Altered Expression of Angiotensinogen and Mediators of Angiogenesis in Ileal Crohn’s Disease

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INTRODUCTION

The renin-angiotensin-aldosterone-system (RAAS) is of interest in human Crohn’s disease (CD) for several reasons. Angiotensin II (AII) is a pro-inflammatory, pro-fibrotic cytokine in numerous human diseases [1]. In addition, it has a particularly important role in the mesenteric vasculature controlling local blood supply via potent arteriolar and pre-capillary sphincter contraction [2]. Inhibition of the effects of AII has beneficial effects on endothelial function [3]. Endothelial dysfunction is implicated in the perpetuation of the chronic human inflammatory bowel disease (IBD and its systemic complications, including arterial thromboembolic diseases) [4]. Loss of endothelial-generated nitric oxide (NO) impairs vasorelaxation, contributes to enhanced leukocyte adhesion in the inflammatory milieu [5] and increases oxidative stress [6]. Resulting local ischemia contributes to angiogenesis, a hallmark of chronic inflammatory disorders.
with the expansion of an abnormal dysfunctional vascular bed further contributing to the influx of inflammatory cells [7, 8]. Most evidence suggests that AII inhibition is anti-angiogenic in nature and therefore has therapeutic potential in CD [9].

Better known as a potent arteriolar vasoconstrictor, AII also effects a multifunctional role as a local cytokine. It is chemotactic for inflammatory cells, increases vascular permeability and increases expression of a variety of proinflammatory molecules [10-14]. AII is profibrotic with an important role in the accumulation of extracellular matrix (ECM), inducing time and dose dependent increases in transforming growth factor-β (TGF-β) mRNA and activation [15]. TGF-β1 is a powerful fibrogenic cytokine whose expression is increased in active Crohn’s colitis [16]. Angiotensin Converting Enzyme (ACE) inhibition is therapeutic in animal models of colitis [17]. Administration of the ACE inhibitor captopril lowered TGF-β1 over-expression in the trinitrobenzene sulfonic acid (TNBS)-induced colitis model [17].

Chronic inflammatory disorders are associated with angiogenesis, resulting from a combination of tissue hypoxia and inflammation [18, 19]. Hypoxia inducible factor (HIF)α regulates the tissue response to hypoxia including the upregulation of genes involved with epithelial barrier function. Intestinal mucosa in IBD becomes hypoxic, particularly the epithelium, via increased metabolic activity of resident and infiltrating cells, as well as reduced perfusion through a local vasculopathy [20]. Angiogenic growth factors including vascular endothelial growth factor (VEGF) are potently stimulated by hypoxia, mediated via HIF1α [21]. Angiogenesis may perpetuate disease by increasing recruitment of inflammatory cells and dysfunctional new vessel architecture [22]. Hence, inhibiting angiogenesis is a therapeutic strategy of interest in chronic inflammatory disorders including IBD. AII receptor blockade can suppress inflammatory corneal neovascularisation or angiogenesis in C57 BJ6 mice [9]. Hence, AII receptor blockers (ARBs) and ACE inhibitors have potential anti-angiogenic activity in IBD.

AII is a potent vasoconstrictor of the splanchnic circulation and can therefore reduce mesenteric perfusion [23]. Animal models of circulatory shock of variable aetiology have shown that AII receptor blockade can suppress inflammatory neovascularisation or angiogenesis in C57 BJ6 mice [9]. Hence, AII receptor blockers (ARBs) and ACE inhibitors have potential anti-angiogenic activity in IBD.

In this study we hypothesise that the mucosal expression of AGT, HIF1α, and MCAM will be increased in view of the inflammatory and fibrotic nature of ileal CD. HIF1α expression may also be increased in CD due to the hypoxic nature of inflammatory tissue. Mucosal expression was investigated using quantitative real-time polymerase chain reaction (RT-PCR) in human ileal CD and in human ileal controls. AGT expression was also examined using immunohistochemistry.

**MATERIALS AND METHODS**

**Patient samples**

Seventy-nine subjects (59 CD, 20 healthy controls) were recruited for the study. The recruited cases were a consecutive series of 52 CD patients undergoing colonoscopic assessment of disease activity or for disease surveillance. In addition, 12 resection specimens from 7 CD patients undergoing surgery were included. The 20 healthy controls underwent colonoscopy screening because of a family history of colorectal neoplasia. Colonoscopy was performed at the Royal Brisbane and Women’s Hospital, a major metropolitan teaching hospital. The diagnosis of CD was based on standard criteria [27]. Informed consent was obtained from all patients and the RBWH Human Research Ethics Committee approved the study protocol.

**Biopsy samples**

Colonoscopic specimens were mucosal biopsy samples obtained from the terminal ileum. Where possible, five pinch biopsy samples were retrieved for RNA and DNA extraction from both affected (inflamed, cases) ileum and unaffected ileum (non-inflamed, cases and controls). These samples were immediately snap frozen in dry ice following withdrawal of the biopsy forceps from the colonoscope, then stored at −80°C until use. Additional samples from the same area were retrieved and placed in formalin for histological examination and characterisation of inflammation by a gastrointestinal pathologist.

**Resection samples**

Resection specimens were included to represent CD patients with more severe, complicated disease. Samples comprised the mucosal layer only, carefully dissected from the submucosa by an experienced pathologist prior to snap-freezing in liquid nitrogen. The depth of dissection was confirmed histologically by a gastrointestinal pathologist.

**Grading inflammation**

The presence or absence of disease involvement in the mucosa being biopsied was determined by the colonoscopist at the procedure. This initial assessment was confirmed at histology, and inflammation further graded as non-inflamed, mild, moderate and severe inflammation, based upon a semi-quantitative scale, similar to that used in the grading of ulcerative colitis [28]. The samples were previously assessed by quantitative RT-PCR for expression of interleukin-8 (IL-8; NM_000584), a major inflammatory mediator, to validate the accuracy of the histological assessment of inflammation [29]. In CD, focal expression of IL-8 is related to the extent of histological inflammation and mucosal destruction [30].

**RNA purification and cDNA preparation**

A total of 20 samples from 20 healthy controls and 81 samples from 59 CD patients were subjected to RNA and DNA isolation. Total RNA was extracted and its integrity assessed as described with a minimum RIN score requirement of 5.0 [29]. First-strand cDNA synthesis was performed using Superscript III First-strand cDNA Synthesis Supermix for quantitative RT-PCR (Invitrogen). Each reaction contained 500 ng total RNA as determined by the Bioanalyser (Agilent Technologies, USA). Total volume of the reaction was 20 uL subjected to 25°C for 10 min, 42°C for 50 min and 85°C for 5 min. The cDNA samples were then diluted 1:20 for use in the RT-PCR reactions.

**Quantitative Real-Time PCR**

Primers for angiotensinogen (AGT; Accession number NM_000029), HIF1α (Accession number NM_001530) and
MCAM (Accession number NM_006500), were designed using PrimerQuest (Integrated DNA Technologies, Iowa, USA; http://www.idtdna.com/Scitools/Applications/Primerquest) (Table I). The primer concentrations were optimised as previously described [29]. Quantitative RT-PCR amplification was performed using platinum SYBR Green RT-PCR Supermix UDG (invitrogen) in a total volume of 15 uL containing 4 uL cDNA (10 ng), 2x RT-PCR Supermix, 300 nmol/L each primer (except MCAM at 900 nmol/L) and H2O. Reactions were amplified using Rotor-Gene 6000 (Corbett Life Science) and quantified using the manufacturer’s software. The thermocycling conditions comprised 3 min at 95°C and then 40 cycles at 95°C for 15 s, 50°C for 15 s, and 72°C for 20 s, followed by standard melt curve analysis to validate the specificity of the PCR products. All samples were amplified in triplicate from the same RNA preparation to validate the accuracy of the method. A calibrator sample [BD RT-PCR Human Reference Total RNA (BD Biosciences, Clontech, Palo Alto, CA, USA)] was included in each run, as well as 3 no-template controls to detect PCR contamination. Quantitative RT-PCR efficiencies were determined for each gene and relative quantification of target mRNA was carried out using the Pfaffl method [31]. Six referencing genes were previously evaluated for normalising quantitative RT-PCR data from ileal CD samples [29, 32]. B2-microglobulin (B2M; Accession No. NM_004048), the most stably expressed gene, was selected for this study.

Genotyping of the common disease-associated NOD2 variants (R702W, G908R and 100FS) was performed by high resolution melt analysis on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) as published [33]. The angiotensinogen-6 (AGT-6) polymorphism was genotyped for CD cases, using PCR-restriction fragment polymorphism (RFLP), as previously described [34]. ATG16L1 genotyping for the T300A variant was performed for CD cases as previously described [35].

### Statistical analysis

Sixty-nine tissue (biopsy) samples from 52 CD patients were analysed in comparison with 20 tissue samples from 20 controls (Table II). Gene expression from patients that contributed more than one tissue sample was not statistically different from those patients contributing only one sample. As such the patient sample was considered the statistical unit. All statistical analyses, except where genotype was assessed in comparison with expression, were conducted using the 69 CD and 20 control samples. Expression differences were assessed using the non-parametric Mann Whitney-U test. To further investigate the expression data, we conducted analyses stratified by NOD2 and inflammation status. Genotype associations were analysed via the Chi square test; Fisher’s exact p-values were used where necessary. Two tailed p-values of <0.05/5 (Bonferroni correction) were considered statistically significant, except for the Chi square associations, where p<0.05 was considered statistically significant. All statistical analyses were conducted using the R statistical software package (R Development Core Team, R: A Language and Environment for Statistical Computing, Vienna, Austria).

### RESULTS

#### Patient phenotype

Crohn’s disease phenotype was according to the Montreal Classification [36] (Table II). Of the 59 CD patients recruited, 37 (62.7%) were female compared to 13 (65%) controls. The mean age of CD patients was 33.1 years (SD 12.3 years). The control subjects, undergoing colorectal cancer surveillance were slightly older (52.7 years, SD 17.7 years). Thirty-five

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**Table I. Primer sequences and product sizes for real-time polymerase chain reaction**

<table>
<thead>
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<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
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<td>CGGATGGAATGGAACCCGACAGCAT</td>
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<td>AAGGCTTACCTCTCTCCTGGAG</td>
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</tr>
<tr>
<td>TGFB1</td>
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<td>CTCGGTGAGCTGAAAGCAATAGTT</td>
<td>114</td>
</tr>
<tr>
<td>C16F</td>
<td>GCACAGAAGGCGCCTATGTCATCT</td>
<td>ACTGATC136GAGCTGCTGCT</td>
<td>143</td>
</tr>
<tr>
<td>HIF1a</td>
<td>ATGAGAGGATTTGACACAGG</td>
<td>GATGGTGGAGAATGGGGGTCACAA</td>
<td>129</td>
</tr>
<tr>
<td>MCAM</td>
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<tr>
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<td>GGTGTGAAAAGCTTGGAGATGTTCCT</td>
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</tr>
</tbody>
</table>

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**Immunohistochemistry**

Immunohistochemistry was performed using ATG antibodies (ab97380, 1:100; Abcam, Cambridge, USA) for use in paraffin-embedded human tissue. The staining protocol was optimised using known positive controls (human kidney and liver) and the human colon. A variety of antigen retrieval protocols were trialled with the optimal being citrate, 30 seconds at 100 degrees. Five micron thick sections of control ileal resection samples and ileal resection samples from a CD patient in inflamed, non-inflamed and strictured ileum were deparaffinised and hydrated through a graded series of alcohol. Following antigen retrieval, the addition of endogenous peroxidase activity by immersion into 3% H2O2/methanol solution was performed. The sections were then incubated with the primary antibodies followed by washing in phosphate buffered solution (PBS), incubation with biotinylated secondary antibody and then with avidin-biotinylated horse radish peroxidase complex and finely developed using DAB (3, 3’- Diaminobenzidine Tetrachloride) as the chromagen. The stained sections were examined by an experienced gastrointestinal pathologist (I.B.).
patients had isolated ileal disease (L1) and 19 ileocolonic disease (L3). The patients demonstrated a mixture of disease behaviour, 15 non-stricturing, non-penetrating (B1), 17 stricturing (B2) and 21 penetrating (B3).

Expression of IL-8 confirms the presence and degree of inflammation

**Biopsy samples**

The normalised expression of IL-8 was higher in ileal CD biopsy samples compared to controls (p=0.015) (Fig. 1). Expression was also higher in inflamed CD biopsies compared to non-inflamed CD samples (p <0.0001).

**Resection samples**

Mucosal expression of IL-8 was higher in ileal resection specimens than in both control samples (p=0.0003) and CD biopsy samples (p<0.0001).

**Inflammation grade**

According to histological grading, IL-8 expression was significantly increased in moderately inflamed CD biopsy samples compared with mildly inflamed CD biopsies (p=0.0097). IL-8 expression was also higher in severely inflamed samples compared to mild and moderately inflamed samples (p=0.0014) (Fig. 1).

**Ileal expression of AGT and HIF1α is reduced in CD biopsy samples**

Normalised ileal expression of AGT was significantly lower in CD cases compared to controls (p<0.0001) (Fig. 2, B). mRNA expression of AGT and HIF1α was lower in CD biopsy samples when compared to control biopsy samples (p<0.0001 and p=0.026, respectively) (Fig. 2 B, C).

**Resection samples**

Ileal expression of HIF1α and MCAM was higher in CD resection samples compared to CD biopsy samples (all p<0.0001), and controls (p=0.0008, p<0.0001, respectively) (Fig. 2 C, D).

**Inflamed vs non-inflamed CD cases**

**Biopsy samples**

AGT expression was reduced in inflamed CD biopsy samples compared to non-inflamed samples (p=0.017) (Fig. 3, A). In contrast, the expression of HIF1α was increased in inflamed CD biopsy samples compared to non-inflamed samples (p=0.009) (Fig. 3, B). Expression of HIF1α was lower in non-inflamed CD biopsy samples compared to controls (p=0.006) (Fig. 3, B).
NOD2 Genotype

Biopsy samples

The presence of a disease-associated NOD2 variant was associated with increased expression of HIF1α (p=0.009) in inflamed CD compared to non-inflamed CD biopsy samples (Fig. 3, B). Patients’ wild type for NOD2 did not demonstrate these relationships. The expression of HIF1α (p=0.35) was similar in non-inflamed and inflamed biopsy samples in wild type patients. The expression of MCAM (p=0.007) (Fig. 3, C) was increased in inflamed samples in the presence of disease-associated NOD2 variants. The presence of NOD2 variants did not impact upon AGT expression (p=0.92) (Fig. 3, A).

ATG16L1 Genotype

Resection samples

MCAM expression was higher in CD resection samples in patients homozygous for ATG16L1 T300A (p=0.0182) (Fig. 4).

AGT Genotype

The presence of one or more AGT-6 variant alleles resulted in a non-significant increase in AGT expression (p=0.07).

Cigarette smoking

Current smoking resulted in lower AGT expression than in never and ex-smokers combined (p=0.027) (Fig. 5). There was no difference in AGT expression between never and ex-smokers (p=0.39).

Patient phenotype and gene expression

No significant differences were found in the expression of IL-8, AGT, HIF1α nor MCAM according to sex, age and disease location. No relationship was demonstrated between Montreal disease behaviour and gene expression.

Immunohistochemistry supports AGT mRNA quantitative RT-PCR expression results

The highest concentration of AGT in the ileum appeared to be in the blood vessel walls. A small amount of expression was seen in the vessel endothelium and mesenchymal cells of the submucosa and lamina propria. Staining was stronger in the control ileal samples compared to inflamed and non-inflamed CD ileum (Figs. 6-10).

DISCUSSION

Demonstrating reduced AGT expression in ileal CD was unexpected given that CD is a chronic inflammatory disease associated with fibrosis, and that AGT is the precursor molecule to AII, a pro-inflammatory, pro-fibrotic oligopeptide. There was a further reduction in AGT expression in inflamed CD.
biopsy samples. The mechanism for this down regulation of AGT expression in ileal CD is therefore speculative but may implicate a vascular contribution to disease pathogenesis. The immunohistochemistry findings support this hypothesis as the highest concentration of AGT was seen in blood vessel walls.

Arteriolar and precapillary sphincter smooth muscle regulate total blood flow to the gut and its capillary blood flow. Arteriolar tone accounts for over half the resistance to blood flow in the gut with small changes in diameter equating to large changes in blood flow through the superior mesenteric artery [2]. The splanchnic vasculature contains...
high concentrations of AII receptors. AII is a powerful splanchnic vasoconstrictor ensuring diversion of blood flow to vital organs in periods of hypotension and shock [25, 37]. The mesenteric circulation receives approximately 20-30% of the cardiac output in normal conditions, most distributed to the small intestine. In response to feeding, there is a further striking increase in blood flow, increase in oxygen uptake and consumption, primarily in the mucosa [38]. In shock, selective, disproportionate mesenteric vasoconstriction is mediated via AII, at the level of the mesenteric vascular bed. This response is due to the hypersensitivity of the mesenteric resistance vessels to AII mediated by the extremely high affinity of the AII receptors in the mesenteric circulation, which in rat mesenteric arterial vessels is 13-55 times that of receptors in the aorta [39]. Numerous models of systemic hypotension and shock demonstrate a therapeutic benefit from ACE inhibitors and ARBs which ameliorate the associated disproportionate intestinal vasoconstriction, subsequent hypoperfusion, increased permeability and bacterial translocation associated with this clinical scenario [23, 25, 26]. These unique circulatory conditions with respect to AII in the intestine, and in the terminal ileum itself, are likely contributors to its importance and that of ischemia in ileal CD. Ileal CD ulcers tend to occur along the mesenteric margin of the bowel. Short mural blood vessels supply the mesenteric margin of the terminal ileum and these have almost no communication with the larger long vessels supplying the anti-mesenteric margin [40]. This renders the mesenteric ileal margin particularly susceptible to ischemic ulceration and supports an ischemic contribution to this characteristic CD lesion [40]. Hence the vasoactive properties of AII may predominate in the terminal ileum compared to other major organs.

These factors may also contribute to the particularly deleterious effect of cigarette smoking in ileal CD compared to colonic disease [41]. In Sprague-Dawley rats, cigarette smoking produces a dose-related vasoconstrictive effect on the mesenteric blood flow [42]. In addition, smoking cessation at diagnosis reduces the risk of complicated CD and any active smoking is deleterious [43]. Our results support these findings with current smokers demonstrating reduced expression of AGT compared to never and ex-smokers. This suggests that active cigarette smokers are at increased risk of mucosal hypoxia due to enhanced mesenteric vasoconstriction.

Early studies observed similarities in the progressive submucosal fibrosis associated with ulcers and strictures due to CD, and that due to ischemia [44]. Recently, it was demonstrated that the highly secretory Paneth cells at the base of small intestinal crypts are the most susceptible epithelial cells to ischemia-reperfusion injury which results in both unfolded protein response (UPR) activation and Paneth cell apoptosis in human small intestine. This study provides a novel link between ischemia and the pathogenesis of CD through Paneth cell dysfunction, in particular ischemia and ileal CD given the ileal location of these highly specialised cells [45].

Hence this combination of factors including the anatomy of the ileal vasculature, the mesenteric vasculature's particular sensitivity to AII, the high concentration of Paneth cells in the terminal ileum, and the particular sensitivity of Paneth cells to ischemia, implicates ischemia in the pathogenesis of ileal CD. It also questions the potential protective role of AII blockade in this process [45]. Given that inflammatory diseases typically result in tissue hypoxia and angiogenesis, we propose that intestinal mucosal ischemia early in the development of CD results in local down regulation of AGT in an attempt to reduce local vasoconstriction and ischemia. Transcriptional regulation of AGT mRNA varies between organs in human subjects. A recent study demonstrated reduced AGT mRNA expression in adipose tissue in obese subjects, due to chronic low grade inflammation [46]. The resection samples were included to represent more severe disease. It was striking that all the molecules assessed had significantly increased expression in the resection samples when compared to controls except AGT. AGT expression was lower in the resection samples compared to controls but this did not reach statistical significance; possibly because the number of resection samples was much lower (n=12) compared to biopsy samples (n=69), hence lacking the same statistical power. Also, the expression of the other molecules appeared to be driven by the level of inflammation (per the IL-8 levels) and its consequences (e.g. mucosal hypoxia). This was in contrast to the results for AGT, suggesting that the predominant regulator of AGT expression in the ileum is not inflammation, but mucosal hypoxia and ischemia resulting in down regulation of its expression, rather than up regulation (which might be expected if inflammation was the more dominant influence).

This hypothesis requires further investigation in view of the potential therapeutic benefit of ACE inhibitors and ARBs. These agents also benefit endothelial function, locally and systemically. IBD patients have generalised endothelial dysfunction, which contributes to their increased risk of vascular events [47]. In cardiovascular disease, ACE inhibition but not other anti-hypertensive agents, improves endothelial NO-related vasodilator function, which is lost in CD [37, 48]. Hence these agents may benefit both the local and the systemic vasculature.

Animal models confirm a therapeutic benefit in colitis through both ACE inhibition and knockdown of the AGT gene [17, 49]. Administration of the ACE inhibitor captopril to rats with TNBS colitis lowered the over expression of TGF-β1 noted in TNBS rats. This evidence also indicates a potential role for ACE inhibitors in the reduction of fibrosis and prevention of strictures in CD [17]. Increased HIF1α expression was observed in inflamed ileal CD compared to non-inflamed biopsies. Regulation of HIF1α levels occurs predominantly at the protein level via an oxygen-dependent degradation domain, where hypoxia permits its stabilisation and accumulation, resulting in the transcription of numerous genes involved in barrier function, energy metabolism and angiogenesis. However, numerous in vivo models have shown increased HIF1α mRNA levels in response to hypoxia [50]. The NF-κB pathway is also activated by hypoxia, and regulates HIF1α transcription under both basal and hypoxic conditions, by binding to its promoter [50, 51]. Hence both inflammation and hypoxia may contribute to the detection of increased HIF1α mRNA in inflamed CD ileal mucosa. These findings were exaggerated in cases carrying a NOD2 disease associated mutation. The additional increase in MCAM, a marker of endothelial junctions and angiogenesis,
in inflamed CD samples carrying a NOD2 mutation suggests that angiogenesis is more prominent in CD patients carrying NOD2 variants in the presence of inflammation. The potential anti-angiogenic activity of ARBs and ACE inhibitors could be of therapeutic interest in these patients in particular.

HIF1α expression was reduced in non-inflamed ileal CD biopsy samples compared to healthy controls. HIF1α regulates a number of barrier protective genes including intestinal trefoil factor, CD73 and multidrug resistance gene-1 (MDR1) and their expression contributes to innate immune defence through maintenance of mucosal integrity [8]. In transgenic murine models, loss of epithelial HIF1α expression resulted in more severe inflammatory colitis and constitutively active HIF1α expression was protective [8]. Hence reduced basal expression of HIF1α could contribute to impaired intestinal epithelial barrier function in CD patients.

Although this is an observational study, its strengths are the inclusion of a large number of CD patient tissue samples and the high quality of the RNA required to participate in a strict quantitative RT-PCR protocol. The demonstration of reduced AGT expression, both mRNA and protein in ileal CD is an important novel result and highlights the therapeutic potential of ACE inhibitors and ARBs in ileal CD. Their potential role is supported by the demonstration of an inverse association of co-therapy with ARBs and peptic ulcer in human subjects taking low dose aspirin which mediates gastric damage by suppression of mucosal prostaglandins and ischemia-reperfusion [52].

CONCLUSIONS

Increasing evidence implicates the intestinal vasculature and local hypoxia in CD pathogenesis. Reduced AGT expression in ileal CD might represent a local response to ischemia or hypoxia. The unique and potent role of AII in splanchic hemodynamics and in the pathogenesis of inflammation and fibrosis contributes to the therapeutic potential of ACE inhibitors and ARBs in CD, in addition to their anti-angiogenic effects. Generalised endothelial dysfunction in IBD patients also supports the rationale for their use. Further investigations are required to explore their therapeutic role in human ileal CD.

Conflicts of interest: None to declare.

Authors’ contribution: G.H.: concept and design of the project, laboratory work, data analysis and interpretation, drafting and writing of the manuscript; J.D.: statistical analysis and data interpretation, critical review of the manuscript; N.H.: laboratory work; E.E.: critical review of the manuscript, supervision of genotyping work; I.B.: responsible for immunohistochemistry, assistance with and critical review of the manuscript; L.S.: concept and design of the work, principal laboratory supervisor, data analysis and interpretation, drafting, writing and critically reviewing the manuscript; G.R.S.: supervision of the project and laboratory work, critical review of the manuscript.

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