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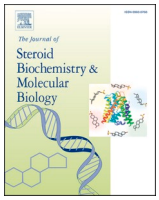

Unique sterol metabolite shifts in inflammatory bowel disease and primary sclerosing cholangitis

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ABSTRACT

Inflammatory bowel disease (IBD) triggers chronic intestinal inflammation and is linked to primary sclerosing cholangitis (PSC). Cholesterol homeostasis, tightly regulated under normal conditions, becomes disrupted in both inflammation and chronic liver disease. We analyzed fecal and serum levels of cholesterol synthesis precursors, oxysterols, and phytosterols in 87 patients with IBD (81 for serum analysis) including patients with Crohn's disease (CD) and ulcerative colitis (UC), 11 patients with PSC, 21 patients with PSC-IBD (18 for serum analysis), and 16 healthy controls (17 for serum analysis). Cholesterol was analysed by flow injection analysis on a high-resolution hybrid quadrupole-Orbitrap mass spectrometer and further serum sterols and all fecal sterols were analysed by a gas chromatograph mass spectrometer. Serum levels of lanosterol, 7-dehydrocholesterol, 7-beta-hydroxycholesterol, 27-hydroxycholesterol, and the plant sterols campesterol, stigmasterol, and sitosterol were similar across control and patient groups. Notably, serum lathosterol was elevated in CD patients compared to those with UC, PSC, PSC-IBD, and healthy controls. All other serum and fecal sterols showed no differences between CD and UC. Cholesterol synthesis precursors in serum, serum cholesterol levels, and both serum and fecal plant sterol levels decreased with increasing IBD severity. Consequently, serum cholesterol, campesterol, sitosterol, and fecal 5-beta sitostanol and 5-alpha sitostanol were negatively correlated with C-reactive protein and fecal calprotectin. The conversion of cholesterol to coprostanol in feces was impaired in IBD, PSC, and PSC-IBD, independent of bowel inflammation severity or liver disease extent. Patients with PSC, and to a lesser extent PSC-IBD, had elevated serum plant sterol levels, positively correlating with liver disease markers. In conclusion, in patients with IBD, cholesterol biosynthetic precursors, serum cholesterol levels, and fecal plant sterols decrease with intestinal inflammation. An inverse association of serum plant sterols with intestinal inflammation was observed in patients with IBD and a direct association of serum phytosterols with liver injury in patients with PSC. The conversion of fecal cholesterol to coprostanol was impaired in all patient cohorts. IBD and PSC alter serum sterol levels differently, whereas changes in fecal sterols are not disease specific and are moderate.

1. Introduction

Inflammatory bowel disease (IBD) is a serious condition with rising global incidence [1–3]. The pathogenesis of its most common forms, Crohn's disease (CD) and ulcerative colitis (UC), remains largely unknown [1–3]. Mucosal inflammation is a hallmark of active IBD, with C-reactive protein being used as a key serological marker, often elevated in IBD cases. Fecal calprotectin is another clinically relevant marker, indicating disease activity in UC and CD patients, with levels increasing during active disease [4].

Cholesterol homeostasis is disturbed in systemic inflammatory diseases, and serum cholesterol levels are low in patients with active IBD [5,6]. Cholesterol levels in the circulation are tightly regulated. Dietary intake, endogenous synthesis and fecal excretion of bile acids and cholesterol are coordinated to achieve cholesterol homeostasis [7]. The mechanisms that contribute to low serum cholesterol in patients with chronic inflammation are not yet understood [6,8].

In IBD, fecal levels of primary bile acids are elevated, while

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secondary bile acids are reduced compared to healthy controls [9,10]. This reduction in secondary bile acids results from dysbiosis in the gut microbiota. Fecal sterols originate from biliary cholesterol excretion and the trans-intestinal cholesterol elimination pathway [11]. Bacteria can convert cholesterol to coprostanol and coprostanone [7]. The fecal levels of cholesterol and coprostanol are higher in patients with UC compared with healthy controls [12], suggesting that low serum cholesterol in IBD is achieved by reduced intestinal absorption and/or increased biliary excretion of cholesterol [5].

Serum levels of plant sterols are commonly used as an indicator of cholesterol absorption, and low levels are supposed to reflect reduced intestinal absorption of sterols [13]. Plant sterols, also known as phytosterols, are integral components of the membrane lipid bilayer of plant cells. Each plant species has its own characteristic distribution of phytosterols, with the three most abundant natural phytosterols being campesterol, sitosterol and stigmasterol. Humans cannot synthesize plant sterols, so they must be obtained from the diet [14]. However, the reliability of systemic plant sterol levels as an indicator of dietary cholesterol uptake is questionable, raising doubts about their use as a marker for cholesterol absorption [13].

Oxysterols are highly bioactive molecules that are derived from the oxidation of cholesterol [15]. These molecules have many physiological activities and, depending on their structure and concentration, can have beneficial and harmful effects. Oxysterols have been implicated in many diseases, including cardiovascular disease and IBD [14]. It has been shown that acute and chronic colitis in mice affects plasma, liver and colon oxysterol levels [16].

Primary sclerosing cholangitis (PSC) is a cholestatic liver disease with a high preponderance of concurrent IBD [17]. Blood cholesterol levels in PSC are often above normal [18,19]. Serum plant sterols sitosterol and campesterol as well as cholestanol, a metabolite formed by oxidation of cholesterol and an intermediate in the biosynthesis of chenodeoxycholic acid, are increased in PSC in comparison to healthy controls [20,21]. It has also been described that cholesterol and cholesterol precursors (cholestenol, lathosterol and desmosterol) in serum are reduced in PSC compared to healthy controls. The controls in this study were significantly older than the PSC patients and increased levels of cholesterol and its precursors may be in part related to the higher age of the control cohort [21,22]. It has also to be taken into consideration that a significant number of PSC patients may suffer from IBD, which is related to alterations in lipid metabolism [23,24]. These authors concluded that the inverse correlation of plant sterols and markers of cholesterol synthesis among PSC patients and healthy controls indicated normal cholesterol metabolism in PSC [21]. Patients with PSC have been shown to exhibit increased plasma bile acid levels, and a higher primary-to-secondary bile acid ratio relative to healthy controls [25]. In as far as these changes are related to PSC or underlying IBD has not been clarified in this study [25].

To better understand the changes in cholesterol metabolism in IBD and PSC, we analyzed plant sterols, cholesterol precursors and cholesterol derivatives in the serum and feces of patients with IBD, PSC, PSC-IBD and controls.

2. Materials and methods

2.1. Patients

Patients diagnosed with IBD, PSC-IBD, or PSC were recruited from the outpatient/inpatient clinic of a German university hospital between December 6, 2021, and January 31, 2023. Diagnoses were based on clinical, histological, and endoscopic criteria [26–28]. Patients with coagulopathy were excluded. Controls for this retrospective study included students, their relatives, hospital staff, and partners of the patients, all living in the same area as the patients.

2.2. Serum collection

Serum cholesterol levels in fasting and non-fasting serum are not much different [29] and non-fasting serum (10 ml) of patients and controls was collected at different times between 8 am and 4 pm. The venous blood samples were gathered in S-Monovette® Serum CAT, 7.5 ml, from Sarstedt in Nürnbrecht, Germany. These tubes have beads coated with a coagulation activator. It took about 20–30 minutes for the blood to clot. Following this, the samples underwent centrifugation at 2000 g for 10 minutes at room temperature. Serum was aliquoted into cups and stored at −80 °C until use.

2.3. Stool collection

Feces were collected in 70 % isopropanol and stored at −80°C until use. Feces were homogenized in a gentleMACS™ dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The dry weights were determined by drying 1.0 ml of the homogenate in a vacuum centrifuge. The fecal homogenates were diluted to a final concentration of 2.0 mg dry weight/ml.

2.4. Measurement of sterol metabolites in serum and feces

Sample preparation for the analysis of sterols and stanols was performed as described [30–32]. Briefly, 200 µL calibrator, control or diluted feces homogenate, respectively, were added to 100 µL deuterated internal standard mix in 15 ml tubes. To break up the ester bonds of the esterified sterols alkaline hydrolysis with 500 µl 1 M KOH was performed for 30 min at 60 °C. 40 µL of an aqueous solution of orthophosphoric acid (50 % w/w) was used to adjust the solutions to pH 7.0. Liquid-liquid extraction was performed with 3 ml isooctane. The isooctane layer was pipetted into an autosampler vial and evaporated to dryness in a vacuum concentrator. 50 µL N-methyl trifluoroacetamide were added to the residue to perform derivatization at 60 °C for 60 min. 450 µL n-hexane were added for dilution, and 1 µL was injected. 100 µL calibrator, control or serum, respectively, were pipetted in 15 ml tubes and 50 µL deuterated internal standard mix were added. Alkaline hydrolysis, liquid-liquid extraction, and derivatization with N-methyl trifluoroacetamide was similar to the analysis of feces.

Gas chromatography-mass spectrometry was performed on a triple quadrupole mass spectrometer TQ8050 equipped with a multifunctional autosampler AOC-6000, an SH Rxi-5Sil MS column (30 m, 0.25 mm, 0.25 µm) (all Shimadzu Deutschland GmbH, München, Germany) and a multi-mode inlet system OPTIC 4 (GL Sciences, Eindhoven, Netherlands). All compounds were monitored as N-methyl trifluoroacetamide derivatives in the multiple reaction monitoring mode.

2.5. Quantification of serum cholesterol

For quantitative lipidomics, a volume of 10 µL serum was extracted by the protocol described by Bligh and Dyer [33] in the presence of non-naturally occurring internal standards (CE 17:0, CE 22:0 and FC [D7]). The measurement of free cholesterol (FC) and cholesteryl ester (CE) was performed by flow injection analysis (FIA) on a high-resolution hybrid quadrupole-Orbitrap mass spectrometer [34,35]. The CE species were recorded in positive ion mode m/z 500–1000 as [M+NH4]+ at a target resolution of 140,000 (at m/z 200) and were corrected for their species-specific response [36]. The analysis of FC was performed by multiplexed acquisition (MSX) of the $[M+NH_4]^+$ of FC and the deuterated internal standard (FC[D7]) [36]. Quantification was performed by multiplying the spiked internal standard amount with the analyte to internal standard ratio. Total cholesterol levels are the sum of all CE species and FC.

2.6. Analysis of laboratory parameters and the calculation of the MELD score

C-reactive protein (CRP) was determined in a particle-enhanced immunoturbidimetric assay The enzyme aspartate aminotransferase (AST) facilitates the movement of an amino group between L-aspartate and 2-oxoglutarate, resulting in the production of oxaloacetate and L-glutamate. Oxaloacetate then combines with malate dehydrogenase and NADH to yield L-malate and NAD+. The rate of NADH oxidation is directly linked to the catalytic activity of AST and is determined by measuring the extinction rate. The enzyme alanine aminotransferase (ALT) facilitates the transfer of an amino group from L-alanine to 2-oxoglutarate, resulting in the production of pyruvate and L-glutamate. Subsequently, pyruvate, in the presence of lactate dehydrogenase, reacts with NADH to generate L-lactate and NAD+. The rate at which NADH is oxidized is directly linked to the catalytic activity of ALT and can be determined by measuring the extinction rate. The measurement of total bilirubin involves coupling it with 3,5-dichlorophenyldiazonium in a strongly acidic solution using a suitable solvent. The resulting red azo dye's color intensity is directly linked to the total bilirubin concentration and can be quantified photometrically. The substrate p-nitrophenyl phosphate is cleaved by alkaline phosphatase to form phosphate and p-nitrophenol. Magnesium and zinc ions are required for this reaction. The p-nitrophenol formed is measured photometrically. Gamma-glutamyltransferase (gamma-GT) transfers the gamma-glutamyl from L-gamma-glutamyl-3-carboxy-4-nitroanilide to glycylglycin to form 5-amino-2-nitrobenzoate, which is measured photometrically. The assays described above are from Roche (Penzberg, Germany) and are run on the Cobas Pro C analyser.

Fecal calprotectin was measured by the Quanta Flash Calprotectin test from Inova Diagnostics (San Diago, CA, USA). This chemiluminescence sandwich immunoassay is operated at the Bio-Flash machine. When citrate-plasma is incubated with a standardized amount of thromboplastin and calcium ions, it triggers the clotting process, and the time until the fibrin core forms is measured using optical methods. The international normalized ratio (INR) is determined by using the prothrombin time ratio (PR) and the International Sensitivity Index (ISI). In this case, PRISI = INR, as calculated by the BCS XP system from Siemens in Forchheim, Germany.

The analysis for all laboratory parameters mentioned above was performed at the Institute of Clinical Chemistry and Laboratory Medicine at University Hospital Regensburg.

The MELD is a composite score derived from three laboratory values: INR, serum total bilirubin and serum creatinine [55].

2.7. Statistical analysis

Data are shown as boxplots and circles and asterisks mark outliers. Outliers are indicated with circles (when the level was >1.5× the interquartile range from either quartile) and asterisks (when the level was >3.0× the interquartile range from either quartile). The data in the tables display the median values, the 25 % quartile and 75 % quartile. Data in Fig. 3 C are given as mean ± standard deviation. Mann Whitney U-test, Kruskal-Wallis Test, and Spearman correlation were the statistical tests used (SPSS Statistics 26.0 program, IBM, Leibniz Rechenzentrum, München, Germany). Kolmogorov-Smirnov and Shapiro-Wilk test showed that except serum cholesterol levels the steroids were not normally distributed in at least two of the four patient cohorts. Therefore, we decided to use non-parametric tests, which can be used for all kinds of data [37], for the analysis of all data. A value of $p < 0.05$ was regarded as significant.

3. Results

3.1. Patients and control cohorts

The cohort included 87 patients with IBD (55 patients with Crohn's disease (CD) and 32 patients with ulcerative colitis (UC)), 11 patients with PSC, 21 patients with PSC-IBD and 16 healthy controls. Sex distribution, age and body mass index (BMI) were similar between controls, patients with IBD, PSC and PSC-IBD (Table 1). PSC and PSC-IBD patients had higher levels of aspartate aminotransferase (AST), gamma-glutamyl transferase (gamma-GT), alkaline phosphatase and bilirubin compared to IBD patients (Table 1).

Alanine aminotransferase (ALT), alkaline phosphatase (AP), aspartate aminotransferase (AST), body mass index (BMI), gamma-glutamyl transferase (gamma-GT), not determined (n.d.)). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparison between IBD and PSC patients, % $p < 0.05$, %% $p < 0.01$ and %%% $p < 0.001$ for comparison between IBD and PSC-IBD patients.

3.2. Fecal cholesterol metabolites of patients and controls

Female and male controls, IBD, PSC and PSC-IBD patients had similar levels of fecal cholesterol, coprostanol, cholestanol, 5-beta sitostanol, campesterol, stigmasterol, sitosterol and 5-alpha sitostanol. Fecal cholesterol negatively correlated with age of the IBD patients ($r = -0.265$, $p = 0.014$) and fecal cholestanol ($r = -0.492$, $p = 0.023$) and stigmasterol ($r = -0.448$, $p = 0.042$) with age in PSC-IBD patients. These sterol metabolites were not related to BMI in any cohort ($p > 0.05$ for all).

Details of the 55 patients with CD and the 32 patients with UC are listed in Table S1. These cohorts had similar age, BMI, fecal calprotectin and CRP levels (Table S1). Fecal cholesterol ($p = 0.170$), coprostanol ($p = 0.552$), cholestanol ($p = 0.631$), 5-beta sitostanol ($p = 0.961$), campesterol ($p = 0.101$), stigmasterol ($p = 0.748$) and 5-alpha sitostanol ($p = 0.909$) did not differ between the 55 patients with CD and the 32 patients with UC and further calculations were made in the entire group of IBD patients.

Fecal cholesterol levels of IBD and PSC-IBD patients were higher in

Table 1
Characteristics of the study groups. Data are presented as the median, 25 % quartile and 75 % quartile. The Model for End Stage Liver Disease (MELD) score was documented for PSC and PSC-IBD patients. Statistical test used: Kruskal-Wallis test.

Characteristics	IBD	PSC	PSC-IBD	Controls
Number (females / males)	87 (43 / 44)	11 (5 / 6)	21 (7 / 14)	16 (10 / 6)
Age (years)	41.7 (32.5–53.4)	53.8 (40.4–62.0)	46.1 (18.8–54.0)	48.0 (27.7–58.4)
BMI (kg/m^2)	24.7 (22.1–28.0)	19.9 (18.0–19.9)	24.4 (18.5–30.4)	n.d.
C-reactive protein (mg/L)	2 (1.0–8.6)	0 (0–0)	3 (1.0–16.2)	n.d.
Fecal calprotectin (µg/g)	57 (25–158)	n.d.	38 (17–146)	n.d.
AST (U/L)	24 (20–28)*,%%	31 (21–85)*	32 (22–66)%%	n.d.
ALT (U/L)	20 (14–26)	27 (15–59)	30 (16–58)	n.d.
Gamma-GT (U/L)	25 (16–37) **,%	78 (46–191) **	43 (22–176)%	n.d.
AP (U/L)	65 (54–83) ***,%%%	158 (75–330) ***	112 (72–209)%%	n.d.
Bilirubin (mg/dL)	0.4 (0.3 – 0.6)**,%	0.8 (0.6–5.8) **	0.6 (0.4–1.1)%	n.d.
MELD Score	n.d.	7 (6–13)	6 (6–8)	n.d.

comparison to controls, who had similar levels as PSC patients (p = 0.353, Fig. 1A). Fecal coprostanol of IBD and PSC-IBD patients was low compared to healthy controls and tended to be reduced in patients with PSC compared to controls (p = 0.058, Fig. 1B). The sum of fecal cholesterol and coprostanol (p = 0.384, Fig. 1C) as well as the level of cholestanol (p = 0.342) did not differ between these groups.

The concentration of 5-beta sitostanol was highest in stool of controls and this difference was significant for IBD and PSC-IBD patients but not for PSC patients (p = 0.094) (Fig. 1D). Campesterol (p = 0.193), stigmasterol (p = 0.476), sitosterol (p = 0.128) and 5-alpha sitostanol (p = 0.391) in feces did not differ between the groups.

3.3. Associations of fecal sterol metabolites with inflammation

Serum C-reactive protein (CRP) levels positively correlated with fecal cholesterol and negatively with coprostanol, cholestanol, 5-beta sitostanol and 5-alpha sitostanol in patients with IBD (Table 2 and Fig. 2 A, B). Fecal calprotectin levels were negatively related with cholestanol, 5-beta sitostanol, campesterol, stigmasterol and 5-alpha sitostanol in IBD (Table 2). Except for a negative correlation of 5-alpha sitostanol with CRP, these fecal sterol metabolites did not significantly correlate with CRP and fecal calprotectin in PSC-IBD (Table 2). Associations of fecal sterol metabolites with serum CRP and fecal calprotectin were not significant in the PSC cohort (data not shown).

3.4. Serum sterol metabolites of patients and controls

Conversion of cholesterol to coprostanol in the intestine has been suggested to cause hypocholesterolemic effects [38]. Serum cholesterol levels were measured in 58 IBD, 7 PSC, 13 PSC-IBD patients and 7 controls. Serum cholesterol levels were similar between the patient and control cohorts (p = 0.156, Fig. 3A). The 40 CD and 18 UC patients showed similar serum cholesterol levels (p = 0.201).

Serum of 81 IBD, 11 PSC, 18 PSC-IBD and 17 controls of our cohort described in Table 1 was available to measure the sterol metabolites of cholesterol biosynthesis pathways (lathosterol, lanosterol, 7-dehydrocholesterol), oxysterols (7-beta-hydroxycholesterol and 27-hydroxycholesterol) and the plant sterols campesterol, stigmasterol and sitosterol.

The 10 female controls had lower levels of 27-hydroxycholesterol (p = 0.011), campesterol (p = 0.011) and sitosterol (p = 0.008) compared to the 7 male controls. A gender difference in the patient cohorts was only observed for 27-hydroxycholesterol, which was lower in the serum of female IBD patients compared to male patients (p = 0.041).

Table 2
Spearman correlation of fecal sterol metabolites with C-reactive protein and fecal calprotectin in IBD and PSC-IBD (Calprotectin data of 7 patients with IBD were not documented).

Correlation	C-reactive Protein	Fecal Calprotectin	C-reactive Protein	Fecal Calprotectin
	IBD		PSC-IBD	
Cholesterol	0.294*	0.037	−0.214	0.293
Coprostanol	−0.317**	−0.167	−0.122	−0.337
Cholestanol	−0.259*	−0.236*	−0.430	−0.119
5-beta Sitostanol	−0.391***	−0.239*	−0.374	−0.319
Campesterol	−0.044	−0.220*	−0.340	0.282
Stigmasterol	−0.108	−0.225*	−0.184	0.014
Sitosterol	−0.060	−0.193	−0.157	0.162
5-alpha Sitostanol	−0.368**	−0.306**	−0.691**	−0.445

* p < 0.05, ** p < 0.01, *** p < 0.001.

Serum 7-dehydrocholesterol (r = 0.226, p =0.041), 27-hydroxycholesterol (r = 0.282, p = 0.010), campesterol (r = 0.239, p = 0.030), stigmasterol (r = 0.273; p = 0.013), sitosterol (r = 0.293, p = 0.008) and cholesterol (r = 0.313, p = 0.018) positively correlated with age in patients with IBD. Correlations with age were not significant in controls, patients with PSC and patients with PSC-IBD.

The 53 CD and 28 UC patients had comparable serum levels of lanosterol (p = 0.120), 7-dehydrocholesterol (p = 0.168), 7-beta-hydroxycholesterol (p = 0.509), 27-hydroxycholesterol (p = 0.965) and the plant sterols campesterol (p = 0.807), stigmasterol (p = 0.953) and sitosterol (p = 0.822). Serum lathosterol of CD patients was higher in comparison to patients with UC (p = 0.029).

Lathosterol was higher in CD in comparison to controls, PSC and PSC-IBD patients (Fig. 3B). Accordingly, IBD patients had increased lathosterol levels in comparison to all other cohorts (Fig. 3C). Lanosterol was increased in IBD compared to PSC-IBD. Serum 7-dehydrocholesterol of controls and IBD patients was higher in comparison to PSC-IBD patients (Table 3 and Fig. 3C).

Levels of 7-beta-hydroxycholesterol were increased in IBD in comparison to healthy controls and PSC patients. Levels of serum 27-hydroxycholesterol were similar between the groups. Campesterol, stigmasterol and sitosterol were increased in PSC and PSC-IBD in

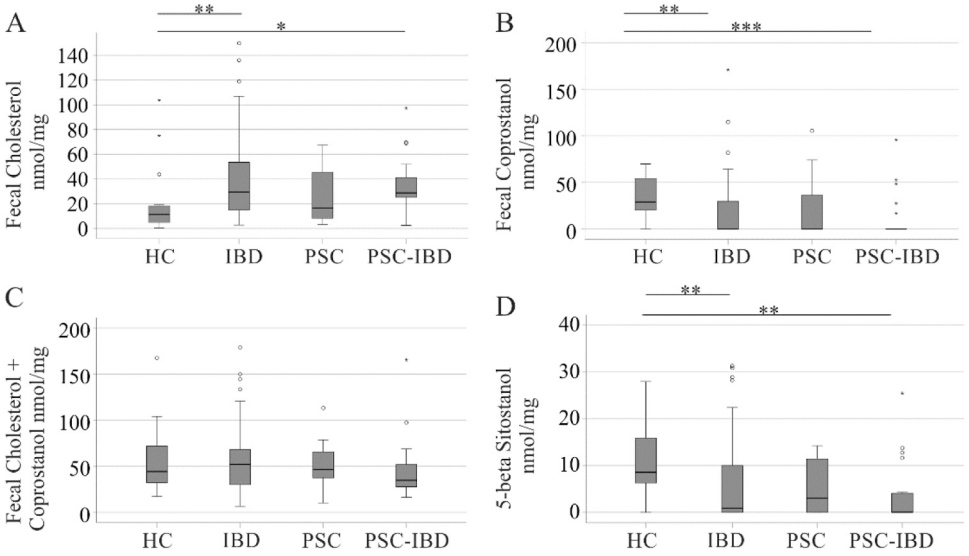

Fig. 1. Fecal levels of sterols of healthy controls (HC), patients with inflammatory bowel disease (IBD), patients with primary sclerosing cholangitis (PSC) and patients with PSC-IBD. A Fecal cholesterol level. B Fecal coprostanol level. C Sum of fecal cholesterol and coprostanol. D 5-beta sitostanol level of HC, IBD, PSC and PSC-IBD. * p < 0.05, ** p < 0.01, *** p < 0.001.

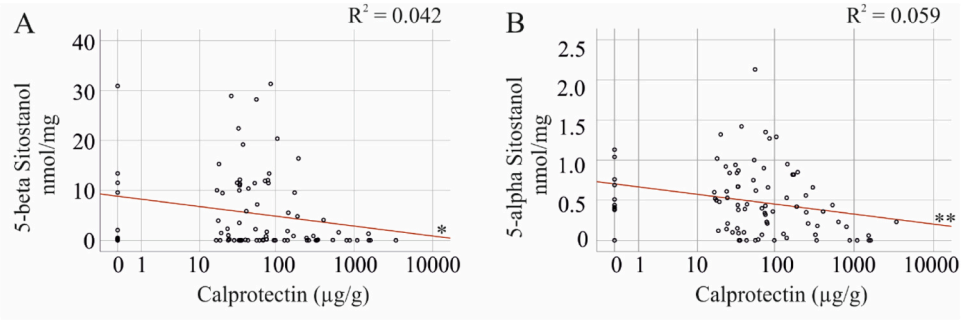

Fig. 2. Fecal plant sterols of patients with inflammatory bowel disease in relation to fecal calprotectin. A 5-beta sitostanol. B 5-alpha sitostanol. * $p < 0.05$, ** $p < 0.01$.

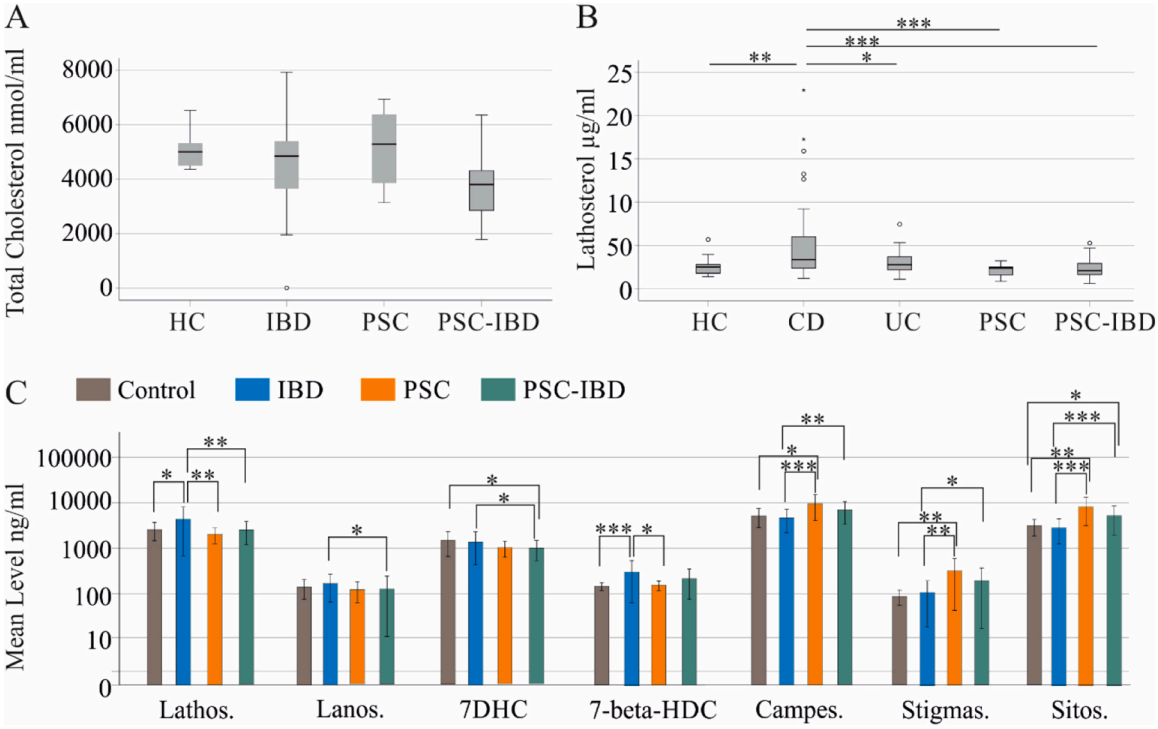

Fig. 3. Serum levels of sterols of healthy controls (HC), patients with inflammatory bowel disease (IBD), patients with primary sclerosing cholangitis (PSC) and patients with PSC-IBD. A Serum cholesterol levels of HC, IBD, PSC and PSC-IBD patients. B Serum lathosterol levels of HC, Crohńs disease (CD), ulcerative colitis (UC), PSC and PSC-IBD patients. C Serum sterols of HC, IBD, PSC and PSC-IBD patients (lathosterol: Lathos., lanosterol: Lanos., 7-dehydrocholesterol: 7DHC, 7-beta-hydroxycholesterol: 7-beta-HDC, campesterol: Campes., stigmasterol: Stigmas., sitosterol: Sitos.). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3
Concentration (ng/ml) of sterols in serum of controls, patients with IBD, PSC and PSC-IBD.

Sterol	Control	IBD	PSC	PSC-IBD
Lathosterol	2499 (1759–2788)$^{\S\ \%\%}$	3067 (2280–5332) $^{**\%\%\ \S}$	2289 (1206–2458)$^{\%\%}$	2051 (1477–3331)**
Lanosterol	129 (70–175)	140 (101–214)*	102 (79–154)	88 (59–143)*
7-Dehydrocholesterol	1150 (950–1396)$^{\$}$	1177 (832–1496)*	779 (669–1334)	836 (677–1021)*$^{\$}$
7-beta hydroxycholesterol	143 (123–154)§§§	195 (154–414)$^{\S\S\S\ \%}$	161 (111–176)$^{\%}$	163 (126–278)
27-hydroxycholesterol	78 (65–115)	78 (66–100)	81 (64–100)	65 (58–82)
Campesterol	5340 (3408–6262) $^{\%}$	4370 (2889–5562) ** &&&	8890 (5291–12368) $^{\&\&\&\%}$	6321 (4497–7745)**
Stigmasterol	91 (64–106)$^{\%\%}$	89 (47–142)* &&	222 (78–525)$^{\%\%}$ &&	145 (75–221)*
Sitosterol	3110 (2344–3813)$^{\%\%\ \$}$	2660 (1804–3936) *** &&&	7643 (3895–12097) $^{\&\&\&\%\%}$	4877 (3351–6752)***$^{\$}$

*, **, *** $p < 0.05$, $p < 0.01$, $p < 0.001$ for comparison of IBD and PSC-IBD.
$^{\$}$ $p < 0.05$, for comparison of controls and PSC-IBD.
§, §§§ $p < 0.05$, $p < 0.001$ for comparison of controls and IBD.
$^{\%}$, $^{\%\%}$ $p < 0.05$, $p < 0.01$ for comparison of controls and PSC.
&&, &&& $p < 0.01$, $p < 0.001$ for comparison of IBD and PSC.

comparison to IBD patients. PSC patients had higher levels than healthy controls (Table 3 and Fig. 3C).

3.5. Associations of serum cholesterol metabolites with inflammation

In IBD a positive association of serum CRP with 7-beta hydroxycholesterol and negative correlations with cholesterol (Fig. 4 A), campesterol, stigmasterol and sitosterol were observed. Serum cholesterol (Fig. 4B), 27-hydroxycholesterol (Fig. 4 C), 7-dehydrocholesterol (Fig. 4D), campesterol (Fig. 5 A) and sitosterol (Fig. 5B) negatively correlated with fecal calprotectin. In PSC-IBD 27-hydroxycholesterol positively correlated with calprotectin (Table 4).

Campesterol and sitosterol levels of IBD patients with high fecal calprotectin were also low (Fig. 5A, B).

3.6. Associations of fecal and serum cholesterol metabolites with markers of liver disease

In IBD, AST was positively correlated with serum 7-dehydrocholesterol and 27-hydroxycholesterol. The latter also correlated with gamma-GT. Serum campesterol and sitosterol correlated positively with bilirubin. Fecal 5-alpha-sitostanol was negatively associated with AP (Table 5).

In PSC-IBD fecal sterols were not correlated with measures of liver disease. Serum lathosterol negatively correlated with bilirubin. Campesterol positively correlated with the MELD score. Stigmasterol was positively correlated with bilirubin and the MELD score. Serum sitosterol positively correlated with gamma-GT, AP, bilirubin and the MELD score (Table 6).

3.7. Correlations of fecal and serum cholesterol metabolites

Fecal cholesterol negatively correlated with serum stigmasterol. Fecal coprostanol and 5-beta sitostanol positively correlated with serum cholesterol, and negatively with serum lathosterol and lanosterol. Serum campesterol was positively associated with fecal 5-beta sitostanol. Fecal cholestanol positively correlated with serum cholesterol and campesterol, and negatively with lathosterol. Fecal and serum campesterol were positively correlated. Fecal stigmasterol and sitosterol positively correlated with serum lanosterol, campesterol and sitosterol. Fecal 5-alpha sitostanol positively correlated with serum cholesterol and negatively with lathosterol. Fecal 5-alpha sitostanol positively correlated with serum campesterol and sitosterol (Table 7).

In patients with PSC-IBD and patients with PSC fecal and serum sterols were not associated.

4. Discussion

This analysis provides evidence that sterol metabolism is disrupted at multiple levels in IBD and PSC: 1. In IBD, cholesterol biosynthesis precursors, serum cholesterol, and serum and fecal plant sterols decrease with intestinal inflammation. 2. The conversion of cholesterol to coprostanol by the gut microbiota is impaired in IBD, PSC, and PSC-IBD. 3. PSC, and to a lesser extent PSC-IBD, are associated with increased serum plant sterol levels.

Gut dysbiosis in IBD has been extensively studied [39–41]. The current analysis confirmed that the conversion of cholesterol to coprostanol is impaired in IBD compared to healthy controls. Recent studies described low fecal coprostanol levels in IBD and concluded that bacterial cholesterol modification is impaired, but these studies did not measure fecal cholesterol in parallel [42,43]. Furthermore, our studies suggested that altered fecal cholesterol metabolism was not related to the degree of intestinal inflammation in IBD patients as assessed by fecal calprotectin levels.

Fecal cholestanol levels negatively correlated with CRP and fecal calprotectin indicating reduced concentrations in inflammation.

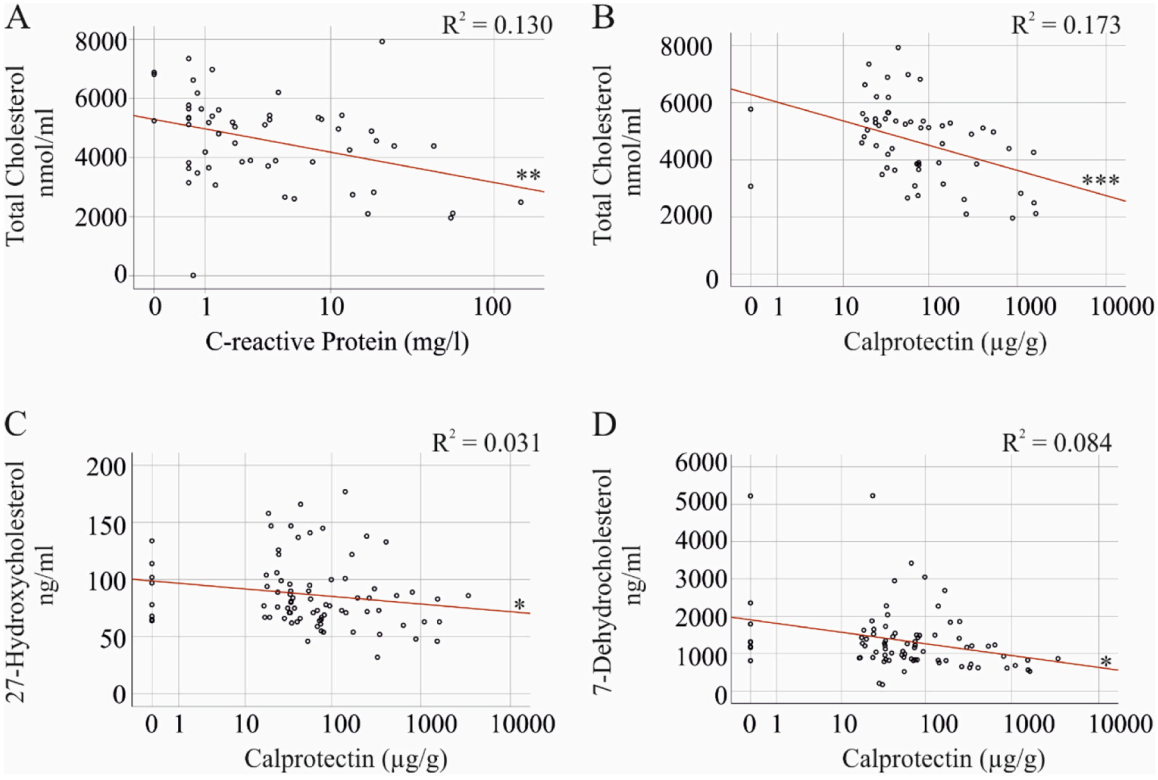

Fig. 4. Serum sterols of IBD patients with inflammatory bowel disease in relation to serum C-reactive protein and fecal calprotectin. A Correlation of serum cholesterol with C-reactive protein. B Cholesterol, C 27-hydroxycholesterol and C 7-dehydrocholesterol in relation to fecal calprotectin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

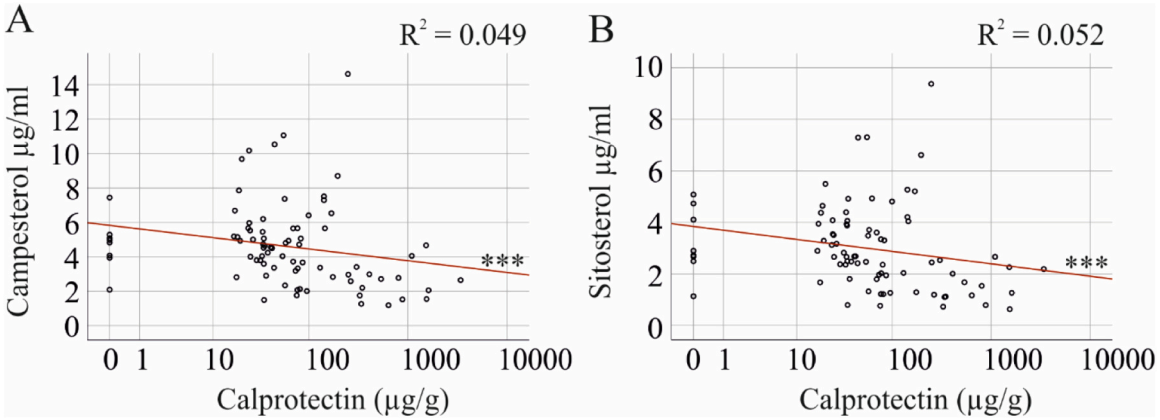

Fig. 5. Serum campesterol and sitosterol of IBD patients with inflammatory bowel disease in relation to fecal calprotectin. A Campesterol in relation to fecal calprotectin. B Sitosterol in relation to fecal calprotectin. *** $p < 0.001$.

Table 4
Spearman correlation of serum sterol metabolites with C-reactive protein and fecal calprotectin in IBD and PSC-IBD (Calprotectin data of 2 patients with IBD were not documented).

Correlation	C-reactive Protein	Fecal Calprotectin	C-reactive Protein	Fecal Calprotectin
	IBD		PSC-IBD	
Cholesterol	−0.377**	−0.485***	−0.329	0.070
Lathosterol	0.158	−0.104	0.087	0.003
lanosterol	0.050	−0.183	0.050	−0.183
7-dehydrocholesterol	−0.195	−0.286*	−0.053	−0.040
7-beta-hydroxycholesterol	0.275*	−0.010	−0.382	0.133
27-hydroxycholesterol	−0.105	−0.240*	−0.033	0.545*
Campesterol	−0.389**	−0.402***	−0.169	0.289
Stigmasterol	−0.335**	−0.202	0.146	−0.154
Sitosterol	−0.373**	−0.386***	0.202	0.068

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 5
Spearman correlation of fecal and serum sterol metabolites with laboratory parameters of liver function in IBD.

Correlation	AST	ALT	Gamma-GT	AP	Bilirubin
Feces					
Cholesterol	−0.226	−0.085	−0.053	−0.136	−0.257
Coprostanol	0.019	−0.027	0.008	−0.128	−0.013
Cholestanol	0.033	0.009	0.114	−0.163	−0.009
5-beta sitostanol	0.065	0.025	0.030	−0.168	0.094
Campesterol	−0.078	−0.022	0.075	−0.079	0.140
Stigmasterol	−0.092	−0.097	−0.138	−0.156	0.094
Sitosterol	−0.139	−0.066	−0.029	−0.074	0.078
5-alpha sitostanol	0.014	0.068	0.005	−0.241*	0.106
Serum					
Cholesterol	0.257	0.084	0.035	0.088	0.029
Lathosterol	0.091	−0.029	0.149	0.141	0.049
Lanosterol	0.156	−0.061	0.050	0.104	0.068
7-Dehydrocholesterol	0.319*	0.159	0.208	0.124	0.254
7-beta hydroxycholesterol	−0.052	−0.090	0.011	−0.104	0.008
27-hydroxycholesterol	0.315*	0.220	0.289*	−0.103	−0.016
Campesterol	0.199	0.039	−0.054	−0.243	0.400**
Stigmasterol	0.112	−0.064	−0.020	−0.070	0.073
Sitosterol	0.119	−0.064	−0.149	−0.229	0.338*

Alanine aminotransferase (ALT), alkaline phosphatase (AP), aspartate aminotransferase (AST), gamma-glutamyl transferase (gamma-GT)). * $p < 0.05$, ** $p < 0.01$

Cholestanol is formed by the oxidation of cholesterol and is an intermediate in the biosynthesis of the primary bile acid chenodeoxycholic acid, whose levels do not differ between patients with IBD and healthy controls, and are not altered in active IBD [Fiorucci, 2019 #2726; Sommersberger, 2023 #2740]. Serum cholesterol and fecal cholestanol levels were positively correlated with each other, consistent with a decrease in both metabolites in inflammation. This indicates that biliary secretion and/or synthesis of cholestanol rather than production of chenodeoxycholic acid are affected by intestinal inflammation.

According to our findings, patients with PSC-IBD also have a reduced ability to convert cholesterol to coprostanol, whereas patients with PSC without underlying IBD show a lesser degree of impairment. A recent study showed that PSC patients without IBD have dysbiosis compared to healthy individuals [17]. These patients also have subclinical inflammation, mainly in the distal colon. PSC-IBD patients may have inflammation in the distal colon, the right side of the colon or the terminal ileum [17]. The localization of inflammation in IBD varies depending on whether CD or UC is present [1,2]. Similar defects in cholesterol conversion in CD, UC and PSC patients suggest that dysfunction of these pathways is not related to the localization and extent of intestinal inflammation.

Although intestinal cholesterol conversion was described over a century ago, only a few bacterial strains that convert cholesterol to coprostanol have been characterized [7,44,45]. In the human population, most individuals are high cholesterol to coprostanol converters, while a minority are low converters [45]. The conversion patterns were found to be equally distributed between the sexes [45] and accordingly, fecal cholesterol and coprostanol levels of female and male controls and patients in our cohorts were similar. The synthesis of coprostanol was not related to age [45] consistent with our observation. Obesity did not alter the pattern of high/low cholesterol to coprostanol converters in humans [44] and these metabolites were not related to BMI in our patient cohorts.

It is worth noting that impaired conversion of cholesterol to coprostanol is associated with hypercholesterolaemia [45]. However, in patients with IBD serum cholesterol levels positively correlated with fecal coprostanol. Serum cholesterol levels in patients with IBD, PSC and PSC-IBD were quite normal and even decreased in patients with IBD and severe inflammation. This indicates that the correlation of serum cholesterol and fecal coprostanol is related to intestinal inflammation. With inflammation, there is a consistent decrease in low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels, and consequently, total blood cholesterol [8]. Blood cholesterol and HDL levels were reduced in patients with active IBD compared to healthy controls, whereas the difference between patients with quiescent IBD and controls was not significant [46]. Lanosterol and 7-dehydrocholesterol were reduced in IBD patients with active disease compared with patients with

Table 6
Spearman correlation of fecal and serum sterol metabolites with laboratory parameters of liver function in PSC-IBD.

Correlation	AST	ALT	Gamma-GT	AP	Bilirubin	MELD Score
Feces						
Cholesterol	0.306	0.299	0.176	0.195	0.262	0.270
Coprostanol	−0.162	−0.171	0.098	−0.243	−0.163	−0.132
Cholestanol	0.139	0.041	0.135	0.009	0.018	−0.039
5-beta sitostanol	−0.161	−0.261	0.037	−0.172	−0.093	−0.253
Campesterol	0.255	0.311	0.160	0.162	0.230	0.223
Stigmasterol	0.334	0.302	0.134	0.080	0.308	−0.077
Sitosterol	0.292	0.312	0.147	0.183	0.222	0.083
5-alpha sitostanol	−0.153	−0.169	−0.251	−0.180	0.013	−0.292
Serum						
Cholesterol	0.303	0.303	0.303	0.303	0.303	0.303
Lathosterol	−0.447	−0.379	−0.451	−0.395	−0.726**	0.009
Lanosterol	−0.011	0.064	−0.077	−0.169	−0.282	−0.028
7-Dehydrocholesterol	−0.016	0.021	0.050	0.001	−0.317	0.401
7-beta-hydroxycholesterol	0.095	0.096	0.421	0.142	0.054	0.358
27-hydroxycholesterol	0.043	0.076	0.271	0.009	−0.051	0.441
Campesterol	0.097	0.058	0.257	0.194	0.244	0.615*
Stigmasterol	0.308	0.177	0.295	0.392	0.482*	0.514*
Sitosterol	0.480	0.410	0.543*	0.559*	0.609**	0.698*

Alanine aminotransferase (ALT), alkaline phosphatase (AP), aspartate aminotransferase (AST), gamma-glutamyl transferase (gamma-GT), Model for End Stage Liver Disease (MELD). * $p < 0.05$, ** $p < 0.01$

Table 7
Spearman correlation of fecal and serum sterol metabolites in IBD.

Feces Serum	Chole sterol	Copro stanol	Chole stanol	5-beta sitostanol	Campe sterol	Stigma sterol	Sito sterol	5-alpha sitostanol
Cholesterol	−0.224	0.448***	0.421**	0.475***	0.015	0.114	0.078	0.356**
Lathosterol	0.156	−0.296**	−0.244*	−0.325**	0.088	0.116	0.125	−0.233*
Lanosterol	0.148	−0.263*	−0.117	−0.254*	0.197	0.287**	0.261*	−0.107
7-Dehydro cholesterol	−0.024	−0.016	0.014	−0.017	0.068	0.135	0.121	0.023
7-beta-hydroxy cholesterol	0.137	0.005	−0.027	0.027	−0.157	−0.103	−0.151	0.031
27-hydroxy cholesterol	0.002	0.137	0.178	0.112	0.062	0.152	0.096	0.185
Campesterol	−0.070	0.162	0.252*	0.274*	0.269*	0.329**	0.280*	0.393**
Stigmasterol	−0.250*	0.111	0.093	0.119	−0.023	0.181	0.044	0.135
Sitosterol	−0.125	0.146	0.159	0.207	0.141	0.342**	0.237*	0.319**

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

quiescent IBD, suggesting that active disease is associated with lower cholesterol biosynthesis.

Studies have shown that consuming high doses of plant sterols reduces the ability of the gut microbiota to metabolize cholesterol [45,47]. Although plant sterols interact with the gut microbiome and appear to be metabolized by gut bacteria, these pathways have received little attention. High doses of plant sterols effectively reduce intestinal cholesterol absorption, resulting in increased fecal cholesterol excretion and decreased blood cholesterol [48]. Patients with IBD mostly had normal levels of serum and fecal plant sterols, ruling out phytosterols as a cause of impaired fecal cholesterol to coprostanol conversion. Serum cholesterol was positively correlated with fecal 5-beta-sitostanol and 5-alpha-sitostanol levels in IBD patients, further demonstrating that at least the levels of excreted plant sterols are not associated with lower serum cholesterol levels. These sterols all decrease with intestinal inflammation, explaining the observed correlations. The negative correlation between fecal cholesterol and serum stigmasterol is consistent with a hypocholesterolaemic effect of phytosterols. In patients with IBD, fecal and serum levels of campesterol and sitosterol were positively correlated, suggesting that fecal excretion is related to serum levels of these sterols. Of note, fecal levels of 5-beta-sitostanol were reduced in IBD patients compared with healthy controls. In addition, fecal levels of 5-beta-sitostanol and 5-alpha-sitostanol negatively correlated with CRP and fecal calprotectin and were correspondingly lower in the feces of patients with active IBD compared to those with quiescent disease. Serum levels of both campesterol and sitosterol correlated negatively with CRP and fecal calprotectin and were reduced in the serum of patients with active IBD compared with patients with inactive disease. In experimental colitis, plant sterol supplementation improved colitis severity [49,50] and the negative correlations of phytosterols with IBD disease activity are consistent with the protective properties of these metabolites.

Of clinical relevance, serum plant sterol levels were elevated in PSC patients and, to a lesser extent, in PSC-IBD patients. Higher serum levels in PSC compared to healthy controls have been described previously, but this study did not differentiate between patients with PSC and PSC-IBD [21]. In our PSC-IBD cohort, serum plant sterol levels correlated positively with laboratory measures of liver injury. Plant sterols are minimally absorbed in the small intestine and efficiently excreted in the bile [51,52], suggesting impaired biliary excretion as a cause of systemic accumulation. The biliary ATP binding cassette transporter G5/G8 pumps plant sterols and cholesterol into the bile, but normal serum cholesterol levels in PSC patients largely rule out a defect in this transporter [53]. Patients with primary biliary cirrhosis also show significantly higher serum plant sterol levels [54]. The biliary secretion rate of these PBC patients was normal and the reason for the higher serum levels was not clarified in this study [54]. It is worth noting that stigmasterol can induce cholestasis in mice when administered intravenously [55]. This suggests that high serum levels of plant sterols, which may not be achieved by a normal diet, contribute to liver injury and cholestasis.

Serum levels of plant sterols, 7-dehydrocholesterol, 27-

hydroxycholesterol and total cholesterol were positively correlated with age. Patients with PSC were older than controls, patients with IBD and patients with PSC-IBD. Thus, older age may contribute to increased levels of phytosterols in the serum of patients with PSC. These sterol metabolites were also induced in patients with PSC-IBD, who were similar in age to patients with IBD and controls, and it is unlikely that age is a major factor here.

The previously described increase in systemic cholesterol levels in PSC [18,19] was not seen in our cohort. Serum cholesterol levels in patients with PSC-IBD may decrease with intestinal inflammation, preventing the identification of higher cholesterol levels in cholestatic liver disease, and our PSC cohort may have been too small to detect significant differences.

Lathosterol, lanosterol and 7-dehydrocholesterol were lower in patients with PSC-IBD than in patients with IBD, suggesting that cholesterol synthesis pathways are less active in cholestatic liver disease [54]. Notably, lathosterol was negatively correlated with bilirubin in PSC-IBD but not in patients with IBD showing that this association is specific to PSC.

Lathosterol levels were higher in CD compared to UC, PSC, PSC-IBD and healthy controls. However, lanosterol and 7-dehydrocholesterol levels were similar in controls and IBD patients. At present, we cannot explain the selective increase in serum lathosterol in CD.

The oxysterol 7-beta-hydroxycholesterol was elevated in the serum of IBD patients and correlated positively with CRP in this patient cohort, consistent with the inflammatory effects of this metabolite [56]. Levels of 27-hydroxycholesterol correlated negatively with fecal calprotectin in IBD and positively in PSC-IBD patients. Female patients with IBD and female controls had higher serum 27-hydroxycholesterol levels, consistent with data from healthy volunteers [57]. Sex-specific effects of 27-hydroxycholesterol on inflammation dependent on 17beta-estradiol have been described [58]. In our study, sex-specific analysis did not identify associations of 27-hydroxycholesterol levels with fecal calprotectin in IBD and PSC-IBD patients (data not shown), and further studies are needed to clarify the role of this metabolite in intestinal inflammation. Positive correlations of 27-hydroxycholesterol with AST and gamma-GT were observed in IBD but not in PSC-IBD patients, and it is possible that the latter cohort was too small to detect significant associations. 27-hydroxycholesterol is one of the most abundant oxysterols in the circulation and in the liver a substrate for bile acid synthesis [59]. The associations of systemic 27-hydroxycholesterol levels with liver function in patients with PSC and PSC-IBD require further analysis.

This single-centre study has limitations. Feces and serum were collected at different times of the day and dietary habits of patients and controls were not documented. Non-fasting serum was collected and although cholesterol does not greatly differ between fasted and non-fasted serum [29] other metabolites may vary. BMI of controls was not known.

In conclusion, our study demonstrates that cholesterol synthesis decreases with increasing intestinal inflammation. Notably, patients with PSC accumulate plant sterols in serum, which positively correlated with markers of liver injury. The conversion of cholesterol to coprostanol is impaired in IBD, PSC-IBD, and PSC patients, independent of disease severity as assessed by serum CRP, fecal calprotectin, and liver disease markers. While phytosterols might benefit bowel inflammation, they could exacerbate cholestatic liver disease and can thus not generally be recommended.

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Institutional Review Board Statement

The study was approved by the Ethics Committee of the University Hospital Regensburg (protocol no. 19–1309–101, approval date: 20.02.2019 and protocol no. 21–2390–101, approval date: 19.05.2021) and all participants gave written informed consent. The study was conducted in accordance with the updated guidelines for good clinical practice and the updated Declaration of Helsinki.

Informed Consent Statement

Written informed consent was obtained from all subjects involved in the study.

CRediT authorship contribution statement

Johanna Loibl: Writing – review & editing, Resources. **Marcus Höring:** Writing – review & editing, Formal analysis. **Gerhard Liebisch:** Writing – review & editing, Formal analysis. **Muriel Huss:** Writing – review & editing, Resources. **Tanja Elger:** Writing – review & editing, Resources. **Silke Matysik:** Writing – review & editing, Investigation, Conceptualization. **Christa Buechler:** Writing – review & editing, Writing – original draft, Conceptualization. **Hauke Christian Tews:** Writing – review & editing, Conceptualization. **Martina Müller:** Writing – review & editing. **Arne Kandulski:** Writing – review & editing, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2024.106621.

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