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Colitis Alters Oxysterol Metabolism and is Affected by 4β-Hydroxycholesterol Administration

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Abstract

Background and Aims: Inflammatory bowel diseases [IBD] represent a challenging health issue with a complex aetiology involving genetic and environmental parameters. Although our understanding of the pathophysiology of IBD has improved, much remains to be explored. In this context, bioactive lipids, more specifically oxysterols, i.e. oxygenated derivatives of cholesterol, represent an interesting avenue to investigate. Indeed, oxysterols or their receptors are involved in inflammation and immune regulation. Therefore, we set out to study the oxysterome in IBD.
Methods: We used both high-performance liquid chromatograph/mass spectroscopy and molecular biology tools to quantify oxysterol levels and the expression of their metabolic enzymes in several models of murine colitis [both acute and chronic], as well as in colon biopsies from patients with Crohn's disease and ulcerative colitis.
Results: We found that the oxysterome is altered in IBD, in both acute and chronic murine models as well as in human IBD. Two of the oxysterols quantified, 4β-hydroxycholesterol and 25-hydroxycholesterol, were consistently altered in all our models and therefore could be of interest in this context. Hence, we administered them to mice with colitis. While 25-hydroxycholesterol had no effect, 4β-hydroxycholesterol worsened colon inflammation.
Conclusions: Our study addresses the potential involvement of oxysterols in colitis and clearly points towards an active role as well as a clinical relevance for these bioactive lipids.

Key Words: 25-OHC; LXR; Cyp3A; 4β-OHC

1. Introduction

Ulcerative colitis [UC] and Crohn's disease [CD] are the two major forms of inflammatory bowel diseases [IBD] and share similar aetiopathogenic pathways and present symptoms such as diarrhoea, rectal bleeding, abdominal pain and weight loss.[1,2] These diseases affect the gastrointestinal tract and result from a dysregulated immune

response in genetically predisposed patients.[1] However, their full pathogenesis remains incompletely understood.[2] Bioactive lipids represent an interesting research avenue as they could be implicated in the pathogenesis, and thus the treatment, of IBD.[3,4] Among these lipids, cholesterol and bile acids have been suggested to play a role in the pathology.[5]

Oxysterols are early oxygenated metabolites of cholesterol or of its precursors.[6] The oxidation of cholesterol occurs via enzymatic reactions or via free radical reactions to form more polar compounds [Supplementary Figure 1]. These reactions result in different oxysterols such as monohydroxylated [OHC] or di-hydroxylated [di-OHC] derivatives, ketone derivatives [ketochol] and epoxycholesterols [epoxychol].[7,8] Oxysterols act on different receptors [nuclear receptors, G protein-coupled receptors or regulatory proteins] which have been implicated in many physiological and pathological processes, including IBD.[7,9,10] For instance, some oxysterols [7α,25-di-OHC and 7α,27-di-OHC] were identified as potent endogenous agonists of the nuclear receptor ROR-γ.[11] This receptor is required in the differentiation of Th17 cells,[11] which are suggested to have a pivotal role in the immune response of patients with CD and UC.[12] 7α,25-di-OHC and 7α,27-di-OHC are also ligands of the GPR183 membrane receptor.[13] Its activation is involved in chemotactic processes of B cells,[13] which are significant players in mucosal immunity. In addition, using LXRα$^{-/-}$, LXRβ$^{-/-}$ and LXRαβ$^{-/-}$ mice [receptors activated by 25-OHC, 27-OHC and 4β-OHC[14–16]], it was shown that LXRβ protects against dextran sulphate sodium [DSS]-induced and trinitrobenzene sulfonic acid [TNBS]-induced colitis.[17] Moreover, the expression of LXRs was decreased in colonic biopsies of CD and UC patients.[17] Additionally, risk loci associated with UC or CD were identified in genes coding for ROR-γ and GPR183.[18]

Together, these and other elements suggest a possible role for oxysterols and their receptors in the pathogenesis of IBD. However, nothing is known about the modulation of these bioactive lipids in the context of IBD or in the models mimicking the disease. In addition, no study has yet administered an oxysterol in an *in vivo* model of colitis in order to explore its impact on IBD markers.

Therefore, in this study, using several models of acute and chronic murine colitis, we analysed variations of oxysterol levels in several tissues and biological fluids [colon, liver, portal vein and vena cava plasma] of mice with colitis in comparison with control mice. We also analysed expression of the key enzymes involved in oxysterol metabolism in the colon of the same mice. We additionally measured oxysterol levels in colonic biopsies of patients with active CD and UC to determine whether oxysterol levels could also be altered in patients. Based on quantitative data, we selected two oxysterols, 4β-OHC and 25-OHC, to be administered to mice with colitis and we analysed their effects on the key markers of the disease.

2. Material and Methods

2.1. Mice

Studies involving animals were performed in compliance with European Directive 2010/63/EU, transformed into the Belgian Law of May 29, 2013 regarding the protection of laboratory animals [agreement number LA1230365] and were approved by the Université catholique de Louvain's animal studies ethics committee [2014/UCL/MD/001]. Male C57BL/6 mice [Janvier], aged 8–10 weeks old and of mean weight 23 g, were housed in filter-top cages in a controlled environment with a 12-h light–dark cycle, environmental enrichment, and free access to food and water. Upon arrival, they were randomly split into groups [with weight matching between the control and colitis groups] of eight mice each [four mice per cage] for DSS-induced colitis and oxazolone-induced colitis, or ten mice [five mice per cage] for TNBS-induced colitis and acclimatized for 1 week before induction of colitis. The number of mice per group was based on our own expertise in these models of colitis and their induction, as well as the literature. Mice wellbeing was monitored at least daily throughout the experiments.

2.2. Induction of experimental colitis

2.2.1. DSS-induced colitis

2.2.1.1. Acute colitis

To induce colitis, 5% DSS [MW 40 kDa, TdB Consultancy AB] was added to the drinking water over a 5-day period, and this solution was replaced with normal drinking water for an additional 2 days. Mice [eight per group] were killed on day 7.[19] Control mice received only normal drinking water. Body weight and food and water intake were monitored daily.

For experiments where oxysterols were administered, 4β-OHC and 25-OHC [10 mg/kg each] in a mixture of saline–ethanol–Tween 80 [18:1:1, v/v/v], or vehicle, were administered intraperitoneally once daily, starting on the day of addition of DSS to the drinking water. Mice were killed 5 h after the last intraperitoneal injection.

2.2.1.2. Chronic colitis

Chronic colitis was induced by three cycles consisting of 7 days of DSS [2.5%] in the drinking water followed by 3 weeks of normal drinking water.[20] Control mice received only normal drinking water for the duration of the study. Body weight as well as food and water intake were monitored daily for the duration of the study. Mice [eight per group] were killed during the recovery phase, 18 days after the end of the third DSS cycle.

2.2.2. TNBS-induced colitis

Food [but not water] was withdrawn 24 h before administration of TNBS [Sigma-Aldrich]. Colitis was induced as previously described.[21] Briefly, mice [ten per group] were anaesthetized by injection of a mixture of ketamine [100 mg/kg i.p.] and xylazine [10 mg/kg i.p.]. TNBS [100 mg/kg, in ethanol–0.9% NaCl [50:50, v/v]] was administered intrarectally [50 µL] into the colon using a cannula inserted 4 cm from the anus. Control mice received 50 µL of an ethanol–0.9% NaCl [50:50, v/v] solution. To help to provide a consistent distribution and retention of TNBS [or vehicle] in the colon, mice were held vertically by the tail for 60 s after administration. Body weight as well as water and food intake were monitored daily, and mice were killed 3 days after TNBS administration.

2.2.3. Oxazolone-induced colitis

Colitis was induced by intrarectal administration of oxazolone (i.e. 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one [Sigma-Aldrich]).[20] Briefly, mice [eight per group] were pre-sensitized by application of 150 µL of a solution of 3% [w/v] oxazolone in a mixture of acetone and olive oil [4:1] on a shaved patch of skin. One week later, mice were re-challenged by intrarectal administration of oxazolone. Food [but not water] was withdrawn 24 h before administration of oxazolone. Mice were anaesthetized by injection of a mixture of ketamine [100 mg/kg i.p.] and xylazine [10 mg/kg i.p.]. Using a cannula inserted 4 cm from the anus, 100 µL of a solution of 1.5% [w/v] oxazolone (in 50:50 (v/v) ethanol–0.9% NaCl) was administered into the colon. Control mice received 100 µL of an ethanol–0.9% NaCl [50:50, v/v] solution. As with TNBS, mice were held vertically by the tail for 60 s after administration to provide a consistent distribution and retention of oxazolone [or vehicle] in the colon. Body weight

and water and food intake were monitored daily, and mice were killed 3 days after intrarectal administration of oxazolone.

For all colitis models, mice were anaesthetized [using isoflurane] and blood was collected before killing by cervical dislocation. Colons were excised, measured and weighed before freezing in liquid nitrogen. Tissues were stored at −80°C until analysis.

2.3. Human samples

Twenty patients referred to the endoscopic unit of the CHU UCL Namur for colonic surveillance or follow up of their IBD were included in the study. The study, which was approved by the Ethics Committee of the CHU UCL Namur [Reference number EC: 80/2016] and declared in September 2016 to the Belgian authorities [No. B039201629251] was conducted between September 2016 and November 2016. All patients gave informed consent to participate to the study. In a first experiment, five were non-IBD patients and five had colonic CD. In a second set of experiments, five were non-IBD patients and five had UC. On average, three or four colonic biopsies were obtained for each patient during the endoscopic procedure, either from a macroscopic inflamed segment in IBD patients or normal appearing segments in controls and stored at −80°C. Histological assessment confirmed the normal or inflamed mucosa of each sample [data not shown].

2.4. Oxysterol quantification

2.4.1. Oxysterol standards and reagents

4β-Hydroxycholesterol [4β-OHC], 5α,6α-epoxycholesterol [5α,6α-epoxychol], 5α,6β-dihydroxycholesterol [5α,6β-di-OHC], 5β,6β-epoxycholesterol [5β,6β-epoxychol], 7α-hydroxycholesterol [7α-OHC], 7α-hydroxycholestenone [7α-OHCnone], 7-ketocholesterol [7-ketochol], 22(*R*)-hydroxycholesterol [22(*R*)-OHC], 24(*S*)-hydroxycholesterol [24(*S*)-OHC], 25-hydroxycholesterol [25-OHC], 7α,25-dihydroxycholesterol [7α,25-di-OHC], 27-hydroxycholesterol [27-OHC], 7α,27-dihydroxycholesterol [7α,27-di-OHC], 24(*S*),25-epoxycholesterol [24(*S*),25-epoxychol], [25,26,26,26,27,27,27-$^{2}H_{7}$]4β-hydroxycholesterol [d_{7}-4β-OHC] and [25,26,26,26,27,27,27-$^{2}H_{7}$]24[*R/S*]-hydroxycholesterol [d_{7}-24-OHC] were purchased from Avanti Polar Lipids. High-performance liquid chromatograph/mass spectroscopy [HPLC-MS] grade dichloromethane, isopropanol, hexane and methanol were purchased from VWR Belgium. Butylated hydroxytoluene [BHT] and ethylene diaminetetraacetic acid [EDTA] were purchased from Sigma Aldrich.

2.4.2. Oxysterol quantification

Free oxysterols were quantified using a validated HPLC-MS method developed in our laboratory.[22] Frozen portions of tissues [mouse colon: 50 mg; mouse plasma: 50 µL; human colon biopsies: 10 mg] were weighed and directly homogenized in a glass vial containing dichloromethane [8 mL]. Then, internal standards [d_{7}-4β-OHC and d_{7}-24-OHC] were added as well as BHT [10 µg per vial] and EDTA [20 ng per vial] to avoid artefactual formation of oxysterols due to cholesterol oxidation during the process. Methanol [4 mL] and double distilled water [2 mL] were then added to the vials. The resulting mixture was mixed vigorously and sonicated [10 min, 4°C]. After centrifugation, the organic layer [containing the oxysterols] was removed and brought to dryness using a nitrogen stream. The samples were then pre-purified over silica using hexane–isopropanol to remove cholesterol, after which oxysterols were recovered by increasing the proportion of isopropanol. The resulting fraction was analysed on an LTQ-Orbitrap mass spectrometer coupled to an Accela HPLC device [ThermoFisher Scientific]. Oxysterol separation was obtained using a kinetex LC-18 column [4.6 × 150 mm, 5 µm] [Phenomenex]. The gradient [400 µL/min] started at 100% MeOH–$H_{2}O$–acetic acid 75:24.9:0.1 [v/v/v] and reached linearly 100% MeOH–acetic acid 99.9:0.1 [v/v] in 15 min, followed by a 10-min plateau before re-equilibrating at the initial conditions. An atmospheric pressure chemical ionization [APCI] source operated in positive mode was used for ionization of analytes. Xcalibur [ThermoFisher Scientific] was used for data acquisition and processing. Oxysterols were quantified by isotope dilution using as internal standards, d_{7}-24-OHC for those oxidized on the lateral side chain and d_{7}-4β-OHC for those oxidized on the sterol backbone. Calibration curves were built using the same protocol.[22]

2.5. Gene expression by RT-qPCR

Total RNA from tissues was obtained using TriPure reagent [Roche] according to the manufacturer's instructions. cDNA was synthesized from total RNA [1 µg] using a reverse transcription kit [Promega, Reverse Transcription System]. Qunatitative PCR [qPCR] was performed with a STEPone PLUS Real-Time PCR System [Applied Biosystems] as previously described.[23] PCRs were run using SYBR Green mix [Promega, GoTaq qPCR Master Mix]. Each sample was measured in duplicate during the same run. Products were analysed by developing a melting curve at the end of the PCR. Data are normalized to the 60S ribosomal protein L19 [RPL19] used as reference gene. RPL19 mRNA expression was not affected by any of the conditions. The sequences of the primers used are listed in Table 1.

2.6. Myeloperoxidase activity

A tissue-associated myeloperoxidase [MPO] assay was performed as previously described.[19] In brief, colon sections were snap-frozen in liquid nitrogen and stored for later assessment. For determination of MPO activity, tissue was placed in hexadecyltrimethylammonium bromide [HTAB] buffer [0.5% HTAB in 50 mM potassium phosphate buffer [pH 6]] on ice and gently homogenized. The homogenate was centrifuged at 18000 *g* for 20 min at 4°C. A total of 7 µL of supernatant was then added to 96-well plates together with 200 µL of a solution of 0.167 mg/mL *o*-dianisidine and 500 ppm hydrogen peroxide in potassium phosphate buffer [50 mM, pH 6]. Samples were analysed in duplicate. Total protein concentration was determined using the DC protein assay [Bio-Rad]. MPO activity in the supernatant was measured at 460 nm and normalized for protein concentration. Results are expressed as the percentage (mean ± SEM) of the respective control group.

2.7. Statistical analyses

Comparisons between two groups were done using Student's t test or the Mann–Whitney non-parametric test when the distribution was not normal. Comparisons between three groups were done using a one-way ANOVA followed by Dunnett's post hoc test or the Mann–Whitney test followed by a Dunn's post hoc test when the requirements for ANOVA were not met. Statistical significance is identified using $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Acute colitis alters oxysterol levels in the mouse colon

We focused our analysis on ten oxysterols that were detected and quantified by HPLC-MS in the colons of control mice, albeit at quite different amounts [Figure 1A]. 4β-OHC was found to be the most abundant oxysterol in the colon [3663.6 ± 241 pmol/g of tissue],

Table 1. Primer sequences

Gene	Product	Forward primer [5′ to 3′]	Reverse primer [5′ to 3′]
Mouse primers			
Ccl3	MIP-1α	AGATTCCACGCCAATTCATC	CTCAAGCCCCTGCTCTACAC
Ch25h	Cholesterol-25-hydroxylase	CTGACCTTCTTCGACGTGCT	GGGAAGTCATAGCCCGAGTG
Cyp27a1	CYP27A1	GGCTACCTGCACTTCCT	CTGGATCTCTGGGCTCTTTG
Cyp3a11	CYP3A11	GAAGCATTGAGGAGGATCACA	GTGCCTAAAAATGGCAGAGG
Cyp3a13	CYP3A13	TTTGATCCACTGTTCCTCTC	TCTCTTGGAAACAAGGAGAC
Cyp7b1	CYP7B1	TAGGCATGACGATCCTGAAA	TCTCTGGTGAAGTGGACTGAAA
Ebp [D8D7I]	Cholesterol-epoxide hydrolase [ChEH]	AGGGCTGGTTCTCTCTCTAC	CGACGAAGCTGTCACTAAGG
Hsd3b7	HSD3B7	CAGTCCACAGCCATCCCTACC	CGTGGGTCGAAGGGCACA
Hsd11b1	11B-HSD1	CCTTGGCTGGGAAAATGACCC	AGGACACAGAGAGTGATGGACA
Il1b	IL-1B	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC
Il6	IL-6	ACAAGTCGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC
Cxcl1	KC	GGCTGGGATTCACCTCAAG	GAGTGTGGCTATGACTTCGG
Nr1h3	LXRalpha	ACCAGCTCCAAGTAGAGAGG	GCATCCGTGGGAACATCAG
Nr1h2	LXRbeta	TGAAGGCGTCCACCATTG	CGTCCTTGCTGTAGGTGAAG
Ccl2	MCP-1	GCAGTTAACGCCCCACTCA	TCCAGCCTACTCATTGGGATCA
Mmp9	MMP9	CACACGACATCTTCCAGTACCA	TGGAAACTCACACGCCAGAAGA
Rpl19	RPL19	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT
Tnf	TNFalpha	AGCCCCCAGTCTGTATCCTT	GGTCACTGTCCCAGCATCTT
Human primers			
CH25H	Cholesterol-25-hydroxylase	CTTCCCTTGGTCCACTCAC	GGAGCGAAGTTGCAGTTAAAG
CYP11A1	CYP11A1	CAGCATCAAGGAGACACTAAGAC	TGGCAGGAATCATGTAATCTCG
CYP27A1	CYP27A1	GGCTCAACTCTTCTACTACTTTGC	CGTTTCTCGAACAGGATGTAGC
CYP3A4	CYP3A4	AAGGGATGGCACCGTAAGTG	GGTCTCTGGTGTTCTCAGGC
CYP3A5	CYP3A5	GGACCCGTACACATGGACTTT	CCAGAGACCCTGACGATAGGA
CYP7A1	CYP7A1	GTCATACCATAAGGTGTTGTGC	CAAATGCCTTCGCAGAAGTAG
CYP7B1	CYP7B1	GACCTTGAAATAGGAGCACATC	GCCCAGAACATAGTTGGAATAG
EBP	Cholesterol-epoxide hydrolase [ChEH]	TCTGGAAAGAGTATGCCAAGG	CATGCACACTGTGAAGTTGTC
HSD3B7	HSD3B7	GAACACGGTTCCTGGTCTAC	TGCACTGCTTCGTATGGG
IL-1b	IL-1B	ACGCTCCGGGACTCACAGCA	TGAGGCCCAAGGCCACAGGT
IL-8	IL-8	GGCAGCCTTCCTGATTTCTG	GGGTGGAAAGGTTTGGAGTATG
NOS2	iNOS	TGCTTTGTGCGGAATGCCAG	AGGCCCGATGAGGATGCAAG
CCL2	MCP-1	CTCAGCCAGATGCAATCAATG	CTTGCTGCTGGTGATTCTTC
RPL19	RPL19	CACATCCACAAGCTGAAGGCA	CTTGCGTGCTTCCTTGGTCT

followed by 5α,6α-epoxychol, 5β,6β-epoxychol, 7-OHC [pool of 7α- and 7β-OHC] and 7-ketochol, which had levels around 500 pmol/g of tissue. The levels of 5α,6β-di-OHC, 7α-OHCnone and 25-OHC ranged between 100 and 200 pmol/g of tissue, while 7α,25-di-OHC, 27-OHC and 7α,27-di-OHC were present in smaller amounts [around 30 pmol/g of tissue].

To assess the effect of colon inflammation on oxysterol levels, we used two murine acute colitis models, DSS-induced colitis and TNBS-induced colitis. In both cases, colon inflammation was assessed by the colon weight/length ratio, MPO activity and pro-inflammatory cytokine expression. All these characteristic markers of colitis were increased upon DSS or TNBS administration [Supplementary Figure 2].

Both models of acute colitis altered the levels of several oxysterols in the colon, although more oxysterols were altered in the DSS model. In both cases, 4β-OHC and 25-OHC were increased in the inflamed colon [Figure 1B] and were correlated with mRNA expression of pro-inflammatory cytokines and chemokines [IL-1β, IL-6, TNF-α and MCP-1] [Supplementary Table 1]. 7α-OHCnone was increased in DSS-induced colitis and decreased in the TNBS model while 5α,6α-epoxychol and 7α,25-di-OHC were only affected in the DSS model [Figure 1B].

3.2. Effect of acute colitis on oxysterol metabolizing enzymes

As the levels of some oxysterols were affected by inflammation of the colon, we assessed the effect of inflammation on mRNA expression of oxysterol-metabolizing enzymes to see whether this could explain the changes in oxysterol levels. Some oxysterols, such as 7-ketochol and the epoxycholesterols, are formed through non-enzymatic reactions by reactive oxygen species [ROS]-driven oxidation, while other oxysterols are formed through enzymatic reactions [Supplementary Figure 1]. It would therefore be expected that the levels of these ROS-produced oxysterols increase during an acute inflammatory reaction. However, this was not the case here.

Regarding the enzymes, Chol25OHase mRNA was increased both in DSS-induced and in TNBS-induced colitis [Figure 2] consistent with the increased levels of 25-OHC. 7α,25-di-OHC is a metabolite of 25-OHC formed by the action of CYP7B1. The mRNA expression of this enzyme was increased in both colitis models, although to a greater extent in the DSS model [Figure 2]. The levels of its product 7α,25-di-OHC were accordingly increased in DSS-induced, but not in TNBS-induced colitis. Surprisingly, the mRNA expression of CYP3A11 and CYP3A13, responsible for 4β-OHC formation, was decreased [Figure 2] although the levels of this oxysterol were increased in colitis. The mRNA expression of HSD3B7, which converts 7α-OHC into 7α-OHCnone, was decreased in both colitis models [Figure 2], consistent with the decrease in 7α-OHCnone in the TNBS model, but not with its increase in the DSS model. However, HSD3B7 is also implicated in the catabolism of other oxysterols. Finally, CYP27A1 mRNA levels were unchanged in DSS-induced colitis and decreased in TNBS-induced colitis [Figure 2], while the levels of its product, 27-OHC, were unchanged in both models.

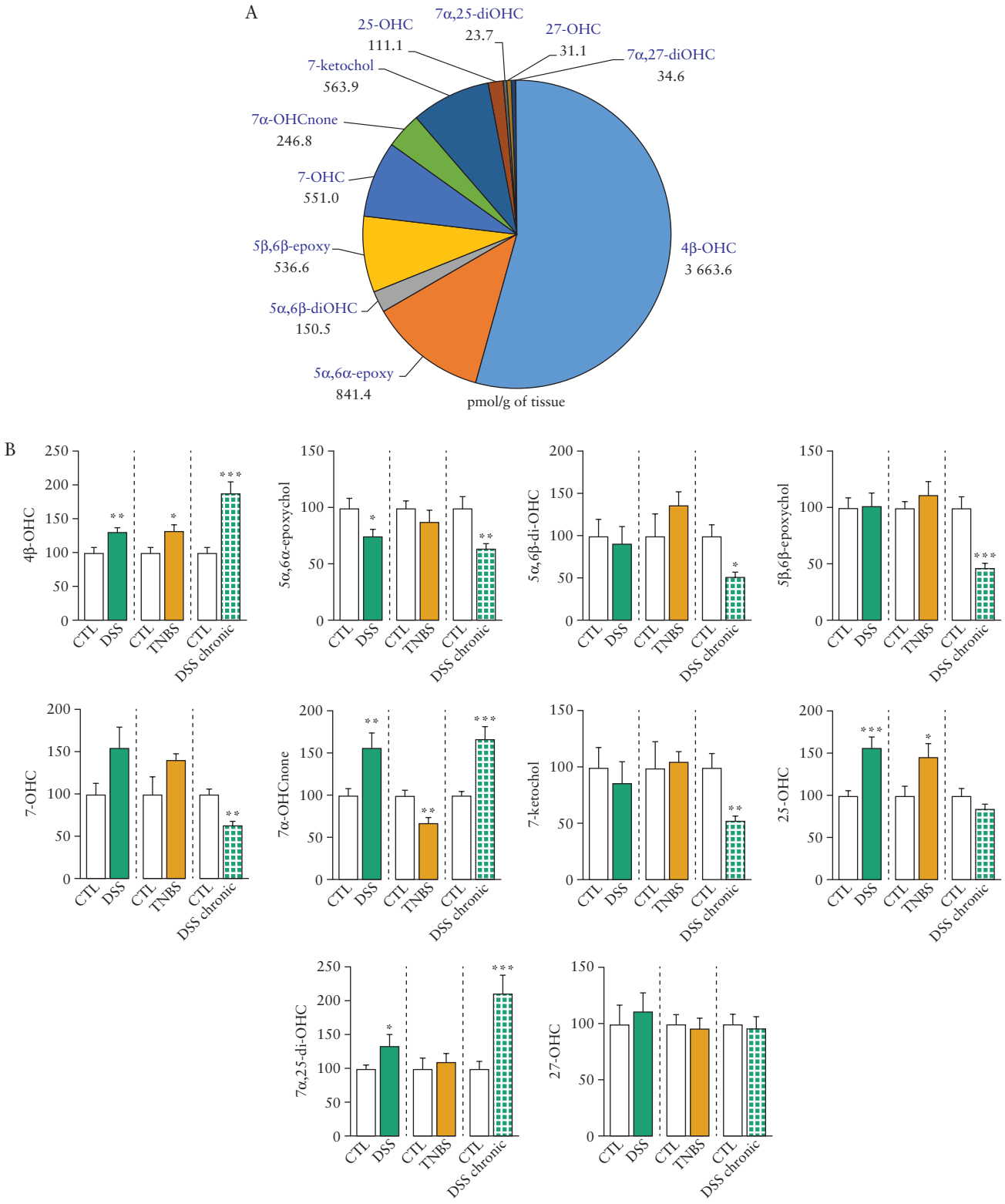

Figure 1. Colitis alters colon oxysterol levels. [A] Oxysterol levels [in pmol/g of tissue] measured by HPLC-MS in the colon of control mice. [B] Variations of oxysterol levels measured by HPLC-MS in the colon of mice with dextran sulfate sodium-induced colitis [DSS], trinitrobenzene sulfonic acid-induced colitis [TNBS] and chronic DSS-induced colitis [DSS chronic]. Data are expressed as percentage [mean ± SEM] of the respective control [CTL] group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ for colitis vs CTL.

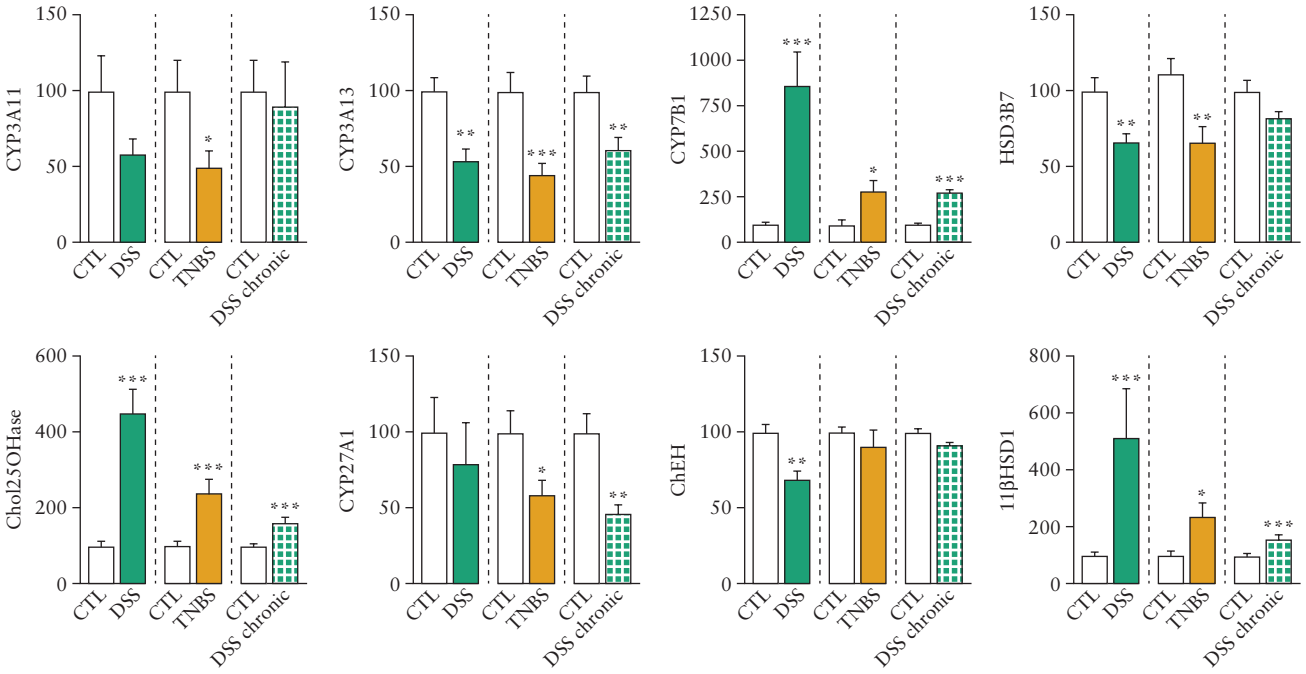

Figure 2. Colitis alters mRNA levels of oxysterol-metabolizing enzymes. mRNA levels, measured by RT-qPCR, of oxysterol-metabolizing enzymes in the colon of the mice from Figure 1: DSS-induced colitis [DSS], TNBS-induced colitis [TNBS] and chronic DSS-induced colitis [DSS chronic]. RPL19 was used as a reference gene. Data are expressed as percentage [mean ± SEM] of the respective control group [CTL]. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ for colitis vs CTL. Chol25OHase: cholesterol 25 hydroxylase. ChEH: cholesterol epoxyde hydrolase. The mouse metabolic pathways are depicted in Supplementary Figure 1.

Thus, while mRNA expression of some of these enzymes could explain the variations in levels we observed [such as for 25-OHC and 7α-25-di-OHC] in both acute colitis models, some discrepancies remain which point to a more complex pathway for oxysterol synthesis and/or post-transcriptional regulation of these enzymes.

3.3. Mouse acute colitis affects oxysterol levels in the plasma

To assess if colitis also affects oxysterol levels in the plasma, we quantified oxysterols in plasma from the portal vein and the vena cava of the same mice with DSS-induced colitis. Concerning plasma oxysterol levels in control mice, we found the same general profile as in the colon. 4β-OHC was the most abundant oxysterol in the plasma and the oxysterols oxidized on the side-chain were the least abundant. 22(*R*)-OHC was below our quantification limit.

Colon inflammation led to variations in plasma oxysterol levels, which were generally the same for plasma from the portal vein and vena cava. Some of these variations, such as the increase in 4β-OHC and 7α-OHCnone, were similar to the variations observed in the colon [Figures 1 and 3]. Plasma levels of 5β,6β-epoxychol, 5α,6β-di-OHC and 7-ketochol were not altered by colitis, as was the case in the colon [Figures 1 and 3]. However, the levels of some oxysterols that were altered in the colon, such as 5α,6α-epoxychol and 7α,25-di-OHC, remained constant in the plasma [Figures 1 and 3]. Conversely, 7-OHC and 27-OHC levels were increased in the plasma, while they were not affected by inflammation in the colon [Figures 1 and 3].

3.4. Mouse acute colitis affects oxysterol levels in the liver

Colon inflammation, and the subsequent alteration in epithelial permeability, leads to a peripheral inflammation, which is responsible for some of the extraintestinal manifestations of IBD.[24] Systemic inflammation is also present in murine models of IBD as we previously showed.[21] This can be observed in the liver with increased expression of pro-inflammatory cytokines.[19] Moreover, the liver is an important organ for cholesterol metabolism and oxysterol synthesis.[6,25] Therefore, we also quantified oxysterols in the liver of mice with DSS-induced colitis. Colitis induced changes in oxysterol levels in the liver, namely an increase in the levels of 5α,6β-di-OHC, 7-OHC, 7-ketochol and 25-OHC [Supplementary Figure 3]. Apart from the increase in 7-OHC and 25-OHC, these variations are not similar to those found in the colon or plasma. For instance, 4β-OHC, which was increased in the colon and plasma, is not altered in the liver. The opposite is true for 7-ketochol, which was unaltered in the colon and plasma. The levels of 5α,6α-epoxychol and 7α,25-di-OHC were unaltered in the liver and plasma but decreased and increased, respectively, in the colon. Together, these data suggest that the observed alterations in oxysterol levels in the colon and liver are due to changes in the local production of these oxysterols.

3.5. Effect of chronic colitis on oxysterol levels in the colon and plasma of mice

The above observations were made in the setting of acute colitis. To determine if such variations were also present in a more chronic model, we analysed oxysterol levels in the colon and in the portal vein plasma of mice with a chronic form of DSS-induced colitis [Supplementary Figure 2]. In this case, mice were killed during the remission phase, 18 days after the end of the third DSS cycle, i.e. when colitis is established but not during an acute flare. Concerning the colon, we observed similar variations in the levels of 4β-OHC, 7α-OHCnone, 7α,25-di-OHC and 5α,6α-epoxychol in the acute and chronic models of DSS-induced colitis, with an increase in 4β-OHC, 7α-OHCnone and 7α,25-di-OHC levels, and a decrease in

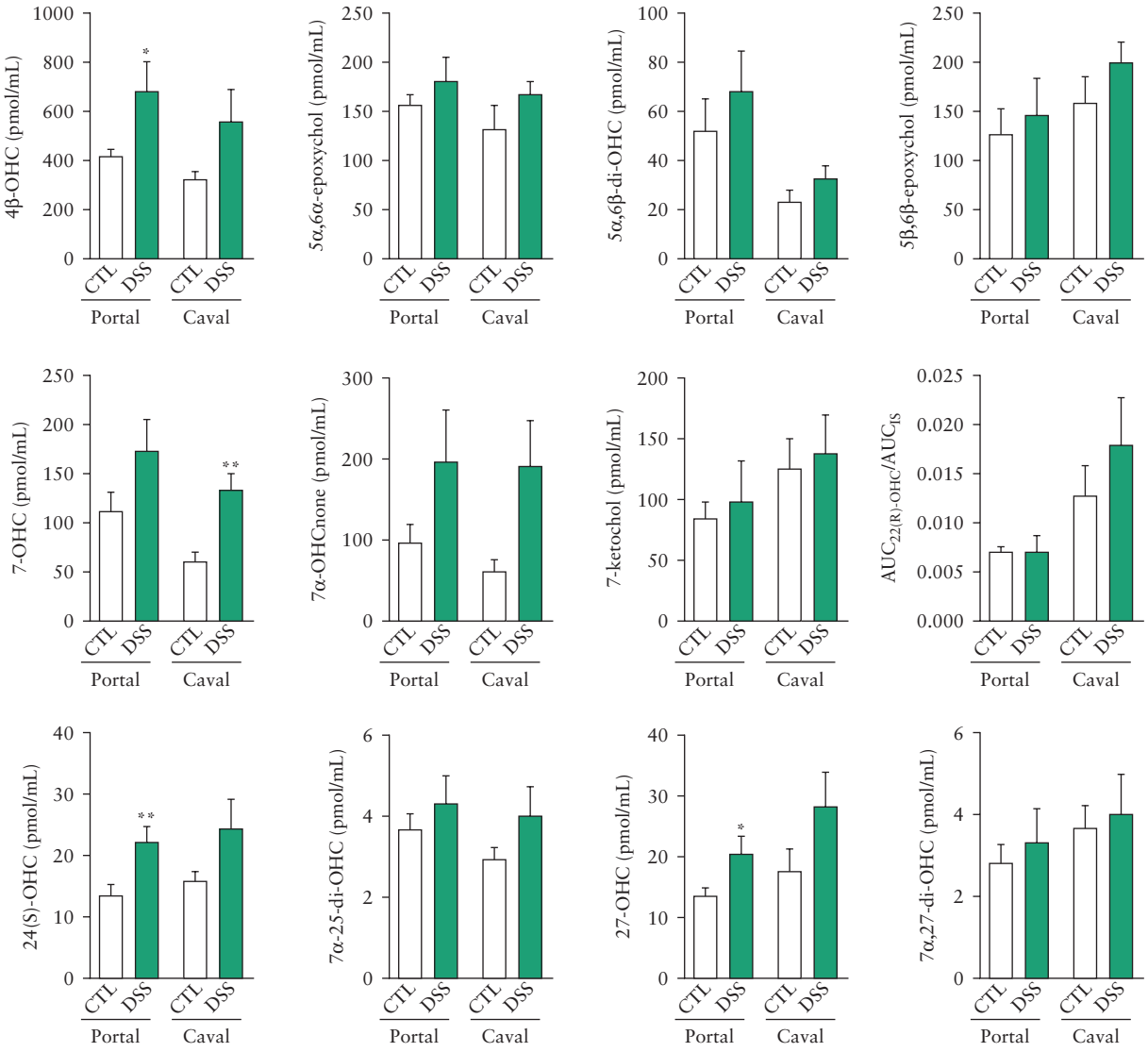

Figure 3. Dextran sulfate sodium [DSS]-induced colitis alters plasma oxysterol levels. Oxysterol levels [in pmol/mL] in the plasma from the portal vein [portal] and the vena cava [caval] of mice with DSS-induced colitis were measured by HPLC-MS. Data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$ for DSS vs CTL.

5α,6α-epoxychol levels [Figure 1B]. However, we also observed a decrease in the levels of 5β,6β-epoxychol, 5α,6β-di-OHC, 7-OHC and 7-ketochol in the chronic model of colitis [Figure 1B]. Interestingly, we found here that oxysterol levels in the colon were positively or negatively correlated with colitis markers [colon weight/length ratio] or inflammatory cytokine expression [IL-1β and TNF-α]. Indeed, the oxysterols that were increased with the colitis were positively correlated with inflammatory markers, while the oxysterols that were decreased by the colitis [with the exception of 7-OHC] were negatively correlated with these same markers [Supplementary Table 2].

As with the acute models, we assessed mRNA expression of the enzymes involved in oxysterol metabolism in the colon. CYP27A1 and CYP3A13 were decreased in the colitis group compared to the control group while CYP7B1 and Chol25OHase were increased [Figure 2]. Expression of 11β-HSD1, responsible for the interconversion of 7β-OHC and 7-ketochol,[26,27] was also increased in the colitis group compared to the control group [Figure 2]. However, as was the case in the acute setting, the only variations in enzyme expression that could explain variations in oxysterol levels are the increases in Chol25OHase and CYP7B1 leading to increased levels of 7α,25-di-OHC.

In contrast to the colon, we did not observe many variations in oxysterol levels in the portal plasma in this chronic model of colitis. The only variations observed were an increase in 4β-OHC levels and, in contrast to what we observed in the acute setting, a decrease in 27-OHC levels [Supplementary Figure 4].

3.6. Effect of oxazolone-induced colitis in mice on oxysterol levels

Both the DSS- and the TNBS-induced colitis models are mainly characterized by a Th1 immune response, sometimes accompanied by a Th17 response.[28,29] Therefore, to determine whether a different activation of the immune system in colitis would also affect oxysterol levels, we quantified oxysterols in the oxazolone-induced model of colitis, which is characterized by a Th2 immune response.[28,29] Following oxazolone administration to the mice, we assessed the

colitis by measuring colon length and mRNA expression of cytokines [Supplementary Figure 5]. In contrast to what we observed in the acute DSS- and TNBS-induced colitis models, 25-OHC levels were decreased in oxazolone-induced colitis while the levels of 4β-OHC were not altered [Supplementary Figure 5]. The levels of 7-OHC, which were unchanged in both the DSS- and the TNBS-induced colitis, were increased in oxazolone-induced colitis [Supplementary Figure 5]. Finally, we observed a decrease in 7α-OHCnone levels, as was observed in TNBS-induced colitis [Supplementary Figure 5].

3.7. Oxysterol levels and metabolism are altered in colonic biopsies of patients with CD and UC

From a pathophysiological point of view, it is important to know whether oxysterol levels and metabolism are also altered in IBD patients. Therefore, we measured oxysterol levels in biopsies from patients with active CD or UC and compared them to biopsies from control patients [Figure 4]. Additionally, in the same biopsies we measured the mRNA expression of the enzymes involved in oxysterol metabolism [Figure 5] as well as pro-inflammatory cytokines to assess colon inflammation [Supplementary Figure 6]. We found that the levels of 7α-OHCnone and 25-OHC were increased in human IBD whereas 27-OHC levels were decreased [Figure 4]. 4β-OHC levels were decreased in human IBD, but this was not statistically significant for UC patients as 4β-OHC was decreased to levels that were below the quantification limit in some of the biopsies from patients with active disease. We then investigated the mRNA expression of the oxysterol-metabolizing enzymes. We found a decrease in mRNA expression of CYP3A4 and CYP3A5, responsible for the production of 4β-OHC in humans, in both forms of IBD compared to controls [Figure 5], in accordance with the decreased levels of this oxysterol. CYP27A1 mRNA expression was also decreased [Figure 5], as were the levels of its product 27-OHC. However, more variations were observed in the biopsies of patients with UC compared to CD. Indeed, mRNA expression of CYP7A1, CYP8B1 and Chol25OHase were also increased in the biopsies from patients with UC but not CD [Figure 5]. These variations are in line with the increase of their products 7α-OHCnone and 25-OHC, respectively, in UC [Figure 4].

3.8. Effect of administration of 4β-OHC and 25-OHC on DSS-induced colitis

Following our observations on the effect of colon inflammation on oxysterol levels, we assessed the effect of administration of selected

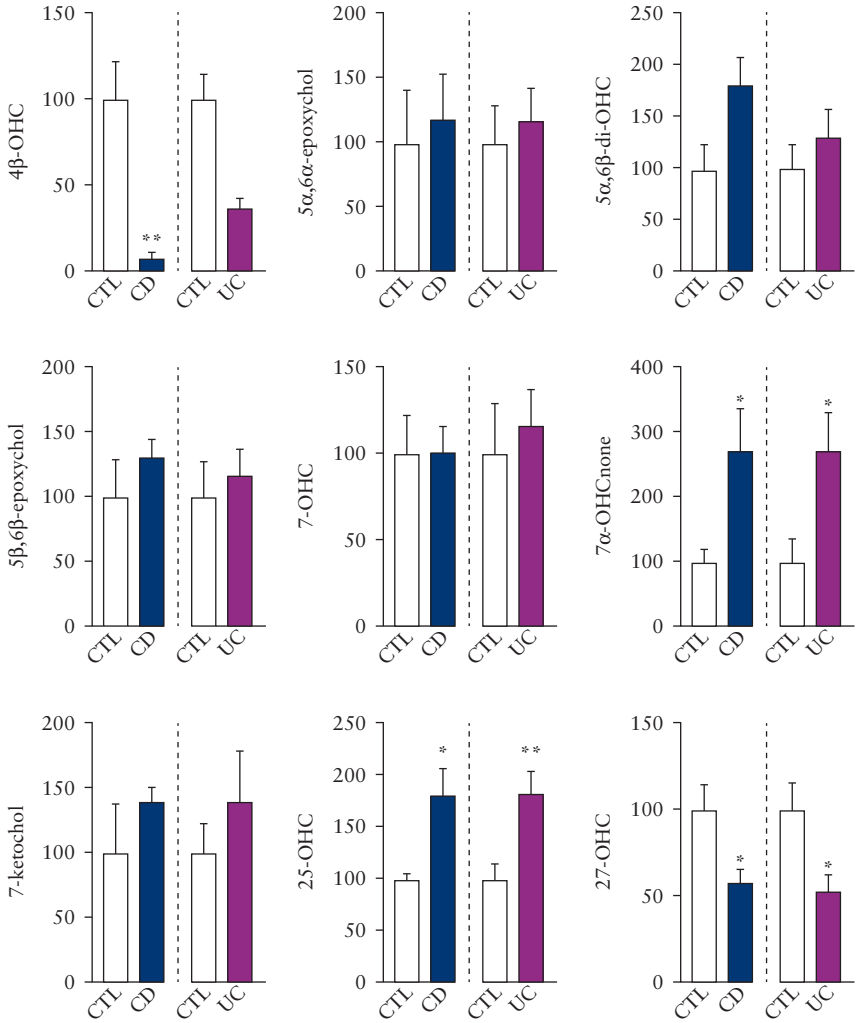

Figure 4. Crohn's disease and ulcerative colitis alter colon oxysterol levels. Oxysterol levels in colon biopsies were measured by HPLC-MS for two cohorts of subjects: patients with Crohn's disease [CD] and control subjects [CTL], and patients with ulcerative colitis [UC] and control subjects [CTL]. Data are expressed as percentage [mean ± SEM] of the control group. *$P < 0.05$, **$P < 0.01$ for CD or UC vs CTL.

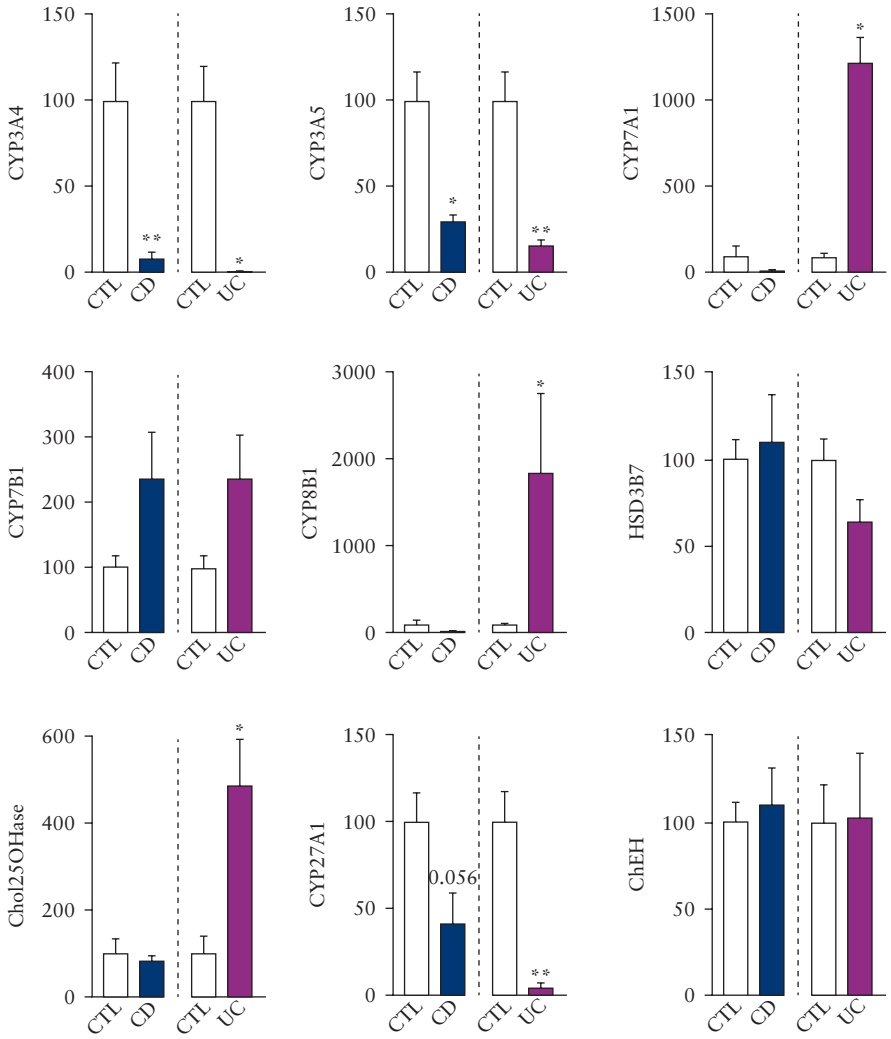

Figure 5. IBD alters mRNA expression of oxysterol-metabolizing enzymes. mRNA levels of oxysterol-metabolizing enzymes were measured by RT-qPCR in colon biopsies for two cohorts of subjects: patients with Crohn's disease [CD] and control subjects [CTL], and patients with ulcerative colitis [UC] and control subjects [CTL]. RPL19 was used as a reference gene. Data are expressed as percentage [mean ± SEM] of the control group. $*P < 0.05$, $**P < 0.01$ for CD or UC vs CTL. Chol25OHase: cholesterol 25 hydroxylase. ChEH: cholesterol epoxyde hydrolase.

oxysterols on colitis. Indeed, there are very few reports on the effects of oxysterol administration *in vivo*. We administered 4β-OHC and 25-OHC, which had altered levels in all the colitis models as well as in IBD biopsies. The former is the most abundant oxysterol in the mouse colon but besides being an LXR ligand,[15] little is known about its biological effects in inflammation. On the other hand, 25-OHC is the most studied oxysterol in inflammation[30–33] and is an agonist of GPR183, LXRα and LXRβ, receptors known to be involved in IBD.[34,35]

As these oxysterols are potential LXR and GPR183 ligands, we analysed mRNA expression of LXRα, LXRβ and GPR183 in the mouse colon and in the human biopsies. All three receptors were expressed in the colon [Supplementary Figures 6 and 7], with LXRα decreased by DSS-induced colitis [Supplementary Figure 7], LXRβ in UC biopsies and both LXRs in CD biopsies [Supplementary Figure 6]. Oxysterols have been reported to be very labile compounds when administered *in vivo*, and therefore we quantified the levels of 4β-OHC and 25-OHC, as well as its metabolite 7α,25-diOHC, in the colon of mice receiving these compounds and found a drastic increase in their respective levels when they were administered [Supplementary Figure 7].

Administration of 4β-OHC to mice with DSS-induced colitis did not affect the colon weight/length ratio or MPO activity compared to the vehicle-treated DSS group [Figure 6A, B]. However, it increased the mRNA expression of several markers of inflammation, such as the pro-inflammatory cytokines IL-1β and IL-6 as well as the chemokines MCP-1, MIP-1α and KC, together with MMP-9, an enzyme involved in breakdown of the extracellular matrix [Figure 6C]. On the other hand, 25-OHC administration had no effect on colitis in our study [Figure 6].

4. Discussion

In recent decades, there has been an increased interest in exploring the role of oxysterols in inflammatory conditions.[30,36,37] In the colon, the early studies were limited to *in vitro* systems mainly using the Caco-2 cell line.[38,39] Although the importance of these bioactive lipids is currently recognized in many pathophysiological conditions,[7] the large number of oxysterol species, the complexity of their metabolism and the multitude of molecular targets involved complicate our understanding of their mechanisms of action. The analysis

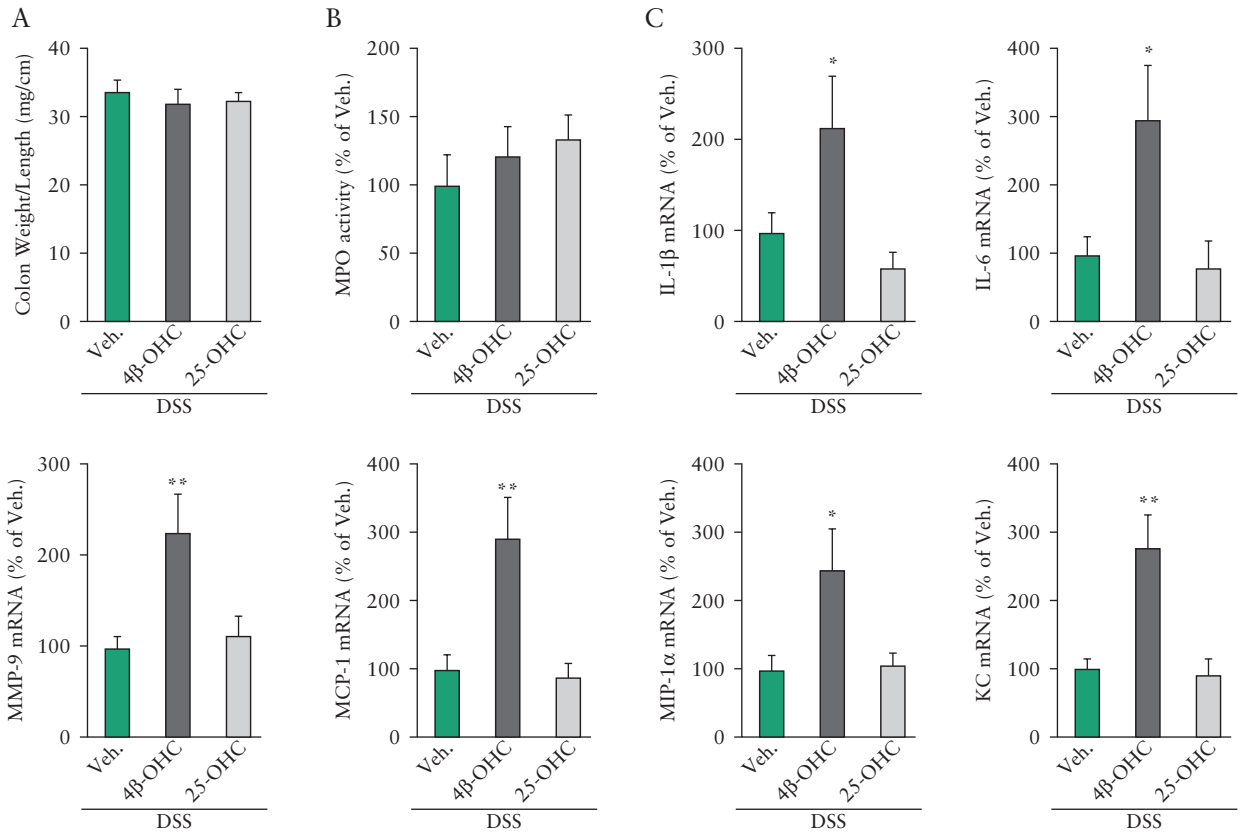

Figure 6. 4β-OHC modulates inflammation in dextran sulfate sodium [DSS]-induced colitis. C57BL/6 mice received 5% DSS in the drinking water for 5 days, followed by water for 2 days to induce colitis. Mice received 10 mg/kg of 4β-OHC, 25-OHC or vehicle [Veh.] i.p. daily starting on the day of addition of DSS in the water. [A] Colon weight/length ratio. [B] Myeloperoxydase [MPO] activity in the colon, expressed as percentage of the DSS-untreated group. [C] mRNA expression of inflammation markers measured by RT-qPCR, with RPL19 used as a reference gene. Data are expressed as percentage [mean ± SEM] of the DSS-untreated group [Veh.]. $*P < 0.05$, $**P < 0.01$ vs the DSS-untreated group [Veh.]

of a large number of oxysterol species, rather than quantifying a single oxysterol molecule, should help to resolve the role of oxysterols in a given condition.

In the present work, we quantified oxysterol levels in the colon, plasma and liver of mice with acute and chronic colitis. To our knowledge, this is the first report of oxysterol quantification in the colon. We found that 4β-OHC is the most abundant unesterified oxysterol in the mouse colon, similarly to what we measured in the liver. In fact, when considering the proportion of the different oxysterols, the profile found in the colon was very similar to that in the liver, which is considered as the main tissue in terms of cholesterol metabolism. In the plasma, we found that 4β-OHC was also the most abundant oxysterol [although to a lesser extent than in the colon and liver]. This is consistent with the data we previously obtained in the serum from the portal vein.[40]

Our data show that colitis, in mice and in humans, is associated with a general disturbance in the enzymatic metabolism of oxysterols in the colon, as most oxysterols with altered levels during colitis are 'enzymatically produced'. Those oxysterols mainly formed by free radical-induced oxidation were much less affected by acute colitis, but were reduced in the chronic model.

When looking at the most abundant oxysterol in the colon, both acute [DSS- and TNBS-induced colitis] and chronic [DSS-induced colitis] models led to a significant increase of 4β-OHC in the colon and in the plasma. Besides for obesity,[22,40,41] this is the only report showing consistently altered levels of 4β-OHC in a pathological condition.

In mice, 4β-OHC is enzymatically formed from cholesterol by two enzymes, CYP3A11 and CYP3A13.[42,43] Contrasting with the increased levels of their product, mRNA expression of CYP3A11 and CYP3A13 was decreased in the colon of both DSS- and TNBS-induced colitis. The observed decrease in the expression of the CYP3A subfamily in colitis is in line with the literature.[44–46] The mRNA expression of CYP27A1, an enzyme catabolizing 4β-OHC, was also decreased by colitis. Although speculative, if CYP27A1 activity is more extensively reduced compared to CYP3A11 [or CYP3A13] this could explain the increased levels measured for 4β-OHC. Another explanation could be that other CYP3A isoenzymes [eight are described in mice] are able to synthetize 4β-OHC and are less subject to changes in expression during colitis.[47]

Although mRNA expression is an interesting tool to try and explain changes in oxysterol levels as the activity of individual cytochromes is not quantifiable in tissues, here it did not allow us to unravel all the pathways involved in the observed variations. Nevertheless, mRNA expression of Chol25OHase and CYP7B1 in the colon support the changes found for 25-OHC and 7α,25-di-OHC and was correlated with the expression of inflammatory markers in both the acute models of colitis [DSS and TNBS] and the chronic DSS-induced colitis [Supplementary Table 3].

Additionally, we found that oxysterol levels were altered differently depending on the model of colitis used. Indeed, DSS-induced colitis and TNBS-induced colitis are more closely related to CD while the immune response found in oxazolone-induced colitis is

more akin to UC.[29] Also, more oxysterol levels were altered in the chronic DSS-induced colitis compared to the acute colitis, supporting the fact that over time oxysterol metabolism could be markedly altered during colitis. The relevance of altered oxysterol levels due to IBD is further supported by our data obtained with colon biopsies from CD and UC patients. While these data are still preliminary in nature due to the limited number of biopsies we assessed, they show that oxysterol levels are altered in human IBD. Whether this participates in the pathogenesis of IBD remains to be elucidated.

4β-OHC and 25-OHC are increased in both DSS- and TNBS-induced acute colitis [Figures 1 and 2]. Moreover, 4β-OHC is increased in chronic DSS-induced colitis [Figure 5]. This is also the case for 7α,25-di-OHC [Figure 5], the metabolite of 25-OHC. This increase could represent a homeostatic response to decrease the inflammatory reaction, or an intrinsic part of the pathogenesis of the disease. Therefore, we administered these two oxysterols to test these hypotheses. 25-OHC, which is able to bind to a large number of receptors, including LXRα and LXRβ,[34] RORα and RORγ,[11] and GPR183,[35] had no effect on the markers of colitis we measured. This is quite surprising considering the previous reports on 25-OHC in models of inflammation.[30–33] However, only a limited number of papers described the effect of the administration of 25-OHC.[33,48] Nevertheless, this lack of effect is not due to metabolism that would have removed 25-OHC from the organism [as we measured higher levels of 25-OHC 5 h after its last administration] nor to the absence of its targets [as we found significant expression of LXRα and LXRβ in the control and inflamed colon].

Regarding 4β-OHC, unfortunately very few elements are known about its molecular targets. It was reported to activate LXRs but it is not clear whether, similarly to other oxysterols, multiple receptors could mediate its effects.[15] Its administration to mice with DSS-induced colitis led to a further increase in the expression of the cytokines and chemokines classically associated with the disease. However, it had no significant effects on the macroscopic signs of colitis, such as the colon weight to length ratio. Nevertheless, the fact that 4β-OHC levels are increased during colitis, that they are correlated with inflammatory markers and that its administration increases the expression of pro-inflammatory cytokines and chemokines suggests that this oxysterol may participate in the pathophysiology of colitis. As we lack information on all the targets of 4β-OHC, we can only speculate on the receptors mediating these effects. 4β-OHC has been proposed as an LXR ligand. However, this could probably be excluded as the mechanism of action as LXRβ was shown to have a protective role in DSS-induced colitis.[17] Furthermore, because the metabolism of oxysterols is quite complex [Supplementary Figure 1], we cannot exclude the possibility that the observed effects are due to a metabolite of 4β-OHC.

An alternative strategy to the administration of the oxysterol could have been to use knockout models of the enzymes involved in the synthesis of the oxysterols of interest [i.e. CYP3A and chol25OHase]. However, these models are not completely characterized yet in terms of changes in oxysterol levels. For instance, CYP3a$^{-/-}$ mice have decreased levels of 25-OHC and increased levels of 7α-OHCnone, but have not been characterized yet regarding the levels of 4β-OHC.[49]

In conclusion, we have shown that colitis, whether acute or chronic, affects oxysterol levels in the colon [as well as in the plasma and liver]. We also show that the administration of 4β-OHC, a poorly studied oxysterol, increases the expression of inflammatory markers in the colon, and thus that it potentially participates in the pathophysiology of the disease. Moreover, the fact that we found altered oxysterol levels in human biopsies supports the need for further studies to unravel the role of oxysterols during colitis.

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Conflict of Interest

None declared.

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Author Contributions

OGL and VM share first authorship. MA and GGM conceived and designed the study, conducted experiments, analysed the data and wrote the manuscript; OGL and VM designed and conducted experiments, analysed the data and wrote the manuscript; BB, VP, AP and PB performed experiments and analysed the data; J-FR and JL provided the clinical samples; GGM obtained funding and supervised the whole study. All authors discussed the experiments and results and approved the final version of the manuscript.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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