side/adverse effects were registered during the study. After one-year follow-up, no significant changes in anthropometric data (weight, waist circumference, or BMI) were observed between groups. These results are in accordance with the objective/meaning of our study, where the goal was not to intervene any habits of patients, but only add n-3 PUFA supplementation. Most of the published papers concerned to simple n-3 PUFA supplementation without scheduled physical activity have comparable results (de Castro and Calder, 2018). On the other hand, we have observed a significant decrease in GGT activity in the n-3 PUFA group compared to the placebo group. Elevated activity of GGT is typical feature of NAFLD patients and has positive correlation with mortality in type 2 diabetes (Williams *et al.*, 2016). It is also associated with subclinical myocardial injury (Lazo *et al.*, 2015), (Ndrepepa and Kastrati, 2016) and predict all-cause cardiovascular and liver mortality (Loomba *et al.*, 2013), (Williams *et al.*, 2016). The reduction of GGT activity may

represent important prognostic factor for patients with NAFLD. Other observed biochemical parameters remained unchanged in both groups. During the follow-up liver elastography did not change in either group. These results are in accordance with other published studies with n-3 PUFA (de Castro and Calder, 2018) and we can assume that one-year follow-up is a too short time period for the development or regression of liver fibrosis (and only small portion of patients enrolled in the study had significant liver fibrosis).

MRS as a non-invasive gold-standard technique for assessing liver fat content (Szczepaniak *et al.*, 2005), (Machann *et al.*, 2006) (Hajek *et al.*, 2011) was performed at the start and end of follow-up. After n-3 PUFA administration the percentage of fat in hepatic tissue measured by MRS in both groups stayed unchanged (respectively was lower in n-3 PUFA group without significance). Possible explanations of this result is recent knowledge, that liver fat restriction during n-3 PUFA administration was shown especially when combined with dietary restrictions (Argo *et al.*, 2015), (Boyraz *et al.*, 2015). Also a higher percentage of DHA in relation to EPA seems to be favourable (Scorletti *et al.*, 2014), (Pacifico *et al.*, 2015) and evaluation of patient´s compliance is needed especially in those subjects with metabolic syndrome.

Despite these facts, we conclude that n-3 PUFA could represent a potential agent in preventing the development of NAFLD in patients with metabolic syndrome and may play a causal role in the pathophysiology of NAFLD/NASH. We observed significant decrease in GGT serum activity after 12 months of n-3 PUFA administration and they seem to be effective especially when combined with dietary restriction and proper dose and treatment duration.

5. SUMMARY

In presented thesis I have focused on the role of lipids in the pathogenesis of selected liver diseases, specifically in cholestatic liver disease and NAFLD. Many lipid molecules play a significant role in the development of liver injury and subsequent structural changes of liver parenchyma which are considered a crucial factor in liver-related mortality.

The first major aim was to clarify the changes in liver ganglioside metabolism in various types of cholestasis and to elucidate the role of heme oxygenase-1 (HMOX1) and associated oxidative stress. High concentrations of BA and other bile components affect the plasma membrane and are responsible for hepatocyte damage during cholestasis. Stability of plasma membrane is determined primarily due to lipid rafts, which consist dominantly of gangliosides representing the cornerstone in mechanical and chemical resistance of whole plasma membrane. Well known is also their function in membrane stabilization during oxidative stress. Taken together, modulation of antioxidative mechanisms seems to be a logical response to cholestatic liver injury. A potent antioxidative defence mechanism represents HMOX1 induction, which might also regulate ganglioside biosynthesis and subsequent membrane stabilization. Despite these facts, detailed analysis of ganglioside metabolism during various types of cholestasis focusing on oxidative stress-related changes was missing. Firstly, we have defined optimal conditions for fixation techniques of gangliosides at the light microscopy level. Using this knowledge, we have determined the detailed pattern of ganglioside biosynthesis and re-distribution within the liver during various types of cholestasis with HMOX modulation. Our results suggest that increased ganglioside biosynthesis and their re-distribution during EE-induced as well as obstructive type of cholestasis might protect hepatocytes against deleterious effect of accumulated BA. The loss HMOX1 activity and subsequent pro-oxidative state of the liver tissue potentiates pathological changes in liver parenchyma as well as changes in metabolism and

re-distribution of gangliosides. Contrary to it, HMOX1 activation has an opposite effect and may represent a general hepatoprotective mechanism. The apparent relationship between HMOX1 and GSL metabolism prompted us to analyse GSL pattern *in vivo* (using *Hmox1* knockout animals) and *in vitro* (using hepatoblastoma HepG2 and neuroblastoma SH-SY5Y cells) with the aim of identifying possible underlying mechanisms. Lack of HMOX1 and subsequent oxidative stress resulted in tissue specific modulation of synthesis and distribution of gangliosides in both experimental models (*in vivo* and *in vitro*). We have proven that observed changes might be, at least partly, mediated through modulation of PKC activity. Based on our results, we conclude that an enhancement of the antioxidative defence mechanisms by HMOX1 induction might be a principal feature regulating ganglioside biosynthesis and membrane stabilization during cholestasis *in vitro* and *in vivo* and might represent a supportive therapeutic measure under cholestatic conditions.

The most prevalent chronic liver disease of nowadays is NAFLD, a condition that increases risk of chronic liver disease, type 2 diabetes, and cardiovascular diseases. The basic pathophysiological mechanism common for NAFLD is the imbalance between increased energy intake and decreased energy expenditure. The hepatocellular injury is driven by an overload of primary metabolic substrates (FA, glucose and fructose) in the liver resulting in increased oxidative stress and lipid peroxidation.

Liver biopsy is considered the gold-standard diagnostic tool especially in advanced form of NAFLD – NASH. It´s use has many disadvantages – limited risk of morbidity and mortality, sampling error and cost. Due to these facts, several non-invasive scoring systems have been developed to predict NASH, or to quantify liver fibrosis, but validation on large number of patients is lacking. In our prospective study we have compared the relevance of serum hyaluronic acid levels and other non-invasive scoring systems in the distinguishing of

NASH, as well as the staging of liver fibrosis in 112 patients with NAFLD. The most promising non-invasive parameter seems to be the cytokeratin-18. Also other tests (serum concentrations of hyaluronic acid, fibrosis scoring indexes) had very good outcome. Our results support the effort to use non-invasive parameters in diagnosis of NASH/fibrosis in daily practice. The stage of NAFLD as well as the presence of fibrosis is crucial prognostic factor and the major goal in the arrangement of appropriate follow-up and screening for further serious complications.

Despite the high and still increasing prevalence of NAFLD, there is no licensed treatment. The aim of our further research was to investigate the impact of substituting n-3 PUFA on the development of NAFLD in murine model of NAFLD using HFMCD diet as well as in patients with NAFLD. Existing studies with n-3 PUFA supplementation in both animal and clinical studies are inconclusive despite many papers have shown various positive effects of n-3 PUFA on NAFLD development. Our *in vivo* experiments revealed the favourable effects of n-3 PUFA on histopathological changes, serum markers of liver damage, FA compound of plasma and liver and show anti-inflammatory properties in the liver tissue. These findings might be related to complex influence of lipid metabolism by reduction of FA availability and normalization of n-6/n-3 plasma ratio. Our next goal was to determine effects of n-3 PUFA administration in development of NAFLD in patients. A double-blind randomised placebo-controlled study with n-3 PUFA supplementation was performed. After one-year follow-up of n-3 PUFA administration, we have observed significant decrease in GGT serum activity, which represents important prognostic factor of cardiovascular- and liver-related mortality. We conclude that n-3 PUFA can modulate FA metabolism, may play a causal role in the pathophysiology of NAFLD/NASH, and might be useful as a potential agent in preventing the development of NAFLD.

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7. ABBREVIATIONS

ABCB11/Abcb11, gene for BSEP/Bsep human/rat

ABCC/Abcc 2/3/4 gene for MRP2/MRP3/MRP4 Mrp2/Mrp3/Mrp4 human/rat

aBDL, BDL with HMOX activation

aC, control with HMOX activation

Akt, protein kinase B

ALP, alkaline phosphatase

ALT, alanine aminotransferase

APRI score, AST to Platelet Ratio Index score

ASBT, apical sodium-dependent bile acid transporter

AST, aspartate aminotransferase

BA, bile acids

Bach1, BTB and CNC homology 1 or basic leucine zipper transcription

BDL, bile duct ligation

BSA, bovine serum albumin

BSEP/Bsep bile salts export pump, human/rat

cAMP, cyclic adenosine monophosphate

C, control

CO, carbon monoxide

CTB, cholera toxin B-subunit

CTB-biotin, cholera toxin B-subunit biotin labeled

CTB-Px, cholera toxin B-subunit (CTB) peroxidase conjugated

DHA, docosahexaenoic acid

DRM, detergent-resistant membranes/microdomains

EDTA, ethylenediaminetetraacetic acid

EE, 17α-ethinylestradiol

ELF, Enhanced Liver Fibrosis score

EPA, eicosapentaenoic acid

ER, endoplasmic reticulum

ERK, extracellular signal-regulated kinase

FA, fatty acid(s)

FW, fresh weight

FXR, farnesoid X receptor

GlcCer, glucosylceramide

GSL, glycosphingolipids

HA, hyaluronic acid

HFMCD, high fat methionine- and choline-deficient

HMOX/Hmox, heme oxygenase

iBDL, BDL with HMOX inhibition

iC, control with HMOX inhibition

ICP, intrahepatic cholestasis of pregnancy

iNOS, inducible nitric oxide synthase

Keap1, Kelch-like ECH associating protein 1

LacCer, lactosylceramide

MAPK, mitogen-activated protein kinase

MRP/Mrp, multidrug resistance-associated protein, human/rat

MRS, magnetic resonance spectroscopy

n-3 PUFA, n-3 polyunsaturated fatty acids

NADPH, nicotinamide adenine dinucleotide phosphate

NAFLD, non-alcoholic fatty liver disease

NAS, NASH activity score

NASH, non-alcoholic steatohepatitis

Nrf2, nuclear factor erythroid 2-related factor 2

NF-κB, nuclear factor-kappa B

NTCP/Ntcp, Na^+-taurocholate cotransporting protein, human/rat

OATP/Oatp, organic anion transporting polypeptide, human/rat

OELF, Original European Liver Fibrosis

PBS, phosphate buffer saline

PI3K, phosphatidylinositol 3-kinase

PKC, protein kinase C

PNPLA3, patatin-like phospholipase domaincontaining-3

PPAR (α, γ), peroxisome proliferator-activated receptors

ROS, reactive oxygen species

SnMP, tin-mesoporphyrin

SREBP-1c, sterol regulatory element-binding transcription factor 1

STAT, signal transducers and activators of transcription

StRE, stress-responsive element

TAG, triacylglycerols

TBA, total bile acids

TE, transient elastography

TM6SF2, Transmembrane 6 superfamily member 2

TNF, tumor necrosis factor

VLDL, very-low density lipoprotein

GSL are abbreviated according to recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (Chester, 1998).

Glycosyltransferases: *GlcT (Glc-T)*, UDP-glucose ceramide glucosyltransferase; *GalTI (Gal-T1)*, UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase; *ST3GalV (Sial-T1)*, ST3 beta-galactoside alpha-2,3-sialyltransferase 5; *ST8SiaI (Sial-T2)*, ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1; *B4GalNTI (GalNAc-T)*, beta-1,4-N-acetyl-galactosaminyl transferase 1; *B3GalTIV (Gal-T2)*, UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase; *Sial-T3,* CMP-NeuAc: GD3 sialyltransferase; *Sial-T4*, CMP-NeuAc: GM1/GD1b/GT1c sialyltransferase.

8. LIST OF PUBLICATIONS

8.1 Publications related to the theme of this thesis

Petr T, **Šmíd V**, Šmídová J, Hůlková H, Jirkovská M, Elleder M, Muchová L, Vítek L, Šmíd F. Histochemical detection of GM1 ganglioside using cholera toxin-B subunit. Evaluation of critical factors optimal for in situ detection with special emphasis to acetone pre-extraction. *European Journal of Histochemistry*. 2010; May 12;54(2):e23.

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Šmíd V, Petr T, Váňová K, Jašprová J, Šuk J, Vítek L, Šmíd F, Muchová L. Changes in Liver Ganglioside Metabolism in Obstructive Cholestasis - the Role of Oxidative Stress. *Folia Biologica (Praha)*. 2016; 62(4):148-59.

Šmíd V, Šuk J, Kachamakova-Trojanowska N, Jašprová J, Valášková P, Józkowicz A, Dulak J, Šmíd F, Vítek L, Muchová L. Heme Oxygenase-1 May Affect Cell Signalling via Modulation of Ganglioside Composition. *Oxid Med Cell Longev.* 2018; 3845027.

8.2 Publications not related to the theme of this thesis

Dvořák K, **Šmíd V**, Jakša R. Granulomatous hepatitis as a rare therapeutic complication. *Gastroent Hepatol*. 2016; 70(2): 141-144.

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