Mitochondrial DNA Is a Pro-Inflammatory Damage-Associated Molecular Pattern Released During Active IBD

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Mitochondrial DNA is a pro-inflammatory damage-associated molecular pattern (DAMP) released during active IBD

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Background: Due to common evolutionary origins, mitochondrial DNA (mtDNA) shares many similarities with immunogenic bacterial DNA. MtDNA is recognised as a pro-inflammatory damage-associated molecular pattern (DAMP) with a pathogenic role in several inflammatory diseases. We hypothesised that mtDNA is released during active disease serving as a key pro-inflammatory factor in IBD.

Methods: Between 2014-2015, we collected plasma separated within 2 hours of sampling from 97 prospectively recruited IBD patients (67 ulcerative colitis [UC] and 30 Crohn's disease [CD]), and 40 non-IBD controls. We measured circulating mtDNA using qPCR (amplifying mitochondria *COXIII/ND2* genes) and also in mouse colitis induced by dextran sulfate-sodium (DSS). We used mass spectometry approach to detect free plasma mitochondrial formylated peptides. Furthermore, we examined for mitochondrial damage using electron microscopy (EM) and TLR9 expression, the target for mtDNA respectively, in human intestinal IBD mucosa.

Results: Plasma mtDNA levels were increased in UC and CD (both $p<0.0001$) compared to non-IBD controls. These levels were significantly correlated to blood (CRP, albumin, white cell count), clinical and endoscopic markers of severity; and disease activity. In active UC, we identified 5 mitochondrial formylated peptides (the most abundant, fMMYALF with known chemoattractant function) in plasma. We observed mitochondrial damage in inflamed UC mucosa and significantly higher fecal MtDNA levels (vs. non-IBD controls [$p<0.0001$]), which support gut mucosal mitochondrial DAMP release as primary source. In parallel, plasma mtDNA levels increased during induction of acute DSS colitis and were associated with more severe colitis ($p<0.05$). In active IBD, TLR9+ lamina propria inflammatory cells were significantly higher in UC and CD compared to controls ($p<0.05$).

Conclusions: We present the first evidence to show that mtDNA is released during active IBD. MtDNA is a potential mechanistic biomarker and our data point to mtDNA-TLR9 as a therapeutic target in IBD.

Keywords: Mitochondrial DNA, DAMPs**,** TLR9.

INTRODUCTION

The most widely accepted hypothesis underpinning the aetiology of Crohn's disease (CD) and ulcerative colitis (UC) involves four pathogenic components: a dysregulated immune response, genetic susceptibility, abnormal microbial composition and environmental triggers[1]. There are clear differences between CD and UC. However, failure to resolve mucosal inflammation (which commonly reactivates upon withdrawal of anti-inflammatory treatments) is a notable shared clinical feature. In 1994, the "danger hypothesis" was proposed, in which immune responses are geared toward recognizing danger, irrespective of whether these signals arise endogenously or exogenously[2]**.** Endogenous damage-associated molecular patterns (DAMPs) are 'danger signals' or 'alarmins' released during host cellular stress or injury. Along with exogenous pattern associated molecular patterns (PAMPs) of microbial origins, DAMPs can initiate and perpetuate an inflammatory response typically via germline encoded pattern recognition receptors (PRR). We (and others) have recently suggested that DAMPs represent underexplored but potentially important pathogenic stimuli that maintain the state of abnormal mucosal inflammation in inflammatory bowel disease (IBD)[3].

We recently showed that gut mitochondrial dysfunction can result in loss of epithelial barrier function and the development of colitis[4]. In this study, we focus on mitochondria as a source of DAMPs in IBD. Mitochondria are intracellular double-membrane-bound organelles with many essential physiological roles such as energy production, regulation of cell death and immune responses[5]. Mitochondria are evolutionarily derived from energy-producing alpha-bacteria, engulfed by archezoan cells approximately 2 billion years ago leading to a symbiotic relationship that forms the basis of the eukaryotic cells[6]. As such, mitochondria share several features with their bacterial ancestors, notably with a double-membrane structure and an independently replicating genome rich in hypomethylated CpG motifs, very similar to bacterial DNA. Just as the innate immune system recognizes conserved bacterial molecules, mitochondrial constituents are similarly immunogenic, acting as DAMPs during their uncontrolled release[7].

The mitochondrial DAMP which has thus far attracted the most attention is mitochondrial DNA (mtDNA). Collins *et al*, first reported the inflammatory potential of mtDNA in 2004, when they found that mtDNA (and not nuclear DNA) induced TNFα and caused inflammatory arthritis when injected into the joints of the mice[8]. MtDNA activates multiple pathways, notably toll-like receptor (TLR)-9 when it is released extracellularly, as well as the cytosolic NLRP3-inflammasome and STING pathways[7]. It is proposed that extracellular mtDNA mainly (but not exclusively) mediates its pro-inflammatory action via TLR9 which then proceeds through

MyD88 triggering MAPK- and NFκβ-signalling[9], or through interferon regulatory factor 7 (IRF7) to enhance type 1 interferon response in dendritic (DC) or other immune cells. Uncontrolled mtDNA release is evident during conditions associated with acute tissue injury such as systemic inflammatory response syndrome (SIRS), fulminant liver failure and sepsis[9-11]; and in chronic inflammatory states such as systemic lupus erythematosus (SLE)[12]. Given the significant tissue injury burden typically observed in active IBD, we hypothesised that such pathogenic release is present and that mtDNA can act as a pro-inflammatory DAMP potentiating and perpetuating the abnormal inflammatory response.

MATERIAL AND METHODS

IBD and control cohorts

Individuals were recruited from outpatient and inpatient settings from the Gastrointestinal Unit, Western General Hospital, Edinburgh between April 2014 and November 2015. For the IBD cohort, recruited patients fulfilled the Lennard-Jones criteria of CD or UC[13]. IBD patients were classified into CD or UC based on clinical, endoscopic and histologic criteria. In addition, individuals with irritable bowel syndrome (IBS) or with no history of IBD and no GI symptoms were recruited as non-IBD controls. IBS individuals had altered bowel habit and were defined following normal ileo-colonoscopy, stool calprotectin and blood parameters. Individuals were excluded if they were younger than 18 or were unable to give written consent. Individuals with indeterminate colitis and non-IBD colitis (e.g. infective and microscopic) were excluded.

Biological material from subsets of prospectively recruited patients were used for:

1. Faecal mtDNA analysis (n=12 active UC vs. 12 non-IBD controls)
2. Mass spectrometry analysis (n=5 acute severe UC vs. 5 non-IBD controls)
3. Electron microscopy (n=6 active UC vs. 6 non-IBD controls)
4. UC patients with longitudinal plasma sampling pre- and post-colectomy (n= 9).

All clinical and biological material/data acquisition were carried out under Lothian Bioresource ethics approval 15/ES/0094.

Clinical phenotype: Disease activity

For UC and CD, disease activity was classified using the Simple Colitis Clinical Activity Index (SCCAI) and Harvey Bradshaw Index (HBI) respectively. Clinical remission was defined as SCCAI of less than or equal to 2 for ambulatory ulcerative colitis patients, and HBI of less than 5 for ambulatory patients with Crohn's disease. "Ambulatory" patients were defined as outpatients. Inpatients were further classified into acute severe disease if they required intravenous steroids for CD or fulfilling the Truelove & Witts criteria for UC. Endoscopic

assessment of disease severity was obtained from endoscopic reports generated by GI physicians in charged of patients at time of sample collection. Further clinical (stool frequency, temperature and pulse) and disease severity characteristics including labarotory assessments (FBC, C-reactive protein and serum albumin) were collected at the time of recruitment and blood sampling.

Human plasma processing

Venepuncture was performed by a trained clinician with a 21 gauge butterfly needle. 12-18mL of blood including at least 9mL in an ethylenediaminetetraacetic acid tube (Vacuette ®) was collected and processed within 2 hours. EDTA blood was centrifuged at 1000g for 10 minutes at 4°C and the plasma fraction was transferred to a 15mL Falcon ® tube; this was then centrifuged at 5000g for 10 minutes at 4°C to remove platelets and microparticals, and achieve 'cell free plasma'. Plasma was divided into 0.5mL aliquots and stored at -80°C until further use.

Extraction of mtDNA from plasma

DNA was isolated using the QIAamp DNA Blood Mini Kit as per manufacturer's instructions[14]. 200µl of plasma was used for each sample. Purity of the DNA was determined using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and all DNA samples had OD260/OD280 values of 1.7-2.0.

Stool processing

DNA was isolated using the QIAamp DNA Stool Mini Kit as per manufacturer's instructions. To minimise cellular disruption (and thus minimise processing-related liberation of DAMPs), phosphate buffered saline (PBS) was used instead of Buffer ASL in step 2 of the protocol.

qPCR protocols

Creation of Standard Curves

Standard curves for absolute quantification of circulating free mtDNA were developed using a modified protocol of that described previously[14]. Mitochondria were extracted from cultured HepG2 cells using the Sigma Mitochondrial Isolation kit for cultured cells using the manufacturer's instructions. Isolated mitochondria were stored at -20°C until further use. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, Valencia CA, USA) as per the manufacturer's instructions. Primer sequences (cytochrome C oxidase subunit III (*COXIII*): forward ATGACCCACCAATCACATGC, reverse ATCACATGGCTAGGCCGGAG; NADH-dehydrogenase 2 (*ND2*): forward CACAGAAGCTGCCATCAAGTA; reverse CCGGAGAGTATATTGTTGAAGAG) were blasted against human genome as well as known

bacteria in order to ensure selectivity for human mtDNA (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

mtDNA primer products were amplified by conventional PCR. PCR conditions were Stage 1 - 95ºC for 2 minutes; Stage 2 - 40 cycles of 95ºC for 30 seconds, 58ºC for 30 seconds; Stage 3 - 72ºC for 5 minutes. The PCR product was then run on a 4% agarose gel (2g of agarose resuspended in 50ml 1x TBE with 5µl Gel Red added to the solution once the agarose dissolved). 10µl of PCR product used and samples run next to a 100bp DNA ladder. PCR product size was as expected (103bp for *COXIII*, 90bp for ND2). A QIAgen PCR Purification kit was used in accordance with manufacturer's instructions. The DNA was then eluted with 50µl Buffer EB into a sterile 1.5ml Eppendorf and stored at -20°C until use. Isolated DNA was quantified by nanodrop (ThermoScientific). Standards were created from serial 10 fold dilutions of the PCR primer product.

Absolute Quantification

Primers (MWG Eurofins) were suspended at 100µM stock solution with DEPC-treated water and stored at -20°C prior to use. Subsequently, 20x primer solution (1.8µM) was made (3.6µL forward, 3.6µL reverse, 192.8µL DEPC-H_2O). In MicroAmp® Optical 384-Well Reaction Plates (Applied Biosytems) 7µl of master mix containing 5µL 2x SYBR Green Fast mix (Applied Biosystems), 0.5µL 20x primer mix and 1.5µl DEPC-treated water was mixed with 3µl of isolated DNA sample or standard. All reactions were carried out in duplicate, and discordant results retested. All plates contained wells with no DNA as a negative, no template control. qPCR reactions were conducted in an ABI7900 Fast Real-Time PCR System (Applied Biosystems) with the following settings: Stage 1 - 95°C for 20 sec; Stage 2 - 40 cycles of 95°C for 3 sec & 60°C for 30 sec; Melt curve: 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 15 sec. Absolute quantification of mtDNA was determined relative to the standard curve based on the following equation (as described by Chiu et al [14]):

$C = Q \times V_{DNA}/Q_{PCR} \times 1/V_{ext}$

- C target concentration in plasma or serum (copies per millilitre);
- Q target quantity (copies) determined by sequence detector in PCR;
- V_{DNA} total volume of DNA obtained after extraction, typically 50µl per extraction;
- V_{PCR} volume of DNA solution used for PCR, typically 3µl
- V_{ext} volume of plasma, typically 200µl

Amplification efficiency of between 90 and 110% was taken as acceptable where slope refers to the gradient of the standard curve: (Efficiency = $10^{(-1/slope)}$ – 1). The coefficient of determination value was also calculated with r^2>0.985. Meltcurves were run to identify the presence of a primer dimer peak.

Mass Spectrometry

Plasma samples (100µL) were acetone precipitated, dried down under vacuum and reconstituted in 0.5% acetic acid. Peptides were then analysed by LC-MS/MS in positive ion mode using a Thermo LTQ-Orbitrap XL mass spectrometer (Hemel Hempstead, UK) coupled to a Waters nanoAcquity UPLC system (Manchester, UK) with a linear gradient over 39 min (mobile phase A: 0.5% acetic acid in water; mobile phase B: 0.5% acetic acid in acetonitrile). N-formylated hexapeptides were identified on the basis of their accurate mass, retention times and characteristic fragmentation patterns compared to custom synthesised standards (Peptide Protein Research Ltd, Fareham, UK). Quantification was achieved using a corresponding stable isotope labelled internal standard and calibration curve for each N-formylated hexapeptide.

Mouse experiments and induction of colitis

C57/BL6 wild-type were used in experiments carried out under Home Office Project Licence PPL 70/8847 (Dr G-T.Ho). Acute colitis was induced by 2% DSS (MP Biomedicals Ltd) in drinking water *ad libitum* for 7 days. Mice were monitored daily for weights, presence of diarrhoea and blood. Euthanasia was performed using CO_2 and blood collection was carried using direct cardiac puncture (~500ul) into EDTA eppendorfs. Like human plasma processing, EDTA blood was centrifuged at 1000g for 10 minutes at 4°C and the plasma fraction was transferred further centrifuged at 5000g for 10 minutes at 4°C to remove platelets and microparticles.

Transmission Electron Microscopy

Colonic pinch biopsies from IBD and non-IBD individuals were obtained from distal colon during colonoscopy, briefly washed with sterile PBS and immediately transferred into 3% EM grade glutaraldehyde solution in 0.1M Sodium Cacodylate buffer, pH 7.3, for 2 hours before further processing (details available on request). For mouse studies, colons were flushed with PBS before transfer into EM solution as above. All TEMs were carried out at Electron Microscopy Unit, King's Building, University of Edinburgh.

Immunohistochemistry

Immunohistochemistry for anti-TLR9 (1 in 50 following Tri-EDTA antigen retrieval; Abcam ab52967) were performed on pseudo-anonymised human IBD and non-IBD colonic resection samples provided by pathology department (Dr J-L) using the Scottish Tissue Bank via Scottish Academic Health Sciences Collaboration (SAHSC) SR493. All IBD samples were coded and matched (sex, age and tissue location) with a non-IBD control group.

Statistical analysis

Data are presented as numbers, percentages, means±SEM and medians±IQR for parametric and non-parametric data respectively. Student t- and Mann-Whitney statistics were used for parametric and non-parametric data respectively. Receiver operating characteristic (ROC) analyses were carried out using paramaters: mtDNA, C-reactive protein and albumin levels to predict need for colectomy. Multivariate logistic regression were performed to assess variables predictive of high mtDNA. Wilcoxon matched-pairs signed rank test was used to determine the difference in matched pre- and post-colectomy mtDNA levels. Spearman's correlations were calculated to evaluate the relationship between mtDNA level and other biochemistry, and between *COXIII* and *ND2* qPCR results. Statistical analyses were performed using Graphpad version 7 (Graphpad Software, San Diego, California, USA) and SPSS version 22 (IBM Corp., Chicago, USA). Two-sided p values of <0.05 were considered statistically significant.

RESULTS

Increased circulating plasma mtDNA in UC and CD

We prospectively recruited and collected plasma from 97 IBD patients (67 UC and 30 CD patients), and 40 non-IBD controls (20 healthy [HC] and 20 irritable bowel syndrome [IBS] controls) (**Table 1**). In all our samples, we performed qPCR using 2 sets of primers flanking *COXIII* and *ND2* genes of the mitochondrial genome. *COXIII* and *ND2* data were highly correlated (r=0.84; p<0.0001)(**Supplementary Figure 1**) and *COXIII* data indicative of mtDNA release is presented from hereon. Overall, we found significantly higher levels of circulating cell-free plasma mtDNA in IBD (167.8 copies/µl [interquartile range, IQR 78.06-387.2]) compared to HC (64.6 copies/µl [IQR 51.6-104]) (p=0.0002) and IBS (44.6 copies/µl [IQR 27.9-134.7]) (p<0.0001). There was no difference between HC and IBS, and these groups were combined as non-IBD controls (**Figure 1A**). Plasma mtDNA levels were significantly higher in both UC (172.3 copies/µl [IQR 74.4-393.2]) (p<0.0001) and CD (136.7 copies/µl [IQR 88.0-370.9]) (p<0.0001) compared to non-IBD controls (61.5 copies/µl [IQR 32.8-104]) (**Figure 1B**).

In UC, individuals with acute severe disease as defined by Truelove and Witts criteria and severe endoscopic appearances, have the highest mtDNA levels (**Figure 2A and B**): clinical remission (53.77 copies/µl [IQR 30.56 – 86.8] vs. severe UC (234.7 copies/µl [115.3 – 723.4]) ($p<0.0001$); and endoscopically mild (33.11 copies/µl [IQR 28.71 – 44.9] vs. severe UC (255.4 copies/µl [96.71 – 641.4]) ($p<0.001$). In the smaller cohort of CD, higher mtDNA levels were observed in those with severely active CD (159.1 copies/µl [IQR 90.17-421]) compared to those in remission(79.92 copies/µl [IQR 30.94 – 145.9] ($p=0.04$) (**Figure 2C**). In acute severe UC who were hospitalised, mtDNA levels on admission in patients who went on to require subsequent emergency colectomy were higher than those who responded to medical therapy (colectomy: 302.5 copies/µl [IQR 139 – 1553] vs no colectomy group 165 copies/µl [66.75 – 253]; $p=0.04$). Comparative ROC analysis of mtDNA, C-reactive protein and albumin demonstrated an area under curve (AUC) of 0.71, 0.76 and 0.82 in predicting colectomy ($p=0.04$, 0.01 and 0.002) respectively (**Supplementary Figure 2**). In CD, only one individual had surgery during recruitment, hence similar analyses were not performed. In our overall IBD cohort, we found that mtDNA levels were significantly correlated with severe disease markers C-reactive protein ($r=0.33$, $p<0.0001$), albumin ($r=-0.32$, $p<0.0001$), and white cell count ($r=0.37$, $p<0.0001$) (**Supplementary Figure 3**). We identified a group of individuals with very high mtDNA levels (n=18, >600 copies/µl) and investigated if there were unique phenotypic characteristics that defined the group. Multivariate analysis showed that C-reactive protein was independently associated with very high mtDNA levels ($p=0.007$, **Supplementary Table 1**). Collectively, our data show that circulating plasma mtDNA can be detected and are significantly increased in UC and CD; and mtDNA levels were associated with disease activity and severity.

Mitochondrial *N*-formylated peptides are also increased in active IBD

A further shared feature of the mitochondria with bacteria, is the production of short *N*-formylated peptides. Bacterial and mitochondrial proteins are the only source of *N*-formylated peptides in nature. Mitochondrial *N*-formylated peptides are functional similar to their bacterial counterparts acting as primarily as neutrophil chemoattractants[15]. To provide further corroborative evidence, we employed a mass spectrometric approach to identify and quantify *N*-mitochondrial formylated peptides in a subset of 5 acute severe UC vs. 5 non-IBD controls. A screen for the free *N*-terminal hexapeptides of the thirteen known mitochondrial encoded proteins confirmed the presence of five *N*-formylated termini (fMMYALF, fMTPMRK, fMNPLAQ, fMNFALI and fMTMHTT) in acute severe UC samples. These were not detected in non-IBD controls. When quantified with synthentic standards, we found that the concentrations of each of these formylated peptides was significantly elevated ($p<0.01$) in acute severe UC (**Figure 3**). Of the mitochondrial *N*-formylated peptides, fMMYALF was the

most abundant. This is highly relevant as fMMYALF is the most biological active mitochondrial *N*-formylated peptide[16].

Increased plasma mitochondrial DNA release in acute DSS colitis

In order to provide further supportive data, we used the acute dextran sulfate sodium (DSS) colitis mouse model to test if plasma mtDNA can be detected as colitis developed. Similar to human IBD, we found significantly higher plasma mtDNA following acute DSS colitis (**Figure 4A**) and high levels of mtDNA were associated with more severe colitis (>10% vs. <10% weight loss at day 7 of DSS colitis; p=0.03) (**Figure 4B**). These findings in an acute colitis model supported our recent findings that also demonstrated increased plasma mtDNA in the chronic *mdr1a*-deficient colitis model[4]. Hence, these data indicate that gut inflammation is associated with the release of mtDNA during active colitis.

Intestinal mucosal mitochondrial damage and mtDNA release in active IBD

We recently showed that loss of mitochondrial protective mechanism at the intestinal mucosal level rendered the mitochondria susceptible to damage, and triggered the onset of colitis in *multidrug resistant-1* (*mdr1*) deficient mice[4]. Other relevant IBD mice models with primary autophagy (*Irgm* and *Atg16l1*)[17,18]; including those with secondary autophagy impairments due to defective ER-stress[19] and NLRP6 inflammasome activity[20] all exhibited similar accumulations of damaged mitochondria within the gut epithelium as seen in *mdr1*-deficient mice. The findings of abnormal mitochondria have been demonstrated in human IBD[21-23]. We confirmed these when we prospectively sampled pinch biopsies from affected colon in individuals with active UC and from non-IBD controls (**Supplementary Table 2**). Transmission EM of the colon showed evidence of mitochondrial damage (with loss of inner cristae structure, increased lucency with swollen rounded appearances) in areas of cellular injury and were also extravasated within sub-epithelium in affected UC mucosa (n=6/group; active UC vs. non-IBD). (**Figure 5A**) (**Supplementary Figure 4A and B**). The nature of EM did not allow more detailed discrimination of whether specific enterocyte or inflammatory cell types displayed a predilection towards mitochondrial damage. We hypothesised that mitochondrial DAMPs are released primarily from affected IBD mucosa. We prospectively sampled faecal samples from individuals with active severe UC and found significantly higher mtDNA levels (p<0.0001) compared to non-IBD controls (**Figure 5B**). Overall, faecal mtDNA were higher than plasma levels (~1000-fold) although this maybe explained by inherent nature of the different biological material. In a longitudinal series of acute severe UC with paired samples pre- and post-colectomy (median Δ107 days, IQR 89-189), plasma mtDNA fell to normal following colectomy (n=9, p=0.008) (**Figure 5C).** Many lines of evidence suggest that mtDNA's DAMP effect is

mediated via TLR9, which is expressed in many immune cells including dendritic cells, B-cells as well as the intestinal epithelium[24]. We analyzed TLR9 protein expression in human IBD colonic resection specimens and found increased frequency of lamina propria TLR9+ve cells in active UC and CD (**Figure 6A, B**). Collectively, these data suggest that extravasation of mtDNA primarily occurs at the inflamed mucosa level in active IBD where TLR9 expression, its primary cellular target is increased. Presently, the latter is associative and more work is necessary to confirm the downstream effects of mtDNA on gut mucosal immune response. Taken together, indicate that mtDNA-TLR9 signalling is an attractive therapeutic target and mtDNA itself, may have a role as a mechanistic biomarker.

DISCUSSION

We present data to show for the first time that significantly increased levels of mtDNA are found in active human IBD and in mouse colitis. Furthermore, plasma mtDNA levels correlate with disease activity and severity. We corroborated our findings with a second known DAMP, by demonstrating the presence of *N*-formylated peptides arising from the mitochondria in the plasma. In this context, we posit that increased mitochondrial damage occur at the inflamed IBD mucosa (supported by human and mouse studies); they are released in a pathogenic manner (both quantitatively and qualitatively) where they can be detected in the circulation and in the stools. Given that many lines of evidence implicate their functional pro-inflammatory actions, these findings have direct translational importance.

MtDNA's role as a TLR9 agonist has received most attention where many inflammatory models show better outcomes when TLR9-signalling is abolished. *Tlr9*–deletion is protective against SIRS following systemic administration of mitochondrial DAMPs[25]; and in lung[26], liver[27] and kidney[28] injury models characterised by high mtDNA release. Blocking TLR9 using inhibitory ligands have been shown to improve mtDNA-driven mouse models of cardiac failure[29] and non-alcoholic steatohepatitis (NASH)[30]. The role of mtDNA-TLR9 in IBD and intestinal inflammation however, is more complex. TLR9 is expressed both in the epithelium and, in resident and recruited lamina propria immune cells. Whereas NFKB-activation is pro-inflammatory[31], intestinal epithelial NFKB-activation is cytoprotective and important to maintain barrier integrity[32]. Hence in *tlr9*-deficiency, the intestinal epithelium is postulated to have lower protective NFKB-activation and is more susceptible to injury. CpG oligonucleotide treatment in mouse studies using low dose DSS colitis showed either no difference or a protective role with the beneficial effects seen before the induction of colitis [33-35]. Furthermore, CpG oligonucleotide treatment given during colitis worsened inflammation[36]. A recent clinical study on TLR9-agonist in moderate-to-severe UC failed to show overall clinical improvement[37].

Hence, translating anti-TLR9 therapy in IBD is likely dependent on the stage of inflammation and the cellular context of where TLR9 is blocked.

Our data show a promising future role for mtDNA (and DAMPs) as a mechanistic biomarker. We recently highlighted the importance of such biomarkers to identify sub-mechanisms that drive the heteregenous clinical presentations and disease progression in IBD, where specific therapeutic interventions can be stratified accordingly. Clearly, more detailed studies are necessary to fully test mtDNA's utility as a biomarker. There are limitations to our current data. First, we have not studied mtDNA in non-IBD intestinal inflammatory conditions such as infectious colitis or diverticulitis. It is conceivable that high mtDNA release is also present. Second, our cohort was predominantly individuals with active UC (data for mitochondrial formylated peptides, EM and faecal mtDNA were drawn from this group). It is not clear if similar findings are found in CD. It would be of interest, to study a wider cohort of gut inflammatory conditions to determine whether mtDNA data is more specific for IBD or if it is a general marker for gut inflammation (like calprotectin).

The are many translational opportunities offered by targeting mitochondrial DAMPs. These includes inhibiting mtDNA release (e.g. diverting the manner of cellular death or active extrusion of mitochondrial DNA using pro-apoptotic caspases), reducing the inflammatory potential of mtDNA (e.g. mitochondrial anti-oxidant treatment or DNAases to disgest NET-bound mtDNA), augmenting damaged mitochondrial clearance mechanisms (e.g. mitophagy activation), interfering with mtDNA-TLR9 activation (using inhibitory CpG ligands) and targeting downstream NFKB- and MAPK-pathways in the relevant inflammatory cell groups (e.g. neutrophils) (reviewed in[38]). Similarly, much is known about the biological effects of *N*-formylated peptides and its cognate receptors (FPR1, 2 and 3). Activation of FPR1 drives neutrophil chemotaxis and stimulates a variety of antimicrobial responses, including degranulation, reactive oxygen species production and cytokine release. We recently show that both FPR1 gene-deletion and pharmacologic inhibition are protective in inflammatory lung disease[6].[39,40]. There are other DAMPs such as calprotectin (s100a8/9), high mobility group box-1 (HMGB1) and interleukin (IL)-33 (reviewed in[3]) with known pro-inflammatory mechanisms found in active IBD. The relative importance of these DAMPs is not yet clarified but as it stands, offers a rich ream of further translational opportunities.

In conclusion, our study suggests that mtDNA acts as a DAMP promoting inflammation in IBD. More broadly, our findings open up a new mechanistic layer and further expands the current model of IBD pathogenesis to incorporate the 'danger' model. Hence DAMPs as 'enemies

within', represents a potential major player in addition to established data implicating genetic susceptibility, and exogenous microbial and environmental factors in the pathogenesis of IBD.

Contribution of authors:

Study concept and design: GTH, RKB, DAD; Acquisition of data: RKB, RK, AT, NTV, MKD, PDW; Analysis and interpretation of data: RKB, DAD, GTH; Drafting of the manuscript: RKB, DAD, AGR, JS, GTH; Critical revision of the manuscript for important intellectual content: MG, JL, AGR, JS, GTH. Technical and Material support: MG, JL, MKD and PDW.

Declaration of Conflict Interests:

No authors have any conflicts of interests to declare.

Figure 1A: Plasma mtDNA (copy/µl) in IBD, HC and IBS (n= 97, 20 and 20 samples respectively; p=<0.001); **B:** Plasma mtDNA (copy/µl) in CD, UC and non-IBD (n= 30, 67 and 40 samples respectively). Median±Interquartile Range (IQR).

Figure 2A: Plasma mtDNA (copy/µl) in UC in clinical remission (ambulatory), active (ambulatory) and severe active (hospitalized) (n= 13, 18 and 44 samples respectively; 8 UC individuals had samples taken more than 1 time point during active disease and in remission); **B**: Categorised according to mild, moderate and severe endoscopic appearances (n= 4, 41 and 23 samples espectively). **C:** Plasma mtDNA(copy/µl) in CD, clinical remission (ambulatory), active (ambulatory) and severe disease (hospitalized) (n= 10, 5 and 16 samples respectively. One CD individual had samples taken more than 1 time point during active disease and in remission).

Figure 3: Mitochondrial formylated peptide quantification in 5 UC vs. 5 non-IBD controls (*p=<0.01 for fMMYALF, fMTPMRK, fMNPLAQ, fMNFALI and fMTMHTT).Mean±Standard Error of Mean

Figure 4A: Plasma mtDNA (copies/ml) following acute DSS colitis vs. controls (n= 12 and 14, p=0.01)†. **B:** Plasma mtDNA (copies/ml) stratified to < or >10% weight loss at Day 7 of DSS colitis (p=0.03). Median±Interquartile Range (IQR)

Figure 5A: Representative transmission electron microscopy of distal colonic epithelium from active UC vs. non-IBD controls (n= 6/group; bar = 5µm). Annotated image: Purple – damage mitochondria (DM), Blue – healthy mitochondria (HM) and yellow – lipid droplets (LD). Black scale bar 2µm. Yellow insert – Damaged and healthy mitochondria from UC and controls respectively (Orange bar 0.5 µm); **B:** Faecal mtDNA (copy/ml) in active UC and non-IBD controls (n=12/group). **C:** Longitudinal analysis of plasma mtDNA(copy/µl) in UC (n=9 patients) during active disease and the same patient post colectomy in clinical remission. All data represent median ± IQR.

Figure 6A: Immunohistochemistry for anti-TLR9 in human IBD colon (UC and CD; n=7/group vs. non-IBD control; n=14). Red arrows – TLR9 positive cells. Black scale bar 100 µm. **B:** TLR9+ve cell counts in lamina propria (LP) of human IBD colon per $2mm^2$ (UC and CD; n=7/group vs. non-IBD control; n=14)‡. Mean±Standard Error of Mean.

Table 1: Baseline characteristics of IBD and controls. IBD – Inflammatory bowel disease, CD – Crohn's disease, UC – Ulcerative colitis, HC – Healthy controls and IBS – Irritable Bowel Syndrome. Data presented as median (±IQR).

	IBD		Controls	
	CD	UC	HC	IBS
n	30	67	20	20
Age	37 (27-44)	36 (28-51)	36 (32-46)	33 (27-42)
M / F	17 / 13	44 / 23	10 / 10	13 / 7
Current smoker	26%	21%	15%	25%
Crohn's Disease				
	Clinical Remission & Ambulatory	Clinically Active & Ambulatory	Hospitalized (IV Steroids)	
Hb (g/dl)	145 (137-151)	156 (138-158)	130 (125-137)	
WCC ($x10^9$/L)	7.4 (5.4-15.1)	10.9 (7.7-11.9)	8.75 (7.4-11.6)	
Platelets ($x10^9$/L)	253 (218-295)	509 (300-414)	334 (279-378)	
C-reactive protein	3.5 (1-7.5)	5 (3.5-8)	26 (12-62)	
Albumin	38 (33-40)	38 (37-38)	29 (27-34)	
HBI	1 (0-2)	7 (6-9)	7 (4-14)	
Ulcerative Colitis				
	Clinical Remission & Ambulatory	Clinically Active & Ambulatory	Hospitalized (IV Steroids)	
Hb (g/dl)	136 (128-151)	131 (123-146)	114 (104-130)	
WCC ($x10^9$/L)	6.3 (4.7-7.5)	7.7 (6.3-8.8)	11.3 (8.3-14.6)	
Platelets ($x10^9$/L)	292 (252-303)	305 (256-335)	414 (288-501)	
C-reactive protein	2 (2-5)	3 (2-17)	21 (10-54)	
Albumin	39 (38-40)	38 (35-40)	30 (25-34)	
SCCAI	0 (0-0)	6 (4-8)	7 (4-10)	

Supplementary Table 1 – Multivariate logistic regression to analyse parameters associated with high mtDNA levels in 160 samples with complete paired data with mtDNA levels (mtDNA >600 vs. ≤600 copies/μL).

	p-value	95% CI for Exp (B)
Hb	0.621	0.973-1.046
WCC	0.083	0.984-1.310
Platelets	0.343	0.998-1.007
CRP	**0.007***	1.084-1.672
Albumin	0.861	0.360-2.346
Age	0.630	0.974-1.045
Smoking status	0.918	0.123-10.311
Sex	0.645	0.416-4.126

Supplementary Table 2 – Clinical characteristics of individuals for TEM studies of distal colon

Case	Age / Sex	Clinical Description
UC 1	23 yrs, Female	Active proctitis (UC), Mayo 2
UC 2	44 yrs; Male	Active left sided UC, Mayo 1
UC 3	41 yrs, Female	Active pan-UC, Mayo 2
UC 4	20 yrs, Male	Active left sided UC, Mayo 2
UC 5	33 yrs, Male	Active severe UC, to point of insertion (descending), Mayo 3
UC 6	49 yrs, Female	Active proctitis (UC), Mayo 1
HC 1	56 yrs; Male	Investigated for abdominal pain and weight loss; normal
HC 2	45 yrs; Female	Investigated for iron deficiency anaemia; internal haemorrhoids only
HC 3	62 yrs, Male	Investigated for PR bleeding; sigmoid diverticular disease found otherwise normal
HC 4	55 yrs, Male	Investigated for altered bowel habit and family history bowel cancer; normal
HC 5	39 yrs, Female	Investigated for iron deficiency anaemia; normal
HC 6	71 yrs, Male	Surveillence scope post polypectomy 2 years ago; left sided diverticular disease otherwise normal

Supplementary Figure 1: Correlation between plasma mtDNA (copy/µl) for all samples using 2 different primers *COXIII* and *ND2* (n=200, r=0.84, p=<0.0001).

Supplementary Figure 2: Receiver operating characteristic (ROC) analysis of mtDNA, C-reactive protein and albumin in acute severe UC in-patients and emergency colectomy.

Supplementary Figure 3: Spearman correlation between paired mtDNA plasma (copy/µl) and C-reactive protein, serum albumin and white cell count in IBD (n= 180 samples; all p<0.0001 with r values of 0.33, -0.32 and 0.37 respectively). CRP – C-reactive protein, WCC – white cell count. Median±Interquartile Range (IQR).

Supplementary Figure 4: Representative colonic EM images of 6 UC vs. 6 non-IBD individuals. Panel A – UC; Panel B – Non-IBD controls. Black bar - 2µm. Red arrows denote damaged mitochondria.

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