EXTENDED REPORT

Th2 cytokine genotypes are associated with a milder form of primary Sjögren's syndrome

M Pertovaara, J Antonen, M Hurme

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See end of article for authors' affiliations

Correspondence to: Dr Marja Pertovaara, Department of Internal Medicine, Section of Rheumatology, Tampere University Hospital, PO Box 2000, FIN-33521 Tampere, Finland; marja.pertovaara@pshp.fi

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Background: Immunohistological studies on salivary and lacrimal glands have yielded conflicting results on the Th1/Th2 balance in primary Sjögren's syndrome (pSS).
Objective: To establish whether pSS is a Th1 or Th2 directed autoimmune disease by analysing the polymorphism of the genes encoding for cytokines involved in the regulation of Th1/Th2 differentiation.
Methods: The polymorphisms of the genes encoding for interleukin 4 (IL4) −590 C/T, interleukin 13 (IL13) +2044 G/A, and interferon γ (IFNG) +874 T/A were analysed in 63 white Finnish patients with pSS (61 female, two male) and in 63 healthy controls. The clinical and immunological data on the pSS patients were analysed in relation to these cytokine gene polymorphisms.
Results: There were no significant differences in the genotype or allele frequencies of IL4 −590, IL13 +2044, or IFNG +874 between pSS patients and controls. The erythrocyte sedimentation rate and concentrations of serum IgA and serum β2 microglobulin were lower in pSS patients carrying the IL4 −590 T allele or the IL13 +2044 A allele than in those not carrying the respective alleles. The IL4 −509 T allele and IL13 +2044 A allele carriers less often had purpura than the corresponding non-carriers.
Conclusions: The frequencies of the cytokine genotypes regulating Th1/Th2 differentiation did not differ between pSS patients and controls. However, the presence of cytokine genotypes with increased susceptibility to atopic and other Th2 diseases was associated with signs of a milder form of pSS. This finding would favour a hypothesis envisaging pSS as primarily a Th1 mediated autoimmune disease.

Primary Sjögren's syndrome (pSS) is a chronic autoimmune exocrinopathy presenting clinically with dry eyes and mouth and various extraglandular symptoms. Hypergammaglobulinaemia and autoantibody production are characteristic features of the disease. Histologically, lymphocyte and plasma cell infiltration in the affected organs is observed. T cell mediated autoimmune responses are currently considered to be key factors in organ destruction in Sjögren's syndrome.[1]

CD4 helper T (Th) cells differentiate into Th1 and Th2 cells, of which the former are primarily responsible for the development of cell mediated immunity, while Th2 cells are involved in antibody production and humoral immune responses. Cytokines direct the immune responses toward a Th1 or Th2 type. Interleukin (IL) 4 and IL13 are key mediators in the Th2 pathway, while interferon gamma (IFNγ), IL2, IL12, and IL18 act as Th1 cytokines. Organ specific autoimmune diseases such as rheumatoid arthritis and diabetes are Th1 mediated, whereas asthma and atopic diseases are examples of classical Th2 diseases.[2 3]

In pSS the data concerning Th1/Th2 differentiation are somewhat conflicting. Although the greater part of the evidence on the cytokines expressed in labial salivary gland tissue samples supports the view that pSS is a Th1 mediated disease,[4–9] studies questioning the predominance of a Th1 response in SS have also been reported.[9 10] Also, Th2 dominance has been shown in lacrimal autoimmune disease in Sjögren's syndrome, albeit only in mouse models.[11 12]

To clarify whether the Th1 or the Th2 responses play the main role in pSS, we decided to study the polymorphisms of the genes encoding cytokines involved in the regulation of Th1/Th2 differentiation. In a well characterised group of white Finnish patients with pSS we examined the polymorphisms of the genes encoding the cytokines regulating the Th1/Th2 balance (IL4 −590 C/T in the promoter region, IL13 +2044 G/A in the coding region, and IFNγ (IFNG) +874 T/A in the promoter region). We chose particularly genes which have been shown to be associated with Th1/Th2 differentiation in several studies (IL4 and IL13 towards Th2 and IFNG towards Th1 differentiation) and are known to be functional, influencing either the serum levels of the corresponding cytokine or the transcriptional activity of the gene.[13–16]

Of these, the allele T of the IL4 −590 gene has been shown to be associated with increased production of IL4,[13] the allele A of the IL13 +2044 gene results in the replacement of the amino acid arginine to glutamine at position 130, which may affect the function of the IL13 protein,[14] and the allele T of IFNG +874 is associated with high production of IFNγ.[15 16]

METHODS

Subjects

All patients fulfilling three or more modified Californian criteria for primary Sjögren's syndrome[17] were selected from the records of patients with sicca symptoms examined in the Department of Internal Medicine, Section of Rheumatology, at Tampere University Hospital, Finland, during the years 1977 to 1992 (n = 111). Salivary flow determinations were not done, and histological findings were graded on the Chisholm–Mason scale,[18] grades 3 and 4 being regarded as diagnostic. Those alive were invited by letter to attend for gene polymorphism determinations and samples for genotyping were obtained after informed consent from 63 pSS patients (61 female, two male; mean (SD) age 60 (12) years, range 29 to 82). The mean disease duration was 9 (4) years (range 3 to 18 years). Sixty one of the subjects also fulfilled the revised American–European consensus group criteria for pSS.[19]

Abbreviations: ANA, antinuclear antibodies; IFNγ, interferon gamma; IL, interleukin; PSS, primary Sjögren's syndrome; RF, rheumatoid factor

Table 1 Demographic, clinical, and immunological characteristics of 63 patients with primary Sjögren's syndrome

Characteristic	
Demographic	
Female:male	61:2
Age (years) (mean (SD))	60 (12)
Disease duration (years) (mean (SD))	9 (4)
Clinical	
Labial salivary gland histological grade 3–4*	46 (73%)
Arthralgia	42 (67%)
Raynaud's symptom	36 (57%)
Recurrent salivary gland swelling	28 (45%)
Latent dRTA	16/45 (36%)
Proteinuria (⩾0.15 g/24 h)	24 (41%)
Arthritis (non-erosive)	13 (21%)
Purpura	12 (19%)
Peripheral nervous system symptoms	11 (18%)
Alveolitis or pulmonary fibrosis	7 (11%)
Pleuritis	7 (11%)
Lymphadenopathy	7 (11%)
Central nervous system symptoms	5 (8%)
Myositis	0
Associated diseases	
Hypothyroidism	9 (14%)
Coeliac disease	7 (11%)
Immunological	
ANA positive	53/62 (86%)
RF positive	45/61 (74%)
anti-SSA antibody positive	43/61 (71%)
anti-SSB antibody positive	32/61 (52%)

Values are n (%) unless specified otherwise.
*Chisholm–Mason scale.[18]
ANA, antinuclear antibodies; dRTA, distal renal tubular acidosis (abnormal ammonium chloride loading test)[20]; RF, rheumatoid factor.

Clinical methods

A careful clinical examination together with an in-depth interview with the patients covering previous and concurrent diseases, previous and current drug treatment, and the duration of pSS had recently been conducted with these patients.[20] Special emphasis was laid on possible extraglandular symptoms of pSS (dermatological, endocrine, gastrointestinal, lymphoproliferative, musculoskeletal, neurological, renal, respiratory, and vascular symptoms). Purpura was defined as a history of typical episodic palpable purpuric lesions in the lower limbs or skin biopsy histology. Lymphadenopathy was defined as lymph node enlargement persistent enough to be an indication for node biopsy. Arthritis was defined as articular swelling observed by a clinician. Peripheral and central neurological symptoms were recorded from the history given by the patients as well as from data on possible neurological investigations from the case histories. The diagnosis of pulmonary fibrosis was based on findings in chest radiographs; the diagnosis of alveolitis had been established by thorough investigations in a pulmonary unit. The presence of distal renal tubular acidosis had been checked for by ammonium chloride loading tests in 45 of the patients and urinary total protein excretions had been measured in 61.[20]

Normal controls

Sixty three healthy Finnish Red Cross Transfusion Service blood donors matched for sex (61 female, two male), ethnic origin (white Finnish), and area of residence (Tampere, Finland) served as a control group for DNA studies of pSS patients. The mean (SD) age of the control subjects was 53 (7) years.

Standard laboratory tests

The standard laboratory tests included erythrocyte sedimentation rate (ESR) and serum concentrations of immunoglobulins IgA, IgG, and IgM. Rheumatoid factor (RF) was determined by laser nephelometry and antinuclear antibodies (ANA) by indirect immunofluorescence using Hep-2 cells. Anti-SS-A and anti-SS-B antibodies were determined by enzyme immunoassay. Serum complement levels (C3 and C4) were measured by laser nephelometry. Serum beta-2 microglobulin was measured by radioimmunoassay (Pharmacia beta-2-micro RIA kit, Pharmacia Diagnostics Uppsala, Sweden).

Genotyping

DNA specimens from whole blood samples were prepared using standard methods. To analyse the base exchange polymorphism at −590 of the IL4 gene (C to T base exchange) the region containing the AvaII polymorphic site at position −590 of the IL4 gene was amplified by polymerase chain reaction (PCR).[21] The oligonucleotides 5′TAA ACT TGG GAG AAC ATG GT 3′ and 5′TGG GGA AAG ATA GAG TAA TA 3′ were used as primers. Fragments were analysed by electrophoresis on 3% agarose stained with ethidium bromide.

Genotyping of the IL13 polymorphism at position +2044 (G/A) was undertaken using the ABI Prism 7000 sequence detection system for both PCR and allelic discrimination (Applied Biosystems, Foster City, California, USA). A commercial kit from Applied Biosystems was used (Assay on Demand, Purchase number 185118655)

The base exchange polymorphism at +874 of the IFNG gene (T/A) was analysed by the amplification refractory mutational system–PCR method (ARMS-PCR) described previously.[15] Genomic DNA was amplified with Thermoprime*PLUS* DNA polymerase (Abgene, Surrey, UK) in two different PCR reactions. Each reaction employed a generic antisense primer 5′-TCAACAAAGCTGATACTCCA-3′ and one of the two allele specific sense primers 5′-TTCTTACAACACAAAATCAAATCT-3′. To confirm the success of PCR amplification, one internal control of 426 base pairs (bp) was amplified using a pair of primers designed from the nucleotide sequence of the human growth hormone (5′-CCTTCCCAACCATTCCCTTA-3′ and 5′-TCACGGATTTCTGTTGTGTTTC-3′). The amplified products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide.

All genotype distributions studied followed the Hardy–Weinberg equilibrium.

Statistical analysis

Unpaired Student's *t* test and χ^2 tests were used in comparisons of continuous and dichotomous variables, respectively. Findings were considered statistically significant at $p<0.05$. Statistical analyses were done with SPSS 10.1 for Windows.

Ethical approval

The study protocol was approved by the ethics committees of Tampere University Hospital and the Finnish Red Cross Transfusion Service, Helsinki, Finland.

RESULTS

Allele and genotype frequencies of the cytokines regulating the Th1/Th2 balance

The demographic and clinical characteristics of the patients are set out in table 1. The allele or genotype frequencies of IL4 −590, IL13 +2044, and IFNG +874 did not differ between pSS patients and healthy control subjects (table 2).

IL4 −590 gene polymorphism

The level of blood ESR was lower in pSS patients with the IL4 −590 T allele than in those without it. The frequencies of

Table 2 Genotype, carrier, and allele frequencies of IL4 −590, IL13 +2044, and IFNG +874 in 63 patients with primary Sjögren's syndrome and 63 healthy control subjects

Genotype	pSS patients	Control subjects	p Value
***IL4 −590* genotypes**			
CC	26 (41)	32 (51)	
CT	31 (49)	26 (41)	
TT	6 (10)	5 (8)	0.563
T allele carrier	37 (59)	31 (49)	0.284
Allele frequencies			
Allele C	0.659	0.714	
Allele T	0.341	0.286	0.342
***IL13 +2044* genotypes (n = 59)**			
GG	24 (41)	28 (45)	
AG	30 (51)	31 (49)	
AA	5 (8)	4 (6)	0.859
A allele carrier	35 (59)	35 (56)	0.674
Allele frequencies			
Allele A	0.339	0.310	
Allele G	0.661	0.690	0.623
***IFNG +874* genotypes**			
AA	27 (43)	23 (37)	
AT	28 (44)	31 (49)	
TT	8 (13)	9 (14)	0.767
T allele carrier	8 (13)	9 (14)	0.794
Allele frequencies			
Allele A	0.651	0.611	
Allele T	0.349	0.389	0.269

Statistical analysis by χ^2 test.
IFNG, interferon γ gene; IL, interleukin; pSS, primary Sjögren's syndrome.

positive results for RF, ANA, or anti-SSA or anti-SSB antibodies did not differ between pSS patients carrying the IL4 −590 T allele and those not carrying this determinant. There were no differences in the levels of serum immunoglobulins IgG or IgM between the patient groups. Serum IgA concentration was significantly lower ($p = 0.049$) and serum β2 microglobulin concentration tended to be lower ($p = 0.063$) in pSS patients carrying the IL4 −590 T allele than in non-carriers (table 3).

Purpura occurred in only 11% of the pSS patients carrying the IL4 −590 allele T *v* 31% of non-carriers ($p = 0.047$). No differences were observed in the frequencies of salivary gland swelling or such extraglandular manifestations of pSS as a history of arthralgia, arthritis, Raynaud's symptom, lymphadenopathy, pleuritis, alveolitis or pulmonary fibrosis, peripheral or central nervous system symptoms, or the presence of overt distal renal tubular acidosis. However, when the pSS patients were grouped by the total number of extraglandular manifestations present into those with 0–2 and those with more than 2, the pSS patients carrying the IL4 −590 T allele had significantly fewer extraglandular manifestations than those not carrying this allele (table 3). Neither the histological grade in labial salivary gland biopsies nor the number of diagnostic criteria for pSS differed in patients with or without the IL4 −590 T allele. There were no significant differences in the previous use of disease modifying

Table 3 Clinical and laboratory characteristics of patients with primary Sjögren's syndrome according to IL4 −590 allele T carriage or IL13 +2044 allele A carriage

	IL4 T+* (n = 37)	IL4 T−† (n = 26)	p Value	IL13 A+* (n = 35)	IL13 A−† (n = 24)	p Value
Age (years)	60 (11)	61 (12)	0.716	59 (11)	60 (13)	0.576
Disease duration (years)	9 (4)	10 (4)	0.243	8 (4)	10 (4)	0.064
ESR (mm/h)	26 (18)	39 (22)	**0.010**	27 (16)	39 (24)	**0.031**
Serum IgA (g/l)	2.8 (1.1)	3.9 (2.4)‡	**0.049**	2.8 (1.0)	3.8 (2.5)¶	0.084
Serum IgG (g/l)	18.2 (6.4)	19.6 (8.1)‡	0.428	18.8 (6.2)	19.4 (8.4)¶	0.793
Serum IgM (g/l)	1.4 (0.6)	1.5 (1.0)‡	0.629	1.5 (0.5)	1.5 (1.0)¶	0.838
Serum C3 (g/l)	1.12 (0.22)	1.16 (0.29)‡	0.544	1.12 (0.21)	1.16 (0.29)¶	0.534
Serum C4 (g/l)	0.18 (0.08)	0.19 (0.07)‡	0.776	0.17 (0.07)	0.19 (0.07)¶	0.255
Serum β2 microglobulin (mg/l)	2.7 (0.8)	3.2 (1.3)‡	0.063	2.6 (0.7)	3.3 (1.4)¶	**0.026**
RF positivity	26 (70%)	19 (79%)§	0.440	26 (74%)	16 (73%)**	0.897
ANA positivity	32 (87%)	21 (84%)‡	0.785	30 (86%)	20 (87%)¶	0.893
Anti-SSA positivity	25 (69%)	18 (72%)‡	0.830	25 (74%)††	16 (70%)¶	0.744
Anti-SSB positivity	18 (50%)	14 (56%)‡	0.644	19 (56%)††	12 (52%)¶	0.783
>2 Extraglandular manifestations‡‡	2 (5%)	8 (31%)	**0.007**	4 (11%)	5 (21%)	0.324

Values are mean (SD) or n (%). Statistical analysis by Student *t* test and χ^2 test. Significant values in bold.
*Number of pSS patients carrying the IL4 −590 T allele or the IL13 +2044 A allele, respectively.
†Number of pSS patients not carrying the IL4 −590 T allele or the IL13 +2044 A allele, respectively
‡n = 25, §n = 24, ¶n = 23, **n = 22, ††n = 34.
‡‡Of the following: arthritis, Raynaud's symptom, purpura, peripheral or central nervous system symptoms, alveolitis or pulmonary fibrosis, pleuritis, pericarditis, myositis, lymphadenopathy, overt renal tubular acidosis
ANA, antinuclear antibodies; C, complement; ESR, erythrocyte sedimentation rate; IFNG, interferon γ; IL, interleukin; pSS, primary Sjögren's syndrome; RF, rheumatoid factor.

Table 4 Clinical and laboratory characteristics of patients with primary Sjögren's syndrome carrying or not carrying the combination of IL4−590 allele T and IL13+2044 allele A

Characteristic	IL4T+/IL13A+ (n = 28)*	IL4T−/IL13A− (n = 17)†	p Value
Age (years)	59 (12)	61 (14)	0.608
Disease duration (years)	8 (4)	10 (4)	0.123
ESR (mm/h)	25 (18)	44 (25)	**0.005**
Serum IgA (g/l)	2.8 (1.1)	4.4 (2.9)‡	**0.045**
Serum IgG (g/l)	18.3 (6.4)	19.6 (9.0)‡	0.621
Serum IgM (g/l)	1.5 (0.6)	1.6 (1.2)‡	0.658
Serum C3 (g/l)	1.11 (0.21)	1.19 (0.32)‡	0.360
Serum C4 (g/l)	0.17 (0.07)	0.20 (0.08)‡	0.255
Serum β2 microglobulin (mg/l)	2.7 (0.7)	3.6 (1.5)‡	**0.025**
RF positivity	21 (75%)	12 (80%)§	0.711
ANA positivity	24 (86%)	13 (81%)‡	0.697
Anti-SSA positivity	19 (70%)¶	11 (69%)‡	0.911
Anti-SSB positivity	15 (56%)¶	9 (56%)‡	0.965
>2 Extraglandular manifestations**	2 (7%)	5 (29%)	**0.046**

Values are n (%) or mean (SD). Statistical analysis by Student *t* test and χ^2 test. Significant values in bold.
*Number of pSS patients carrying simultaneously the IL4 −590 T allele and the IL13 +2044 A allele.
†Number of pSS patients not carrying the IL4 −590 T allele and not carrying the IL13 +2044 A allele.
‡n = 16, §n = 15, ¶n = 27.
**Of the following: arthritis; Raynaud's symptom, purpura, peripheral or central nervous system symptoms, alveolitis or pulmonary fibrosis, pleuritis, pericarditis, myositis, lymphadenopathy, or overt renal tubular acidosis.
ANA, antinuclear antibodies; ESR, erythrocyte sedimentation rate; IL, interleukin; pSS, primary Sjögren's syndrome; RF, rheumatoid factor.

antirheumatic drugs or corticosteroids between the groups. The frequencies of associated diseases such as hypothyroidism and coeliac disease did not differ between the patient groups (data not shown).

IL13 +2044 gene polymorphism

The patients carrying the IL13 +2044 allele A had significantly lower ESRs and serum β2 microglobulin than those not carrying this allele (table 3). Purpura occurred in only 9% of the pSS patients carrying the IL13 allele A *v* 38% of non-carriers (p = 0.007).

Carriers of IL4 −590 T allele and IL13 +2044 A allele

The IL13 +2044A and the IL4 −590 loci are known to be in strong linkage disequilibrium.[22] In our material, 80% of the pSS patients carrying the IL13 +2044 A allele carried the IL4 −590 T allele compared with 29% of non-carriers, p<0.0001.

The laboratory findings in the pSS patients carrying both the IL4 −590 T and the IL13 +2044 A allele are presented in table 4. Purpura occurred in only 14% of the pSS patients carrying the IL4 allele T and the IL13 allele A (n = 28) *v* 35% of the corresponding non-carriers (n = 17) (p = 0.004). They also had a lower total number extraglandular manifestations than those not carrying these alleles (table 4).

IFNG +874 gene polymorphism

There were no significant differences in any of the laboratory variables or the clinical features described above between pSS patients with the IFNG +874 allele T compared with those without (data not shown).

DISCUSSION

The data concerning the Th1/Th2 balance in pSS patients are somewhat conflicting. Both Th1 and Th2 cytokine levels have been found to be raised in the peripheral blood of pSS patients compared with normal subjects.[23] On the other hand, a decreased ratio of peripheral blood Th1/Th2 producing cells has been reported in pSS patients.[24 25] In normal subjects it has been shown that the lymphocytes infiltrating the labial salivary glands have the potential to produce both Th1 and Th2 cytokines.[6] In the majority of studies on patients with pSS the expression of Th1 cytokines has dominated in labial salivary gland biopsies and tissue samples have totally lacked the expression of Th2 cytokines IL4 and IL13.[4–9] However, in two studies IL4 expression in some and IL13 expression in the majority of Sjögren's syndrome salivary gland biopsies were also found, arguing against the predominance of a Th1 response in Sjögren's syndrome.[9 10]

Instead of studying levels of Th1 and Th2 cytokines in peripheral blood, we chose here another approach—that is, we investigated the genetics of cytokines which have previously been shown to be associated with Th1/Th2 differentiation in several studies and are known to be functional.[13–16] We found that neither the allele or genotype frequencies of classical Th2 cytokines (IL4 and IL13) nor the Th1 cytokine (IFNG) were different in patients with pSS compared with healthy control subjects. Hence we did not establish firm and conclusive evidence for a clear Th1 or Th2 predilection in pSS. Previously, an association of IL10 genetics with a predisposition to pSS has been observed, the GCC haplotype (G at position −1082, C at −819, and C at −592) being associated with increased risk for pSS[26 27] and higher plasma IL10 concentrations.[26] IL10 is a cytokine known to modulate both Th1 and Th2 activities in man and to induce both anti-inflammatory and proinflammatory responses. IL10 produced by regulatory T cells suppresses the effector immune responses[3] and as it was the high IL10 producer genotype which was found to be increased in patients with pSS,[26] the IL10 dependent regulatory function is probably more potent in them.

The genes encoding IL4 and IL13 are known to be associated with the Th2 mediated diseases asthma and atopy, as well as with IgE production.[14 28] Within the group of patients with pSS the genetics of IL4 and IL13 were associated in the present study with laboratory indices indicative of a mild disease (for example, a low ESR and low IgA concentrations) and seemed to protect against the extraglandular manifestation of purpura. As the presence of purpura has been found to be an adverse predictor of the development of lymphoma in pSS,[29] this finding further indicates that Th2 genotypes are associated with a milder form of pSS. Furthermore, the concentration of serum β2

microglobulin, which is known to reflect activation of the lymphopoietic system and to be increased in lymphoproliferative complications of pSS,[30 31] was found to be lower in carriers of the more rare genotypes of IL4 and IL13. The IL4 −590 and IL13 +2044 genotypes are known to be in strong linkage disequilibrium[22] and were also here significantly associated with each other. When grouping together the pSS patients carrying the more rare alleles IL4 −590 T and IL13 +2044 A and comparing them with a subgroup not carrying either of these alleles it was noted that the observed effects were even stronger. As features of a milder pSS were significantly associated with genotypes which contribute to a Th2 disease, one might postulate that pSS would be a predominantly Th1 dominated disease. A shift towards Th2 differentiation seems at least clearly to elicit features of milder pSS. Similarly, the rare allele of IL4 VNTR, which is known to be increased in atopic diseases, has been found to be associated with less severe disease in rheumatoid arthritis, a clearly established Th1 disease.[32] However, we observed no association with the polymorphisms of the Th1 cytokine IFNG and the severity of pSS.

To conclude, the frequencies of cytokine genes regulating the Th1/Th2 differentiation did not differ between pSS patients and healthy control subjects. However, the presence of cytokine genes with increased susceptibility to Th2 diseases was associated with signs of a milder form of pSS, as in rheumatoid arthritis, a classical Th1 disease. This would favour a hypothesis envisaging pSS as a primarily Th1 mediated autoimmune disease, but the findings need to be confirmed in independent cohorts of pSS patients.

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Authors' affiliations

M Pertovaara, Department of Internal Medicine, Section of Rheumatology, Tampere University Hospital, Tampere, Finland
J Antonen, Department of Internal Medicine, Tampere University Hospital, and University of Tampere Medical School, Tampere, Finland
M Hurme, Department of Microbiology and Immunology, University of Tampere Medical School, Tampere, Finland and Centre for Laboratory Medicine, Tampere University Hospital

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