Biliary polyunsaturated fatty acids and telocytes in gallstone disease.

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Running header: PUFA and telocytes in gallstone disease
Abstract

It has been reported that intake of ω-3 polyunsaturated fatty acids (PUFA) reduces the risk of coronary heart disease. It also influences the bile composition, decreasing biliary cholesterol saturation in the bile of patients with gallstones. In addition to bile composition disturbances, gallbladder hypomotility must be co-factor in the pathogenesis of cholelithiasis as it leads to the prolonged nucleation phase. Our current knowledge about gallbladder motility has been enhanced by the study of a population of newly described interstitial (stromal) cells - telocytes (TCs). The purpose of this study was to determine whether TC loss, reported by our team recently, might be related to bile lithogenicity, expressed as cholesterol saturation index or to the difference in biliary PUFA profiles in patients who suffer from cholecystolithiasis and those not affected by this disease. We determined biliary lipid composition including the fatty acid composition of the phospholipid species in bile. Thus, we investigated whether differences in biliary fatty acid profiles (ω-3 PUFA and ω-6 PUFA) in gallbladder bile may influence its lithogenicity, and the quantity of TCs within the gallbladder wall. We conclude that the altered polyunsaturated fatty acid concentrations in the gallbladder bile, with elevation of ω-6 PUFA constitute important factors influencing TC density in the gallbladder wall, being one of the possible pathophysiological components for the gallstone disease development. This study established that altered bile composition in patients with cholelithiasis may influence TC quantity within the gallbladder muscle and we concluded that reduction in TC number may be a consequence of the supersaturated bile toxicity, while some other bile components (ω-3 PUFA, glycocholic and taurocholic acids) may exert protective effects on TC and thus possibly influence the mechanisms regulating gallbladder and extrahepatic bile duct motility. Thus ω-3 PUFA may represent a possible option to prevent cholesterol gallstones formation.
INTRODUCTION

Gallstone disease constitutes a significant health problem in developed societies, affecting 10% to 15% of the adult population\(^1\). The prevalence of cholesterol gallstones has increased in recent years, especially in the Western world\(^2\). Cholelithiasis (gallstone formation) results from a combination of several factors, including supersaturation of bile with cholesterol, accelerated nucleation of cholesterol monohydrate in bile, biliary stasis and delayed gallbladder emptying due to impaired gallbladder motility. Cholesterol supersaturation can result from an excessive concentration of cholesterol in bile, a deficiency of substances that keep cholesterol in solution (i.e., bile salts and phospholipids), or a combination of these factors\(^3\). Western diet, characterized by a high total calories content, high level of cholesterol, saturated fatty acids, refined carbohydrates, proteins, and low fiber, has been proven to be lithogenic\(^4,5\). It has been reported that intake of \(\omega-3\) polyunsaturated fatty acids (PUFA) reduces the risk of coronary heart disease and decreases biliary cholesterol saturation in the bile of gallstone patients\(^6\). \(\omega-3\) PUFA prevent gallstone formation in mice being on a lithogenic diet by increasing the levels of bile phospholipids and suppressing bile mucin formation\(^7\). Polyunsaturated fatty acids are a specific family of fatty acids. The most bioactive \(\omega-3\) PUFAs are C20:5\(\omega3\) eicosapentaenoic acid (EPA) and C22:6\(\omega3\) docosahexaenoic acid (DHA), which are found predominantly in oily fish such as salmon, mackerel, and sardines\(^8,9\). Whereas \(\omega-6\) PUFA promote the production of inflammatory cytokines which, in turn, can stimulate multiple pathways such as cell proliferation, apoptosis, and angiogenesis which favour tumour growth. The \(\omega-6\) PUFA, arachidonic acid, is found in the diet or is formed from the conversion of the \(\omega-6\) PUFA, linoleic acid. Linoleic and \(\alpha\)-linolenic acids are the fatty acids designated as ‘‘essential’’ since they are not synthesized by mammalian cells and
must be provided in the diet. Increased dietary $\omega$-6 polyunsaturated triglycerides accelerate cholesterol gallstone formation in the prairie dog\(^{10}\). In addition to bile composition disturbances, gallbladder hypomotility must be co-factor in the pathogenesis of cholelithiasis as it leads to the prolonged nucleation phase i.e. time for cholesterol microcrystals to precipitate from lithogenic bile\(^{11,12,13}\). Recently, our knowledge about gallbladder motility has been enhanced by the study of a population of newly described cells, the so-called telocytes (TCs). TCs are a novel interstitial (stromal) cell type described in many tissues and organs (www.telocytes.com). TC is characterized by a small cell body (9-15 μm) and a variable number (one to five) of extremely long and thin telopodes (Tps), with alternating regions of podomers ($\sim$80 nm) and podoms (250-300 nm)\(^{14}\). Tps are interconnected by homo- and heterocellular junctions and form three-dimensional networks. TCs are suggested to be involved in signalling processes\(^{15,16}\). TCs were already discovered in the wall of the human gallbladder\(^3,17\). Previously, we reported a significant decrease in c-Kit – positive cells density (TCs) in the gallbladder wall in patients suffering from cholelithiasis\(^{18}\). The purpose of this study was to determine whether TC loss was related to bile lithogenicity, which was expressed as a lithogenic index (cholesterol saturation index, CSI), or to the difference in biliary PUFAs profiles in patients who suffer from cholecystolithiasis and those who are not affected by this disease. We determined biliary lipid composition including the fatty acid composition of the phospholipid species in bile. Thus, we investigated whether differences in biliary fatty acid profiles ($\omega$-3 PUFA, $\omega$-6 PUFA) in gallbladder bile may influence its lithogenicity, and the quantity of TCs within the gallbladder wall.
MATERIALS AND METHODS

Subjects

Twenty-five consecutive patients with symptomatic gallstone disease were scheduled for elective surgery (laparoscopic cholecystectomy) and selected for the study group (5 males, mean age 54.2 ± 13.7 y; 20 females, mean age 55.9 ± 16.9 y). Gallstones were visualized in the gallbladder on ultrasound examination before the operation. Patients presented with mild, recurrent episodes of biliary colic. None of these patients had associated choledocholithiasis or acute cholecystitis. The control group consisted of 15 consecutive patients (8 males, mean age 62.0 ± 7.6 y; 7 females, mean age 61.0 ± 9.8 y), who were electively treated for pancreatic head tumors and had no pre- or intraoperative signs of cholelithiasis and jaundice. Pancreaticoduodenectomy was performed according to the standard Whipple procedure or the pylorus-sparing Traverso-Longmire technique in patients with resectable lesions. For patients with non-resectable lesions, bypass (gastroenterostomy) was carried out for palliative purposes. Gallbladders that were not affected by primary tumors and did not contain any concrements were removed. Serum bilirubin levels were measured preoperatively and were normal in both groups. All patients were surgically treated in the First Department of General, Oncological and Gastrointestinal Surgery at the Jagiellonian University Medical College from 2010-2011.

Ethical approval

The study was conducted in accordance with the moral, ethical, regulatory and scientific principles governing clinical research. Every patient gave informed consent to the cholecystectomy procedure and to the clinical study. All surgical samples were retrieved with the approval of the Jagiellonian University Bioethical Committee using procedures that conformed to the Declaration of Helsinki guidelines (protocol number - KBET/30/B/2010).
**Tissue processing**

Tissue samples from fresh cholecystectomy specimens were collected and rinsed thoroughly with PBS (phosphate-buffered saline, 0.01 M, pH = 7.4), fixed in 4% phosphate-buffered paraformaldehyde, routinely processed and embedded in paraffin. Serial sections were cut and mounted on poly-L-lysine-coated glass slides and stained with hematoxylin and eosin (H&E) for routine histopathology.

**Immunohistochemistry**

Indirect double immunofluorescence after heat-induced epitope retrieval was used to allow the simultaneous visualization of two antigens. A preincubation step was performed with 5% normal goat serum and 0.5% Triton X-100 for 20 min to reduce non-specific binding and to increase penetration of the antibodies. For the simultaneous visualization of two antigens, an indirect double immunofluorescence procedure was used. The sections were incubated for 17 h at room temperature in humidified chambers with a mixture of either a rabbit polyclonal anti-c-Kit antibody (anti-CD117; A4502; Dako, Glostrup, Denmark; diluted 1 : 150) and a mouse monoclonal anti-mast cell tryptase antibody (M7052; Dako; 1 : 800) or the anti-CD117 antibody and a mouse monoclonal anti-CD34 antibody (NCL-ENDO; Novocastra, Newcastle, UK; 1 : 50). The sections were rinsed in PBS and incubated for 1 h at room temperature with a mixture of a Cy3-conjugated goat anti-rabbit antibody (111-165-144; Jackson ImmunoResearch, West Grove, PA, USA; 165-144; 1 : 600) and a biotinylated goat anti-mouse antibody (115-065-146; Jackson IR; 1 : 600). The primary and secondary antibodies were diluted in the same solution used in the preincubation step. After washing in PBS, the slides were incubated with DTAF-conjugated streptavidin (016-010-084; Jackson IR; 1 : 500 in PBS) for 1 h. After a final rinse in PBS, the nuclei were counterstained with DAPI (D9542; Sigma, St. Louis, MO, USA; 1:30,000) for 30 s. The sections were mounted in Vectashield
medium (H-1000; Vector Laboratories, Burlingame, CA, USA) to minimize fluorophores photobleaching.

**Microscopic examination and quantification of TCs**

Slides were examined using an Olympus BX50 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP71 digital CCD camera. The use of mast cell tryptase staining enabled c-Kit-positive mast cells to be distinguished from TCs. The distribution of TCs in the gallbladder corpus was quantitatively assessed. TCs were considered to be c-Kit positive and tryptase negative concurrently. These cells were counted in 10 consecutive high-power fields (400×). The data are expressed as the mean number of cells per 1 field of view (FOV) of gallbladder *muscularis propria*. The thickness of the *muscularis propria* in the same region of the gallbladder was also measured using image analysis software (Multiscan v.18, Computers Scanning System II, Warsaw, Poland).

**Biliary lipid composition**

During surgery, bile samples were aspirated under sterile conditions by puncture of the gallbladder after ligation of the cystic duct. Cholesterol, phospholipid and bile acid concentrations as well as percentage of individual fatty acids (FA) of phospholipids fraction (PL) in bile samples were determined.

Bile samples were extracted on SEP-PACK-NH₂ columns (500 mg, Waters, USA). Each column was first activated using 6 ml of n-hexane; then, 0.1 ml of centrifuged bile sample was applied to the column, and the flow-through was discarded. The samples were eluted in three 1-ml volumes of a chloroform-isopropanol mixture (3:1, v/v), followed by three 1-ml volumes of methanol. The eluted fractions from each column were collected and dried at 50°C under nitrogen. Dry residues were reconstituted in 0.5 ml of isopropanol and mixed vigorously. Cholesterol (Randox Laboratories Ltd., UK) and phospholipid (Wako Chemicals, Neuss, Germany) concentrations were measured by enzymatic methods. The intra- and
interassay coefficient of variations were 3% and 4.8% for cholesterol and 5% and 6% for phospholipids, respectively. All determinations were performed with a Cobas-Bio analyzer (Roche). Individual bile acids were measured by reverse-phase high-performance liquid chromatography with an isocratic solvent system (Waters, USA). Prior to chromatographic separation, bile acids were extracted from bile samples using SEP-PACK C18 columns (Waters, USA). All columns were activated using 5 ml of methanol and 5 ml of water, after which 0.1 ml of bile mixed with phosphate buffer (0.07 mmol/l, pH 7.0) and 0.1 ml of internal standard were applied. Then, the columns were washed with 10 ml of water, 3 ml of 10% acetonitrile and another 10 ml of water. Bile acids were eluted in 3 ml of methanol. The eluates were dried at 37°C under nitrogen, and dry residues were re-dissolved in 1 ml of an acetonitrile-water mixture (1:1, v/v). Each sample was filtered using a Millex GN filter (13 mm) and separated chromatographically using an XTERRA RPC-18 column (18.5 μm × 3.9 mm × 150 mm, Waters, USA) with detection at 200 nm. The mobile phase (flow rate 2 ml/min) contained 10% acetonitrile in a mixture of methanol and 0.1 M monobasic potassium phosphate (60:40, v/v, pH 4.50). Before use, the solvent was filtered through a 0.45 µm filter (type HV, Millipore, Bedford, MA, USA). An elution profile of conjugated bile acid standards (Sigma, St. Louis, MO, USA) was obtained by injecting 20 µl of a standard bile acid mixture in methanol that contained glycocholic acid (1.640 mmol/l), taurocholic acid (1.488 mmol/l), glycochenodeoxycholic acid (1.696 mmol/l), glycodedoxycholic acid (1.696 mmol/l), taurochenodeoxycholic acid (1.532 mmol/l) and taurodeoxycholic acid (1.532 mmol/l).

Cholesterol saturation index (CSI) was calculated by dividing the cholesterol concentration by the maximum cholesterol solubility according to Carey and Small¹⁹ and corrected for the total lipid content of each individual bile²⁰. Bile samples with a CSI equal to 1 or more were considered supersaturated.
Total FA, total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), n-6 PUFA, and n-3 PUFA concentrations, as well as the ratio of n-6 PUFA to n-3 PUFA in bile, were calculated. The analytical procedure for FA of phospholipids fraction in bile was consisted of few separate steps: extraction of bile total lipids by Folch et al. method (1957); separation of lipid fraction on Sep-PakNH₂ columns; methylation and separation of the fatty acid from phospholipids fraction by gas chromatography (GC) (Agilent Technologies 6890N Network GC Systems) equipped with HP-88 capillary column (100m, 0.250mm, 0.20μm). 1,2-dipentadecanoil-sn-glicero-3-phosphocholine (Sigma-Aldrich, Germany) was used as an internal standard. Levels of n-7 (palmitoleic, C16:1), n-9 (oleic, C18:1), n-3 (alfa-linolenic, C18:3; eicosapentanoic, C20:5, docosahexaenoic, C22:6), n-6 (linoleic, C18:2; eicosadienoic C20:2; arachidonic, C20:4) and saturated (lauric, C12; myristic, C14; palmitic, C16; stearic, C18) acids of phospholipids fraction were measured. The results of individual FA were expressed as the percentage of total FA.

Data analysis and statistical evaluation

The data were expressed as the mean and standard deviation (SD). The results were analyzed using a one-way analysis of variance (ANOVA), followed by a post-hoc LSD test. Pearson’s correlation test was used to examine the relationship between continuous variables. P values less than 0.05 were considered to indicate statistical significance. All statistical analyses were performed using STATISTICA 9.0 software (StatSoft, Tulsa).

RESULTS

Histopathological findings

The histopathological evaluations showed chronic cholecystitis of varying intensity in both groups of patients. The inflammation was assessed and designated as mild, moderate or severe (Fig.1B). In the study group, severe inflammation was predominant. In contrast, only mild to
intermediate inflammation was observed in the control group (Fig.1A). In the study group the thickness of the gallbladder *muscularis propria* was significantly increased compared with the control group (4.35 ± 0.6 mm vs. 3.2 ± 0.3 mm; P < 0.01). In immunostained slides, the c-Kit-positive/mast cell tryptase-negative cells were considered to be TCs. We found them predominantly located in the corpus, but these cells were also observed in the gallbladder fundus and neck. TCs had a centrally located nucleus and were mostly fusiform in shape with small branches, that were rarely visible in some sections; however, sparse, round tryptase/c-Kit-positive cells were also present. Numerous TCs were detected, mostly in the *muscularis propria*, and some TCs were observed in the connective tissue separating the smooth muscle bundles (Fig.2A).

The number of TCs in the wall of gallbladder corpus was significantly lower in the study group than in the control group (2.77 ± 1.21 vs. 6.72 ± 1.82 cell/FOV in the *muscularis propria*; P<0.001) (Fig. 2B, Fig.4B). CD-34-positive cells were visualized in the gallbladder; however, most of them appeared to be mainly of vascular origin. However, rare CD-34-positive cells were encountered in the *muscularis propria*. Based on the CD34 and CD117 staining these sparse CD-34-positive cells were c-Kit negative concurrently and no prolongations were observed. Unfortunately, reliable quantitative analysis was not possible due to the limited number of cells.

**Bile composition analysis**

No significant difference for concentrations of cholesterol, bile salts and phospholipids was obtained between bile samples with and without cholesterol crystals. However, the CSI was significantly higher (1.45 ± 0.21 vs.0.81 ± 0.12; P<0.01) in gallbladder bile with crystals than in bile without crystals. We observed significantly lower mean concentrations of glicocholic acid (GCA) (35.66 ± 16.66 vs. 43.44 ± 32.40; P <0.02) and taurocholic acid (TCA) (9.00 ±
7.66 vs. 15.18 ± 11.09; P<0.05) in the bile from cholelithiatic gallbladder, as compared to the control group, respectively.

The study revealed a significant disturbances of the fatty acids content in phospholipid fraction of gallbladder bile. The specific fatty acids assessed in this work are listed in the Methods section. Saturated fatty acid (SFA) concentrations in the phospholipid fraction of the bile in gallstone patients were slightly decreased as compared with control (47.7% ±3.0 vs. 50.7 % ±7.5; P=0.07, respectively). The monounsaturated fatty acid (MUFA) concentrations in the phospholipid fraction of the bile in gallstone patients remained unchanged between both groups (13.7% ± 2.5 for cholelithiasis group vs. 15.2% ± 54.1 for controls; P=0.17). Surprisingly however, the polyunsaturated fatty acid concentrations in the phospholipid fraction of the bile in gallstone patients were significantly higher in patients suffering from cholelithiasis compared with the control subjects (38.6 % ± 4.7 vs. 34.0 % ± 6.8; P=0.0046) (Fig.3A and 3B). The ω-3 PUFA levels did not differ between both groups: 3.68 % ± 1.1 for gallstone patients, 3.79 % ± 1.6 for controls respectively, P=0.81, whereas the ω-6 PUFA concentrations were significantly elevated in gallstone patients (35.8 % ± 4.3 for gallstone patients, 30.2 % ± 2.6 for controls, P=0.0015) (Fig.4A). Such increase originated mainly from the significant elevation of the C18:2(n-6) (linoleic acid) serum concentrations (28.8 % ± 4.8 vs. 22.8 % ± 4.9, respectively for cholelithiasis and controls; P=0.0005). In addition, the ω-6 PUFA to ω-3 PUFA ratio was significantly higher in gallstone patients compared to controls (11.0 ± 3.1 vs. 8.41 ± 2.3; P=0.0121). The study revealed also the moderate correlation of TCs count in the gallbladder muscularis propria with the total PUFA concentrations (r= - 0.543; P<0.05), ω-6 PUFA levels (r= - 0.415; P<0.05) and ω-6 PUFA to ω-3 PUFA ratio (r= - 0.559; P<0.05). The detailed data from the fatty acid concentrations assessment in the phospholipid fraction of the bile in gallstone patients are presented in the Table 1.
DISCUSSION

In observational studies, higher intake of saturated fat or trans fatty acids was associated with an increased incidence of gallstones\textsuperscript{26,27,28}. In contrast, higher intake of polyunsaturated or monounsaturated fatty acids was associated with decreased risk\textsuperscript{29}. The apparent protective effect of polyunsaturated fatty acids is consistent with experimental observations, in which hamsters fed an essential fatty acid-deficient diet had a high incidence of cholesterol gallstones and lithogenic bile (diets low in essential fatty acids are, in general, also low in polyunsaturated fatty acids)\textsuperscript{30,31}. In addition, in patients with gallstones, supplementation with 11.3 g per day of fish oil (which is high in polyunsaturated fatty acids) decreased the cholesterol saturation of bile by 25 percent\textsuperscript{32}. While both \(\omega-3\) and \(\omega-6\) polyunsaturated fatty acids may be protective or noxious, further research is needed to determine the optimal amounts and ratios of these fatty acids. Hence, this study was undertaken to evaluate the biliary lipid composition and its relation to TC density in gallbladder wall. Our results revealed that the biliary \(\omega-6\) PUFA concentrations are strongly related to the number of TCs within human gallbladder. Significant loss of TCs in the gallbladder wall in patients with gallstones was associated with an increased CSI of bile and high biliary \(\omega-6\) PUFA concentrations. We assume that, in humans, \(\omega-6\) PUFAs could increase CSI and prolong nucleation time contributing to TC loss and gallstone formation. The recent dietary shift towards the consumption of n-6 at the expense of n-3 PUFAs is thought to be a primary cause of many diseases related to the Western diet\textsuperscript{33}. The body converts linoleic acid to arachidonic acid and derives eicosapentaenoic acid from \(\alpha\)-linolenic acid. Ideally the effects of these fatty acids and their eicosanoid derivatives are tailored to the specific biological needs of the body. The balance between \(\omega-3\) and \(\omega-6\) PUFAs is essential for metabolism and maintenance of the functions of both classes. Derangement of biliary \(\omega-6:\omega-3\) PUFA ratio may impact on the histological pattern of cholelithiatic gallbladder through modulation of the amount of biliary
lipids. Moreover, derangement of ω-6:ω-3 PUFA ratio could influence the synthesis of various eicosanoids. In the presence of large amounts of dietary ω-6 PUFAs, the eicosanoids derived from arachidonic acid are synthesized in larger quantities than those from eicosapentaenoic acid. The arachidonic acid-derived eicosanoids are biologically active even in small quantities. In high concentrations they contribute to the formation of thrombi and the development of inflammatory disorders. The influence of PUFAs and their eicosanoid products on organ microcirculation and ischemia/reperfusion injury has been demonstrated in many studies and it could be also related to gallbladder microcirculation and TC injury.

Another important mechanism underlying TC loss concerns the chronic inflammatory processes involving the gallbladder wall. Portincasa et al. (Portincasa et al. 2000) described impaired gallbladder motility caused by mild inflammation. Indeed, we observed inflammatory infiltration, predominantly localized in the lamina propria, in the gallbladders of patients with gallstones, which was associated with a significant increase in the mast cell count. However, as we reported previously, TC loss does not correlate with inflammatory grade or mast cell count.

We realize that additional stainings need to be considered in better understanding the role of TCs in the human gallbladder. For example, we observed CD34-positive cells in the gallbladder wall, including muscularis propria; however, they appeared to be predominantly of vascular origin. These CD34-positive cells were c-Kit negative, and no prolongations were observed. Unfortunately, we were unable to present reliable quantitative analysis due to the limited number of cells. Newly described markers, like CD34 and vimentin or PDGFRα (Platelet-Derived Growth Factor Receptor α) co-expression are of great interest recently. A growing number of publications on telocytes along with discovery of new markers for their identification attracted an increasing interest among researchers regarding TC function and
their potential applications in the treatment of various diseases including cholelithiasis. This knowledge will certainly help to explain the pathogenesis of this disease. ω-3 PUFAs can inhibit cholesterol nucleation and cholesterol monohydrate crystal precipitation without affecting mixed micelles; they do this by stabilizing the phospholipid vesicle, which is another solubilizer of cholesterol. ω-3 PUFAs inhibit cholesterol monohydrate crystal precipitation and gallstone formation in prairie dogs by increasing the stability of biliary phospholipid vesicles through decreasing ionized calcium and protein in bile of the gallbladder without changing either the concentration of biliary lipids or the CSI. ω-3 PUFAs might alter vesicular stability and cholesterol-carrying capacity by modifying the composition of phospholipids in bile, thereby suppressing cholesterol precipitation.

Derangement of ω-6:ω-3 PUFA ratio could negatively influence this protective effect. Our results showed that there were no significant differences between two groups of patients in the mean concentrations of total bile acids, phospholipids, or cholesterol in vesicular bile, except for the lower mean concentrations of glycocholic and taurocholic bile acids in the cholelithiatic group. The lower amounts of these compounds were associated with the increased lithogenicity index (CSI). Moreover, in patients with gallstones, a significant positive correlation between the mean number of TCs and the concentrations of glycocholic and taurocholic bile acids was found. We conclude that glycocholic and taurocholic acids are somehow protective to TCs. However, it is not clear whether this is an artefact of the statistical analysis or whether TCs are indeed preserved by these acids, and the exact mechanism of this possible protective effect should be examined further, including in an experimental model. We acknowledge that possible mechanisms underlying the destructive influence of bile on TCs remain speculative rather than empiric, as reports on the role of TCs in gallstone pathophysiology are sparse. A study by Xu and Shaffer reported that gallbladder hypomotility was impaired by the increased bile cholesterol level. A study by Lavoie et al.
proved that excess cholesterol in the smooth muscle of the gallbladder attenuates the ability of the muscle to contract as a result of changes in signal transduction and ion channel activity, decoupled membrane receptor—ligand interactions and disturbances in contractile protein activity. Moreover, in a subsequent study on guinea pigs fed a lithogenic diet, Lavoie et al.\textsuperscript{40} reported cholesterol accumulation in gallbladder smooth muscles in the plasma membrane, especially membrane caveolae, leading to a decrease in membrane fluidity and a subsequent change in rhythmic electric activity.

We conclude that the disrupted polyunsaturated fatty acid concentrations in the gallbladder bile, with elevation of $\omega$-6 PUFA constitute important factors influencing TCs density in the gallbladder wall, being one of the possible pathophysiological components for the gallstone disease development. This study established that altered bile composition in patients with cholelithiasis may influence TC quantity within the gallbladder muscle. We conclude that a reduction in TC number may be a consequence of the toxicity of the supersaturated bile, while some other bile components ($\omega$-3 PUFAs, glycocholic and taurocholic acids) may exert protective effects on TCs and thus possibly influence the mechanisms regulating gallbladder and extrahepatic bile duct motility. We are aware, that present study only indicates a potential relationship between altered bile acid composition and reduced TCs count in cholelithiasis using Pearson’s correlation analysis. The direct evidence needs to be further obtained with gain of loss-of-function assays. However, the $\omega$-3 PUFAs may represent a new option for the cholesterol gallstone disease management.
Disclosure

The authors declare no conflict of interest.

Funding:

This study was financed from the Jagiellonian University grant: K/ZDS/005450.
REFERENCES


is an early feature in the development of cholesterol gallstone disease.


FIGURE LEGENDS:

**Figure 1.** Cross section of the gallbladder wall from the control group (A) and cholelithiatic group (B) showing signs of severe chronic inflammation. Note intense infiltration with inflammatory cells and thickened muscular layer. H&E staining. l.p. - lamina propria; m.p. - muscularis propria.

**Figure 2.** Cross section of the gallbladder wall from control (A) and cholelithic group (B) presenting CD117-positive and tryptase-negative telocytes (red color, *arrows*) and mast cells (CD117-positive and tryptase-positive, orange/green, *arrowheads*). Immunofluorescence, CD-117 (Cy3, red), tryptase (FITC, green), nuclei counterstained with DAPI (blue).

**Figure 3.** Concentrations of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) in gallbladder bile from control subjects (A); n=15, and from patients with cholelithiasis (B); n=25. The results of individual fatty acids expressed as the percentage of total fatty acids. The data are expressed as the mean values ± standard deviation.

**Figure 4.** The ω-6 polyunsaturated fatty acid PUFA concentrations (4A) and the number of telocytes – TCs (4B) in control subjects (n=15) and in patients with cholelithiasis (n=25). The results of ω-6 PUFA expressed as the percentage of total fatty acids. The data are expressed as the mean values ± standard deviation.
Figure 1
Figure 2
Figure 3
Figure 4
Table 1

The polyunsaturated fatty acid, monounsaturated fatty acid and saturated fatty acid concentrations in the phospholipid fraction of the bile in cholelithiasis patients (n=25) and control subjects (n=15).

<table>
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<th>The polyunsaturated fatty acids (PUFA)</th>
<th>Group: C18:2(n-6)</th>
<th>C20+CC18:3(n-6)</th>
<th>C18:3(n-3)</th>
<th>C20:2(n-6)</th>
<th>C20:4(n-6)</th>
<th>C20:5(n-3)</th>
<th>C22:6(n-3)</th>
<th>Total PUFA</th>
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<td>Controls</td>
<td>22.88 ± 4.93</td>
<td>0.33 ± 0.20</td>
<td>0.38 ± 0.19</td>
<td>0.12 ± 0.03</td>
<td>6.92 ± 3.26</td>
<td>1.24 ± 0.95</td>
<td>2.16 ± 0.75</td>
<td>34.04 ± 6.83</td>
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<td>Cholelithiasis</td>
<td>28.85 ± 4.84</td>
<td>0.34 ± 0.21</td>
<td>0.59 ± 0.20</td>
<td>0.18 ± 0.09</td>
<td>6.46 ± 1.25</td>
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<th>C18:1</th>
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<td>Controls</td>
<td>2.36 ± 1.09</td>
<td>12.84 ± 3.17</td>
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<td>Cholelithiasis</td>
<td>2.09 ± 0.96</td>
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<tbody>
<tr>
<td>Controls</td>
<td>0.03 ± 0.02</td>
<td>0.43 ± 0.18</td>
<td>44.22 ± 6.79</td>
<td>6.04 ± 1.383</td>
<td>50.74 ± 7.46</td>
</tr>
<tr>
<td>Cholelithiasis</td>
<td>0.03 ± 0.02</td>
<td>0.50 ± 0.18</td>
<td>40.74 ± 1.46</td>
<td>6.43 ± 2.17</td>
<td>47.7 ± 3.00</td>
</tr>
</tbody>
</table>

Results of individual fatty acids are expressed as the percentage (%) of total fatty acids in the phospholipid fraction of the bile. Data expressed as the mean and standard deviation (SD).