[Frontiers in Bioscience 6, e154-167, November 1, 2001]

ROLE OF BILIARY PROTEINS AND NON-PROTEIN FACTORS IN KINETICS OF CHOLESTEROL CRYSTALLISATION AND GALLSTONE GROWTH

Milan Jirsa [1,2], Albert K. Groen [2]

[1] 1st Department of Internal Medicine, 1st Faculty of Medicine, Charles University, Praha, Czech Republic, [2] Laboratory of Experimental Hepatology, Department of Gastrointestinal and Liver Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

TABLE OF CONTENTS

1. ABSTRACT

Numerous biliary proteins as well as non-protein factors influence kinetics of cholesterol crystallisation from supersaturated bile. Cholesterol crystallisation pathways, methods used for quantitative evaluation of liquid-to-solid transition of cholesterol in bile and the classical concept of nucleation defect in pathogenesis of gallstones are reviewed in the first part of this article. The relevance of individual biliary proteins and non-protein factors is discussed in the second part. Finally, a new concept according to which accelerated crystallisation of cholesterol inhibits gallstone growth and protects the gallbladder wall from toxic injury resulting from the increased absorption and accumulation of cholesterol from supersaturated bile is suggested.

2. INTRODUCTION

The primary event on the way from gallstone free bile to cholesterol gallstones is supersaturation of bile with cholesterol. Additional defects appearing simultaneously in patients with cholesterol gallstones are gallbladder hypomotility, accelerated cholesterol crystallisation in bile and mucus hypersecretion. These additional defects may be balanced differentially in patients with solitary stones growing for many years (1, 2) and in patients with multiple small stones.

This review focuses on the role of protein factors influencing kinetics of cholesterol crystallisation in supersaturated bile. Composition of bile, physiology of bile secretion and physical-chemistry of bile are summarised briefly and the reader is referred to numerous recent reviews on these topics.

3. BILIARY LIPIDS

Bile salts, phospholipids, sterols and lipopigments are the major lipid species in bile (3, 4). Changes in their composition exceeding their physiological range are of crucial importance in pathogenesis of all types of gallstones. Biliary lipids are secreted from hepatocytes that are equipped with a series of transport systems

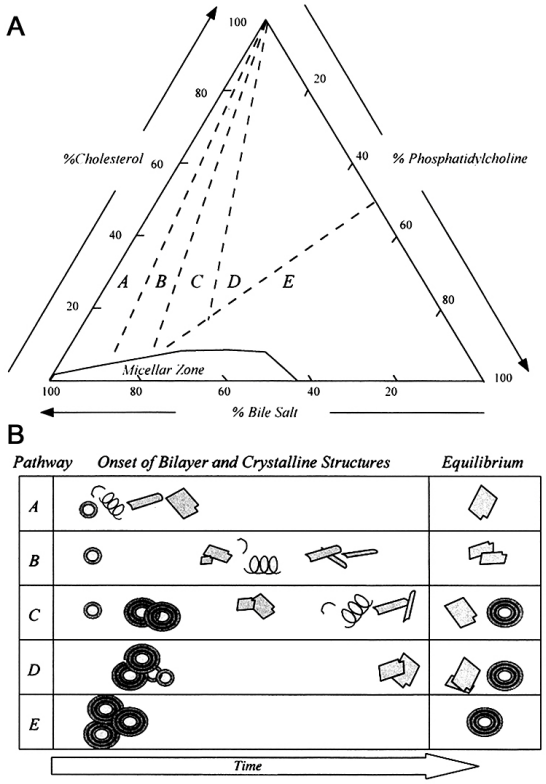

Figure 1. A: Quaternary phase diagram showing relative concentrations for which various crystallisation pathways occur. From ref. (164). B: Sequence of structures appearing during these pathways. For pathway A, arc-like crystals appear, followed by helices, tubules and plates. For pathway B, plates appear initially, followed by transient appearances of helices, tubules and plates. In pathway C, formation of microscopically observable vesicles precede the same sequence as in B. In pathway D, liquid crystals precede the development of plate. Pathway E denotes regions of the phase diagram where only liquid crystals are present. From ref. (164).

specialised for secretion of many kinds of compounds against a steep concentration gradient (see the recent reviews (5-8)).

Free water insoluble cholesterol (solubility of cholesterol in water is 10^{-8} mol/L) is present in bile in four types of liposomes (lipid aggregates) (3, 9): simple micelles, mixed micelles, unilamellar vesicles and multilamellar vesicles. The type of, the ratio between and the chemical composition of biliary lipid aggregates in their equilibrated water solution are influenced by multiple factors. The most important factors are the total concentrations of bile salts, phospholipids and cholesterol (3, 10-12), the concentrations of individual bile salts (13) and the degree of saturation of biliary phospholipids (14-16).

Phase and chemical composition of lipid aggregates have been studied extensively in model systems (17-19) and equilibrium phase diagrams have been constructed. Based on these studies, maximal equilibrium concentrations of cholesterol in the liquid phase were calculated, and the cholesterol saturation index (CSI) was defined to express the saturation rate of the liquid phase (20-22).

Bile is never in equilibrium *in vivo*. Unilamellar "nascent" vesicles composed of phospholipids and cholesterol are co-secreted with monomeric bile salts into canalicular bile and lipid secretion is followed by postcanalicular formation of micelles. Cholesterol and, to a greater extent, phospholipids are shifted into mixed micelles in the gallbladder (23), and a continuous change in the composition of gallbladder bile is accompanied by rearrangement of biliary liposomes. Biliary liposomes are thought to be the main source of cholesterol excess in bile, supplying the sterol for both crystal formation and gallstone growth.

4. CHOLESTEROL CRYSTALLISATION IN BILE

4.1. Cholesterol crystallisation pathways

A classical description of the cholesterol crystallisation pathway based on the observation of Halpern *et al*. (24) involves two steps. Aggregation and fusion of vesicles supersaturated with cholesterol leads to formation of liquid crystals and plate-like crystals of cholesterol monohydrate nucleate and grow from liquid crystals. This concept has several weak points:

Firstly, a body of older literature (24-29), as well as several more recent studies (30-32), support the concept that biliary vesicles are the main precursors of newly formed cholesterol crystals. Phospholipid/cholesterol vesicles are enriched with cholesterol during post-canalicular formation of micelles, and, thus, may become the main cholesterol carriers in supersaturated bile. Owing to their size (33), cholesterol nuclei are most likely formed within the layers of vesicles, perhaps in special cholesterol rich domains (34), and then are fed with cholesterol from either vesicles or micelles (32). However, aggregation and/or fusion of vesicles has not been directly visualised in any of the most recent studies.

Secondly, liquid crystals do not always precede formation of solid cholesterol crystals. Classical plates with the missing edge birefringent in polarised light are not the only form of cholesterol crystals because, in some biles, transient forms of non-plate-like filamentous, helical, and tubular crystals of anhydrous cholesterol may appear under certain circumstances. Wang and colleagues (35) described 5 different pathways, named A to E, in their study on model biles (Figure 1). Sequences B, C and D have also been observed in native human biles (36). There is no explanation for these phenomena in the classical concept.

4.2. Measurement of cholesterol crystallisation in bile

Crystal observation time, inappropriately called nucleation time (NT) as critical cholesterol nuclei are too small to be detected, was the first method used in studies on the early phases of cholesterol crystallisation (45). Bile samples are ultracentrifuged to remove the already present

cholesterol crystals, the microscopically isotropic upper phases are incubated at 37°C, and aliquots are checked every 24 hours by polarisation microscopy for the presence of newly formed crystal(s). It is clear that the point at which cholesterol crystallisation occurs cannot be precisely defined, and this method thus possesses an intrinsic error. In addition, this method is only qualitative, which implicates another possible source of error. Attempts to quantify cholesterol crystallisation have been made, and a number of methods reflecting crystal occurrence as a function of time have been elaborated. Counting the number of crystals in a Burker's chamber in a polarisation microscope is a simple, more reproducible method than nucleation time, but it is also much more laborious. Less laborious but less sensitive turbidimetric assays (37, 38) and nephelometric assays (39) have been used in some studies. The spectrophotometric assay was also modified for use in microtiter plates (40). The weak point of turbidimetry or nephelometry is the assumption that all crystals are of approximately the same size. To overcome this problem, measurement of cholesterol crystal mass obtained by sequential gradient density ultracentrifugation of aliquots was reported as an alternative approach (41). A novel procedure for the measurement of gallstone growth rate has recently been described by van den Berg and co-workers (42). In this procedure, the growth rate is expressed as the change of the mass of multiple small human gallstones originating from the same patient during their incubation in the tested bile.

4.3. Kinetics of cholesterol crystallisation

Supersaturation of bile with cholesterol is not sufficient to produce cholesterol gallstones because highly supersaturated bile was found in many gallstone free subjects (43). In a study on Pima Indians, a population with the highest prevalence of cholesterol gallstones, it appeared to take many years to develop gallstones from highly supersaturated bile (44). Holan and co-workers (45) first reported striking differences between cholesterol crystal observation times of gallbladder biles of the same CSI. Crystals occurred over 5 (mean 15) days in biles from gallstone free subjects but in less than 4 days in biles from gallstone patients. No correlation was found between CSI and NT in the gallstone group and rapid nucleation was observed in several gallstone free patients with undersaturated bile. These findings indicated that CSI values calculated for the model system did not reflect completely the situation in native bile. Presence of factors influencing kinetics of cholesterol nucleation was suggested to explain the discrepancy.

Another argument supporting the concept of the role of a nucleation defect in the pathogenesis of gallstones is that nucleation of cholesterol in native bile is heterogenous (46), indicating that additional compound(s) or substance(s) promoting nucleation and/or growth of nuclei over their critical diameter must be involved to accelerate the process. For homogenous nucleation, higher CSI values (>3) have been predicted that are almost never found in native bile (46).

Nucleation defects play an important role in a subgroup of patients with multiple gallstones. While solitary gallstones develop over a time period of several years (1, 2, 47) and have been suggested to grow from free-floating crystal plates, formation of multiple stones requires an abrupt crystallisation event (47). Jungst and co-workers (48) found, indeed, that nucleation was faster in patients with multiple gallstones than in those with solitary gallstones.

There have always been arguments against the study of *in vitro* nucleation in the pathogenesis of gallstone disease. Firstly, it was argued that there is no method to completely remove critical nuclei or undetectable microcrystals of cholesterol from rapidly nucleating bile (49). The question arises why these nuclei or crystals do appear in biles of gallstone patients but do not appear in biles of the same CSI from controls (50-52). Secondly, if cholesterol nuclei are completely removed, measurement of *de novo* (re)nucleation represents an artificial condition irrelevant to the *in vivo* situation in which the growth rate of the yet existing crystals seems to be more important (49). Finally, there is the question of whether the nucleation influencing factors are independent of or just induced by supersaturation of bile with cholesterol.

5. CHOLESTEROL CRYSTALLISATION PROMOTING AND INHIBITING FACTORS

A search for the principal nucleation-promoting factor was started by the observation of Burnstein and colleagues (53) that addition of a small amount (2-10%) of rapidly nucleating bile to a slowly crystallising bile decreased significantly nucleation time of the mixture. The role of a protein factor was postulated by Holzbach *et al*. (54), who reported an increase in nucleation time by a mixture of biliary proteins supplemented with biliary lipids. Subsequently, a long list of biliary constituents has been checked for their possible role in the acceleration or inhibition of cholesterol crystallisation.

5.1. Non-protein regulators of cholesterol crystallisation

Cholesterol nucleation regulating factors can be divided into three groups:

Firstly, certain parameters of lipid composition of bile such as total lipid concentration, hydrophobicity of bile salts, bile salt/lecithin ratio and cholesterol/lecithin ratio of vesicles have been proven to influence kinetics of cholesterol crystallisation (55).

Secondly, calcium salts, contamination of bile by microorganisms and biliary sludge have been proposed as non-protein regulators of cholesterol crystallisation.

Calcium salts, mostly bilirubinates and phosphates, are regularly found in centres of cholesterol gallstones (56-58), and, together with mucin and other biliary proteins, in concentric shell-like layers (59). Formation of these layers is explained by precipitation of calcium carbonate in an organic matrix, a process similar to biomineralisation seen in other tissues, such as bones and teeth. Low molecular weight biliary calcium-binding proteins, known as APF/CBP (anionic protein

fraction/calcium binding protein), have been reported to play a regulatory role in the biomineralisation of cholesterol gallstones (for review, see (60)). Ostrow proposed a hypothesis about the role of both calcium and APF/CBP in cholesterol nucleation, incorporating both the involvement of calcium in the process of formation of the gallstone nidus and the effect of APF/CBP as a regulator of calcium precipitation (57). Indeed, in model biles, APF inhibited cholesterol mass precipitation rate but not cholesterol crystal occurrence time, while calcium had the opposite effect (61). A rapid precipitation of calcium phosphates, seeded in mucin-containing model bile, induced rapid crystallisation of cholesterol (62). Whether calcium also plays a role in native bile is questionable, because addition of EDTA to native bile did prevent precipitation of calcium bilirubinate but did not influence cholesterol crystal occurrence time (36, 63).

The finding of calcium palmitate in the core of some cholesterol gallstones implicated a potential role of bacteria, in particular *Escherichia coli*, in the initial phase of cholesterol gallstone disease (64). Swidsinski and colleagues have reported the presence of bacterial DNA in the centres, but not in the peripheries of cholesterol gallstones (65, 66). Though doubted by some (66, 67), the presence of bacteria in gallbladder bile in the early phase of stone formation is not unlikely. It can be concluded either that bacteria directly participate in the formation of the nucleus of cholesterol gallstone or that the nidus appears as a consequence of infection or inflammation induced by these bacteria. On the other hand, simultaneous occurrence of both events cannot be excluded.

Biliary sludge represents an amorphous substance composed of crystals of cholesterol monohydrate, calcium salts of unconjugated bilirubin and mucus (68). Sludge can be visualised by ultrasonography in subjects under different conditions, such as pregnancy (69), rapid weight loss, ceftriaxone therapy, octreotide therapy, bone marrow or solid organ transplantation (70) and, importantly, in patients on long-term parenteral nutrition (71). It may be a transient phenomenon, but, in a subgroup of individuals, pigment or cholesterol gallstones may occur (72). Sludge is important for stabilisation of aggregated vesicles and crystals, and it may act as a nidus during formation of the gallstone nucleus (73).

The third group of cholesterol nucleation regulatory factors is represented by mucin and non-mucin biliary proteins, which are discussed in the following section.

5.2. Biliary proteins involved in regulation of cholesterol nucleation and crystal growth

5.2.1. Mucin

Mucin is a highly glycosylated glycoprotein made up of approximately 80% carbohydrate and 20% protein. Mucin glycans are mostly O-linked, range from 8-20 sugars in length, and consist of neutral sugars which form a backbone to which sialic acid and sulfate esters might be attached at the periphery (74-79). All mucins also possess N-glycosylation sites. Nine *MUC* genes coding different mucin proteins, numbered MUC1 to MUC4, MUC5A, MUC5B, and MUC6 to MUC8, have been reported (77, 80, 81). The ubiquitously expressed (82) MUC1 is the main membrane-associated and non-gel-forming mucin. MUC5B and MUC3 are the major gel-forming mucins present in the gallbladder (83). Primary structure of MUC5B is organised into a N-terminal, a tandem repeat and a C-terminal domain. The N-terminal domain is composed of three cysteine enriched, poorly-glycosylated, von-Willebrand factor-like D1-D3 domains, containing motifs resembling sequences from adhesion molecules, the LDL receptor, and scavenger receptors (84). The tandem repeat domains contain 7 extensively glycosylated R domains, divided with 7 short cysteine-rich Cys subdomains (85). The C-terminal domain is again less glycosylated and contains a von-Willebrand factor-like D4 domain (86, 87). The structure of MUC3 is similar, but the cysteine-rich D domains resemble epidermal growth factor motifs (88-90). Gel-forming mucins polymerise through intermolecular disulfide linkages between cysteine-rich domains.

The primary function of gallbladder mucin is probably to protect the gallbladder mucosa from chemical or microbial injury (91). There are several reasons to believe that gallbladder mucin is also involved in early stages of the pathogenesis of cholesterol gallstone disease.

Firstly, as already mentioned in the previous section, mucin is frequently found in core parts of cholesterol gallstone (56).

Secondly, mucin hypersecretion is known to precede the formation of stones in both experimental animals (92-94) and humans (95) fed with cholesterol-enriched diet. The stimulus for hypersecretion must be present in bile, because, in prairie dogs fed with a lithogenic diet after cystic duct ligation, no hypersecretion of mucin in the gallbladder could be observed (92). Prostaglandins and their precursor, arachidonic acid, were the first pro-inflammatory mediators suggested to induce increased secretion of mucin (96-98). Despite these studies, the role of prostaglandins remains controversial, since no effect on mucin secretion was reported by others (99) and, in human clinical studies in which non-steroidal anti-inflammatory drugs were administered to patients on rapid-weight reducing diets (100) or after gallstone dissolution therapy (101), no reduction of the risk of gallstone development was observed. The influence of other biliary constituents, such as lysophosphatidylcholine (98), tumour necrosis factor alpha (102, 103) and cholesterol, on mucin secretion has also been studied. Accumulation of cholesterol in gallbladder wall has been shown to decrease membrane fluidity of biliary epithelia and, perhaps, increase generation of NO (83) by induction of inducible NO synthase, which is the principle isoform of NO synthetase expressed in the gallbladder (104). The role of NO as a mucin secretagogue has been demonstrated in other tissues (83).

Thirdly, mucin forms a gel layer, which serves as a matrix for biliary sludge, described above, in which cholesterol crystals nucleate (73).

Fourthly, in a number of studies, gallbladder mucin was found to act as promoter of cholesterol

crystallisation. This effect appeared to be both time and dose dependent (105, 106). No differences were found between cholesterol crystallisation activities of mucin purified from gallbladder of gallstone patients and control subjects, suggesting that there was no qualitative difference between the isolated mucin samples (107). An important property of mucin is its capability to bind biliary lipids (106) and a series of other hydrophobic compounds (108, 109). This property is lost after treatment of mucin with pronase that destroys selectively poorly glycosylated domains and abolishes its cholesterol crystallisation promoting activity (106). Afdhal and co-workers (110) demonstrated in model biles with fluorescently labeled vesicles that mucins accelerate both vesicle fusion and aggregation. While glycan domains reversibly promoted vesicle aggregation, hydrophobic domains facilitated fusion of vesicles. However the second half of the hypothesis that large multilamellar vesicles generate crystals more rapidly in presence of mucin remained unproven.

5.2.2. Non-mucin proteins in bile and how do they get there

Biliary proteins represent about 4% of biliary solutes by weight (3). The most abundant biliary proteins are albumin, mucin, APF/CBP and IgA.

There are several sources of biliary proteins (111). The majority of biliary proteins originate from serum. Serum proteins are thought to be secreted into bile via a paracellular pathway across tight junctions between hepatocytes and biliary epithelia (111, 112). The driving force for this secretion is the concentration gradient. The total protein concentration in hepatic bile is about 50 times lower than in serum. The diffusion rate of individual biliary proteins is inversely proportional to their molecular weight (113). A significant part of biliary albumin is secreted into bile directly from hepatocytes (114). The same may be true for other biliary serum derived proteins.

Transcytosis is another pathway by which serum proteins can get into bile. This process is started by pinocytosis or receptor-mediated endocytosis of certain proteins at the sinusoidal pole of hepatocytes. Polymeric IgA receptor-mediated endocytosis is a well studied pathway responsible for clearance of serum IgA in rats (115-119). A part of the extracellular domain of polymeric IgA receptor, known as secretory component, is cut by a specific protease and remains bound to secreted IgA (120).

A minor part of biliary proteins are canalicular membrane proteins that are either extracted from the canalicular membrane by bile acids or co-secreted into bile with phospholipids (121).

Finally, lysosomal enzymes are secreted into bile via exocytosis (122-124).

5.2.3. Cholesterol crystallisation inhibitors

Since the bile of patients without gallstones was frequently found to be supersaturated with cholesterol, the presence of cholesterol crystallisation inhibitors was expected in such biles. Holzbach *et al*. (54) found indeed a protein fraction that inhibited cholesterol crystallisation. Apolipoproteins A-I and A-II appeared to be good candidates in model systems (125). Busch *et al*. (126) confirmed that Apo A-I both increased the cholesterol crystal occurrence time and reduced the rate of crystal growth. The molecular mechanism of these effects was partially elucidated by Yamashita and co-workers (127, 128), who observed the interaction of commercially available Apo A-I with vesicles, but not with micelles in model systems. The effect of Apo A-I on cholesterol nucleation and crystal growth was explained as stabilization of the cholesterol-rich vesicles and prevention of their fusion. These findings further supported the conclusions of the earlier sequential transmission electron microscopy study by Tao *et al*. (129). To circumvent the objections that all the above listed studies were made either with commercial or partially denatured biliary Apo A-I, Apo A-I was isolated from bile under non-denaturing conditions, its vesicle stabilising effect was confirmed, and a novel function of Apo A-I as promoter of cholesterol absorption in the gallbladder was proposed (130). However, one argument against the role of apolipoproteins A-I and A-II in cholesterol gallstone formation in vivo still remains: no differences were found between apoprotein concentrations in gallstone patients and controls (131).

Holzbach's group has reported two additional cholesterol crystallisation inhibitors. A 128kDa glycoprotein composed of 58- and 63-kDa subunits was isolated by affinity chromatography using a *Helix Pomatia* lectin affinity column (132). A low-molecular (15kDa) biliary protein that forms dimers and tetramers appeared to be capable of inhibiting cholesterol crystallisation (133). In both studies, the reported proteins were not fully characterised, and no data on their biliary concentrations are available.

Finally, the inhibitory effect of IgA (134) and its components (135) on cholesterol crystal growth has been reported. While the relevance of the earlier study (135) seems to be low, because denaturation of IgA occurred during the isolation procedure, the crystal growth inhibitory effect of biliary IgA was clearly demonstrated in the later study (134), although only at low (1 and 10 mcg/ml) concentrations. In contrast, others (136, 137) reported an opposite effect of IgA on cholesterol nucleation. The explanation of these controversial findings may be that Upadhya *et al.* (136) used a different testing system, and Busch *et al.* (134) and Ahmed *et al.* (137) used IgA from different sources: Busch *et al*. utilized a sub-fraction of biliary IgA bound to *Helix Pomatia* and Ahmed *et al.* used IgA from colostrum.

5.2.4. Cholesterol crystallisation promoters

The first evidence for the presence of cholesterol crystallisation promoters in bile has been already mentioned (53). Using lectin affinity chromatography with Concanavalin A (Con A) Sepharose, a non-mucin cholesterol crystallisation-promoting biliary glycoprotein fraction was isolated by Groen *et al.* (138). This activity of the Con A-binding biliary glycoproteins was pronase-resistant and specific for biliary glycoproteins, because no

Table 1. Putative pro-nucleation proteins. Only well characterised proteins were included

Protein	Pronase resistance	Reference(s)
α_1-antichymotrypsin	yes	154
α_1-acid glycoprotein	no	141, 148
α_1-antitrypsin	no	154
CEACAM1-85	yes	155, 156
Aminopeptidase N	no	152, 153
Fibronectin	no	144
Haptoglobin	no	128, 147
Mucin	no	105, 106, 107, 108, 109
Phospholipase C	yes	149

activity could be observed after treatment of serum glycoproteins with pronase. The concept of the pronase-resistant crystallisation promoter was supported by the observation of Pattinson and Willis (139) that kinetics of cholesterol crystallisation in bile was totally unresponsive to digestion with pronase. However, in the same paper, the authors demonstrated that pronase destroyed all biliary proteins visible on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

A number of biliary glycoproteins contained in CABF have been found to possess the cholesterol crystallisation promoting activity (table 1).

Immunoglobulins IgM and IgG were found to be the most active candidates, shortening crystal detection time in both heat-inactivated bile (136, 140) and model bile (137, 141). The levels of immunoglobulins in bile were found to be increased in cholesterol gallstone patients (136, 140), and up to a twelvefold increase was reported in Chilean patients (142). However, immunoextraction of immunoglobulins from Con A-binding biliary glycoprotein fraction did not influence crystallisation activity (143). Moreover, immunoglobulins are sensitive to treatment with pronase (139).

A role for fibronectin as a promoter of cholesterol nucleation was suggested by Chijiiwa *et al* (144), but others have not confirmed their results (145).

The group of Holzbach found cholesterol crystallisation-promoting activity in several acute phase proteins (146, 147). The concentration of alpha-1-acid glycoprotein was increased in the bile of gallstone patients (148), and its extraction by immunoaffinity chromatography decreased the activity by 33% (141). Unfortunately, no effect of immunoextraction of alpha-1-acid glycoprotein from Con A-binding fraction of biliary glycoproteins could be observed (143).

Pattinson and Willis (149) found a pronase-resistant C-like phospholipase activity that revealed high cholesterol crystallisation-promoting activity. In opposition to that, no activity of the immunoaffinity purified phospholipase C was detected (141).

A set of biliary glycoproteins associated with biliary vesicles has been described by Miquel and co-workers (150, 151). Beside albumin and immunoglobulins, four hydrophobic glycoproteins, with molecular weights, which were 130, 114, 85 and 62-67kDa on SDS-PAGE gel under non-reducing conditions, were found. Three of them had a significant crystallisation-promoting activity in model bile. The 130kDa glycoprotein was later characterised as aminopeptidase N (152). However, no differences were found in concentration of aminopeptidase N in biles of gallstone patients versus controls, nucleation time correlated only with relative concentrations of aminopeptidase N in the gallstone group (153) and no immunoextraction experiments have been reported to our knowledge.

Finally, Ziljstra and co-workers performed a series of experiments in order to isolate and characterise the pronase-resistant glycoprotein crystallisation promoters from bile (154). The Con A-binding fraction of biliary glycoproteins was digested with pronase and subsequently fractionated on a superose gel-permeation column. Two peaks, in the void volume and at 60kDa, contained the activity. The 60kDa peak contained a number of fragments derived from the protease inhibitors alpha-1-antitrypsin and alpha-1-antichymotrypsin. The void volume peak contained biliary lipids and an 85kDa protein similar in composition to the so-called low- density lipid-protein particle isolated from the Con A-binding fraction of biliary glycoproteins by de Bruijn *et al*. (143). The 85kDa pronase-resistant glycoprotein has recently been identified as the 85kDa isoform of carcinoembryonic antigen-related cell adhesion molecule 1 (155). Surprisingly, concentrations of total carcinoembryonic antigen (CEA) cross-reacting protein in gallbladder bile were found to be lower in bile of patients with either cholesterol and pigment gallstones than in controls without gallstones who underwent operations for pancreatic cancer, ampullomas and chronic pancreatitis (156). When biliary CEA cross-reacting proteins were fractionated by SDS-PAGE and their relative concentrations were measured densitometrically on immunoblots, two isoforms of CEA-related cell adhesion molecule 1 (115 and 85kDa, respectively) and two, as-yet unreported CEA cross-reacting proteins of MW 40 and 50kDa were found. The increase of these proteins, specifically of the 50kDa protein, could partially explain the difference in total CEA cross-reacting protein in gallstone and non-gallstone patients.

6. RELEVANCE OF PRO- AND ANTI-NUCLEATING FACTORS

To evaluate the role of individual biliary proteins in this process, Harvey and Strasberg (157) formulated criteria that must be met by any substance functioning as promoter of cholesterol nucleation: (a) the substance should be present in fast-crystallising bile with either a quantitative or qualitative difference from normal bile; (b) upon

extraction of the substance from fast-crystallising bile, the crystallisation should be delayed; and (c) when the substance is added to slowly crystallising bile, the crystallisation should be accelerated. A fourth criterion that applied specifically to proteins was added by van den Berg and Groen (42): (d) the protein must be resistant to digestion with pronase.

The first criterion, a difference in concentration or quality of protein, has never been tested strictly, for ethical reasons. The only proteins that have been checked against the remaining criteria and have been subjected to all available tests are biliary mucin and IgG. Although these proteins clearly stimulated crystallisation in model systems, extraction from native bile failed to inhibit crystallisation (107, 142). In addition, the promoting activity of both proteins was sensitive to pronase digestion (154). Keulemans *et al*. (158) demonstrated that extraction of the combined Con A-binding fraction in bile does decrease crystallisation strongly in biles from patients with rapidly crystallising biles. Addition of this fraction has been shown to stimulate crystallisation in native bile (138), and the activity was absent in biles from control patients (159). In addition, the activity was fully resistant to pronase.

Despite intensive research, the individual factors responsible for this activity have not been fully identified. Potential candidates are alpha-1-antichymotrypsin, phospholipase C-like activity and a CEACAM1-85-containing low density particle. Since the concentarions of these factors did not differ significantly between biles from stone patients and controls, the quantity is probably not the discriminating factor. Whether the quality is different remains to be established. It can also not be excluded that the promoting activity is the result of an interaction between the different factors.

It has generally been assumed that accelerated crystallisation of cholesterol in bile plays an imporatnt role in cholesterol stone formation. The recent work of van den Berg *et al.* (160) has shed a different light on this paradigm. In studies on regulation of gallstone growth, promoters were found to inhibit crystallisation on the stone surface, probably because the accelerated crystallisation in the aqueous phase rapidly reduces cholesterol supersaturaion. Apparently, crystallisation on the stone surface and crystallisation in the aqueous phase are two independent processes that compete for the excess cholesterol present in bile. Supersaturated bile has been shown to be toxic to the gallbladder (161). Stone formation and production of crystallisation promoters may be a response of the gallbladder to dispose of the toxic agent. Irritation of the galbladder wall by supersaturated bile may induce local (insoluble) mucin hypersecretion which provides a nidus on which excess cholesterol can readily crystallise. A subsequent production of cholesterol crystallisation promoters may represent an additional response of the liver and/or gallbladder to cholesterol supersaturated bile. By stimulating precipitation of cholesterol in the aqueous phase, deposition in the gallbladder wall or in stones is prevented. The crystallised cholesterol is probably not reabsorbed, and such a mechanism would serve to efficiently remove cholesterol from the body. This pathway of cholesterol elimination may explain the negative correlation which has been found between occurence of gallstone disease and levels of serum HDL, the preferred source of biliary cholesterol. Furthemore, production of crystallisation promoters limits stone growth, so that the small stones can still be expelled from the gallbladder together with floating crystals. Clearly, this attractive scheme is highly speculative, and much additional research is required to investigate these hypotheses further.

7. PERSPECTIVES

Biliary proteins do play a role in kinetics of cholesterol crystallisation particularly in the bile of patients with multiple gallstones. Research based on the classical concept of cholesterol nucleation promoters and inhibitors evaluating their individual role according to the criteria postulated by Harvey and Strasberg (157) led to the identification of a long list of putative important factors. However, none of these factors has been unequivocally linked to either cholesterol gallstone disease or to a subgroup of patients with multiple and rapidly developing gallstones in which the defect of nucleation should play a very important role. The failure of this classical approach can be explained by the fact that formation of bile is an extremely complex process under control of numerous genetic and environmental factors. The relative importance of the individual factors might thus be different between patients and/or groups of patients suffering from phenotypically the same gallstone disease. A rigorous genetic approach, quantitative trait loci mapping for cholesterol gallstones, applied on gallstone resistant AKR/J and gallstone sensitive C57L/J mouse strains fed with lithogenic diet, has led to identification of an array of candidate Lith genes which seem to play a role in the disease, at least in mice (162, 163). A similar approach based on gene linkage studies in carefully selected populations, in which environmental factors should be standardised as much as possible, is clearly required for successful analysis of the etiology of such a complex disease in humans. Understanding of the genes involved will probably lead to identification of the key players involved in the management of the lipid composition of bile and the most relevant regulator(s) of cholesterol crystallisation. Subsequent elucidation of the interaction with environmental factors may point the way to the development of novel strategies for the prevention of gallstone disease.

8. ACKNOWLEDGEMENT

M. Jirsa was supported by the Research Fellowship from the European Association for the Study of the Liver (EASL).

9. REFERENCES

1. Lowenfels A. B, A. M. Walker, D. P. Althaus, G. Townsend & L. Domellof: Gallstone growth, size, and risk of gallbladder cancer: an interracial study. *Int J Epidemiol* 18, 50-54 (1989)

2. Mok H. Y, E. R. Druffel & W. M. Rampone: Chronology of cholelithiasis. Dating gallstones from atmospheric radiocarbon produced by nuclear bomb explosions. *N Engl J Med* 314, 1075-1077 (1986)

3. Hay D. W. & M. C. Carey: Pathophysiology and pathogenesis of cholesterol gallstone formation. *Semin Liver Dis* 10, 159-170 (1990)

4. Hay D. W. & M. C. Carey: Chemical species of lipids in bile. *Hepatology* 12, 6S-14S (1990)

5. Kullak-Ublick G. A, U. Beuers & G. Paumgartner: Hepatobiliary transport. *J Hepatol* 32, 3-18 (2000)

6. Jansen P. L. & M. Muller: Genetic cholestasis: lessons from the molecular physiology of bile formation. *Can J Gastroenterol* 14, 233-238 (2000)

7. Cohen D. E.: Hepatocellular transport and secretion of biliary lipids. *Curr Opin Lipidol* 10, 295-302 (1999)

8. Elferink R. P. & A. K. Groen: The mechanism of biliary lipid secretion and its defects. *Gastroenterol Clin North Am* 28, 59-74 (1999)

9. Carey M. C. & J. T. Lamont: Cholesterol gallstone formation. 1. Physical-chemistry of bile and biliary lipid secretion. *Prog Liver Dis* 10, 139-163 (1992)

10. Somjen G. J. & T. Gilat: Changing concepts of cholesterol solubility in bile. *Gastroenterology* 91, 772-775 (1986)

11. Donovan J. M. & M. C. Carey: Physical-chemical basis of gallstone formation. *Gastroenterol Clin North Am* 20, 47-66 (1991)

12. Carey M. C.: Pathogenesis of gallstones. *Recenti Prog Med* 83, 379-391 (1992)

13. Donovan J. M, A. A. Jackson & M. C. Carey: Molecular species composition of inter-mixed micellar/vesicular bile salt concentrations in model bile: dependence upon hydrophilic-hydrophobic balance. *J Lipid Res* 34, 1131-1140 (1993)

14. Halpern Z, M. Moshkowitz, H. Laufer, Y. Peled & T. Gilat: Effect of phospholipids and their molecular species on cholesterol solubility and nucleation in human and model biles. *Gut* 34, 110-115 (1993)

15. Jungst D, T. Lang, P. Huber, V. Lange & G. Paumgartner: Effect of phospholipids and bile acids on cholesterol nucleation time and vesicular/micellar cholesterol in gallbladder bile of patients with cholesterol stones. *J Lipid Res* 34, 1457-1464 (1993)

16. Cohen D. E. & M. C. Carey: Lecithin acyl chain unsaturation modulates distribution of biliary lecithin between micelles and vesicles as well as micellar cholesterol solubility and cholesterol/lecithin ratio of vesicles on bile. *Gastroenterology* 98, A133 (1990)

17. Small D. M, M. C. Bourges & D. G. Dervichian: The biophysics of lipidic associations. I. The ternary systems: lecithin-bile salt-water. *Biochim Biophys Acta* 125, 563-580 (1966)

18. Bourges M, D. M. Small & D. G. Dervichian: Biophysics of lipidic associations. II. The ternary systems: cholesterol-lecithin-water. *Biochim Biophys Acta* 137, 157-167 (1967)

19. Small D. M, M. Bourges & D. G. Dervichian: Ternary and quaternary aqueous systems containing bile salt, lecithin, and cholesterol. *Nature* 211, 816-818 (1966)

20. Carey M. C.: Critical tables for calculating the cholesterol saturation of native bile. *J Lipid Res* 19, 945-955 (1978)

21. Metzger A. L, S. Heymsfield & S. M. Grundy: The lithogenic index - a numerical expression for the relative lithogenicity of bile. *Gastroenterology* 62, 499-501 (1972)

22. Redinger R. N. & D. M. Small: Bile composition, bile salt metabolism and gallstones. *Arch Intern Med* 130, 618-630 (1972)

23. Little T. E, S. P. Lee, H. Madani, E. W. Kaler & K. Chinn: Interconversions of lipid aggregates in rat and model bile. *Am J Physiol* 260, G70-79 (1991)

24. Halpern Z, M. A. Dudley, A. Kibe, M. P. Lynn, A. C. Breuer & R. T. Holzbach: Rapid vesicle formation and aggregation in abnormal human biles. A time-lapse video-enhanced contrast microscopy study. *Gastroenterology* 90, 875-885 (1986)

25. Harvey P. R, G. Somjen, M. S. Lichtenberg, C. Petrunka, T. Gilat & S. M. Strasberg: Nucleation of cholesterol from vesicles isolated from bile of patients with and without cholesterol gallstones. *Biochim Biophys Acta* 921, 198-204 (1987)

26. Schriever C. E. & D. Jungst: Association between cholesterol-phospholipid vesicles and cholesterol crystals in human gallbladder bile. *Hepatology* 9, 541-546 (1989)

27. Halpern Z, M. A. Dudley, M. P. Lynn, J. M. Nader, A. C. Breuer & R. T. Holzbach: Vesicle aggregation in model systems of supersaturated bile: relation to crystal nucleation and lipid composition of the vesicular phase. *J Lipid Res* 27, 295-306 (1986)

28. Peled Y, Z. Halpern, R. Baruch, G. Goldman & T. Gilat: Cholesterol nucleation from its carriers in human bile. *Hepatology* 8, 914-918 (1988)

29. Peled Y, Z. Halpern, B. Eitan, G. Goldman, F. Konikoff & T. Gilat: Biliary micellar cholesterol nucleates via the

vesicular pathway. *Biochim Biophys Acta* 1003, 246-249 (1989)

30. Kaplun A, F. M. Konikoff, A. Eitan, M. Rubin, A. Vilan, D. Lichtenberg, T. Gilat & Y. Talmon: Imaging supramolecular aggregates in bile models and human bile. *Microsc Res Tech* 39, 85-96 (1997)

31. Gantz D. L, D. Q. Wang, M. C. Carey & D. M. Small: Cryoelectron microscopy of a nucleating model bile in vitreous ice: formation of primordial vesicles. *Biophys J* 76, 1436-1451 (1999)

32. Konikoff F. M, D. Danino, D. Weihs, M. Rubin & Y. Talmon: Microstructural evolution of lipid aggregates in nucleating model and human biles visualized by cryogenic transmission electron microscopy. *Hepatology* 31, 261-268 (2000)

33. Duax W. L, J. F. Wawrzak, J. F. Griffin & C. Cheer: Sterol conformation and molecular properties. In: Biology of cholesterol. Eds: P. L. Yeagle, CRC Press, Boca Raton, 1-18 (1988)

34. MacDonald R. C.: Surface chemistry of phospholipid vesicles relevant to their aggregation and fusion. *Hepatology* 12, 56S-60S (1990)

35. Wang D. Q. & M. C. Carey: Complete mapping of crystallization pathways during cholesterol precipitation from model bile: influence of physical-chemical variables of pathophysiologic relevance and identification of a stable liquid crystalline state in cold, dilute and hydrophilic bile salt-containing systems. *J Lipid Res* 37, 606-630 (1996)

36. Wang D. Q. & M. C. Carey: Characterization of crystallization pathways during cholesterol precipitation from human gallbladder biles: identical pathways to corresponding model biles with three predominating sequences. *J Lipid Res* 37, 2539-2549 (1996)

37. Busch N, H. Tokumo & R. T. Holzbach: A sensitive method for determination of cholesterol growth using model solutions of supersaturated bile. *J Lipid Res* 31, 1903-1909 (1990)

38. Harvey P. R. & G. A. Upadhya: A rapid, simple high capacity cholesterol crystal growth assay. *J Lipid Res* 36, 2054-2058 (1995)

39. de Bruijn M. A, C. Noordam, B. G. Goldhoorn, G. N. Tytgat & A. K. Groen: The validity of the cholesterol nucleation assay. *Biochim Biophys Acta* 1138, 41-45 (1992)

40. Somjen G. J, Y. Ringel, F. M. Konikoff, R. Rosenberg & T. Gilat: A new method for the rapid measurement of cholesterol crystallization in model biles using a spectrophotometric microplate reader. *J Lipid Res* 38, 1048-1052 (1997)

41. Konikoff F. M, H. Laufer, G. Messer & T. Gilat: Monitoring cholesterol crystallization from lithogenic model bile by time-lapse density gradient ultracentrifugation. *J Hepatol* 26, 703-710 (1997)

42. van den Berg A. A, J. D. van Buul, J. D. Ostrow & A. K. Groen: Measurement of cholesterol gallstone growth in vitro. *J Lipid Res* 41, 189-194 (2000)

43. Holzbach R. T, M. Marsh, M. Olszewski & H. K.R.: Cholesterol solubility in bile. Evidence that supersaturated bile is frequent in healthy man. *J. Clin. Invest.* 52, 1467-1479 (1973)

44. Bennion L. J, W. C. Knowler, D. M. Mott, A. M. Spagnola & P. H. Bennett: Development of lithogenic bile during puberty in Pima indians. *N Engl J Med* 300, 873-876 (1979)

45. Holan K. R, R. T. Holzbach, R. E. Hermann, A. M. Cooperman & W. J. Claffey: Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology* 77, 611-617 (1979)

46. Lamont J. T. & M. C. Carey: Cholesterol gallstone formation. 2. Pathobiology and pathomechanics. *Prog Liver Dis* 10, 165-191 (1992)

47. Wolpers C. & A. F. Hofmann: Solitary versus multiple cholesterol gallbladder stones. Mechanisms of formation and growth. *Clin Investig* 71, 423-434 (1993)

48. Jungst D, T. Lang, C. von Ritter, E. Pratschke & G. Paumgartner: Cholesterol nucleation time in gallbladder bile of patients with solitary or multiple cholesterol gallstones. *Hepatology* 15, 804-808 (1992)

49. Apstein M. D. & M. C. Carey: Pathogenesis of cholesterol gallstones: a parsimonious hypothesis. *Eur J Clin Invest* 26, 343-352 (1996)

50. Sedaghat A. & S. M. Grundy: Cholesterol crystals and the formation of cholesterol gallstones. *N Engl J Med* 302, 1274-1277 (1980)

51. Whiting M. J. & J. Watts: Supersaturated bile from obese patients without gallstones supports cholesterol crystal growth but not nucleation. *Gastroenterology* 86, 243-248 (1984)

52. van Erpecum K. J, G. P. van Berge Henegouwen, B. Stoelwinder, M. F. Stolk, W. F. Eggink & W. H. Govaert: Cholesterol and pigment gallstone disease: comparison of the reliability of three bile tests for differentiation between the two stone types. *Scand J Gastroenterol* 23, 948-954 (1988)

53. Burnstein M. J, R. G. Ilson, C. N. Petrunka, R. D. Taylor & S. M. Strasberg: Evidence for a potent nucleating factor in the gallbladder bile of patients with cholesterol gallstones. *Gastroenterology* 85, 801-807 (1983)

54. Holzbach R. T, A. Kibe, E. Thiel, J. H. Howell, M. Marsh & R. E. Hermann: Biliary proteins. Unique

inhibitors of cholesterol crystal nucleation in human gallbladder bile. *J Clin Invest* 73, 35-45 (1984)

55. Holzbach R. T. & N. Busch: Nucleation and growth of cholesterol crystals. Kinetic determinants in supersaturated native bile. *Gastroenterol Clin North Am* 20, 67-84 (1991)

56. Malet P. F, C. E. Williamson, B. W. Trotman & R. D. Soloway: Composition of pigmented centers of cholesterol gallstones. *Hepatology* 6, 477-481 (1986)

57. Ostrow J. D.: Unconjugated bilirubin and cholesterol gallstone formation. *Hepatology* 12, 219S-224S (1990)

58. Taylor D. R, R. S. Crowther, J. C. Cozart, P. Sharrock, J. Wu & R. D. Soloway: Calcium carbonate in cholesterol gallstones: polymorphism, distribution, and hypotheses about pathogenesis. *Hepatology* 22, 488-496 (1995)

59. Malet P. F, N. E. Weston, B. W. Trotman & R. D. Soloway: Cyclic deposition of calcium salts during growth of cholesterol gallstones. *Scan Electron Microsc* 775-779 (1985)

60. Lafont H. & J. D. Ostrow: Calcium salt precipitation in bile and biomineralization of gallstones. In: Gallbladder and biliary tract diseases. Ed: N. H. Afdhal, Marcel Dekker, New York, 317-360 (2000)

61. Konikoff F. M, P. Lechene de la Porte, H. Laufer, N. Domingo, H. Lafont & T. Gilat: Calcium and the anionic polypeptide fraction (APF) have opposing effects on cholesterol crystallization in model bile. *J Hepatol* 27, 707-715 (1997)

62. van den Berg A. A, J. D. van Buul, G. N. Tytgat, A. K. Groen & J. D. Ostrow: Mucins and calcium phosphate precipitates additively stimulate cholesterol crystallization. *J Lipid Res* 39, 1744-1751 (1998)

63. Gallinger S, P. R. Harvey, C. N. Petrunka & S. M. Strasberg: Effect of binding of ionised calcium on the in vitro nucleation of cholesterol and calcium bilirubinate in human gall bladder bile. *Gut* 27, 1382-1386 (1986)

64. Vitetta L, A. Sali, V. Moritz, A. Shaw, P. Carson, P. Little & A. Elzarka: Bacteria and gallstone nucleation. *Aust N Z J Surg* 59, 571-577 (1989)

65. Swidsinski A, W. Ludwig, H. Pahlig & F. Priem: Molecular genetic evidence of bacterial colonization of cholesterol gallstones. *Gastroenterology* 108, 860-864 (1995)

66. Swidsinski A, M. Khilkin, H. Pahlig, S. Swidsinski & F. Priem: Time dependent changes in the concentration and type of bacterial sequences found in cholesterol gallstones. *Hepatology* 27, 662-665 (1998)

67. Soloway R. D. & R. S. Crowther: Bacteria and cholesterol gallstones: molecular biology comes to gallstone pathogenesis. *Gastroenterology* 108, 934-936 (1995)

68. Lee S. P. & J. F. Nicholls: Nature and composition of biliary sludge. *Gastroenterology* 90, 677-686 (1986)

69. Maringhini A, M. Ciambra, P. Baccelliere, M. Raimondo, A. Orlando, F. Tine, R. Grasso, M. A. Randazzo, L. Barresi, D. Gullo, M. Musico & L. Pagliaro: Biliary sludge and gallstones in pregnancy: incidence, risk factors, and natural history. *Ann Intern Med* 119, 116-120 (1993)

70. Ko C. W, J. H. Sekijima & S. P. Lee: Biliary sludge. *Ann Intern Med* 130, 301-311 (1999)

71. Fleming C. R.: Hepatobiliary complications in adults receiving nutrition support. *Dig Dis* 12, 191-198 (1994)

72. Werlin S. L. & J. P. Scott: Is biliary sludge a stone-in-waiting? *J Pediatr* 129, 321-322 (1996)

73. Carey M. C. & M. J. Cahalane: Whither biliary sludge? *Gastroenterology* 95, 508-523 (1988)

74. Tabak L. A.: Structure and function of human salivary mucins. *Crit Rev Oral Biol Med* 1, 229-234 (1990)

75. Strous G. J. & J. Dekker: Mucin-type glycoproteins. *Crit Rev Biochem Mol Biol* 27, 57-92 (1992)

76. Devine P. L. & I. F. McKenzie: Mucins: structure, function, and associations with malignancy. *Bioessays* 14, 619-625 (1992)

77. Seregni E, C. Botti, S. Massaron, C. Lombardo, A. Capobianco, A. Bogni & E. Bombardieri: Structure, function and gene expression of epithelial mucins. *Tumori* 83, 625-632 (1997)

78. Nieuw Amerongen A. V, J. G. Bolscher, E. Bloemena & E. C. Veerman: Sulfomucins in the human body. *Biol Chem* 379, 1-18 (1998)

79. Klein A, G. Strecker, G. Lamblin & P. Roussel: Structural analysis of mucin-type O-linked oligosaccharides. *Methods Mol Biol* 125, 191-209 (2000)

80. Shankar V, M. S. Gilmore, R. C. Elkins & G. P. Sachdev: A novel human airway mucin cDNA encodes a protein with unique tandem-repeat organization. *Biochem J* 300, 295-298 (1994)

81. Shankar V, P. Pichan, R. L. Eddy, Jr, V. Tonk, N. Nowak, S. N. Sait, T. B. Shows, R. E. Schultz, G. Gotway, R. C. Elkins, M. S. Gilmore & G. P. Sachdev: Chromosomal localization of a human mucin gene (MUC8) and cloning of the cDNA corresponding to the carboxy terminus. *Am J Respir Cell Mol Biol* 16, 232-241 (1997)

82. Ho S. B, G. A. Niehans, C. Lyftogt, P. S. Yan, D. L. Cherwitz, E. T. Gum, R. Dahiya & Y. S. Kim: Heterogeneity of mucin gene expression in normal and neoplastic tissues. *Cancer Res* 53, 641-651 (1993)

83. Offner G. D.: Gallbladder mucin. In: Gallbladder and biliary tract diseases. Ed: N. H. Afdhal, Marcel Dekker, New York, 211-233 (2000)

84. Offner G. D, D. P. Nunes, A. C. Keates, N. H. Afdhal & R. F. Troxler: The amino-terminal sequence of MUC5B contains conserved multifunctional D domains: implications for tissue-specific mucin functions. *Biochem Biophys Res Commun* 251, 350-355 (1998)

85. Desseyn J. L, V. Guyonnet-Duperat, N. Porchet, J. P. Aubert & A. Laine: Human mucin gene MUC5B, the 10.7-kb large central exon encodes various alternate subdomains resulting in a super-repeat. Structural evidence for a 11p15.5 gene family. *J Biol Chem* 272, 3168-3178 (1997)

86. Keates A. C, D. P. Nunes, N. H. Afdhal, R. F. Troxler & G. D. Offner: Molecular cloning of a major human gall bladder mucin: complete C-terminal sequence and genomic organization of MUC5B. *Biochem J* 324, 295-303 (1997)

87. Desseyn J. L, J. P. Aubert, I. Van Seuningen, N. Porchet & A. Laine: Genomic organization of the 3' region of the human mucin gene MUC5B. *J Biol Chem* 272, 16873-16883 (1997)

88. Gum J. R, Jr, J. J. L. Ho, W. S. Pratt, J. W. Hicks, A. S. Hill, L. E. Vinall, A. M. Roberton, D. M. Swallow & Y. S. Kim: MUC3 human intestinal mucin. Analysis of gene structure, the carboxyl terminus, and a novel upstream repetitive region. *J Biol Chem* 272, 26678-26686 (1997)

89. Crawley S. C, J. R. Gum, Jr, J. W. Hicks, W. S. Pratt, J. P. Aubert, D. M. Swallow & Y. S. Kim: Genomic organization and structure of the 3' region of human MUC3: alternative splicing predicts membrane-bound and soluble forms of the mucin. *Biochem Biophys Res Commun* 263, 728-736 (1999)

90. Gum J. R, J. W. Hicks, D. M. Swallow, R. L. Lagace, J. C. Byrd, D. T. Lamport, B. Siddiki & Y. S. Kim: Molecular cloning of cDNAs derived from a novel human intestinal mucin gene. *Biochem Biophys Res Commun* 171, 407-415 (1990)

91. Lamont J. T.: Mucus: the front line of intestinal mucosal defense. *Ann N Y Acad Sci* 664, 190-201 (1992)

92. Lee S. P.: Hypersecretion of mucus glycoprotein by the gallbladder epithelium in experimental cholelithiasis. *J Pathol* 134, 199-207 (1981)

93. Pemsingh R. S, B. R. MacPherson & G. W. Scott: Mucus hypersecretion in the gallbladder epithelium of ground squirrels fed a lithogenic diet for the induction of cholesterol gallstones. *Hepatology* 7, 1267-1271 (1987)

94. Malet P. F, S. Q. Deng & R. D. Soloway: Gallbladder mucin and cholesterol and pigment gallstone formation in hamsters. *Scand J Gastroenterol* 24, 1055-1060 (1989)

95. Lee S. P, J. T. LaMont & M. C. Carey: Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones. *J Clin Invest* 67, 1712-1723 (1981)

96. Lee S. P, M. C. Carey & J. T. LaMont: Aspirin prevention of cholesterol gallstone formation in prairie dogs. *Science* 211, 1429-1431 (1981)

97. LaMont J. T, B. S. Turner, D. DiBenedetto, R. Handin & A. I. Schafer: Arachidonic acid stimulates mucin secretion in prairie dog gallbladder. *Am J Physiol* 245, G92-98 (1983)

98. LaMorte W. W, J. T. LaMont, W. Hale, M. L. Booker, T. E. Scott & B. Turner: Gallbladder prostaglandins and lysophospholipids as mediators of mucin secretion during cholelithiasis. *Am J Physiol* 251, G701-709 (1986)

99. Cohen B. I, E. H. Mosbach, N. Ayyad, M. Yoshii & C. K. McSherry: Aspirin does not inhibit cholesterol cholelithiasis in two established animal models. *Gastroenterology* 101, 1109-1116 (1991)

100. Broomfield P. H, R. Chopra, R. C. Sheinbaum, G. G. Bonorris, A. Silverman, L. J. Schoenfield & J. W. Marks: Effects of ursodeoxycholic acid and aspirin on the formation of lithogenic bile and gallstones during loss of weight. *N Engl J Med* 319, 1567-1572 (1988)

101. Hood K, D. Gleeson, D. C. Ruppin & R. H. Dowling: Prevention of gallstone recurrence by non-steroidal anti-inflammatory drugs. *Lancet* 2, 1223-1225 (1988)

102. Cheung C. H, E. W. Moore, J. B. Prystowsky & R. V. Rege: TNFa stimulates mucus secretion in gallbladder epithelial cells. *Gastroenterology* 114, A1224 (1998)

103. Eder M. I, D. P. Nunes, N. H. Afdhal & G. D. Offner: TNFa stimulates mucin secretion, but not mucin synthesis, in human gallbladder epithelial cells. *Gastroenterology* 114, A1238 (1998)

104. Keaveny A. P, G. D. Offner & N. H. Afdhal: Inducible nitric oxide synthase (iNOS) is the principle isoform expressed in human gallbladder. *Gastroenterology* 114, A526 (1998)

105. Levy P. F, B. F. Smith & J. T. LaMont: Human gallbladder mucin accelerates nucleation of cholesterol in artificial bile. *Gastroenterology* 87, 270-275 (1984)

106. Smith B. F.: Human gallbladder mucin binds biliary lipids and promotes cholesterol crystal nucleation in model bile. *J Lipid Res* 28, 1088-1097 (1987)

107. Gallinger S, R. D. Taylor, P. R. Harvey, C. N. Petrunka & S. M. Strasberg: Effect of mucous glycoprotein on nucleation time of human bile. *Gastroenterology* 89, 648-658 (1985)

108. Smith B. F. & J. T. LaMont: Hydrophobic binding properties of bovine gallbladder mucin. *J Biol Chem* 259, 12170-12177 (1984)

109. Smith B. F. & J. T. LaMont: Bovine gallbladder mucin binds bilirubin in vitro. *Gastroenterology* 85, 707-712 (1983)

110. Afdhal N. H, N. Niu, D. P. Nunes, R. Bansil, X. X. Cao, D. Gantz, D. M. Small & G. D. Offner: Mucin-vesicle interactions in model bile: evidence for vesicle aggregation and fusion before cholesterol crystal formation. *Hepatology* 22, 856-865 (1995)

111. LaRusso N. F.: Proteins in bile: how they get there and what they do. *Am J Physiol* 247, G199-205 (1984)

112. Reuben A.: Biliary proteins. *Hepatology* 4, 46S-50S (1984)

113. Thomas P, C. A. Toth & N. Zamcheck: The mechanism of biliary excretion of alpha 1-acid glycoprotein in the rat: evidence for a molecular weight-dependent, nonreceptor-mediated pathway. *Hepatology* 2, 800-803 (1982)

114. Saucan L. & G. E. Palade: Differential colchicine effects on the transport of membrane and secretory proteins in rat hepatocytes in vivo: bipolar secretion of albumin. *Hepatology* 15, 714-721 (1992)

115. Fisher M. M, B. Nagy, H. Bazin & B. J. Underdown: Biliary transport of IgA: role of secretory component. *Proc Natl Acad Sci U S A* 76, 2008-2012 (1979)

116. Renston R. H, A. L. Jones, W. D. Christiansen, G. T. Hradek & B. J. Underdown: Evidence for a vesicular transport mechanism in hepatocytes for biliary secretion of immunoglobulin A. *Science* 208, 1276-1278 (1980)

117. Mestecky J. & J. R. McGhee: Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv Immunol* 40, 153-245 (1987)

118. Scott L. J. & A. L. Hubbard: Dynamics of four rat liver plasma membrane proteins and polymeric IgA receptor. Rates of synthesis and selective loss into the bile. *J Biol Chem* 267, 6099-6106 (1992)

119. Barr V. A. & A. L. Hubbard: Newly synthesized hepatocyte plasma membrane proteins are transported in transcytotic vesicles in the bile duct-ligated rat. *Gastroenterology* 105, 554-571 (1993)

120. Hughes G. J, S. Frutiger, L. A. Savoy, A. J. Reason, H. R. Morris & J. C. Jaton: Human free secretory component is composed of the first 585 amino acid residues of the polymeric immunoglobulin receptor. *FEBS Lett* 410, 443-446 (1997)

121. Groen A. K, M. J. Van Wijland, W. M. Frederiks, J. J. Smit, A. H. Schinkel & R. P. Oude Elferink: Regulation of protein secretion into bile: studies in mice with a disrupted mdr2 p-glycoprotein gene. *Gastroenterology* 109, 1997-2006 (1995)

122. Godfrey P. P, L. Lembra & R. Coleman: Effects of colchicine and vinblastine on output of proteins into bile. *Biochem J* 208, 153-157 (1982)

123. Rahman K. & R. Coleman: Effect of chloroquine on biliary lipid and lysosomal enzyme output in the isolated perfused rat liver at low bile salt output rates. *Biochim Biophys Acta* 922, 395-397 (1987)

124. Barnwell S. G. & R. Coleman: Abnormal secretion of proteins into bile from colchicine-treated isolated perfused rat livers. *Biochem J* 216, 409-414 (1983)

125. Kibe A, R. T. Holzbach, N. F. LaRusso & S. J. Mao: Inhibition of cholesterol crystal formation by apolipoproteins in supersaturated model bile. *Science* 225, 514-516 (1984)

126. Busch N, N. Matiuck, S. Sahlin, S. P. Pillay & R. T. Holzbach: Inhibition and promotion of cholesterol crystallization by protein fractions from normal human gallbladder bile. *J Lipid Res* 32, 695-702 (1991)

127. Yamashita Y, S. Tazuma & G. Kajiyama: Method for quantitative assessment of transformation of non-micellar cholesterol carriers in model bile systems. *J Gastroenterol Hepatol* 11, 864-869 (1996)

128. Yamashita G, R. Secknus, A. Chernosky, K. A. Krivacic & R. T. Holzbach: Comparison of haptoglobin and apolipoprotein A-I on biliary lipid particles involved in cholesterol crystallization. *J Gastroenterol Hepatol* 11, 738-745 (1996)

129. Tao S, S. Tazuma & G. Kajiyama: Fatty acid composition of lecithin is a key factor in bile metastability in supersaturated model bile systems. *Biochim Biophys Acta* 1167, 142-146 (1993)

130. Secknus R, G. H. Darby, A. Chernosky, T. Juvonen, E. W. Moore & R. T. Holzbach: Apolipoprotein A-I in bile inhibits cholesterol crystallization and modifies transcellular lipid transfer through cultured huma1n gall-bladder epithelial cells. *J Gastroenterol Hepatol* 14, 446-456 (1999)

131. Sewell R. B, S. J. Mao, T. Kawamoto & N. F. LaRusso: Apolipoproteins of high, low, and very low density lipoproteins in human bile. *J Lipid Res* 24, 391-401 (1983)

132. Ohya T, J. Schwarzendrube, N. Busch, S. Gresky, K. Chandler, A. Takabayashi, H. Igimi, K. Egami & R. T. Holzbach: Isolation of a human biliary glycoprotein inhibitor of cholesterol crystallization. *Gastroenterology* 104, 527-538 (1993)

133. Secknus R, G. Yamashita, S. Ginanni Corradini, A. Chernosky, C. Williams, L. Hays, M. A. Secknus & R. T. Holzbach: Purification and characterization of a novel human 15 kd cholesterol crystallization inhibitor protein in bile. *J Lab Clin Med* 127, 169-178 (1996)

134. Busch N, F. Lammert & S. Matern: Biliary secretory immunoglobulin A is a major constituent of the new group of cholesterol crystal-binding proteins. *Gastroenterology* 115, 129-138 (1998)

135. Busch N, F. Lammert, H. U. Marschall & S. Matern: A new subgroup of lectin-bound biliary proteins binds to cholesterol crystals, modifies crystal morphology, and inhibits cholesterol crystallization. *J Clin Invest* 96, 3009-3015 (1995)

136. Upadhya G. A, P. R. Harvey & S. M. Strasberg: Effect of human biliary immunoglobulins on the nucleation of cholesterol. *J Biol Chem* 268, 5193-5200 (1993)

137. Ahmed H. A, M. L. Petroni, M. Abu-Hamdiyyah, R. P. Jazrawi & T. C. Northfield: Hydrophobic/hydrophilic balance of proteins: a major determinant of cholesterol crystal formation in model bile. *J Lipid Res* 35, 211-219 (1994)

138. Groen A. K, J. P. Stout, J. A. Drapers, F. J. Hoek, R. Grijm & G. N. Tytgat: Cholesterol nucleation-influencing activity in T-tube bile. *Hepatology* 8, 347-352 (1988)

139. Pattinson N. R. & K. E. Willis: Nucleation of cholesterol crystals from native bile and the effect of protein hydrolysis. *J Lipid Res* 32, 215-221 (1991)

140. Harvey P. R, G. A. Upadhya & S. M. Strasberg: Immunoglobulins as nucleating proteins in the gallbladder bile of patients with cholesterol gallstones. *J Biol Chem* 266, 13996-14003 (1991)

141. Abei M, J. Schwarzendrube, H. Nuutinen, T. A. Broughan, P. Kawczak, C. Williams & R. T. Holzbach: Cholesterol crystallization-promoters in human bile: comparative potencies of immunoglobulins, alpha 1-acid glycoprotein, phospholipase C, and aminopeptidase N1. *J Lipid Res* 34, 1141-1148 (1993)

142. Miquel J. F, L. Nunez, L. Amigo, S. Gonzalez, A. Raddatz, A. Rigotti & F. Nervi: Cholesterol saturation, not proteins or cholecystitis, is critical for crystal formation in human gallbladder bile. *Gastroenterology* 114, 1016-1023 (1998)

143. de Bruijn M. A, K. S. Mok, C. P. Nibbering, T. Out, J. Van Marle, F. Stellaard, G. N. Tytgat & A. K. Groen: Characterization of the cholesterol crystallization-promoting low-density particle isolated from human bile. *Gastroenterology* 110, 1936-1944 (1996)

144. Chijiiwa K, A. Koga, T. Yamasaki, K. Shimada, H. Noshiro & F. Nakayama: Fibronectin: a possible factor promoting cholesterol monohydrate crystallization in bile. *Biochim Biophys Acta* 1086, 44-48 (1991)

145. Miquel J. F, C. Von Ritter, R. Del Pozo, V. Lange, D. Jungst & G. Paumgartner: Fibronectin in human gallbladder bile: cholesterol pronucleating and/or mucin "link" protein? *Am J Physiol* 267, G393-400 (1994)

146. Abei M, H. Nuutinen, P. Kawczak, J. Schwarzendrube, S. P. Pillay & R. T. Holzbach: Identification of human biliary alpha 1-acid glycoprotein as a cholesterol crystallization promoter. *Gastroenterology* 106, 231-238 (1994)

147. Yamashita G, S. Ginanni Corradini, R. Secknus, A. Takabayashi, C. Williams, L. Hays, A. L. Chernosky & R. T. Holzbach: Biliary haptoglobin, a potent promoter of cholesterol crystallization at physiological concentrations. *J Lipid Res* 36, 1325-1333 (1995)

148. Nuutinen H, M. Abei, J. Schwarzendrube, S. Ginanni Corradini, R. M. Walsh, P. Kawczak & R. T. Holzbach: Biliary alpha 1-acid glycoprotein concentrations in gallstone-free controls and in patients with multiple or solitary cholesterol gallstones. *Dig Dis Sci* 40, 1786-1791 (1995)

149. Pattinson N. R. & K. E. Willis: Phospholipase C and diacylglycerol lipase in human gallbladder and hepatic bile. *Gastroenterology* 99, 1798-1806 (1990)

150. Miquel J. F, A. Rigotti, E. Rojas, E. Brandan & F. Nervi: Isolation and purification of human biliary vesicles with potent cholesterol-nucleation-promoting activity. *Clin Sci (Colch)* 82, 175-180 (1992)

151. Miquel J. F, L. Nunez, A. Rigotti, L. Amigo, E. Brandan & F. Nervi: Isolation and partial characterization of cholesterol pronucleating hydrophobic glycoproteins associated to native biliary vesicles. *FEBS Lett* 318, 45-49 (1993)

152. Nunez L, L. Amigo, A. Rigotti, L. Puglielli, G. Mingrone, A. V. Greco & F. Nervi: Cholesterol crystallization-promoting activity of aminopeptidase-N isolated from the vesicular carrier of biliary lipids. *FEBS Lett* 329, 84-88 (1993)

153. Nunez L, L. Amigo, G. Mingrone, A. Rigotti, L. Puglielli, A. Raddatz, F. Pimentel, A. V. Greco, S. Gonzalez, J. Garrido, J. F. Miquel & F. Nervi: Biliary aminopeptidase-N and the cholesterol crystallisation defect in cholelithiasis. *Gut* 37, 422-426 (1995)

154. Zijlstra A. I, G. D. Offner, N. H. Afdhal, M. van Overveld, G. N. Tytgat & A. K. Groen: The pronase resistance of cholesterol-nucleating glycoproteins in human bile. *Gastroenterology* 110, 1926-1935 (1996)

155. Jirsa M, L. Muchova, L. Draberova, P. Draber, F. Smid, Z. Marecek, M. Kuroki & A. K. Groen: Identification of the 85 kDa glycoprotein in the cholesterol crystallization promoting low density particle as carcinoembryonic antigen like cell adhesion molecule 1 (CEACAM1). Abstract. *J. Hepatol.* 32, Suppl. 1, 120 (2000)

156. Jirsa M, L. Muchova, L. Draberova, P. Draber, P. Smid, Z. Marecek, M. Kuroki & A. K. Groen: Carcinoembryonic antigen-related cell adhesion molecule 1 is the 85 kDa pronase resistant biliary glycoprotein in the cholesterol crystallization promoting low density particle. Abstract. *J. Hepatol.* 34, Suppl. 1, 181 (2001)

157. Harvey P. R. & S. M. Strasberg: Will the real cholesterol-nucleating and -antinucleating proteins please stand up? *Gastroenterology* 104, 646-650 (1993)

158. Keulemans Y. C, K. S. Mok, J. F. Slors, M. A. Brink, D. J. Gouma, G. N. Tytgat & A. K. Groen: Concanavalin A-binding cholesterol crystallization inhibiting and promoting activity in bile from patients with Crohn's disease compared to patients with ulcerative colitis. *J Hepatol* 31, 685-691 (1999)

159. Miquel J. F, J. Van Der Putten, F. Pimentel, K. S. Mok & A. K. Groen: Increased activity in the biliary con A-binding fraction accounts for the difference in crystallization behavior in bile from Chilean gallstone patients compared with Dutch gallstone patients. *Hepatology* 33, 328-332 (2001)

160. van den Berg A. A. & A. K. Groen: Role of proteins in cholesterol crystallization in bile. In: Gallbladder and biliary tract diseases. Eds: N. H. Afdhal, Marcel Dekker, New York, 235-249 (2000)

161. Chen Q, J. Amaral, P. Biancani & J. Behar: Excess membrane cholesterol alters human gallbladder muscle contractility and membrane fluidity. *Gastroenterology* 116, 678-685 (1999)

162. Paigen B, N. J. Schork, K. L. Svenson, Y. C. Cheah, J. L. Mu, F. Lammert, D. Q. Wang, G. Bouchard & M. C. Carey: Quantitative trait loci mapping for cholesterol gallstones in AKR/J and C57L/J strains of mice. *Physiol Genomics* 4, 59-65 (2000)

163. Lammert F, M. C. Carey & B. Paigen: Chromosomal organization of candidate genes involved in cholesterol gallstone formation: a murine gallstone map. *Gastroenterology* 120, 221-238 (2001)

164. Konikoff F. M. & J. M. Donovan: Cholesterol crystallization in bile. In: Gallbladder and biliary tract diseases. Eds: N. A. Afdhal, Marcel Dekker, New York, 185-209 (2000)

Key Words: Cholesterol, Crystallization, Gallstones, Nucleation, Pronucleation Factors, Antinucleation Factors, Calcium, Biliary Proteins, Mucin, Non-Mucin Proteins, Review

Send correspondence to: Dr. Albert K. Groen, Laboratory of Experimental Hepatology, Department of GI and Liver Diseases, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands, Tel: 3120-566-4174 , Fax: 3120-566-9190, E-mail: a.k.groen@amc.uva.nl