Association study of the IL18RAP locus in three European populations with coeliac disease

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Coeliac disease is caused by dietary gluten, triggering a chronic inflammation of the small intestine in genetically predisposed individuals. Recently, a risk locus on chromosome 2q11–q12, harbouring interleukin 18 receptor accessory protein (IL18RAP) and three other genes, was suggested for coeliac disease. IL18 has been shown to play an important role in T helper type 1 activity in coeliac disease, making this locus a highly interesting candidate. In this study, two previously indicated risk variants at the IL18RAP locus (rs13015714 and rs917997) were tested for genetic association in 1638 cases with coeliac disease and 1385 control individuals from the Finnish, Hungarian and Italian populations. The protein expression level of IL18RAP was also compared between risk allele carriers and non-carriers by Western blotting. Furthermore, immunohistochemical analysis was performed to study IL18RAP protein expression in small intestinal biopsies of untreated and treated coeliac patients and controls. We confirmed genetic association and dose effects of variants at the 2q12.1 locus with coeliac disease in the Hungarian population. The GA haplotype of the markers rs13015714 and rs917997 showed the strongest association (P = 0.0001, odds ratio = 1.475, 95% confidence interval 1.21–1.80). Two putative isoforms of IL18RAP were detected and the ratios and total levels of these isoforms may contribute to the aetiology of coeliac disease. Our study supports IL18RAP as a novel predisposing gene for coeliac disease and highlights the need for further functional studies on this relatively unknown gene in coeliac disease pathogenesis.

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INTRODUCTION

Coeliac disease, also known as gluten intolerance, is a chronic inflammatory disease of the small intestine with autoimmune features. It is triggered in genetically susceptible individuals by dietary exposure to gluten from wheat, barley or rye. Approximately 1% of the Caucasian population has coeliac disease, although the disease is often asymptomatic or exhibits mild or extraintestinal symptoms. Similarly to atopic diseases and type I diabetes, the prevalence of coeliac disease is increasing, being already 2% in Finland (1).

To date, the only confirmed and functionally characterized genetic risk factors for coeliac disease are the HLA-DQA1 and DQB1 alleles coding for the disease-associated DQ2 or DQ8 heterodimers at the MHC class II locus (CELIAC1) (2). These genes are necessary for antigen presentation of gluten in coeliac disease, but are alone not sufficient for disease onset (3,4). Recently, a whole-genome association scan was performed to identify novel risk factors for coeliac disease in the UK population (5). The strongest findings were replicated in the Dutch and Irish populations, and this scan identified and confirmed the IL2–IL21 gene cluster on 4q27 as a risk factor in coeliac disease and recently also in other autoimmune diseases (5–7). Further studies are needed to determine the primary risk variant, and its role in the expression or function of either IL2 or IL21.

A systematic follow up study of the most highly associated non-HLA single-nucleotide polymorphisms (SNPs) in the UK, Irish and Dutch populations revealed several loci that are likely to be important for coeliac disease susceptibility (8). One of these regions, on 2q12, harbours four genes, of which one encodes for the interleukin 18 receptor associated protein (IL18RAP, ACPL). Allele A of rs917997, a SNP 1.5 kb downstream of IL18RAP, was strongly associated with coeliac disease susceptibility in the Dutch and an independent UK sample set, but not in the Irish population. This allele also had a significant allele dosage effect on IL18RAP mRNA expression in whole blood, with the coeliac disease risk allele A correlating with lower mRNA levels. IL18RAP was also recently shown to be associated with inflammatory bowel disease (9).

As this gene constitutes an important novel coeliac disease candidate gene, we studied the association of SNPs in this gene to coeliac disease in case–control datasets from the Finnish, Hungarian and Italian populations. Population heterogeneity may strongly affect the frequency and importance of risk genes for complex diseases, and therefore new findings need to be confirmed in several populations. Since protein level data on IL18RAP are very limited, we also performed preliminary expression analyses of IL18RAP to gain a better understanding of this molecule and its possible isoforms.

RESULTS

Genetic association

Table 1 shows the results of the association analysis of the two SNPs at the IL18RAP locus and their haplotypes to Finnish, Hungarian and Italian cases and controls (A) as well as a comparison with results from (i) Van Heel et al. (5), (ii) Hunt et al. (8) and (iii) Romanos et al. (17). (B) Carriership of rs13015714*G and rs917997*A was found to be significantly associated with increased risk for coeliac disease in the Hungarian case–control material \( P = 0.0012, \) odds ratio (OR) 1.38, 95% confidence interval (CI) 1.14–1.68 and \( P = 0.0011, \) OR 1.39, 95% CI 1.14–1.70, respectively. The corresponding GA haplotype showed significant risk association \( P = 0.0001, \) OR 1.48, 95% CI 1.21–1.80) in Hungarian cases, whereas the TG-haplotype showed protection \( P = 0.0040, \) OR 0.75, 95% CI 0.62–0.91. No statistically significant association to coeliac disease was seen in either the Finnish or Italian populations, although the frequency differences between cases and controls showed the same tendency as in the Hungarian samples, as well as the previous UK and Dutch studies (Table 1). Table 1C shows the association of individual genotypes in the Hungarian population. A significant dose effect was seen with the risk allele of both SNP markers. For rs13015714, the OR was 1.89 (95% CI 1.14–3.14) for the GG risk genotype and 1.29 (95% CI 1.00–1.66) for the GT genotype, whereas for the TT genotype it was 0.69 (95% CI 0.54–0.89). A similar pattern was seen with the rs917997 alleles. A meta-analysis of our Finnish, Hungarian and Italian datasets, as well as previously published Dutch, Irish, the UK and Italian datasets, showed strong association of the IL18RAP locus SNPs with coeliac disease (Fig. 1). For carriership of rs917997 (A), the OR was 1.25 \( (P = 1.89 \times 10^{-11}, \) 95% CI 1.17–1.33). When the Finnish, Hungarian and Italian sample sets were studied separately of the other sample sets, the meta-analysis yielded an OR of 1.23 \( (P = 0.0010, \) 95% CI 1.09–1.39).

Genetic linkage was also studied in Finnish and Hungarian families, partly overlapping with the case material. No linkage was seen in the analysis (data not shown). The information content of the family material and studied markers for linkage analysis was as low as 18–27%.

IL18RAP expression and function in blood leucocytes

We used western blot analysis to study the expression levels of IL18RAP in peripheral blood mononuclear cells (PBMCs) from anonymous blood donors. Figure 2A shows IL18RAP protein in PBMCs from 16 blood donors genotyped for rs917997. Western blotting revealed two bands of different size specific for IL18RAP. A band at 70 kDa, corresponding to the estimated full-length IL18RAP, was visible in 15 out of 16 donors. In one sample, there were two separate bands at 70 kDa (lane 1). Western blotting also revealed a band at 37 kDa in PBMCs from 14 out of 16 donors. A non-significant trend was observed indicating that carriers of the risk allele rs917997*A (AA and AG) may express higher levels of the 37 kDa band than non-carriers and lower expression levels of the 70 kDa band (Fig. 2B). This is supported by an independent experiment in an additional 10 blood donors, using 20 μg of protein, half of the amount previously used. The 70 kDa band was not visible because of the low amount of protein used, but the same trend was seen between the levels of the 37 kDa protein and genotype (data not shown). As the two experiments are not directly comparable, we show the results of only the larger one.
IL18 was shown to induce IFN-gamma secretion in a dose-dependent manner in anti-CD3/CD28 activated PBMC (Supplementary Material, Fig. S1), but not in unactivated cells (data not shown). However, no significant difference was found between the rs917997 IL18RAP genotypes among 38 healthy donors.

To study the expression of IL18RAP in coeliac disease, immunohistochemical analysis of small intestinal biopsy samples from five untreated coeliac patients (one homozygous for the risk haplotype and four homozygous for the protective haplotype), nine treated coeliac patients (five homozygous for the risk haplotype and four homozygous for the protective haplotype) and five controls (two homozygous for the risk haplotype and three homozygous for the protective haplotype) was performed. The analysis revealed staining in mononuclear cells in the lamina propria, the entero-endocrine cells in the crypts in two other samples, as well as some of the Paneth cells in the crypts (Supplementary Material, Fig. S2A, B and C, respectively). No consistent differences between untreated and treated coeliac patients, or controls, or different genotypes, were observed (data not shown).

**DISCUSSION**

Our study aimed to investigate the recently reported genetic association between coeliac disease and polymorphisms at the IL18RAP locus. IL18RAP codes for the beta-chain of the IL18 receptor, and is therefore a functionally relevant candidate gene for coeliac disease. A T-helper type 1 (Th1) inflammatory response characterized by the expression of IFN-gamma and active IL18 cytokine has been described in mucosal samples of untreated coeliac patients (10). In addition, *in vitro* gluten stimulation of biopsy samples from coeliac patients induces the expression of IL18 (11). Furthermore, increased levels of IL18 have also been found in peripheral blood of patients with coeliac disease, and IL18 serum levels are higher in untreated coeliac patients than after commencement of a gluten-free diet (12). Taken together, these findings strongly suggest that IL18 contributes to the

### Table 1. Association of rs13015714 and rs917997 with coeliac disease

(A) Allele and haplotype association

<table>
<thead>
<tr>
<th>Marker</th>
<th>Frequency cases, controls</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finnish</td>
<td>rs13015714</td>
<td>0.212, 0.186</td>
<td>0.0729</td>
</tr>
<tr>
<td></td>
<td>rs917997</td>
<td>0.213, 0.188</td>
<td>0.0916</td>
</tr>
<tr>
<td>Hungarian</td>
<td>rs13015714</td>
<td>0.306, 0.242</td>
<td>0.0012</td>
</tr>
<tr>
<td></td>
<td>rs917997</td>
<td>0.304, 0.239</td>
<td>0.0011</td>
</tr>
<tr>
<td>Italian (Trieste)</td>
<td>rs13015714</td>
<td>0.281, 0.256</td>
<td>0.4718</td>
</tr>
<tr>
<td></td>
<td>rs917997</td>
<td>0.281, 0.240</td>
<td>0.2309</td>
</tr>
</tbody>
</table>

(B) Results from previous studies

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<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
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<td>UK GWAS</td>
<td>rs13015714</td>
<td>0.261, 0.214</td>
<td>0.0004</td>
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<td></td>
<td>rs917997</td>
<td>0.267, 0.215</td>
<td>9.0636 × 10^{-5}</td>
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<tr>
<td>UK</td>
<td>rs13015714</td>
<td>0.243, 0.202</td>
<td>0.0018</td>
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<tr>
<td></td>
<td>rs917997</td>
<td>0.245, 0.208</td>
<td>0.0048</td>
</tr>
<tr>
<td>Irish</td>
<td>rs13015714</td>
<td>0.207, 0.202</td>
<td>0.7621</td>
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<tr>
<td></td>
<td>rs917997</td>
<td>0.214, 0.204</td>
<td>0.5488</td>
</tr>
<tr>
<td>Dutch</td>
<td>rs13015714</td>
<td>0.288, 0.214</td>
<td>9.717 × 10^{-6}</td>
</tr>
<tr>
<td></td>
<td>rs917997</td>
<td>0.293, 0.215</td>
<td>3.6108 × 10^{-6}</td>
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</table>

(C) Genotype association in the Hungarian population

<table>
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<th>Marker</th>
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<th>Frequency cases, controls</th>
<th>P-value</th>
<th>OR (95% CI)</th>
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<tr>
<td>rs13015714</td>
<td>GG</td>
<td>0.0119</td>
<td>1.89</td>
<td>1.14–3.14</td>
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<tr>
<td></td>
<td>GT</td>
<td>0.0483</td>
<td>1.29</td>
<td>1.00–1.66</td>
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<td></td>
<td>TT</td>
<td>0.0040</td>
<td>0.70</td>
<td>0.54–0.89</td>
</tr>
<tr>
<td>rs917997</td>
<td>AA</td>
<td>0.0224</td>
<td>1.81</td>
<td>1.10–3.00</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>0.1236</td>
<td>1.23</td>
<td>0.95–1.58</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>0.0046</td>
<td>0.70</td>
<td>0.54–0.89</td>
</tr>
</tbody>
</table>

Association of rs13015714 and rs917997 alleles and haplotypes is shown in the Finnish, Hungarian and Italian case–control materials (A), and the previously published results from UK, Dutch, Irish and Italian populations are shown for comparison (B). The haplotype cut-off >1% for the controls was used. The genotype association in the Hungarian cases and controls (C). OR refers to OR of allele carriership except in (C).

Van Heel et al. (5).
Hunt et al. (8).
Romanos et al. (17)
induction and maintenance of a Th1-mediated inflammatory response to gluten in patients with coeliac disease.

The IL18 receptor is comprised of IL18RAP together with the IL18 receptor 1 protein (IL18R1 or IL1Rrp) (13,14). IL18RAP forms the signalling chain of this receptor complex and has been shown to be crucial for IL18 signalling, resulting in the production of IFN-gamma (15). The two subunits of the IL18 receptor are expressed preferentially on T cells and natural killer cells (13,14), and are mainly expressed on Th1 cells in response to IFN-alpha and/or IL12 (16). Salvati et al. (10) reported that the transcription of both IL18 receptor chains is consistently upregulated in the intestinal mucosa of untreated coeliac patients when compared with healthy controls. In light of these previous findings, IL18RAP is a highly relevant candidate gene for coeliac disease and its different genetic variants may have distinct effects on IL18 signalling.

In this study, we genotyped two previously identified IL18RAP locus coeliac disease risk SNPs from a total of 1638 coeliac disease patients and 1385 controls from the Finnish, Hungarian and Italian populations. We replicated the association of the Finnish, Hungarian and Italian populations. We replicated 1638 coeliac disease patients and 1385 controls from the IL18RAP locus coeliac disease risk SNPs from a total of killer cells (13,14), and are mainly expressed on Th1 cells in response to gluten in patients with coeliac disease.

This appears somewhat contradictory to the strong IFN-gamma response seen in active coeliac disease. We attempted to directly correlate function with genotype by measuring IFN-gamma levels from IL18 stimulated activated PBMCs. Although we saw increased IFN-gamma production upon IL18 stimulation, there were no significant differences association (OR 1.25, \( P = 1.89 \times 10^{-11} \)). This indicates that the IL18RAP locus is a true risk factor for coeliac disease in multiple populations. Confirming previously reported risk factors in multiple populations increases confidence in novel findings, as demonstrated by the current and previous studies on the IL18RAP locus in coeliac disease. The frequencies of the associated alleles may also vary between populations and affect the power of the study. The risk variants at the IL18RAP locus are relatively common in all currently studied populations, but do demonstrate clear Northern-Southern Europe variation, being the least frequent in Finland and the most frequent in Hungary and Italy.

In addition to the allelic association, we found a significant dose effect of the associated alleles, which has not been described in previous studies. In the Hungarian population, a single copy of the risk allele rs917997*A showed an OR of 1.39 (95% CI 1.14–1.68), but homozygosity for this allele increased the OR to 1.89 (95% CI 1.14–3.14). The GA risk haplotype showed stronger association than any single marker in the Hungarian population. In contrast, the rare GG and TA haplotypes showed no risk, or perhaps a protective effect. Due to the strong LD between the two markers, the additional information gained from haplotypic analysis is limited. Detailed haplotype analysis of the LD block containing IL18RAP in the original genome-wide UK dataset (5) also showed that the two risk variants are only found in the common risk haplotype and two rare haplotypes derived from it (Supplementary Material, Fig. S3). Therefore, genotyping of other tagging markers would not help in further identification of risk and non-risk haplotypes. Identification of the primary risk-conferring variant in this haplotype is likely to require both re-sequencing in this LD block and clear functional data.

It is not yet clear how IL18RAP locus risk variants would contribute to coeliac disease susceptibility. IL18RAP is contained within a ~400 kb LD block that also contains three other genes (IL1RL1, IL18R1 and SLC9A4). IL1RL1 and SLC9A4 are not expressed in small intestinal tissue and therefore were not considered as high a priority to examine for this study, but their role in pathogenesis cannot be excluded and they should be investigated further in future studies. As mentioned earlier, IL18R1 and IL18RAP come together to form the receptor for IL18, making both of them very good candidate genes for coeliac disease susceptibility. Further work is needed to conclusively confirm IL18RAP as the primary risk gene and its role in pathogenesis, and also to exclude IL18R1. It is also possible that these two neighbouring genes are co-regulated. However, in the study by Hunt et al. (8), the disease-associated genotypes correlated with lower mRNA expression of IL18RAP, but not of IL18R1, pointing to a primary role for IL18RAP.

The mRNA correlation shown by Hunt et al. (8) suggests that individuals with the risk allele have reduced IL18RAP expression, possibly leading to lower IFN-gamma secretion. This appears somewhat contradictory to the strong IFN-gamma response seen in active coeliac disease. We attempted to directly correlate function with genotype by measuring IFN-gamma levels from IL18 stimulated activated PBMCs. Although we saw increased IFN-gamma production upon IL18 stimulation, there were no significant differences
between groups stratified by rs917997 genotype. This experiment may have been limited by small sample size, the heterogeneous nature of PBMCs and the supra-physiological T cell activation induced by anti-CD3–anti-CD28 stimulation.

As IL18RAP has been relatively poorly characterized to date, we studied its protein expression in PBMCs. In addition to the expected 70 kDa protein corresponding to the estimated full-length protein, we saw expression of a ~37 kDa protein, suggesting a putative alternative isoform of IL18RAP. Although not reaching significance, these results suggest a correlation of the risk allele with higher expression levels of the 37 kDa variant and lower expression levels of the 70 kDa variant. Hunt et al. (8) show that the risk allele correlates with lower levels of mRNA expression, consistent with our levels of the full-length (70 kDa) protein. This may be a result of the design of the mRNA assay by Hunt et al. (8), which identifies the full-length transcript only.

Given that little is known about the IL18RAP protein and its alternative splicing or functional isoforms, future studies should concentrate on their systematic characterization in coeliac disease-specific tissues, cells and inflammatory conditions. Indeed, alternative splicing of IL18RAP mRNA has been described in human and rat, coding for putative-truncated proteins and soluble forms of the receptor (18,19). Theoretically, such isoforms could suppress IL18 activity and IFN-gamma induction by competing for binding to IL18R1, thereby explaining the association of the risk allele with low mRNA levels. However, the expression results in our study and that of Hunt et al. (8) still contradict the known IFN-gamma-mediated Th1 response in coeliac disease. We can, therefore, not exclude neither novel alternative roles of IL18 signalling nor putative coupling of IL18RAP with other cytokine receptor chains, forming new receptor complexes with unknown or even opposite functions than the IL18 pathway. The pathogenicity of IFN-gamma and Th1 responses in coeliac disease may also need a revision, as there is increasing evidence of Th17 cells involved in autoimmunity (20).

Immunohistochemical analysis of IL18RAP showed a wide variety of expression patterns and revealed no consistent differences between the untreated and treated coeliac patients and the controls. Furthermore, no differences were observed between different IL18RAP genotypes (data not shown). This analysis does, however, confirm that IL18RAP is expressed in the gut inflammatory mononuclear cells thus further arguing for the involvement of this protein in coeliac disease pathogenesis.

In conclusion, our study independently confirms the association results of Hunt et al. (8) and supports IL18RAP as a novel coeliac disease risk gene in the Hungarian population and possibly also in the Finnish and Italian populations. The unclear nature of the risk genotype effects on protein levels highlights the need for future studies pertaining to IL18RAP mRNA stability, alternative splicing and receptor isoforms, as well as novel protein interactions or functions that will elucidate the role of this relatively poorly studied gene in coeliac disease pathogenesis.

MATERIALS AND METHODS

Study subjects

The Finnish material consisted of 844 patients with coeliac disease (68.9% females) and 698 population controls (38.1%
females). The Finnish cases were collected at the University of Tampere and described previously (21,22) with the exception of an additional novel material consisting of only biopsy-proven and/or anti-endomysial antibody positive patients (ca. 1% of patients).

The Finnish controls were selected to be representative for the Finnish population and were part of three larger datasets. One of them was a population-based sample of Finnish monozygotic twins. The second was part of a national cohort, originally collected to provide a comprehensive picture of the health state of the Finnish population (23). The third was part of a dataset chosen to be representative for the Finnish population density excluding Lapland and Northern Karelia. The controls matched geographically well with the patients which represented the general-mixed Finnish population with a slight bias to Southern Finland.

The Hungarian material consisted of 607 individuals with coeliac disease (64.2% females) and 448 population controls (54.8% females). The collection of the Hungarian samples has been described previously (22,24,25).

The Italian coeliac disease dataset consisted of 187 cases (72.3% females) and 239 geographically matched controls (35.0% females) from the Trieste region. Untreated coeliac patients were diagnosed in accordance with the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (26) criteria and intestinal biopsies were analyzed using Oberhuber’s classification (27). The serum samples of the patients were positive for both anti-transglutaminase and anti-endomysium antibodies.

The collection of the patient and control materials were approved by the ethical committees of the Tampere and Helsinki University Hospitals, Finnish National Public Health Institute, Heim Pal Children’s Hospital, Budapest, the University of Debrecen, and by the Independent Local Ethical Committee of the ‘Burlo Garofolo’ Children’s Hospital in Trieste, Italy. All enrolled participants were informed about the study according to the study protocol and gave written informed consent.

DNA extraction and genotyping
Genomic DNA was extracted from whole blood samples by a standard non-enzymatic method, using the Flexigene DNA kit or the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). Samples were genotyped for the SNPs rs13015714 (C__31439507__10) and rs917997 (C__345197__1__) using TaqMan SNP Genotyping Assays on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) at the Department of Medical Genetics, of the University of Helsinki, Finland. Part of the Finnish control genotypes were derived from an earlier genome-wide study performed on the Illumina HumanHap300Duo bead chip platform genotyped at the Center for Comparative Genomics, Uppsala, Sweden.

Genetic analysis
The genotyping success rates for rs917997 and rs13015714 were between 96.9 and 100% in both cases and controls in each population. Neither marker deviated significantly from Hardy–Weinberg equilibrium in the cases or in the controls in any of the studied populations (P > 0.05). Allele and haplotype frequency calculations, as well as association analysis, were performed using Haploview (v. 4.0) (28). Two-sided P-values are reported and OR (for carriage of risk allele unless otherwise noted) were calculated. A haplotype cut-off of >1% was used in the controls. Combined ORs from different populations were calculated using the Mantel–Haenszel meta-analysis approach. Forest plots were constructed using meta v2.14 on the R platform available from http://cran.r-project.org/web/packages/meta/index.html. The Breslow-Day test for rs917997 was significant (P = 0.03) in a meta-analysis of our Finnish, Hungarian and Italian datasets combined with the previously published Dutch, UK, Irish (8) and Italian (17) datasets, indicating heterogeneity of ORs. However, Cochran’s Q statistic revealed no evidence of allelic heterogeneity in this data (P-value = 0.50). The random effects variance (taut) was less than or equal to zero validating the use of a fixed effects model. Effect variance was calculated using the R library Catmap (29).

Non-parametric linkage was analysed using the NPLall statistics with Merlin (v. 2 beta) (30). One hundred and seventeen Finnish families and 107 Hungarian families informative for linkage were used in the analysis.

Power calculations
We estimated the power of our study to detect association of IL18RAP to coeliac disease in our sample sets using the Genetic Power Calculator program (http://pngu.mgh.harvard.edu/~purcell/gpc/) (31). We assumed a disease prevalence of 0.01, a risk allele frequency of 0.215, an OR of 1.34 and alpha 0.05. Our power to detect association of IL18RAP to coeliac disease was 66% in the Hungarian dataset; 74% in the Finnish dataset and 27% in the Italian dataset.

PBMC cultures, mRNA analysis and western blotting
PBMCs were purified from buffy coats of anonymous blood donors by density gradient centrifugation and cultured for 72 h in complete RPMI-1640 media. Western blotting was carried out from the collected samples according to standard protocols with reducing (SDS) conditions. Forty microgram of cell lysate from each sample was used and treated with the Complete Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Germany) according to the manufacturer’s instructions. Membranes were stained with 1 μg/ml mouse anti-human IL18RAP (clone 4G4, Abnova, Taiwan), and after stripping with anti-beta-actin antibody for loading control (clone 8226, Abcam, UK). HRP-conjugated goat anti-mouse IgG (Abcam, UK) was used as secondary antibody for detection. Statistical comparison between the carriers and non-carriers of rs917997 allele A was performed using GraphPad Prism 4. A single sample had the genotype AA, it was grouped with the heterozygotes in the analysis.

In a separate experiment, cryopreserved PBMCs from 38 healthy donors were cultured for 22 h in X-VIVO15 media (Lonza, USA) with anti-CD3–anti-CD28 beads (Invitrogen, UK) at a ratio of 1:5 beads:cell with/without IL18 (R&D systems, UK) at doses 0, 1, 10, 100 ng/ml. All donor
samples were set up in culture in a single experiment on day 1, to eliminate experiment to experiment, and batch to batch variation. Cell cultures were triplicate for each donor/condition in 96 well plates. IFN-gamma was measured by ELISA in supernatants (E-Bioscience), and mean of triplicate culture wells analysed. Statistical comparison between the three rs917997 genotype stratified groups was performed using a curve fit regression analysis accounting for dose-response (GraphPad Prism 4).

**Immunohistochemistry**

IL18RAP protein expression was analysed by staining formalin-fixed, paraffin-embedded biopsy samples from untreated and treated patients with coeliac disease and controls with 5 μg/μl mouse anti-human IL18RAP (clone 4G4, Abnova, Taiwan). 5 μg/μl mouse IgG2a kappa (clone MG2a-52, Abcam, UK) was used as an isotype control on each slide. The slides were heated in a 95°C water bath in 10 mmol/l citrate buffer, pH 6.0, for 1 min+1 min, and immunohistochemistry was performed using the ABC method with Vectastain Elite ABC kit (Vector Laboratories, CA, USA).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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