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**Formylated Peptide Receptor-1 (FPR1) mediated gut inflammation as a therapeutic target in Inflammatory Bowel Disease**

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**Summary**

Despite significant advances, IBD is associated with high rates of suboptimal drug response. We present evidence of excessive innate immune activation via neutrophilic inflammation mediated by the Formylated Peptide Receptor (FPR)-1, with several lines of human and mouse data as a new tractable therapeutic approach in IBD.

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**Abstract**

**Background:** Formylated peptide receptor (FPR)-1 is a G-coupled receptor that senses foreign bacterial and host-derived mitochondrial formylated peptides (FPs), leading to innate immune system activation.

**Aim:** We sought to investigate the role of FPR1-mediated inflammation and its potential as a therapeutic target in IBD.

**Methods:** We characterized FPR1 gene and protein expression in 8 human IBD (~1000 patients) datasets with analysis on disease subtype, mucosal inflammation and drug response. We performed *in vivo* dextran-sulfate sodium (DSS) colitis in C57/BL6 *FPR1* knockout mice. In *ex vivo* studies, we studied the role of mitochondrial FPs and pharmacological blockade of FPR1 using Cyclosporin H in human peripheral blood neutrophils. Finally, we assess mitochondrial FPs as a potential mechanistic biomarker in the blood and stools of patients with IBD.

**Results:** Detailed *in silico* analysis in human intestinal biopsies showed that *FPR1* is highly expressed in IBD (n=207 IBD vs 67 non-IBD controls, p<0.001), and highly correlated to gut inflammation in UC and CD (both p<0.001). FPR1 receptor is predominantly expressed in leukocytes, and we showed significantly higher FPR1+ve neutrophils in inflamed gut tissue section in IBD (17 CD and 24 UC; both p<0.001). Further analysis in 6 independent IBD (data available under Gene Expression Omnibus accession numbers GSE59071, GSE206285, GSE73661, GSE16879, GSE92415 and GSE235970) showed an association with active gut inflammation and treatment resistance to Infliximab, Ustekinumab and Vedolizumab. *FPR1* gene deletion is protective in murine DSS colitis with lower gut neutrophil inflammation. In the human *ex vivo* neutrophil system, mitochondrial FP, nicotinamide adenine dinucleotide dehydrogenase subunit-6 (ND6) is a potent activator of neutrophils resulting in higher CD62L shedding, CD63 expression, ROS production and chemotactic capacity; these effects are inhibited by Cyclosporin H. We screened for mitochondrial ND6 in IBD (n=54) using ELISA and detected ND6 in stools with median values of 2.2 gg/ml (IQR 0.0-4.99; range 0-53.3) but not in blood. Stool ND6 levels, however, were not significantly correlated with paired stool calprotectin, C-reactive protein and clinical IBD activity.

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**Conclusions:** Our data suggest that FPR1-mediated neutrophilic inflammation is a tractable target in IBD; however, further work is required to clarify the clinical utility of mitochondrial FPs as a potential mechanistic marker for future stratification.

**Keywords:** IBD, neutrophils, FPR1, mitochondria, DAMPs

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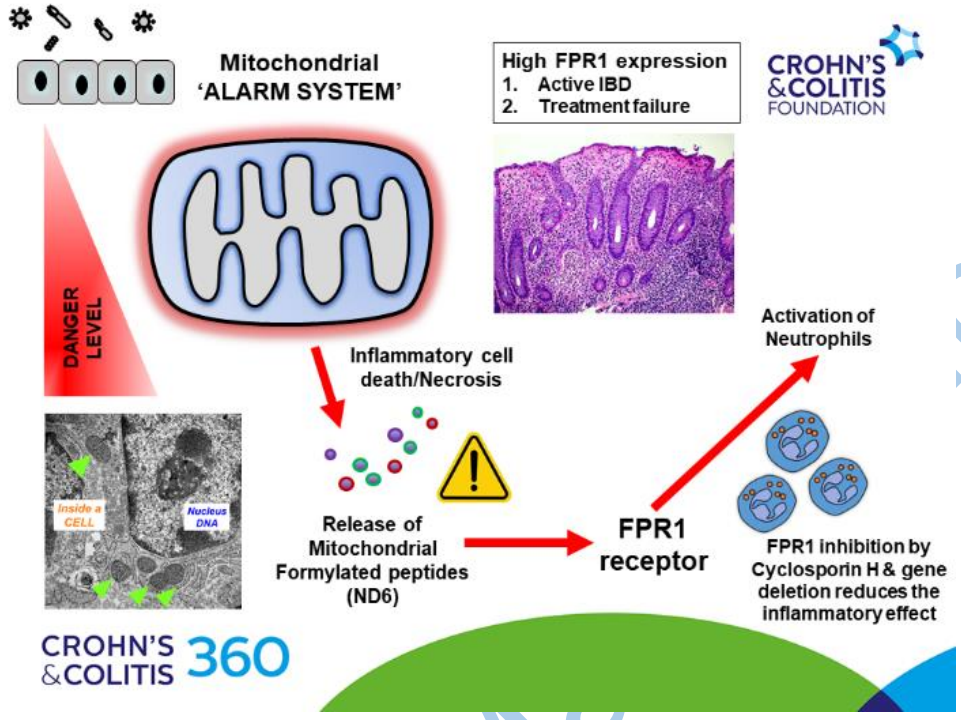
Graphical Abstract

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**Key messages**

**What is already known?**

Despite advances in immune-suppressive treatments for IBD, many patients fail to achieve complete mucosal healing. In the inflamed IBD mucosa, there are increased levels of damage-associate molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) that can drive the persistence of inflammation in IBD.

**What is new here?**

We provide evidence to show the importance of Formylated-peptide receptor (FPR)-1 mediated neutrophilic inflammation in IBD.

High expressions of FPR1 are associated with active IBD and treatment resistance.

Loss of FPR1 and/or pharmacologic inhibition of FPR1 reduce inflammation in mouse and human experimental models.

Mitochondrial DAMPs, ND6 are released during active gut inflammation and can activate FPR1-receptor.

**How can this study help patient care?**

FPR1-mediated inflammation can be therapeutically targeted in IBD as an adjunctive approach in IBD with potential stratification with mitochondrial DAMP biomarkers.

**Lay Summary**

Our study shows that a receptor called FPR1 that 'calls in inflammatory cells' to the gut might explain why there is too much inflammation in IBD. 'Switching off' FPR1 might be useful as a new way to treat IBD.

**Data Availability:** Microarray data available via access to Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo) microarray/gene expression databases GSE11223, GSE20881, GSE16879, GSE23597, GSE59071, GSE73661, GSE92415 and GSE206285. Single-cell RNA sequencing data is available via Gene Expression Omnibus (GEO) GSE134809.

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## Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are immune-mediated conditions with complex and overlapping pathogenic factors that can initiate and perpetuate a non-resolving pattern of mucosal inflammation[1]. Most current therapies inhibit the downstream inflammatory response yet complete mucosal healing is difficult to achieve and is seen in ~50% of treated severe IBD. There remains a need to identify new therapeutic targets as part of a wider strategy to achieve deep mucosal healing and remission in IBD. In this context, there is an increasing focus on the upstream inflammatory factors, which can potentiate the abnormal gut inflammatory process observed in IBD. Here, both exogenous Pathogen-Associated Molecular Patterns (PAMPs) by binding to germ-line encoded Pathogen Recognition Receptors (PRRs); and Damage-Associated Molecular Patterns (DAMPs) that are endogenous host molecules that are released during tissue injury can act as danger signals that activate the innate immune system[2]. Several lines of data suggest that high levels of and the persistence of PAMPs and DAMPs may be an important hitherto under-recognized contributory driver to the failure of IBD-associated inflammation to resolve completely in response to medical therapies[3]. Presently, there is an increasing focus on targeting DAMP-mediated inflammation in many human inflammatory diseases[4].

We recently showed that mitochondrial DAMPs (mtDAMPs), particularly mitochondrial DNA (mtDNA) are increased in IBD with significant correlation with disease activity and severity[5]. Several lines of evidence show that uncontrolled extracellular release of mtDAMPs can drive the development of inflammation and auto-immunity[6, 7]. MtDAMPs express at least two critical inflammatory molecular signatures: mitochondrial *N*-formyl peptides (mtFPs) and mtDNA. In the latter, mtDNA shares similar immune-activating properties as bacterial DNA due to their shared ancestry. Whilst the effects of mtDNA via a complex network of intracellular nucleic acid receptors such as TLR9, STING and AIM3 can result in a graduated immune response involving different immune cell types[8], We recently showed that circulating blood mtFPs (FMMYALF, FMTPMRK, FMNPLAQ, FMNFALI, FMTMHTT) could be detected in severe IBD, by using a targeted LC-MS mass spectrometry approach screen[5]. Of these five mtFPs, FMMYALF or nicotinamide adenine dinucleotide dehydrogenase subunit-6 from hereon, ND6 was the most abundant in our IBD subset; and interestingly, also the most pro-inflammatory mtFP[9].

Mitochondrial formylated peptides have long been considered an important chemoattractant for neutrophils[10]. Recently, blood mitochondrial ND6 has been shown to be elevated in human

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diseases, pertinently in systemic inflammatory response syndrome (SIRS), severe Covid19, stroke and rheumatoid arthritis[11-14]. The uncontrolled release of mitochondrial ND6 can result in the rapid triggering of inflammation via formylated peptide receptor (FPR)-1, a G protein-coupled chemoattractant receptor [10, 15]. FPR1 is highly expressed in neutrophils; and also on monocytes, macrophages, dendritic cells and epithelial cells[16]. Of interest, FPR1 recognizes both N-formyl peptides that are contained in bacteria or mitochondria[17], and other relevant DAMP ligands including cathepsin G, annexin A1 (ANXA) and FAM19A4[18-20]. FPR1-mediated signaling therefore is relevant in human inflammatory diseases and is an attractive druggable pathway[15]. In addition to more established FPR1 inhibitors such as Cyclosporin H[21], there are now several small molecule antagonists that are more potent and specific for FPR1 that may have promise in inflammatory diseases[22-24]. Hence, in our study, we sought to firstly characterize the importance of FPR1 in gut inflammation and IBD. And, secondly, we explore the potential of measuring mitochondrial ND6 levels as a biomarker that may facilitate future stratification for FPR1-blockade as a therapeutic option in IBD.

## Results

### Formylated peptide receptor-1 is highly expressed in inflamed intestinal tissue in human IBD

We performed *in silico* analysis of our previously published colonic gene microarray dataset (99 CD, 129 UC and 56 non-IBD controls; data available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) [accessed September 2022] accession[25] (GSE11223 and GSE20881). Overall, we showed that *FPR1* gene is highly expressed in the IBD colon compared to non-IBD controls (n=207 [124 UC and 83 CD] vs. n=67 colonic biopsies in each respective group; p=0.0018) (Figure 1a). We analyzed and presented *FPR1* gene expressions from colonic biopsies (as each patient has more than one colonic biopsy) to allow paired analyses with gut inflammation status and other relevant genes of interest. Here, *FPR1* gene expression was higher in colonic biopsies from inflamed gut mucosa in UC and CD compared to non-inflamed respective sections (both p<0.0001) (Figure 1b). As our microarray gene expression data were obtained from whole pinch gut biopsies, we performed paired analyses in each colonic biopsy sample and showed negative *FPR1* correlation with epithelial gene markers *EpCAM* and *CHD1*; suggesting that the epithelial *FPR1* gene is less dominant (Supplementary Figure 1a and b). Mitochondrial and bacterial formylated peptides also bind to formylated peptide receptors 2 and 3 (FPR2 and FPR3) where FPR1 shares high homology with. In

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contrast with FPR1, both FPR2 and FPR3, were not differentially expressed in inflamed vs. non-inflamed IBD mucosa (Supplementary Figure 1c and d). FPR2 receptor has a low affinity for formylated peptides and instead recognizes lipoxin A; and is more important in the resolution of inflammation[26]. The function of FPR3 is unclear, of interest FPR3 does not interact with formylated peptides or ligands for FPR1 or FPR2[27]. Using immunohistochemistry, we demonstrated that inflamed mucosa in both UC and CD have significantly higher FPR1+ve lamina propria immune cell infiltration compared with non-inflamed IBD gut ($p<0.0001$ and 0.0002 respectively) (Figures 1d and e). Immunofluorescence co-staining with elastase identified these immune cells as predominantly neutrophils (Figures 1d and f). In UC, transmigrating neutrophils across gut endothelial vessels and crypt abscesses (typically a collection of dead neutrophils in the gut lumen) are notably FPR1+ (Supplementary Figure 1c). FPR1-3 are expressed in many cell types but FPR1 expression is highest in neutrophils[16]. We accessed the publically available single-cell RNA sequencing data as published by Martin et al.[28] to determine the cell types that expressed *FPR1-3* in the gut epithelial, immune and stromal compartments out with the neutrophil population. Of interest, we found the highest expressions of *FPR1-3* in the macrophage population with no difference in inflamed and non-inflamed CD gut (data available under Gene Expression Omnibus accession number GSE134809 and in Supplementary Figure 1d).

**Formylated peptide receptor-1 expression is associated with multiple biologic treatment resistance in IBD**

We further analysed 6 independent IBD microarray gene expression Gene Expression Omnibus (GEO) datasets of the gut (data available under Gene Expression Omnibus accession numbers GSE59071, GSE206285 [UNIFI], GSE73661, GSE16879, GSE92415 and GSE23597 – comprising of 858 IBD and 85 non-IBD patients) (Table 1). In three datasets (except for GSE16879 and GSE92415 $p=0.056$ and 0.587 respectively, Figure 2d, e) with non-IBD groups for comparison, *FPR1* expression was significantly higher in IBD (Figure 2a-e). In agreement with our data, GSE59071 comprising of UC subjects showed higher *FPR1* expression in inflamed gut mucosa compared with non-inflamed UC mucosa ($p<0.001$, n=73 vs. 23 patients respectively) (Figure 2a). We further investigated if *FPR1* intestinal mucosal gene expression is associated with therapeutic response of biologic treatment in 6 IBD datasets namely – Ustekinumab (GSE206285 [UNIFI]), Infliximab (GSE16879 and GSE23597), Vedolizumab (GSE73661) and Golimumab (GSE92415) (all data available in the aforementioned GEO series

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accession numbers). There is a consistent pattern of higher *FPR1* gene expression in the non-responders with significant associations seen in Ustekinumab, Infliximab and Vedolizumab therapy in UC (all p<0.05; Figures 3a-d; Table 2). Taken together, we demonstrate consistently high *FPR1* in the inflamed IBD gut, mainly on neutrophils in the lamina propria of actively inflamed IBD mucosa with an association with poor response to several current biologic treatments that target different inflammatory mechanisms in IBD.

**Genetic deletion of *FPR1* reduces the severity of mouse experimental DSS colitis**

Constitutive gene deletion of *FPR1* in mice does not result in overt spontaneous clinical phenotype however, in systemic *Listeria monocytogenes* infection, *FPR1*-deficiency results in increased bacterial burden and mortality[29]. In sterile lung injury models, *FPR1*-deficiency resulted in lower levels of neutrophilic inflammation[30, 31]. We investigated the effects of experimental colitis induced by dextran-sulphate sodium (2% DSS) over 7 days in *FPR1*-deficient and wild-type C57/BL6 mice. Here, we found that *FPR1*-gene deletion is protective in DSS colitis, with lower weight loss, histological and clinical evidence of colitis; and neutrophil infiltration of the colonic mucosa (Figures 4a-f). This is of interest, given the importance of FPR1-mediated signalling in response to bacterial formylated peptides (fMLF), which is abundant in the colon and likely an important host defence against gut luminal bacteria. This line of data points towards a key role in FPR1-mediated inflammatory signalling in mouse DSS colitis and raises the potential to target this pathway in IBD.

**Mitochondrial ND6 activates peripheral blood human neutrophils via FPR1**

As we recently detected circulating blood mitochondrial ND6 in our IBD cohort, we synthesised mitochondrial ND6 and investigated its effects on human neutrophil activation and used the dose range as published by Rabiet et al[9]. Using peripheral blood leukocytes from healthy donors, CD45+ cells were selected and gated, followed by CD16+, a marker of functional and non-apoptotic neutrophils (Figure 5a) and CD11b+, a neutrophil migration marker (Figure 3B). Following a 2-hour stimulation, ND6 (10nM) increased CD11b+ expressing migratory neutrophils with similar effects seen with bacterial formylated peptide, fMLF (10nM) (Figure 5b). CD11b+CD16+ neutrophils were further gated for and assessed based on their CD62L and CD63 cell surface expression. Following ND6 stimulation, neutrophils shedded CD62L (p<0.001) and significantly increased their surface expression of CD63 (p<0.001), a marker of full neutrophil activation when combined with loss of CD62L (Figure 5c). Cyclosporin H (CsH), a potent FPR1 inhibitor, inhibited the effects of neutrophil activation. Prior to stimulation, human

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neutrophils were pre-treated for 10 minutes with 2.5μM CsH which significantly reduced ND6 and fMLF-induced CD11b+CD63+CD62L- neutrophil surface expression when compared to similar groups not treated with CsH ($p<0.001$) (Figure 5d). Both ND6 and fMLF increased the transmigration of neutrophils towards these respective stimuli that are again, blocked by CsH (Figure 5e). FPR1 engagement stimulates the effector function of neutrophils as evidenced by the production of extracellular reactive oxygen species (ROS). ND6 (10nM) stimulation of peripheral human neutrophils over 30 minutes resulted in a significant increase of ROS production with a similar magnitude seen in fMLF-treated neutrophils as a positive control (Figures 5f-h).

**Mitochondrial ND6 is present in stools in IBD but not in circulation**

We performed an initial screen for ND6 in blood plasma using an ELISA approach in our patient cohort with highly active disease (n=16, with extensive evidence of endoscopic moderate to severe colitis and/or stool calprotectin of >500μg/g and/or CRP >30mg/l) and 16 non-IBD controls. Of interest, ND6 was undetectable with standard curve dilution to the picogram range (data not shown). Given the higher likelihood of mitochondrial DAMP release from IBD gut mucosa into stools, we further investigated for presence of mitochondrial ND6 here. Our ELISA approach can detect measurable ND6 levels in stool supernatants extracted using a method optimized for stool calprotectin measurement. Calprotectin (s100a8/9) is a neutrophilic protein that is released during uncontrolled cell death and is a widely used biomarker for gut inflammation in the clinic. Here, the overall median stool ND6 was 2.2 ng/ml (IQR 0.0-4.99; range 0-53.3) (Table 3). We first tested if stool ND6 levels were associated with the clinical severity of IBD inflammation but found no difference between the groups with active vs. highly active disease (median 1.6 vs. 3.2 ng/ml, $p=0.51$) (Figure 6a). In addition, there was no statistical difference in stool ND6 levels between IBD patients with active disease and those in remission (median 2.2 vs. 4.8 ng/ml respectively; $p=0.78$) (Figure 6b). In each stool sample, we performed paired stool calprotectin s100a8/0 ELISA measurements. Here, stool calprotectin levels were statistically higher in the active IBD vs. remission groups (median 1163.0 vs. 160.6 μg/g; $p=0.0008$) and compared to non-IBD stools (median 1163.0 vs. 86.8 μg/g; $p=0.0007$) (Figure 6c). We further investigated whether subgroups of IBD patients with more active disease, using blood C-reactive protein measurement at a cut-level of 10mg/l. Here, IBD patients with CRP >10mg/l have higher stool ND6 but this was not statistically significant (median 5.9 vs. 9.6 ng/ml; $p=0.46$) (Figure 6d). There was no significant correlation between stool ND6 with blood C-reactive and calprotectin s100a8/0 (both $r=-0.09$, $p=0.5$). These lines of

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data suggest that although ND6 is present in stool supernatants, our ELISA data using methodology optimized for stool calprotectin, did not show an association with clinically active IBD states.

## Discussion

In our study, we presented several lines of evidence to support an important role of FPR1-mediated inflammation in IBD. We found that FPR1 is highly expressed in neutrophilic inflammation in the IBD gut mucosa. Importantly, high *FPR1* expression is associated with treatment resistance to several current IBD therapies with different mechanisms of action, namely anti-TNF (infliximab), anti-$\alpha 4\beta 7$ (vedolizumab) and anti-IL23p40 (Ustekinumab). This is further supported by recent studies that showed an upregulation of FPR1 in UC patients who do not achieve mucosal healing[32, 33].

Genetic deletion of *FPR1* was protective in acute DSS-colitis and *in vitro* blockade of FPR1-receptor against mitochondrial ND6 using Cyclosporin H reduced human peripheral blood neutrophil activation. Given the unique organ juxtaposition with gut bacteria where FPR1 can sense bacterial formylated peptides in this rich environment, it is of interest to investigate if loss of FPR1 will result in worse or better colitis outcomes. Here using experimental mouse DSS-colitis, we showed that genetic deletion of *FPR1* resulted in less inflammation and neutrophil recruitment in the gut. This is of key interest as this suggests that mtDAMP (vis-à-vis PAMP) may play a relatively more important functional role, at least in the acute murine colitis setting. Although our findings agree with recent studies showing a protective effect of *FPR1*-gene deletion in murine colitis[34, 35], the dominant mechanistic context of FPR1-mediated signalling in governing neutrophil trafficking and survival specifically in the gut and IBD have not been fully elucidated. Overall, it is noteworthy that *FPR1* gene deletion in other injury/inflammatory models of lung and brain is also protective and associated with lower inflammation[30, 31, 36]. *FPR1* knockout mice develop normally and do not display signs of spontaneous colitis; but they display an increased bacterial burden and mortality in models of systemic *Listeria monocytogenes* infection[29, 37]. A recent study shows that FPR1 may have an additional role in regulating host metabolism with an effect of gut microbiome and luminal fMLF activation of FPR1 – thus implicating a role in homeostasis[38].

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In the context of IBD, FPR1 has recently identified as a key driver gene in the inflammatory process associated with IBD in a functional genomic predictive network study[39]. Our combined human IBD and mouse *in vivo* data indicate that the FPR1-blockade could be particularly relevant in patients who do not respond to conventional advanced medical therapies and hence, offer a novel angle to address the current therapeutic ceiling in IBD. Collectively, they suggest that FPR1-blockade is a tractable approach in IBD and there is now a need to investigate this in more detail in human studies. Recent development of small molecules that may target FPR1[22-24], including the early phase clinical trial EudraCT Number: 2021-000035-31 [23].

In our previous study, we have shown that blood and stool mitochondrial DNA are elevated in active IBD[5]. Hence, we further investigated if mitochondrial FP, ND6 can serve as a mechanistic biomarker to identify patients with a potential dominant ND6 DAMP-mediated inflammatory endophenotype within IBD. Recently, Kwon et al. showed that high levels of blood ND6 in patients admitted with septic shock in the intensive care unit (ICU) were independently associated with increased infection and mortality[11]. A further human study in intracerebral haemorrhage showed that blood ND6 levels correlated to the severity of tissue damage[13]. Using a similar ELISA methodology, we could not detect circulating blood ND6 in the initial screening cohort of 16 patients with highly active IBD. In the significantly more unwell and compromised ICU patient cohort by Kwon et al, blood ND6 levels were measured at a range of 0.5-5 ng/ml, this magnitude difference is in stark contrast to our undetectable levels in blood. Notwithstanding, we found detectable levels of ND6 in stool supernatants in IBD with a range of 0-53.3 ng/ml which is much lower. There were no associations with disease activity and no correlation with the gut inflammation biomarker, calprotectin s100a8/9. Our initial data suggests that blood and stool ND6 measurements are not useful as potential biomarkers to stratify IBD patients.

There are limitations in our studies. Firstly, whilst it is pertinent that ND6 is not linked to IBD disease severity, our study is not geared towards the testing of the clinical utility of ND6 as a biomarker; and further testing in a much larger IBD cohort is required. This data although negative, provides a useful basis for further work to explore DAMP-based biomarkers with the potential to stratify patients in future FPR1 interventional drug studies. We present our data as a key comparator to the currently used ‘DAMP’ biomarker in IBD. Secondly, the role of circulating ND6 formed the focus of our investigation based on data from other studies. However, in IBD mitochondrial ND6 is likely to impart its effects in the local gut environment. Notwithstanding

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this, we do not know the degradation profile of mitochondrial ND6 which may contribute to the absent/low signal in blood using our ELISA approach. Thirdly, from a conceptual angle, many other ligands can activate neutrophils and we do not know the relative importance of all these factors compared to ND6. Finally, we have used CsH as an FPR1 antagonist. Although this is widely used, CsH has off-target effects[40, 41]. More specific pharmacologic agents or experiments from *FPR1*$^{-/-}$ mouse neutrophils can provide clearer data.

There remains a significant unmet need in improving the medical management of IBD. Despite more treatment options, there is a 'therapeutic ceiling' of 50% particularly in severe IBD. Recent attention has increasingly turned to exploring adjunctive therapeutic options that may augment current therapies in IBD. Targeting DAMP/PAMP-mediated inflammation in this context, specifically FPR1 neutrophilic-mediated inflammation has been explored in inflammatory diseases of the lungs, liver and brain[13, 30, 36, 42]. Recent studies have now established neutrophils as a major component in complex inflammatory gene modules that are associated with medical treatment failure in IBD[43, 44]. Our data suggest that FPR1 is an attractive drug target in IBD however; FPR1-inhibition strategies in clinical trial settings need careful appraisal. Such approaches are likely implemented in a time-defined window (at peak inflammation in acute severe IBD flare-up) and potentially as an adjunct to rescue therapy in conjunction with established IBD medical management.

## Methods

### Patients and Healthy Donors

For IHC work, colonic sections from 17 patients diagnosed with IBD (9 CD and 8 UC) from Western General Hospital, Edinburgh, UK were obtained via NHS Lothian Bioresource (South East Scotland Ethics Reference 16/ES/0084). Each patient had an age- and sex-matched non-IBD uninflamed section for comparison. Human peripheral blood samples were collected from healthy individuals in the Centre for Inflammation Research Blood Donor Register under the provision of Ethics Reference 21-EMREC-041. For blood and stool ND6 work, biological samples were obtained from IBD patients and non-IBD controls at the Western General Hospital, Edinburgh as part of the GI-DAMPs study (South East Scotland Ethics Reference 18/ES/0090). All clinical and NHS laboratory data were entered in a coded-anonymised fashion linked to the study patient ID in the Edinburgh Gut Research Unit RedCap Database (2020-present).

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## Gene expression analysis

Details of publically available IBD gene datasets accessed are shown in Table 1 and accessed via http://www.ncbi.nlm.nih.gov/geo/. The gene expression units are (log2) normalised gene expression. Four of the six, microarray datasets (data available under Gene Expression Omnibus accession numbers GSE59071, GSE73661, GSE92415, GSE206285) were already in Robust Microarray Analysis (RMA) normalised format (log2 normalised). GSE16879 and GSE23597 were log2 RMA normalised for our data analysis. For GSE11223 and GSE20881, gene expression was normalised to Agilent Stratagene Universal Human Reference. The difference in log2 fold change was calculated using linear models for microarray data (LIMMA) https://bioconductor.org/packages/release/bioc/html/limma.html. For single-cell RNA sequencing data analysis, we accessed raw sequencing reads of scRNA-seq samples as well as UMI tables are available on the Gene Expression Omnibus under GEO Series accession number GSE134809 which was previously published by Martin et al.[28]

## Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin embedded patient and mice sections. In brief, sections were dewaxed in xylene and rehydrated in graded alcohols. Heat-mediated antigen retrieval was achieved using either Citrate Buffer pH6 (2mM Sodium Citrate and 8mM Citric Acid) or Tris/EDTA Buffer pH 9 (1mM Ethylenediaminetetraacetic acid (EDTA) and 5mM Tris Base). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide and non-specific binding was blocked using 2% horse serum diluted in 1 x Tris Buffer Saline (TBS) (0.1M Tris/HCL, 1.5M NaCl). Ly6G (Sigma-Aldrich), neutrophil elastase (Novus biologicals) and FPR1 (Sigma-Aldrich) antibodies were incubated overnight at 4°C at 1:1000 and 1:500 respectively. A negative control was included with the absence of the primary antibody. Subsequently, sections were incubated with the secondary antibody ImmPRESS detection kit (Vector Laboratories, UK). Finally, the detection of the secondary antibody was achieved using 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako UK Ltd.), counterstained using haematoxylin and Scott's tap water, and dehydrated through a series of graded alcohols. Samples were then placed in xylene and mounted using a DPX mounting medium.

## Immunofluorescence

Dual immunofluorescence for FPR1 and neutrophils was performed on formalin-fixed paraffin embedded patient samples as follows; sections were dewaxed in xylene for 3 x 10 minutes and rehydrated through a series of graded alcohols. Heat-mediated antigen retrieval was performed

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using Tris/EDTA Buffer pH 9. Sections were blocked in 2% foetal calf serum (FCS) diluted in TBS and incubated overnight in neutrophil elastase antibody (Novus biologicals) and FPR1 antibody (Sigma-Aldrich) at 1:1000 and 1:500 respectively at 4$^{o}$C. A negative control was included with the absence of both primary antibodies. Alexa Fluor® 488 and Alexa Fluor® 647 secondary antibodies (ThermoFisher, UK) were combined at 1:500 in 1xTBS and incubated for 1 hour at room temperature. Sections were thoroughly washed and mounted using VECTASHIELD anti-fade mounting media with DAPI (Vector Laboratories, UK) and stored in the dark at 4$^{o}$C until analysed.

**Tissue imaging**

Brightfield images were obtained and visualised for analysis using the Carl Zeiss Zen 2 Blue edition programme (Zeiss, Germany). Immunohistochemistry for neutrophil infiltration and the presence of FPR1+ infiltrating cells were counted. Immunofluorescence staining was captured using the Zeiss LSM 780 Confocal, visualised using Carl Zeiss ZEN 2 blue edition software (Zeiss, Germany), and categorised based on absence and/ or presence of FPR1 and/ or neutrophil antibody. Tissue staining was analysed in three representative 0.6mm X 0.6mm areas within the lamina propria and scored by two independent observers.

**Neutrophil isolation**

Human peripheral blood was collected from healthy volunteers under local Ethics Approval Reference 21-EMREC-041. Blood was collected into 3.8% sodium citrate and centrifuged for 20 minutes at 350xg before discarding the plasma. Peripheral blood mononuclear cells and polymorphonuclear cells were isolated from red blood cells using 6% dextran sedimentation. A discontinuous (72.9, 63.0, and 49.5%) Percoll gradient was then used to separate the polymorphonuclear fraction from the PBMCs. Isolated cell fractions were then washed and resuspended in their appropriate culture media for further experimental analysis. A neutrophil preparation of >95% purity, as determined via cytospin centrifugation followed by Diff-Quik (Gentaur Molecular Products, Belgium) staining, was deemed acceptable for use within our study.

**Flow cytometry**

Isolated neutrophils were resuspended in PBS free from calcium and magnesium ions (PAA, UK) at 10 x $10^{6}$ /ml and stimulated for 2 hours with 100nM fMLF (Sigma-Aldrich, UK) or 100nM fMMYALF (GenScript) or pre-treated for 10 minutes with 2.5µM FPR1 antagonist cyclosporin H

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(Enzo Scientific, UK) before stimulation. Neutrophils were incubated for 1 hour at 4°C with antibodies to Brilliant Violet 421™ anti-human CD45 (Clone: HI30), APC/Fire™ 750 anti-human CD11b (activated) (Clone: CBRM1/5), Alexa Fluor® 488 anti-human CD16 (Clone: 3G8), PE anti-human CD62L (Clone: DREG-56), and APC anti-human CD63 (Clone: H5C6) (BioLegend, UK). Samples were washed and resuspended in 2% FCS and incubated briefly with DAPI (1:1000) before analysis using a BD Bioscience LSR Fortessa flow cytometer and FlowJo software (version 10.1).

**ROS production**

ROS production was determined using a lumino-based approach by measuring chemiluminescence. In brief, $12.5x10^6$ neutrophils/ml were pre-treated with/without 2.5µM cyclosporin H for 10 minutes at 37°C before being incubated for 10 minutes with luminol (150 µM) and HRP (18.75 U/ml) at 37°C in a 96 well round bottom plate. Neutrophils were transferred to a pre-coated (1% fat-free milk in PBS) 96-well chemiluminscence white plate with/ without 100nM fMLF or 100nM fMMYALF. Light emission production was recorded immediately using a plate reader and Synergy H1 plate reader (BioTek Instruments).

**Stool supernatant preparation**

Stored stool samples were defrosted and 2 supernatants were made from each sample. 1 was diluted 1:50 with extraction buffer using an Easy Extract device (Firefly Scientific, Worsley) and vortexed for 3 minutes. For the second supernatant (used for ND6 detection), this process was repeated but 1xPBS without Ca/Mg was used as the diluent. Supernatant samples were then stored at -80°C until use.

**Plasma preparation for ELISA**

Blood samples were obtained from patients as part of the GI-DAMPs study. Samples were taken in EDTA tubes and processed within 6 hours. Whole blood was centrifuged at 1000xg for 10 minutes and plasma was transferred to sterile tubes. Plasma was further centrifuged at 3000 x g for 10 minutes and divided into 1ml aliquots for storage at -80°C, until use.

**ELISA**

A human MT-ND6 ELISA kit (MyBioSource) was used for both plasma and stool supernatant ND6 quantification. This kit is not optimised for use with stool samples. Prepared plasma samples were defrosted and diluted 1:2 with sample diluent as per product instructions. Prepared stool supernatants were defrosted and diluted at 1:50 with sample diluent. A

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Calprotectin ELISA kit (CalproLab) was used for the quantification of calprotectin in stool supernatants. Supernatants were defrosted and diluted 1:100 with sample dilution buffer as per manufacturer instructions.

**Statistical analysis**

Analysis was performed using the Mann-Whitney test with two-tailed p-values in non-parametric continuous datasets. Wilcoxon signed-rank test. FlowJo (Tree Star, USA) was used to analyse flow cytometry data to assess the percentage of positively/ negatively labelled neutrophils and statistical analysis was performed using one-way ANOVA with Bonferroni correction and Dunnett's test. ROS assay readouts. All statistical analysis was performed using GraphPad Prism 9 (La Jolla, USA). For microarray data analysis of normalized gene expression was carried out and to correct for multiple testings, the false discovery rate (FDR) was calculated for p-values using GraphPad Prism 9.

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| GEO Dataset | Description | Year |
|---|---|---|
| GSE11223<br><br>GSE20881 | Transcriptional profiling of colon epithelial biopsies from ulcerative colitis patients and healthy control donors[25].<br>Colon biopsies from Crohn's patients and healthy controls[45] | 2008 |
| GSE16879 | Mucosal expression profiling in patients with inflammatory bowel disease before and after first infliximab treatment (anti-TNF)[46] | 2009 |
| GSE23597 | Expression data from colonic biopsy samples of infliximab treated UC patients (anti-TNF)[47] | 2011 |
| GSE59071 | Mucosal gene expression profiling in patients with inflammatory bowel disease[48] | 2015 |
| GSE73661 | The effect of vedolizumab (anti-α4β7-integrin) therapy on colonic mucosal gene expression in patients with ulcerative colitis (UC)[49] | 2016 |
| GSE92415 | Characterization of molecular response to Golimumab in Ulcerative Colitis by mucosal biopsy mRNA expression profiling: results from PURSUIT-SC induction study (anti-TNF)[50] | 2018 |
| GSE206285<br>UNIFI | Efficacy and safety of ustekinumab treatment in patients with ulcerative colitis[44] | 2022 |

**Table 1:** Summary of all IBD Gene Expression Omnibus (GEO) microarray/gene expression databases accessed for FPR1 gene analysis.

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| GSE_number | Inflammation Status | ΔLog2 FPR1 |
|---|---|---|
| GSE59071 | UC Inflamed vs UC Non-inflamed | 1.66 |
| GSE59071 | UC Inflamed vs Non-IBD | 1.67 |
| GSE206285 | UC vs Non-IBD | 0.28 |
| GSE73661 | UC vs non-IBD | 1.26 |
| GSE16879 | IBD vs non-IBD | 0.43 |
| GSE92415 | UC vs non-IBD | 0.07 |
| | **Drug Response** | |
| GSE206285 | Responder vs Non-responder | -0.16 |
| GSE73661 | Responder vs Non-responder | -1.40 |
| GSE16879 | Responder vs Non-responder | -0.64 |
| GSE92415 | Responder vs Non-responder | -0.07 |
| GSE23597 | Responder vs Non-responder | -0.40 |

**Table 2:** Difference in log2 FPR1 expression in IBD, data available under Gene Expression Omnibus (GEO) databases above accessed for FPR1 gene analysis.

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| | Active (n=45) | Remission (n=9) | Non-IBD (n=5) |
|---|---|---|---|
| UC<br>CD<br>IBD-U | 24<br>18<br>3 | 3<br>5<br>1 | |
| Age (years) | 37.6 (2.0) | 42.6 (5.3) | 48.0 (3.9) |
| Female/Male | 23/22 | 3/6 | 3/2 |
| C-Reactive Protein (mg/dl) | 31.7 (8.7) | 3.6 (0.9) | NA |
| Stool Calprotectin (µg/g) | 1255.0 (147.4) | 227.9 (97.2) | 115.4 (44.7) |
| No. of in-patients for active IBD treatment (%) | 35 (77%) | | |

**Table 3:** Characteristics of IBD patient cohort for stool ND analysis. Continuous data is presented as mean ± standard error of the mean (SEM).

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**Figures**

**Figure 1: (a)** Overall *in silico* analysis of *FPR1* in colonic pinch biopsies using Gene Expression Omnibus (GEO) GSE11223 and GSE20881 comparing IBD (n=207 colonic pinch biopsies; comprising of UC and CD [n=124 and 83 respectively]) vs. non-IBD controls (n=67) (p=0.0018). **(b)** *FPR1* gene expression in UC non-inflamed vs. inflamed pinch biopsies (n=57 and 67 respectively); and CD non-inflamed vs. inflamed pinched biopsies (n=41 and 42 respectively) (both p<0.0001) Mann-Whitney test. *FPR1* gene expression expressed as relative units to Stratagene Universal Human Reference Manual: Universal Human Reference RNA (chem-agilent.com). **(c)** Representative immunohistochemistry sections of inflamed and non-inflamed colonic sections of IBD (n=17 CD and 24 CD respectively), FPR1 is marked by horse-radish peroxide red (HRP) and neutrophils, elastase (DAB-stained). **(d)** Quantification of FPR1+ve cells in CD and UC – average count/mm2 of colonic section. Mann-Whitney statistics. **p=0.0002, ***p<0.0001. **(e)** Representative immunofluorescence of UC and CD colonic sections – DAPI (blue), FPR1 (green), Neutrophil elastase (red) and merged images (magenta).

**Figure 2**: **(a)** *FPR1* gene expression in inflamed and non-inflamed UC tissue from GEO dataset GSE59071. **(b-e)** *FPR1* gene expressions from GEO datasets GSE206285, GSE73661, GSE16879 and GSE92415 respectively. The gene expression units are (log2) normalised gene expression in Robust Microarray Analysis (RMA) normalised format. Statistics: Mann-Whitney test with False Discovery Rate (FDR) p-value correction.

**Figure 3: (a-e)** *FPR1* gene expression in responders and non-responders to **(a)** Ustekinumab GSE206285; **(b, c)** Infliximab GSE16879 and GSE23596 respectively; **(d)** Vedolizumab GSE73661 and **(e)** Golimumab GSE92415. Statistics: Mann-Whitney test and gene expression units are (log2) normalised gene expression in Robust Microarray Analysis (RMA) normalised format. Statistics: Mann-Whitney test with False Discovery Rate (FDR) p-value correction.

**Figure 4: (a)** Percentage of weight loss in *FPR1-/-* and wild type C57/BL6 in 2% dextran sulphate sodium (DSS) in drinking water *ad libitum*. **(b)** Representative H&E cross-section of distal colon in *FPR1-/-* and wild type following 7 days of DSS colitis. Bar is 500uM. **(c)** % of inflamed distal colonic mucosa (ulcerated and loss of colonic epithelium/preserved non-inflamed colonic epithelium with preserved crypt architecture) in *FPR1-/-* and wild type following 7 days of DSS colitis. **(d)** Colon length in *FPR1-/-* and wild type following 7 days of DSS colitis. **(e)** Quantification of LyG6+ve cells in the distal colon of *FPR1-/-* and wild type following 7 days of

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DSS colitis – average count/mm2 of colonic section. **(f)** Representative immunohistochemistry sections of in distal colonic lamina propria of *FPR1-/-* and wild type following 7 days of DSS colitis neutrophils Ly6G (DAB-stained).

**Figure 5: (a)** Neutrophils were isolated from healthy human peripheral blood and separated using density gradient centrifugation. Purified neutrophils were labelled for multicolour flow cytometry. Expression of DAPI (dead cells), CD45 (general leukocytes), CD16 (neutrophils), CD11b (activated neutrophils), CD62L (primed neutrophils), and CD63 (activated neutrophils) were analysed and a representative gating strategy was applied to identify activated neutrophils. **(b)** Quantification of CD11b+ neutrophils. **(c)** Quantification of CD63+/CD62L- neutrophils. **(d)** Percentage of activated CD11b+/CD62L-/CD63+ neutrophils in response to fMLF/ synthetic ND6 stimulation and/or cyclosporin H (CsH) treatment. **(e)** Number of migrated neutrophils in response to fMLF/ synthetic ND6 stimulation and/or CsH treatment. **(f)** Extracellular neutrophil ROS production (with HRP to detect extracellular ROS) in response to fMLF/ synthetic ND6 stimulation; **(g)** Extracellular neutrophil ROS production (with HRP to detect extracellular ROS) in response to fMLF/ synthetic ND6 stimulation with CsH treatment. **(h)** Extracellular neutrophil ROS production (with HRP to detect extracellular ROS) in response to fMLF/ synthetic ND6 stimulation and CsH treatment (Figure H is Figure F and G combined). Data are means +/- standard error (SEM) from n=3 experiments performed in triplicate. Two-tailed t-test, Mann-Whitney and one-way ANOVA with Bonferroni correction and Dunnet's tests were considered significant if $p<0.05$ with an asterisk (*) indicating $p<0.05$, double asterisks (**) indicating p<0.001 and triple asterisks (***) indicating ***p<0.0001.

**Figure 6: (a)** Stool ND6 ELISA in IBD patients with active vs. highly active disease (n=16 and 27 respectively). **(b)** Stool ND6 ELISA in IBD patients with active disease, in remission and non-IBD subjects (n=45, 9 and 5 respectively). **(c)** Stool calprotectin s100a8/9 in IBD patients with active disease, in remission and non-IBD subjects (n=45, 9 and 6 respectively). **(d)** Stool ND6 ELISA in IBD patients with active disease stratified according to C-reactive protein < or > 10mg/l (n=22 and 21 respectively). **(e)** Correlation analyses of paired stool ND6 and blood C-reactive protein levels; 42 paired measurements. **(f)** Correlation analyses of paired stool ND6 and calprotectin s100a8/9 levels; 53 paired measurements. Data presented as mean±SEM. Mann-Whitney statistical test between groups. Spearman correlation paired analyses. Significance level p<0.05. NS – not significant. **p=0.0008; ***p=0.0007

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Fig 1

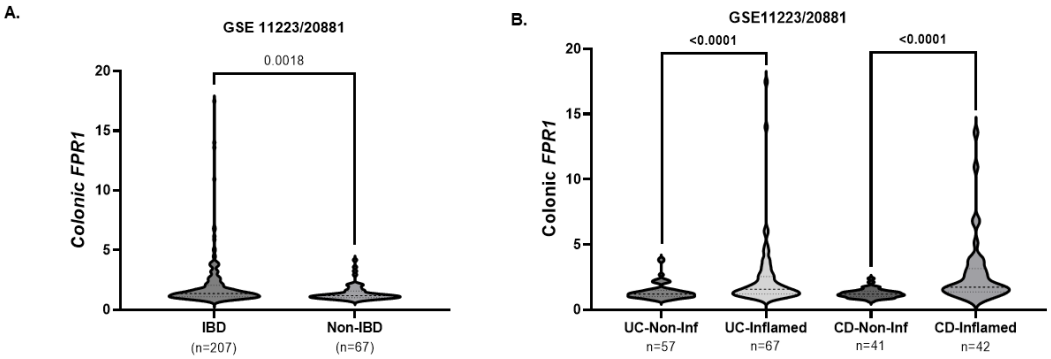


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Fig 1

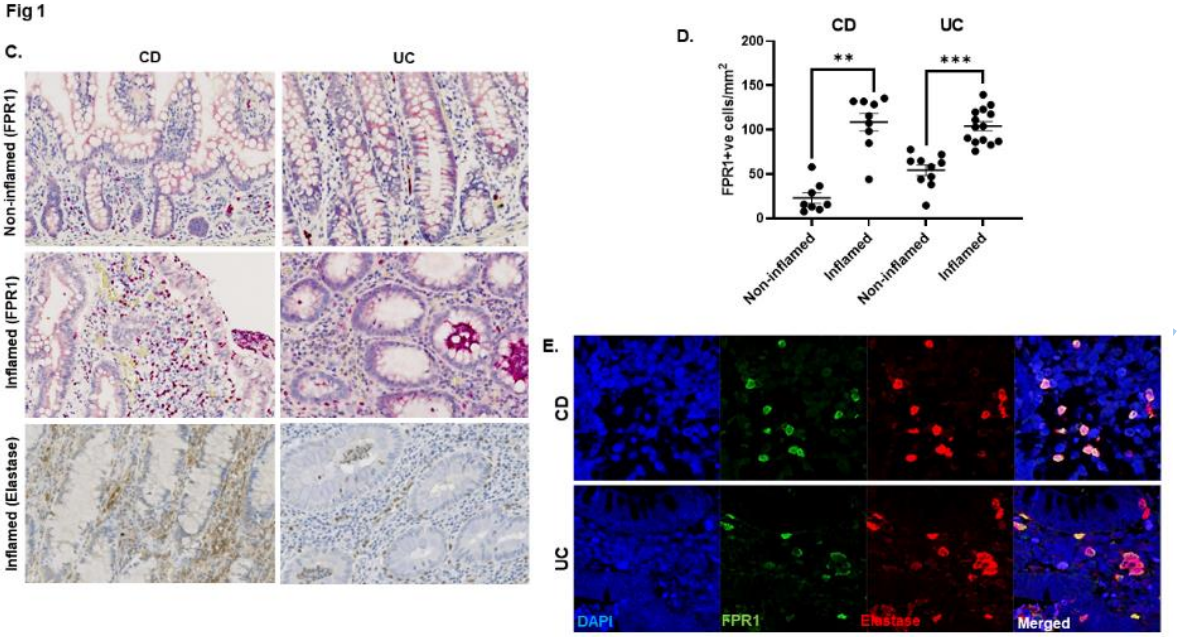


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Fig 2

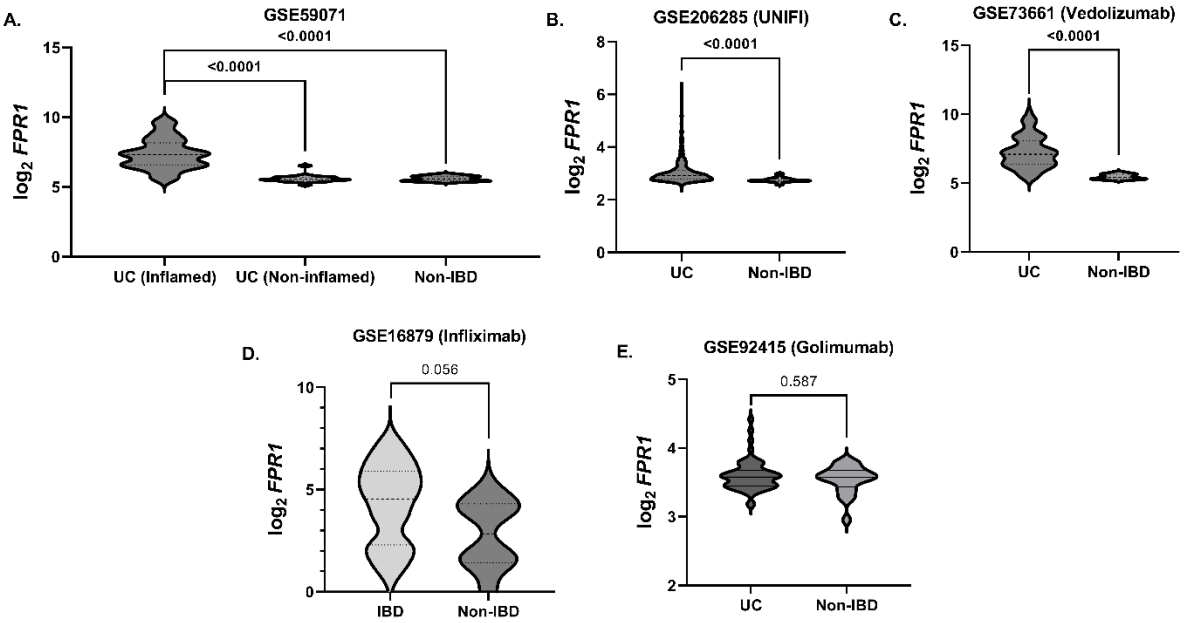


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Fig 3

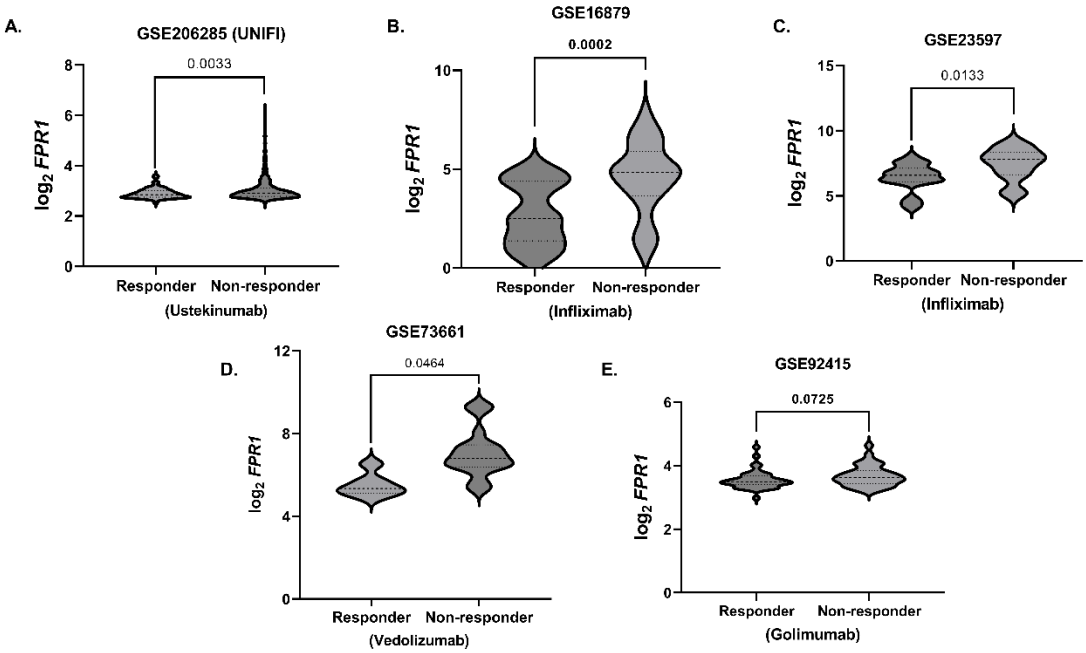


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Fig 4

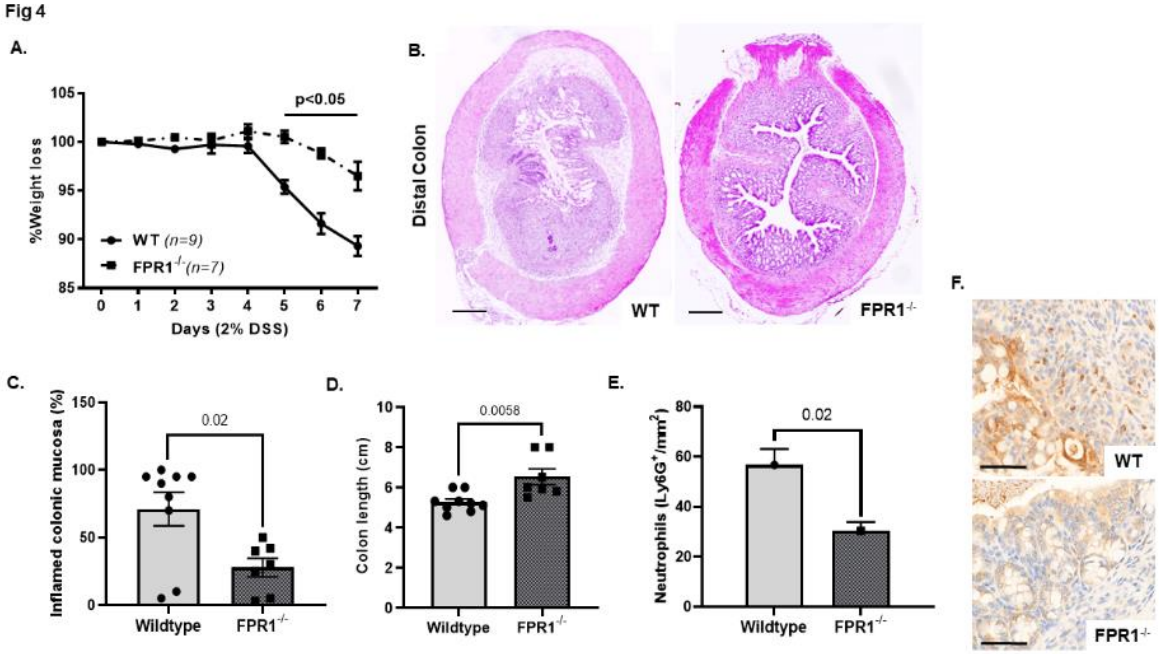


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Fig 5

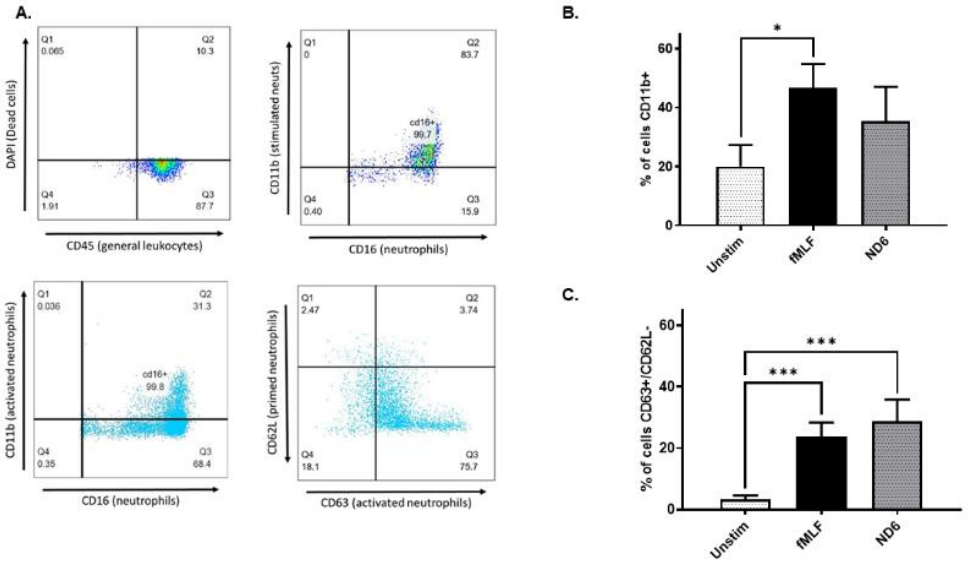


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Fig 5

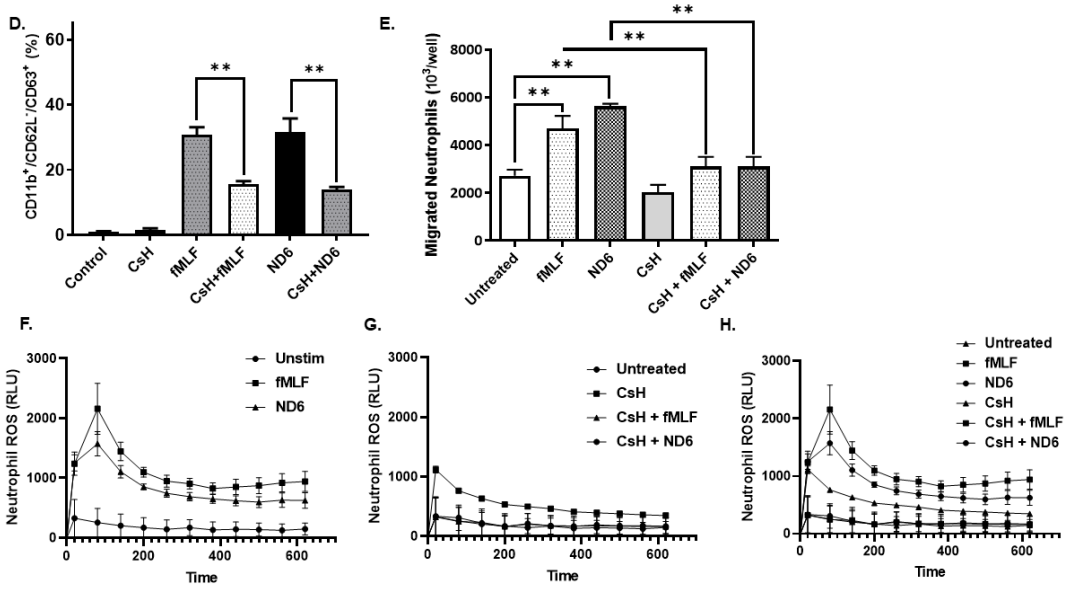


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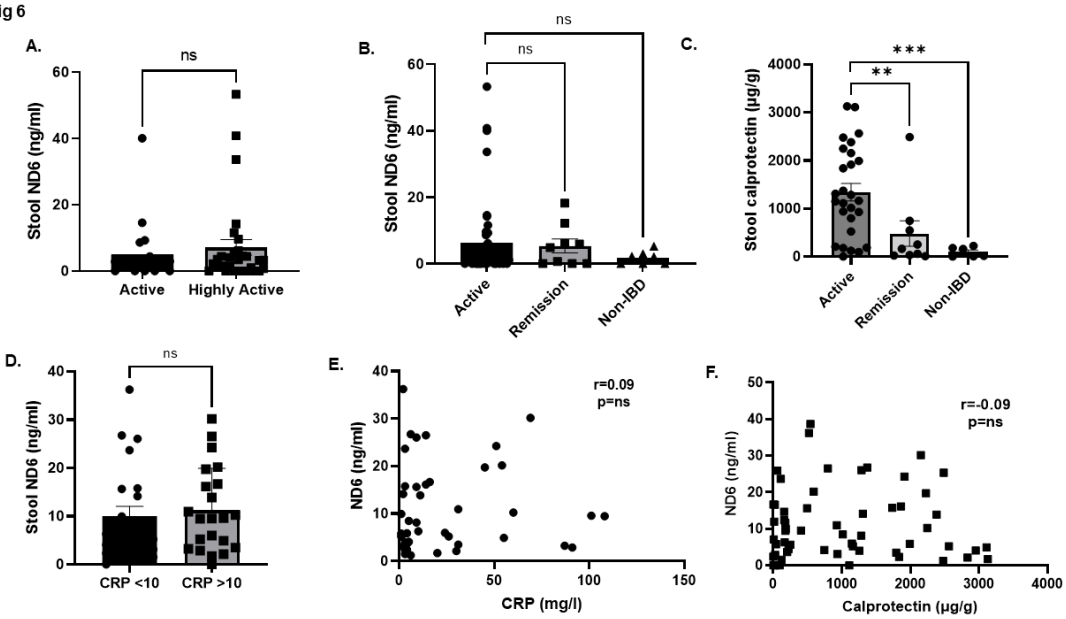
Fig 6

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