New Determinants for Gallstone Disease?

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This review has been accepted as a thesis together with four previously published papers by University of Copenhagen 30th of June 2017 and defended on 24th of November 2017

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Dan Med J 2018;65(2):B5438

THE FOUR STUDIES INCLUDED IN THIS THESIS

I. Shabanzadeh DM, Sorensen LT, Jorgensen T. Determinants for gallstone formation - a new data cohort study and a systematic review with meta-analysis. Scand J Gastroenterol 2016;51:1239-48.
II. Shabanzadeh DM, Holmboe SA, Sorensen LT, Linneberg A, Andersson A-M, Jorgensen T. Are incident gallstones associated to sex-dependent changes with age? A cohort study. Andrology 2017;5:931-8.
III. Shabanzadeh DM, Jorgensen T, Linneberg A, Sorensen LT, Skaaby T. Vitamin D and gallstone disease-A population-based study. Endocrine 2016;54:818-25.
IV. Shabanzadeh DM, Skaaby T, Sorensen LT, Eugen-Olsen J, Jorgensen T. Metabolic biomarkers and gallstone disease - a population-based study. Scand J Gastroenterol 2017;52:1270-7.

BACKGROUND

Gallstones are abnormal stone masses formed in the gallbladder or the intrahepatic bile ducts and infrequently also migrate to the common bile duct or the intestines(5, 6). The presence of gallstones in humans has been identified in the mummy of an Egyptian priestess dated back to about 1500 BCE(7). The first observation of gallstones in humans was reported by the Florentine physician Antonio Benivenius towards the end of the fifteenths century at an autopsy of a lady that had deceased with abdominal pain(8). Historical writings and autopsy findings indicate that Catherine the Great of Russia and the emperor Alexander the Great both suffered from gallstone disease with death of the latter ascribed to acute cholecystitis(7).

Gallstones are classified by their composition of major constituents into pure cholesterol stones, pure pigment stones or mixed stones(9). Cholesterol gallstones have been estimated to account for 75-90% of gallstones prevalence in Western countries(10). A number of studies throughout the decades that have analyzed the composition of surgically removed gallstones indicate that cholesterol gallstones have been the dominating cause of clinical gallstone disease for long. Studies from the 1970'ies including x-ray and chromatography analyses of gallstones show that cholesterol constituted 89% of the weighted occurrence in Swedish populations(11, 12). In a study from 1987-88, cholesterol stones with a cholesterol content above 70% or mixed stones with cholesterol contents of 20-69% at chromatography accounted for 77% of gallstones identified at cholecystectomy or autopsy in a Danish population(13). In a more recent study in a German clinical population, cholesterol was the main constituent in 93.3% and pigment was in 5.5% of gallstones at spectrometry(14). Due to the dominance of cholesterol gallstones in the western countries, most of the determinants for gallstone disease identified in epidemiological studies are assumed to apply to cholesterol or mixed stones(10).

Pigment stones contain calcium bilirubinate as the main component and can further be divided into black and brown stones(15). Black pigment stones may be associated with physiological conditions including hemolysis and increased production of unconjugated bilirubin(16) such as clinical conditions of hepatic origin like cirrhosis(17) or of pre-hepatic origin like spherocytosis, sickle cell disease, thalassemia, and malaria(15). Higher prevalence of black pigment than cholesterol gallstones are found in developing countries and in Asian populations(18-23). Although black pigment gallstones still are highly prevalent in Asia, the prevalence of cholesterol gallstones has been ever rising since the late 1960'ies – a trend ascribed to a westernized lifestyle(22). Brown pigment stones are found in the hepatic ducts and believed to be caused by biliary stasis and cholangitis(24-27) due to anaerobic and aerobic bacterial infection, parasitic infestations, or bile seeking worms(15). They contain more cholesterol and fatty acids than black pigment stones(25, 26, 28). Brown pigment stones are uncommon in Western countries and reported with higher prevalence in Asia(18, 29, 30).

The true presence or absence of gallstones can only be confirmed through surgery or autopsy. However, non-invasive radiological examinations have been developed in order to examine patients with suspected gallstone disease(31). Oral cholecystography was the examination of choice in the pre-ultrasound era, but somewhat unpractical since it required two days preparation, ingestion of tablets, exposure to radiation, and the cholecystogram was often inconclusive due to failed visualization of the gallbladder(32, 33). When comparing detection of gallstones at surgery with radiology, sensitivity and specificity for oral cholecystography is 90% and 95%, computed tomography is 79% and 99%, and for ultrasound is 97% and 95%, respectively(31). The superiority of gallstone detection with ultrasound has been reproduced in the morbidly obese patients with sensitivity 91% and specificity 100%(34). Inter-observer agreement for both detection and exclusion of gallstone disease is good (Kappa scores 0.78 and 0.73,

respectively) (35). Due to the many advantages, ultrasound examination has become the preferred non-invasive examination for gallstone disease.

Gallstone disease prevalence was traditionally determined through autopsy studies, but the selective approach of autopsies performed caused biased estimates(36). Truer estimations of gallstone disease prevalence in larger populations became possible with the introduction of real-time ultrasound throughout 1980-90'ies which caused a wave of general population screening studies(10, 37). Gallstone disease prevalence was found to have a significant ethno-geographic variation with the highest in the Native American Indian populations and the lowest in Black Africans(10, 37). In Western cultured countries of Europe and the US, intermediate high overall prevalence of about 10-20% have been found with the highest prevalence in Northern Europe. Female sex and higher age were significantly associated with gallstone disease in all countries(37, 38).

Mechanisms of gallstone formation

The very first theories about gallstone formation were based on chemical studies of ox bile. Thudichum (1863) left ox bile to decompose for years and suggested the acidified bile as the necessary environment for gallstone formation(39). He found that human bile was rich in cholesterol and he theorized that the acid of putrefaction would set free cholesterol to crystallize and deposit upon any particle that would happen to be within easy distance(39). During the following decades, the composition and appearance of the human gallstone and its central nucleus were studied and more complex theories of gallstone formation were suggested. In 1892, Naunyn theorized cholesterol gallstone formation to be a disease of the gallbladder caused by a local bacterial infection(40). This gallbladder wall infection would cause a desquamation of epithelium with the waste serving as the primary source of bile cholesterol and forming a pultaceous mass with primary cholesterol crystallization occurring from the central nucleus or secondarily occurring through an infiltration of bile cholesterol. Bile stasis was also emphasized as part of the gallstone formation process(40). During the coming century, conflicting mechanisms were suggested. Boysen (1900) and Rovsing (1924) emphasized the pigment gallstone formation and the importance of the black pigmented nucleus. They thought pigment stones were the product of a disease in the hepatic ducts under aseptic conditions which was in conflict with Naunyn's theory of infection. Once the pigment nucleus reached the gallbladder, the stone would grow through infiltration and crystallization of cholesterol in layers, through a process where the pigment nucleus was possibly dissolved which would explain the presence of cholesterol stones with little or no pigment(41, 42). Aschoff and Bacmeister (1909) addressed the existing controversies through review of literature and through performing a number of clarifying experimental studies with human bile. They concluded that bile stasis was the most important mechanism of gallstone formation(43). Bile stasis was defined as a mechanical obstruction of the physiological bile drainage such as tight female clothing, pregnancy, altered anatomy, or intra-abdominal pathologies such as appendicitis or tumors. The stasis would cause a higher bile pressure, with changes in the gallbladder wall including a thickened muscular layer, deposition of lipids, and an infiltration of lymphocytes. Bile cholesterol was considered a product of liver metabolism which also could be enhanced through a number of altered physiological conditions. Infection of the gallbladder was considered only secondary to gallstone formation but could change gallstone size, appearance, and composition with a coating of calcium and pigment causing formation of mixed or pigment gallstones.

Bile cholesterol and its crystallization were central to all of these primary theories. The chemical properties of bile and the transport function of the gallbladder mucosa earned much focus in the research performed during the following decades. It became evident that bile salts and phospholipids were necessary to keep cholesterol soluble(44-46). A cholesterol-rich diet was found correlated with cholesterol gallstone formation in animal models – such as in prairie dogs(47) – and increased cholesterol excretion to bile was observed in humans with cholesterol gallstones(48, 49). Through continuous chemical experiments with bile, the primary focus became the solubility of bile cholesterol relative to the other two bile constituents including phospholipids and bile salts produced by hepatocytes. Based on in vitro studies, a ternary diagram was developed, which defined the physical state of bile cholesterol into an ascending order on its way to cholesterol crystallization which included 1. micelles (liquid), 2. vesicles (liquid crystals), and 3. crystals(50) (**Figure 1**). The ternary diagram has since been reproduced in several experimental models including human bile(51-53) and constitutes the theory of bile cholesterol supersaturation, which still is believed to be the leading mechanism for cholesterol gallstone formation.

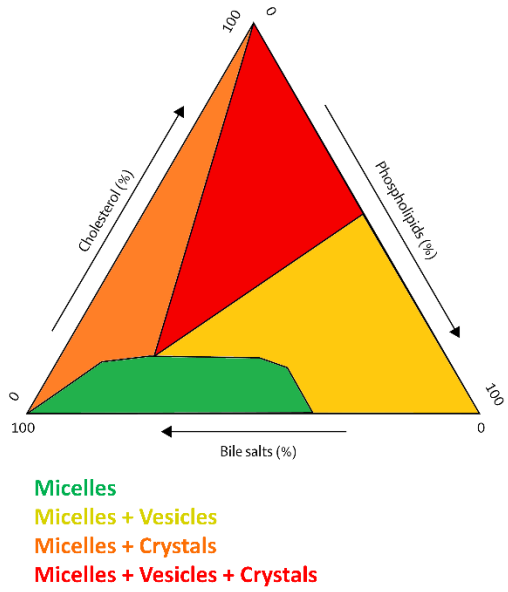

Figure 1: The ternary diagram defining the physical state of bile cholesterol

The enterohepatic circulation of bile salts also involves important mechanisms contributing to cholesterol gallstone formation. Hepatic bile salts such as cholate and chenodeoxycholate are synthesized from cholesterol in hepatocytes under normal physiological conditions(6). Secondary bile salts such as deoxycholate and lithocholate are produced by fecal microbiota containing the enzyme 7α-dehydroxylase, through a degradation process of hepatic bile salts in the large bowel, where they are reabsorbed to the bile pool with the enterohepatic circulation(6). Both secondary bile salts and the fecal microbiota producing them are found in high amounts in persons with gallstone disease compared with gallstone free controls(54-58). When compared to

the primary hepatic bile salts, the secondary bile salts have inverse functions in the formation of gallstones through being hydrophobic, increasing bile cholesterol saturation, increasing cholesterol in vesicles, and thereby promoting cholesterol crystallization in gallbladder bile(59). A slower orocecal transit and slower large bowel transit are contributing mechanisms to an increased absorption of fecal secondary bile salts to the enterohepatic circulation during gallstone disease formation(56, 60).

Impaired gallbladder motor function is the third mechanism contributing to the formation of cholesterol gallstones(61). Under normal physiological conditions, gallbladder contractions are stimulated by cholecystokinin, a hormone released by the duodenum as a response to food ingestion. Whether impaired gallbladder motor function causes bile stasis and cholesterol crystallization or whether it is a secondary process to cholesterol crystallization in gallbladder wall is debatable(61).

The quest for gallstone disease determinants

The "female, fat, fair, fertile, and forty year old," patient that sometimes also is "flatulent" and "flabby" has been the subject of the five or seven "F" clinical stereotype for gallstone disease. However, this clinical aphorism is not based on empirical research. Following the initial autopsy studies for assessment of gallstone disease prevalence, case-control studies were some of the first to explore possible differences in risk factors for gallstone carriers (cases) and non-carriers (controls)(62-65). However, most of these studies suffer from inadequate sample size, and comparability was hampered by unrepresentative controls, causing selection biased and confounded estimates of association(38). The Framingham Heart Study began in 1948 and started a new tradition for studying cardiovascular disease etiology through an epidemiological approach in larger general populations. A decade later, the Framingham study also included the study of gallstone disease epidemiology(66). The impact of cardiovascular disease determinants for gallstone disease was explored with the wave of ultrasound screening studies, and gallstone disease was found associated with obesity, diabetes, pregnancies, familial aggregation, oral contraceptives, dietary habits, smoking, alcohol or coffee abstinence, and serum lipids – just to mention a few(65, 67-77). Although numerous studies were published, they were all limited by their cross-sectional design with the inability to establish causal temporal associations.

More recent cohort studies have included large populations with assessment of clinical gallstone disease such as self-reported, hospitals admissions, or cholecystectomy. However, when studying the natural history of gallstone disease, only a small fraction of gallstone carriers will experience a clinical detection of their gallstones during long-term follow-up(78-82). Thereby, only assessing clinical gallstone disease will include a selected part of the gallstone disease population and the majority will be misclassified as not having gallstone disease. Further, these studies are unable to distinguish determinants for gallstone formation from determinants for clinical gallstone disease. Studies assessing clinical gallstone disease as outcome have identified temporal associations for cardiovascular disease determinants such as obesity, diabetes, oral contraceptives, hormone replacement therapy, dietary habits, smoking, alcohol and coffee abstinence, and physical activity(65, 83-94). However, some identified associations from these studies may be biased through the selective approach of assessing clinical gallstone disease.

The superior design in exploring gallstone disease determinants is the cohort study including larger general populations and with ultrasound assessment of gallstone disease both at baseline and at follow-up. Only a few cohort studies have explored incident gallstone disease through ultrasound examinations of general populations, and only few determinants have been identified(79, 95-100). Such studies are needed in order to identify determinants of gallstone disease in order to improve future prevention or treatment of this highly prevalent disease.

Definitions of gallstone disease in thesis

For the remaining part of this PhD thesis, the term screen-detected gallstones refers to ultrasound detected gallstones when screening an entire population, cholecystectomy refers to the surgical removal of the gallbladder already performed at the time of screening, and screen-detected gallstone disease is defined as the composite definition for gallstones and cholecystectomy. Clinical gallstone disease as defined above is characterized by not being a result of a systematic screening for gallstones of an entire population. Gallstone disease detected in cohort studies will be defined as incident and if detected in cross-sectional studies will be defined as prevalent.

AIMS

The overall aim of the PhD thesis was to investigate new determinants for screen-detected gallstone disease assessed through ultrasound examination in a Danish general population sample. Specifically, the thesis explored the following objectives:

- determinants of incident gallstone disease in the study population and in other cohort studies of general populations including ultrasound screening
- whether sex-dependent changes in determinants over a decade determined incident gallstone disease
- whether circulating levels of vitamin D or determinants thereof were associated with gallstone disease prevalence
- if genetic susceptibility or metabolic changes of obesity such as insulin resistance, systemic inflammation, or vascular dysfunction were associated with gallstone disease prevalence

HYPOTHESIS

The hypothesis that generated the objectives of this PhD thesis included:

- that cardiovascular disease determinants including factors of metabolism and lifestyle also determined incident gallstone disease
- that female predominance of gallstone disease is caused by sex-dependent changes in metabolism, pregnancies, and lifestyle with advancing age
- that the high prevalence of gallstone disease in northern European countries is caused by lower in vivo production of vitamin D due to lower sun exposure
- that the metabolic changes and genetic susceptibility of obesity are associated with gallstone disease

MATERIAL AND METHODS

A random sample from the general population, comprising 4807 persons, aged 30–60 years, and living in 11 municipalities in the western part of the urban Copenhagen was studied. The sample was drawn from the Civil registration system in October 1982. The study was part of the international collaboration MONICA (Multinational mONItoring of trends and determinants in CArdiovascular disease) with the aim to examine cardiovascular determinants in the general population. Participants were informed

about the aim of the study including the screening for cardiovascular disease risk factors, but were not informed about findings of gallstone disease or other benign conditions in the gallbladder following ultrasound examination to avoid unnecessary treatment and worrying. Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was accepted by the local research ethic committee(101).

People were invited to examination through mail and non-responders were re-invited. Those who still did not respond were contacted by telephone and, if not reached, a third letter asking them to take contact by telephone was sent. Examination outside working hours and free transportation were offered if necessary. Examinations took place after 12 hours of fasting and included an abdominal ultrasound, physical examination (blood pressure, weight, and height), blood samples, and questionnaires about medical history including previous cholecystectomy, lifestyle, and socioeconomic factors. Participants were interviewed if errors or omissions had occurred in the questionnaire responses. Baseline examination took place 1982-84 and the cohort was re-examined twice with similar protocols in 1987–1988 and 1993–1994 (**Figure 2**). Prevalence studies from the baseline examination including a detailed description of the cohort have been published before(69, 101). Parts of the re-examinations have been published as incidence studies with exploration of the effect of age and sex(96, 102).

Blood or serum samples from examinations were stored at minus 20°C. New analyses were performed in 2004-11, including vitamin D, hepatic function, renal function, male reproductive hormones, genetic variations, and biomarkers of systemic inflammation and insulin resistance.

Logistic regression analyses with gallstone disease as the outcome were chosen for inferential statistics since the study population was examined and re-examined at fixed time-points. Thereby, the study design did not include the effects of long-term follow-up as seen in time-to-event cohort studies where every participant contributes with different lengths of observation time. Sex was adjusted for due to the known sex-differences in gallstone prevalence or otherwise addressed with separate analyses. Age was adjusted for due to the known association with gallstone disease prevalence and due to the delayed entry design of the cohort study. Multiple adjusted models were performed in order to control the associations under study for confounders identified in previous studies or identified in the studies included in this PhD thesis. Models were built including at least 10 outcome cases for every parameter as a rule of thumb. In multiple models, interactions with sex were tested for and continuous variables were explored for quadratic and cubic polynomial associations with gallstone disease.

PRESENTATION OF STUDIES

Study I (1)

Determinants for gallstone formation – a new data cohort study and a systematic review with meta-analysis

Aim

To identify determinants for incident gallstone disease in a Danish cohort and to perform a meta-analysis of results from existing cohorts.

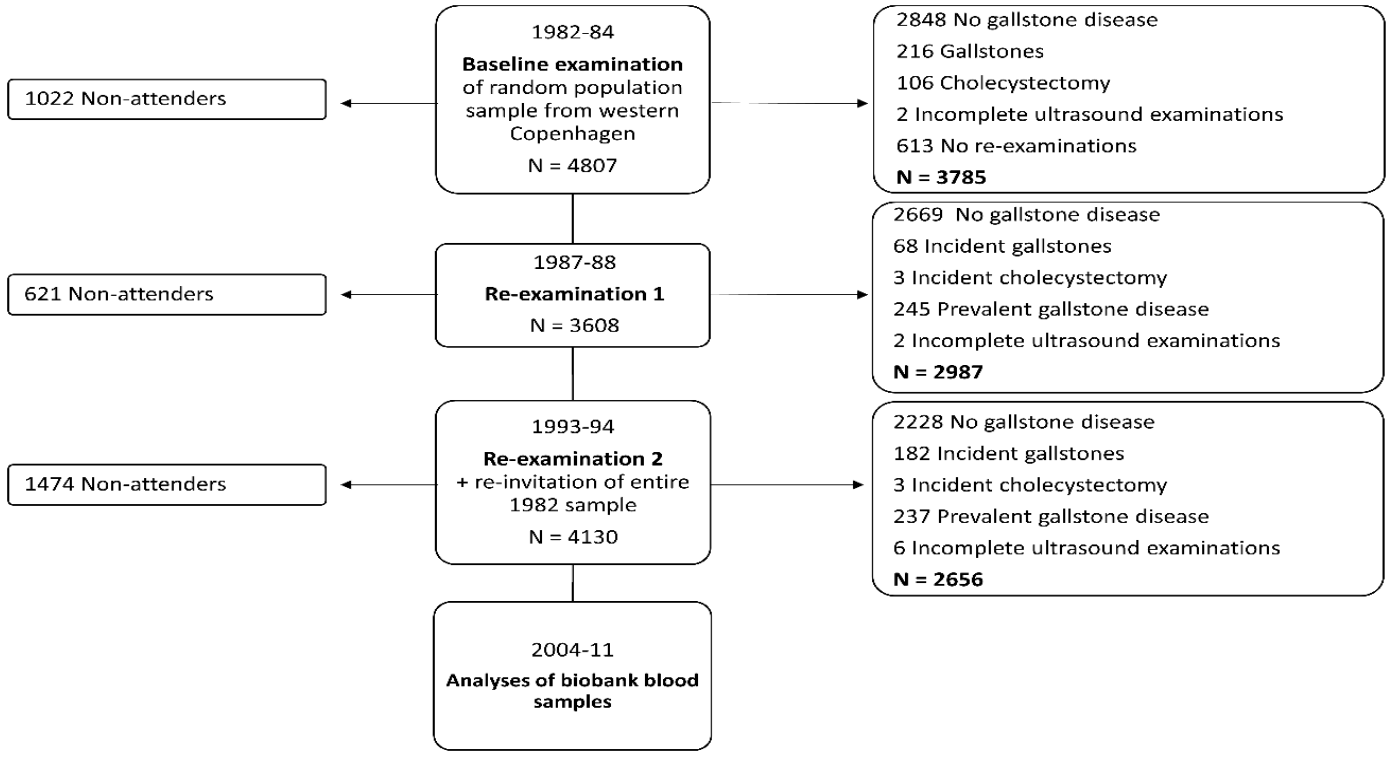

Figure 2: Participant flow and description of examinations

Methods
Data from a cohort study was used. Gallstone incidence was assessed through repeated ultrasound examinations (baseline examination and re-examinations 1 and 2, **Figure 2**). Body mass index (BMI), blood pressure, self-rated health, lifestyle variables, blood lipids, and use of female sex hormones were measured at the baseline examination (**Figure 2**). Statistical analyses included logistic regression. Based on a prospective protocol, a systematic review of the literature was performed identifying all articles dealing with determinants of incident gallstone disease. Meta-analyses of comparable determinants were performed through fixed effect models.

Results
Participants with no gallstones at baseline and with at least one re-examination were followed up completely (mean 11.6 years, N = 2848). The overall cumulative incidence of gallstones was 0.60% per year. Independent determinants for incident gallstone disease were high age, female sex, high non-high density lipoprotein (non-HDL) cholesterol, and gallbladder polyps. In addition, high BMI was associated in men. The systematic review additionally identified associations for comorbidities, parity, and dietary factors. Meta-analyses confirmed the significant associations for incident gallstone disease and high age, female sex, high BMI, and high non-HDL cholesterol. No significant associations were found for blood pressure, smoking, alcohol consumption, HDL cholesterol, or triglycerides in meta-analyses.

Strengths and limitations
The strengths of this cohort study were the multiple ultrasound examinations during the long-term follow-up period. The limitations were the inability to explore temporal associations with other important metabolic variables such as fasting blood glucose, insulin, and glycated hemoglobin which were not measured at baseline examination. Due to the long-term follow-up, time-dependent changes of the explorative variables could, potentially, have an influence on gallstone formation. The latter has been addressed in Study II(2).

The strength of the systematic review was the restricted inclusion of cohort studies performed in general populations assessed for screen-detected gallstone disease, thereby avoiding the various selection bias seen in studies assessing clinical gallstone disease. Limitations in the meta-analyses included the incomparable data between variables reported in the identified studies, thereby restricting meta-analyses of adjusted estimates to include only four studies. Although significant determinants identified in the incidence study were confirmed in meta-analyses, the statistical heterogeneity was high which may restrict the generalizability of our findings. Heterogeneity could not be explored with sensitivity analyses due to the low number of studies included.

Study II (2)
Are incident gallstones associated to sex-dependent changes with age? A cohort study
Aim
To determine if changes with age in physiology, lifestyle, or reproductive hormones were associated with incident gallstones or cholecystectomy.

Methods
A cohort study of a general population random sample (N=2366) aged 30-60 years. Participants were ultrasound screened for gallstones in 1982-84 and again in 1993-94 (baseline examination and re-examination 2, **Figure 2**). Lifestyle data and blood samples were obtained and re-analyzed in 2004. Changes with age in physiology (body mass index, blood pressure, blood lipids, self-rated health), lifestyle (smoking, alcohol and coffee consumption, dietary habits, physical activity level), and indices of reproductive function (number of births, oral contraceptive use, hormone replacement therapy, male reproductive hormones) were explored in females and males separately. Adjusted logistic regression analyses were performed.

Results
Incident gallstones or cholecystectomy at ultrasound examination in participants initially free of gallstones at baseline occurred in 9.9% of the study population. In females, increasing alcohol consumption (odds ratio (OR) 0.94, 95% CI [0.90;0.98]) and the cessation of hormone replacement therapy (OR 0.29, 95% CI [0.10;0.83]) inversely determined incident gallstone disease. In males, increasing levels of sex hormone-binding globulin (SHBG) (OR 0.97, 95% CI [0.94;0.998]) inversely determined incident gallstone disease. Other changes with age in physiology, lifestyle, or reproductive hormones were not associated with incident gallstone disease. High baseline free testosterone determined incident gallstone disease in males (OR 1.15, 95% CI [1.02;1.30]).

Strengths and limitations
The uniqueness of this cohort study included the assessment of changing determinants over long-term follow-up and the novelty of assessing male reproductive hormones. Not assessing serum female endogenous reproductive hormones or cumulative lifetime exposures to both female and male reproductive hormones in this study were the main limitations. These could not be measured due to a lack of data on menopausal status and menstrual cycle at times of blood sampling. Further, changes in determinants were measured as the difference between baseline examination and re-examination making the latter an assessment of both explorative variable and of outcome. This lack of distinction between induction and latency period could possibly have caused a misclassification bias, which may have limited the interpretation of a temporal association in analyses of changes with age. Further, a time-related issue was demonstrated through hormone replacement therapy in females with the identification of the protective effect of cessation of hormone replacement therapy without finding any significant effects of hormone use on incident gallstone disease. If estrogens truly are associated to incident gallstone disease, one would suspect current estrogen users to have a significant association with incident gallstone disease as well. Such diverging results may be caused by left truncation, a bias due to non-inclusion of participants with the outcome of interest before being able to be included in a study(103).

Study III (3)
Vitamin D and gallstone disease – A population-based study
Aim
To determine whether circulating levels of 25-hydroxyvitamin D were associated to ultrasound proven gallstones or cholecystectomy in a general population sample. Determinants of vitamin D status were also explored.

Methods
A re-invitation of the 4130 people that were still alive from a random sample of the population of Copenhagen with ages 41–71 years was performed and 2650 participated and had complete ultrasound examinations (re-examination 2, **Figure 2**). Ultrasound examinations were performed to assess gallstone status and blood samples were drawn to assess 25-hydroxyvitamin D and biomarkers of renal and hepatic function. Gallstone disease was found in 422 participants. Associations were estimated by logistic regression models.

Results
Levels of 25-hydroxyvitamin D were not significantly associated with gallstone disease. Time of birth during low vitamin D exposure was associated with gallstone disease (gallstone prevalence 18.0 versus 14.4%, OR 1.33, 95% CI [1.07;1.65]). Highest quartile of cystatin C was significantly associated with gallstone disease (gallstone prevalence 22.1 versus 12.0%, OR 1.53, 95% CI [1.08;2.18]). Serum levels of creatinine and alanine amino transferase were not associated with gallstone disease. Sensitivity analyses excluding participants with cholecystectomy did not alter results significantly.

Strengths and limitations
The novelty of this study was the exploration of associations for vitamin D, associated biomarkers, and gallstone disease in a large population sample from Denmark which is a country known to have a high prevalence of seasonal vitamin D insufficiency. The most important limitations were the inability to study temporal associations due to the cross-sectional study design and the use of time of birth during low vitamin D exposure in utero as a proxy for maternal levels of vitamin D during the third trimester of pregnancy.

Study IV (4)
Metabolic biomarkers and gallstone disease – a population-based study
Aim
To examine associations for metabolic biomarkers of obesity such as insulin resistance, vascular dysfunction, systemic inflammation, genetic susceptibility and ultrasound proven gallstone disease or cholecystectomy in a population-based cross-sectional study.

Methods
A total of 2650 participants with complete ultrasound examinations were included, of whom 422 had gallstone disease (re-examination 2, **Figure 2**). Associations to gallstone disease were estimated by multivariable logistic regression models and expressed as OR and 95% CI.

Results
Gallstone disease was associated with high fasting glucose (OR 1.14, 95% CI [1.05;1.24]), high fasting insulin (OR 1.03, 95% CI [1.01;1.05]), high homeostasis model assessment (HOMA) insulin resistance (OR 1.18, 95% CI [1.02;1.36]), the metabolic syndrome (OR 1.51, 95% CI [1.16;1.96]), high white blood cell count (OR 1.07, 95% CI [1.00;1.15]), and high C-reactive protein (OR 1.03, 95% CI [1.01;1.05]). A non-significant tendency towards an association to high soluble urokinase plasminogen activator receptor was also found (OR 1.08, 95% CI [0.99;1.18]). The *MC4R*(rs17782313) (OR 1.27, 95% CI [1.02;1.58]), *MAP2K5*(rs2241423) (OR 1.80, 95% CI [1.04;3.41]), *NRXN3*(rs10146997) (OR 1.26, 95% CI [1.01;1.57]), *HHEX*(rs1111875) (OR 1.29, 95% CI [1.03;1.62]), *FAIM2*(rs7138803) (OR 0.66, 95% CI [0.48;0.91]), and apolipoprotein E4 allele (OR 0.76, 95% CI [0.59;0.98]) were associated with gallstone disease. Urinary albumin was not associated with gallstone disease. Addition of biomarkers of insulin resistance to multivariable models removed the association between BMI and gallstone disease prevalence.

Strengths and limitations
The novelty of this study was the exploration of inflammatory biomarkers, genetic risk alleles for obesity and diabetes type 2, and gallstone disease. The association between BMI and gallstone disease seemed to be mediated through insulin resistance. The inability to identify temporal associations in this study is the main limitation just as with Study III(3). The exploration of the 32 genetic risk alleles included in this study may have lacked power to show associations with gallstone disease and the identified associations would not withhold adjustment for multiple testing.

DISCUSSION
Principal findings
Through an exploration of determinants for screen-detected gallstone disease in a Danish population, the following was identified:

- Age, female sex, BMI, non-HDL cholesterol, and gallbladder polyps are independent determinants for incident gallstone disease. These significant determinants were confirmed in meta-analysis including similar designed cohort studies performed in Italy, Sweden, and Taiwan.
- Changes with age in increasing alcohol consumption and in cessation of hormone replacement therapy in females, and in increasing SHBG in males inversely determine incident gallstone disease.
- High free testosterone at baseline determines incident gallstone disease in males.
- No association between 25-hydroxyvitamin D and gallstone disease prevalence was identified. Time of birth during low vitamin D exposure in utero and renal failure were associated with gallstone disease prevalence suggesting that vitamin D might have an impact on gallstone disease.
- Biomarkers of insulin resistance are associated with gallstone disease prevalence and seem to mediate the association between BMI and gallstone disease.
- Biomarkers of systemic inflammation and genetic risk alleles for obesity or diabetes type 2 seem associated with gallstone disease prevalence.

Gallstone disease epidemiology in a Danish cohort
The incidence rate of gallstone disease in the study population was found to be 0.60% per year in Study I(1). Similar designed cohort studies performed in Italy and Taiwan report incidence rates of 0.46-0.97% per year(79, 97, 98, 100, 104). In a Swedish study, a higher incidence rate of 1.39% per year was found. This may have been caused by an older cohort(99). However, higher gallstone disease prevalence has also been found in northern compared to southern Europe(37, 38) which may be caused by a lower exposure to vitamin D in utero as found in Study III(3). The hypothesis of a relationship between seasonal vitamin D insufficiency caused

by lower sun exposure and higher gallstone disease prevalence in Denmark could not be confirmed in Study III(3) of this PhD thesis, but should be explored further in future studies.

Only a few studies have compared gallstone disease prevalence between different ethnic populations and only one study has been performed for European populations including cohorts from Denmark and northeastern Germany(105). The German cohort had about twice the odds for gallstone disease when compared to the Danish, which only partly was explained by higher BMI, unfavorable lipid profiles, higher prevalence of diabetes, and a more frequent use of oral contraceptives and hormone replacement therapy in German subjects. The study concluded that these classical cardiovascular disease determinants were unable to fully explain the higher German prevalence and that other factors including genetic components should be explored in future studies(105). The inability to explain the large differences in gallstone disease prevalence between Hispanics and non-Hispanic blacks or whites through environmental factors was also the conclusion based on the gallstone disease prevalence screening-studies performed in the US(106).

More speculative theories have proposed the ethnic predominance of gallstone disease in northern Europe and in the Native Indian populations of North and South America to be the cause of survival advantages in acquiring a low metabolic rate during periods of cold climates with marginal food supplies. This evolutionary promotion of ''thrifty genes'' and the factors associated with a Western lifestyle such as diet and a sedentary physical activity level are thought to be linked to obesity and development of gallstone disease(107). Only a few studies based on empirical research exploring the genetic epidemiology of gallstone disease have been performed. American-Indian genetic admixture was associated with gallstone disease when comparing mitochondrial DNA from high prevalence gallstone disease populations of Chilean Hispanics and Mapuche Indians with the lower prevalence population of Chilean Maoris(108). Likewise, when comparing 92 ancestry informative single nucleotide polymorphisms in Hispanic American women, American-Indian genetic admixture was associated with cholecystectomy and both European and sub-Saharan African genetic admixture was inversely associated with cholecystectomy(109). Pathways in the human endogenous synthesis of bile salts and of cholesterol have been suggested as mechanisms for the high American-Indian prevalence of gallstone disease(110). No studies have compared the genetic epidemiology of gallstone disease in populations from Denmark or other northern European countries to Southern European populations or to other lower prevalence populations.

Lifestyle

An increase in alcohol consumption inversely determined incident gallstone disease in females in Study II(2) while alcohol consumption at baseline was not identified a determinant in Study I(1). No other cohort study has explored associations for change in alcohol consumption. Similar designed cohort studies confirm the findings of an inverse association for incident gallstone disease, but only with baseline alcohol consumption – these studies explored weekly alcohol consumption compared to alcohol abstainers(99) and linear trend for wine consumption(97). Clinical gallstone disease has also been inversely associated with alcohol consumption(83, 94).

Inverse associations for alcohol consumption and gallstone disease have previously been suggested due to the protopathic bias in observational studies caused by a reduced alcohol use in patients with abdominal symptoms related to clinical gallstone disease(111). Such a bias is unlikely in Study II(2) and other population-based cohort studies due to the exploration of temporal associations and the inclusion of an unselected and non-clinical population. A causal association for the protective effects of alcohol consumption on gallstone formation is supported by a lowering of bile cholesterol saturation(112-114) and an increase in bile salt production and excretion to gallbladder bile(115, 116). The inverse association between alcohol consumption and cardiovascular disease(117) further emphasizes the protective effects of alcohol consumption on cholesterol metabolism. These benefits have been attributed to a cardio-protective rise in blood HDL cholesterol(118) which, similar as stated above for alcohol consumption, also has been associated with an increase in bile salts(119). Further preventive mechanisms of alcohol consumption on gallstone formation may include a changed gallbladder motor function with stimulation of contractions, thereby inhibiting bile stasis and gallstone formation(120). However, the effects of alcohol consumption or chronic alcoholism on gallbladder motor function are controversial(120-122).

The effects of chronic and acute alcohol consumption on proximal bowel transit in humans may be controversial based on experimental studies(122-124). However, a higher everyday alcohol consumption has been associated with a faster self-reported whole gut transit in the general population(125) and an acute administration of alcohol has been shown to suppress impeding Type I pressure waves in the jejunum and to stimulate propulsive Type III pressure waves in the ileum(126), indicating that alcohol consumption speeds up distal bowel transit. The protective effects of alcohol consumption on gallstone formation may thereby also be exerted on the enterohepatic circulation by impeding the entry of secondary bile acids. The sex differences in the effects of rising alcohol exposure identified in Study II(2) are somewhat unexplained, but could also be caused by an inability to detect significant associations due to the lower prevalence of gallstone diseased in males.

Physical activity level did not determine incident gallstone disease (Studies I(1) and II(2)). In support of this finding, an intervention of moderate to vigorous physical activity in pregnant women has also been shown to have no impact on incident gallstone disease measured through ultrasound examination in a randomized controlled trial(127). When exploring the subgroup of the study population that had gallstones and was unaware of its gallstone status, a physical activity including light, moderate, and vigorous levels compared to a sedentary level inversely determined clinical gallstone disease hospital admissions(128). The current evidence therefore indicates that gallstone formation is not determined by physical activity, but that a sedentary physical activity level determines clinical gallstone disease in persons with gallstone disease. Further supporting this hypothesis, physical activity has also been inversely associated with clinical gallstone disease in larger cohort studies(93). Physical activity increases plasma cholecystokinin, which stimulates gallbladder contractions(129) and an impaired gallbladder motor function with ejection fraction below 40% has also been associated with recurrence of pain attacks in clinical gallstone disease(130). These mechanisms may explain the protective effects of physical activity on clinical gallstone disease. Due to the current evidence and conflicting results in incident clinical versus screen-detected gallstone disease, the impact of objectively measured physical activity

through accelerometers on gallstone formation should be subject to future studies in order to explore this hypothesis further.

Other lifestyle factors were found unrelated to gallstone disease in the studies included in this thesis. The systematic review of existing literature identified that a similar designed cohort study associated incident gallstone disease with tobacco smoking, consumption of milk and oils, and inverse associations with consumption of coffee, fish, and whole meal(97). However, associations for tobacco smoking and coffee consumption could not be confirmed in the meta-analysis of Study I(1). Clinical gallstone disease has been associated with tobacco smoking(92), consumption of fatty acids(87), and inversely associated with coffee consumption(84, 85). However, bias caused by selected populations, by between study heterogeneity in exposure assessment, or by the inability to distinguish gallstone formation from clinical disease just like in the exploration of physical activity above, may explain these discrepancies in study results.

BMI, cholesterol metabolism, and insulin resistance

BMI was associated with incident gallstone disease in males, but the association was found for both females and males in meta-analysis in Study I(1). Similarly designed cohort studies have identified associations for incident gallstone disease with BMI(79, 97, 98, 100). BMI has also been identified as a determinant for incident gallstone disease in pregnancy and early post-partum period in a cohort study including only pregnant women(131). Meta-analysis including studies with incident clinical gallstone disease have also identified associations for BMI and waist circumference(90). A number of alternative body fat tissue measures have been associated with gallstone disease prevalence independently of BMI, such as waist-to-hip circumference ratio with screen-detected gallstone disease and computed tomography measured visceral or subcutaneous fat with clinical gallstone disease(132, 133).

Spontaneous changes in BMI over a decade had no association with incident gallstone disease in Study II(2). Another similar study identified spontaneous weight gain and not weight loss with incident screen-detected gallstone disease(97), which is in accordance with the known association between BMI and gallstone disease. Other cohort studies have associated weight loss and weight cycling with clinical gallstone disease when compared to weight maintainers(134-137). Excessive weight loss during calorie restricting diets has also been associated with incident screen-detected gallstone disease(138, 139). Patients undergoing bariatric surgery and with subsequent rapid weight loss have also been associated with incident screen-detected gallstone disease(140). Suggested mechanisms for gallstone formation during rapid weight loss have included an initial increase in bile cholesterol saturation(141-143) and an impaired gallbladder motor function(142, 143). Current evidence therefore suggests that spontaneous changes in weight or BMI in the general population do not seem to be associated with incident gallstone disease contrary to rapid weight loss. The latter risk can be significantly reduced through interventions with ursodeoxycholic acid or high-fat weight loss diets as demonstrated in randomized controlled trials(144). Weight loss as a preventive intervention for incident gallstone disease has – to the best of the author's knowledge – not been tested in a randomized controlled trial yet.

The identified determinant of blood non-HDL cholesterol with incident gallstone disease in Study I(1) has been identified in a similarly designed cohort study before which assessed low density lipoprotein cholesterol(99). The association may be controversial since HDL rather than non-HDL cholesterol has been considered as the major source for reverse cholesterol transport from tissue and into the liver(145). Although transport rates for non-HDL cholesterol are lower, transport of both HDL and non-HDL cholesterols to liver and bile has been demonstrated in humans(146). Further, bile cholesterol saturation has been associated with higher blood non-HDL cholesterol and inversely associated with higher blood HDL cholesterol in healthy humans(147). Although no association for HDL cholesterol and incident gallstone disease was identified in Study I(1), the association for non-HDL cholesterol seems biologically feasible. Further supporting the identified association of Study I(1), non-HDL cholesterol lowering statins have been inversely associated with screen-detected gallstone disease prevalence and with incident clinical disease in observational studies(148, 149).

Biomarkers of insulin resistance were associated with gallstone disease prevalence and the association for BMI seemed to depend on insulin resistance. This suggests that insulin resistance possibly mediates the association between BMI and gallstone disease in Study IV(4). The associations for biomarkers of insulin resistance such as blood glucose, insulin, impaired glucose tolerance, HOMA, or diabetes, and screen-detected gallstone disease have previously been found in several cross-sectional and case-control studies(75, 150-153). Diabetes or elevated blood glucose has also been associated with incident screen-detected gallstone disease(97, 98, 100), however not consistently(79, 99). Meta-analysis including incident clinical gallstone disease has also been associated with diabetes(91).

Insulin resistance may increase cholesterol supersaturation of bile through a number of mechanisms including stimulation of the low density lipoprotein-receptor activity and of the rate limiting enzyme for endogenous cholesterol synthesis, the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG Co-A reductase)(154, 155). The most important cholesterol transporter facilitating cholesterol excretion into both gallbladder bile and bowel lumen is the ABCG5/8 heterodimer(156), and its expression has been demonstrated promoted by insulin resistance in mice(157). Further, an impaired gallbladder motor function in favor of bile stasis has been demonstrated in dynamic ultrasound studies including fasting and stimulated gallbladders in persons with obesity, insulin resistance, and induced hyperglycemia or hyperinsulinemia(158-161). Slower orocecal transit with the hydrogen breath test has also been demonstrated under induced hyperglycemia and hyperinsulinemia, and in patients with diabetes(162, 163) supporting an effect on the enterohepatic circulation and secondary bile salts. Suggested causes of impaired gallbladder function and impaired bowel function under conditions of insulin resistance include a suppressing effect of hyperglycemia on the vagal-cholinergic system which causes reduced gallbladder sensitivity to cholecystokinin or the effects of autonomous neuropathy in diabetes(61, 159). Fair amounts of experimental and observational evidence thereby support a causal association for gallstone formation with BMI and insulin resistance.

Sex-differences in incident gallstone disease

Female sex determined(1) and the cessation of hormone replacement therapy in females inversely determined incident gallstone disease(2). Although many cross-sectional screening-studies have

associated female sex with gallstone disease prevalence, the association has only been identified in one other cohort study(100). Association for oral contraceptives or hormone replacement therapy has only been identified for clinical gallstone disease(83, 86, 88, 89). Number of births was not associated to incident gallstones in Study II(2), but has been found associated with prevalent and incident screen-detected gallstone disease before(97, 164). A high cumulative incidence of screen-detected gallstone disease including gallbladder sludge of 7.9% at third trimester with regression to 4.2% in the post-partum period has been identified in a cohort study including pregnant women, indicating that the higher risk of gallstone disease during pregnancy only is transient(131). This temporality of an increased risk during pregnancy and early post-partum period may explain the discrepancies in existing studies' results when only total number of births is explored without including time since birth.

The female predominance of gallstone disease may be explained by the effects of female reproductive hormones on cholesterol metabolism. The binding of 17β-Estradiol to the nuclear estrogen receptor in the liver stimulates excretion of cholesterol into bile increasing cholesterol saturation(165). Estrogens also stimulate the activity of HMG-CoA reductase facilitating endogenous cholesterol synthesis(165). A case-control study found a significantly higher urinary estrone in females aged over 50 years and with gallstone disease when compared to controls which also supports the associations for endogenous estrogens and gallstone formation(166). Increased bile cholesterol saturation has also been identified in a randomized controlled trial after interventions of hormone replacement therapy in postmenopausal women(167). Bile cholesterol saturation may thereby be the most important mechanism involved in the female predominance of gallstone disease. Use of oral contraceptives does not seem to influence fasting gallbladder volume(168).

A higher baseline free testosterone in males determined incident gallstone disease and an increase of SHBG in males inversely determined incident gallstone disease in Study II(2). Associations for reproductive hormones have only been explored in two case-control studies previously, where luteinizing hormone was identified to have an inverse association with gallstones in males(169) and no other associations for female or male reproductive hormones were identified(169, 170). On a population level, there is an age-related decline in testosterone levels in males, which is paralleled by an age-related increase in SHBG(171). The results of Study II(2) may, therefore, suggest that male testosterone levels determine incident gallstones.

Experimental research has demonstrated that bile cholesterol saturation increases in female rodents with administration of testosterone while castration of male rodents decreases it(172, 173). These findings indicate analogous effects of female and male reproductive hormones on bile cholesterol saturation and analogy is further supported by the similar steroid hormone structures, intracellular pathways through nuclear receptors, and by regulation of gene expression. A number of studies have also explored the effects of administered female reproductive hormones on gallstone disease in males. Male sex offenders treated with progesterone had higher clinical gallstone disease prevalence(174). Further, estrogen treatment of males with prostate cancer when compared to placebo or orchiectomy was associated with cholecystectomy detected at autopsy(175) and screen-detected gallstone disease(176), respectively. These studies all were limited by insufficient designs and completion of follow-up and are therefore only preliminary. Future cohort studies should explore hormone analogy further and the impact of lifetime exposure to reproductive hormones in both females and males in order to detect possible targets for gallstone disease prevention or treatment.

Systemic inflammation

Biomarkers of systemic inflammation such as C-reactive protein were associated with gallstone disease prevalence in Study IV(4). Previous cross-sectional and case-control studies have explored the impact of C-reactive protein or white blood cell count on gallstone disease without identifying any significant associations(153, 177, 178). The identified associations in Study IV(4) are therefore novel findings. Case-control and cohort studies including clinical populations have only associated gallstone disease with immunological diseases such as rheumatoid arthritis or incident psoriasis without exploring biomarkers of systemic inflammation(179, 180).

The possible role of the immune system in gallstone formation has only recently been reviewed(181). In animals fed by cholesterol-rich diets, the appearance of cholesterol crystals in gallbladder bile has been associated with local inflammation of the gallbladder wall(182) with infiltration of inflammatory cells(183), suggesting that local inflammation is an early event in gallstone formation. Further, epithelial cell proliferation and increasing gallbladder wall thickness caused by cell infiltration appear before stone formation(183, 184) and has been associated with impaired gallbladder motility(183). These observations all suggest that local gallbladder inflammation might cause impaired gallbladder motor function. Whether the local inflammatory changes seen in the gallbladder during gallstone formation are associated with the systemic inflammation identified in Study IV(4) should be explored in future cohort studies.

Genetic susceptibility for gallstone disease

The single nucleotide polymorphisms of genes *MC4R* (rs17782313), *MAP2K5* (rs2241423), *NRXN3* (rs10146997), *HHEX* (rs1111875) were positively associated, while *FAIM2* (rs7138803) was inversely associated with gallstone disease prevalence in Study IV(4). A study of a Danish population found no association for single nucleotide polymorphisms *FTO* (rs9939609) or *MC4R* (rs17782313) and incident clinical gallstone disease, but an association was found for increasing number of *FTO* (rs9939609), *MC4R* (rs17782313), and *TMEM18* (rs6548238)(185). No other studies have explored associations for single nucleotide polymorphisms for *MAP2K5*, *NRXN3*, *HHEX*, *FAIM2* and gallstone disease yet. These findings are therefore novel to the existing literature and should be explored further in other cohorts in the future. Since the understanding of these genes in the regulation of obesity and diabetes type 2 is limited, it is preliminary to suggest biological mechanisms involved in the potential association with gallstone disease.

The apolipoprotein E4 allele was found inversely associated with gallstone disease prevalence in a dominant model exploring E4 allele homo- and heterozygote, but not associated in a recessive model exploring the E4 allele homozygote in Study IV(4). In a previous meta-analysis of observational studies including predominantly Chinese Han populations, the E4 allele was directly associated with gallstone disease in a dominant model(186). Another study performed in a Danish population found no association between apolipoprotein E genotypes and gallstone

disease(187). Meta-analysis performed in mixed ethnic populations or in subgroup meta-analysis restricted to white populations also found no significant associations for E4 allele carriers(187). Conflicting results are reported for the E4 association in Spanish and Hispanic populations(188-191). Biologically, the apolipoprotein E plays a critical role in controlling the response to dietary cholesterol and in cholesterol excretion to bile as demonstrated in knock-out mice(192). However, no impact on bile cholesterol excretion has been found for the E4 carrier state in Caucasians with gallstone disease(193). Results from the human studies and from Study IV(4) seem somewhat conflicting and may indicate that the effect of the E4 allele on gallstone disease depends on ethnicity. The E4 allele may have an association in Chinese or Hispanic populations, but probably only minor or no importance in northern European Caucasian populations such as the Danish. This suggested population dependent hypothesis should be explored further through a meta-analysis of existing studies.

Only one genome wide association study from 2007 has compared sequenced whole genomes of persons with and without gallstone disease to date. This study only identified association for one single nucleotide polymorphism D19H (rs11887534) for the gene *ABCG8* of the above mentioned cholesterol transporter with gallstone disease in a German population(194). This strong association has been replicated in both Danish and other populations(195, 196). Since then, only studies exploring associations for genes linked to other diseases or pathways with possible links to gallstone disease based on knowledge or suspicion have been performed without sequencing whole genomes – such as Study IV(4) of this PhD thesis. These studies have identified associations for the single nucleotide polymorphism of the bilirubin conjugating enzyme *UGT1A1* (rs6742078) with bilirubin content of gallstone and with gallstone disease in males(197, 198). A number of other single nucleotide polymorphisms associated with cholesterol metabolism and transport have also been found(199, 200).

METHODOLOGICAL CONSIDERATIONS

Biobanks are unique for exploration of new determinants based on knowledge obtained since original examinations were performed – the stored serum and blood samples from the included cohort's baseline and re-examinations enabled the exploration of new determinants for gallstone disease in the included study population. However, repeated freeze-thaw cycles during long-term storage of biological material may potentially cause denaturation or water evaporation. Such a bias could potentially cause both under- and overestimation of results(201). But analyses of blood or serum following multiple freeze-thaw cycles are reported reliable and reproducible for vitamin D(202), genetic material(203), reproductive hormones, and a number of biomarkers(201, 204, 205). Significant changes have only been identified for selected biomarkers with 30 freeze-thaw cycles or more(201), making the risk of such bias negligible in the studies performed for this PhD thesis.

Other potential bias when analyzing biobank material or data sampled decades ago may be the inability to identify or account for changing disease trends in the population that have occurred since the original examination. Although gallstone composition may differ with the underlying disease or with ethnicity, cholesterol gallstones have been the cause of clinical gallstone disease in both Denmark and other northern European countries for the past decades including the period of examinations of the study population(11-13). The epidemic of obesity has been present for the past decades and a study population sampled today would therefore have a higher BMI(206) which, presumably, would cause higher estimates of both prevalent and incident gallstone disease in absolute numbers. However, these changes would have no influence on the relative estimates obtained in the studies included in this PhD thesis.

A number of potential outcome and exposure misclassifications may be present in the material. At the outcome level, ultrasound examinations cannot discriminate cholesterol from pigment gallstones. With cholesterol gallstones being the dominating composition of gallstone disease in Denmark, a bias in the identification of determinants for gallstone disease is unlikely. At the exposure level, the assessment of lifestyle relied on participant self-report, which must be suspected to underreport detrimental lifestyle. Participants were uninformed about gallstone disease status following ultrasound examination and the assessment of lifestyle variables may therefore only have caused non-differential misclassification bias. Such bias are generally thought to cause estimates towards the null(207), which may have caused non-significant associations for tobacco smoking, physical activity level, and incident gallstone disease in the studies performed. Newer objective measures of lifestyle factors avoid information bias due to self-report and, presumably, would also improve the interpretation of results.

A general problem when performing population-based studies is non-participation which may cause a selected study population. In the studies of this PhD thesis, participation was 74-85% of people invited and alive (**Figure 2**). After the first re-examination, a follow-up of non-responders was performed and information was obtained in 78% through interview by telephone, postal questionnaire, or through autopsy reports on deceased. Clinically diagnosed gallstone disease in non-responders was no different than in responders and no gallstones were found in performed autopsies(96). Selection bias due to non-participation in studies of this thesis therefore seems unlikely.

The inability to explore temporal associations in the cross-sectional *studies III and IV* is the most important limitation of the studies in this PhD thesis. The identified temporal associations for BMI, non-HDL cholesterol, baseline free testosterone, and inverse temporal associations for alcohol consumption, SHBG and incident gallstone disease (*studies I and II*) may be weak from a statistical perspective since 95% CI were close to one. But all of these associations were for a low-unit increase of the variable on a continuous scale, i.e. increase in 1 kg/m2 for BMI or 1 mmol/L in non-HDL cholesterol, and causal associations are therefore still supported by these findings.

PERSPECTIVES FOR FUTURE RESEARCH

Cholecystectomy is currently considered the definitive treatment of clinical gallstone disease(208, 209) and laparoscopic cholecystectomy is one of the most common surgical procedures performed in the Nordic countries(210). High rates of approximately 6200 and 12900 laparoscopic cholecystectomies are performed every year in Denmark and Sweden, respectively(211, 212). Due to the ongoing obesity epidemic, an escalation of both incident and clinical gallstone disease is suspected in the coming years and the need of gallstone disease prevention will evidently be increasing. Although prevalence and incidence of gallstone disease are higher in northern compared to southern European populations,

currently no evidence of specific determinants explaining this difference exists. Preventive strategies should therefore be based on the known determinants confirmed in this PhD thesis and with ongoing research efforts focusing on identifying local and modifiable determinants.

Gallstone disease seems to be associated with increased mortality overall and due to cardiovascular disease, but cholecystectomy does not seem to alter this risk in cohort studies exploring screen-detected gallstone disease(213, 214). Screening for gallstone disease can therefore not be justified based on survival benefits of treatment. Further, a cohort study including only participants with screen-detected gallstone disease found that awareness of gallstone disease was independently associated with clinical gallstone disease(82). This study has been performed in a subgroup of the included study population of this thesis. Other cohort studies including screen-detected gallstone disease in aware populations have reported higher occurrence of clinical gallstone disease compared to the previously mentioned study(82). Although no randomized controlled trials of gallstone disease screening have been performed on a population level, one can assume that screening for gallstone disease will cause a rise in clinical gallstone disease without causing a survival benefit. Screening general populations for gallstone disease is therefore currently not justified.

Determinants for incident gallstone disease identified in this thesis have similarities with some of the determinants of cardiovascular disease. Interventions on dietary habits at the population level have included legislative changes such as taxation of unhealthy foods, which has been estimated to reduce cardiovascular disease mortality(215). Other promising legislative cardiovascular disease prevention strategies include a ban of junk food commercials, removing trans-fatty acids, increasing consumer awareness of unhealthy foods by food labelling, and nutritional criteria for schools and other institutions(215). Larger clinical or population-based studies including pharmacological lowering of non-HDL cholesterol with statins may also seem feasible in a near future(148, 149).

Future large-scale clinical or population based interventional trials for primary or secondary prevention of cardiovascular disease with aims of lowering weight or non-HDL cholesterol as mentioned above should be monitored for incident gallstone disease through abdominal ultrasound examinations. The assessment of only clinical gallstone disease is insufficient and may lead to biased conclusions as discussed in this PhD thesis. Based on the findings of this thesis, it is assumed that such preventive interventions for cardiovascular disease may decrease incident gallstone disease.

Based on findings in this PhD thesis, future cohort studies should explore associations for male reproductive hormones preferably in both males and females, systemic inflammation, genetic variations, and vitamin D, with the latter preferably being performed in mother-child cohorts with assessment of fetal exposure of vitamin D. Such population-based cohort studies or experimental research is required before preventive or interventional strategies can be suggested based on the identified cross-sectional associations. Randomized controlled trials aiming at lowering BMI or non-HDL cholesterol with use of statins or future weight-lowering drugs would be the next necessary step in order to strengthen the evidence of causality of the identified associations of this PhD thesis. Interventions aiming to increase alcohol consumption in alcohol abstainers or low consumers would also be relevant for prevention of both cardiovascular and gallstone disease, however such a trial may not be ethically feasible.

Besides exploring the identified cross-sectional associations prospectively, other emerging pathways should also be explored in the future studies. The role of the fecal microbiota in bile acid metabolism and the enterohepatic circulation has been discussed in this thesis, and a further and more detailed exploration of microbiota will become possible in the future. With the decreasing cost and increasing speed of DNA sequencing, new emerging sequencing methods such as 16S rRNA amplicon sequencing can be used to explore between-individual differences in fecal microbiota community structure(216). Studies using these techniques for exploration of gallstone disease have already been published(58). These studies continue the ongoing quest for determinants for gallstone disease and may represent the initiation of identifying new targets for future preventive strategies of gallstone disease.

CONCLUSIONS

It was possible to both confirm previously identified determinants and identify new determinants for incident gallstone disease in a non-selected Danish general-population cohort screened for gallstone disease with multiple ultrasound examinations. The previously identified determinants for incident gallstone disease including higher age, female sex, higher BMI, higher non-HDL cholesterol, and the inversely associated determinants including increasing alcohol consumption and cessation of hormone replacement therapy in females were confirmed. Newly identified determinants included free testosterone and increasing SHBG, the latter with an inverse association. Other lifestyle factors were not identified as determinants for incident gallstone disease. New associations for gallstone disease prevalence included biomarkers for systemic inflammation, genetic risk alleles for obesity or diabetes type 2, and fetal exposure to vitamin D.

Exploration of reproductive hormones should be repeated in other cohorts and the other newly identified associations for gallstone disease prevalence should be explored with cohort study designs in the future. This is necessary in order to identify new preventive strategies for gallstone disease. Future population based interventional studies including lowering of weight, lowering of non-HDL cholesterol, or alcohol consumption is supported by the findings of this PhD thesis. Population-based screening for gallstone disease is not recommended due to an assumed increase in clinical disease without survival benefit. Population-based interventional studies aiming to prevent cardiovascular disease should also include screening for gallstone disease with ultrasound examination. Other emerging targets for gallstone disease prevention or treatment such as the fecal microbiota should also be explored in the future.

Due to the ongoing epidemic of obesity and a foreseeable escalation of gallstone disease in the future, preventive strategies for gallstone disease should be developed in order to avoid a mass disease with escalation of health-care costs. This PhD thesis has suggested a number of new determinants and associations that through continuous research efforts may represent targets for prevention and treatment for gallstone disease one day.

SUMMARY

Gallstone disease is highly prevalent in Denmark and other countries of northern Europe, and cholecystectomy for the treatment of clinical gallstone disease is one the most frequently performed surgical procedures. Research efforts for the identification of

mechanisms involved in gallstone formation have a long history and the most established include bile cholesterol saturation, gallbladder motor function, and the enterohepatic circulation of secondary bile salts produced by fecal microbiota. A small number of determinants that are believed to affect these mechanisms have been identified until now. However, much of this research on determinants for gallstone disease has been hampered by insufficient study designs and by insufficient assessment of gallstone disease by only assessing the selected minority of people with clinical gallstone disease.

In a Danish general-population cohort screened for gallstone disease with multiple ultrasound examinations, it was possible to both confirm previously identified determinants and to identify new determinants for gallstone disease. Temporal associations for incident gallstone disease and female sex, BMI, non-HDL cholesterol, and inverse associations for increasing alcohol consumption and cessation of hormone replacement therapy in females were confirmed. New determinants included testosterone and increase in SHBG in males which had directly and inverse associations with incident gallstone disease, respectively. All of the identified determinants for incident gallstone disease found in this thesis can be linked to the three biological mechanisms of gallstone formation.

Other modifiable factors such as tobacco smoking, coffee consumption, dietary habits, physical activity, and blood pressure were not identified as determinants of incident gallstone disease in this thesis. Previous findings from other studies may be hampered by study design without exploration of temporal associations or due to selective assessment of gallstone disease. A common information bias for all existing literature exploring lifestyle habits and gallstone disease is the self-reported exposures which may cause misclassification bias. If explored in future studies, assessment of lifestyle habits should include objective measures in order to contribute any further to existing evidence on determinants for gallstone disease.

Associations for biomarkers of insulin resistance and gallstone disease prevalence were found. Insulin resistance probably mediates the association between BMI and gallstone disease. Although only cross-sectional, the association for both BMI and insulin resistance with gallstone disease seems well established based on existing experimental and observational evidence.

New cross-sectional associations for gallstone disease prevalence were identified for biomarkers of systemic inflammation, genetic risk for obesity or diabetes type 2, and for biomarkers of renal function. Levels of vitamin D were not identified as the cause of the higher northern European gallstone disease prevalence, although birth during season of low sun and vitamin D exposure seemed associated.

Future clinical or larger population-based interventional trials aiming at changing body weight, circulating levels of non-HDL cholesterol, or alcohol consumption are supported by the findings of this PhD thesis. Screening of gallstone disease through ultrasound examinations should be performed in future interventional trials aiming at preventing cardiovascular disease in order to monitor the effects of such interventions on gallstone formation and, further, to avoid the selection bias caused by just assessing clinical gallstone disease. Screening for gallstone disease on the population-level is not recommended due to an assumed increase in clinical gallstone disease without a survival advantage of treatment. Explorations of male reproductive hormones, biomarkers of systemic inflammation, circulating levels of vitamin D, and genetic risk alleles should be repeated in future cohort studies before these possible determinants may be subject for future strategies for prevention or treatment of gallstone disease.

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