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ORIGINAL ARTICLE

# Investigation of bacterial and viral agents and immune status in Behcet's disease patients from Iran

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## Abstract

**Aim:** Behçet's disease (BD) is an autoimmune disorder associated with HLA-B51 positivity. Serologic/genomic findings have suggested microbes as possible causative agents and the geographical distribution suggests environmental influences.

**Methods:** We performed comparative analyses of 40 patients with BD or related symptoms not fulfilling BD criteria. Patients originating from different regions of Iran were tested by molecular/serological methods for human herpes viruses and parvovirus B19, two Chlamydiae species, as well as *Coxiella, Listeria, Yersinia, Leptospira* and *Mycobacterium* paratuberculosis. Human leukocyte antigen-typing was performed: testing of cytokine profiles and immune mediators representative for the cellular immune system, including neopterin/kynurenine production.

**Results:** No apparent differences in interleukin (IL)-4, 6, 8 and 10 were observed, whereas production of soluble IL-2-receptor and tumor necrosis factor (TNF)-alpha were more pronounced in the BD group. Neopterin/kynurenine production was comparable, although both groups showed twice the levels of healthy people. No significant differences of herpes simplex virus (HSV) antibody titres were observed but higher titres against *Chlamydophila pneumoniae* were found in the controls. In 20 BD patients and controls neither parvovirus B19 DNA was detected nor bacterial DNA. Viral DNA of Epstein–Barr virus (EBV), cytomegalovirus (CMV) and human herpes virus (HHV)8 was detected more frequently in the BD group, whereas HSV DNA was only found in the controls, indicating that stomatitis might be caused by HSV.

**Conclusion:** Although no significant association of BD was detected with a single pathogen, our findings suggest that detection of HSV DNA or Chlamydiae would rather argue against classic BD. Whether there is a discriminative potential of the tested immune mediators/receptors has to be elucidated in further studies.

**Key words:** bacteria, Behcet's disease, cytokines, HLA-B51 subtyping, neopterin, virus.

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## INTRODUCTION

Behçet's disease (BD) is a multi-system autoimmune disorder. Previous serological and genomic findings suggest bacteria and viruses as possible causative agents of BD, but until today these data remain inconclusive. Furthermore, human leukocyte antigen (HLA)-B*51 positivity seems to play a role in the development of the disease as well as geographical factors.[1]

A viral cause of BD was first postulated by Behcet himself,[2] but even today this hypothesis remains unproven. Akaogi *et al.*[3] postulated a possible triggering role of hepatitis G virus and hepatitis B virus, but another study group[4] did not find an association between BD and hepatitis viruses. Parvovirus B19 may play a role in the development of the disease.[5] Currently HSV1 is the only virus associated with BD. Herpes simplex virus (HSV)1 DNA was detected by several authors in the blood of patients with BD.[6,7] In imprinting control region (ICR)-mice BD-like symptoms can be induced by HSV1 infection.[8] Furthermore, several bacteria were associated with BD, especially Streptococci.[9] Peripheral T-cells of patients with BD exposed to *Streptococcus sanguis* produced higher amounts of interleukin (IL)-6 and interferon (IFN)-γ compared to T-cells of healthy controls.[10]

Genomic findings suggest that to-date HLA-B51 positivity is the only known risk factor for the development of BD; 50–70% of patients with BD are positive for this HLA subtype.[11] Interestingly, there are different populations with a high prevalence of HLA-B51, like several Amerind-speaking American native tribes which do not have BD, suggesting the existence of other genetic or environmental risk factors which may cause BD only in combination with HLA-B51.[12]

Other authors focused on vascular injury seen in BD. Zouboulis *et al.*[13] found more frequent high titers against lupus anticoagulant than in healthy controls. In BD patients activated protein C resistance (APCr) is significantly higher than in a control group.[14] Furthermore, high titers of anti-endothelial-cell antibodies (AECA) were found during disease activity.[15] These findings suggest that endothelial damage due to immunological processes, as well as disturbed blood coagulation, may be involved in BD.

Although single observations exist in BD patients, there are very few papers concerning more than four parameters using the same patient group. We used polymerase chain reaction (PCR) technique for detecting virus and bacteria and various other methods for detecting specific antibodies against possible trigger mechanisms. Additionally, we determined immunological reactions in BD patients and their HLA-B51 status.

## MATERIAL AND METHODS

### Patient characteristics

In this study we included 40 patients with BD according to the criteria of the International Study Group for Behcet's Disease and 40 controls with clinical symptoms similar to BD like oral aphthosis, inflammation of the joints or inflammation of the eyes. BD was clinically excluded among the controls.

We used ethylenediaminetetraacetic acid (EDTA)-whole blood samples of 20 patients and 20 controls. Furthermore we analysed EDTA-plasma and serum samples of another 20 patients and 20 further controls (Tables 1–4).

The protocol for the research project was approved by the Ethics Committee of the University of Medical Sciences Teheran and this study was performed according to the Declaration of Helsinki. Informed consent was obtained for all investigations on human subjects and patient anonymity has been preserved.

## METHODS

### DNA preparation

For the preparation of DNA we used the NucleoSpin® Blood L kit (Macherey-Nagl, Düren, Germany). Two milliliters of each whole blood sample were processed, following the manufacturer's instructions. DNA was eluted in 200 μL of Tris-hydromethyl-aminomethane-hydrochloric acid (TRIS-HCL)-buffer (pH 8.5). To specify the eluted amount of DNA, photometry was performed at a wavelength of 280 nm using the Ultrospec 2100 *pro* UV/Visible Spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK).

### PCR detecting virus

One reaction consisted of 10 μL JumpStart™ ReadyMix™ REDTaq™ DNA Polymerase (Sigma, St Louis, MO, USA) containing polymerase, dideoxynucleotides (dNTPs) and reaction buffer, 8 μL formamide (5%), 1 μL virus-specific primer pair (5 pm/μL) and 1 μL DNA, making up 20 μL in total. Reaction was performed using the Perkin Elmer GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA). Additionally 'nested'-PCR was performed to increase the sensitivity to nine virus copies per reaction for HSV and to

**Table 1** Clinical characteristics of Behçet's disease (BD) patients (blood samples, $n$ = 20)

| Sample number | IBDDAM score (ocular) | IBDDAM score (non-ocular) | Gender | Age at sampling time | Age at diagnosis | Age at onset of disease | Organ manifestation from onset of disease until time of sampling |
|---|---|---|---|---|---|---|---|
| 1 | 42.6 | 3 | M | 32 | 25 | 21 | Oral aphthosis, erythema nodosum, skin aphthosis, panophthalmitis |
| 2 | 97.6 | 0 | M | 31 | 28 | 27 | Oral aphthosis, erythema nodosum, panophthalmitis |
| 3 | 0 | 0 | M | 18 | 17 | 16 | Oral and genital aphthosis, pseudofolliculitis, retinal vasculitis |
| 4 | 34 | 0.2 | F | 17 | 17 | 12 | Oral aphthosis, panophthalmitis |
| 5 | 0 | 0 | M | 34 | 34 | 30 | Panophthalmitis, oral aphthosis, pseudofolliculitis |
| 6 | 0 | 0 | F | 46 | 35 | 29 | Oral and genital aphthosis, pseudofolliculitis, erythema nodosum, anterior uveitis, CNS involvement |
| 7 | 0 | 8 | M | 30 | 30 | 27 | Oral and genital aphthosis, DVT |
| 8 | 0 | 3 | M | 33 | 33 | 32 | Oral and genital aphthosis, pseudofolliculitis, anterior and posterior uveitis, monoarthritis |
| 9 | 49.4 | 2 | F | 35 | 35 | 30 | Oral and genital aphthosis, panophthalmitis |
| 10 | 26.2 | 3.2 | M | 25 | 25 | 21 | Panophthalmitis, oral aphthosis |
| 11 | 22.8 | 3.2 | M | 12 | 12 | 11 | Oral aphthosis |
| 12 | 16 | 2 | M | 28 | 28 | 25 | Oral aphthosis, uveitis |
| 13 | 8 | 3.5 | F | 27 | 27 | 19 | Oral and genital aphthosis |
| 14 | 21 | 1 | M | 28 | 28 | 23 | Oral aphthosis, panophthalmitis |
| 15 | 27 | 6 | M | 45 | 45 | 43 | Panophthalmitis, oral and genital apthosis |
| 16 | 21.1 | 2.5 | M | 42 | 38 | 38 | Panophthalmitis, oral and genital aphthosis, erythema nodosum |
| 17 | 3 | 2 | F | 42 | 42 | 5 | Ocular involvement, oral aphthosis, epilepsy |
| 18 | 46 | 2.4 | M | 24 | 24 | 18 | Panophthalmitis, oral and genital aphthosis |
| 19 | 15 | 3 | F | 21 | 21 | 20 | Retinal vasculitis, oral and genital aphthosis |
| 20 | 16.6 | 3 | F | 24 | 24 | 21 | Panophthalmitis, oral and genital aphthosis |

IBDDAM, Iranian Behçet's disease dynamic activity measure; M, male; F, female; CNS, central nervous system; DVT, deep vein thrombosis.

88 copies per reaction for the other herpes viruses and to verify the results of the 'first-round' PCR.

## PCR detecting bacteria

Detection of bacterial DNA in the whole blood samples was done using real time PCR technology from NucleoSpin® Blood L Kit (Macherey-Nagl) and (TaqMan® Universal PCR Master Mix; Applied Biosystems, Branchburg, NJ, USA) as described by Corless *et al.*[16] Additional primers for the detection of additional Gram-positive and mycobacterial 16 rRNA genes were chosen using a 16S rRNA alignment representing a total of 111 bacterial species. The lower detection limit could be deduced to be 1200 bacteria/mL whole blood, clearly distinguishable from the negative control.

## PCR for HLA-subtyping

Low-resolution typing for HLA-B genes was carried out applying two different methods: sequence-specific

**Table 2** Clinical characteristics of Behçet's disease (BD) patients (plasma samples, $n = 20$)

| Sample number | IBDDAM score (ocular) | IBDDAM score (non-ocular) | Gender | Age (years) | Time since onset of symptoms (months) | Positive pathergy test | Involvement of the eyes | Oral aphthae | Genital aphthae | Involvement of the joints | Erythema nodosum | Involvement of the vessels |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | M | 25 | 60 | Yes | No | Yes | Yes | No | No | No |
| 2 | 0 | 0.25 | F | 45 | 180 | Yes | No | Yes | Yes | No | Yes | No |
| 3 | 59.5 | 0.58 | M | 39 | 96 | No | Yes | Yes | Yes | No | No | No |
| 4 | 6 | 0.25 | F | 20 | 24 | No | Yes | Yes | No | No | No | No |
| 5 | 24 | 2.58 | M | 27 | 84 | Yes | Yes | Yes | Yes | No | No | No |
| 6 | 0 | 2.16 | F | 37 | 60 | Yes | No | Yes | Yes | No | No | No |
| 7 | 44 | 1.58 | M | 33 | 240 | Yes | Yes | Yes | No | No | Yes | No |
| 8 | 0 | 2.08 | F | 21 | 24 | Yes | No | Yes | Yes | No | No | No |
| 9 | 11 | 0.16 | F | 47 | 12 | No | Yes | Yes | No | No | No | No |
| 10 | 2 | 2.08 | F | 37 | 96 | Yes | No | Yes | Yes | No | No | No |
| 11 | 0 | 1.08 | F | 28 | 86 | Yes | No | Yes | Yes | No | No | No |
| 12 | 40 | 0.58 | M | 52 | 72 | Yes | Yes | Yes | No | No | No | No |
| 13 | 14 | 3.33 | M | 31 | 60 | No | Yes | Yes | Yes | No | No | No |
| 14 | 0 | 0.5 | F | 35 | 108 | Yes | No | Yes | Yes | Yes | No | No |
| 15 | 24.9 | 1.08 | M | 23 | 36 | No | Yes | Yes | Yes | No | No | No |
| 16 | 6 | 0.16 | F | 17 | 12 | Yes | Yes | Yes | No | No | No | No |
| 17 | 0 | 0.83 | M | 35 | 36 | No | No | Yes | Yes | No | Yes | Yes |
| 18 | 0 | 0.91 | F | 43 | 72 | Yes | No | Yes | No | No | Yes | No |
| 19 | 0 | 0.5 | M | 23 | 12 | No | No | Yes | Yes | No | Yes | No |
| 20 | 6 | 0.25 | F | 26 | 12 | No | Yes | Yes | No | No | No | No |

IBDDAM, Iranian Behçet's disease dynamic activity measure.

**Table 3** Clinical characteristics of the controls (blood samples, $n = 20$)

| Sample number | Age in years | Gender | Time since onset of symptoms (months) | Organ manifestation from onset of disease until time of sampling |
|---|---|---|---|---|
| 1 | 20 | M | 12 | Oral aphthosis |
| 2 | 28 | M | 24 | Oral aphthosis |
| 3 | 35 | M | 3 | Oral aphthosis |
| 4 | 28 | F | 36 | Oral aphthosis |
| 5 | 34 | F | 24 | Involvement of the joints |
| 6 | 38 | M | 40 | Oral aphthosis |
| 7 | 34 | F | 50 | Oral aphthosis |
| 8 | 22 | M | 100 | Oral aphthosis |
| 9 | 34 | F | 60 | Oral aphthosis |
| 10 | 32 | M | 216 | Pemphigus vulgaris |
| 11 | 56 | M | 2 | Lichen plane |
| 12 | 44 | F | 60 | Bullous pemphigoid |
| 13 | 27 | M | 36 | Oral aphthosis |
| 14 | 45 | F | 36 | Oral aphthosis, involvement of the eyes |
| 15 | 34 | M | 36 | Oral aphthosis |
| 16 | 21 | M | 60 | Oral aphthosis |
| 17 | 24 | F | 120 | Oral aphthosis |
| 18 | 28 | F | 48 | Oral aphthosis |
| 19 | 21 | M | 12 | Oral aphthosis |
| 20 | 40 | M | 180 | Oral aphthosis |

M, male; F, female.

oligonucleotides (SSO) and sequence-specific priming (SSP). We used commercially available kits from Olerup SSP AB (Saltsjobaden, Sweden) and Reli SSO (Dynal, Wirral, UK). The pattern of distribution of the bands was analysed by HELMBERG-SCORE™ Virtual Sequencing software (GenoVision Inc., Vienna, Austria). All HLA-B types have slightly different genomic sequences, most often single-base exchanges. They are called 'single nucleotide polymorphisms' (SNPs). These small differences can be detected using the SSP method (Olerup SSP™ HLA-B low M26 kit; GenoVision Inc.). The same method was used to carry out the HLA-B*51 subtyping.

## Immunofluorescence staining for detection of anti-HSV1-antibodies

To detect antibodies against HSV1 we used dialysed EDTA whole blood samples from patients and controls. Monkey kidney cells (MA-104) were infected with HSV1 and were used as the test system. We used two different human sera with known antibody titers against HSV1 (1 : 12 500 and 1 : 10 000) as positive controls and anti-HSV1 immunoglobulin G (IgG)-free rabbit serum as a negative control. Optical examination was performed with a fluorescence microscope (Leica DMLB; McBain Instruments, Simi Valley, CA, USA).

## Detection of antibacterial antibodies in the serum samples

Serological analysis for the detection of antibodies directed against the following bacteria were performed: microagglutination tests for *Leptospira* ssp. (*L. australis, L. autumnalis Akyani A, L. bataviae, L. Jez bratislava, L. canicola, L. copenhageni, L. gryppotyphosa, L. hardjö, L. hebdomadis, L. icterohaemorrhagiae, L. pomona, L. saxköbing, L. sejrö, L. tarassovi* and *L. wolffi*). Antibodies against *Brucella abortus* (*B. abortus*), *Brucella melitensis* (*B. melitensis*), *Chlamydia psittacii* (*C. psittacii*), *Listeria monozytogenes* (*L. monozytogenes*) and *Mycobacterium paratuberculosis* (*M. paratuberculosis*) were determined by complement-binding reaction (CBR). Against *Yersinia enterocolitica* (*Y. enterocolitica*) and *Coxiella burnetti* (*C. burnetti*, phase 1 and phase 2) an enzyme-linked immunosorbent assay (ELISA) test was carried out.

## Detection of anti-HSV1-IgG in the plasma samples

For the detection of anti-HSV1-IgG in the plasma samples we used the Serion ELISA classic-kit [Serion ELISA classic Herpes Simplex Virus 1 IgG (quant.); Institut Virion\Serion, Würzburg, Germany] following the manufacturer's instructions.

**Table 4** Clinical characteristics of the controls (plasma samples, $n$ = 20)

| Sample number | IBDDAM score (ocular) | IBDDAM score (non-ocular) | Gender | Age (years) | Time since onset of symptoms (months) | Positive pathergy test | Involvement of the eyes | Oral aphthae | Genital aphthae | Involvement of the joints | Erythema nodosum | Involvement of the vessels |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | – | – | F | 34 | 192 | – | No | Yes | No | No | – | – |
| 2 | – | – | M | 35 | 48 | – | No | Yes | No | No | – | – |
| 3 | – | – | F | 52 | 36 | – | No | Yes | No | No | – | – |
| 4 | – | – | M | 39 | 30 | – | Yes | No | No | No | – | – |
| 5 | – | – | F | 42 | 180 | – | No | Yes | No | No | – | – |
| 6 | – | – | F | 41 | 86 | – | No | Yes | No | No | – | – |
| 7 | – | – | M | 23 | 12 | – | No | Yes | No | No | – | – |
| 8 | – | – | M | 43 | 72 | – | No | Yes | No | No | – | – |
| 9 | – | – | F | 34 | 60 | – | No | Yes | Yes | No | – | – |
| 10 | – | – | F | 45 | 0 | – | No | Yes | No | No | – | – |
| 11 | – | – | F | 35 | 48 | – | No | Yes | No | No | – | – |
| 12 | – | – | F | 28 | 36 | – | No | Yes | No | Yes | – | – |
| 13 | – | – | F | 23 | 12 | – | No | Yes | No | No | – | – |
| 14 | – | – | F | 32 | 36 | – | No | Yes | No | No | – | – |
| 15 | – | – | M | 21 | 60 | – | Yes | No | No | No | – | – |
| 16 | – | – | M | 31 | 180 | – | No | Yes | No | Yes | – | – |
| 17 | – | – | M | 37 | 96 | – | No | Yes | No | No | – | – |
| 18 | – | – | M | 27 | 48 | – | No | Yes | No | No | – | – |
| 19 | – | – | F | 29 | 48 | – | No | Yes | No | No | – | – |
| 20 | – | – | M | 25 | 24 | – | No | Yes | No | No | – | – |

IBDDAM, Iranian Behçet's disease dynamic activity measure; M, male; F, female.

**Table 5** Viral DNA in BD patients ($n$ = 20) and control samples ($n$ = 20) using PCR

| Viruses | PCR-positive patients | PCR-positive controls | *P*-value |
|---|---|---|---|
| HSV1/2 | 0 | 4 | 0.106 |
| EBV | 2 | 0 | 0.487 |
| CMV | 2 | 0 | 0.487 |
| HHV6 | 2 | 1 | 1.000 |
| HHV7 | 4 | 3 | 1.000 |
| HHV8 | 1 | 0 | 0.999 |
| VZV | 0 | 0 | 1.000 |
| Parvovirus B19 | 0 | 0 | 1.000 |

PCR, polymerase chain reaction; HSV1/2, human herpes virus 1/2; EBV, Epstein–Barr virus; CMV, cytomegalovirus; HHV6/7/8, human herpes virus 6/7/8; VZV, varicella zoster virus.

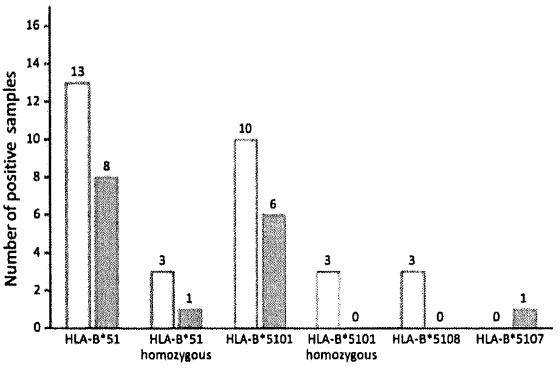


**Figure 1** HLA-typing and subtyping of Behçet's disease (BD) patients and controls. Note that more patients (white, $n$ = 20) than controls (grey, $n$ = 20) were positive for HLA-B51 and its subtypes (except HLA-B 5107).

## ELISA method for the detection of cytokines

Commercially available ELISA kits were used for detecting cytokines. The tests were performed according to the instructions. For soluble IL-2R we used the test from Coulter-Immunotech Diagnostics, Hamburg, Germany, for IL-10 the ultrasensitive EASIA kit from BioSource Europe, Nivelles, Belgium and for IL-4, IL-6, IL-8 and TNFalpha the EASIA test kits from BioSource Europe S.A.

## Microimmunofluorescence method for detection of anti-*Chlamydophila pneumoniae* antibodies

Immunoglobulin G and IgA antibody titers to *C. pneumoniae* were analyzed with a microimmunofluorescence test (MIKRO-IFT; Labsystems, Helsinki, Finland). The resulting antibody titers were classified into titers from 16 to 1024 on the basis of their serial dilution. *A priori*, we agreed on classifying *C. pneumoniae* IgG or IgA antibodies ≥ 16 as positive. IgG titers of ≥ 512 and IgA titers ≥ 64 were considered elevated.

## Neopterin, tryptophan and kynurenine

Neopterin is a marker for the activation of the cellular immune system. After stimulation with antigens, T-cells produce IFNγ which activates macrophages and induces the production of neopterin. Tryptophan is catabolized by indoleamine (2,3)-dioxygenase (IDO) to kynurenine. This enzyme is upregulated by cortisole and especially by IFNγ. The kynurenine/tryptophan ratio reflects the activity of the enzyme. ELISAs were carried out to determine the plasma levels of neopterin, tryptophan and kynurenine.

## Statistics

Results of the patients and the controls were compared using the chi-square test or Fisher's exact test.

# RESULTS

## Detection of viral and bacterial DNA

Although the sensitivity of our nested PCR method for all tested viruses was very high, we found four out of 20 HSV1/2 positive samples in the control group but none in the patient group. In the BD samples there were two positive for Epstein–Barr virus (EBV) and also two for cytomegalovirus (CMV) compared to none of the controls. Comparable results in BD samples and in controls were obtained for human herpes virus (HHV)6, HHV7, HHV8 varicella zoster (VZV) and parvovirus B19 (Table 5).

For bacterial PCR testing we could not obtain any positive results although we reached a high sensitivity of about 50 colony-forming units (CFU) per reaction.

## HLA-typing and subtyping using PCR

In the patient group we found 13/20 HLA-B*51-PCR positive samples, whereas only 8/20 controls had the same phenotype.

In the patient group PCR subtyping we found three to be homozygous for HLA-B*51, 10 were HLA-B*5101 and three were HLA-B*5101 homozygous. Three were positive for HLA-B*5108 and none for B*5107. In the control group only eight were positive for HLA-B*51, one was homozygous for this locus. For the subgroup HLA-B*5101 only six controls were positive but none of them homozygous. One was

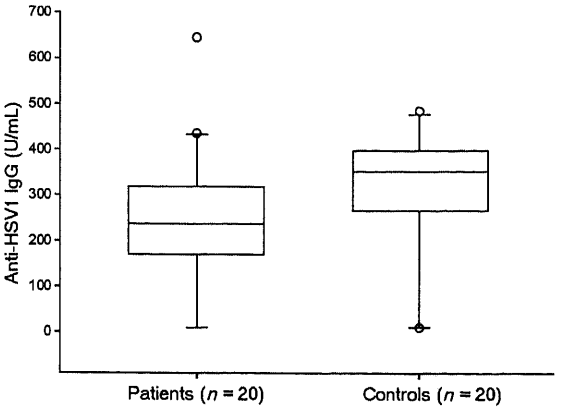


Figure 2 Anti-herpes simplex virus (HSV)1 antibody titer in patients and controls. Plasma samples were tested for HSV1 antibodies using enzyme-linked immunosorbent assay technique. Interestingly we more often found high titers against HSV1 in controls compared to Behçet's disease patients.

HLA-B*5107-positive but none of the controls were B*5108-positive (Fig. 1).

## Anti-HSV1 antibodies

In the immunofluorescence test for whole blood samples, 12/20 patients had an anti-HSV1-IgG titer of 1 : 100 and 2/20 titers of 1 : 500. In the controls we found 6/20 with 1 : 100 and 2/20 with 1 : 500. None of the BD and control samples were positive for HSV1-IgM.

Using the ELISA technique for plasma samples we found elevated levels in the control group compared to the BD patients (Fig. 2).

## Detection of antibacterial antibodies

In 5/20 BD and in one control sample we found antibody titers of 1 : 10 against *M. paratuberculosis*, and in one control this was 1 : 20. Against *Y. enterocolica* two patients had antibodies of 51–100 U/mL, four controls reached 31–50 U/mL. Three patients and one control had antibody concentrations of 20–30 U/mL. One patient and two controls were positive against *C. psittaci* (1 : 10). Antibodies against *L. Jez bratislava* (1 : 50) were detectable in one patient and two controls, and *L. hardjö*, *L. saxköbing* and *L. tarassovi* were detectable in one control. None of the patients had antibodies against *C. burnetti* (phase 1), but two controls were positive. Against *C. burnetti* (phase 2) one patient showed antibodies in a concentration of 101–200 U/mL; a further patient and one control had titers of 51–100 U/mL, two patients and three controls had 31–50 U/mL and two patients still had 20–30 U/mL.

## Neopterin, tryptophan and kynurenine titers

For neopterin BD patients reached 10.9 ± 3.2 and the control group 11.6 ± 2.8 nmol/L. Although the patients showed about twice of the level of the healthy population (5.2 ± 2.5 nmol/L) there was no statistical significance between BD patients and the controls (Fig. 3a).

The serum levels of tryptophan of healthy persons differed between 40 µmol/L and 100 µmol/L; the serum levels of kynurenine are usually at 1.92 ± 0.58 µmol/L. In our study, plasma levels of tryptophan were 47.2 ± 9.1 µmol/L in the patient group and 45.3 ± 8 µmol/L in the control group; plasma levels of kynurenine were 1.49 ± 0.55 µmol/L

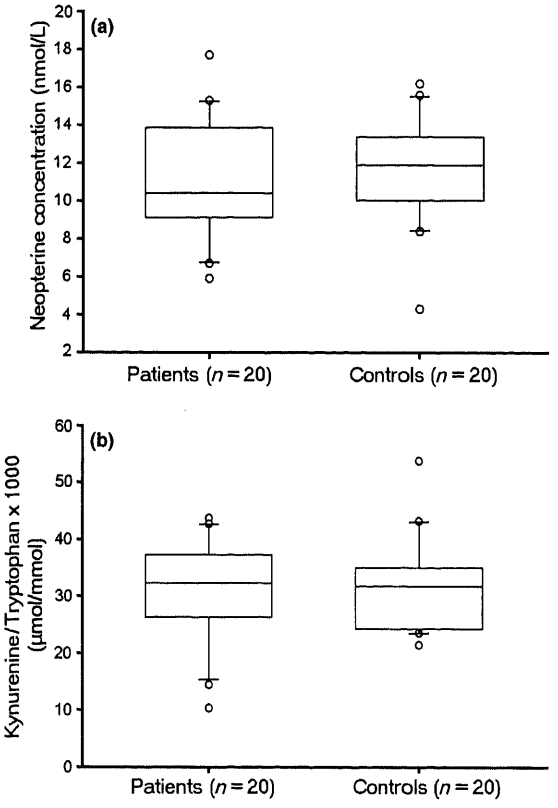


Figure 3 Neopterin levels (a) and the kynurenine/tryptophan-ratio (b) of patients and controls are shown. The box plot figures show the values of patients (left) and of controls (right). Boxes represent the interquartile range (25th–75th percentile); the line across the box indicates the median. Neither in the neopterin tests nor in the kynurenine/tryptophan-ratio were there any statistically significant differences.

in the patient group and 1.41 ± 0.29 μmol/L in the control group, which means slightly decreased values compared to healthy persons. The kynurenine/tryptophan ratio is indicative for the activity of this enzyme and it is quoted at 26.9 ± 8.1 μmol/L in healthy persons. In the patient group an average value of 31.7 ± 8.7 μmol/L was calculated, in the control group this was 3.8 ± 8.0 μmol/L. Interestingly, serum plasma levels of tryptophan were within the normal range in both groups; kynurenine levels were slightly decreased, which militates against an increased fermentative activity of tryptophan-2,3-dioxygenase.

The kynurenine/tryptophan ratio of the patients and the controls were about the same level (Fig. 3b).

## Detection of cytokines IL2-R, IL-4, IL-6, IL-8, IL-10 and TNFα

We found an activation of T-cells, which is more expressed in BD patients than in the control group, reflected in a higher TNFα serum level (Fig. 4f, $P = 0.450$). The median values of both groups reached higher values than healthy controls (which is between 4.6–12.4 pg/mL according to the data from the kit provider). IL-8 activity was not elevated in both groups (Fig. 4d) and was in the range of healthy controls (up to 50 pg/mL according to the kit provider's data). Soluble IL-2 receptor was only slightly elevated in the BD group compared to the rheumatic control group and the other tested cytokines did not show any differences between the BD group and the controls (Fig. 4a–c,e).

## Quantitative IgG and IgM measurements using immunofluorescence

In 12/20 BD samples we found IgG titers of 1 : 100 whereas in the controls there were only 6/20 with this titer ($P = 0.987$). An IgG titer of 1 : 500 was found in both, the BD patients and the controls each 2/20. IgM

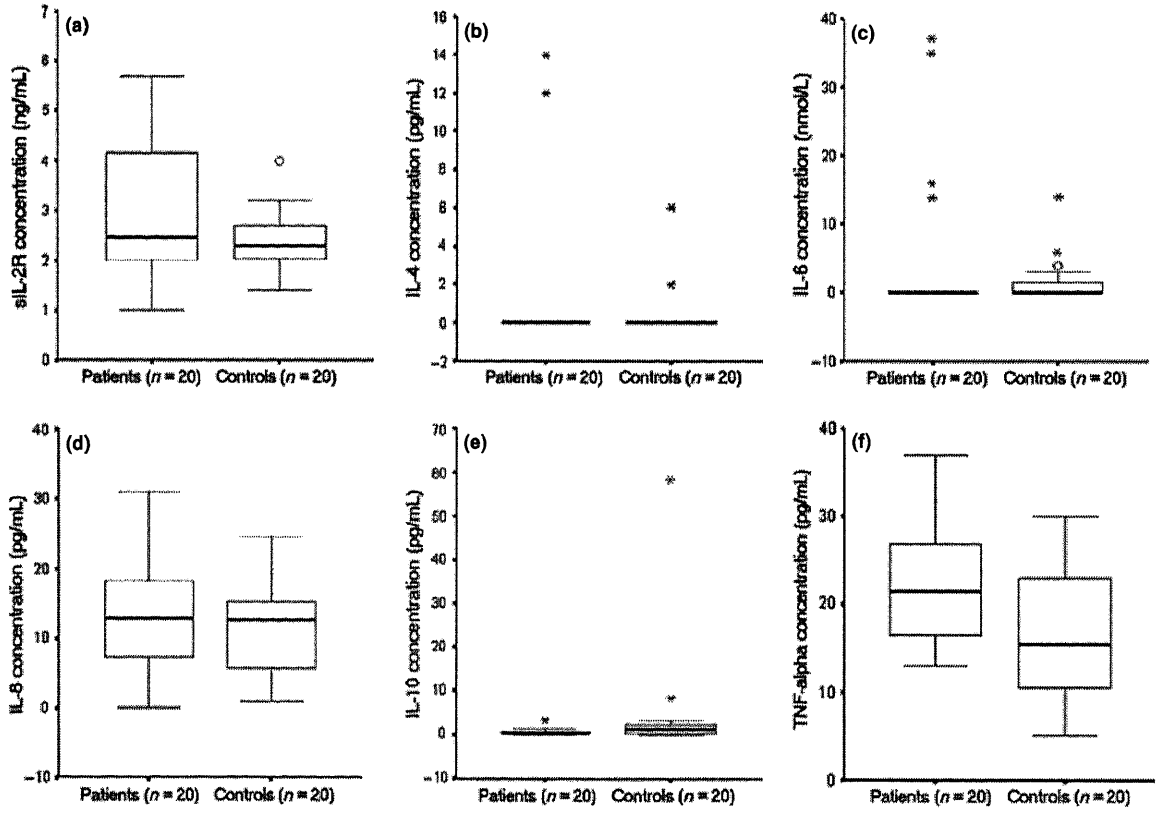


Figure 4 Cytokines from plasma samples from patients and controls are shown: (a) soluble IL-2 receptor (sIL-2 R); (b) IL-4; (c) IL-6; (d) IL-8; (e) IL-10; (f) TNFα. The box plot figures show the values of the patients (left) and of the controls (right). Boxes represent the interquartile range (25th–75th percentile); the line across the box indicates the median. Although there was no statistically significant difference in the measured cytokines seen, TNFα was elevated in the Behçet's disease patient group.

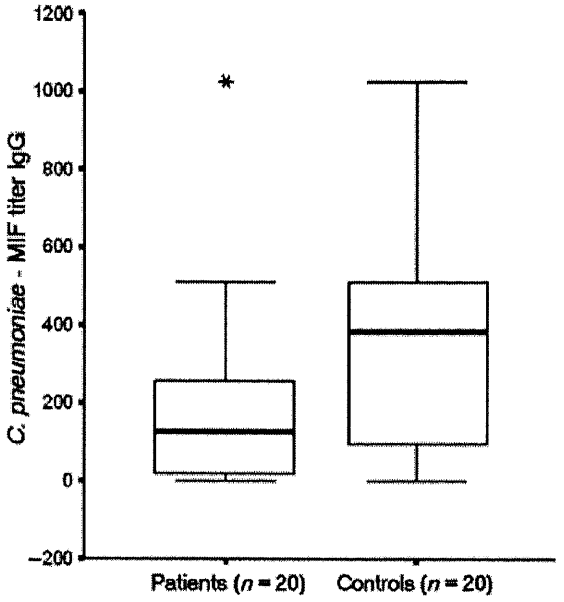


Figure 5 IgG antibody titers against *C. pneumoniae* are shown in Behçet's disease (BD) patients and in controls. The box plot figures show the values of the patients (left) and of the controls (right). Boxes represent the interquartile range (25th–75th percentile); the line across the box indicates the median. Interestingly, the median value of the controls is higher than for the BD patients.

titers were measured in HSV-PCR-positive samples but in none of these blood samples was IgM detectable.

### Detection of *Chlamydia pneumoniae* antibodies

We observed only in 4/20 BD patients with IgG titers against *C. pneumoniae* ≥ 512, whereas in the control group there were 10/20 with this titer. The numbers of IgA titers ≥ 64 were similar in both groups (in the BD group $n = 5/20$ and in the controls $n = 4/20$, see Fig. 5).

## DISCUSSION

Although there exist numerous suggestions for bacterial or viral trigger mechanisms for BD, data are contradictive. Most of the reports refer only to one or two parameters concerning BD trigger organisms.

Using PCR techniques, Akaogi *et al.*[3] detected DNA of hepatitis G virus and hepatitis B virus more often in BD patients than in healthy controls, suggesting that hepatitis viruses might be possible causative agents of BD, but other authors were not able to acknowledge these findings.[4,17] Although there are a few hints that parvovirus B19 may be involved in the development of BD,[7] HHVs, especially HSV1, seem to be one of the relevant factors underpinning the theory of a viral cause of BD. For example, HSV1-DNA was more often isolated from the saliva of patients with BD than in controls.[9] HSV1-DNA could also be found in genital aphthae and erythemanodosum-like lesions of BD. Studd *et al.* detected HSV1-DNA in peripheral blood mononuclear cells of BD patients in significantly higher amounts than in healthy controls.[6] There also exists an animal model where HSV1 can induce BD-like symptoms, especially skin, genital ulcers and eye lesions.[8] We investigated the whole panel of the herpes virus group and even parvovirus B19 in BD patients and controls. Interestingly, no HSV1-DNA was detected among the BD patients, but it was detectable in some controls. Our results are similar to those of others,[18] who were not able to detect HSV1-DNA in BD patients. We also could not detect DNA of parvovirus B19, neither in BD patients nor in controls.

There are also various findings referring to antibodies against HSV1 in patients with BD. Hamzaoui *et al.* found more often high IgG titers against HSV1 in patients with BD compared to controls.[19] In our study we used immunofluorescence to test the blood samples for anti-HSV1-IgG-antibodies and obtained similar results. Interestingly, in the serum samples we found more often high titers against HSV1 in the control group than in the BD group, which would fit to the more frequent detection of HSV DNA in the non-BD group.

In summary, we did not find an association between herpes simplex viremia and BD, suggesting that also patients with clinical symptoms of HSV can be found in the BD group, as an immunosuppressive therapy might lead to an HSV reactivation. A possible association between BD and HSV cannot be excluded at present considering the serological data, but more extended studies are needed.

Also various bacteria have been discussed as possible trigger mechanisms for BD. Delayed-type hypersensitivity against streptococcal antigens is more often seen in BD patients than in healthy controls.[9] After eradication of *Helicobacter pylori* a significant reduction of oral aphthae in patients with BD was achieved.[20] Zouboulis *et al.* found more often high titers against *Mycoplasma fermentans* in BD patients compared to healthy controls, so maybe these two bacteria may also serve as trigger mechanisms for the disease.[21] In our study, we tested serum samples of 20 BD

patients and 20 controls with rheumatic diseases other than BD for antibodies against many bacteria. We did not find a significant difference between the two groups, only antibodies against *Mycobacterium paratuberculosis* were found more often in the BD patients. This bacterium is suspected to play a role in the aetiology of Crohn's disease[22,23] as well as in the genesis of sarcoidosis.[24] Crohn's disease is the most important differential diagnosis to gastrointestinal lesions caused by BD. Erythema nodosum can often be seen in patients with sarcoidosis and in patients with BD.[25] Until today no case of a direct transmission of *M. paratuberculosis* between man and beast has been described,[26] but a possible trigger role of this bacterium in the aetiology of BD cannot be excluded. There was no positive correlation between previous *C. pneumoniae* infection and BD. In fact, antichlamydial titers against this respiratory and vascular pathogen were even more prominent among controls. The significance of these remains to be determined.

In the majority of cases serological methods were used. In our study we used real-time PCR to test whole blood samples of BD patients for the presence of bacterial 16S rDNA. The limit of our sensitivity was 50 CFU; this limit is also described by others.[27] Bacterial DNA was neither detectable in the patients nor in the controls. A limiting factor for the non-specific detection of bacterial DNA is the contamination of polymerases with this kind of DNA, because bacterial cultures are used to produce these enzymes. For this reason polymerase extracted from such cultures always contains few contaminating bacterial DNA which will be co-amplified during the PCR process. This may lead to false positive results. Even today there is no procedure to eliminate this impurity, even though different methods have been tested to improve the detection limit. For example, restriction enzymes like SAU 3A1,[28] Alu1[29] or HaeIII[16] were used to reduce the level of contamination but results were inconsistent. In our study we tried to improve the sensitivity also using SAU 3A1 cutting short DNA fragments, but we were not able to improve the sensitivity. Also the usage of a microfilter to clean the polymerase of contaminating DNA or the usage of highly purified polymerase (low-DNA polymerase) could not solve this problem.[30]

We further used AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Branchburg, NJ, USA), containing 10 copies of 16S rDNA per 5 units of enzyme, but failed to lower the detection limit below 50 CFU. At present, there is no method to cleanse the polymerase of contaminating bacterial DNA in an efficient way. The production of polymerase using fungal cultures may be a way to obtain a product free of bacterial DNA.

An association of BD with HLA-B*51 positivity in 50–70% of patients[11] is well documented.[31–33] Even today the presence of HLA-B*51 seems to be the only relevant pathogenic factor for BD.[12] It is also possible that the presence of HLA-B*51 may only be a risk factor for an aggravated course of disease.[34] In our study we performed HLA typing with consecutive subtyping of the whole blood samples. Thirteen out of 20 patients (65%) were HLA-B*51 positive. Similar results were achieved by other authors.[35] Eight out of 20 controls (40%) were positive for HLA-B*51; this corresponds to the prevalence of HLA*B51 in Iran.[36,37] The subtype HLA-B*5101 was detected in 10 out of 13 HLA-B*51 positive patients and in six out of eight HLA-B*51-positive controls. HLA-B*5101 is the most common subtype in patients with BD.[12,33,38] The subtype HLA-B*5108 ranks second to HLA-B*5101 in patients with BD[12,39,40] with a frequency of 5.5–26.7%, depending on the geographical region.[40] In our study three out of 20 patients were positive for HLA-B*5108, but this subtype was not present in the control group. The role of HLA-B*51, especially of the subtypes HLA-B*5101 and HLA-B*5108 in the aetiology of BD is still unclear but it is the object of intensive research.

Only a few authors investigated serum neopterin levels of patients with BD.[41] Neopterin levels were elevated in BD and rheumatoid patients and were twice as high as normal controls. The elevated levels are due to the inflammatory activity in both groups, which has been described earlier by other authors.[42] This is due to elevated plasma levels of interferon-γ in the control group and in the patient group.[43] Normally an increased activity of this enzyme leads to decreased plasma levels of tryptophan.[43,44] Interestingly, serum plasma levels of tryptophan were in the normal range in both groups; kynurenine levels were slightly decreased, which militates against an increased fermentative activity of tryptophan-2,3-dioxygenase.

From cytokine measurements it was obvious that especially TNFα was elevated in BD patients. This was in concordance with findings of other authors,[45] whereas IL-8 serum levels in BD, and also in the rheumatic control group, were not elevated and lay within the range of healthy controls. Both markers reflect the activity of the disease.[45,46]

In our study we included 40 BD patients which is only a small cohort. However, no other study investigated such a broad range of possible causative agents, serological parameters and immunologic features.

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