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## Absence of ABCG2-mediated mucosal detoxification in patients with active inflammatory bowel disease is due to impeded protein folding

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**Ethics:** All authors declare that this study is conducted with the approval of the Erasmus MC University Medical Centre, Rotterdam, ethics committee.

**Abbreviations:** IBD, inflammatory bowel disease, CD, Crohn's disease, UC, ulcerative colitis, IEC, intestinal epithelium cells, NO, nitric oxide, ER, endoplasmic reticulum, PDI, protein-disulfide isomerase, GFP, green fluorescent protein, RFP, red fluorescent protein, 293t-AB, HEK293t cells transiently transfected with GFP-ABCG2, FFPE, formalin fixed paraffin embedded, IHC, immunohistochemistry.

**Abstract**

1 Xenotoxic damage in inflammatory diseases, including inflammatory bowel disease (IBD),  
2 is importantly compounded by reduced activity of the xenobiotic transporter ABCG2 during  
3 the inflammatory state. An association between activation of the unfolded protein response  
4 pathway and inflammation prompted us to investigate the possibility that reduced ABCG2  
5 activity is causally linked to this response. To this end we correlated expression of ABCG2  
6 and the unfolded protein response marker GRP78 in colon biopsies from healthy  
7 individuals (n=9), and patients with inactive (n=67), or active (n=55) IBD, ischemic colitis  
8 (n=10), or infectious colitis (n=14). In addition, tissue-specimens throughout the small  
9 bowel from healthy individuals (n=27), and from patients with inactive (n=9) or active  
10 (n=25) Crohn's disease were co-stained for ABCG2 and GRP78. In all biopsies from  
11 patients with active inflammation, irrespective of the underlying disease, an absolute  
12 negative correlation is observed between epithelial ABCG2 expression and GRP78  
13 expression, suggesting that inflammation-dependent activation of the unfolded protein  
14 response is responsible for suppression of ABCG2 function. The link between the unfolded  
15 protein response and functional ABCG2 expression is further corroborated by live-imaging  
16 of ABCG2 expressing cells which show that various inflammatory mediators, including  
17 nitric oxide, activate the unfolded protein response and concomitantly reduce plasma  
18 membrane localisation as well as transport function of ABCG2. Thus a novel mechanism  
19 for explaining xenobiotic stress during inflammation emerges, in which intestinal  
20 inflammation activates the unfolded protein response in turn abrogating defences against  
21 xenobiotic challenge by impairing ABCG2 expression and function.

## 1 Introduction

2

3 The intestinal epithelium is a single-cell physical barrier between the gut luminal content  
4 and mucosa, important for detection [1] and defence [2-3] against microbes. Intestinal  
5 epithelial cells (IEC) are constantly exposed to a large variety xenobiotic substances,  
6 including microbes and their products as well as exogenous (e.g. drugs or food) and  
7 endogenous (e.g. bile acids) toxic substances present in the luminal content. IEC utilize  
8 various mechanisms to maintain epithelial integrity in face of the xenobiotic challenge.  
9 They are protected against exogenous and endogenous toxic compounds by, among  
10 others, proteins from the ATP-binding cassette (ABC) transporter family. This family  
11 includes the P-glycoprotein/Multidrug Resistance (P-gp/MDR1/ABCB1), Multidrug  
12 Resistance-associated Protein-1 (MRP1/ABCC1), Multidrug Resistance-associated  
13 Protein-2 (MRP2/ABCC2) and Breast Cancer Resistance Protein (BCRP/ABCG2) [4-5].  
14 Especially the homodimeric xenobiotic transporter ABCG2, which specifically localises to  
15 the IEC apical membrane, is widely considered to be especially important in combating  
16 luminal-derived xenobiotic stress [6] [7].

17 Disruption of IEC integrity leads to inflammatory lesions, which are a hallmark of active  
18 inflammation in patients with inflammatory bowel disease (IBD). Appropriate response to  
19 such disruption requires rapid mucosal healing by cell division, differentiation and  
20 maturation to restore barrier function [8-9]. Active inflammation in patients with IBD has  
21 been associated with dramatically reduced expression of ABC-transporters in IEC and the  
22 resulting diminished capacity to deal with luminal xenobiotic challenge is thought to  
23 interfere with the efficiency of regenerative responses [7, 10-12]. Further compounding this  
24 problem is that several medications used in IBD are specific substrates of ABCG2. Hence  
25 understanding of the mechanisms governing ABCG2 expression is highly relevant for  
26 devising improved therapy [13-14]. Nevertheless, the molecular mechanisms driving  
27 ABCG2 expression during inflammatory responses remain obscure at best.

28 Generally, excreted and membrane-bound proteins are translated in the endoplasmic  
29 reticulum (ER). If ER function is disrupted, proteins can accumulate inside the ER and this  
30 can initiate a specific ER stress syndrome denoted the unfolded protein response.  
31 Recently, abnormalities in the ER stress response pathways have been associated with  
32 the pathogenesis of IBD [15-17]. Proper function of the ABCG2 protein depends on N-  
33 glycosylation and the formation of inter/intra molecular disulfide bonds [18-19]. These  
34 bonds are formed inside the ER by protein-disulfide isomerases (PDI). Inflammatory  
35 conditions, such as increased nitric oxide production, can dramatically reduce the  
36 efficiency of these PDI, which can initiate misfolding of various proteins leading to ER  
37 stress [20-21]. As correct ABCG2-protein folding is necessary for its functionality, we  
38 hypothesized that mucosal inflammation in the setting of IBD alters proper ABCG2-protein  
39 folding and thereby reduces its apical expression. In this study we describe a novel  
40 mechanism by which ABCG2 protein expression is decreased during active inflammation.

## 1 **Experimental procedures**

### 3 *Patient materials*

4 Formalin-fixed paraffin-embedded (FFPE) colonic biopsies were collected from nine  
5 healthy individuals and from 76 patients: 25 with Crohn's disease (CD), 36 with ulcerative  
6 colitis (UC), 10 with ischemic colitis and 5 with infectious colitis. Biopsies throughout the  
7 small intestine were collected from 17 other healthy individuals and 34 CD patients. For  
8 mRNA isolation freshly-frozen biopsies were collected from six healthy individuals and  
9 from 15 patients: 8 with inactive IBD and 7 with active IBD.

10 The demographic characteristics, number of biopsies per group, duration of disease and  
11 medication use are described in supporting Tables 1, 2 and 3 (included in the supporting  
12 section).

### 14 *Immunohistochemistry and scoring*

15 The FFPE sections were immunohistologically stained using the primary antibodies BXP-  
16 21 anti-ABCG2 (Santa Cruz Biotechnology, Santa Cruz, CA) and BiP anti-GRP78 (Cell  
17 Signalling, Boston, USA) according to the manufacturers' protocol (included in the  
18 supporting section). The expression of ABCG2 and GRP78 in IEC was scored by intensity  
19 over the whole slide by two independent observers, and discrepancies were re-assessed  
20 to come to a final agreement. Individual scores were used for statistical analyses, whereas  
21 they were combined into three subgroups for graphic representation. For ABCG2 positive  
22 staining at the apical membrane of the epithelial cells was defined as positive if >50%  
23 of the IEC were positive, mild 15-50%, or negative <15%. Immunoreactivity for GRP78 was  
24 also defined in terms of positive IEC, but now the percentage were >70% defined as  
25 positive, 20-70% mild, <20% negative.

### 27 *mRNA isolation and quantitative PCR*

28 mRNA was isolated from colonic biopsies, which were taken from CD and UC patients,  
29 using the NucleoSpin Extract II mRNA isolation kit (Macherey Nagel, Düren, Germany).  
30 Gene expression of GapdH, IL8, IL1beta, ABCG2, villin, and ER stress associated gene  
31 CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) [20] were  
32 measured using quantitative PCR (qPCR, IQ5, Bio-Rad). Villin and ABCG2 are both IEC  
33 specific, thus ABCG2 mRNA is corrected for Villin to prevent underestimation in biopsies  
34 with less IEC. A detailed protocol and the primer sequences involved are presented in the  
35 supporting section.

### 37 *Cell culture*

38 HEK293t (ATCC# CRL-1573) cells were cultured according to routine procedures.  
39 Transient transfection was performed using Polyethylenimine (PEI, Polysciences,  
40 Eppelheim, Germany). S-nitroso-N-acetylpenicillamine (SNAP, Sigma-Aldrich) was used  
41 as a NO donor. Tunicamycin from *Streptomyces* sp. (Sigma-Aldrich) was used as a  
42 positive control for ER stress induction. The cell culture supernatant nitrate concentrations  
43 were measured using Griess Reagents (Cayman Chemical, Michigan, USA) according to  
44 the manufacturers protocol. Additional cell culture methods are described in the supporting  
45 section.

### 47 *XBP1 splicing*

48 ER stress and unfolded protein response activity was assessed by identify mature XBP1  
49 mRNA and its spliced variant [16] (included in the supporting section).

1 *Construct cloning*

2 To investigate ABCG2 functionality we created a green fluorescent protein (GFP)-ABCG2  
3 fusion construct (included in the supporting section).

4  
5 *Fluorescent microscopy*

6 The GFP-tagged proteins were examined using a confocal microscope (LSM510META,  
7 Zeiss). For the transport assay, nuclear Hoechst 33342 (4  $\mu$ M, Sigma-Aldrich)  
8 accumulation was determined at 30 s intervals during a total of 12 min. The ER was  
9 stained with ER-RFP (Red Fluorescent Protein) using ER-Tracker (Molecular Probes,  
10 Paisley, UK) according to the manufacturer's instruction. We analyzed the GFP tagged  
11 proteins with a 488 nm laser, Hoechst with a 405 nm laser and the ER-RFP with 543 nm  
12 laser. A specific ABCG2 transport inhibitor Ko143 (2  $\mu$ M, Sigma-Aldrich) was used to  
13 identify ABCG2 transport specificity.

14  
15 *SDS-page and fluorescent immunoblotting*

16 For checking correct length of fusion proteins, whole cell lysates were analysed on  
17 immunoblot from standard SDS-page gel, using antibodies against ABCG2 (BXP-21,  
18 Santa Cruz Biotechnology) and GFP (polyclonal, rabbit IgG, Invitrogen) (included in the  
19 supporting section).

20  
21 *Statistics and software*

22 Statistical analyses were performed using the SPSS 11.0 software package for Windows.  
23 Data on different patient groups were compared using the Mann-Witney *U* test with  
24 Kruskal-Wallis post test. Using univariate and multivariate multinomial logistic regression  
25 analysis, we examined the association between colitis and GRP78 and ABCG2  
26 expression, adjusting for age and duration of disease. A two-tailed *p* value <0.05 was  
27 accepted as statistically significant. Images were composed using Adobe Photoshop CS3.  
28

## 1 Results

### 2 3 *Colonic inflammation reduces apical ABCG2 expression in IEC*

4 To investigate ABCG2 expression during human intestinal inflammation, ABCG2  
5 expression in colonic biopsies was qualitatively assessed employing  
6 immunohistochemistry (IHC). As expected IEC in biopsies of control subjects as well as of  
7 IBD patients in remission (with no active inflammation) show strong specific staining for  
8 ABCG2 at the apical surface (Figure 1A). In contrast, apical IEC ABCG2 staining is  
9 dramatically reduced in patients with active IBD, in the colon as well as in the small  
10 intestine, and also in biopsies from ischemic colitis and infectious colitis patients (Figure  
11 1A). Thus diverse colonic inflammatory responses are uniformly characterised by down  
12 regulation of apical ABCG2 levels, suggesting that processes common to these alternative  
13 modes of inflammation underlie the absence of apical ABCG2 intestinal inflammation.

14 In apparent agreement, quantitative study of ABCG2 levels in the human colon reveals  
15 significantly less ABCG2 expression in active colitis (24% positive expression, 13/54),  
16 ischemic colitis (10% positive staining, 1/10) and infectious colitis (0% positive staining  
17 0/14) as compared to either inactive colitis (80% positive expression, 53/67) or control  
18 tissues (100% positive expression, 9/9), all  $p < 0.001$  (see Figure 1B). There is no  
19 significant difference in ABCG2 levels between active CD (27%, 6/22) vs. active UC (22%,  
20 7/32) and inactive CD (67%, 18/27) vs. inactive UC (88%, 35/40). As a control, we  
21 examined left- and right-sided biopsies from IBD patients and controls to investigate a  
22 confounding possible differential expression of ABCG2 based on position in the colon, but  
23 this analysis demonstrates that only the degree of inflammation is an important factor  
24 guiding apical ABCG2 levels. Thus inflammation *per se* is associated with a marked  
25 reduction of apical ABCG2 expression in the human colon.

### 26 27 *Small bowel inflammation is associated with down regulation of apical ABCG2 expression*

28 Mirroring the findings obtained in the human colon, small bowel biopsy specimens  
29 obtained from CD patients with active disease by double balloon endoscopy exhibit  
30 markedly less ABCG2 staining when compared to biopsies obtained from patients with  
31 inactive disease or no intestinal inflammatory phenomena (see Figure 1A). In active CD,  
32 there is significantly less small bowel ABCG2 staining (75% negative staining, 19/25) as  
33 compared to inactive CD (11% negative staining, 1/9) and controls (8% negative staining,  
34 2/25), both  $P < 0.05$  (see Figure 1C). We find no significant differences between inactive  
35 CD and controls. In all cases current drug use did not influence the expression of ABCG2.  
36 We conclude that human intestinal inflammation is accompanied by reduced expression of  
37 xenobiotic transporter ABCG2 and experiments were initiated to identify the underlying  
38 molecular mechanisms.

### 39 40 *Intestinal inflammation acts to reduce ABCG2 via a post-transcriptional mechanism*

41 To delineate the molecular events reducing the capacity of the intestine to cope with  
42 xenobiotic challenge we first determined whether genomic or post-transcriptional  
43 processes are involved in this effect. To this end, we measured ABCG2 mRNA levels in  
44 freshly frozen colon biopsies from healthy individuals, inactive and active IBD patients.  
45 Importantly, ABCG2 mRNA, levels in IEC, do not differ between these three groups (all  
46  $p > 0.8$ ). The expression, however, of the pro-inflammatory cytokine IL8 and IL1beta are 10-  
47 and 8-fold higher ( $p < 0.05$ ) in biopsies with active mucosal inflammation (Figure 2) and  
48 thus technical issues do not underlie the absence of intestinal inflammation-induced  
49 effects on apical ABCG2 expression. Hence we are forced to conclude that down  
50 modulation of ABCG2 in human intestinal inflammation is brought about by a post-  
51 transcriptional process.

1 *Activation of the protein unfolded protein response accompanies human intestinal*  
2 *inflammation*

3 As it is becoming increasingly clear that activation of the unfolded protein response  
4 accompanies inflammatory processes and that ER stress may interfere with proper  
5 production of complexly folded proteins (the “half” transporter ABCG2 transverses the  
6 membrane 6 times), we examined whether the ER stress pathway is activated in intestinal  
7 inflammation and to which extent this activation mirrors the down regulation of ABCG2. To  
8 this end we employ the expression of the *bona fide* ER stress marker GRP78.  
9 Representative images of the GRP78 immunohistochemical analyses of the same  
10 biopsies as stained for ABCG2 are shown in Figure 3A. There is a significant increase in  
11 GRP78 expression in active colitis (32% positive expression, 16/54), ischemic colitis (90%  
12 positive staining, 9/10) and infectious colitis (79% positive staining 11/14), compared to  
13 inactive colitis (0% positive expression, 0/67) and control tissues (0% positive expression,  
14 0/9), all  $p < 0.001$  (see Figure 3B). There are no significant differences in GRP78  
15 expression between active CD and active UC or between inactive CD and inactive UC.  
16 Also there are no differences in GRP78 expression in left- and right-sided colon biopsies  
17 taken from the same patient. We conclude that colonic inflammation is accompanied by  
18 ER stress response which is absent in the un-inflamed colon.

19 In the small bowel biopsies of active CD patients there is increased GRP78 expression  
20 (29% positive expression, 6/21) compared to inactive CD patients (0% positive staining,  
21 0/9) and controls (8% positive expression, 2/25)  $p < 0.05$  (see Figures 3A and 3C). In all  
22 actively inflamed tissue samples elevated GRP78 expression is evident, irrespective of  
23 disease type or location. ER stress during intestinal inflammation is further confirmed  
24 measuring the mRNA expression of ER stress associated gene, CHOP, in colonic  
25 biopsies. In biopsies taken from actively inflamed mucosa a significant increase in CHOP  
26 mRNA expression was measured,  $p < 0.01$  (see Figure 2B). Moreover, an increased ratio of  
27 spliced XBP1 mRNA was detected in patients with active intestinal inflammation (Figure  
28 2C). We conclude that activation of the unfolded protein response and intestinal  
29 inflammation are highly associated processes and we initiated experiments to investigate  
30 whether ER stress explains down regulation of ABCG2 in intestinal inflammation.

31  
32 *Impaired ABCG2 function in ER stressed cells*

33 To investigate the effect of activated ER stress on ABCG2 protein expression and  
34 function, we created a GFP-ABCG2 fusion protein. To this end, ABCG2 cDNA was cloned  
35 into a pEGFP-C1 vector which led to the production of GFP-ABCG2 after transfection  
36 (Figure 4A). The NO-donor SNAP was used to ascertain whether NO was able to induce  
37 ER stress in GFP-ABCG2 transfected HEK293t (293t-AB) cells. The Griess assay reveals  
38 SNAP application induces robust increases in  $\text{NO}_2$  concentration, a good proxy measure  
39 for NO production (see Figure 4B). The validity of assay is demonstrated by experiments  
40 showing an increase in the ratio of unspliced XBP1 mRNA over spliced XBP1 mRNA nine  
41 h after application of 0.625 mM SNAP (see Figure 4C). In agreement administration of  
42 tunicamycin, a well established ER stress response inducer, increases the XBP1 splicing  
43 ratio to approximately the same level as SNAP application (supporting Figure 1B).  
44 Consistent with high ABCG2 transport activity, administration of Hoechst 33342 does not  
45 stain the nuclei of the 293t-AB cells (Figure 5A), also demonstrating the functionality of the  
46 ABCG2-GFP fusion protein in xenobiotic transport. Nevertheless, 16 h after addition of the  
47 ER-stress inducers SNAP or tunicamycin, the nuclei of 293t-AB cells become Hoechst  
48 33342 positive (Figure 5A and supporting movies 1, 2 and 3). As shown in Figure 5B  
49 Hoechst 33342 also stains the nucleus blue in 293t-AB transfected cells in the presence of  
50 a specific ABCG2 inhibitor Ko143 (see also supporting movie 4). No altered GFP-ABCG2  
51 protein function is found when SNAP or tunicamycin is added 48 h after transfection (see



1 supporting Figure 1C). Hence, in this over expressing model system ER stress provokes a  
2 very strong decrease in ABCG2 function, in agreement with a scheme in which  
3 inflammation-dependent induction of ER stress is causatively related to a reduced capacity  
4 to deal with xenobiotic challenge.

5 In support for such a model, all images from 293t-AB cells with ER stress reveal reduced  
6 apical membrane expression of GFP-ABCG2, with remaining intracellular expression. In  
7 order to ascertain whether GFP-ABCG2 remains in the ER after stress induction, we  
8 stained the ER with ER-RFP. When ER stress was present some GFP-ABCG2 co-  
9 localized with ER-RFP, whereas the majority was present in other cellular compartments  
10 (Figure 5C). As shown in supporting Figure 2 the localization and expression of non-ER  
11 dependent GFP-tagged proteins (GFP-betaCatenin, GFP-H2B) was not changed during  
12 ER stressed conditions. Thus protein folding difficulties seems incompatible with functional  
13 expression of ABCG2.

## 1 Discussion

2  
3 The data presented in the current study are consistent with a model in which intestinal  
4 inflammation drives an epithelial misfolded protein response, which in turn interferes with  
5 the ABCG2 expression and function leading to a diminished capacity of the epithelial  
6 compartment to cope with xenobiotic stress. Colonic and small bowel biopsies from IBD  
7 patients with active disease and those from patients with ischemic and infectious colitis all  
8 show reduced apical ABCG2 expression in IEC compared to controls and IBD patients in  
9 remission. The decline of ABCG2 expression in the presence of active inflammation in IBD  
10 patients corresponds with the findings of two studies done exclusively on UC patients [7,  
11 22]. In addition, we found that ABCG2 expression is not affected by medication use,  
12 patient age, or location of the inflammation.

13 Expression of ABCG2, like that of many other proteins, can be regulated at various levels.  
14 In addition to a decline in ABCG2 protein expression, others have described a reduction in  
15 ABCG2 mRNA expression in actively inflamed intestine [7, 22]. We corrected the ABCG2  
16 mRNA expression for Villin to prevent underestimation of ABCG2 expression, since both  
17 genes are specific for IEC. Our results show no reduction of ABCG2 mRNA expression in  
18 biopsies with active mucosal inflammation. However, since the sample size used here is  
19 smaller than those in previous studies, we cannot completely rule out the contribution of  
20 reduced mRNA expression to the decline in ABCG2 protein expression. As such our  
21 findings merely imply that the decline in mRNA expression probably does not solely  
22 account for the complete loss of ABCG2 protein expression discernable on our IHC  
23 images and that post-transcriptional mechanisms must be involved. The observation that  
24 unfolded protein responses are both a general and specific to intestinal inflammation  
25 suggest a causal link between ER stress and diminished apical ABCG2 levels. Indeed,  
26 ABCG2 is a "half" transporter, 6 times membrane spanning protein, functionally critically  
27 dependent on homodimerisation, N-linked glycosylation and various disulfide bonds and is  
28 thus a likely early victim of ER stress responses [18-19]. Disrupted protein-folding  
29 mechanisms that can lead to ER stress during active inflammation have recently been  
30 linked to IBD [16, 23-24] and may well involve reduced capacity of the IEC compartment to  
31 deal with the xenobiotic stress and thus in turn interfere with demanding regenerative  
32 responses that an adequate response to IBD-related intestinal challenges requires.

33 Our inflammation links to unfolded protein responses remains unclear, but associated with  
34 inflammatory responses is an excessive production of NO, which in turn is predicted to  
35 disturb the function of redox-state sensitive disulfide-bonding chaperones like PDI that are  
36 necessary for proper protein folding [25]. In all biopsies with active inflammation (i.e. IBD  
37 and non-IBD related), the GRP78 expression is significantly higher than in the biopsies of  
38 IBD patients in remission, and in those of healthy controls. Furthermore, expression of  
39 CHOP mRNA (an ER stress marker) is increased in biopsies with active mucosal  
40 inflammation. In addition to this, biopsies with active inflammation reveal an increased  
41 ratio of spliced XBP1 mRNA expression. Thus factors common to multiple types of  
42 inflammation must be involved here and as NO production is one of the few cellular  
43 responses described to generally accompany inflammation, it is tempting to attribute a  
44 important role to excessive NO generation in inflammation-related induction of ER stress  
45 responses [23, 25-27]. Indeed, we observe elevated XBP1 splicing [16] following  
46 application of the NO-donor, suggesting that NO can directly affect protein-folding  
47 mechanisms [20] and induce ER stress. In the current study we show that the reduction in  
48 ABCG2 expression is inflammation-associated, which fits with the observations that  
49 ABCG2 expression is reduced in IBD-unrelated inflammatory conditions in other organs  
50 [10, 12, 28]. Other IBD associated proteins are also reduced in protein expression during  
51 active intestinal inflammation [29-31]. Further research is needed to investigate the role

1 impeded protein folding and the effect of this on cellular processes. Especially, ABCG2-  
2 mediated efflux of toxic components is thought to protect the epithelium of the gut and the  
3 underlying tissue to xenobiotic challenge. Importantly, as xenobiotic stress is directly  
4 linked to genotoxicity our results may also help explaining the still poorly understood  
5 relation between intestinal inflammation and colorectal cancer [32]. Decreased ABCG2  
6 expression may have other deleterious consequences as well. Sulfasalazine, a derivate of  
7 mesalazine (5-ASA) that is commonly used in the treatment of IBD patients, is actively  
8 excluded from the epithelial compartment by ABCG2 [33]. Rodents with reduced IEC  
9 ABCG2 expression have increased 5-ASA trough levels [34]. In addition to its anti-  
10 inflammatory effect, accumulation of sulfasalazine can be toxic for epithelial cells [35-36].  
11 Thus reduced expression and function of ABCG2 may therefore greatly enhance the  
12 sensitivity of IEC to sulfasalazine and limit the use of this compound for the treatment of  
13 IBD. Hence understanding the mechanisms governing ABCG2 expression are highly  
14 relevant for efforts with respect to rational design of novel therapy.  
15 In conclusion, IEC ABCG2 protein expression is decreased in the inflamed bowel probably  
16 as a result of incorrect protein folding due to inflammation-driven ER dysfunction. Thus a  
17 novel paradigm explaining xenobiotic stress during inflammation emerges, in which  
18 intestinal inflammation activates the unfolded protein response in turn abrogating  
19 defences against xenobiotic challenge by impairing ABCG2 expression and function.

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2

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12

## 1 Figure legends

2  
3 **Figure 1:** Reduced ABCG2 expression in actively inflamed intestinal biopsies.

4 **A)** Representative images of intestinal biopsies IHC stained for ABCG2. *Colon, Ctr*) colon  
5 biopsy from healthy individual. *Colon, IA*) colon biopsy from an IBD patient in remission.  
6 *Colon, A*) colon biopsy from an IBD patient with active inflammation. *Colon, ISC*) colon  
7 biopsy from a patient with ischemic colitis. *Colon, IFC*) colon biopsy from a patient with  
8 infectious colitis. *SB Ctr*) small bowel biopsy from a healthy individual. *SB, IA*) small bowel  
9 biopsy from a Crohn's disease patient in remission. *SB, A*) small bowel biopsy from a  
10 Crohn's disease patient with active inflammation. The ABCG2 expression is represented  
11 by red-brown staining on the apical membrane of IEC. Original magnification is 400x.

12 **B)** Decrease in the IEC fraction of colonic biopsies with ABCG2 expression during active  
13 inflammation. **C)** Actively inflamed small bowel displays reduced expression of ABCG2. **B)**  
14 **and C)** All intestinal biopsies were double blindly examined as negative, moderate or  
15 positive for ABCG2 membrane immune reactivity. Mann–Whitney U tests and Kruskal-  
16 Wallis tests were used for statistical analysis.

17  
18 **Figure 2:** mRNA expression from intestinal biopsies.

19 **A)** No difference in ABCG2 mRNA expression in colonic biopsies. Relative mRNA  
20 (deltadeltaCt) expression corrected for Villin. Villin and ABCG2 are both IEC specific, thus  
21 ABCG2 mRNA is corrected for Villin to prevent underestimation in biopsies with less IEC.

22 **B)** Increased pro-inflammatory cytokine expression in colonic biopsies from patients with  
23 active inflammation. Relative mRNA expression (deltadeltaCt) corrected for GapdH. IL8  
24 and IL1beta are pro-inflammatory cytokines and CHOP is an ER stress related gene. .

25 **A) and B)** Control are healthy individuals (n=6), ia IBD are biopsies with inactive  
26 inflammation (n=8), a IBD are biopsies with active inflammation (n=7). Mann–Whitney U  
27 tests and Kruskal-Wallis tests were used for statistical analysis. **C)** XBP1 mRNA splicing.  
28 Agarose gel image of XBP1 splicing PCR products. Spliced XBP1 is an indicator for  
29 increased ER stress. A is active IBD, IA is inactive IBD and H is healthy control.  
30

31 **Figure 3:** Increased GRP78 expression in actively inflamed intestinal biopsies.

32 **A)** Representative images of intestinal biopsies IHC stained for GRP78. *Colon, Ctr*) colon  
33 biopsy from healthy individual. *Colon, IA*) colon biopsy from an IBD patient in remission.  
34 *Colon, A*) colon biopsy from an IBD patient with active inflammation. *Colon, ISC*) colon  
35 biopsy from a patient with ischemic colitis. *Colon, IFC*) colon biopsy from a patient with  
36 infectious colitis. *SB Ctr*) small bowel biopsy from a healthy individual. *SB, IA*) small bowel  
37 biopsy from a Crohn's disease patient in remission. *SB, A*) small bowel biopsy from a  
38 Crohn's disease patient with active inflammation. Expression of GRP78 is detected as red-  
39 brown colour and is present in the cytoplasm of IEC and mononuclear cells in the lamina  
40 propria. Original magnification is 400x. **B)** Increased in the IEC fraction of colonic biopsies  
41 with elevated GRP78 expression during active inflammation. **C)** Actively inflamed small  
42 bowel displays elevated GRP78 expression. **B) and C)** The biopsies were double blindly  
43 examined as positive, moderate or negative according the amount of positive GRP78  
44 immune reactivity. Mann–Whitney U tests and Kruskal-Wallis tests were used for  
45 statistical analysis.  
46

47 **Figure 4:** Nitric oxide induced ER stress reduces ABCG2 protein expression and function.

48 **A)** Western blot analysis showing the expression of GFP-ABCG2 protein using the  
49 antibodies BXP21 (anti-ABCG2) and anti-GFP. Tubulin (Tub) and beta-actin (b-Act) were  
50 used as loading control. MM is molecular marker.

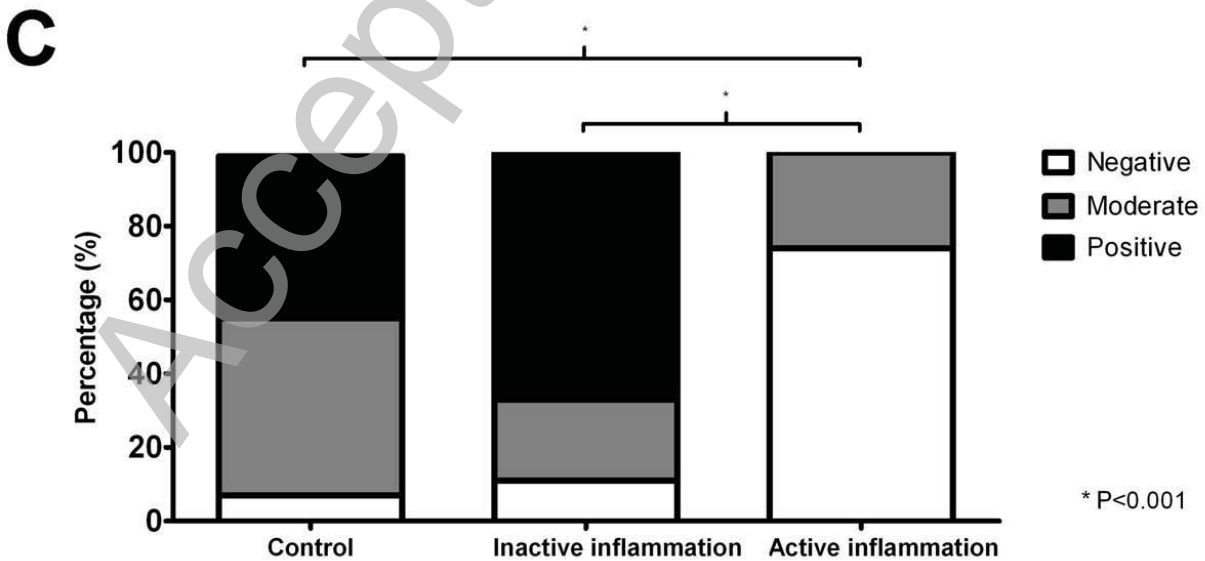
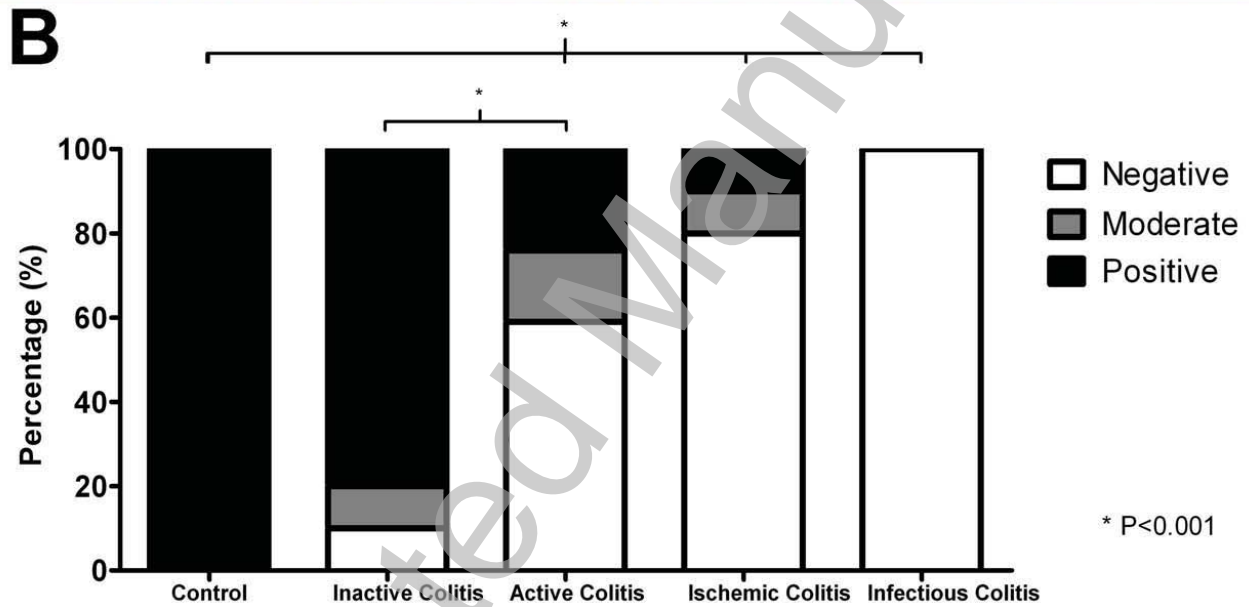
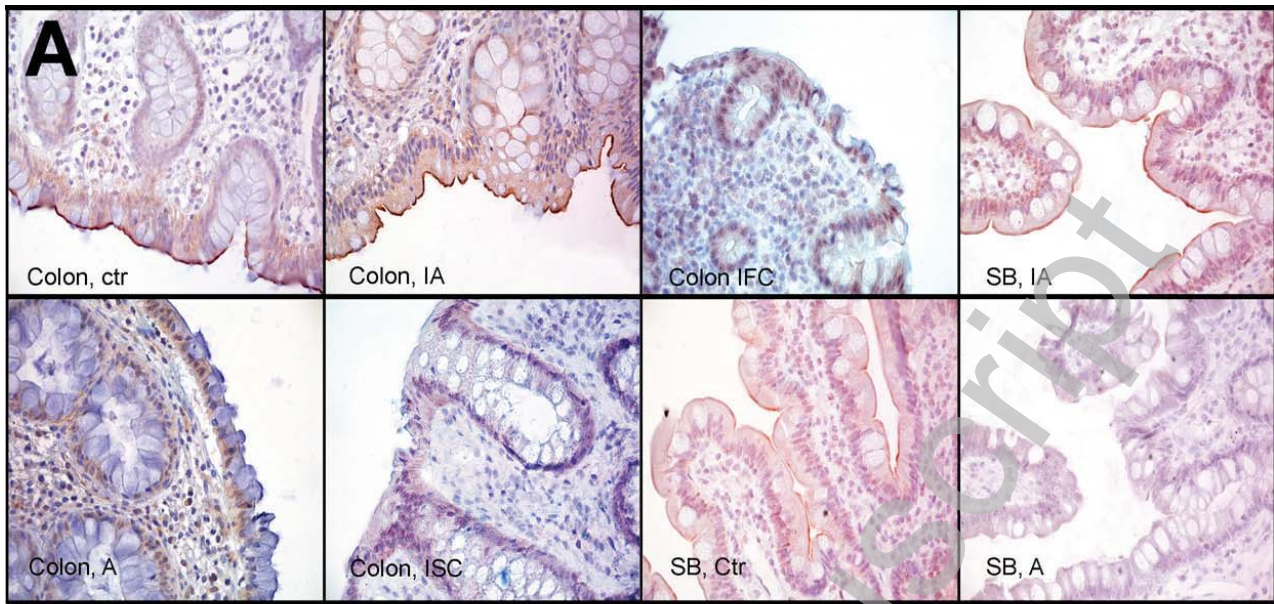


1 **B)** SNAP induces increasing levels of nitrite, a stable derivative of NO, in time.  
2 Concentrations are measured from supernatants of HEK293t cells transiently transfected  
3 with GFP-ABCG2 (293t-AB cells). **C)** After transfection with GFP-ABCG2, HEK293t cells  
4 were stimulated with 0.625 mM SNAP and XBP1 splicing was determined, and the ratio of  
5 un-spliced (U) and spliced (S) XBP1 were plotted. Ctr depict HEK293t cells only, 0 are  
6 GFP-ABCG2 transfected cells without SNAP stimulation and other time points represents  
7 GFP-ABCG2 transfected cells with 0.625 mM SNAP. Gel picture is representative of three  
8 independent experiments, graph represents the average of these three individual  
9 experiments and error bars are SD.

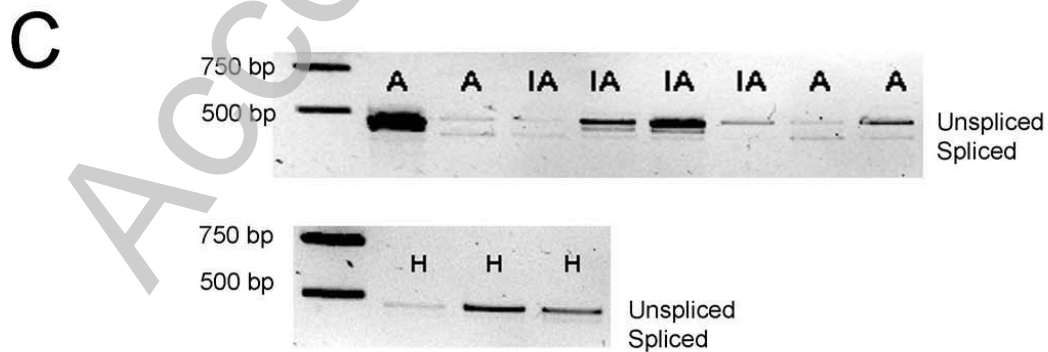
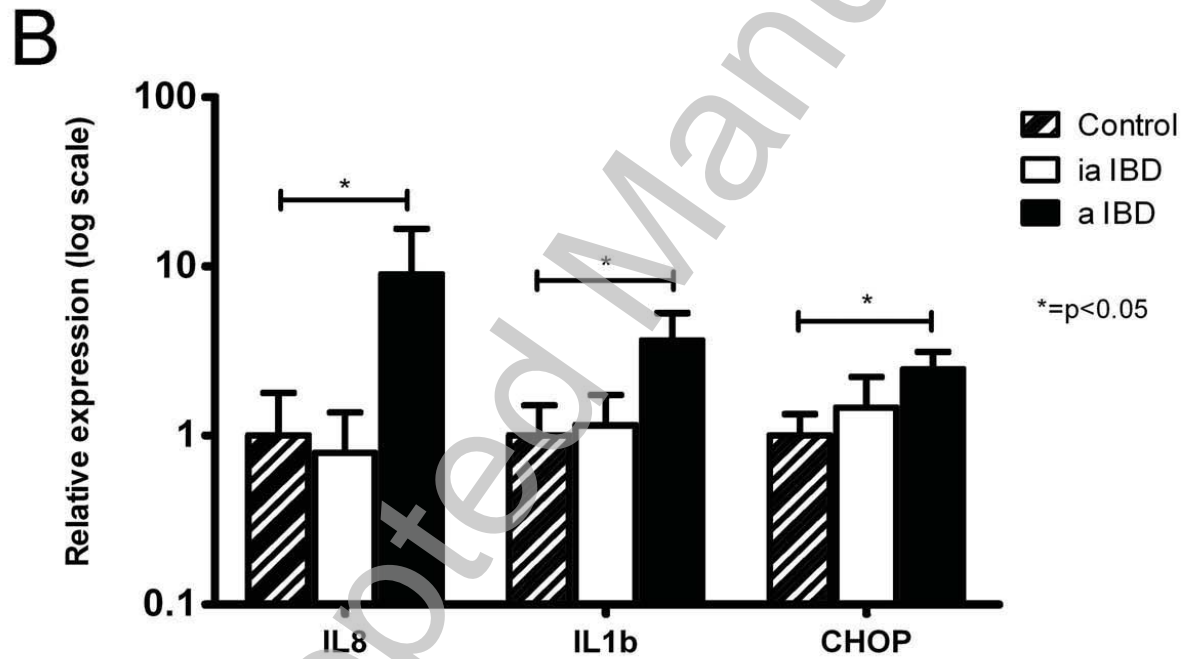
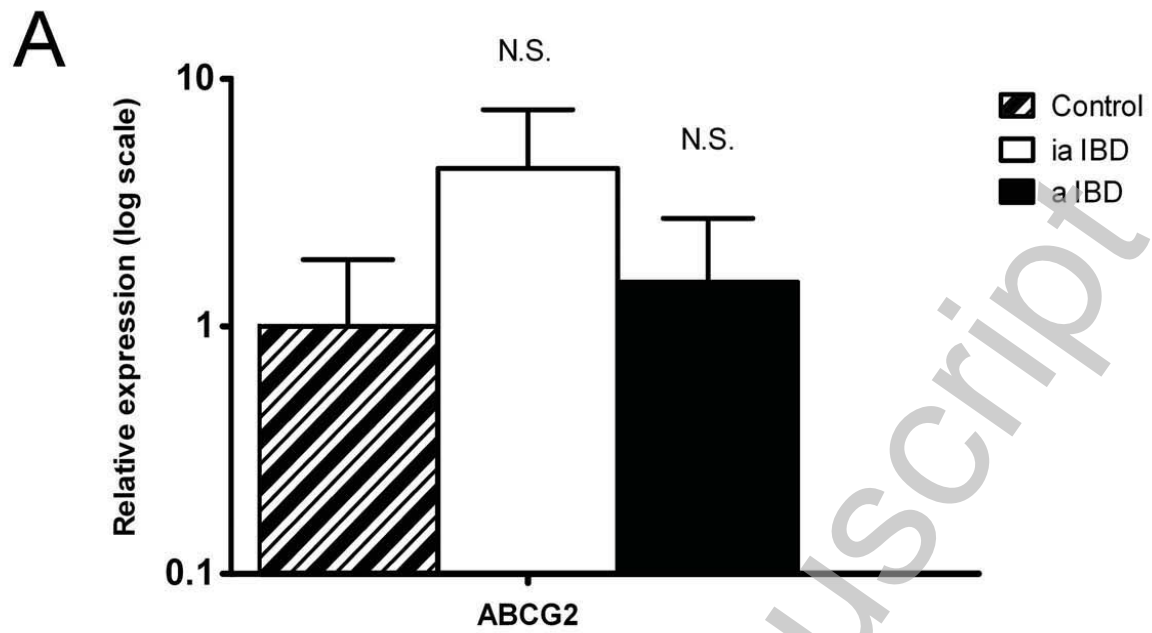
10  
11 **Figure 5:** Reduced ABCG2 activity and less membrane localization in ER stressed cells.  
12 Hoechst 33342 (4  $\mu$ M) DNA binding was measured to study the activity of ABCG2.

13 **A)** ABCG2 transfected cells were exposed to SNAP, tunicamycin or solvent for 16 h after  
14 which Hoechst 33343 binding was studied. See supporting Movies 1, 2 and 3. Original  
15 magnification 400x. **B)** The specific inhibitor of ABCG2, Ko143 (2  $\mu$ M) added 12 min after  
16 Hoechst 33342 (4  $\mu$ M)), was used as a positive control for ABCG2 activity. The  
17 accompanying movie is supporting Movie 4. Original magnification 400x. **C)** 293t-AB cells  
18 were stained with ER-RFP. ER stress was induced by addition of SNAP or tunicamycin 16  
19 h before ER-RFP staining.

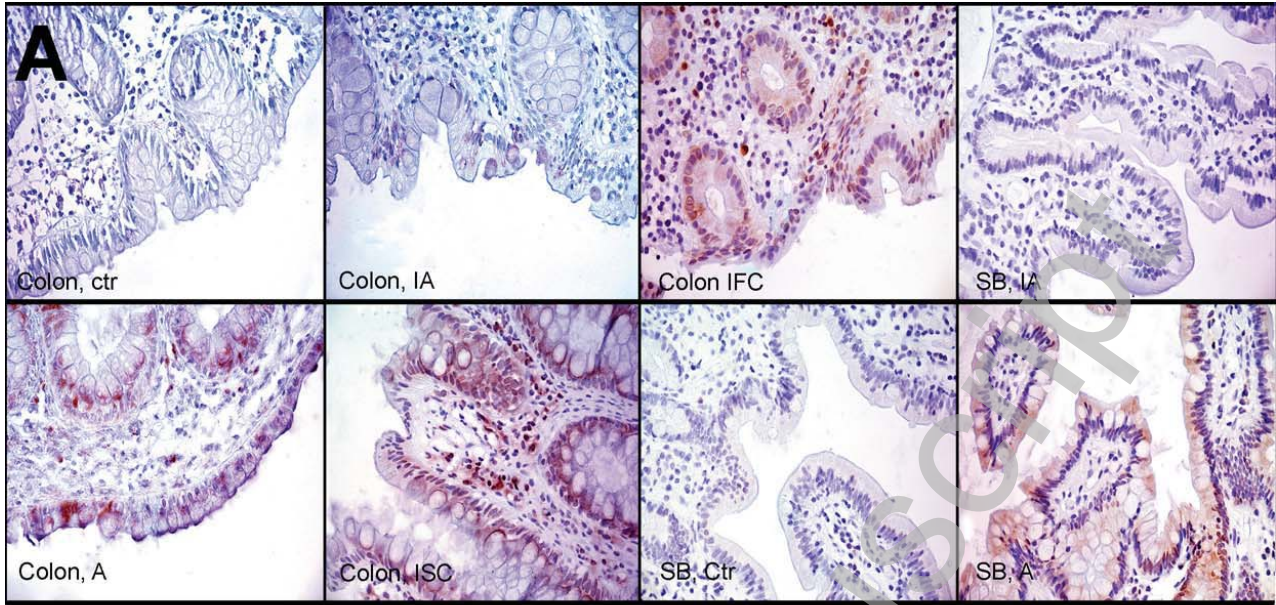
20 All confocal images are representative of at least three independent experiments. Original  
21 magnification 400x.



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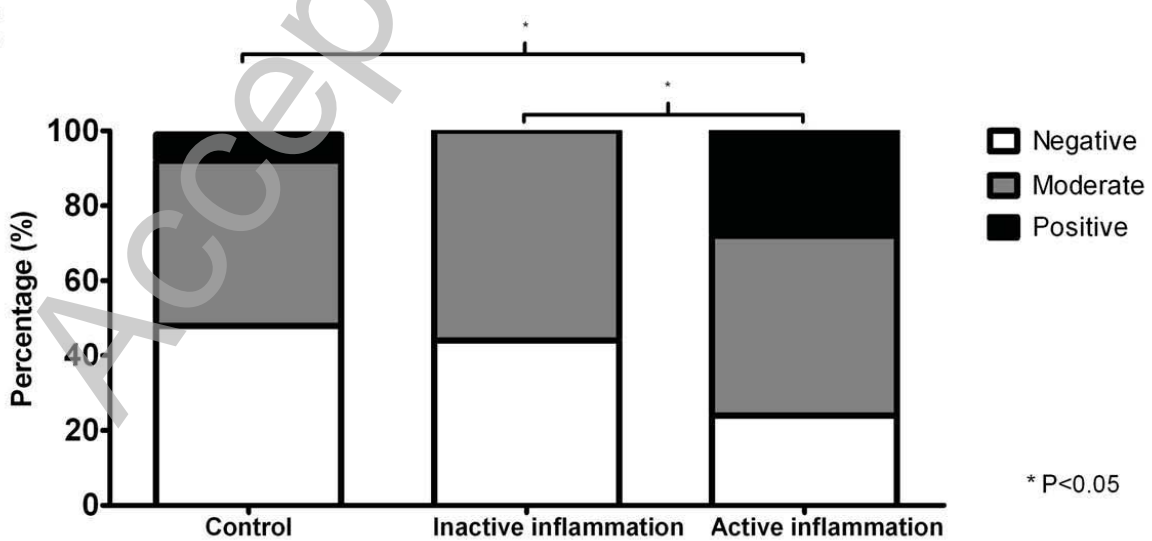
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**B**



**C**



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