Abstract

Aim of the study: The decrease in estrogen levels in the postmenopausal period changes the lipid profile by the expression of hepatic genes related to metabolism of cholesterol and bile acid synthesis that could be important in the pathogenesis of cholelithiasis. The aim of the study was to determine the APOB gene 7673C>T and 12669G>A polymorphisms in the pathogenesis of gallstones and analysis of the composition of gallstones in pre- and postmenopausal women.

Material and methods: The study group consisted of 94 women qualified to the laparoscopic cholecystectomy while the control group consisted of 81 women in whom gallstones and other changes in the bile ducts were excluded. Gallstones composition analysis was performed using commercially available assays. The prevalence of the APOB gene polymorphisms was determined using the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP).

Results: When assessing the composition of gallstones in pre- and postmenopausal women, we observed differences in the studied parameters. Analysis of genetic variants of APOB gene 7673C>T and 12669G>A polymorphisms showed no significant statistical differences between studied groups and controls.

Conclusions: Analysis of 7673C>T and 12669G>A polymorphisms showed no relationship between specific genetic variants and the risk of gallstones in pre- and postmenopausal women, pointing to the fact that the investigated polymorphisms are not relevant as prognostic factors in gallstone disease in the Caucasian population. Because of the possible contribution of a variety of factors in gallstones pathogenesis the studies are required to take account of additional environmental factors, what may indicate different occurrence between investigated polymorphisms, gallstone disease development and gallstones composition in Caucasians.

Key words: menopause, gallstones, apolipoprotein B, genetic polymorphism.

Introduction

Menopause is associated with significant hormonal changes in the female body. These processes may predispose to many diseases, such as osteoporosis, cardiovascular diseases, obesity and type 2 diabetes. In perimenopausal women, estrogen deficiency also leads to lipid disorders, increase in total cholesterol, triglycerides and low-density lipoprotein (LDL) cholesterol as well as decrease in the high-density lipoprotein (HDL) cholesterol level [1, 2]. The progressive decrease in estrogen level changes the lipid profile through expression of genes encoding apoproteins and may be
important in the pathogenesis of gallstones especially in the case of the formation of cholesterol stones. In addition, estrogens have the suppressant effects for bile acid secretion and influence on the composition and function of the gallbladder [3, 4].

In Poland, gallstones account for about 20% of the population and are more common in women than in men. The most important abnormality associated with lithogenesis is supersaturation of bile with cholesterol, increased formation of cholesterol deposits and impaired contractility of the gallbladder [5, 6]. It was shown that bile becomes lithogenic due to disorders of the proportions between its components, i.e. cholesterol, bile acids and phospholipids [7-9]. Disorders of cholesterol metabolism may result from the existence of different isoforms responsible for transport of lipid compounds. Apolipoprotein B plays an important role in the formation of gallstones [10] and occurs in different isoforms that control the hepatic uptake and cholesterol level [11].

The most studied genetic variant of APOB gene encoding apolipoprotein B is the XbaI polymorphism in exon 26 (conversion of cytosine to thymine at position 7673 (7673C>T, Thr2488Thr) [12]. Due to the presence of a mutant variant there is no conversion of the threonine residue at position 2488 of ApoB polypeptide chain [13]. However, the XbaI polymorphism of the APOB gene is responsible for the changes in plasma cholesterol levels [14] and probably plays a role in the pathogenesis of gallstones [11, 13]. Another variant of the APOB gene is the EcoRI polymorphism (12669G>A, Glu4154Lys), in which the presence of mutant allele is associated with the altered level of apolipoprotein B, and reduced risk of gallstone formation [13-15].

There is a growing amount of studies that the formation of gallstones may be genetically determined [16, 17]. The candidate genes include the apolipoprotein B gene involved in cholesterol transport and controlling the amount of excretion of cholesterol [18, 19].

The aim of the study was to determine the contribution of APOB gene 7673C>T and 12669G>A polymorphisms in the pathogenesis of gallstones and analysis of the composition of gallstones in pre- and postmenopausal women.

Material and methods

Patients

The study group consisted of 94 Caucasian women (35 premenopausal women, mean age: 39 ± 6.4 years and 59 postmenopausal women, mean age: 59 ± 5.5 years) qualified for laparoscopic cholecystectomy. The control group consisted of 81 women (23 premenopausal women, mean age: 37 ± 5.4 years and 58 postmenopausal women, mean age: 61 ± 5.3 years) in whom gallstones and other changes in the bile ducts were excluded. All patients were enrolled in the study in the Department of Laparoscopic Surgery at the Pomeranian Medical University in Szczecin. Blood samples were collected from all patients, while gallstones were also collected from women with cholelithiasis during laparoscopy. In the study group, the parameters of the lipid profile such as total cholesterol, HDL, LDL and triglycerides were also determined.

Analysis of the composition of gallstones

Assessment of the composition of gallstones (total cholesterol, bile acids, calcium ions and bile pigments) with a powder deposition mass was performed in accordance with the method described by Steen and Blijenberg [20]. Total cholesterol was determined by Color Test – Cholesterol Oxidase/Peroxidase (BioSystems). Determination of total bile acids was performed using the enzyme assay – Mercoktest Bile Acids (Merck). The content of bilirubin was determined by spectrophotometry using a Bilirubin Diazotized Sulfanilic-Color Kit (BioSystems). The composition of the fatty acids was evaluated by liquid chromatograph Hewlett Packard 1100 HPLC.

The concentration of cholesterol and bile acids in the tested samples was calculated in relation to the reference test in accordance with the Beer-Lambert law. The obtained cholesterol values (mmol/cm³), bile acids (µmol/cm³) and bilirubin (pmol/dm³) were converted to the amount of these compounds in the analyzed samples of gallstones (mg/100 mg of deposit). Calcium carbonate content was determined according to the method described by Scheibler. The volume of evolved CO₂ was converted to standard conditions, then the amount of calcium carbonate was expressed as mg/100 mg of deposit.

Genetic analysis

Determination of APOB gene 7673C>T and 12669G>A polymorphisms was performed using the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). For PCR the following primers were used: ApoBXbaF: 5’-GGA GAC TAT TCA GAA GCT AA-3’, ApoBXbaR: 5’-GAA GAG CCT GAA GAC TG A CT-3’, ApoBEcoRF: 5’-CTG AGA GAA GTG TCT TCA GAA GCT AA-3’, ApoBEcoRR: 5’-CTG AGA GAA GTG TCT TCA GAA GCT AA-3’. The polymorphic sites were defined using restriction enzymes for the APOB gene, such as Xbal (7673C>T, Thr2488Thr, rs693) and EcoRI (12669G>A, Glu4154Lys, rs1042031) (Table I).

Statistical analysis

Statistical analysis was carried out using the Statistica program (StatSoft). Compatibility of distribution
of genotypes with Hardy-Weinberg law, and the frequencies of genotypes and alleles between groups were analyzed using Fisher's exact test. Confidence intervals (95% CI) were determined using the Newcombe-Wilson method. In the analysis of differences of the gallstones composition between the studied genotypes Mann-Whitney U test was used. The values of \( p < 0.05 \) were considered as a statistical significant difference.

**Results**

**Analysis of lipid parameters and composition of gallstones**

In the group of pre- and postmenopausal women with cholelithiasis it was observed a slight increase in the total cholesterol (210.1 ± 34.7 mg/dl) and normal values of HDL lipid fraction (58.4 ± 15.3 mg/dl), LDL lipid fraction (121.8 ± 31.5 mg/dl) and triglycerides (134.1 ± 81.8 mg/dl). Furthermore, in the postmenopausal women analysis of the composition of the gallstones showed a statistically significant lower level of cholesterol (61.7 mg/100 mg) compared to the premenopausal women (85.7 mg/100 mg, \( p < 0.05 \)) (Table II). Additionally, comparing the contents of calcium carbonate (CaCO\(_3\)) in the gallstones significantly higher levels of carbonate (15.5 mg/100 mg) in women after menopause were noted compared to the premenopausal group (8.9 mg/100 mg, \( p < 0.05 \)). Moreover, in postmenopausal women the analysis of the total calcium content of gallstones showed its higher level (7.4 vs. 4.2, NS). Also, higher levels of bile acids were observed in postmenopausal women (2.3 vs. 1.4, NS) (Table II). The composition of all bile acids in the analyzed gallstones in women after menopause also showed higher levels of acids tested, except cholic and chenodeoxycholic acid (Table III).

**Analysis of the APOB gene 7673C>T polymorphism**

Analyzing the APOB gene 7673C>T polymorphism, frequencies of CC, TT and CT genotypes in premenopausal women were comparable to the group of healthy women and women with cholelithiasis. In both these groups, the heterozygous genotype was observed with higher frequency compared to the other genotypes (Table IV). In addition, analysis of homozygous CC and TT genotypes showed comparable frequencies of their occurrence in postmenopausal women both in healthy patients as well as with cholelithiasis. In both groups (pre- and postmenopausal women) heterozygous genotype CT was also performed with the similar frequency (Table V).

**Analysis of the APOB gene 12669G>A polymorphism**

In the premenopausal women the frequencies of GG, AA and GA genotypes of APOB gene 12669G>A polymorphism were compared to the group of healthy women and women with cholelithiasis. In both these groups, the heterozygous genotype was observed with higher frequency compared to the other genotypes (Table IV). Furthermore, in postmenopausal women the analysis of the composition of the gallstones showed a statistically significant lower level of cholesterol (61.7 mg/100 mg) compared to the premenopausal women (85.7 mg/100 mg, \( p < 0.05 \)) (Table II). Additionally, comparing the contents of calcium carbonate (CaCO\(_3\)) in the gallstones significantly higher levels of carbonate (15.5 mg/100 mg) in women after menopause were noted compared to the premenopausal group (8.9 mg/100 mg, \( p < 0.05 \)).

**Table I.** Restriction enzymes and product size after hydrolysis

| Polymorphism | Enzyme | Recognition sequence | PCR product (bp) | Product size after hydrolysis (bp) |
|--------------|--------|----------------------|------------------|----------------------------------|
| 7673C>T      | XbaI   | 5’...T↓C T A G A...3’ | 710              | CC (710) CT (710, 433, 277) TT (433, 277) |
| 12669A>G     | EcoRI  | 5’...G↓A A T T C...3’ | 480              | AA (480) AG (480, 253, 227) GG (253, 227) |

**Table II.** The composition of gallstones in pre- and postmenopausal women (mg/100 mg of deposit)

| Study group                  | Total cholesterol | CaCO\(_3\) | Total calcium | Bile acids |
|------------------------------|-------------------|------------|--------------|------------|
| Women with cholelithiasis, n = 94 |                   |            |              |            |
| Before menopause, n = 35      | 85.7 ± 5.9        | 8.9 ± 5.6  | 4.2 ± 2.6    | 1.4 ± 0.4  |
| After menopause, n = 59       | 61.7 ± 11.5*      | 15.5 ± 4.2*| 7.4 ± 2.1    | 2.3 ± 0.9  |

*\( p < 0.05 \)

**Table III.** The composition of bile acids in the pathogenesis of gallstones in pre- and postmenopausal women

| TCA | TCDCA | GCA | GCDCA | CA | CDCA | LCA |
|-----|-------|-----|-------|----|------|-----|
|     |       |     |       |    |      |     |
| Women with cholelithiasis, n = 94 | 0.10 ± 0.19 | 0.18 ± 0.27 | 0.52 ± 0.56 | 0.34 ± 0.41 | 0.12 ± 0.32 | 0.17 ± 0.31 | 2.08 ± 1.17 |
| Premenopausal women, n = 35      | 0.02 ± 0.05  | 0.03 ± 0.07 | 0.24 ± 0.17 | 0.28 ± 0.27 | 0.08 ± 0.09 | 0.21 ± 0.17 | 0.58 ± 0.33 |
| Postmenopausal women, n = 59     | 0.21 ± 0.22  | 0.19 ± 0.20 | 0.58 ± 0.33 | 0.23 ± 0.26 | 0.03 ± 0.05 | 0.02 ± 0.09 | 0.64 ± 0.52 |

*\( p < 0.05 \)

TCA – taurocholic acid, TCDCA – taurochenodeoxycholic acid, GCA – glycocholic acid, GCDCA – glycochenodeoxycholic acid, CA – cholic acid, CDCA – chenodeoxycholic acid, LCA – lithocholic acid
Tab. IV. The prevalence of genotypes and alleles of the tested polymorphism of the *APOB* gene in premenopausal women with cholelithiasis and in the control group

| Genotype | Control group (healthy women) | Study group (women with cholelithiasis) | OR     | 95% CI      | p   |
|----------|-------------------------------|------------------------------------------|--------|-------------|-----|
|          | Observed n (%)                | Expected %                               | Observed n (%) | Expected % |
| APOB 7673C>T |                                 |                                           |        |             |     |
| CC       | 5 (21.7)                      | 27.2                                     | 9 (25.7) | 31.0        | 1.24 | 0.31-5.54 | 0.49 |
| TC       | 14 (60.9)                     | 49.9                                     | 21 (60.0) | 49.4        | 0.96 | 0.28-3.20 | 0.58 |
| TT       | 4 (17.4)                      | 22.9                                     | 5 (14.3) | 19.6        | 0.79 | 0.15-4.54 | 0.51 |
| Total    | 23 (100)                      | –                                        | 35 (100) | –           |     |           |     |
| C        | 24 (52.2)                     | –                                        | 39 (55.7) | –           | 1.15 | 0.51-2.60 | 0.43 |
| T        | 22 (47.8)                     | –                                        | 31 (44.3) | –           | 0.85 | 0.38-1.96 | 0.43 |
| Total    | 46 (100)                      | –                                        | 70 (100) | –           |     |           |     |
| APOB 12669G>A |                               |                                           |        |             |     |
| GG       | 13 (56.5)                     | 54.6                                     | 20 (57.1) | 55.2        | 1.03 | 0.31-3.36 | 0.58 |
| GA       | 8 (34.8)                      | 38.6                                     | 12 (34.3) | 38.2        | 0.98 | 0.28-3.47 | 0.59 |
| AA       | 2 (8.7)                       | 6.8                                      | 3 (8.6)  | 6.6         | 0.98 | 0.10-9.72 | 0.66 |
| Total    | 23 (100)                      | –                                        | 35 (100) | –           |     |           |     |
| G        | 34 (73.9)                     | –                                        | 52 (74.3) | –           | 1.02 | 0.39-2.57 | 0.57 |
| A        | 12 (26.1)                     | –                                        | 18 (25.7) | –           | 0.98 | 0.39-2.54 | 0.57 |
| Total    | 46 (100)                      | –                                        | 70 (100) | –           |     |           |     |

*p < 0.05

Tab. V. The prevalence of genotypes and alleles of the tested polymorphism of the *APOB* gene in postmenopausal women with cholelithiasis and in the control group

| Genotype | Control group (healthy women) | Study group (women with cholelithiasis) | OR     | 95% CI      | p   |
|----------|-------------------------------|------------------------------------------|--------|-------------|-----|
|          | Observed n (%)                | Expected %                               | Observed n (%) | Expected % |
| APOB 7673C>T |                                 |                                           |        |             |     |
| CC       | 15 (25.9)                     | 27.6                                     | 17 (28.8) | 29.4        | 1.16 | 0.47-2.85 | 0.44 |
| TC       | 31 (53.4)                     | 49.9                                     | 30 (50.9) | 49.7        | 0.90 | 0.41-1.98 | 0.46 |
| TT       | 12 (20.7)                     | 22.5                                     | 12 (20.3) | 20.9        | 0.98 | 0.36-2.66 | 0.57 |
| Total    | 58 (100)                      | –                                        | 59 (100)  | –           |     |           |     |
| C        | 61 (52.6)                     | –                                        | 64 (54.2) | –           | 1.07 | 0.62-1.84 | 0.45 |
| T        | 55 (47.4)                     | –                                        | 54 (45.8) | –           | 0.93 | 0.54-1.61 | 0.45 |
| Total    | 116 (100)                     | –                                        | 118 (100) | –           |     |           |     |
| APOB 12669G>A |                               |                                           |        |             |     |
| GG       | 38 (65.5)                     | 62.9                                     | 37 (62.7) | 59.5        | 0.89 | 0.39-2.02 | 0.45 |
| GA       | 16 (27.6)                     | 32.8                                     | 17 (28.8) | 35.3        | 1.06 | 0.43-2.58 | 0.52 |
| AA       | 4 (6.9)                       | 4.3                                      | 5 (8.5)  | 5.2         | 1.25 | 0.25-6.64 | 0.51 |
| Total    | 58 (100)                      | –                                        | 59 (100)  | –           |     |           |     |
| G        | 92 (79.3)                     | –                                        | 87 (73.7) | –           | 0.84 | 0.43-1.64 | 0.35 |
| A        | 24 (20.7)                     | –                                        | 27 (26.3) | –           | 1.19 | 0.61-2.33 | 0.35 |
| Total    | 116 (100)                     | –                                        | 118 (100) | –           |     |           |     |

*p < 0.05*
Discussion

Cholelithiasis is a major problem, especially in the developing countries. Its pathogenesis is still subject of research [21, 22]. It is suggested that genetic predisposition and environmental factors are the cause of cholesterol gallstone disease [18, 23]. Currently, data are lacking for the existence of a link between the occurrence of gallstones, the chemical composition of gallstones and certain genetic factors [21, 22].

The conducted studies concern mainly the polymorphisms of genes determining the incidence of cholesterol gallstones [24]. Recent studies have found that changes within some polymorphic genes encoding proteins involved in the transport of cholesterol and bile acids can be correlated with the risk of cholelithiasis [25-27]. Also, in the Caucasian population the APOB genetic variants have been studied in aspect of linkages with the lipid profile [12, 28, 29].

In our study, we conducted an analysis of APOB gene 7673C>T and 12669G>A polymorphisms in pre- and postmenopausal women, as well as analysis of the potential relationship of these variants with the transport and metabolism of cholesterol. The results suggest a lack of correlation between analyzed APOB polymorphisms and risk of cholelithiasis in pre- and postmenopausal women. We only observed a change in the composition of gallstones between the analyzed groups which concerned the particularly low level of total cholesterol and increase in calcium carbonate in postmenopausal women compared to premenopausal women. These observations also concern an increase of level of certain bile acids in the composition of gallstones in postmenopausal women.

Studies on the APOB gene 7673C>T and 12669G>A polymorphisms and their significance in the pathogenesis of cholelithiasis are inconclusive. The presence of the mutated allele of 2669G>A polymorphism is mainly associated with altered level of apoB lipoprotein [30, 31]. Tan et al. conducted interesting observations on this genetic variant in the Chinese population. They showed a 4-fold decrease in the risk of gallstone formation in carriers of the mutated AA genotype and 1.75-fold reduction of risk in all carriers of the mutant A allele of the APOB gene 12669G>A polymorphism [32]. These observations were not confirmed in the Caucasian population [33]. In the present study, when analyzing the APOB gene 7673C>T polymorphism we observed that the frequency of genotypes and alleles showed no significant differences between women with cholelithiasis and a group of healthy women before and after menopause. Similarly, in the Hindu and Polish population, there was no relationship between the presence of polymorphic variants and the development of gallstone disease [33, 34]. In addition, Hegele et al. [35] suggested that the observed frequencies of alleles of APOB gene 7673C>T polymorphism are different between the studied populations. Furthermore, in the Caucasian population, a much higher frequency of 7673T allele was noted compared to Asians belonging to the Chinese population [36, 37]. Moreover, in some studies, there was no correlation between the 7673T allele and cholesterol level [35, 38].

In contrast to the above studies, analysis conducted in Mexico suggest the relationship between the concentration of apolipoprotein B in serum and gallbladder disease pathogenesis [39]. It was also found that the presence of the mutant 7673T allele is associated with higher levels of cholesterol and LDL and thus may be a marker of the increased risk of gallstone disease in the population [13]. Also, Law et al. [40] and Rajput-Williams et al. [41] showed a positive correlation between the mutant 7673T allele and the increase in the cholesterol level.

So far, the results on the relationship between APOB polymorphism and plasma lipids and lipoproteins related to the risk of gallstone disease in the studied populations are ambiguous. Hence, further analysis on large populations is necessary taking into account other factors, such as ethnicity, age, gender, hormonal status, smoking or dietary differences.

Conclusions

Our study did not indicate a significant association of the APOB gene 7673C>T and 12669G>A polymorphisms with the risk of gallstones in women before and after menopause, pointing to the fact that these variants do not matter as prognostic factors for cholelithiasis in the Caucasian population. Due to the possible contribution of a variety of factors in the pathogenesis of gallstones further studies are required to take ac-
count of additional environmental factors, which may point to still other relationship between the occurrence of studied polymorphisms and the development of gallstones in Caucasians.

Disclosure

Authors report no conflict of interest.

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