The Role of Osteopontin (OPN/SPP1) Haplotypes in the Susceptibility to Crohn’s Disease

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Abstract

Background: Osteopontin represents a multifunctional molecule playing a pivotal role in chronic inflammatory and autoimmune diseases. Its expression is increased in inflammatory bowel disease (IBD). The aim of our study was to analyze the association of osteopontin (OPN/SPP1) gene variants in a large cohort of IBD patients.

Methodology/Principal Findings: Genomic DNA from 2819 Caucasian individuals (n = 841 patients with Crohn’s disease (CD), n = 473 patients with ulcerative colitis (UC), and n = 1505 healthy unrelated controls) was analyzed for nine OPN SNPs (rs2728127, rs2835744, rs11730582, rs28357094, rs4754 = p.Asp80Asp, rs1126616 = p.Ala236Ala, rs1126772 and rs9138). Considering the important role of osteopontin in Th17-mediated diseases, we performed analysis for epistasis with IBD-associated IL23R variants and analyzed serum levels of the Th17 cytokine IL-22. For four OPN SNPs (rs4754, rs1126616, rs1126772 and rs9138), we observed significantly different distributions between male and female CD patients. rs4754 was protective in male CD patients (p = 0.0004, OR = 0.69). None of the other investigated OPN SNPs was associated with CD or UC susceptibility. However, several OPN haplotypes showed significant associations with CD susceptibility. The strongest association was found for a haplotype consisting of the 8 OPN SNPs rs2728127-rs2853744-rs11730582-rs11439060-rs28357094-rs112661-rs1126772-rs9138 (omnibus p-value = 2.07 × 10⁻⁶). Overall, the mean IL-22 secretion in the combined group of OPN minor allele carriers with CD was significantly lower than that of CD patients with OPN wildtype alleles (p = 3.66 × 10⁻⁵). There was evidence for weak epistasis between the OPN SNP rs28357094 with the IL23R SNP rs10489629 (p = 4.18 × 10⁻²) and between OPN SNP rs1126616 and IL23R SNP rs2201841 (p = 4.18 × 10⁻²) but none of these associations remained significant after Bonferroni correction.

Conclusions/Significance: Our study identified OPN haplotypes as modifiers of CD susceptibility, while the combined effects of certain OPN variants may modulate IL-22 secretion.

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Introduction

The pathogenesis of inflammatory bowel diseases (IBD) such as Crohn’s disease (CD) and ulcerative colitis (UC) is only partially understood. Currently, these diseases are assumed to be triggered by an exaggerated immune response to intestinal bacteria in a genetically susceptible host. In addition to the nucleotide-binding oligomerization domain 2/caspase recruitment domain-containing protein 15 (NOD2/CARD15) [1,2], various novel susceptibility loci such as the interleukin-23 receptor (IL23R) [3,4], the ATG16L1 (autophagy-related 16-like 1) gene [5,6] and variants in the 5p13.1 region [7] have been identified as susceptibility variants in CD patients. Based on new insights in the genetic background of CD, there is raising evidence for a key role of innate immunity and CD-related inflammatory pathways such as IL-23/IL-17 mediated T cell responses [8]. Recently, osteopontin (OPN, also known as Eta-1), an extracellular matrix glycosylated phosphoprotein produced by immune cells, epithelial cells and osteoblasts has been identified as an important molecule involved in tissue repair, inflammation and autoimmunity as well as tumour growth [9,10,11,12]. So far,
two forms of osteopontin have been identified - secreted osteopontin (sOPN) seems to be involved in the production of pathogenetic Th1 and Th17 cells, while an intracellular form of osteopontin (iOPN) is a key regulator for Toll like receptor-9 (TLR9) and/or TLR7-dependent interferon-α (IFN-α) expression by plasmacytoid dendritic cells (DCs) and Th17 development [13]. There is evidence for a key role of osteopontin in Th1- and Th17-mediated diseases [10,14,15] such as rheumatoid arthritis [16,17,18], psoriasis [19] and multiple sclerosis [20,21,22,23]. In addition, osteopontin has also shown to be involved in granuloma formation [10], cell migration [24,25,26], and IL-12 production [27,28,29].

Osteopontin is expressed in the terminal ileum of CD patients [30] and seems to be closely involved in the Th1 immune response associated with CD [31,32,33,34]. Moreover, it has also been reported to play an important role in the pathogenesis of UC [35,36,37,38]. Analyzing the exact role of osteopontin in a murine model of acute colitis, a recent study demonstrated that Opn−/− mice showed increased serum levels of TNF-α but also reduced mRNA expression of IL-1β and matrix metalloproteinases as well as decreased blood levels of IL-22 [39]. In contrast, in a chronic DSS model, Opn−/− mice were protected from mucosal inflammation showing lower serum IL-12 levels compared to wildtype mice and neutralization of OPN in wildtype mice abrogated colitis [39]. These findings implicate a dual function of osteopontin in intestinal inflammation characterized by activation of innate immunity and Th17 cytokines such as IL-22 initiating mucosal repair in acute inflammation; while under conditions of chronic intestinal inflammation it may promote the Th1 response and thereby enhancing inflammation [39]. Further investigations by daSilva et al. in a DSS model demonstrated that osteopontin administration reduced the disease activity index, improved red blood cell counts, and reduced gut neutrophil activity compared with the DSS-treated wildtype mice [37]. Interestingly, the study by Heilmann et al. demonstrated a significant correlation of osteopontin serum levels with disease activity in human CD [39].

In this study, we aimed to analyze the role of OPN gene variants on IBD disease susceptibility and phenotype. We also investigated potential epistasis with IBD-associated IL23R gene variants. In total, we genotyped nine common single nucleotide polymorphisms (SNPs) in the OPN gene, which were previously shown to be associated with other immune-mediated diseases [40,41,42,43]. Last, based on the important role demonstrated for IL-22 in colitis experiments in Opn−/− mice [39], we analyzed the effect of OPN gene variants on IL-22 serum levels.

Methods

Ethics statement

Written, informed consent was obtained from all patients prior to inclusion into the study. In the case of minors, the consent was provided by the parents. This study was approved by the Ethics committee of the Medical Faculty of Ludwig-Maximilians-University Munich. The study protocol adhered to the ethical principles for medical research involving human subjects of the Helsinki Declaration (as described in detail under: http://www.wma.net/en/30publications/10policies/b3/index.html).

Study population

Our study population comprised 2819 individuals of Caucasian origin including n = 841 patients with CD, n = 473 patients with UC and n = 1505 healthy unrelated controls. All phenotypic data were collected blind to the results of genotyping and included detailed demographic and clinical parameters (disease behaviour, anatomic manifestation of IBD, complications, surgical or immunosuppressive therapy). The diagnosis of CD and UC was based on established guidelines according to endoscopic, radiological, and histopathological parameters. For classification of CD patients, the Montreal classification [44] based on age at diagnosis (A), location (L), and behaviour (B) of disease was used. In patients with UC, anatomic location was also based on the Montreal classification, based on the criteria ulcerative proctitis (E1), left-sided UC (distal UC; E2), and extensive UC (pancolitis; E3). Patients with indeterminate colitis were excluded from the study. The clinical characteristics of the IBD study population are shown in Table 1.

DNA extraction

From all study participants, blood samples were taken and genomic DNA was isolated from peripheral blood leukocytes using the DNA blood mini kit from Qiagen (Hilden, Germany) according to the manufacturer’s guidelines.

Genotyping of OPN gene variants

Nine OPN SNPs (rs2728127, rs2853744, rs11730582, rs11739060, rs28357094, rs4754 = p.Asp80Asp, rs1126616 = p.Ala236Ala, rs1126772 and rs9138) were genotyped by PCR and melting curve analysis using a pair of fluorescence resonance energy transfer (FRET) probes in a LightCycler® 480 Instrument (Roche Diagnostics, Mannheim, Germany) as previously described in detail [45,46,47,48]. The selection of these SNPs was based on previous studies in which associations for several of these OPN variants with autoimmune and Th1- and Th17-mediated diseases...
have been shown \[40,41,42,43,49,50,51,52,53\]. The donor fluorescent molecule (fluorescein) at the 3'-end of the sensor probe (or the anchor probe in the case of rs2835744 and rs11730582) is excited at its specific fluorescence excitation wavelength (333 nm) and the energy is transferred to the acceptor fluorescent molecule at the 5'-end (LightCycler Red 610, 640 or 670) of the anchor probe (or the sensor probe in the case of rs2835744 and rs11730582). The specific fluorescence signal emitted by the acceptor molecule is detected by the optical unit of the LightCycler. The sensor probe is exactly matching to one allele of each SNP, preferentially to the rarer allele, whereas in the case of the other allele, there is a mismatch resulting in a lower melting temperature. The total volume of the PCR was 5 μl containing 25 ng of genomic DNA, 1 × Light Cycler 480 Genotyping Master (Roche Diagnostics), 2.5 pmol of each primer and 0.75 pmol of each FRET probe (TIB MOLBIOL, Berlin, Germany). In the case of rs11739060, the concentration of the forward primer, and in the case of rs1126772, the concentration of the reverse primer was reduced to 0.5 pmol. The PCR comprised an initial denaturation step (95 °C for 10 min) and 45 cycles (95 °C for 10 sec, primer annealing temperature as given in the Supplementary data (Table S1) for 10 sec, 72 °C for 15 sec). The melting curve analysis comprised an initial denaturation step (95 °C for 1 min), a step rapidly lowering the temperature to 40 °C and holding for 2 min, and a heating step slowly (1 acquisition/°C) increasing the temperature up to 95 °C and continuously measuring the fluorescence intensity. The results of the melting curve analysis have been confirmed by analyzing two patient samples for each possible genotype using sequence analysis. For sequencing, the total volume of the PCR was 100 μl containing 250 ng of genomic DNA, 1 × PCR buffer (Qiagen, Hilden, Germany), a final MgCl₂ concentration of 2 mM, 0.5 mM of a dNTP mix (Sigma, Steinheim, Germany), 2.5 units of HotStar Plus Taq™ DNA polymerase (Qiagen) and 10 pmol of each primer (TIB MOLBIOL). The PCR comprised an initial denaturation step (95 °C for 5 min), 35 cycles (denaturation at 94 °C for 30 sec, primer annealing at 60 °C for 30 sec, extension at 72 °C for 30 sec) and a final extension step (72 °C for 10 min). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and 10 pmol of each primer (TIB MOLBIOL). The PCR comprised an initial denaturation step (95 °C for 10 min). The PCR products were also tested with PLINK and the –epistasis command. For analyzing potential differences of IL-22 serum levels between the carriers of the different OPN gene variants, the mean IL-22 serum level of carriers of the wildtype allele of each SNP was compared with the mean IL-22 serum level of carriers of the minor allele (=combined group of heterozygous and homozygous carriers) using Student’s t-test.

**Results**

**Frequency distribution of OPN gene variants and their role in IBD susceptibility**

For all three subgroups (CD, UC, and controls), the minor allele frequencies of the nine OPN SNPs (rs2728127, rs2835744, rs11730582, rs11739060, rs28357094, rs4754 = p.Asp80Asp, rs1126616 = p.Ala236Ala, rs1126772 and rs9138) are summarized in Table 2. With the exception of rs4754, no significant differences in the allele frequencies were observed comparing CD and UC patients to healthy controls (Table 2). Our analysis revealed a weak association of SNP rs4754 (p.Asp80Asp) with CD susceptibility (p = 1.28 × 10⁻²; OR (95% CI) 0.85 [0.74–0.96]); Similar to CD, rs4754 (p.Asp80Asp) decreased susceptibility to UC, although this association did not reach significance in univariate analysis (p = 5.25 × 10⁻²; OR (95% CI) 0.85 [0.70–1.00]) (Table 2). Moreover, both associations of rs4754 (regarding CD and UC susceptibility) were not statistically significant after Bonferroni correction, suggesting that these OPN variants are not major contributors to IBD susceptibility on their own. In addition, rs4754 deviated from the Hardy-Weinberg equilibrium in the control population (p = 0.0005) and was therefore excluded from the haplotype analysis. Haplotype analysis was conducted with PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/) and the –hap-logistic option using a sliding-window approach with 2 up to 8 included SNPs. Interaction between different polymorphisms were also tested with PLINK and the –epistasis command. For analyzing potential differences of IL-22 serum levels between the carriers of the different OPN gene variants, the mean IL-22 serum level of carriers of the minor allele (=combined group of heterozygous and homozygous carriers) using Student’s t-test.
p = 3.67 × 10⁻¹². In contrast, there were no associations of certain OPN haplotypes with UC susceptibility (Table 4).

### Analysis for gender-specific differences in OPN variants

Previous studies demonstrated significant gender-specific effects of OPN variants in systemic lupus erythematosus (SLE) and type 1 diabetes, particularly in male patients [43,50]. Considering the deviation of rs4754 from the Hardy-Weinberg equilibrium, we therefore investigated potential gender-specific effects in IBD susceptibility. For four OPN SNPs (rs4754, rs1126616, rs1126772 and rs9138), we observed significantly different distributions between male and female CD patients. Interestingly, for these SNPs, there was an opposite direction of the association results for males and females (rs4754: p = 0.0004, OR = 0.69 [95% CI: 0.56–0.85]; rs9138: p = 0.1256, OR = 0.85 [males], p = 0.0864, OR = 1.19 [females]). Given that the most pronounced difference between male and female CD patients was found for rs4754, which deviated from the Hardy-Weinberg equilibrium in the control population, we next investigated if the deviation from Hardy-Weinberg equilibrium is based on a gender-specific effect. This analysis revealed that there was significant deviation from Hardy-Weinberg equilibrium in male controls (n = 917; p = 0.0018), but not in female controls (n = 547; p = 0.1347), confirming the gender-specific effect of this OPN SNP found in CD patients.

### Analysis for epistasis between OPN variants and IL23R variants

To investigate if OPN variants modify IBD susceptibility by epistatic interaction with other Th17-related IBD susceptibility genes, we next analyzed for potential epistasis of OPN variants with main IBD-associated IL23R variants. We found evidence of weak epistasis between the OPN SNP rs28357094 with the IL23R SNP rs10489629 (p = 4.18 × 10⁻⁵) and between OPN SNP rs1126616 and IL23R SNP rs2201841 (p = 4.18 × 10⁻⁵) but none of these associations remained significant after Bonferroni correction (Table 5).

### Correlation between OPN variants and IL-22 serum levels in CD patients

Based on the recent data of Heilmann et al. [39] demonstrating decreased blood levels of IL-22 in acute colitis in Opn⁻/⁻ mice, we next investigated a potential association of OPN variants and IL-22 serum levels in a subcohort of CD patients. No correlation was found between OPN SNPs and IL-22 serum levels (Table 6). However, overall the IL-22 serum levels tended to be lower in the carriers of OPN minor alleles, which was statistically significant when the mean IL-22 expression level of carriers of the 9 investigated OPN SNPs minor alleles (homo- and heterozygous carriers) were compared to the homozygous carriers of the wildtype allele (p = 3.6 × 10⁻⁵). Interestingly, for 7 out of 8 OPN SNPs forming the haplotype rs2728127-rs2853744-rs11730582-rs11439060-rs28357094-rs112661-rs1126772-rs9138, which was strongly associated with CD susceptibility (omnibus p-value 2.07 × 10⁻⁶), the IL-22 serum levels were nominally lower in CD carriers of the minor allele than in wildtype carriers, although these differences were for each SNP only small and statistically not significant (Table 6).

### Discussion

The presented study represents the first detailed analysis of OPN gene variants in IBD patients. In this study, there were no significant associations of single OPN SNPs with CD or UC susceptibility after Bonferroni correction for multiple testing; however, several OPN haplotypes were associated with CD susceptibility. The strongest association was found for a haplotype consisting of the 8 OPN SNPs (rs2728127-rs2853744-rs11730582-rs11439060-rs28357094-rs112661-rs1126772-rs9138), which was strongly associated with CD susceptibility (omnibus p-value 2.07 × 10⁻⁶). However, considering the strength of the association signals found for a number of other recently identified IBD susceptibility genes [56,57], this argues against a major role for OPN in the genetic susceptibility for IBD. Given the strong association of osteopontin with Th1- and Th17-mediated diseases, the finding of an association of OPN haplotypes with CD, a Th1- and Th17-mediated disease, but not UC susceptibility is not surprising. In contrast, UC has been associated with a predominantly modified Th2 response but partially also with a Th17 immune response. The results of our haplotype analysis suggest...
that certain rare haplotypes significantly contribute to the genetic risk of CD. This is in agreement with recent results of the International IBD Genetics Consortium which identified a total of 71 CD susceptibility loci [56]. These 71 susceptibility loci explain only slightly more than 20% of CD heritability. Therefore, it is assumed that a number of rare SNPs and haplotypes contribute to the overall CD risk such as recently shown by us for \( PXR \) gene variants [58]. In addition, most likely a high number of common CD risk genes with small effect size are still unidentified but for their identification very large cohorts would be required.

So far, genetic variants in the \( OPN \) gene have shown to be involved in susceptibility to other immune-mediated diseases such as SLE [59, 60], oligoarticular juvenile idiopathic arthritis [61] and sarcoidosis [51]. Despite promising functional data, previous genotype analyses could not confirm \( OPN \) as significant disease-modifying gene in classical Th17-mediated diseases such as multiple sclerosis [62, 63] and rheumatoid arthritis [64]. Investigating the role of \( OPN \) as a susceptibility gene in SLE, a recent study demonstrated a significant association in male patients [50] – a phenomenon also seen in a study investigating \( OPN \) variants in type-1 diabetes, implicating a potential gender-specific mechanism acting in the autoimmune process [43]. Similarly, our analysis demonstrated gender-specific effects for four \( OPN \) SNPs, particularly for rs4754 which deviated from the Hardy-Weinberg equilibrium in male controls. Moreover, there was a significant association of this SNP with CD in male but not in female patients.

While osteopontin is closely involved in the Th1- and Th17-mediated immune response associated with CD [31, 32, 33, 34], its role in murine colitis models is controversially discussed. In one study, osteopontin deficiency protected mice from DSS-induced colitis [38], while in another study, osteopontin administration in \( 2 \)/\( 2 \) mice reduced the disease activity index, improved red blood cell counts, and reduced gut neutrophil activity compared to the mean IL-22 serum level of the carriers of the minor alleles (homo- and heterozygous carriers), \( 2 \)/\( 2 \) mice showed decreased blood levels of IL-22 [39]. Since we recently demonstrated that IL-22 serum levels are increased in CD and correlate with disease activity and the \( IL23R \) genotype [55], we next analyzed a potential association between \( OPN \) genotypes and IL-22 serum levels in CD patients. Overall, we observed lower IL-22 serum levels in the carriers of \( OPN \) minor alleles (homo- and heterozygous carriers), which was statistically significant when the mean IL-22 expression level of carriers of the 9 investigated \( OPN \) SNPs minor alleles was compared to the mean IL-22 serum level of the carriers of the

### Table 3. Haplotypes of \( OPN \) SNPs in Crohn’s disease (CD) case-control sample (846 cases and 1510 controls) and omnibus p-values for association with CD susceptibility.

| Haplotype combination | Omnibus p-value |
|-----------------------|-----------------|
| rs2728127-rs2853744   | 9.09 \( \times 10^{-1} \) |
| rs2853744-rs11730582  | 2.74 \( \times 10^{-1} \) |
| rs11730582-rs11439060 | 6.87 \( \times 10^{-2} \) |
| rs11439060-rs28357094 | 2.25 \( \times 10^{-1} \) |
| rs28357094-rs1126616  | 6.11 \( \times 10^{-1} \) |
| rs1126616-rs1126772   | 1.81 \( \times 10^{-1} \) |
| rs1126772-rs9138      | 4.71 \( \times 10^{-1} \) |
| rs2728127-rs2853744-rs11730582 | 1.95 \( \times 10^{-1} \) |
| rs2853744-rs11730582-rs11439060 | 1.34 \( \times 10^{-1} \) |
| rs11730582-rs11439060-rs28357094 | 5.37 \( \times 10^{-2} \) |
| rs11439060-rs28357094-rs1126616 | 2.72 \( \times 10^{-1} \) |
| rs28357094-rs1126616-rs1126772 | 3.72 \( \times 10^{-1} \) |
| rs1126616-rs1126772-rs9138 | 6.45 \( \times 10^{-1} \) |
| rs2728127-rs2853744-rs11730582-rs11439060 | 2.15 \( \times 10^{-2} \) |
| rs2853744-rs11730582-rs11439060-rs28357094 | 1.62 \( \times 10^{-1} \) |
| rs11730582-rs11439060-rs28357094-rs1126616 | 1.35 \( \times 10^{-1} \) |
| rs11439060-rs28357094-rs1126616-rs1126772 | 2.74 \( \times 10^{-1} \) |
| rs28357094-rs1126616-rs1126772-rs9138 | 6.77 \( \times 10^{-1} \) |
| rs2728127-rs2853744-rs11730582-rs11439060-rs28357094 | 3.77 \( \times 10^{-2} \) |
| rs2853744-rs11730582-rs11439060-rs28357094-rs1126616 | 1.98 \( \times 10^{-1} \) |
| rs11730582-rs11439060-rs28357094-rs1126616-rs1126772 | 6.95 \( \times 10^{-2} \) |
| rs11439060-rs28357094-rs1126616-rs1126772-rs9138 | 3.84 \( \times 10^{-1} \) |
| rs2728127-rs2853744-rs11730582-rs11439060-rs28357094-rs1126616 | 5.03 \( \times 10^{-2} \) |
| rs2853744-rs11730582-rs11439060-rs28357094-rs1126616-rs1126772 | 6.86 \( \times 10^{-2} \) |
| rs11730582-rs11439060-rs28357094-rs1126616-rs1126772-rs9138 | 5.75 \( \times 10^{-2} \) |
| rs2728127-rs2853744-rs11730582-rs11439060-rs28357094-rs1126616-rs1126772-rs9138 | 1.44 \( \times 10^{-7} \) |
| rs2853744-rs11730582-rs11439060-rs28357094-rs1126616-rs1126772-rs9138 | 2.76 \( \times 10^{-5} \) |
| rs2728127-rs2853744-rs11730582-rs11439060-rs28357094-rs1126616-rs1126772-rs9138 | 2.07 \( \times 10^{-8} \) |

Significant p-values < 0.05 are depicted in bold. All significant p-values remained significant after 10,000 permutations.

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Table 4. Haplotypes of OPN SNPs in ulcerative colitis (UC) case-control sample (501 cases and 1510 controls) and omnibus p-values for association with UC susceptibility.

| Haplotype combination                                      | Omnibus p-value |
|------------------------------------------------------------|-----------------|
| rs2728127-rs2853744                                        | 5.62×10⁻¹       |
| rs2853744-rs11730582                                        | 3.72×10⁻¹       |
| rs11730582-rs11439060                                        | 7.01×10⁻¹       |
| rs11439060-rs28357094                                        | 9.54×10⁻¹       |
| rs28357094-rs1126616                                        | 8.08×10⁻¹       |
| rs1126616-rs1126772                                         | 2.80×10⁻¹       |
| rs1126772-rs9138                                            | 2.65×10⁻¹       |
| rs2728127-rs2853744-rs11730582-rs11439060-rs28357094         | 8.26×10⁻¹       |
| rs2853744-rs11730582-rs11439060-rs28357094-rs1126616         | 4.98×10⁻¹       |
| rs11730582-rs11439060-rs28357094-rs1126616-rs1126772         | 8.39×10⁻¹       |
| rs11439060-rs28357094-rs1126616-rs1126772                   | 1.97×10⁻¹       |
| rs28357094-rs1126616-rs1126772-rs9138                        | 5.24×10⁻¹       |
| rs2728127-rs2853744-rs11730582-rs11439060-rs28357094         | 5.02×10⁻¹       |
| rs2853744-rs11730582-rs11439060-rs28357094-rs1126616         | 8.25×10⁻¹       |
| rs11730582-rs11439060-rs28357094-rs1126616-rs1126772         | 5.07×10⁻¹       |
| rs11439060-rs28357094-rs1126616-rs1126772-rs9138             | 3.01×10⁻¹       |
| rs2728127-rs2853744-rs11730582-rs11439060-rs28357094-rs112661 | 7.27×10⁻¹       |
| rs2835744-rs11730582-rs11439060-rs28357094-rs1126616-rs1126772 | 5.85×10⁻¹       |
| rs11730582-rs11439060-rs28357094-rs1126616-rs1126772-rs9138 | 5.36×10⁻¹       |
| rs2728127-rs2853744-rs11730582-rs11439060-rs28357094-rs112661 | 5.86×10⁻¹       |
| rs2853744-rs11730582-rs11439060-rs28357094-rs1126616-rs9138  | 5.95×10⁻¹       |
| rs2728127-rs2853744-rs11730582-rs11439060-rs28357094-rs1126772 | 5.00×10⁻¹       |

None of the haplotypes was significantly associated with UC susceptibility (p>0.05).
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Table 5. Analysis for epistatic interactions between OPN SNPs and IL23R SNPs regarding CD susceptibility (based on 1510 controls and 704 cases).

| OPN SNPs | rs2728127 | rs2853744 | rs11730582 | rs11439060 | rs28357094 | rs1126616 | rs1126772 | rs9138 |
|----------|-----------|-----------|------------|------------|------------|-----------|-----------|--------|
| IL23R SNPs |       |           |            |            |            |           |           |        |
| rs1004819 | 5.45×10⁻¹ | 1.34×10⁻¹ | 3.00×10⁻¹  | 8.83×10⁻¹  | 5.93×10⁻¹  | 3.69×10⁻¹ | 2.86×10⁻¹ | 4.52×10⁻¹ |
| rs7517847 | 4.52×10⁻¹ | 7.94×10⁻¹ | 2.33×10⁻¹  | 5.96×10⁻¹  | 3.98×10⁻¹  | 8.57×10⁻¹ | 4.97×10⁻¹ | 5.79×10⁻¹ |
| rs10489629 | 1.90×10⁻¹ | 3.31×10⁻¹ | 5.54×10⁻¹  | 2.32×10⁻¹  | 4.18×10⁻²  | 8.05×10⁻¹ | 4.31×10⁻¹ | 6.28×10⁻¹ |
| rs2201841 | 2.49×10⁻¹ | 2.18×10⁻¹ | 2.43×10⁻¹  | 1.74×10⁻¹  | 5.91×10⁻²  | 4.71×10⁻² | 6.46×10⁻² | 8.10×10⁻² |
| rs11465804 | 8.02×10⁻¹ | 5.97×10⁻¹ | 5.98×10⁻¹  | 7.45×10⁻¹  | 9.86×10⁻²  | 6.19×10⁻¹ | 4.54×10⁻¹ | 6.18×10⁻¹ |
| rs11209026 | 6.71×10⁻¹ | 8.05×10⁻¹ | 2.46×10⁻¹  | 6.64×10⁻¹  | 5.17×10⁻¹  | 8.87×10⁻¹ | 6.29×10⁻¹ | 9.76×10⁻¹ |
| p.Arg381Gln | | | | | | | | |
| rs1343151 | 6.65×10⁻¹ | 2.25×10⁻¹ | 9.68×10⁻¹  | 7.34×10⁻¹  | 1.23×10⁻¹  | 9.98×10⁻¹ | 3.32×10⁻¹ | 8.79×10⁻¹ |
| rs10889677 | 2.49×10⁻¹ | 3.09×10⁻¹ | 3.29×10⁻¹  | 1.53×10⁻¹  | 6.05×10⁻²  | 6.88×10⁻² | 8.51×10⁻² | 9.73×10⁻² |
| rs11209032 | 4.46×10⁻¹ | 2.92×10⁻¹ | 2.71×10⁻¹  | 3.58×10⁻¹  | 2.71×10⁻¹  | 1.91×10⁻¹ | 3.46×10⁻¹ | 3.75×10⁻¹ |
| rs1495965 | 1.79×10⁻¹ | 2.77×10⁻¹ | 9.52×10⁻²  | 1.34×10⁻¹  | 1.11×10⁻¹  | 1.94×10⁻¹ | 2.82×10⁻¹ | 2.39×10⁻¹ |

Significant p-values<0.05 are depicted in bold. However, these associations did not remain significant after Bonferroni correction.
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Table 6. OPN gene variants modulate IL-22 serum levels in CD patients.

| OPN SNP | IL-22 serum levels in OPN wildtype carriers [pg/ml] | IL-22 serum levels in OPN minor allele carriers* [pg/ml] | p-value |
|---------|-----------------------------------------------|-----------------------------------------------|---------|
| rs2728127 | 39.72 | 37.28 | 0.537 |
| rs2853744 | 38.24 | 39.54 | 0.854 |
| rs11730582 | 42.07 | 37.18 | 0.341 |
| rs11439060 | 39.72 | 37.28 | 0.537 |
| rs28357094 | 39.23 | 37.36 | 0.614 |
| rs4754 = p.As80Asp | 40.19 | 36.78 | 0.380 |
| rs1126616 = p.Ala236Ala | 40.19 | 36.59 | 0.357 |
| rs1126772 | 41.04 | 34.96 | 0.106 |
| rs9138 | 40.35 | 36.59 | 0.333 |
| Mean | 40.08 | 37.06 | 3.66 x 10^-5 |

The mean IL-22 serum level was analyzed for each OPN variant in a subgroup of 151 CD patients for which DNA for genotyping and serum for ELISA analysis was available. P values are given for the comparison of the mean IL-22 serum levels of carriers of the minor allele (homozygous and heterozygous) compared to cytokine levels in homozygous wild-type carriers. doi:10.1371/journal.pone.0029309.t006

In summary, our study identified certain OPN haplotypes to be associated with CD susceptibility. OPN variants may modulate IL-22 secretion which is consistent with data in Opn−/− mice, in which low levels of the epithelial-protective cytokine IL-22 predispose to intestinal inflammation. However, the rather weak association signals found in this study argue against a significant role for OPN as major IBD susceptibility gene which is consistent with the recent IBD meta-analyses [56,57]. Further functional analysis of large cohorts and detailed fine mapping is required to clarify the role of OPN variants in the genetic susceptibility to IBD.
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