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ORIGINAL ARTICLE ***Reproductive genetics***

Effect of *IL12A* and *IL12B* polymorphisms on the risk of *Chlamydia trachomatis*-induced tubal factor infertility and disease severity

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BACKGROUND: Interleukin-12 (IL-12) and related cytokines induce activation and differentiation of T cells. Our aim was to investigate the associations between genetic differences in IL-12-family cytokines and the pathogenesis of chlamydial disease.

METHODS: The final study population consisted of 100 women with *Chlamydia trachomatis*-induced tubal factor infertility (TFI) and 125 pregnant women as controls. Three single nucleotide polymorphisms (SNPs) of *IL12A* and seven SNPs of *IL12B* genes were determined from isolated DNA using the Sequenom system with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

RESULTS: We found that the *IL12B* SNP rs3212227 was associated with both susceptibility and severity of TFI. The minor allele C was rare and only one CC homozygote was found among the controls. AC heterozygotes were more common among TFI cases than among controls ($P = 0.009$) and were associated with increased risk of TFI [odds ratios (OR) = 2.44, 95% confidence intervals (CI) = 1.23–4.87]. Carrying the minor allele C was also associated with disease severity (P for trend = 0.008) and moderate (OR = 2.51, 95% CI = 1.06–5.95) and severe tubal damage (OR = 2.73, 95% CI = 1.15–6.52).

CONCLUSIONS: The results suggest that variation in the *IL12B* gene partly explains inter-individual differences in disease susceptibility and severity.

Key words: IL-12 / disease susceptibility / disease severity / chlamydial infection / *C. trachomatis*

Introduction

Persistent or repeat *Chlamydia trachomatis* infection and consequent inflammation can damage Fallopian tubes and are associated with reproductive disorders, including pelvic inflammatory disease, infertility and ectopic pregnancy (Paavonen and Eggert-Kruse, 1999). Host immunogenetic factors may partly explain why some women are more susceptible to the development of tissue damage during infection while most infected women do not develop clinical complications (Morré *et al.*, 2009; Öhman *et al.*, 2009).

Interferon-gamma (IFN-γ) is critical in the activation of cell-mediated immune responses against viral and intracellular infections (Boehm *et al.*, 1997), and also against *C. trachomatis*, an obligatorily intracellular pathogen (Rottenberg *et al.*, 2002). In a previous study, we found an association between the *IFNG* +874 single nucleotide polymorphism (SNP) and the intensity of the *C. trachomatis*-specific cell-mediated immune response, although the differences between genotype groups were small (Öhman *et al.*, 2011). However, associations between the studied SNP and disease susceptibility or severity were not found. Despite this, the genotype associated with a lower lymphocyte proliferation (LP) response was more common among infertile women with severe tubal damage than among cases with minor or moderate tubal damage (Öhman *et al.*, 2009). These results suggest that other factors that regulate IFN-γ production, such as interleukin-12 (IL-12), may be of interest.

Production of IFN-γ from NK cells and lymphocytes is induced by IL-12 which acts as an antagonist to IL-10 (Boehm *et al.*, 1997). IL-12 and IL-10 are both mainly produced by monocytes, macrophages and

dendritic cells, which are the first leukocytes to interact with pathogens. Through antigen presentation and cytokine expression, they initiate and coordinate the development of antigen-specific cell-mediated immune response (O'Garra and Murphy, 2009). Monocytes have an important role in the regulation of the cell-mediated immune response during *C. trachomatis* infection by secreting IL-10. SNPs in the promoter region of the *IL10* gene are associated with inter-individual variation in IL-10 expression, intensity of the cell-mediated response and susceptibility to severe tubal damage (Öhman *et al.*, 2006, 2009).

Endocervical IL-12 levels are increased in women infected with *C. trachomatis* (Wang *et al.*, 2005). We have previously found an association between the intronic *IL12B* SNP rs2853694 and the intensity of the *C. trachomatis*-specific cell-mediated immune response (Öhman *et al.*, 2010). This suggests that the cytokine has an active role in the immune cascade in chlamydial infection and that polymorphisms within or flanking its coding gene can affect the outcome. IL-12 is a heterodimeric cytokine, encoded by two separate genes; i.e. *IL12A* which encodes subunit p35 and *IL12B* which encodes subunit p40. Subunit p40 is a common building block of IL-12 and IL-23 cytokines (Trinchieri, 2003). SNPs of both *IL12A* and *IL12B* genes have been identified and some of them have been associated with infectious diseases. In particular, the SNP marker rs3212227 of *IL12B* has been linked with recurrent chlamydial infections (Geisler *et al.*, 2010) and with susceptibility to psoriasis (Cargill *et al.*, 2007; Nair *et al.*, 2008).

The role of variation in IL-12 coding genes in the pathogenesis of *C. trachomatis*-induced tubal damage is not known. We therefore selected three SNPs of the *IL12A* gene and seven of the *IL12B* gene to study the associations between these polymorphisms and susceptibility to, and the severity of, *C. trachomatis*-induced tubal damage.

Materials and Methods

The study population

The study population consisted of 163 women (median age 33, range: 23–40 years) who attended the Infertility Clinic of the Department of Obstetrics and Gynecology, Helsinki University Hospital during 1990–2005 and had accurate laparoscopic evaluation of tubal factor infertility (TFI). A history of past *C. trachomatis* infection was analysed in these cases by assessing both cell-mediated immunity and antibody responses to *C. trachomatis* elementary bodies (EB) and to CHSP60 antigens. Serum for antibody analysis was available from all 163 TFI cases and cell samples for LP testing were available from 72 of these cases. However, 26 TFI cases did not have an immune marker of past *C. trachomatis* infection and were excluded. The control group consisted of 179 pregnant women (median age 36, range: 27–44) from the Helsinki area, whose samples were provided by the Finnish Maternity Cohort (FMC) serum bank collected in 2006.

Due to a low quantity of DNA for some of the samples, the genotyping failures accumulated to some subjects. The subjects who had three or more missing SNP genotypes (54 controls and 37 TFI cases) were excluded from further analysis. The study population for genetic association analysis therefore contained 125 controls and 100 TFI cases.

The TFI cases were grouped into three categories according to the severity of tubal damage, following the classification of Hull and Rutherford (Rutherford and Jenkins, 2002). Minor damage with proximal or distal tubal occlusion but no tubal distension and at most flimsy adhesions was found in 24 of the 100 cases with TFI, moderate damage with unilateral sactosalpinx or moderate tubal adhesions was found in 41 cases and severe damage with bilateral sactosalpinx or extensive adhesions was found in 35 cases.

Genotyping of cytokine polymorphism

Leukocytes, blood clots and serum were used as DNA source material. The DNA was extracted from leukocytes by using a guanidine hydrochloride method. A MagNA Pure LC instrument (Roche Diagnostics) was used for DNA extraction from blood clots and serum. For blood clots, a DNA Isolation Kit—Large Volume (Roche Diagnostics) was used, following the DNA LV Cells Protocol, and for serum a Total Nucleic Acid Isolation Kit—Large Volume (Roche Diagnostics) was used, following the Total NA/LV Serum Plasma protocol. The quantity of DNA in serum samples was low and contributed to genotyping failure in some samples.

IL12A and *IL12B* SNPs were determined using the Sequenom system with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Three SNPs of *IL12A* (rs2243123, rs583911, rs568408) and seven of *IL12B* (rs3212227, rs1003199, rs11574790, rs919766, rs2569253, rs2853694 and rs6887695) genes were studied (Table I and Fig. 1).

A candidate gene approach was used and the selected SNPs were chosen because of their potential role effect on IL-12 in antichlamydial immunity and chlamydial pathogenesis. The study included particularly those SNPs for which evidence of functionality or an association with other diseases existed, and a few that were involved in more speculative areas.

Chlamydia trachomatis-specific immune responses

A history of previous *C. trachomatis* infection was evaluated by assessing *C. trachomatis*-specific humoral and cell-mediated immunity, as described earlier (Tiitinen *et al.*, 2006). *Chlamydia trachomatis*- and chlamydial heat shock protein 60 (CHSP60)-specific IgG serum antibodies were assayed by ELISA kits (Medac Diagnostika, Hamburg, Germany). The antibody results were recorded as mean absorbance of duplicate samples at 450 nm. The threshold for a positive antibody level [mean optical density (OD) value of negative controls 0.350] was OD $>$ 0.4. Peripheral blood mononuclear cells (PBMCs) were isolated and cell-mediated immunity was assessed by measuring LP responses to *C. trachomatis* EB strains E and F and to CHSP60 (kindly provided by Professor Richard Morrison), as previously described (Kinnunen *et al.*, 2002). The LP responses were measured as counts per minute (cpm) of incorporated [3H]-methyl thymidine, using a liquid scintillation counter (Wallac, Turku, Finland), and the results were expressed as median stimulation indices (SI = median cpm in the presence of antigen divided by median cpm in its absence) of triplicate cultures. The viability and reactivity of the cultured PBMCs were controlled in each experiment by requiring SI of $>$10 in response to the control Pokeweed mitogen.

Statistical analyses

A χ^2 test for trend was used to test genotype distribution trends. Genotypes conferring susceptibility are presented in terms of odds ratios (ORs) with 95% confidence intervals (95% CIs) (SPSS 18.0 software). The two-sample test of proportions was performed to compare genotype frequencies between cases and controls (Stata 5.0 statistical software). Haploview 4.2 software (Barrett *et al.*, 2005) was used to analyse the linkage disequilibrium (LD) between SNPs in *IL12A* and *IL12B* genes.

Sample size calculations in Stata and Epi Info suggested, for example, that a study of 100 cases and controls would have 80% power to detect a marker which is present in 30% of cases and 10% of controls, with 99% confidence, i.e. allowing for five multiple comparisons. If the marker were present in 40% of cases, this could be detected with 80%

Table I The sequences for the PCR primers, the extension primers and the PCR product length for the SNPs used are shown.

SNP ID	Second PCR primer	First PCR primer	Amplicon (bp)	Extension primer
rs2243123	ACGTTGGATGAGTCTTTCTCATGCTGCTCC	ACGTTGGATGGGTGAATCCAGTGTAAGCAG	100	ctCTGCTCCCTCTGGAC
rs583911	ACGTTGGATGAGCTTGTCTTAAGGGTTTGC	ACGTTGGATGCAAGTATAACTTCTAAAGGG	100	GCATGTTTGTTATATCCATCA
rs568408	ACGTTGGATGGTCAAAAATACTTGATCAG	ACGTTGGATGGGATTAAGAACTAGGGAGGG	97	GTCAAAAATACTTGATCAGAGGTAT
rs3212227	ACGTTGGATGGGATCACAATGATATCTTTGC	ACGTTGGATGCTGATTGTTTCAATGAGC	85	cccTATCTTTGCTGTATTTGTATAGTT
rs1003199	ACGTTGGATGGCCTAGAATGACTTTCTTGAC	ACGTTGGATGAACCTAGAGGAAGAGGTAAG	99	CCATGATATATAAAACACAGATACC
rs11574790	ACGTTGGATGCAGCTTACCCTGTGACTATG	ACGTTGGATGCCGTGAAGACTCTATCTTTC	98	GCCAAGGGGTCTTCA
rs919766	ACGTTGGATGGCTACAATCACTAGGAACTC	ACGTTGGATGGGGTCAGAAGAGCTGAAGT	97	AGGAACTCTCTCCCCAA
rs2569253	ACGTTGGATGAGTTTCTCTGTACAGTTGGC	ACGTTGGATGCTGCCACACAGTAAATTCGG	94	tGTTGGCTGACTCCTC
rs2853694	ACGTTGGATGCAAGGTGCAATTTCAGCAAG	ACGTTGGATGTTCCTGAAGCCTCATAGCAC	100	TTGTAGCTTTGAATTCTCC
rs6887695	ACGTTGGATGGTTTGAGAGAAGCAGTGTAG	ACGTTGGATGGTCACAAGCGTAGTAAATGG	98	GCAGTGTAGTGTAGTGGT

power and 99.9% confidence, i.e. allowing for 50 independent comparisons. For a stronger effect than this, even more comparisons are allowed, for example, a marker present in 50% of cases gives adequate power, even if 500 independent comparisons were made. So we concluded that the study had adequate power to detect polymorphisms that were strongly associated with disease, even allowing for multiple comparisons in a conservative way.

Results

Genotyping

Overall genotyping performance in the final study population was 83.6–99.6% depending on the SNP.

In the control group, all 10 studied polymorphisms were in Hardy–Weinberg equilibrium (HWE). Two of the studied *IL12B* SNPs, rs11574790 ($P = 0.001$) and rs919766 ($P = 0.004$), which exhibited strong LD (Fig. 1), were not in HWE in the TFI cases.

Immune markers

Serum for antibody analysis was available from all 163 TFI cases and from 179 controls. Cells for LP analysis were available from 72 TFI cases. Cells from controls were not available. The immune responses are presented in Table II. We found that 26 of the 163 (16%) TFI cases were not reactive to any of the studied markers and these were excluded from the genetic susceptibility analysis because of lack of *C. trachomatis* attributable evidence.

The prevalence of *C. trachomatis*-specific IgG antibodies was 4.4-fold higher and the prevalence of CHSP60-specific IgG antibodies was 2.9-fold higher in TFI cases than in the controls. Presence of chlamydial antibodies was associated with an increased risk for TFI (Table II).

Genetic associations with disease susceptibility

Genotype distributions in the TFI cases and controls were compared to analyse the possible risk factors of disease susceptibility (Table III). For the *IL12B* gene, a divergent genotype distribution was found in connection with the SNP rs3212227. The minor allele C was rare; among TFI cases no CC homozygotes were found. The AC genotype was more common in the TFI women than in controls ($P = 0.009$) and was associated with an increased risk for TFI (OR = 2.44, 95% CI = 1.23–4.87).

Genetic associations with disease severity

The relationships between *IL12A* and *IL12B* genotypes and the degree of tubal damage were studied. Only the *IL12B* SNP rs3212227 was significantly associated with disease severity (P for trend = 0.008). Data on SNP rs3212227 are shown in Table IV. The AC and CC genotypes were combined because of the small number of CC homozygotes. Subjects carrying the C allele had an increased risk of moderate (OR = 2.51, 95% CI = 1.06–5.95) and severe tubal damage (OR = 2.73, 95% CI = 1.15–6.52).

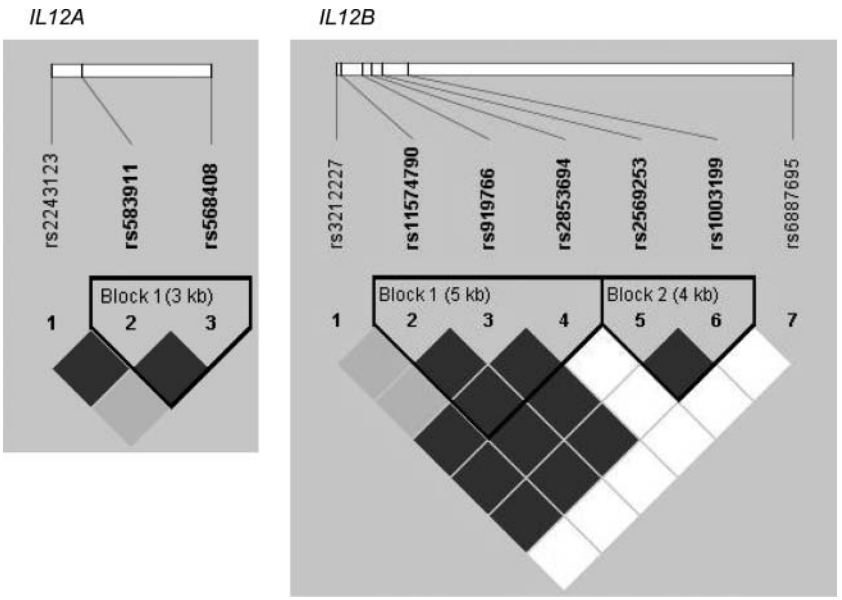

Figure 1 LD maps for the markers genotyped in the *IL12A* and *IL12B* genes. Black squares represent high LD; white squares represent low LD. Locations of SNPs are shown.

Discussion

In the present study, we investigated the role of *IL12A* and *IL12B* polymorphisms in the pathogenesis of *C. trachomatis*-induced TFI in a unique and well-characterised population that included accurate laparoscopic evaluation of the cases and uniform classification of the degree of tubal damage. This setting enabled us to study the association between SNPs and the severity of disease manifestations, in addition to disease susceptibility and gave an excellent opportunity to study the role of genes in pathogenesis.

We found that one of the studied SNPs, rs3212227 of *IL12B*, was associated with TFI and the severity of tubal damage. This SNP is recognized as a susceptibility correlate in many inflammatory conditions from infections to autoimmune diseases (Cargill *et al.*, 2007; Nair *et al.*, 2008; McGovern *et al.*, 2009; Phawong *et al.*, 2010; Wang *et al.*, 2010). The other SNPs studied were not associated with susceptibility to TFI or severity of tubal damage.

The immune response to genital *C. trachomatis* infection is complex (Loomis and Starnbach, 2002). It seems that a delicate balance between pro- and anti-inflammatory cytokines is required for infection clearance and at the same time to avoid immune-mediated pathology (Debattista *et al.*, 2003). *Chlamydia trachomatis* infection increases endocervical IL-12 production *in vivo* (Wang *et al.*, 2005) indicating that IL-12 has an active role in the immune cascade provoked by *C. trachomatis*. The SNP rs3212227 has also been associated with recurrent *C. trachomatis* infections (Geisler *et al.*, 2010). Particularly, the minor allele C, which in our data increased the risk and severity of TFI, was associated with recurrent *C. trachomatis* infections. As repeated infections and continuous inflammation increase the risk of tubal damage, our findings are in agreement with those by Geisler *et al.* In contrast, for inflammatory autoimmune illnesses, such as psoriasis and Crohn's disease, the common genotype AA has been linked to disease susceptibility (Cargill *et al.*, 2007; Nair *et al.*, 2008; McGovern *et al.*, 2009).

The results of this study suggest that *IL12B* is involved in the disease process, but more data are needed to investigate the mechanism

Table II Humoral and cell-mediated reactivity to *C. trachomatis*-specific antigens in cases of TFI and control women.

	Controls, *n* (%)	TFI cases, *n* (%)	OR (95% CI)
IgG *C. trachomatis*			
Negative	145 (86.8)	69 (42.3)	1
Positive	22 (13.2)	94 (57.7)	8.98 (5.20–15.49)
IgG CHSP60			
Negative	135 (80.8)	73 (44.8)	1
Positive	32 (19.2)	90 (55.2)	5.20 (3.17–8.52)
SI *C. trachomatis* E			
Negative	N/A	19 (26.4)	N/A
Positive		53 (73.6)	
SI *C. trachomatis* F			
Negative	N/A	16 (30.8)	N/A
Positive		36 (69.2)	
SI CHSP60			
Positive	N/A	39 (54.2)	N/A
Negative		33 (45.8)	

The risk of TFI among *C. trachomatis*- and CHSP60-IgG-positives was estimated using logistic regression. ORs with 95% CIs are presented.
SI, stimulation index.

Table III Distribution of cytokine genotypes in cases with chlamydial TFI and controls.

Gene, SNP	*n* (%)		*P*-test of proportion	χ^2 for trend (*P*-value)	OR (95% CI)
	TFI women	Controls			
IL12A, rs2243123					
TT	53 (55.2)	64 (54.2)	0.887	0.868	Reference
CT	35 (36.5)	47 (39.8)	0.614		0.90 (0.51–1.59)
CC	8 (8.3)	7 (5.9)	0.494		1.38 (0.47–4.06)
IL12A, rs583911					
AA	33 (38.8)	34 (32.1)	0.331	0.468	Reference
AG	33 (38.8)	47 (44.3)	0.443		0.72 (0.38–1.39)
GG	19 (22.4)	25 (23.6)	0.841		0.78 (0.36–1.68)
IL12A, rs568408					
GG	73 (76.8)	89 (73.6)	0.58	0.664	Reference
AG	18 (18.9)	27 (22.3)	0.545		0.81 (0.42–1.59)
AA	4 (4.2)	5 (4.1)	0.977		0.98 (0.25–3.77)
IL12B, rs3212227					
AA	66 (69.5)	89 (84.0)	0.015	0.028	Reference
AC	29 (30.5)	16 (15.1)	0.009		2.44 (1.23–4.87)
CC	0 (0)	1 (0.9)	0.343		
IL12B rs11574790					
CC	86 (86.9)	106 (84.8)	0.66	0.858	Reference
CT	10 (10.1)	19 (15.2)	0.259		0.65 (0.29–1.47)
TT	3 (3.0)	0 (0)	0.05		
IL12B rs919766					
AA	85 (85.9)	106 (84.8)	0.824	0.716	Reference
AC	11 (11.1)	19 (15.2)	0.372		0.72 (0.33–1.60)
CC	3 (3.0)	0 (0)	0.05		
IL12B rs2853694					
CC	31 (32.6)	36 (31.0)	0.804	0.782	Reference
AC	41 (43.2)	57 (49.1)	0.386		0.84 (0.45–1.56)
AA	23 (24.2)	23 (19.8)	0.443		1.16 (0.55–2.46)
IL12B rs2569253					
CC	28 (34.1)	48 (45.3)	0.123	0.095	Reference
CT	41 (50.0)	47 (44.3)	0.441		1.50 (0.80–2.80)
TT	13 (15.9)	11 (10.4)	0.265		2.03 (0.80–5.13)
IL12B rs1003199					
TT	31 (31.6)	48 (41.4)	0.141	0.315	Reference
CT	52 (53.1)	50 (43.1)	0.146		1.61 (0.89–2.92)
CC	15 (15.3)	18 (15.5)	0.966		1.29 (0.57–2.93)
IL12B rs6887695					
GG	58 (58.6)	70 (56.5)	0.749	0.856	Reference
CG	34 (34.3)	46 (37.1)	0.67		0.89 (0.51–1.57)
CC	7 (7.1)	8 (6.5)	0.855		1.06 (0.36–3.09)

The two-sample test of proportions was performed to compare genotype frequencies in cases and controls. A χ^2 test for trend was used to analyse a possible trend between disease and genotype. ORs with 95% CIs are shown.

behind the genotype association. Differences in *IL12B* may be reflected by individual variation in levels of IL-12 and IL-23 cytokines, which are composed of a common IL-12p40 and distinct IL-12p35 or IL-23p19 subunits, respectively. The IL-12 effect is mediated through the IFN-γ pathway necessary to defend against *C. trachomatis*. The effects of IL-23 are less well known, but it seems to play a key role in the induction of Th17 cells. These cells induce production of proinflammatory cytokines and might be responsible for the inflammation-

Table IV Comparison of genotype distribution and risk estimates of different degrees of tubal damage.

IL12B, rs3212227	Controls, *n* (%)	Severity of tubal damage (Hull and Rutherford)			χ^2 for trend (*P*–value)	Minor damage	Moderate damage	Severe damage
		Minor, *n* (%)	Moderate, *n* (%)	Severe, *n* (%)		OR (95% CI)	OR (95% CI)	OR (95% CI)
AA	89 (84.0)	18 (78.3)	25 (67.6)	23 (65.7)	0.008	1	1	1
AC or CC	17 (16.0)	5 (21.7)	12 (32.4)	12 (34.3)		1.45 (0.48–4.45)	2.51 (1.06–5.95)	2.73 (1.15–6.52)

A χ^2 test for trend was used to analyse the relationship between genotype and disease severity. ORs with 95% CIs are shown.

driven pathogenesis instead of the Th1 subset (Goriely *et al.*, 2009). Besides effects mediated through IL-12 and IL-23, it is also possible that IL-12p40 has a biological activity on its own, as it has been reported that IL-12p40 can act as an antagonist of IL-12 *in vitro* (Zhang and Wang, 2008) and thus mediate an anti-inflammatory effect. This hypothesis is also supported by Müller-Berghaus *et al.* (2004) who showed that elevated levels of IL-12p40 were associated with lower levels of IL-12p70.

Although the mechanism behind the connection is still unsolved, the disease associations and the literature (Cargill *et al.*, 2007; Nair *et al.*, 2008; McGovern *et al.*, 2009; Geisler *et al.*, 2010) suggest that the *IL12B* rs3212227 allele C is linked with a weaker, and allele A with a stronger, inflammatory response. Subjects with the AA genotype seem to cope with *C. trachomatis* infection better than subjects who carry the allele C. This might be a result of more efficient Chlamydia clearance and also protective immunity.

In conclusion, we showed evidence here that a transcribed marker in the *IL12B* gene is associated with susceptibility to *C. trachomatis*-induced TFI and with severe disease manifestation. The results may explain some individual variations in the manifestations of *C. trachomatis* disease. Further studies dealing with the *IL12B* polymorphism and the immune response are still needed to reveal the mechanism behind the disease association and to increase understanding of the pathogenesis of the disease, as this will be needed in the design of new therapeutic strategies.

Authors' roles

The study was designed by H.Ö. and H.-M.S. The study population was collected and laparoscopic evaluations were performed by A.T., M.H. and J.P. The selection of SNPs and design of their analysis was done by A.N., R.B., J.R. and L.-L.J. The SNPs were genotyped by L.-L.J. and H.Ö. The data were analysed by H.Ö. The manuscript was drafted by H.Ö., H.-M.S. and R.B. All authors contributed to the final version of the manuscript. The final version was seen and approved by all authors.

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Conflict of interest

None declared.

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