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Quantitative Assessment of the Impact of Crohn's Disease on Protein Abundance of Human Intestinal Drug-Metabolising Enzymes and Transporters

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ABSTRACT

Crohn's disease affects the mucosal layer of the intestine, predominantly ileum and colon segments, with the potential to affect the expression of intestinal enzymes and transporters, and consequently, oral drug bioavailability. We carried out a quantitative proteomic analysis of inflamed and non-inflamed ileum and colon tissues from Crohn's disease patients and healthy donors. Homogenates from samples in each group were pooled and protein abundance determined by liquid chromatography–mass spectrometry (LC-MS). In inflamed Crohn's ileum, CYP3A4, CYP20A1, CYP51A1, ADH1B, ALPI, FOM1, SULT1A2, SULT1B1 and ABCB7 showed ≥10-fold reduction in abundance compared with healthy baseline. By contrast, only MGST1 showed ≥10 fold reduction in inflamed colon. Ileal UGT1A1, MGST1, MGST2, and MAOA levels increased by ≥2 fold in Crohn's patients, while only ALPI showed ≥2 fold increase in the colon. Counter-intuitively, non-inflamed ileum had a higher magnitude of fold change than inflamed tissue when compared with healthy tissue. Marked but non-uniform alterations were observed in the expression of various enzymes and transporters in ileum and colon compared with healthy samples. Modelling will allow improved understanding of the variable effects of Crohn's disease on bioavailability of orally administered drugs.

Introduction

As an idiopathic inflammatory bowel disease (IBD), aetiology of Crohn's disease (CD) is associated with a combination of genetic, microbial and environmental factors.[1] Patients with CD are prone to kidney, liver, cardiovascular and respiratory disease,[2] hence they may receive many oral drugs other than those used to control CD itself.

Many orally administered drugs undergo first-pass metabolism in the intestine and liver before reaching the systemic circulation. Moreover, efflux transporters residing in the enterocytes play a significant role in modulating the bioavailability of drugs which are their substrates.[3] Hence, changes in the expression of drug-metabolising enzymes and transporters (DMETs) localised in the enterocytes will influence the extent of drug absorption. Some studies have established that inflammation can alter the function and expression of intestinal DMETs, affecting oral drug absorption, activation and clearance.[4,5] Importantly, CD can affect any part of the gastrointestinal tract (GIT) but especially the ileum and colon.[1] The ileum plays a more significant role in determining drug bioavailability than the colon owing to its high surface area and the presence of enzymes and transporters expressed in its epithelial cells.[6–8] Understanding the quantitative changes of these proteins helps with the prediction of alterations in exposure and effect of oral drugs, which may lead to unfavourable clinical outcomes.

Clinical data related to the fate of drugs in patient populations, such as CD, are scarce, particularly for new drugs, because of the lack of such patients in clinical studies during drug development, a problem that is now being addressed by the regulatory authorities.[9] In the

Abbreviations: CD, Crohn's disease; DMETs, Drug-metabolising enzymes and transporters; DMEs, Drug-metabolising enzymes; HN-CD, Histologically normal Crohn's disease tissue; IBD, inflammatory bowel disease; I-CD, inflamed Crohn's disease tissue; PBPK, physiologically-based pharmacokinetics; BSA, Bovine Serum Albumin.

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absence of such data, application of physiological-based pharmacokinetic (PBPK) models to predict oral drug kinetics based on attributes of the patient population can improve dosing in different grades of the disease.[10] The collection of quantitative proteomic measurements of DMETs in CD is therefore an essential step to inform PBPK models and facilitate *in vitro* to *in vivo* extrapolation (IVIVE) of available knowledge on drugs to clinical outcomes in CD. A meta-analysis of available system parameters to build a CD PBPK population demonstrated a gap in the availability of intestinal DMET expression/activity data (Alrubia *et al*, accepted for publication in Clinical Pharmacokinetics).[11]

In most previous studies on CD intestine, either mRNA assays or semi-quantitative immunoassays were employed and the protein and gene expression profiles of DMETs in CD have been reported and compared with control groups in tissue and cell lines.[12,13] Several reports on the mRNA expression of solute carriers (SLCs) demonstrated upregulation of ENT1, ENT2, CNT2, PEPT1, OATP4A1 and OATP2B1 in CD, whereas ASBT, MCT1 and OCTN2 were downregulated.[12,14] Among ATP-binding cassette (ABC) transporters, significant upregulation of MRP1 and downregulation of P-gp (MDR1) and MRP3 gene and protein expression in CD have been observed.[15–17] However, the data related to P-gp were conflicting, with some reports showing non-significant change of gene expression[18,19] and increased protein abundance in CD from histologically normal samples.[20] Genetic mutation of OCTN1 and 2 have been linked with increased risk of CD.[21] Mutation and downregulation of ASBT have been associated with diarrhoea in CD patients.[22]

CYP3A4 is the most studied among cytochrome P450 (CYP) enzymes, with reports of lower mRNA and relative protein expression in CD patients relative to control groups.[16,18] In contrast, immunohistochemistry measurements suggested that it is upregulated in the CD colon.[20] Significant upregulation of several other enzymes, such as CYP2C9, CYP2B6, CYP2E1, CYP1A1, UGT, GST was reported in colon,[20] while reduced CYP2E1 mRNA expression in rectum[18] and SULT2A1 in ileum were reported.[19] Several studies reported no change in the mRNA expression of CYP3A5, MRP2, MRP3, BCRP and OSTα/β in CD relative to control.[15,18,19]

Quantitative studies are still scarce, and focused mainly on P-gp and CYP3A4.[16,17,19,20,23] Uridine 5′-diphosphate glucuronosyltransferases (UGT) are strikingly absent from these reports with only UGT1A3 being reported, while the expression of key enzymes, such as sulfotransferases (SULT) and other non-CYP non-UGT enzymes, has been reported only in non-inflamed CD tissue.[23] Most assays were based on mRNA expression, which is an indirect and not very robust indicator of protein abundance. Despite the importance of the ileum in oral drug disposition, expression of transporters, such as MRP1 and MCT1, has not been reported. Some studies combined ulcerative colitis and CD patients;[18,24] others combined expression value of DMEs from the ileum and colon,[25] impeding differential analysis of the expression data. Further, the control groups were not always healthy subjects and in some studies the control group was lacking altogether[14,15,18,23,24,26] preventing the generation of disease perturbation factors (DPF). The DPF reports alteration in protein expression due to disease as a ratio relative to healthy expression.[27,28] Lack of tissue samples, inadequate proteomic data, high variability caused by differences between intestinal segments[34] and heterogeneity among applied methods hinder building reliable predictive PBPK models for CD.

Liquid chromatography-mass spectrometry (LC-MS) based proteomics offers greater proteome coverage and more accurate quantitative measurements compared with other techniques used previously to characterise CD samples.[29] In this study, we aimed to quantify the abundance of CYPs, UGTs, non-CYP non-UGT DMEs, ABC transporters and SLCs involved in oral drug disposition using LC-MS proteomics. This was carried out in pooled samples of inflamed and histologically normal CD ileum and colon, with subsequent comparison against a healthy control, to enable generation of DPF for each quantified protein.

Materials and Methods

Materials

Unless otherwise indicated, all chemicals were supplied by Sigma-Aldrich (Poole, UK). Solvents were high performance liquid chromatography (HPLC) grade and supplied by ThermoFisher Scientific (Paisley, UK). Lysyl endopeptidase (Lys-C) was purchased from Wako (Osaka, Japan). Sequencing-grade modified trypsin was supplied by Promega (Southampton, UK) and Complete Mini EDTA-free protease inhibitor cocktail tablets by Roche (Mannheim, Germany). BCA protein concentration measuring kit was obtained from ThermoFisher Scientific (Hemel Hempstead, UK).

Intestinal Tissue

Fresh-frozen human intestine mucosal samples included inflamed ileum (n = 6), inflamed colon (n = 7) (I-CD), histologically normal ileum (n = 2), histologically normal colon (n = 5) (HN-CD) from active CD patients undergoing ileocolonic resection. Tissues were obtained with informed consent and supplied by Manchester Biomedical Research Centre (BRC) Biobank, Manchester University NHS Foundation Trust, Manchester, UK. Prior ethics approval was granted by NRES Committee North West - Haydock (19/NW/0644). Histologically normal tissues were taken from macroscopically normal regions away from the inflamed bowel regions. The two types of CD tissue are required to investigate the degree to which the integrity and function of intestinal tissue is compromised because of direct inflammatory effect on the inflamed tissue and the inflammatory environment surrounding the non-inflamed tissue, in order to confirm whether the inflammatory effect is spread in the intestine or localised.

Healthy ileum (n = 5) and colon (n = 5) mucosal samples obtained from healthy but deceased subjects were supplied by Caltag Medsystems Limited (Buckingham, UK). University research ethics committee (UREC), UK (2019-8120-12392), granted prior ethics approval. Demographic information is provided in Supplemental Table 1.

Enterocyte Isolation and Subcellular Fractionation

Enterocytes isolation from mucosal tissues by calcium chelation elution and homogenate fraction processing were adapted from Harwood et al[30] with minor modifications. Briefly, the process was done on ice and solutions were equilibrated at pH 7.4. The base buffer for all solutions used for chelation was 112 mM NaCl, 5 mM KCl, 20 mM HEPES. The mucosa was washed twice in the base buffer and immersed in 27 mM sodium citrate solution with a protease inhibitor cocktail (PI) for 30 min, followed by incubation in EDTA buffer (30 mM EDTA, 10 U/mL heparin, 1 mM dithiothreitol (DTT) and PI) with stirring at 250 rpm for 40 min to initiate chelation. The chelated enterocytes were collected from the mucosa by repeated flushing with EDTA buffer. The chelated material was washed by centrifugation twice at 2000*x g* for 10 min. The resulting enterocyte pellet was re-suspended in homogenisation buffer (10 mM Tris–HCl, 250 mM sucrose, 0.1 mM EGTA, 0.5 mM $MgCl_2$, 5 mM histidine and PI) at 3 ml per g of cells. Homogenisation was carried out with a Dounce handheld homogeniser for a minimum of 75 strokes, followed by treatment with an ultrasonication probe (30 W) for two 10 s bursts to disrupt cell membranes. The homogenate preserves the subcellular fractions (cytosolic, reticular and plasma membrane), in which all DMETs are expressed, avoiding loss of proteins often incurred by

Table 1
Number of Identified Peptides & Proteins and Quantified CYP Enzymes, UGT Enzymes, other DMEs, ABC Transporters and SLCs in Inflamed Crohn's Disease (I-CD), Histologically Normal Crohn's Disease (HN-CD) and Healthy Ileum and Colon Pooled Samples.

	Peptides	Proteins	CYP enzymes	UGT enzymes	Other DMEs	ABC transporters	SLCs
Ileum							
I-CD	13214	2510	7	5	21	6	43
HN-CD	10064	2155	8	3	21	3	39
Healthy	16373	3113	12	4	27	10	46
Colon							
I-CD	12966	2503	4	4	22	5	39
HN-CD	12720	2414	5	4	19	2	39
Healthy	16933	2959	5	3	23	7	41

CYP, Cytochrome P450; UGT, Uridine-5′-diphospho glucuronosyltransferase; DMEs, Drug metabolising enzymes; ABC, ATP-binding cassette; SLC, Solute carriers.

further purification. Homogenates were stored in aliquots at -80°C until required.

Sample Preparation and Proteolytic Digestion

Pooled human ileum and colon homogenates (n = 6) were processed and grouped based on the nature of the tissue and classified as follows: I-CD colon, I-CD ileum, HN-CD colon, HN-CD ileum, healthy colon and healthy ileum. Homogenate protein content was determined using BCA assay before and after pooling (in triplicate) using bovine serum albumin (BSA) as a standard. Individual samples in each group were mixed in equivalent concentration (20 μg/μl for each group, except HN-CD ileum at 50 μg/μl). 70 μg pooled homogenate protein in each sample was spiked with 0.126 μg BSA (26 pmol/mg of protein) as internal standard. The 6 pooled homogenate samples were prepared for proteomics by filter-aided sample preparation (FASP), as previously described,[31] using Amicon Ultra 0.5 mL centrifugal filters at 10 kDa molecular weight cut-off (Merck Millipore, Nottingham, UK).

LC-MS/MS Analysis and Protein Quantification

Digested samples were diluted to a final concentration of 0.5 μg/μl with HPLC water containing 0.1% (v/v) formic acid and 3% (v/v) acetonitrile. 1 μl of each sample was injected into an UltiMate® 3000 rapid separation liquid chromatography (RSLC, Dionex Corporation, Sunnyvale, CA) system coupled to a Q Exactive HF Hybrid Quadrupole Orbitrap mass spectrometer (ThermoFisher Scientific, Waltham, MA). Peptides were eluted over 90 min gradient following the LC-MS methodology described previously.[27]

Data analysis was carried out using MaxQuant version 1.6.1.0. (Max Planck Institute for Biochemistry, Munich, Germany), and absolute protein quantification was performed using Hi-N label-free method based on the collected MS data.[32] The database search was applied against a UniProtKB human proteome of 71,599 proteins (UniProt, May 2017) in addition to BSA. The average intensity of the three most abundant non-conflicting unique peptides were used to quantify the identified target proteins in relation to BSA at known concentration in each sample. Where three unique peptides were not available, two unique peptides were used for quantification. Shared sequences by more than one protein from the same family were used with CYP2C, as no unique peptides for individual proteins were available. In addition, this was the case with CYP3A4 and CYP3A7, where a shared peptide was used to measure CYP3A4 abundance only. This is because CYP3A7 is the lowest abundance (< 1 fmol/μg protein) CYP3A isoform detected by LC-MS/MS,[33] while CYP3A4 is the highest detected CYP enzyme in human intestine.[34] The peptide sequences used for quantification are provided in Supplemental Table 2.

The calculated ileum and colon mucosal abundances were expressed in units of pmol/g of mucosal tissue. This was done by scaling up protein concentrations in homogenates (pmol/mg homogenate) using the amount of tissue prepared for homogenisation (mucosal weight).

Statistical Analysis

Statistical data analyses and abundance comparisons (ratios relative to healthy control) were performed using Microsoft Excel and GraphPad Prism version 8 (La Jola, CA). To assess technical variability of target quantification, two samples representing the disease group (CD ileum and colon samples) were prepared in triplicate and analysed by LC-MS/MS under the same conditions. Variability was evaluated using a coefficient of variation (CV) in replicates, which was within 30% (data not shown), and only changes of at least 2 fold increase/decrease were considered as a result of CD impact.

Results

Proteomic Analysis of Pooled Intestine Homogenates

The number of the identified proteins and quantified DMETs from I-CD, HN-CD and healthy pooled ileum and colon samples are in Table 1. In total, the protein levels of 10 ABC transporters, 48 SLCs, 13 CYPs, 5 UGTs and 28 non-CYP non-UGT drug-metabolising enzymes were measured in the two intestinal regions. The expression of DMETs protein levels were compared in ileum (Fig. 1) and colon (Fig. 2) as fold change in inflamed CD tissue (I-CD/HV) and in non-inflamed CD tissue (HN-CD/HV) from healthy control. From 48 quantified SLCs, PEPT1 (SLC15A1), MCT1 (SLC16A1) and OST-α (SLC51A) were of particular interest because of their known role in determining oral drug bioavailability (see Supplemental Table 3), but several SLCs showed a large expression differences relative to healthy tissue (Supplemental Table 4).

Comparison of Proteomic Measurements Against Literature Data

Different methods, subcellular fractions and unit of measurement make head-to-head comparisons between data generated in this study and previously published data (from healthy samples) challenging. We therefore focused on rank order of abundance. One study covering a large number of DMETs in the ileum reported CYP3A4 and CYP27A1 as the most abundant CYPs and CYP4F2 as the least abundant, consistent with our findings.[35] The rank order of UGTs was also similar to our data, with UGTB17 being highly abundant and UGT1A1 being of low abundance. SULT1B1 and GSTP1 were the highest expressed non-CYP non-UGT enzymes, in line with our findings. Another study reported CES2 as the highest expressed non-CYP enzyme in the ileum, which is consistent with our data, when only compared with the targeted enzymes in the study.[36]

SLC25A were the most abundant SLCs, with SLC25A3 being the highest expressed, similar to our observations in the ileum, while other SLCs varied in rank order.[35] In healthy ileum, our data indicated

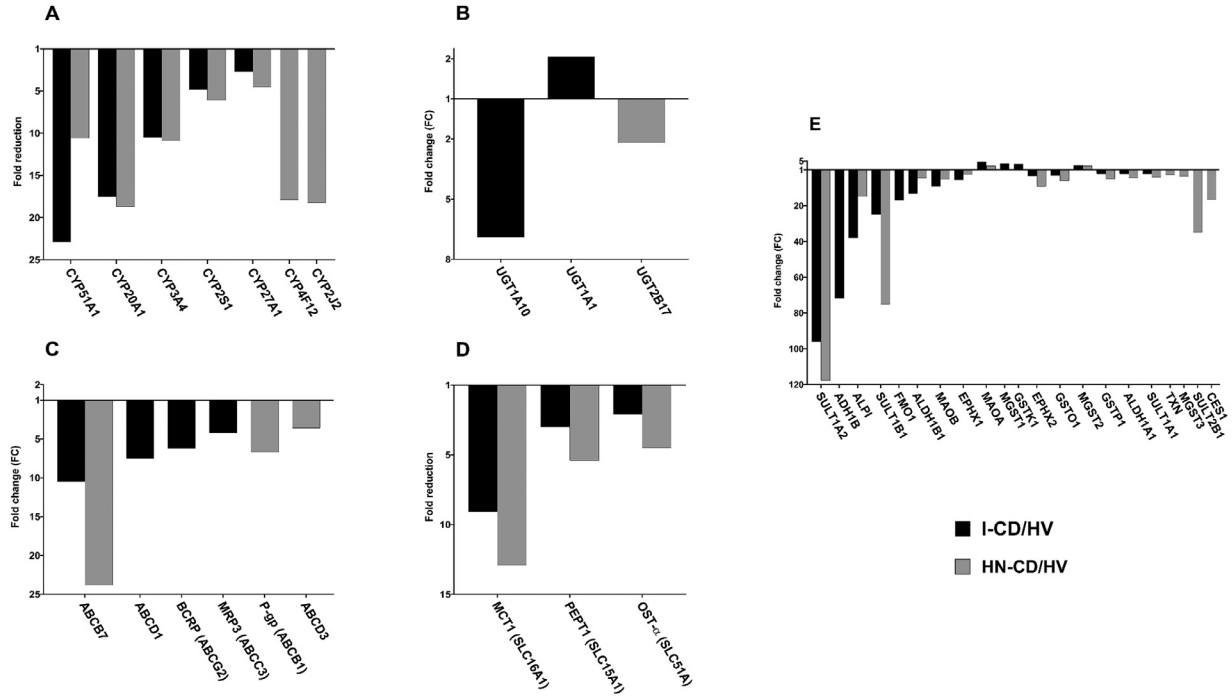

Figure 1. Relative changes in expression of DMETs relative to a healthy pooled sample ($n = 5$) in pooled inflamed CD ileum ($n = 6$) (I-CD/HV) and non-inflamed CD ileum ($n = 2$) (HN-CD/HV). Change in expression is shown for (A) CYP enzymes, (B) UGT enzymes, (C) ABC transporters, (D) SLCs of interest (PEPT1, MCT1 and OST-α) and (E) non-CYP non-UGT drug-metabolising enzymes (DMEs). Only proteins with relative change ≥2 fold are included. Where the protein was not detected in the diseased or healthy pool, no data are shown.

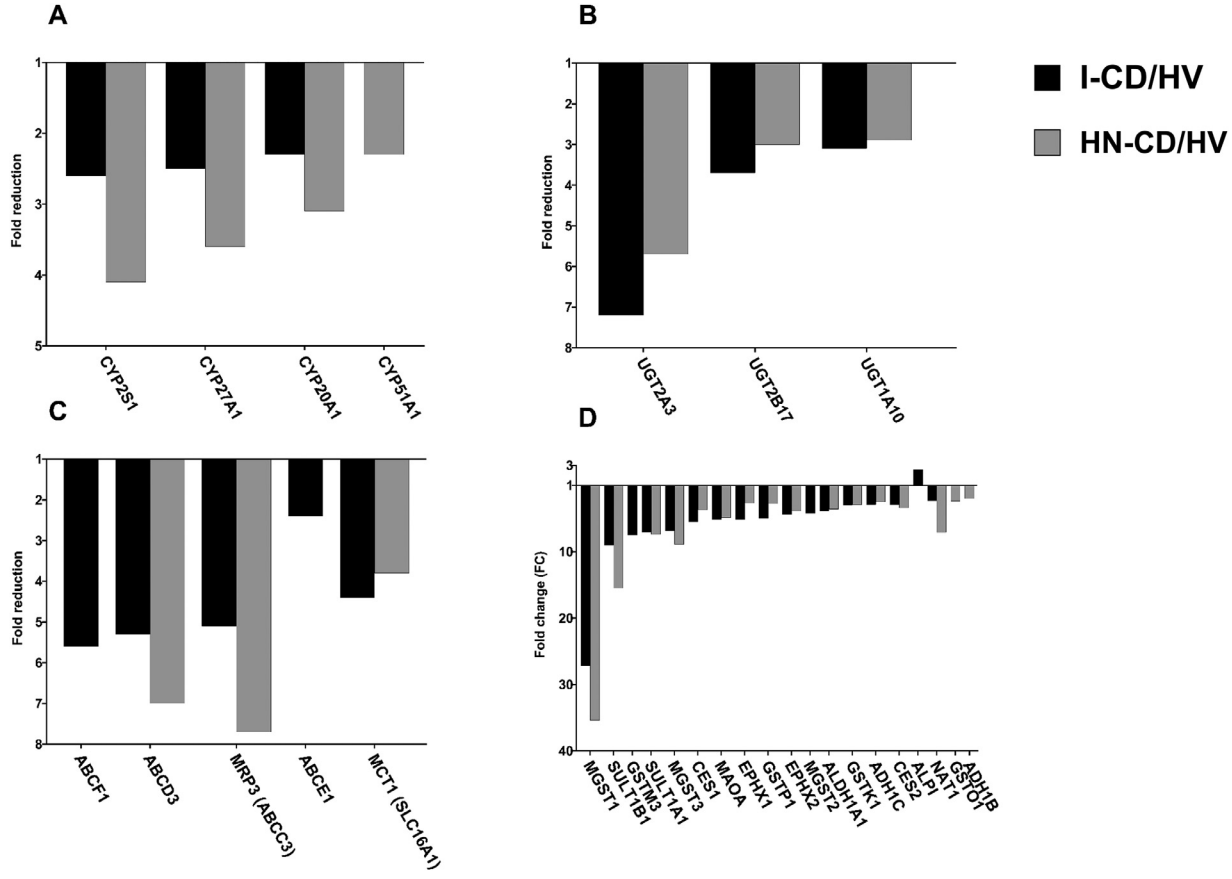

Figure 2. Relative changes in expression of DMETs relative to a healthy pooled sample ($n = 5$) in pooled inflamed CD colon ($n = 7$) (I-CD/HV) and non-inflamed CD colon ($n = 5$) (HN-CD/HV). Change in expression is shown for (A) CYP enzymes, (B) UGT enzymes, (C) ABC transporters and SLCs of interest (MCT1) and (D) non-CYP non-UGT drug-metabolising enzymes (DMEs). Only proteins with relative change ≥2 fold are included. Where the protein was not detected in the diseased or healthy pool, no data are shown.

that SLC16A1 (MCT1) was slightly higher in abundance than SLC15A1 (PEPT1), while SLC15A1 was reported in the literature to have higher abundance.[37] By contrast to the literature, our data also suggest that P-gp is not the highest ABC transporter in healthy ileum,[35,37] but similar trends with the literature are seen with ABCD3, BCRP and MRP3. In a study that focused on CYPs and UGTs in the colon, only UGT1A1 was detected,[38] consistent with our findings. Other work showed that MCT1 and MRP3 were highly abundant SLC and ABC transporters in the colon,[37] which is in line with our findings.

Expression of DMETs in Ileum of CD Patients Compared with Healthy Tissue

We quantified 99 DMETs in healthy ileum compared with 81 and 74 in I-CD and HN-CD, respectively. Expression of DMEs in I-CD and HN-CD ileum was generally lower than in healthy control; fold decrease ranged from 2.7 (CYP27A1) to 96 (SULT1A2) in I-CD. Of the transporters detected, only SLC25A5 showed higher expression in I-CD ileum compared with healthy tissue with a fold change >2. The fold decrease for SLCs ranged from 2 (SLC51A (OST-α)) to 41 (SLC39A5). For the HN-CD ileum, the magnitude of the reduction or increase was not consistent for the same protein with inflamed samples. The fold decrease was in the range 2 (CES2 SLC25A24) to 117.7 (SULT1A2) and increase in the range 2.3 (SLC25A5, MAOA and MGST2) to 8.2 (SLC25A6). Not all identified DMETs in I-CD or HN-CD were detectable in healthy tissue. UGT2B7, SLC1A5, SLC26A2, SLC35A1, SLC30A1, and ADH1C were detected in CD and/or HN-CD but not in healthy tissue, while CYP2D6, CYP3A5, CYP4F11, CYP4F2, ABCE1, ABCF1, ABCF2, ABCF3, SLC23A1, NAT1, CES3, FMO5, SULT1E1 and SULT2A1 were only detected in healthy ileum.

Fig. 1A shows that expression of CYP51A1 in I-CD tissue reflects the highest difference among DMEs (23-fold reduction) compared with healthy control. CYP3A4 is similarly >10-fold reduced in both inflamed and histologically normal CD tissue. The lowest reduction in expression was observed with CYP27A1 in I-CD tissue. Fold change in UGT expression (Fig. 1B) revealed that UGT1A10 had the highest expression difference in I-CD (7-fold reduction) compared with healthy control. Other UGT expression changes of >2 were UGT2B17 (2.2-fold reduction) and UGT1A1 (2.1-fold increase). SULT1A2 showed the highest fold decrease in both I-CD and HN-CD ileum compared with healthy control with ~96- and 118-fold reduction in I-CD and HN-CD, respectively (Fig. 1E).

The most remarkable downregulation of ABC transporters in HN-CD was observed for ABCB7 by ~24 fold (Fig. 1C). I-CD showed a lower magnitude of fold change compared with all quantified ABC transporters, except for ABCD1, MRP3 and BCRP, which were not detected in HN-CD tissue. MCT1 showed the highest fold decrease in both I-CD and HN-CD ileum with ~9 and 13 fold, respectively, compared with healthy control (Fig. 1D).

Expression of DMETs in Colon of CD Patients Compared with Healthy Tissue

A total of 77 DMETs were quantified in healthy colon compared with 73 and 65 in I-CD and HN-CD colon, respectively (Fig. 2). The fold decrease in expression of DMETs in I-CD tissue compared with healthy control was in the range of 2 (SLC1A5 and SLC25A24) to 27.2 (MGST1). A small number of targets showed increased levels, such as SLC2A1 and ALPI. Compared with healthy tissue, nearly all quantified DMETs showed a reduction in expression in HN-CD colon. The fold decrease ranged from 2 (ADH1B, SLC35A4 and SLC44A2) to 35.4 (MGST1). Only SLC44A2 showed a 2 fold increase.

Several DMETs were not detected in healthy colon; UGT2B7, SLC25A22, SLC16A3, OST-α (SLC51A), SLC35A1 and SLC5A1 were only detected in CD and/or HN-CD. On the other hand, ABCF2, ABCF3, SLC35C1, SLC43A2, and SLC2A13 were only detected in healthy tissue. The decrease in CYP2S1 expression was 2.6 and 4 fold (Fig. 2A) in I-CD and HN-CD colon, respectively, compared to healthy colon. For UGTs, UGT2A3 showed the highest fold change, returning a 7-fold decrease in I-CD and 6-fold decrease in HN-CD. UGT1A10 showed the lowest change among UGTs, with ~3 fold decrease in both I-CD and HN-CD from healthy control (Fig. 2B). Among non-CYP non-UGT enzymes, MGST1 was the most downregulated protein in both inflamed and histologically normal CD colon compared with healthy tissue, returning a ~27- and 35-fold reduction, respectively (Fig. 2D).

ABCD3 and MRP3 showed the most reduction among ABC transporters (~5.5 fold change in ABCD3 expression in I-CD and ~7.5 fold reduction in MRP3 in HN-CD) (Fig. 2C).

Relative Distribution of DMET Expression in CD Ileum and Colon

Differences in expression of enzymes and transporters in CD between inflamed and histologically normal tissue from the same intestinal segment were observed.

In I-CD ileum, 81 DMET proteins were quantified compared with 74 in matching histologically normal tissue (HN-CD). In colon, the number was 73 in I-CD tissue compared with 66 in matching HN-CD tissue. The pie charts in Figs. 3–6 show changes in relative distribution of the abundance of DMEs and transporters in I-CD and HN-CD from ileum and colon, compared with healthy tissue.

Inflammation had a greater effect on DMET expression in CD ileum than colon. Whereas CYP27A1 was the most abundant CYP in both I-CD and HN-CD (37% and 24%, respectively) CYP20A1 was most abundant in healthy samples (18%) (Fig. 3A). Among other DMEs, MAOA was the most abundant in I-CD and HN-CD samples (20% in both), while ALPI and SULT1B1 were highest expressed in healthy ileum (14% for both) (Fig. 4A). Among UGT2B17 was most abundant UGT in healthy and I-CD ileum (38% and 50%, respectively), while UGT2A3 was highest in HN-CD tissue (47%) based on UGT expression only.

ABCD3 was the most abundant ABC transporter in I-CD and HN-CD samples (50% and 82%, respectively), but ABCF3 was highest in healthy tissue (23%) (Fig. 5A). SLC25A3 was the most abundant SLC in healthy ileum (36%), while SLC25A5 was highest in I-CD and HN-CD tissue (28% and 17%, respectively) (Fig. 6A).

I-CD and HN-CD showed surprisingly similar DMET expression compared with healthy tissue. For CYPs in all studied tissue groups, CYP2S1 was the highest expressed (39% in I-CD, 26% in HN-CD and 38% in healthy tissue) (Fig. 3B). The highest expressed non-CYP enzymes were MAOA in I-CD (13%), TXN in HN-CD (18%) and MGST1 (44%) in healthy colon (Fig. 4B). UGT2B17 was the highest expressed UGT in all three groups (70% in I-CD, 75% in HN-CD and 66% in healthy).

ABCD3 was the highest expressed ABC transporter (39% in I-CD, 65% in HN-CD and 42% in healthy) (Fig. 5B). The case was different with SLCs; SLC25A5 was the highest expressed in I-CD and healthy tissue (21% and 33%, respectively), while SLC25A6 was the most abundant in HN-CD colon (20%) (Fig. 6B).

DMETs Abundance per g Ileum and Colon Mucosa of CD Patients

In total, 13 CYPs, 5 UGTs, 28 non-CYP non-UGT DMEs, 10 ABC transporters and 48 SLCs were quantified in the two intestinal regions. Table 2 and Table 3 show the expression levels in pmol/g of pooled samples of mucosa from I-CD, HN-CD and healthy ileum and colon, respectively. The expression levels of other SLCs in the ileum and colon are listed in Supplemental Table 4 and Table 5, respectively.

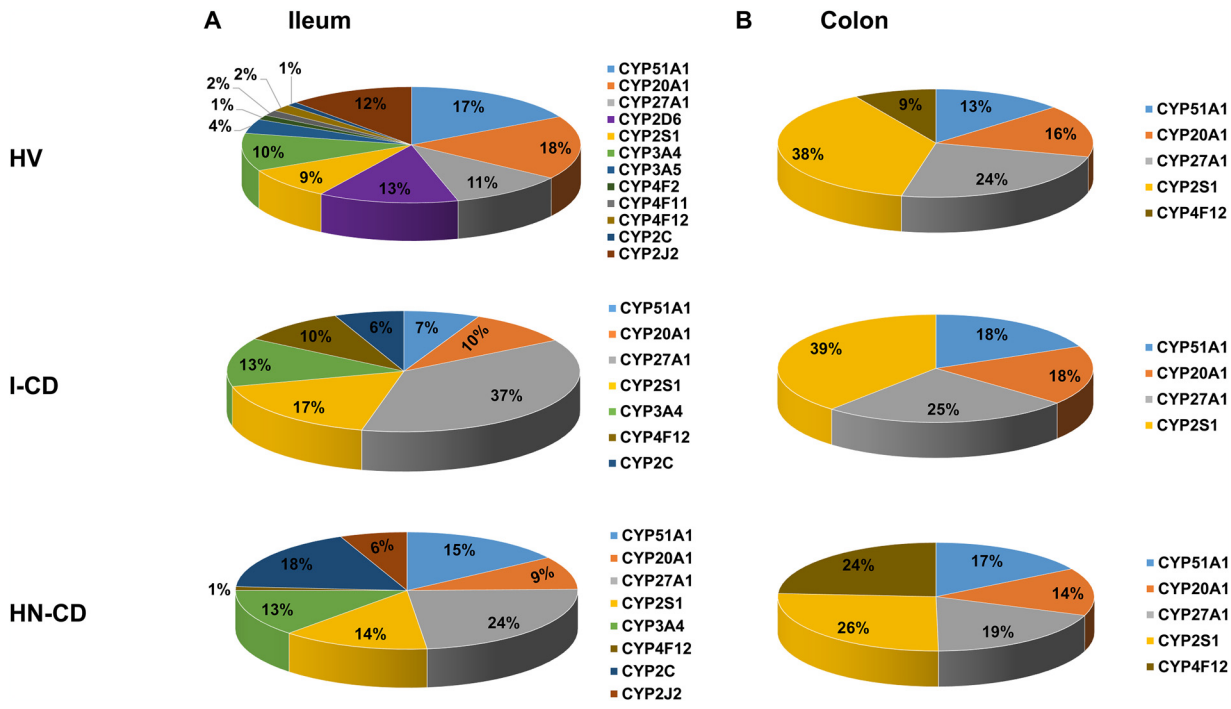

Figure 3. Relative distribution of cytochrome P450 enzymes in inflamed CD (I-CD), histologically normal CD (HN-CD) and healthy (HV) tissue, showing changes in (A) Ileum and (B) Colon pooled samples.

Expression Profile of DMETs in Ileum and Colon of CD Patients

The expression profiles described above are represented in heatmap format in Supplemental Fig. 1. CYPs were overall more abundant in ileum for all the three examined groups compared with colon (Supplemental Fig. 1A). A noticeable exception is CYP4F12 in HN-CD colon vs ileum. In general, UGTs were more abundant in non-inflamed colon than ileum but the opposite was observed in inflamed tissue (Supplemental Fig. 1B). Healthy and I-CD ileum showed a higher abundance of other DMEs for most of the targets compared to colon, but the opposite is noted with HN-CD group (Supplemental Fig. 1C). Noticeably, CES2 and

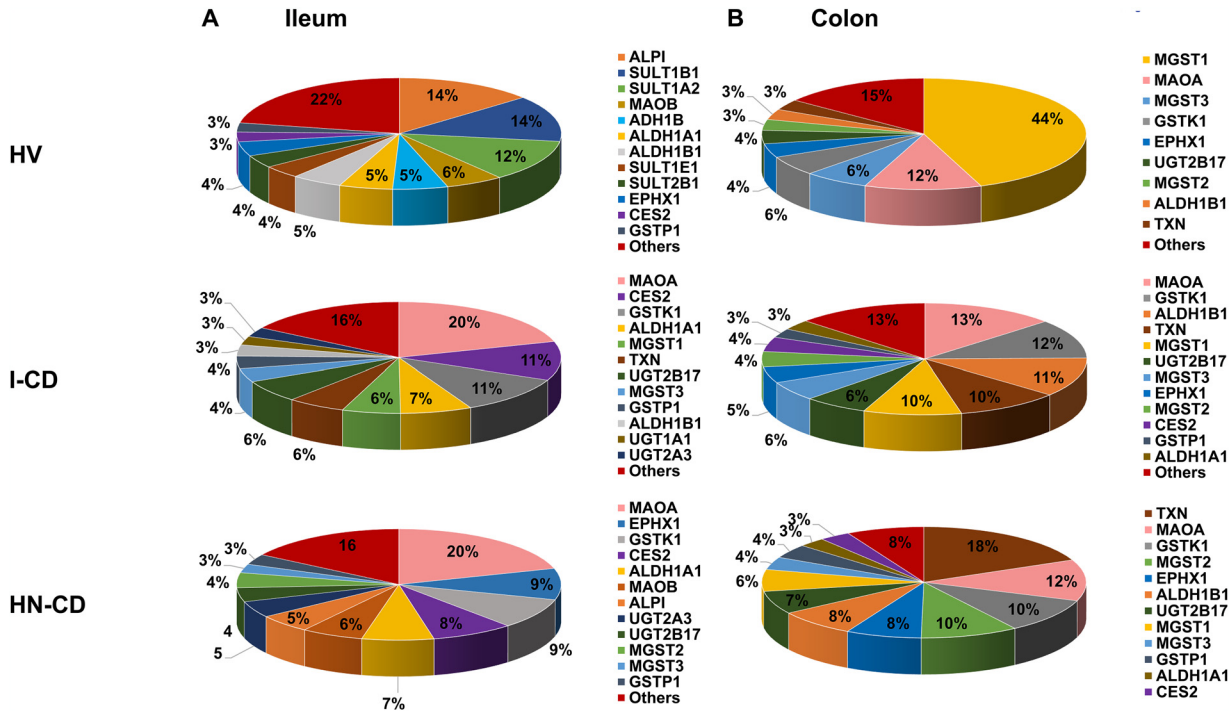

Figure 4. Relative distribution of non-CYP drug-metabolising enzymes in inflamed CD (I-CD), histologically normal CD (HN-CD) and healthy (HV) tissue, showing changes in (A) Ileum and (B) Colon pooled samples. Proteins present at ≥3% of total protein are mentioned individually while the rest are indicated as 'Others' in the pie charts.

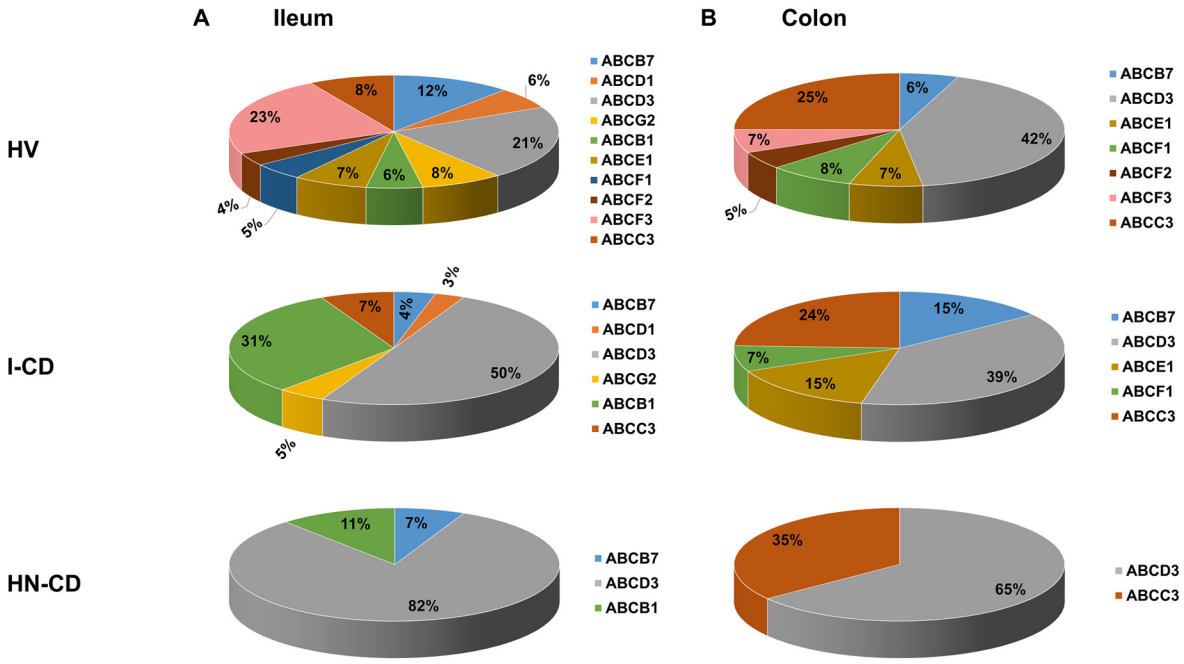

Figure 5. Relative distribution of ABC transporters in inflamed CD (I-CD), histologically normal CD (HN-CD) and healthy (HV) tissue, showing changes in (A) Ileum and (B) Colon pooled samples.

ALDH1A1 expression was much higher I-CD ileum than colon. For the healthy group, MGST1 and ALPI showed the highest inter-segment difference, with the former being more abundant in the colon, the latter in the ileum.

Ileal expression of ABC transporters was generally higher than colonic expression in non-inflamed tissue, but lower in inflammation (Supplemental Fig. 1D). The exceptions were higher ABCC3 and ABCD3 in healthy colon and higher ABCD3 in I-CD ileum. SLCs of interest were more abundant in ileum compared with colon in all three groups, except for MCT1, which was higher in HN-CD and I-CD colon than ileum (Supplemental Fig. 1E).

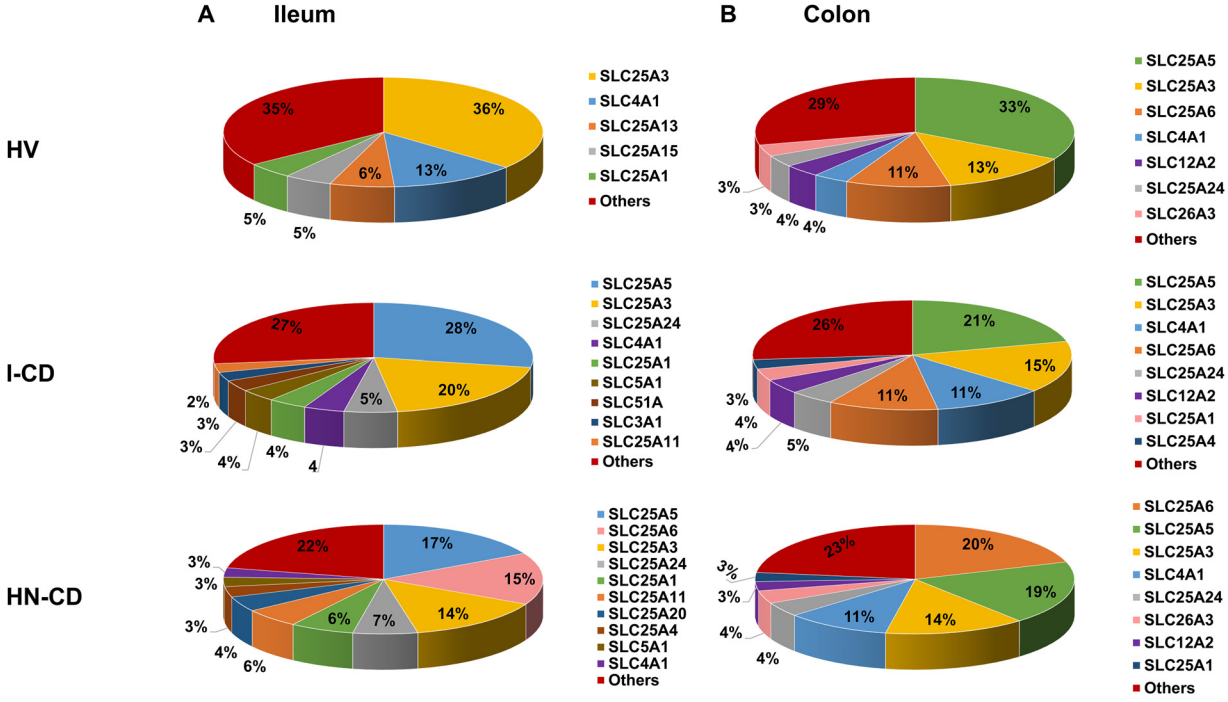

Figure 6. Relative distribution of solute carriers (SLCs) in inflamed CD (I-CD), histologically normal CD (HN-CD) and healthy (HV) tissue, showing changes in (A) Ileum and (B) Colon pooled samples. Proteins present at ≥ 3% of total protein are mentioned individually while the rest are indicated as 'Others' in the pie charts.

Table 2
Abundance (pmol/g mucosa) of CYP Enzymes, UGT Enzymes, other DMEs, ABC Transporters, SLCs of Interest (PEPT1, MCT1 and OST-α) in Inflamed Crohn's Disease (I-CD), Histologically Normal Crohn's Disease (HN-CD) and Healthy Ileum Pooled Samples.

Protein	I-CD	HN-CD	Healthy
Cytochrome P450 enzymes			
CYP2C[b]	1.9	5.2	3
CYP2D6	ND	ND	38
CYP2J2	ND	1.9	34.1
CYP2S1	5.2	4.1	25
CYP3A4	ND	4	30
CYP3A4[a]	4	3.8	41.6
CYP3A5	ND	ND	11.4
CYP4F2	ND	ND	3.7
CYP4F11	ND	ND	4.4
CYP4F12	2.9	0.3	5.5
CYP20A1	2.9	2.7	50.5
CYP27A1	11.4	6.9	31.2
CYP51A1	2.1	4.6	48.3
Uridine-5′-diphospho (UDP)-glucuronosyltransferases (UGT)			
UGT1A1	13.7	ND	6.4
UGT1A10	2	ND	14
UGT2A3	15	13	19.4
UGT2B7	4.1	3.4	ND
UGT2B17	35.1	11.1	24.9
Sulfotransferases (SULT)			
SULT1A1	8.5	4.4	18
SULT1A2	2	1.6	191.9
SULT1B1	8.5	2,8	211.3
SULT1E1	ND	ND	59.1
SULT2A1	ND	ND	20.7
SULT2B1	ND	1.7	57.7
Other transferase, phosphatase and thioredoxin enzymes			
ALPI	5.7	14.6	216.6
GSTK1	60.5	24.2	17.8
GSTO1	9.1	4.9	29.2
GSTP1	19.7	8.3	41.7
MGST1	31.6	5.3	8.6
MGST2	13	11.9	5.1
MGST3	21.2	6.9	25
NAT1	ND	ND	11.9
TXN	31.5	6.7	19.5
Dehydrogenase, hydrolase and esterase enzymes			
ADH1B	1.2	ND	85.8
ADH1C	6.1	3.3	ND
ALDH1A1	38.7	19.5	83.2
ALDH1B1	17.17	6	79.2
CES1	ND	1.1	18.7
CES2	61.2	22.3	44.2
CES3	ND	ND	30.7
EPHX1	11.9	24.7	65.7
EPHX2	7.1	2.5	23.1
Flavin-containing monooxygenases and amine oxidase enzymes			
FMO1	0.8	ND	12.8
FMO5	ND	ND	6.1
MAOA	110.8	55.4	24.6
MAOB	9.8	16.8	90.2
ATP-binding cassette (ABC) transporters			
P-gp (ABCB1)	13.4	1.3	8.7
ABCB7	1.8	0.8	18.5
MRP3 (ABCC3)	3.1	ND	13.3
ABCD1	1.3	ND	9.5
ABCD3	21.7	9.2	33.5
ABCE1	ND	ND	11.5
ABCF1	ND	ND	7.9
ABCF2	ND	ND	5.8
ABCF3	ND	ND	35.9
BCRP (ABCG2)	2	ND	12.6
Solute carriers (SLC)			
PEPT1 (SLC15A1)	5	2.8	15.3
MCT1 (SLC16A1)	1.9	1.3	17
OST-α (SLC51A)	19.3	9	40.9

[a] based on CYP3A4 and CYP3A7 unique peptides;
[b] group specific as no specific peptides for each enzyme were detected; ND, Not detected.

Table 3
Abundance (pmol/g mucosa) of CYP Enzymes, UGT Enzymes, other DMEs, ABC Transporters, SLCs of Interest (MCT1 and OST-α) in Inflamed Crohn's Disease (I-CD), Histologically Normal Crohn's Disease (HN-CD) and Healthy Colon Pooled Samples.

Protein	I-CD	HN-CD	Healthy
Cytochrome P450 enzymes			
CYP2S1	5.5	3.5	14.3
CYP4F12	ND	3.2	3.2
CYP20A1	2.5	1.9	5.9
CYP27A1	3.6	2.5	9.1
CYP51A1	2.6	2.2	5
Uridine-5′-diphospho (UDP)-glucuronosyltransferase (UGT)			
UGT1A10	3.2	3.5	9.9
UGT2A3	4.4	5.5	31.5
UGT2B7	2	ND	ND
UGT2B17	21.8	26.9	79.7
Sulfotransferases (SULT)			
SULT1A1	3	2.8	20.9
SULT1B1	4.7	2.7	41.9
Other transferase, phosphatase and thioredoxin enzymes			
ALPI	4.5	ND	1.8
GSTK1	39.3	40.7	117.1
GSTO1	4.9	3.5	8.2
GSTM3	1	ND	7.5
GSTP1	9.1	16.4	45
MGST1	33.3	25.6	905.9
MGST2	15.2	39.1	64.5
MGST3	19.4	14.9	133
NAT1	3.6	1.2	8.4
TXN	35.4	74	63.3
Dehydrogenase, hydrolase and esterase enzymes			
ADH1B	ND	2.5	5
ADH1C	4.2	4.8	12.2
ALDH1A1	10.1	10.8	39.1
ALDH1B1	38.9	31.6	62.4
CES1	1.4	2.1	7.8
CES2	14.5	12.4	41.9
CES3	3.6	ND	5.3
EPHX1	15.8	30.2	82.3
EPHX2	2.3	2.5	9.9
Flavin-containing monooxygenases and amine oxidase enzymes			
FMO5	2.4	ND	3.6
MAOA	45.6	48.2	237.9
ATP-binding cassette (ABC) transporters			
ABCB7	2.5	ND	4.7
MRP3 (ABCC3)	4	2.7	20.4
ABCD3	6.5	4.9	34.4
ABCE1	2.4	ND	5.9
ABCF1	1.2	ND	6.9
ABCF2	ND	ND	4.1
ABCF3	ND	ND	5.5
Solute carriers (SLC)			
MCT1 (SLC16A1)	2.6	2.9	11.2
OST-α (SLC51A)	2.3	3.3	ND

ND, Not detected.

Some proteins were detectable in only one segment. CYP2C, CYP2D6, CYP2J2, CYP3A4, CYP3A5, CYP4F2 and CYP4F11, UGT1A1, FMO1, MAOB, SULT1A2, SULT1E1, SULT2A1, SULT2B1, ABCB1, ABCD1, ABCG2 and PEPT1 (SLC15A1) were not detected in the colon. GSTM3 was not detected in the ileum.

Discussion

Crohn's disease (CD) affects the PK of oral drugs used to control the disease itself, such as budesonide[39] and mesalamine[40], and medications administered for other medical conditions, such as verapamil[41], midazolam[42] and propranolol.[43] The Crohn's population is susceptible to many other diseases, because of its chronic effect and young age of onset. The incidence of CD has increased steadily in the last 25 years.[44] However, available *in vivo* and *in vitro* data on the effect of CD on pharmacologically relevant intestinal proteins are scarce. This hinders model-based prediction of changes in drug kinetics in CD, with implications for appropriate dose adjustment in these patients.

This study shows clear but non-uniform differences in expression of most DMETs between adult CD and healthy tissue. The largest change in CYP abundance was observed for CYP51A1 and CYP20A1 in inflamed CD ileum. CYP20A1 is an orphan enzyme, without a known role in drug metabolism,[45] while CYP51A1 is involved in steroid biosynthesis and is an antifungal drug target[46] (Supplemental Table 3). Expression levels of these enzymes in CD intestine have not been reported previously.

CYP3A4 expression was 10 fold lower in inflamed and histologically-normal CD ileum compared with healthy control, confirming previous reports of CYP3A4 mRNA and relative protein expression.[16,18] Other conflicting immunohistochemistry data reported significant increased levels in inflamed CD colon compared

to control.[20] This may be dependent on the segment of intestine, disease state of the patient and the techniques used for measurement.[16,18,20]

Despite the importance of UGT enzymes in drug clearance, their expression was reported in a very limited number of IBD studies.[20,23] Our data demonstrated a significant reduction in expression of UGT1A10 and UGT2A3 (by ~7 fold) in inflamed CD ileum. Anticancer drugs (e.g. irinotecan active metabolite SN-38, flavopiridol and genistein) and cardiovascular agents (e.g. losartan, candesartan, and zolarsartan) are substrates of UGT1A10.[47–49] UGT2A3 has no known substrate or biological role (Supplemental Table 3). Previous studies reported a slight increase in UGT1A1 relative expression (~1.4 fold) in CD colon,[20] in line with the (2 fold) increase in UGT1A1 expression in inflamed CD ileum in the current study. UGT1A1 was not detected in colon in our study. Similar to UGT1A10, UGT1A1 participates in metabolising anticancer and cardiovascular agents (Supplemental Table 3).

SULT1A2 showed the largest reduction among non-CYP non-UGT DMEs in CD ileum samples. This transferase facilitates renal excretion of compounds through sulfonation. Like other SULT enzymes (Supplemental Table 3), SULT1A2 has a wide range of substrates, including steroids, bile acids and phenol- and alcohol-containing compounds.[50] A non-significant reduction in SULT1A2 gene expression was reported in CD ileum and colon.[23] ADH1B and ALPI were considerably reduced in CD ileum by ~70 and ~40 fold, respectively, consistent with ALPI mRNA measurments.[51] ALPI activates several prodrugs (Supplemental Table 3) by removing phosphate groups,[52] while ADH1B participates in retinoid catabolism.[53] Abundance of ADH1B is reported herein for the first time.

ABCB7 showed the highest alteration among ABC transporters. This protein transports heme in the cell, therefore reduced expression can contribute to anaemia.[54] P-gp is the most extensively studied transporter because of its role in efflux of a wide range of substrates.[55,56] Its mRNA and relative expression have been reported to significantly decrease in CD ileum and colon.[15–17] In our data, P-gp levels increased by 1.5 fold in inflamed CD ileum but decreased by ~7 fold in histologically-normal CD ileum. As a limitation of this study, the significance level of alteration was not assessed because each pooled sample was analyzed only once. In inflamed CD ileum, BCRP showed a 6 fold reduction from healthy baseline, consistent with a reported 2-fold reduction in mRNA expression.[19] BCRP is a major multidrug resistance transporter, with mitoxantrone, topotecan, irinotecan, flavopiridol, and methotrexate being some of its anticancer substrates.[57] ABCD3 is the highest expressed in all tissues except for the healthy ileum, it transports long and branched chain fatty acids and bile acid intermediates.[58] In a previous report, ABCD3 was found to be very abundant throughout healthy and diseased tissues from multiple organs including the intestine.[59]

We selected three SLCs of interest (PEPT1, MCT1, OST-α) for their role in pharmacology. Expression of all three transporters decreased in CD samples. MCT1, which transports lactate, pyruvate, butyrate, acetoacetate, β-hydroxybutyrate and γ-Hydroxybutyric acid (GHB),[60] showed the largest reduction in both CD ileum and colon. A previous report related a significant decrease in mRNA and relative protein expression in CD colon to butyrate deficiency in IBD patients.[12] PEPT1 was reduced 6 fold in CD and was detected only in the ileum. PEPT1 transports peptide-like substrates, including β-lactam antibiotics and angiotensin-converting enzyme inhibitors.[61] A previous survey reported a significant increase in PEPT1 in CD colon.[14] The bile acid and steroid transporter[62] OST-α was reduced in CD samples in this study. A meta-analysis of SLC expression in distal ileum showed that PEPT1 was more abundant in healthy Caucasian population compared to OST-α, [7] while in our pooled healthy ileum sample, OST-α and PEPT1 accounted for 3% and 1%, respectively, of the identified SLC abundance.

The significant alteration in various DMETs in this study might be the result of compromised integrity of the epithelium layer due to inflammation. Over the course of disease, enterocytes undergo functional and morphological modifications.[63] This can lead to increased intestine permeability and alteration to its cellular composition.[64]

The activity and/or expression reduction in CYP2C, CYP3A4, UGT1A1, UGT2B17, P-gp, and BCRP and upregulation of MRP3 are correlated with increased inflammatory biomarkers (ILs, TNF-α, and INF-γ) in inflammatory conditions.[65–67] Treatment of human Caco-2 cell line with pro-inflammatory cytokines caused downregulation of CYP3A4 and upregulation of P-gp mRNA expression.[68] This is seen with our abundance data in inflamed ileum, where CYP3A4 dropped by ~10 fold and P-gp increased by ~1.5 fold relative to healthy baseline. This correlation was only observed in our ileum samples as CYP3A4 and P-gp were not detected in colon samples. In inflammatory conditions other than IBD, CYP3A4 expression in the liver was reported to be reduced with increased IL-2, IL-1β, IL-6, TNF-α, and INF-α and γ,[66,69,70] while a reduction in liver CYP2C activity was associated with high levels of IL-2.[71] Moreover, the regulatory mechanisms of DMET expression can be affected by CD-related inflammation, including pregnane X receptor (PXR) responsible for regulation of several DMETs, such as CYP3A4, UGT1A1 and P-gp.[72–74] In inflammatory conditions, nuclear factor-κB (NF-κB) activation stimulates the transcription of pro-inflammatory cytokines and inhibits the activity of PXR nuclear receptor, shifting production from DMETs to cytokines.[75] This can cause alterations in inflamed tissue and the surrounding normal tissue driven by the inflammatory environment created by the disease.

CYP3A4 and CYP2S1 both play a role in controlling inflammatory conditions as they metabolise eicosanoids classes, such as prostaglandins.[76,77] CYP2S1 was the highest expressed CYP in healthy colon and its abundance was downregulated in all CD tissues we examined. Reduction in activity of GSTs was previously linked to increased risk of IBD.[78] All of the detected GSTs in our colon and ileum CD tissues were downregulated except GSTK1 in ileum, which was higher in inflamed and non-inflamed CD tissue. GSTK1 is essential to maintain mitochondrial function and homeostasis. Its deficiency induces inflammation and it is upregulated under oxidative stress.[79] Thus, its increase can be the result of a defence mechanism against inflammation. Similar to GSTs, reduction in ALPI activity increases the risk of IBD as it can lead to altered intestine microbiome, inflammation, and changed permeability.[80] Our data show a large drop in ALPI expression in inflamed and non-inflamed CD ileum (38 and 15 fold, respectively), while in the colon, it increases by 2.5 fold in inflamed relative to healthy colon; it was not detected in non-inflamed CD colon. Expression of transporters is also affected by inflammation; treatment of the intestinal epithelial cell line HT-29 with IFN-γ and TNF-α resulted in downregulation of MCT1 mRNA expression in a dose dependent manner.[12]

Differences observed between inflamed and histologically normal CD samples are in general agreement with previous reports. Inflamed CD tissue was reported to show larger changes in expression of CYP3A4,[18] P-gp,[17,18] and MCT1[12] compared to non-inflamed tissue. The degree in alteration correlates proportionally with the severity of mucosal inflammation. Mild and moderate mucosal inflammation exhibited non-significant difference in expression of CYP3A4, P-gp[18] and MCT1[12], while a significant reduction was recorded with severely inflamed mucosa compared with non-inflamed mucosa. Such comparison was not possible with our samples as different grades of inflammation severity were not available. Two studies reported alteration of several DMETs in non-inflamed ileum and colon mucosa from CD patients; a significant downregulation in the gene and protein expression of CYP3A4, SULT2A1, CES2, P-gp and MRP3 was reported compared with control.[16,23] This is similar to the trends revealed by our data.

The highest abundance alteration in our data was observed in the ileum. Many of the DMETs with high impact on drug PK, such as CYP3A4, CYP3A5, UGT1A1, SULT1A2, SULT2A1, SULT1E1, SULT2B1, P-gp, BCRP and PEPT1 were only detected in the ileum. In general, proteins detected in both the ileum and colon had smaller change in abundance in CD colon compared with the ileum. Such observed difference in abundance of DMETs might be attributed to functional and anatomical properties of each segment. The ileum has a large surface area caused by the abundant presence of villi, which expand the surface area by 30–600 fold, allowing it to play a more prominent role in xenobiotic absorption and biotransformation compared to the colon.[81]

The impact of altered abundance of DMEs and transporters on drug bioavailability was estimated using three oral drugs in Simcyp Simulator with ADAM absorption model. Verapamil (CYP3A4 and P-gp substrate), digoxin (P-gp substrate) and rosuvastatin (BCRP substrate) were simulated in CD patients based on active CD population created in *Alrubia et al. (under review)*[11] by changing the abundance of relevant proteins based on the proteomics data generated in this study in inflamed and non-inflamed tissue relative to healthy control. The output focused on changes in AUC in CD population relative to healthy baseline. Verapamil bioavailability increased by two fold while no change was observed with digoxin and rosuvastatin. Drugs that are substrates of more than one protein, especially those suggested to work in synergy, such as CYP3A4 and P-gp,[82–84] are expected to incur higher changes in bioavailability.[85–88]

Limitations of this study include the use of pooled samples, which does not afford a measure of variability between patients. The conclusions should therefore be used with caution when the relative change is close to technical variability. This emphasizes the importance of using of individual samples in future reports, preferably with knowledge of disease severity to enable correlation of the degree of inflammation with observed changes in abundance. In addition, other limitations are the small number of pooled samples and the lack of matching between inflamed and non-inflamed samples. Using matched samples can confirm if inflamed tissue is more susceptible to changes in expression.

The generated data present, for the first time, quantitative profiles of all detected DMETs in CD ileum and colon using LC-MS/MS methodology. The complexity of CD-driven protein alterations is demonstrated with the impact of disease on inflamed and adjacent non-inflamed tissue. The magnitude of change from healthy expression was variable between the different proteins, regions and conditions. Further studies on the upper segments of the intestine and on individual CD subjects, to establish inter-segment and inter-patient variability, are warranted. This should allow correlation of trends with demographic characteristics, severity of inflammation and medication history for better prediction of drug exposure using PBPK models.

Declaration of Interests

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.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2022.07.012.

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