Translational Aspects of Behçet's Disease

Genetics, cytokines and new treatment modalities

Jasper Kappen

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INTRODUCTION

Behçet's disease (BD) is an multifactorial auto-inflammatory vasculitis characterized by recurrent oral -and genital ulcers, uveitis, and skin lesions [1, 2]. The first series of patients with BD was already published in 1937 as a triad of these symptoms [3]. Less frequently, involvement of the gastrointestinal tract, central nervous system and large vessels may occur. As in other immune mediated disorders BD is characterised by attacks rather than by a persistent inflammatory disease [4].

Cases of Behçet's disease cluster along the ancient Silk Road, which extends from eastern Asia to the Mediterranean basin. The reported prevalence in Turkey varies between 70 and 420 per 100.000 [5]. In Northern European countries however the prevalence is probably less than 1 per 100.000 in Caucasians. It is believed that both genetic and environmental factors contribute to the development of the disease [6, 7]. Men might be more frequently and severely affected than women

Susceptibility to BD is strongly associated with the presence of the HLA-B51 allele and its presence is also strongly associated with a more aggressive course [8]. The cause of BD, however, is still unknown. Triggers such as infections, both viral and bacterial, trauma, and other stress factors may play a role.

Although the exact cause of BD remains to be elucidated, it is clear that neutrophils interacting with T-cells play an important role in the pathophysiology. The activated T-cells produce TNF-α, which in turn leads to production of other proinflammatory cytokines like interleukin 1 en interleukin 6 by other immune cells. These proinflammatory cytokines stimulate migration and activation of leucocytes, thereby causing a local inflammatory response [2]. Additionally, a disturbed function of regulatory T-cell may also play a role. Eventually vessel -and subsequently tissue damage occurs, as can be seen in biopsies taken from BD lesions from various sites involved [4].

IMMUNE SYSTEM EN CYTOKINES

The inflammation in BD is characterized by a Th-1 and probably also Th-17 mediated immune response (figure 1) [2, 9]. In the early phase of inflammation granulocytes and gamma-delta T-cells (Tγδ) are identified in the affected tissues. These are believed to be activated by environmental "triggers" [10]. A lymfohistiocytic infiltrate is found in a later phase.

The assumption that granulocytes play an important role in BD is fed by a number of observations: increased prevalence of HLA-B51 in 13-80% of the patients [4] , associated with over-activated neutrophils. increased toll like receptor expressions on granulocytes

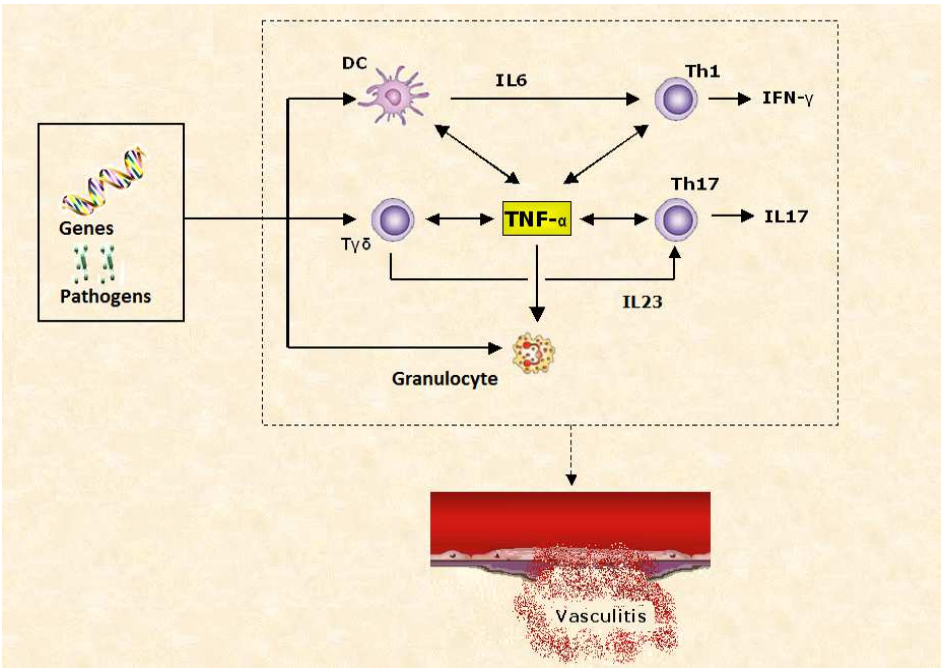

Figure 1; Immunopathophysiology of Behçet's disease. It is assumed that stimulation by pathogens of granulocytes, Tγδ and DCs, in a genetically predisposed host a cascade of inflammatory cytokines eventually lead to vascular damage and vasculitis.
Abbreviations: IL; interleukin, TNF; tumor necrosis factor, Tγδ; Tγδ subset T cells, Th; CD4 + cells Thelper, DC; dendritic cells

of BD patients, frequently occurring hyperactive granulocytes, and observations of hyperactive response of granulocytes to "heat shock proteins" and fragments of the bacterial cell wall [11]. The inflammatory effect of these observations seems that these hypersensitive granulocytes, but also Tγδ cells through antigen presenting dendritic cells start a cascade of pro-inflammatory cytokines. Several cytokines are involved in the pathophysiological mechanisms. IL6, produced by antigen presenting cells, which promotes the differentiation towards Th1 cells and IL23, produced by Tγδ cells that promotes the Th17 differentiation P40 [12, 13]. The central cytokine in this inflammatory process is tumor necrosis factor (TNF) -α, which is produced by virtually all the cells involved. TNF-α subsequently activates the production of other cytokines such as interleukin (IL) -1β, IL-6, IL-8, IL-10 and IL-17 (Figure 1). Other inflammatory cells are attracted to the inflammation site and contribute to an inflammatory infiltrate. Increased tissue (pro) inflammatory cytokines and serum concentrations in various affected tissues from patients with BD are described [14-16]. Adisturbed , hyperactive local immune response seems responsible for the initial inflammatory symptoms that are seen in BD.

Also regulatory T cells (Treg) are of interest in autoimmune diseases. These cells seem essential for the immunological homeostasis in which the T-cell-effector response is inhibited. The involvement of Treg in BD is debated. In summary, in BD tissue damage together with a local hyper-inflammation appears to play an important role.

GENETICS

In patients of Turkish origin there is positive family history in 12% of the cases with a sibling risk ratio between 11–52 [2, 17]. The heritability of BD has been estimated to range between 20 to 60%, with the strongest genetic association embracing variants in HLA-B51, explaining about 20% of the disease heritability [2, 17]. The combination of epidemiological and genetic data suggest causal involvement of both genetic and environmental factors [6].

Over the years numerous single nucleotide polymorphisms (SNPs) have been found associated with BD [7, 18-20]. In 2010 the first genome-wide association study (GWAS) in BD cohorts of Turkish and Japanese origin demonstrated association of various variants in the known HLA-B51 domain, and two new association signals, mapping to the interleukin-10 (*IL10*), and the IL-23 receptor–IL-12 receptor Beta2 (*IL23R–IL12RB2*) locus [21, 22]. These associations later have been confirmed in an Iranian cohort [23]. Yet another study in Algerian individuals only replicated the associations from the IL10 variants [24]. GWAS in Chinese and Korean individuals reported associations of SNPs mapping to two other loci, with regions containing the signal transducer and activator of transcription 4 (*STAT4*) and GTPase of immune associated protein *GIMAP* genes, respectively. Polymorphisms in *IL10* and *IL23R–IL12RB2* were not found to be significantly associated with BD in either of those studies [14,15]. Most recently associations with *CCR1, STAT4* and *KLRC4* were identified by imputation analysis [25]. This study also identified the *IL12A* region as suggestively associated with BD, but genome wide significance was not reached. Discrepancies in association results, as have been shown for the *GIMAP* variants might be explained by the different ethnic origin of the cohorts studied [26, 27].

CLINICAL PICTURE

Diagnosis:

Currently the diagnosis of BD is based on the criteria as proposed by the International Study Group for BD in 1990 [28]. To fulfil these criteria recurrent oral ulcers must be present together with two or more of the following; recurrent genital ulceration, eye lesions, skin lesions or a positive pathergy test (table 1). This test consists of pricking a sterile

Table 1: International Study Group criteria for Behçet's disease

Finding	Definition
Recurrent oral ulceration	Minor aphthous, major aphthous, or herpetiform ulcers observed by the physician or patient, which have recurred at least three times over a 12 month period
Recurrent genital ulceration	Aphthous ulceration or scarring observed by the physician or patient
Eye lesions	Anterior uveïtis, posterior uveïtis, or cells in the vitreous on slit-lamp examination; or retinal vasculitis detected by an ophthalmologist
Skin lesions	Erythema nodosum observed by the physician or patient, pseudofolliculitis, or papulopustular lesions; or acneiform nodules observed by the physician in a post-adolescent patient who is not receiving corticosteroids
Positive pathergy test	Test interpreted as positive by the physician at 24 to 48 hours

20G needle into the skin of a patient's forearm. The results are judged to be positive when the puncture causes an aseptic erythematous nodule or pustule that is more than 2 mm in diameter at 24 to 48 hours. At the reaction site there is initially an accumulation of neutrophils, followed by the accumulation of mononuclear cells [29]. The pathergy test is sometimes considered pathognomonic for the disease but can also be positive in other diseases like pyoderma gangrenosum [30].

Clinical manifestations [2, 4] (Chapter 2):

Oral aphthous ulcers:
Often the oral ulcers are the first and sometimes the only symptom of the disease. An inverse relationship with cigarette smoking exists, in which a worsening of the disease is seen when stopping. The localization to the often very painful and persistent ulcers importantly affect the daily lives of patients, it can even lead to severe emaciation. Various forms of ulcers are described; minor, major and herpetiform. The ulcers are aphtous in nature and can be up to 2 cm in diameter. They occur throughout the intestinal area and tend to form in places where a trauma, such as, for example, occurred after a dental procedure.

Genital ulcers:
Painful genital ulcers are described throughout the urogenital area. Such an ulcer is often mistaken for a sexually transmitted disease (STD). An STD must therefore be excluded before proceeding to treatment. The ulcers are characterized by poor wound healing, in more than 75 percent scar formation occurs.

Skin disorders:
Skin disorders are seen in 75 percent of the BD patients and again described in various forms. The most common skin lesions are sterile papulo-pustular lesions that may be found spread over the whole body. Erythema nodosum is not uncommon, often present in female patients and usually seen on the lower extremities. Other common skin conditions are thrombophlebitis, pyodermal- or acnei-form abnormalities. Folliculitis and pustules often occur in places where skin shaved or minor skin damage can occur. 'Nail-fold lesions' and dermatography are less frequently seen.

Pathergy skin test:
The pathergy skin test is a local inflammatory response to a sterile puncture and has a specificity of almost one hundred percent for BD. The sensitivity of the test varies considerably and is between five and eighty percent. When using sterile needles the frequency of positive pathergy tests seem to be decreased.

Eye involvement:
Untreated eye symptoms can quickly lead to blindness. In non-Western countries twenty percent of the BD patients is blind. Each segment of the eye may be affected. Usually it involves inflammation in the anterior chamber (anterior uveitis). More seriously is posterior uveitis in which the optic nerve may be involved. Scleritis and conjunctivitis have been reported in BD patients, but are not seen often [2].

Intestinal BD:
Because BD can also manifest in the small intestine and colon in the form of aphthous ulcers, intestinal scarring, fistula or perianal lesions, the possibility of confusion with Crohn's disease of is present. Microscopic, however, in BD granulomas are not often encountered. Granulomas are quite often are present in the Crohn's disease.

Joint involvement:
Non-erosive oligoarticular joint manifestationis often present. It usually remains restricted to the large joints such as the knee, ankle and wrist. Erosive defects are rarely seen.

Vasculitis:
Vasculitis plays a central role in the pathophysiological disease process and causes the involvement of various organs. Both veins and arteries can get involved at all levels, microvascular arterial vasculitis is seen the most. In addition, a wide variety of clinical syndromes, such as deep vein thrombosis, (pulmonary arterial) aneurysms, intra-cerebral arterial occlusions, Budd-Chiari syndrome, and thrombophlebitis are described in BD-patients. All these disorders are caused by a local inflammation of the vascular

wall and therefore is treated as such with immunosuppressive medications. Pulmonary aneurysms caused by vasculitis may lead to life-threatening arteriobronchiale fistulas and and present late with hemoptysis.

Neurological manifestations:
Localization in the central nervous system is seen in 5 to 10 per cent of the cases and causes a high mortality rate. The brainstem is the most frequently affected, however also aseptic meningitis and arterial vasculitis is seen. The most common symptoms include cranial nerve dysfunction, ataxia, and bilateral pyramidal symptoms such as hemiparesis, areflexia or blatter dysfunction. Neuropsychiatric manifestations may manifest as behavioural changes, dementia and other psychiatric disorders. Polyneuropathy is not a symptom that is seen in BD. Diagnostics in neuro-BD is often very difficult because of the lack of hard diagnostic criteria. Examination of the CSF the total protein, neutrophil granulocytes and CSF pressure sometimes is slightly increased. However, a normal CSF examination does not rule out a cerebral BD. Various deviations can be seen radiological. High intensive-enhancing parenchymal lesions are often only found using a T1-weighted MRI, whereas CT shows no abnormalities. A (MR) angiography may in some cases be used to demonstrate a cerebral vasculitis or vasculopathy. Cerebral vasculitis can be a rapidly progressive disease. A tight control is desired when a BD patient has striking migraine symptoms and loss of function.

CURRENT TREATMENT OPTIONS

First of all we must state that unfortunately most of the treatment regimens in BD the level of evidence is limited since there are few randomized controlled trials.

Treatment of patients with BD is directed by the presence of organ involvement, or vision –or life-threatening situations (table 2). Topical steroids are usually effective for mucocutaneous involvement. Some patients however respond insufficiently and in these cases additional treatment may be necessary. Colchicine is the mainstay of BD therapy, effective for refractory mucocutaneous disease, although a double blinded study conducted already in 1980 did not show a statistically significant reduction in the recurrence of both oral and genital ulcers as compared to placebo [31, 32]. Thalidomide has also been shown to be effective in patients with mucocutaneous lesions refractory to treatment with colchicine. Thalidomide exerts considerable side effects (neuropathy, constipation) and for obvious reasons should be avoided in women with childbearing potential unless they adhere to stringent anti-conceptive regime. Pentoxifylline, which reduces the production of inflammatory cytokines like TNF-α, was effective in reduc-

Table 2: Current treatment used for Behçet's disease

Medication	Dose	Main indications
Prednisonev	Local Systemic: 0.5 – 1 mg/kg	Uveïtis Mucocutaneous involvement Induction treatment uveïtis Neurological involvement Refractory arthritis Gastro-intestinal ulceration
Colchicine	0.5 - 1.5 mg / day	Skin involvement Arthritis
Dapsone	100 - 200 mg / day	Mucocutaneous involvement Arthritis
Azathioprine	2 - 3 mg / kg / day	Uveitis
Pentoxifylline	1200 mg /day	Mucocutaneous involvement
Sulfasalazine	1- 3 g / day	Mucocutaneous involvement arthritis
Thalidomide	50 - 200 mg / day	Refractory mucocutaneous involvement
Cyclosporine	2 dd 3 - 5 mg/kg / day	Uveïtis Mucocutaneous involvement
Methotrexate	7.5 - 15 mg / week	Arthritis Uveitis (rarely)
Cyclophosphamide	750 mg / m^2 /mo IV	Life threatening involvement (vasculitis, neurological)
IFN-α-2a	9 million units 3 /week for 3 months followed by a low maintenance dose of 3 million units 3 /week)	Uveitis
TNF-α blockers	Various doses	Uveitis Arthritis Gastro-intestinal ulceration

ing the frequency and severity of oral an vaginal ulcers [33]. Alternative treatment for mucocutaneous involvement are dapsone [34] and cyclosporine. Cyclosporine is used infrequently for this indication for fear of side effects [35]. However, being a potent immunosuppressive agent, cyclosporine resulted in significantly more alleviation of oral aphthous ulcers versus colchicine in a study by *Masuda et al.* [36] Sulfasalazine is mainly effective in arthritis and gastrointestinal involvement [4, 37].

Involvement of internal organs, sight-threatening uveïtis, or severe CNS involvement may require much more aggressive treatment consisting of systemic corticosteroids of > 1mg/kg/day combined to DMARDs such azathioprine or cyclosporine. These immunosuppressives are considered effective treatment for patients with BD, especially those with uveitis. One of the newer immunosuppressive drugs, mycophenolic acid derivates, although not effective in mucocutaneous lesions [38] can be effective in therapy refractory gastro-intestinal involvement [39].

Especially (large vessel) vasculitis involving the pulmonary arteries and neurological disease, both notoriously life-threatening call for intensive measures. In these cases the use of cyclophosphamide alone or in combination with prednisone is warranted [40, 41].

Interestingly, although the cause of BD is unknown, treatment with high dose penicillin either alone or in combination with colchicine was effective in reducing symptoms (mucocutaneous and joint manifestations) in several studies. A possible role for streptococcal infection has been proposed [42-44].

In some patients the aforementioned therapies are either ineffective or associated with intolerable adverse effects necessitating alternative treatment strategies. In the slipstream of the success of new developments in the management of immune mediated diseases such as rheumatoid arthritis and IBD, promising designed novel agents targeting key-immunological factors of these immunopathological compatible diseases, have been tried in BD patients with sometimes astonishing results.

EMERGING TREATMENT OPTIONS

As mentioned above, T-cells and cytokines are believed to play an important role in the pathogenesis of BD. Emerging therapies aim at influencing T-cell function or cytokines.

Interferon alpha

Interferon-α (IFN-α) maybe no longer deserves to be called an emerging therapy as it is used for therapy refractory BD for over twenty years [45]. IFN-α has been reported to restore the low natural killer cell activity in patients with BD to a near normal level. A double-blind study assigned 50 patients to receive either IFN-α2a (6 million units 3 times weekly) or placebo for 3 months [46]. Significant decreases in pain and duration of oral ulcers, frequency of genital lesions, and number of pustular papules were noted in patients receiving IFN-α2a. The frequency and duration of erythema nodosum-like lesions and thrombophlebitis were also decreased in the same group. In a systematic review of 32 studies and 4 published abstracts published before 2002, a total of 338 patients received either IFN-α2a or 2b of various doses [47]. Within a few months of therapy, eighty-six percent of the patients with mucocutaneous symptoms, 96% with arthritis, and 94% with uveitis exhibited a partial or complete response. Significant improvements were also found in erythema nodosum-like lesions, skin ulcerations, and superficial thrombophlebitis. Interferon-α-2a seems to be more efficacious than IFN-α-2b. Compared with low-dose regimens, high-dose regimens were more effective and achieved more remissions. However, disease activity generally returned to baseline level either immediately after or up to 7 months after therapy was discontinued. Side effects of IFN-α include mild alopecia, mild leukopenia (generally reversible with discontinuing

interferon), influenza-like symptoms, and depression or psychosis. The recommended regimen is a high dose of IFN-α-2a (9 million units divided in 3 doses per week) for 3 months followed by a low maintenance dose (3 million units divided in 3 doses per week). Since the successful advance of TNF blockers, IFN-α are seldomly used in the daily practice of most clinics, especially in the ErasmusMC.

TNF-α blockers

It is believed that TNF-α plays a central role in the inflammatory process of BD.Th-1 proinflammatory cytokines such as IL-1-β, IFN-γ and IL-12, but most of all, TNF-α are involved in the inflammatory response that is held responsible for disease related symptoms. TNF blockers represent a novel regimen in the treatment of BD. The most often representative of this group is the chimeric TNF-α antibody infliximab. Its use has been reported effective in more than 10 smaller studies including a total of more than 120 patients and 115 other single BD cases of refractory mucocutaneous lesions, severe gastrointestinal -or neurological involvement, and ocular disease [48-53]. Evidence. One of the most significant studies was published in 2004 by Sfikakis et al. [54] In this study 25 patients with ocular disease were treated with a single infusion of 5mg/kg infliximab. In all but one patient ocular inflammation was controlled within one day. In general, remarkable and swift improvement or complete resolution of the oro-genital ulcers, gastrointestinal ulcerations, and other BD related symptoms can be achieved shortly after one or two infusions of infliximab at various doses ranging from 3 -10 mg/kg. A dose of 300 – 500 mg is generally accepted effective, but the time of interval and duration of therapy remain undetermined. In patients with acute, unilateral, posterior uveitis and significant reduction of visual acuity (<0.2), as well as in those cases with inflammation of the macular area, a single infusion of infliximab, or intravitreal steroids, may be superior to other immunosuppressive drugs. In cases of bilateral posterior eye segment inflammation, a single infusion of infliximab could be used as a first-line agent to achieve a fast-onset response, along with the initiation of other immunosuppressive drugs. However, if the ocular disease remains uncontrolled a combination of immunosuppressive regimens with repetitive infusions of infliximab 5 mg/kg every 6–8 weeks for up to 2 years can be initiated.

Another TNF-α blocker used in BD is the fusion protein etanercept. One double-blind, placebo-controlled study has been conducted by Melikoglu et al. [55] This study involved 40 male patients with mainly mucocutaneous disease randomly given either 4 weeks of 25 mg etanercept (subcutaneously twice a week) or placebo. Significant improvements of oral ulcers, nodular and papulopustular lesions were observed in the etanercept group. Within four weeks 45% of the etanercept group remained free of oral ulcers compared with only 5% in the placebo group. No significant improvement in genital ulcers or pathergy positivity was observed. Eighty-five percent of the patients

receiving etanercept were free of nodular lesions compared with 25% of those in the placebo group. Most patients experienced recurrent disease 3 months after etanercept was stopped. Other case reports include in total less than 10 BD patients mainly successful treated with etanercept.

In 2006 two groups reported treatment with adalimumab, a human TNF-α antibody, in 9 refractory BD patients. In both retrospective studies all patients various symptoms resolved swiftly and persistently [56, 57]. Adalumimab could be given for at least 3 years without serious adverse effects. In Chapter 4 we present new data on the use of adalumimab in BD patients.

The role of TNF blockers in patients with uveitis is currently accepted for selected cases. Although reportedly very effective in BD, the place of TNF-α blockers in the therapeutic strategy, its long-term effects and the timing of intervals still has not been determined yet.

Anti IL-1 and anti IL-6

The role of other cytokines like IL-1 and IL-6 in the pathogenesis of BD is becoming more eminent. Blocking the involved pathways can lead to new treatment options in BD. A case report of 2008 already describes a patient with refractory BD responding rapidly to treatment with daily anti IL-1. Lowering the dose to alternate day injections led to an increase in disease activity, again responding to an increase in the dose [58]. A recent overview of biological treatment in BD shows positive effects with no new safety issues [59]. Also nhibition of IL-6 signalling could be promising [59, 60]. Tocilizumab is a humanized monoclonal antibody that binds both to soluble and to membrane-bound IL-6 receptor and has been shown to be effective in rheumatoid arthritis. It goes without saying that more data, on efficacy and indication with regard to these novel treatment modalities is needed.

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Outline of the thesis

"Although the etiology of Behçet's disease (BD) still needs to be unravelled, it is widely considered an excessive T-cell mediated inflammatory response in a genetically susceptible host." This sentence is often the introduction in research articles on BD. It summarises not only the reason for this thesis but also covers the outline.

The chapters in this thesis travel from the demography towards morbidity towards immunopathology and etiology ending with (molecular designed) therapy in BD.

Chapter 2 will focus on the epidemiology of BD in the Rotterdam area. Apart from an overview of patients characteristics and with prevalence data of the different ethnicities, we take place in the camp that challenges, the assumption that the prevalence of BD declines after immigration. In Chapter 3 genetic aspects of BD are presented, starting with a targeted gene approach, the prevalence of NOD variants that are related to Crohn's disease, a disorder with many similarities to BD. Subsequently a non-targeted approaches by use of a Genome wide associations study as well as a whole genome sequence is presented. Chapter 4 cytokines addresses the subject of involved, cytokines and where to measure these, in tissue or in serum? As a result of the immunopathological studies, novel treatment modalities are shown in Chapter 5, with emphasis on the use of biologicals such as the major cytokine (TNF) blocking agents . Finally the results are summarized and discussed in chapter 6.

2

Epidemiology

Behçet's disease, prevalence and manifestations in the Rotterdam area

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Submitted

ABSTRACT

Introduction: Behçet's disease (BD) is most prevalent in countries along the former Silk Road. Prevalence varies from 70-420 per 100,000 in Turkey, and 13.5-20 and 1-2 per 100,000 in Asia and Western Europe, respectively. Additionally, disease severity and morbidity might be correlated with ethnicity. We studied demography and morbidity in the Dutch BD cohort and compared those with known figures.

Patients and Methods: The prevalence of BD patients in the Rotterdam area was determined by comparing the total amount of patients within the ethnic population with the amount of patients diagnosed with BD. Patient files of the Erasmus University Medical Centre (Erasmus MC) were reviewed for morbidity figures and compared with existing data.

Results: In total 84 BD patients of Dutch, Turkish or Moroccan descent were identified in the Rotterdam area. Prevalences of BD differed per ethnicity; 1, 71 and 39 per 100.000 for Dutch-Caucasians, Turks, and Moroccans, respectively. These figures are comparable with the occurrence of BD in West Turkey and Morocco. Within the studied Erasmus MC cohort no significant differences in morbidity appeared between the ethnic groups. However, uveitis and pustules were significantly more present in the Erasmus MC cohort as compared to UK, German, Turkish and Moroccan cohorts.

Discussion and Conclusions: We present the first epidemiologic study of BD in the Netherlands. The prevalence of BD in the studied Dutch region and in countries of ancestry is similar. Morbidity is equally spread, compared to other countries, but uveitis and pustules seem to be more present in the Netherlands.

INTRODUCTION

Behçet's disease (BD), first described by Hulusi Behçet in 1937, is an idiopathic systemic vasculitis with variable clinical manifestations [1]. The diagnosis BD is based on clinical criteria with the presence of recurrent oral ulceration together with genital ulcers, ocular inflammation and skin lesions [2]. Less often arthritis, central nervous system (CNS) -and gastrointestinal tract inflammation is present [3-5]. The etiology still needs to be unravelled. The inflammatory symptoms are considered to be caused by an excessive T-cell mediated inflammatory response, triggered by an environmental antigen in a genetically susceptible host [3, 6, 7]. Over the years several genetic associations with BD are identified [8-14]. Furthermore, a positive family history can be found in 12% of non-Caucasoid patients and a sibling risk ratio of 11–52 in Turkish patients.

BD typically occurs in countries along the former Silk Road, an ancient route of commerce between the Mediterranean (Spain and Portugal) and the Far East (China). The highest prevalences of BD are seen in Turkey (20-420 per 100,000, with about 70 per 100,000 in its European region). In Asian countries, such as Japan, Korea, China, Iran, and Saudi Arabia, prevalence varies from 13.5 to 20 cases per 100,000. In Western countries prevalences of approximately 1 per 100,000 are reported [5] [15, 16]. Morbidity is reported to be lower in Western populations, in comparison with Eastern Mediterranean and Middle and Far Eastern countries [3, 15, 16]. BD can develop at any age, but the disease appears most frequently between the second and fourth decade of life [17]. In countries along the former Silk Road, BD is more frequent among men, whereas in Western countries it is more prevalent in women [17-22]. Demographic and morbidity data of BD in Northern Europe and the Netherlands are sparse. Demographic data from other Northern European countries are restricted to two German and British cohorts [23, 24]. It has been suggested that life-style or environmental factors could be influential on the immuno-etiology of BD hence influencing the prevalence in patients migrating to other countries [20, 25]. In this light countries, such as the Netherlands, to where patients from high incidence countries migrate to can be helpful to address this issue.

Therefore we initiated a demographic study in Dutch BD patients to elucidate variability of prevalence and morbidity amongst different ethnic groups and changing figures upon migration.

MATERIALS AND METHODS

All patients included in our study fulfilled the diagnosis BD by the criteria of the International Study Group for BD [2]. For prevalence analyses the numbers of BD patients were collected from hospital records in the Rotterdam area (Erasmus University Medical

Center (Erasmus MC), Vlietland Hospital, Maasstad Hospital, Sint Franciscus Gasthuis). Only Dutch, Turkish and Moroccan ethnicities were included in this analyses since the occurrence of BD in other ethnic backgrounds in the Netherlands are known to be too low to reach significant power. Public information on populations and ethnicities of inhabitants of the postal area Rotterdam is freely accessible from the governmental institute for statistical data (Centraal Bureau voor de Statistiek).

The morbidity analyses were performed by reviewing files on the presence of disease symptoms from all Erasmus MC visiting BD patients (also from outside the Rotterdam postal area) up to January 2012. To compare our data to other cohorts we performed a PubMed survey on epidemiological data and BD. Statistical analyses were performed with SPSS 17.0 version (Chicago Illinois). Chi-square test method was used, a p-value < 0.05 was considered to be statistically significant.

RESULTS

Prevalence

In total 84 BD patients of Dutch, Turkish or Moroccan descent were identified in the area with the postal code 2900 - 3319 (table 1). The other 16 patients were from different origin. The prevalence of BD in the Rotterdam area differed per ethnicity, 1 per 100,000 for Dutch, 71 per 100,000 for Turkish and 39 per 100,000 for Moroccans. We identified 11 Turkish and 3 Moroccan BD patients that were born in the Netherlands.

Table 1; Prevalence of BD in the Rotterdam area.

Rotterdam area (postal code 2900 - 3319)	Inhabitants	BD patients	per 100,000	Born in the Netherlands
Total	1,319,680	100	8	
Dutch-Caucasian	874,162	12	1	
Turkish	73,028	52	71	11
Moroccan	51,218	20	39	3

Disease features

The Erasmus MC cohort of 110 BD patients was distributed in three subpopulations of Dutch-Caucasian, Turkish and Moroccan patients. The remainders constituted a mix of various ethnicities. Patient characteristics are presented in table 2a. The average age of the patients was 44 years (Table 2a). The male female ratio was 1 for the entire group; 0.64 for the Dutch patients, 1.31 for the Turkish, and 1.66 for the Moroccan patients, respectively. Oral and genital ulcers and skin involvement were most prevalent. No sig-

Table 2a; Ethnic comparison of Dutch BD patients for diagnostic criteria in BD.

n=110	All	The Netherlands	Turkey	Morocco	Others
	100% (110)	37.3% (41)	34.5% (38)	14.5% (16)	13.7% (15)
Average age	44.2	43.0	44.3	46.5	
♂:♀ ratio	1	0.64	1.31	1.66	
Oral ulcers	100% (110)	100% (41)	100% (38)	100% (16)	100% (15)
Genital ulcers	79.1% (87)	85.4% (35)	84.2% (32)	62.5% (10)	66.7% (10)
Erythema nodosum	31.8% (35)	24.4% (10)	36.8% (14)	37.5% (6)	33.3% (5)
Pustules	80.9% (89)	73.2% (30)	81.6% (31)	93.8% (15)	86.6% (13)
Uveitis*	61.8% (68)	51.2% (21)	60.5% (23)	68.8% (11)	86.6% (13)
Positive pathergy Test	57.1% (32/56)	66.7% (16/24)	38.1% (8/21)	40% (2/5)	100% (6/6)

** No distinction was made between anterior, intermediate or posterior uveitis.*

Table 2b; Ethnic comparison of Dutch BD patients for other features present during the disease course.

n=110	All	The Netherlands	Turkey	Morocco	Others
	100% (110)	37.3% (41)	34.5% (38)	14.5% (16)	13.7% (15)
Fatigue	68.2% (75)	61.0% (25)	76.3% (29)	68.8% (11)	66.7% (10)
Headache	60.0% (66)	48.8% (20)	57.9% (22)	75.0% (12)	80% (12)
Arthralgia	67.3% (74)	*51.2% (21)*	*73.7% (28)*	*81.3% (13)*	80% (12)
Arthritis	30.9% (34)	*22.0% (9)*	34.2% (13)	*50.0% (8)*	26.7% (4)
Gastro-intestinal complaints	44.5% (49)	36.6% (15)	42.1% (16)	62.5% (10)	53.3% (8)
Diarrhea	22.7% (25)	24.4% (10)	18.4% (7)	31.3% (5)	20.0% (3)
Neurological involvement	12.7% (14)	12.2% (5)	5.3% (2)	12.5% (2)	33.3% (5)
HLA-B51 positivity	43.3% (13/30)	40.0% (6/15)	44.4% (4/9)	33.3% (1/3)	66.7%(2/3)

Italic/ underlined = statistically significant

nificant differences were observed between the ethnic groups in any of the symptoms of the diagnostic criteria (supplementary table 1).

Other clinical symptoms were again split up, per ethnicity (table 2b). Fatigue, headache and arthralgia were often present. Of all symptoms significant differences between the ethnic cohorts were only demonstrated for the prevalence of arthritis and arthralgia between Dutch and Moroccan patients ($p < 0.038$) and arthralgia between Dutch and Turkish patients ($p < 0.04$); a preference for the Turkish and Moroccan patients was seen (supplementary table 1). There was no significant difference between morbidity in male and female patients (data not shown).

Comparison to other cohorts

We compared our data with Turkish, Moroccan, German and British cohorts (table 3a en 3b [23, 24, 26, 27]. The German cohort consisted of two main ethnicities: German

Table 3a; International comparison between the EMC and 4 other cohorts for diagnostic criteria for BD.

	The Netherlands	Germany (23)	UK (24)	Turkey (26)	Morocco (27)
n	110	590	419	2,147	1,034
♂/♀ ratio	1	1.4	0.5	1.03	2
Oral ulcers	100%	98%	100%	100%	100%
Genital ulcers	*79.1%*	*64%*	*89%*	*88%*	*86%*
Erythema nodosum*	*31.8%*	*42%*	-	-	*16%*
*Pustules**	*80.9%*	*62%*	-	-	*64%*
Uveitis	*61.8%*	53%	68%	*29%*	*44%*
Positive pathergy test	*57.1%***	*34%*	*32%*	56%	53%

Italic/ underlined = statistically significant

** 86% of UK BD patients had skin manifestations; this was not specified in the original study.*

*** Not all Dutch BD patients underwent a pathergy test; therefore this figure was based on all positive tests in 56 patients who underwent this test.*

In the other cohorts, data on other disease features related to BD were limited (table 3b). We did find a significant difference between prevalence of arthralgia (common in the UK), arthritis and neurological involvement. (supplementary table 2).

Table 3b; International comparison between the EMC and 4 other cohorts for other features in the course of BD.

	The Netherlands	Germany (23)	UK (24)	Turkey (26)	Morocco (27)
n	110	590	419	2,147	1,034
Arthralgia	*67.3%*	-	*93%*	-	*32%*
Arthritis	*30.9%*	-	-	-	*45%*
Gastro-intestinal complaints*	48.2%	-	-	-	-
Gastro-intestinal involvement **	-	12%	7%	2.8%	11%
Neurological involvement	*12.7%*	11%	*31%*	*2.2%*	17%

Italic/ underlined = statistically significant

** Gastro-intestional involvement included one of the following; nausea, vomiting, abdominal pain, diarrhea. Ulceration of the gastro-intestinal was not necessarily found.*

*** Ulceration of the gastro-intestinal tract was objectivated.*

(38.5%) and Turkish (45.3%). For all empty cells in the table no data were available in the original articles. The male female ratio was 0.5 in the UK cohort and 1 or higher in the other cohorts. Oral and genital ulcers and skin involvement were most prevalent. In contradiction to observations in our own cohort, disease manifestations differed between the different populations (table 3a and supplementary table 2). Pustules appeared to be more prevalent in the Erasmus MC cohort as compared to Germany and Morocco. Furthermore, uveitis was more prevalent in Western cohorts as compared to Turkish and Moroccan.

DISCUSSION

We present the first epidemiological and morbidity data of BD patients living in the Netherlands. In this population the majority of patients are Dutch-Caucasian, Turkish or Moroccan. The prevalence of BD in the Rotterdam area amongst these groups reflects epidemiological studies in comparable populations and does not appear to shift after migration.

Comparable data from Germany showed that the prevalence of BD in the Turkish population was similar to our findings, and equals the reported prevalence in the European part of Turkey [22]. Thus, our observation of next generation immigrants with BD and the fact that BD can also develop in non-Turkish (or Asian) patients can get BD contradicts with the suggestion that BD does not develop after migration. This suggestion of causative land-based disease was based on the observation that BD did not occur in Japanese immigrants on Hawaii [25] and on the presence of a decreasing BD prevalence after migration from Turkey to Germany [20]. However, the latter could not be confirmed in a second study by the same group in 2012 [22].

We therefore would like to stress that the pathogenesis of BD does not appear to be decisively determined by the country of residence. Whether the disease severity is influenced could not be studied, since no validated BD severity scoring system exists.

The average age of the BD patients in the Erasmus MC cohort was similar amongst various ethnicities [17] [28]. The Dutch-Caucasian male/ female ratio tended to be lower as compared with Moroccans and Turks. Apparently a female prevalence occurs in Western and Asian countries as observed in other cohorts (table 2a) [24, 27]. Saylan et al reported female predominance in the United States, the United Kingdom, Korea, and China, whereas a male predominance was found for almost all Middle Eastern countries [29]. A low male/ female ratio is often seen in auto-immune diseases. However, BD is generally not associated with auto-immunity, rather with auto-inflammation [30]. The latter is not associated with female predominance, leading to uncertainty about the significance of the mentioned observations [31].

Prevalence of many symptoms was significantly different in our EMC cohort as compared to other cohorts, differences in data collection can be of influence. Clinically considered relevant differences in morbidity merely consist of skin lesions and uveitis. In Dutch patients a significantly higher percentage of pustules as compared to Germany and Morocco was seen. Prevalence of erythema nodosum also was statistically significant higher in the Erasmus MC cohort and in the German study, as compared to Moroccan figures. It is widely thought that environmental components are essential in BD patho-

physiology. Therefore it was expected that skin involvement would have been higher in countries with high disease prevalence. Other factors, such as genetic susceptibility could account for our observation.

A significantly higher prevalence of uveitis was seen in Western cohorts compared to Turkish and Moroccan population. Since BD is rare in the Netherlands it is possible that a general physician could miss the diagnosis BD in a patient that presents with oral ulcers [28]. A relative underdiagnoses of the less severe cases of BD might lead to relatively higher prevalence of severe case with uveitis. A second additive reason for the high percentage of uveitis found in the Erasmus MC cohort is the close cooperation between our hospital and the Rotterdam Eye Hospital in respect of referral of patients .

In conclusion, the prevalence of BD in different ethnic groups in the Rotterdam area is similar to those in the countries of origin of these patients. It does not appear to shift after migration. However, a substantial amount of patient data is necessary to elucidate migrational effects on the occurrence and morbidity of BD more robustly. This warrants international cooperation between BD treating physicians.

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SUPPLEMENTARY DATA

Supplementary table 1; P-values for ethnic comparison of Dutch BD patients for diagnostic criteria and other features in BD.

Parameter	*Compared for ethnicity*	*p-value**	*Table*
♂:♀ ratio	Dutch - Turks	0.093	2a
	Dutch - Moroccans	0.110	
	Turks - Moroccans	0.753	
HLA-B51 positivity	Dutch - Turks	0.831	2a
	Dutch - Moroccans	0.829	
	Turks - Moroccans	0.735	
Genital ulcers	Dutch - Turks	0.886	2a
	Dutch - Moroccans	0.057	
	Turks - Moroccans	0.080	
Erythema nodosum	Dutch - Turks	0.229	2a
	Dutch - Moroccans	0.332	
	Turks - Moroccans	0.964	
Pustules	Dutch - Turks	0.373	2a
	Dutch - Moroccans	0.087	
	Turks - Moroccans	0.250	
Uveitis	Dutch - Turks	0.405	2a
	Dutch - Moroccans	0.231	
	Turks - Moroccans	0.568	
Pathergy test positivity	Dutch - Turks	0.055	2a
	Dutch - Moroccans	0.264	
	Turks - Moroccans	0.937	
Fatigue	Dutch - Turks	0.143	2b
	Dutch - Moroccans	0.585	
	Turks - Moroccans	0.562	
Headache	Dutch - Turks	0.417	2b
	Dutch - Moroccans	0.073	
	Turks - Moroccans	0.235	
Arthralgia	Dutch - Turks	**0.040****	2b
	Dutch - Moroccans	**0.038****	
	Turks - Moroccans	0.553	
Arthritis	Dutch - Turks	0.225	2b
	Dutch - Moroccans	**0.038****	
	Turks - Moroccans	0.277	
Gastro-intestinal complaints	Dutch - Turks	0.616	2b
	Dutch - Moroccans	0.076	
	Turks - Moroccans	0.171	
Diarrhea	Dutch - Turks	0.519	2b
	Dutch - Moroccans	0.597	
	Turks - Moroccans	0.300	
Neurological involvement	Dutch - Turks	0.279	2b
	Dutch - Moroccans	0.975	
	Turks - Moroccans	0.354	

** All p-values were calculated using a Chi-square test method in SPSS.*

*** All p-values ≤ 0.05 were considered to be significant and were therefore made **bold**.*

Supplementary table 2; P-values for international comparison between the EMC and 4 other cohorts for diagnostic criteria for BD and other features in the course of BD.

Parameter	*Compared for ethnicity*	*p-value**	*Table*
Genital ulcers	Dutch - German	**0.002****	3a
	Dutch - British	**0.006****	
	Dutch - Turkish	**0.006****	
	Dutch - Moroccan	0.052	
Erythema nodosum	Dutch - German	**0.045****	3a
	Dutch - British	***	
	Dutch - Turkish	***	
	Dutch - Moroccan	**10^{-19}****	
Pustulas	Dutch - German	**10^{-19}****	3a
	Dutch - British	*******	
	Dutch - Turkish	*******	
	Dutch - Moroccan	**10^{-19}****	
Uveitis	Dutch - German	0.090	3a
	Dutch - British	0.219	
	Dutch - Turkish	**10^{-19}****	
	Dutch - Moroccan	**10^{-19}****	
Pathergy test positivity	Dutch - German	**0.001****	3a
	Dutch - British	**10^{-19}****	
	Dutch - Turkish	0.863	
	Dutch - Moroccan	0.545	
Arthralgia	Dutch - German	***	3b
	Dutch - British	**10^{-19}****	
	Dutch - Turkish	***	
	Dutch - Moroccan	**10^{-19}****	
Arthritis	Dutch - German	***	3b
	Dutch - British	***	
	Dutch - Turkish	***	
	Dutch - Moroccan	**0.005****	
Neurological involvement	Dutch - German	0.603	3b
	Dutch - British	**10^{-19}****	
	Dutch - Turkish	**10^{-19}****	
	Dutch - Moroccan	0.250	
Major vessel involvement	Dutch - German	0.536	3b
	Dutch - British	**10^{-19}****	
	Dutch - Turkish	0.095	
	Dutch - Moroccan	**0.021****	

** All p-values were calculated using a Chi-square test method in SPSS.*

*** All p-values ≤ 0.05 were considered to be significant and were therefore made **bold**.*

**** These fields were left blank, because of lack of data in the original study. Therefore no p-value could be determined.*

3

Genetics

Low prevalence of NOD2 SNPs in Behçet's disease suggests protective association in Western Caucasians

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ABSTRACT

Objectives: It has been shown previously that three NOD2 variants (Arg702Trp, Gly908Arg and Lue1007fs) are associated with Crohn's disease (CD), a disorder clinically resembling Behçet's disease (BD). We studied the frequency of these variants in BD patients.
Methods: DNA samples of 200 BD patients (59 Western Caucasian, 139 Middle Eastern of Arab descent (ME) and 2 Asian) and 520 healthy controls (444 Western Caucasian and 76 ME) were genotyped using a Taqman assay.
Results: Both the Arg702Trp and Leu1007fs (=frameshift) variants were significantly less frequently present amongst BD patients compared to healthy controls (0.5% versus 5.8%, $p<1.10\text{-}5$ and 0.0% versus 1.8%, $p<0.007$, respectively). In the Western Caucasian subpopulation Arg702Trp was significantly less frequent in the BD group as compared to the controls ($p = 0.04$). In the ME subpopulation a trend was observed ($p<0.06$).
Conclusion: Of the three CD associated SNPs one of the variant NOD2 alleles was significantly less present in Western Caucasian BD patients.

INTRODUCTION

BD is an idiopathic systemic vasculitis characterized by recurrent oral and genital aphthous ulcers, skin lesions, ocular inflammation and more seldomly, arthritis, CNS -and gastrointestinal tract inflammation [1, 2].

Prevalence varies from 110 to 420 per 100.000 in Turkey to about 2 per 100.000 in western countries [2]. A strong association with HLA-B51, a positive family history in 12% of non-Caucasoid patients and a sibling risk ratio of 11-52 suggest causal involvement of both genetic and environmental factors [3].

The etiology of BD is still to be unravelled but it is widely considered an excessive inflammatory response, possibly triggered by an (infectious) antigen in a genetically susceptible host. The abundance of neutrophils in early lesions and the association of HLA-B51 with neutrophil activation indicate involvement of innate immunity in BD [1-2, 4].

Similar symptoms such as uveitis, arthritis, erythema nodosum and colitis with ileocaecal mucosal inflammation and punched-out fissuring ulcers suggest a genetic overlap between BD and CD [5, 6]. Recently, the latter has been linked with genetic alterations in innate immunity. Increased susceptibility to CD is associated with two SNPs (Arg702Trp, Gly908Arg) and a frameshift mutation (Leu1007fs) in the CARD15 gene [7, 8]. This gene encodes for the nucleotide-binding oligomerization domain containing 2 (NOD2) protein. In short, NOD2 acts by recognition of muramyl dipeptide (MDP) derived from peptidoglycan, present in the cell wall of all bacteria. NOD2 engagement activates nuclear factor-kappaB (NF-κB), a transcription factor with a central role in both innate and adaptive immunity. This transcription factor is also considered to play a key role in many inflammatory diseases including BD and CD[7,9,10].

Since BD and CD share many clinical features and for both diseases innate immune mechanisms appear crucial, we addressed the hypothesis that NOD2 variants are mechanistically operational in both diseases. We therefore determined the occurrence of the aforementioned NOD2 SNPs in two large and independent BD cohorts

MATERIALS AND METHODS

Study population

Two cohorts of in total 200 unrelated BD patients (52 from the Erasmus MC at Rotterdam, 109 from The Jordan Hospital, Amman, Jordan and St John's Ophthalmic Hospital, East Jerusalem, Israel and 39 from St. Thomas' Hospital, London, UK). Of those, 59 were Western Caucasian, 2 of Asian and 139 from ME origin of Arab descent. All patients fulfilled the International Study Group criteria for the diagnosis of BD[5]. In total 520

healthy controls were recruited (444 Caucasian and 77 ME). Dutch controls were healthy Caucasian blood donors, the UK controls were Caucasian transplant donors from which Afro-Caribbean and Far Eastern individuals were removed. Given the high prevalence of BD in the ME, local controls were either hospital staff or otherwise healthy cataract patients matched to the same population as BD patients from each country. ME controls were over 50 years of age as new presentation with BD after this age is extremely unusual in this population. The ethical committees of all centers approved genetic evaluations after written informed consent by the patients.

Genotyping

Genomic DNA was extracted from 5 mL samples of peripheral venous blood using the magnetic bead separation technique from AGOWA (Berlin, Germany) on a Microlab STAR pipetting robot (Hamilton Robotics, Reno, Nevada, USA). 1–2 ng genomic DNA was dispensed into 384-well plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, Ca, USA). Genotypes were determined using a Taqman allelic discrimination assay. The Assay-on-Demand service (www.appliedbiosystems.com) was used for the polymorphisms Arg702Trp and Gly908Arg. For Leu1007fs the Primers and probes for Leu1007fs were designed as described by Inhora *et al*.[8] (Isogen, IJsselstein, The Netherlands).

The PCR reaction mixture for the assay-on-demand assays included 1–2 ng of genomic DNA in a 2 µL volume and the following reagents: FAM and VIC probes (200 nM), primers (0.9 µM), and 2x Taqman PCR master mix (ABgene, Epsom, UK). For Leu1007fs the CPR reaction mixture included 2 ng of DNA in a 2uL volume and the following reagents: FAM probes (250 nM, TET probe (500 nM), primers (300 nM) and 2x Taqman PCR master mix (Applied Biosystems Inc, Foster City, Ca, USA). PCR cycling reaction was performed in 384-well PCR plates in an ABI 9700 PCR system (Applied Biosystems Inc) and consisted of initial denaturation for 15 min at 95 °C, and 40 cycles with denaturation of 15 s at 95 °C and annealing and extension for 60 s at 60 °C. Results were analyzed by the ABI Taqman 7900HT using the sequence detection system 2.22 software (Applied Biosystems Inc).

Statistics

All data were submitted for Hardy Weinberg Equilibrium (HWE) calculations before inclusion in the analyses. Subjects were grouped according to genotype for the polymorphisms of interest. Per subject the allele information of both alleles was added. To detect allele frequency differences between cases and controls Pearson's chi-square test was used. The analyses were performed in Haploview 4.0. A p-value of < 0.05 was considered significant.

RESULTS

Of all alleles less than 2.5% were excluded for further data processing because genotyping failed. In the 200 patients (400 alleles) and 520 controls (1040 alleles) no homozygous variants were present.

Both the Arg702Trp and Leu1007fs variants were significantly less frequently present among BD patients compared to healthy controls. The largest difference was found in the Arg702Trp variant: 0.5% versus 5.8%. For the Leu1007fs variant a difference of 0.0% versus 1.8% was observed. The frequency of the NOD2 Gly908Arg variant was not significantly different between the BD patients and the controls (Table 1).

Differences in frequencies of NOD2 polymorphisms may vary with geographical location [11]. We thus separately re-assessed the SNPs of the Western Caucasian and Middle Eastern subpopulations. Though not powered for this analysis, the Arg702Trp variant remained significantly reduced in the Western Caucasian subpopulation (p=0.042). In the ME group a trend (p=0.058) was seen for a reduced occurrence of Arg702Trp. The Leu1007fs variant was not significantly different in the sub-cohorts (Table 1).

DISCUSSION

In this genetic study consisting of 200 BD patients, three NOD2 variants were genotyped to test the assumption that these variants have a similar distribution as in CD patients. Contrary to this initial hypothesis, we observed a significantly lower frequency of two of the three variant alleles in the BD group as compared to healthy controls.

In the subgroup analysis, a statistically significant difference was obtained for the Arg702Trp variant allele of the Caucasian subpopulation. Our study was appropriately powered to identify frequencies of NOD2 variants in the same magnitude as described for CD. In contrast to this we observed a lower proportion of variant alleles in our BD population. Apparently the other subgroups in our study were too low in number to reach significance.

In our study we focused on individual SNP frequencies in an ethnically mixed cohort and extracted subpopulations. Ahmad *et al.* described haplotypes of three NOD2 variants in combination with other SNPs in 374 patients and 500 controls and could not find an association with BD[11]. Details on the individual variants, however, were not presented. Uyar *et al* used an entirely Turkish cohort (existing of 85 BD patients and 100 healthy controls) and could not demonstrate a significant difference[12]. It has been shown in other studies as well as in our present study that the NOD2 variants are observed at a lower frequency in ME cohorts[11, 12, 13]. Therefore, the size of the Turkish cohort might

Table 1; The frequency of three variant alleles in total cohort of 200 BD patients and 520 healthy controls (left column) and in sub-cohorts of Caucasian and Middle Eastern individuals (right column). Significantly less Arg702Trp and Leu1007fs variants are seen in the BD cohort compared to the healthy controls.

SNP	Allele	Total			Caucasian			Middle East		
		BD (frequency)	Control (frequency)	P-value	BD (frequency)	Control (frequency)	P-value	BD (frequency)	Control (frequency)	P-value
Arg702Trp	Arg	388 (0.995)	961 (0.942)	1,35E-05	112 (0,980)	811 (0.934)	0.0421	272 (1.000)	150 (0.987)	0.0579
	Trp	2 (0.005)	59 (0.058)		2 (0,018)	57 (0.066)		0 (0.000)	2 (0.013)	
Gly908Arg	Gly	387 (0.982)	1011 (0.980)	0.7533	116 (1.000)	864 (0.982)	0.1432	267 (0,974)	147 (0.967)	0.6606
	Arg	7 (0.018)	21 (0.020)		0 (0.000)	16 (0.018)		7 (0.026)	5 (0.033)	
Leu1007fs		392 (1.000)	1017 (0.982)	0.0069	116 (1.000)	866 (0.980)	0.1209	272 (1.000)	151 (0.993)	0.1805
	fs	0 (0.000)	19 (0.018)		0 (0.000)	18 (0.020)		0 (0.000)	1 (0.007)	

be too small to detect the observations made in our larger study. A relation of NOD2 variants and HLA-B51 has not been shown in the past and therefore not expected to be of value in the present study since these genes are located on different chromosomes (16 and 6, respectively) [11, 12].

Assuming that the findings in the current study can be confirmed by future studies, it is of interest to speculate how the low frequency of NOD2 variants in BD might contribute to the understanding of the underlying mechanism of the disease. It is clear that variation in many genes will contribute to BD susceptibility and disease progression. Candidates are the PTPN22 620W, IL-10 and TNF-α genes that support an increased inflammatory responses and a failure of regulation[14]. Similar mechanisms in CD are postulated to explain the involvement of NOD2 variants leading to decreased NF-κB activation (loss of function) and thus reduced defensin production resulting in an increased bacterial load in the gut of CD patients[9, 15, 16]. Moreover, NOD2 activation inhibits stimulation of Toll-like receptors by pathogen products, and as NOD2 function is impaired, TLR signalling is enhanced and generates an exaggerated inflammatory response inducing tissue damage[9, 15].

In a second model transgenic expression of the Leu1007fs variant show a gain of function, with a direct increase in NF-κB, proinflammatory cytokines and tissue damage[9]. A potential connection between these paradoxical results is the kinetics of the NOD2 response. Acute stimulation of human blood-derived macrophages with the NOD2 agonist, muramyl dipeptide (MDP) induces a proinflammatory cytokine response. By comparison, persistent treatment of cells with MDP prior to activation through TLR2 or TLR4 ligands decreases cytokine responses, possibly through induction of IRAK-M, in support of control of TLR signalling. However, this tolerance is lost in cells from Leu1007fs homozygous patients[17]. As gut epithelial cells, dendritic cells and macrophages are in constant contact with bacterial products of the normal gut flora it is proposed that such tolerance is a protective mechanism[18]. Failure to control responses would lead to tissue damage as seen in CD.

Our findings suggest that NOD2 variants react in some cases with a decreased production of inflammatory cytokines to an exogenous stimulus. This is supported by the observation that NOD2 gene-deficient mice show reduced joint inflammation and are protected against early cartilage damage after intraarticular injection of *Streptococcus pyogenes* cell wall fragments[19]. In addition to this, human peripheral blood mononuclear cells of Leu1007fs variant donors exposed to cell wall fragments, produce less TNF-α and IL-1β than healthy controls[19]. However, this is contradictory to macrophage data discussed above, and may be due to the in vivo nature of these experiments, route of challenge, or the type of antigen used. The complex role of NOD2 in response to bacterial challenge in different cell types should be addressed in BD and other conditions.

In conclusion, our observations indicate that at least Western Caucasian carriers of the NOD2 variant allele have a reduced risk of developing BD. Our study contributes to the general assumption that genetic factors are involved in BD. It will therefore be of considerable interest to investigate other variants in BD patients in order to gain more insight into the pathophysiology and potentially identify new leads for treatment options.

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Genome-wide association study in an admixed case series reveals *IL12A* as a new candidate in Behçet disease

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ABSTRACT

Introduction: The etiology of Behçet's disease (BD) is unknown, but widely considered an excessive T-cell mediated inflammatory response in a genetically susceptible host. Recent genome-wide association studies (GWAS) have shown limited number of novel loci-associations. The rarity and unequal distribution of the disease prevalence amongst different ethnic backgrounds have hampered the use of GWAS in cohorts of mixed ethnicity and sufficient sample size. However, novel statistical approaches have now enabled GWAS in admixed cohorts.

Methods: We ran a GWAS on 336 BD cases and 5,843 controls. The cases consisted of Western Europeans, Middle Eastern and Turkish individuals. Participants from the Generation R study, a multiethnic birth cohort in Rotterdam, The Netherlands were used as controls. All samples were genotyped and data was combined. Linear regression models were corrected for population stratification using Genomic Principal Components and Linear Mixed Modelling. Meta-analysis was performed on selected results previously published.

Results: We identified SNPs associated at genome-wide significant level mapping to the 6p21.33 (HLA) region. In addition to this known signal two potential novel associations on chromosomes 6 and 18 were identified, yet with low minor allele frequencies. Extended meta-analysis reveal a GWS association with the *IL12A* variant rs17810546 on chromosome 3.

Discussion: We demonstrate that new statistical techniques enable GWAS analyses in a limited sized cohort of mixed ethnicity. After implementation, we confirmed the central role of the HLA region in the disease and identified new regions of interest. Moreover, we validated the association of a variant in the *IL2A* gene by meta-analysis with previous work. These findings enhance our knowledge of genetic associations and BD, and provide further justification for pursuing collective initiatives in genetic studies given the low prevalence of this and other rare diseases.

INTRODUCTION

Behçet's disease (BD) is a systemic auto-inflammatory occlusive vasculitis presenting with oral and genital aphthous ulcers, skin lesions, ocular inflammation and a pathognomonic pathergy test [1-3]. Although the etiology of the disease is largely unknown, it is considered to be an excessive inflammatory response possibly triggered by an infectious antigen in a genetically susceptible host. Prevalence varies from 110–420 per 100,000 in the Middle East (Turkey) to about 2 per 100,000 in Western countries [3, 4]. In patients of Turkish origin there is a positive family history in 12% of the cases with a sibling risk ratio between 11–52 [3, 4]. The heritability of BD has been estimated to range between 20 to 60%, with the strongest genetic association embracing variants in HLA-B51, explaining about 20% of the disease heritability [3, 4]. The combination of epidemiological and genetic data suggest causal involvement of both genetic and environmental factors [5].

Over the years, numerous single nucleotide polymorphisms (SNPs) have been found associated with BD [6-9]. In 2010 the first genome-wide association study (GWAS) in BD cohorts of Turkish and Japanese origin demonstrated association of various variants in the known HLA-B51 domain, and two new association signals, mapping to the interleukin-10 (*IL10*), and the IL-23 receptor–IL-12 receptor Beta2 (*IL23R–IL12RB2*) locus [10, 11]. These associations later have been confirmed in an Iranian cohort [12]. Yet another study in Algerian individuals only replicated the associations from the IL10 variants [13]. GWAS in Chinese and Korean individuals reported associations of SNPs mapping to two other loci, with regions containing the signal transducer and activator of transcription 4 (*STAT4*) and GTPase of immune associated protein *GIMAP* genes, respectively. Polymorphisms in *IL10* and *IL23R–IL12RB2* were not found to be significantly associated with BD in neither of those studies [14,15]. More recently, associations with *CCR1, STAT4* and *KLRC4* were identified by means of imputation and GWAS meta-analysis [14]. This study also identified the *IL12A* region as suggestively associated with BD, but genome wide significance (GWS) was not reached. Discrepant association results, as those observed for variants mapping to the *GIMAP* locus, might be explained by the different ethnic origin of the cohorts studied [15, 16]. Therefore, studying cohorts of diverse ethnic background may be useful to understand the origin of these differences. Moreover, the inclusion of multiple ethnicities results in larger datasets (representing higher power) crucial for these analyses given the typically small effects of common genetic variants discovered by the GWAS approach [17]. Nonetheless, GWAS results can be confounded by ancestry differences between cases and controls, potentially leading to spurious associations. This challenge in multi-ethnic studies has so far been approached by methods that take into account confounding by genetic ancestry, such as correction for Genomic Principal Components (GPC) [18]. More recently, linear mixed models (LMM) have been introduced as a reliable method to correct not only for ancestry differences, but also for family structure and/or cryptic relationships [19].

We have therefore set up a multi-center GWAS including BD patients of both Middle Eastern and Western background. We demonstrate that by means of novel statistic approaches it is feasible to run a GWAS in a small multiethnic cohort and use these results for meta-analysis.

MATERIALS AND METHODS

Study populations

A total of 369 unrelated BD patients from 18 different geographic origins were included in our study (Table 1).

The age range was 18 to 73 years (mean 47 years). All patients fulfilled the International Study Group (ISG) criteria of BD diagnoses [20]. No exclusion criteria were applied.

Table 1. Cases before and after QC. Collection at [1] Erasmus MC at Rotterdam, The Netherlands, [2] University Hospital of Cukurova, Turkey, [3] St John's Ophthalmic Hospital, Jerusalem, Israel, [4] St Thomas' Hospital, London, UK, [5] University Hospital, Damascus, Syria, Ethnicity of cases after QC.

Ethnicity	Number of patients	Collection	Number of patients after QC
Afghanistan	1	[1]	1
Iranian	4	[1]	4
Lebanese	1	[1]	1
Cape Verde	2	[1]	2
Curacao	1	[1]	1
Dominican Republic	1	[1]	1
Dutch caucasian	24	[1]	20
Greece	1	[1]	1
Israel	1	[1]	1
Jordan	1	[1]	1
Morocco	16	[1]	12
Surinam	2	[1]	1
Thailand	1	[1]	1
China	1	[1]	1
Turkey	35	[1]	32
Turkey	91	[2]	87
Arab Jerusalem ancestry	110	[3]	98
UK caucasian	38	[4]	33
Syrian	38	[5]	38
Total	**369**		**336**

As controls, 87 Syrian healthy volunteers (22-73 years, mean 51 years) and 5,756 participants from the Generation R study, a multi-ethnic birth cohort in Rotterdam (4-9 years, mean 6 years), The Netherlands , were used [21].

Replication was pursued in a cohort of 82 BD patients who met the ISG criteria, and 98 ethnically matched controls in Western European, collected from Birmingham and Midland Eye Centre, Birmingham, UK.

The ethics committees of the involved centers (Erasmus MC (METC), Ethical Committe at Çukurova University and Sandwell and West Birmingham Hospitals Trust Ethics Committee) approved the study, and written informed consent was obtained from all participants.

Genome wide genotyping

Genotyping of cases and controls (including the Generation R Study) was performed using the Illumina HumanHap 610K and/or 660 K arrays, following manufacturers protocols. Quality Control (QC) was performed individually for each set following a standard protocol to exclude samples and SNPs with low quality genotyping. Markers with minor allele frequency (MAF) ≤ 1%, missing genotypes ≥5% or which failed an exact test of Hardy-Weinberg equilibrium proportions in the controls (P<1x10-7) were excluded from the datasets. Samples with gender discrepancy, excess of heterozygosity, duplicates or samples with relatedness or other inconsistencies were also removed. In a subsequent stage, we extracted SNPs common to both platforms which passed individual QC and then applied the QC protocol for the combined dataset.

Phenotype-Genotype Analyses

Heritability

To characterize the extent to which common genetic variants play a role in BD susceptibility, we applied a recently proposed approach for estimating heritability based on genome-wide sharing between distantly related individuals, as implemented in the GCTA software in combination with the determination of ancestry-aware kinship coefficients by REAP software [22, 23] . No pairs exceeded the standard cutoff coefficient of 0.025 for genetic relatedness, confirming that no two individuals in the analysis were closer than third degree cousins. Relatedness coefficients calculated from REAP were used in the relatedness matrix before implementing GCTA. A disease prevalence of 0.42% was used in the modelling [3, 4].

Genome-wide association analysis

Association between BD susceptibility and 553.224 genome-wide SNPs was carried out using a regression framework adjusting for population stratification. GWS threshold for

the association was established at $p<5x10^{-8}$. Two different strategies were followed to adjust for population sub-structure in the data.

Genomic Principal Components

In the first approach, 20 GPC were obtained for all pairs of individuals in the combined dataset based on the similarities of individual genotypic profiles for 37,060 independent autosomal SNPs contained in the HapMap Phase II release 22 build 36 [24] using Multi-Dimensional Scaling (MDS) as implemented in PLINK (http://pngu.mgh.harvard.edu/purcell/plink/). Afterwards, we performed logistic regression adjusting for those 20 GPC.

Linear Mixed models

In the second approach we used LMM in order to account for possible population structure in the association analysis. This methodology estimates the level of relatedness even between independent individuals using the genotyped markers. By modeling the dissimilarity between genotypes of the subjects, it is able to correct the association results for stratification. This approach is implemented in the publicly available EMMA eXpedited (EMMAX) software [25]. Since the effect sizes reported by EMMAX are based on Mantel–Haenszel statistics, the odd ratios for associations of the significant SNPs were estimated from the regular logistic regression models performed in PLINK.

Meta-analysis

Meta-analysis of our own study results with published results for the BD suggestive associated SNP rs17810546 [14] was performed using an inverse variance fixed-effects approach in METAL [26]

Replication analysis

Four SNPs: rs8187722, rs439033, rs11969661 and rs17087141 were genotyped using TaqMan assays designed by Applied Biosystems, Warrington, UK in 82 BD patients and 98 control samples. In short, PCR was performed in 384-well plates with a 10 µl total reaction volume containing 20 pg/ml DNA (samples) or water (controls) and 2x LC-480 probe master (Roche, West Sussex UK) followed by endpoint genotyping analysis using the LC-480 system (Roche, West Sussex UK). Genotypes were determined as either homozygous (e.g. AA or GG) or heterozygous (e.g. AG) according to the presence or absence of fluorescence for each genotype.

RESULTS

An overview of the ethnicity of the included BD cases is presented in Table 1; all controls were collected in St John's Ophthalmic Hospital, Jerusalem and had Arab ancestry. In order to gain power and match all remaining ethnicities, this dataset was combined with the genotyped samples from the multiethnic Generation R Study.

Genome wide Genotyping

Genotyping was performed in 456 samples (369 cases and 87 controls) collected from the different participating hospitals (Erasmus MC at Rotterdam, The Netherlands, University Hospital of Cukurova, Turkey, St John's Ophthalmic Hospital, Jerusalem, Israel and by St Thomas' Hospital, London, UK). After quality control, 553,224 genotyped SNPs in 423 of these samples remained. We excluded 33 patients (including 1 control) because of low Illumina call rate (< 97.5%) and one more individual was excluded for gender mismatch. After QC of this combined dataset, 336 cases and 5843 controls were available for analysis.

Phenotype Genotype Analyses

Heritability

The heritability estimate, based on the 505,454 genotyped SNPs and 4,855 unrelated individuals (296 cases) was 32.0% (95%CI 21.0-44.0%;P= 5.5E-17). Thus a considerable percent of the BD risk is explained by the additive effect of the common SNPs analyzed.

Genomic Principal Components

The representation of the first GPC for the combined dataset projected together with the three reference panels of the International HapMap Project Phase II known as YRI, CEU and CHB/JPT representing populations of African, European and Asian background respectively [27] is shown in Figure 1. As shown by the overlapping distribution across the four first GPC, BD cases (represented in black) are well matched by genetic ancestry to controls (represented in yellow Syrian volunteers and grey for Generation R participants).

Genome wide association analysis

Association analyses were performed for 505,454 SNPs. As expected in such structured population, results from analysis without any type of stratification adjustment, showed association through the whole genome and a Genomic Inflation Factor (λ) of 5.25 (Supplementary Figure 1). After GPC correction, the association analysis showed minimal inflation of the test statistics with a λ of 1.035 (Figure 2).

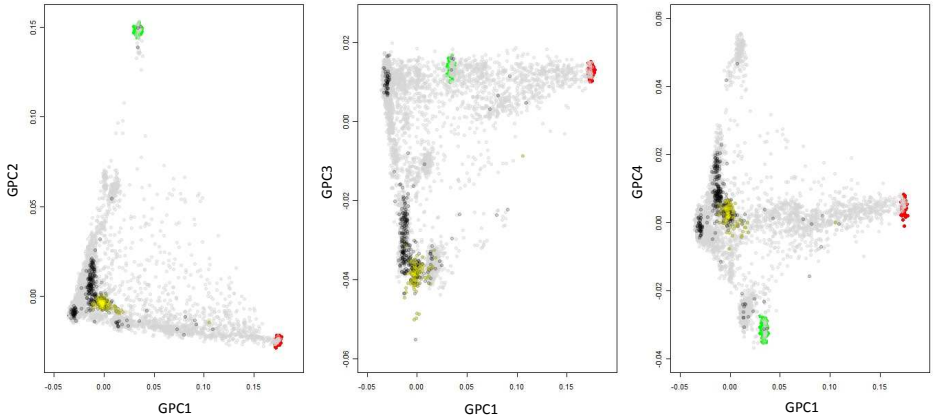

Figure 1. Genetic substructure of the Combined GWAS dataset. Two-dimensional scatterplots from multidimensional scaling analyses of the Generation R Study and Behçet collected data together with the three initial Panels form the HapMap Project. Each dot represents an individual in the dataset. Color codes: Grey=Generation R, Black= Behcet Cases, Yellow = Jordan controls. Blue= CEU, Red=YRB, Green= JPT.

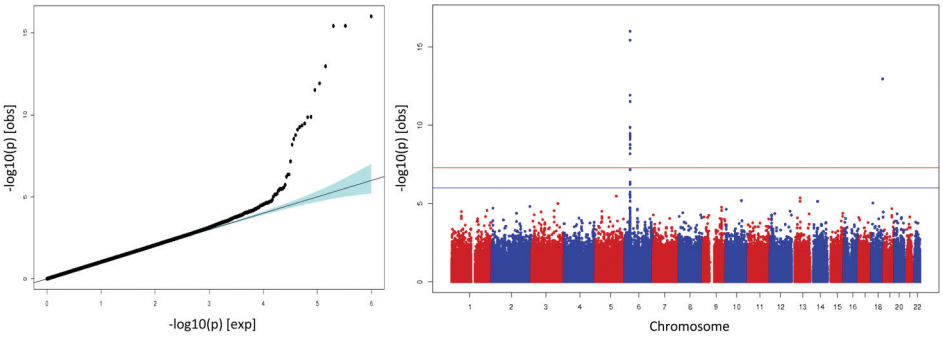

Figure 2. Behçet GWAS results using Genomic Principal Components (GPC) adjustment. Each dot represents an SNP in the dataset. **a. QQ-plot.** Associated SNPs deviating from the null hypothesis of no association (identity line). **b. Manhattan plot.** SNPs showing association with the disease map to chromosome 6 and a singleton in chromosome 18.

The top associated SNPs mapped to the HLA region on chromosome 6 with rs7770216 being the most significant (Table 2). Yet, another GWS signal in chromosome 18 driven by rs17087141 mapped to a region containing the uncharacterized miscRNA LOC400655.

Additional association analyses using different strategies including reduction of number of matching controls (to different case/control ratios) and incorporating different number of genomic principal components in the models yielded similar results for effect coefficients and genomic inflation factors (data not shown).

Table 2. GWS SNPs associated with Behçet's disease susceptibility using GPC and EMMAX approaches. Results for the association analysis when correcting for 20 PCs to adjust for stratification. In italic font the SNPs which show association only by one of the two approaches

CHR	SNP	BP	A1	A2	MAF	N	20PCs		EMMAX*
							OR	P	P
6	rs9380215	31157634	A	G	0.09	6178	2.132	1.32E-10	3.15E-15
6	rs4947296	31166157	C	T	0.09	6178	2.131	1.38E-10	4.98E-15
6	rs9263509	31174398	T	C	0.16	6179	1.82	6.62E-09	2.69E-10
6	rs2233984	31187243	A	G	0.09	6179	2.064	3.35E-10	1.08E-15
6	rs4495304	31188697	C	T	0.09	6028	2.069	7.92E-10	3.84E-14
6	rs4959053	31207556	A	G	0.09	6175	2.348	1.20E-12	3.90E-17
6	*rs2523589*	*31435313*	*C*	*A*	*0.43*	*6177*	*0.5466*	*2.92E-09*	5.83E-08
6	rs2844575	31442924	G	A	0.48	6173	1.81	5.62E-10	5.92E-08
6	rs9266409	31444547	C	T	0.25	6178	2.183	3.66E-16	2.62E-18
6	rs2253907	31444849	A	G	0.48	6175	1.797	4.41E-10	1.46E-09
6	rs7770216	31448590	T	G	0.26	6072	2.198	9.75E-17	2.25E-18
6	rs6933050	31451611	C	T	0.25	6178	2.183	3.69E-16	2.59E-18
6	rs1131896	31487094	A	G	0.31	6141	1.647	6.86E-08	2.66E-09
6	rs2256028	31487177	T	G	0.21	6177	1.784	1.72E-09	7.49E-12
6	rs2848713	31492458	A	G	0.1	6173	2.118	3.03E-12	7.08E-22
6	*rs11969661*	*160626409*	*T*	*C*	*0.02*	*6179*	*2.696*	*1.30E-03*	*5.15E-09*
6	*rs439033*	*160739406*	*C*	*T*	*0.02*	*6163*	*2.016*	*2.30E-03*	*1.14E-08*
6	*rs8187722*	*160784748*	*G*	*A*	*0.01*	*6179*	*1.696*	*1.00E-04*	*2.87E-15*
18	rs17087141	69036707	C	T	0.03	6167	4.925	1.08E-13	2.81E-18

* Odd ratios cannot be calculated by EMMAX approach

Results from the GWAS analysis using EMMAX yielded three independent GWS signals (Table 2). In addition to those mapping to the two previously described BD loci from chromosomes 6 and 18, an additional signal was found on chromosome 6q25.3 6 in the solute carrier family 22 member 3 (*SLC22A3*) gene region. The three most significant markers underlying this GWAS signal included rs11969661, rs439033 and rs8187722 (Table 2). Using EMMAX resulted in adequate adjustment for population stratification as corroborated by λ=1.0 (Table 2) and the absence of early deviation from the identity line in the QQplot (Figure 3).

The most significant SNP identified by the EMMAX approach maps also to the HLA region in chromosome 6. The regional association plot of this HLA-B region is shown in Supplementary Figure 2. This HLA-B region has been associated with BD susceptibility in previous GWAS analyses [10, 22]. Figures 4 and 5 show the association plots for the

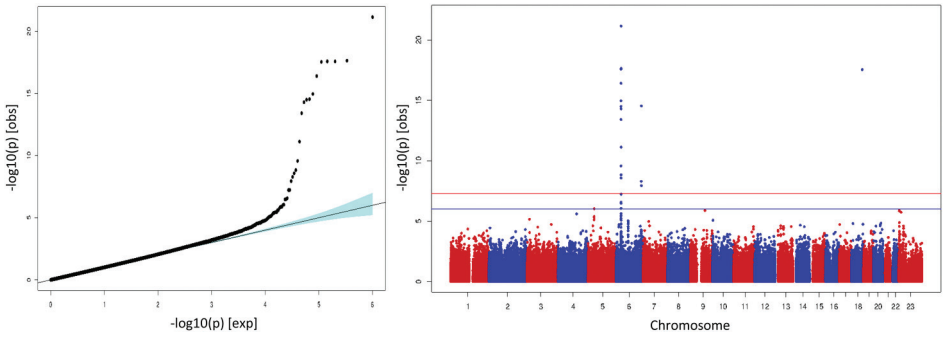

Figure 3. Behcet GWAS results using Linear Mixed Models Genomic approach. Each dot represents an SNP in the dataset. **a. QQ-plot.** Associated SNPs deviating from the null hypothesis of no association (identity line). **b. Manhattan plot.** SNPs showing association with the disease map to two different signals in chromosome 6 and a singleton in chromosome 18.

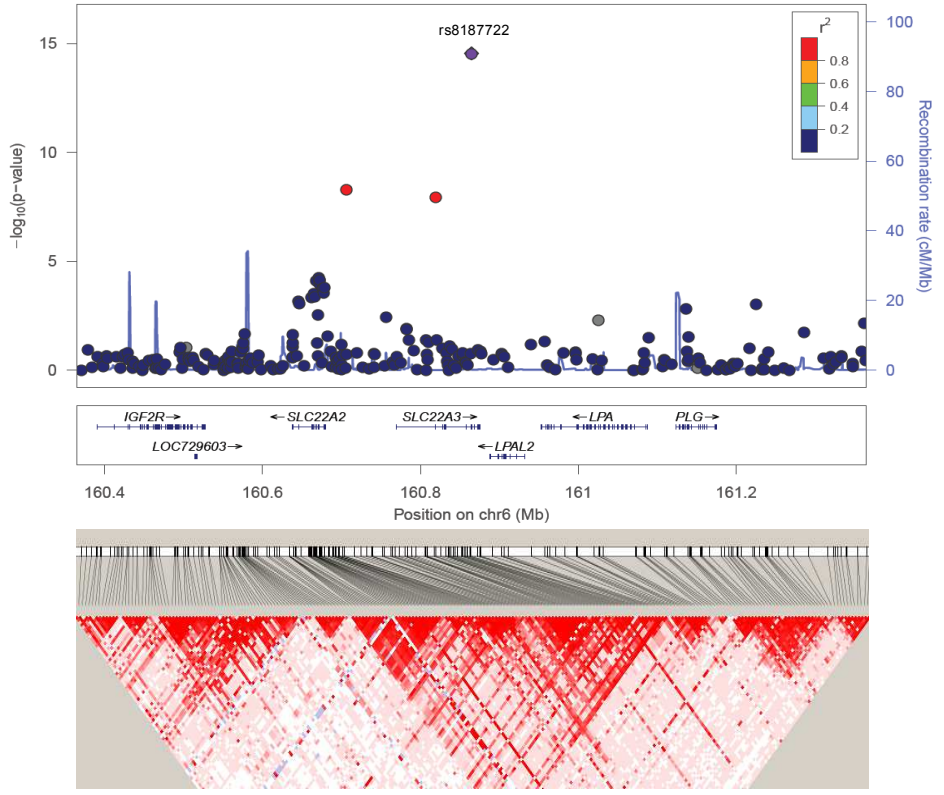

Figure 4. SNP association plot for Behçet's susceptibility-associated region of chromosome 6q25.3. Dots represent GWAS P-values (EMMAX approach) and positions of SNPs found within the 6q25.3 locus. The top SNP, i.e. rs8187722, is denoted by a diamond. Different colours indicate varying degrees of pair-wise linkage disequilibrium (1000 Genomes Nov 2010 CEU) between the top SNP and all other genotyped SNPs. Genetic coordinates are per 1000 Genomes Nov 2010-CEU. Bottom, LD heat map based on D' values from the combined population under study including all SNPs in the 500Kb region.

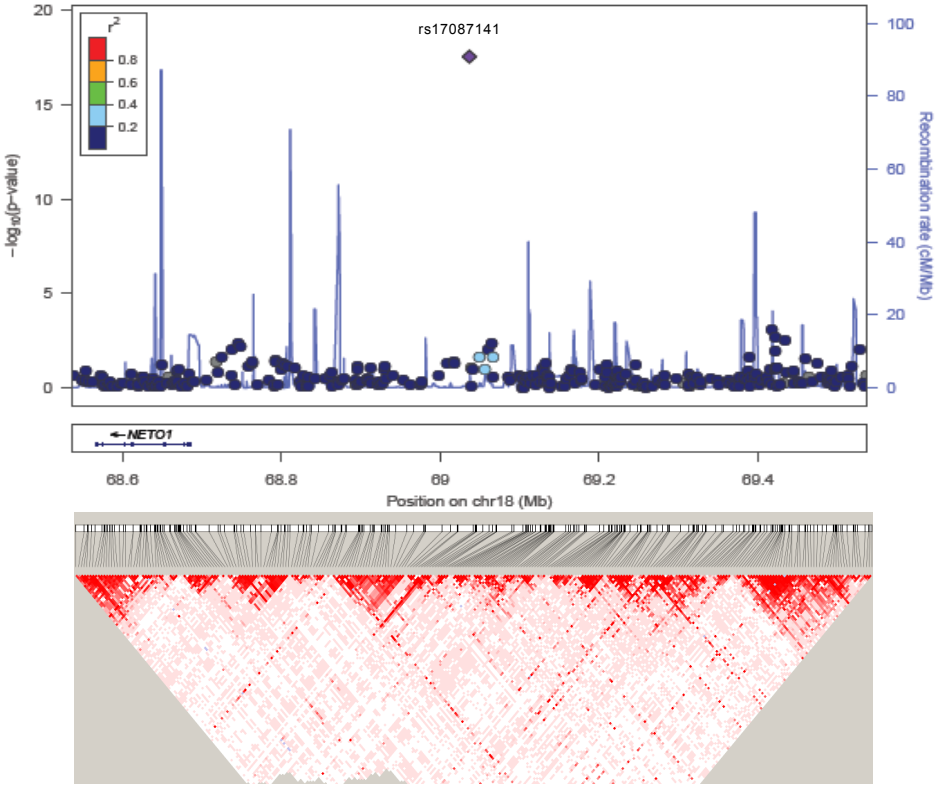

Figure 5. SNP association plot for Behçet's susceptibility-associated region of Chromosome 18q22.3. Dots represent GWAS P-values (EMMAX approach) and positions of SNPs found within the 18q22.3 locus. The top SNP, i.e. rs17087141, is denoted by a diamond. Different colours indicate varying degrees of pair-wise linkage disequilibrium (1000Genomes CEU) between the top SNP and all other genotyped SNPs. Genetic coordinates are per 1000 Genomes Nov 2010-CEU. . Bottom, LD heat map based on D' values from the combined population under study including all SNPs in the 500Kb region.

other two GWS signals in 6q25.3 (*SCLA22A3* locus) and 18q22.3, which have not been previously reported as associated with BD.

Meta-analyses

By scanning published BD GWAS, we identified a variant suggestive of association (P=6.01x10-7) [14], showing strong evidence for association with BD in our study, although not at GWS level. After meta-analysis of our results with those published by Kirino et al. in two Turkish cohorts the leading SNP (rs17810546) in *IL12A* reached GWS. The G-allele conferred a 1.7 increased risk for BD (OR= 1.66; 95%CI [1.42,1.93]; P= 1.12E-10 (Table 3.)).

Table 3. Meta-analysis of leading SNP in IL12A. Association results for rs17810456 (G-allele) in two Turkish cohorts (14) and the current study.

Study	N	N cases	OR	CI 95%	P value
Turkish replication*	2487	1209	1.64	[1.31 - 2.04]	1.20E-05
Turkish discovery*	1447	821	1.41	[1.06 - 1.89]	2.00E-02
Current study	6179	336	2.06	[1.49 - 2.84]	9.31E-06
Combined	10113	2366	1.66	[1.42 - 1.93]	1.12E-10

* From Table 1 Kirino et al. 2013

Replication

No significant evidence for replication of the leading SNPs from the GWAS signals of chromosome 6 and 18 was found, while the MAF of the genotyped markers were very similar to those reported in healthy individuals.

DISCUSSION

Enabled by innovative novel methodology we could run a GWAS in a rare condition like BD within a unique case collection of multiethnic background. We identified variants associated with BD mapping to the well-established MICA-HLA-B locus and to two regions on chromosome 6 in the *SLC22A* gene region and on chromosome 18 in an uncharacterized region. Moreover, meta-analysis with previous published results enabled the identification of a GWS association with variants in the *IL12A* region. All together, these common variants across this four loci explained up to 32% of the variance in BD risk.

Methods for the calculation of heritability estimates from SNP microarray data of population-base studies have recently emerged [22, 23]. We could determine that the narrow sense heritability of BD explained by common variants in our study was 32%, estimate in line with those obtained by previous reports based on family data [3, 4, 28], albeit smaller. This could be explained by the overestimation of heritability estimates in family-based design, as a result of biases due to epistatic interactions or shared environment. In addition, GCTA estimates should be interpreted as the lower bound of the true additive genomic influence on heritability, since the genetic variance is limited to the common SNPs present in the arrays(29). Therefore variance explained by rarer variants (MAF<0.01) and/or causal variants that were not genotyped or are not tagged by the SNPs on the genotyping array will be missed. As GCTA is intended for studies on homogeneous populations it is necessary to use appropriate methods to calculate the relatedness matrix in studies with admixed individuals to avoid overestimation of the heritability. Therefore, to obtain unbiased estimates we used REAP for the estimation of the kinship coefficient [30].

Methods based on LMM enabled the analysis of GWAS data in a cohort of mixed ethnicity of relative small sample size, maximizing power. The fact that our GWAS identified well-established variants in the HLA-B locus, provides certainty that the methodological approach was sound and in fact applicable to other rare diseases. Expanding the current case collection or performing meta-analysis with other collections is warranted. Efforts including datasets of different ancestries, should be analyzed with methodologies which take into account different genomic architecture based on ethnic background. Such methods can also be applied at the meta-analysis level as implemented in MANTRA [31].

In addition to the signal in the well-known HLA locus on chromosome 6, we also identified two genomic regions of interest. Nevertheless, all variants had relatively low MAF (2-3%) and thus their significance should be interpreted with caution. We could not find evidence for replication of the associations in an independent yet underpowered case/control set. Considering the low MAF and the very limited power of the replication further scrutiny in an expanded dataset is required to confirm these variants as real or spurious associations.

Yet another GWS association with rs17810546 in the *IL12A* locus was identified by meta-analyses of our results with those reported previously in the literature. These results support the role of this locus in the susceptibility of BD. Kirino et al., described this locus as suggestive for association with BD. In that study the variants did not were reach GWS in the combined analysis likely due to the variant being monomorphic in the samples of Japanese origin included in the study. Variants in *CCR1-CCR3, STAT4* and *KLRC4* which were polymorphic in both Turkish and Japanese populations surpassed GWS thresholds, suggesting that the lower power likely contributed to the non-significant findings in the *IL12A* locus.

BD is a rare disease, even in countries with the highest prevalence. Collecting the patient material for large multi-ethnic genetic studies proved to be challenging. Not surprisingly, the first two GWAS studies reported in BD were in two separate cohorts with a single ethnicity, a Turkish and a Japanese cohort [10, 11]. After meta-analyses of the data of both studies, polymorphisms in *IL10* and *IL23R–IL12RB2* proved to be associated at GWS level. In our study, these two polymorphisms were not associated with BD, nor in two other recent studies in Chinese and Korean individuals [32, 33]. Moreover, the *GIMAP* variants presented in the Chinese and a Korean cohort were not identified in the other studies. Recently, Ortiz-Fernandez et al., reported no association of *GIMAP* variants in a Spanish study but also acknowledged lack of power as a likely cause for this negative results [16]. Considering that most of our cases are of admixed ethnicity differences in ethnic background, specific pathology or clinical features of the cases are a more plausible explanation for the lack of replication of those variants in our study on top of limited power [32, 33].

In conclusion we have shown that appropriate methodological approaches enable performing GWAS in cohorts of admixed ethnicity, combining cases of different ethnic origin as is often required in rare conditions like BD. As such, previously reported SNPs in the MICA-HLA-B locus reached GWS in the current approach. Additionally, we have shown that results of a mixed cohort can successfully be used in meta-analysis, by which we could establish the involvement of previously putative variant in the *IL12A* locus in the susceptibility of the disease.

Our results emphasize the need of collaborative efforts intending to enlarge the collection of BD cases, which can enable a well-powered setting for detection of genetic associations with the disease, especially when interrogating low-frequency variants.

Potentially novel loci need to be further explored in additional studies, and ultimately coupled with functional studies, allowing a better understanding of the pathophysiology and the genetic architecture underlying the risk for BD.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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DATA AVAILABILITY

Due to restrictions based on privacy regulations and informed consent of participants, phenotype and genotype data cannot be made freely available in a public repository. Data of the Generation R Study can be obtained upon request. Requests should be directed towards Prof.dr. V. Jaddoe (v.jaddoe@erasmusmc.nl). As for the data collected exclusively for this effort, requests should be directed towards the corresponding author.

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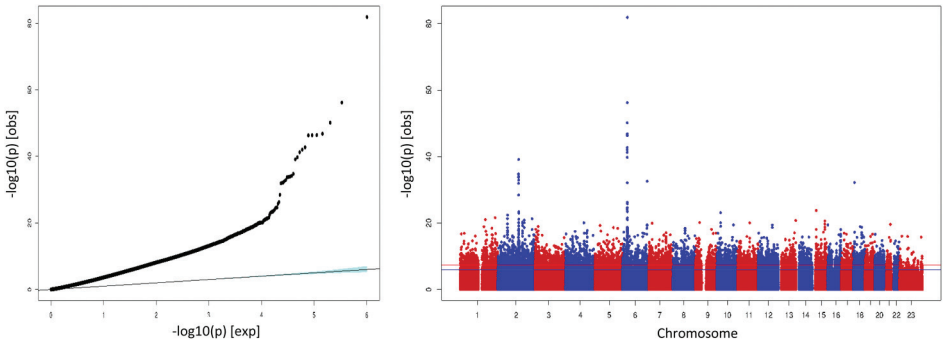
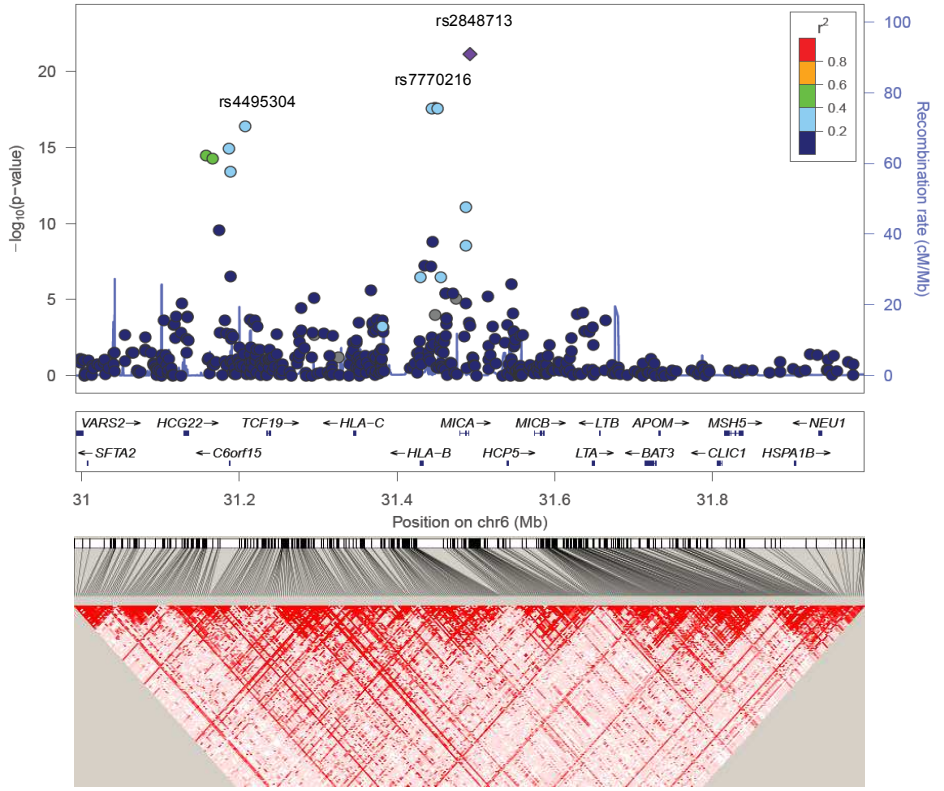


Supplementary Figure 1. **Behçet GWAS results using not adjustment for population stratification.** Each dot represents an SNP in the dataset. **a. QQ-plot.** Associated SNPs deviating from the null hypothesis of no association (identity line) evidence high inflation. **b. Manhattan plot.** SNPs though all chromosomes show association with the disease.



Supplementary Figure 2 HLA Regional Association Plot. GWAS results forBehcet susceptibility using Linear Mixed Models approach. Top SNP rs2848713 EMMAX approach (MAF=0.10, A-risk allele) is denoted by a diamond. Marker rs7770216 is the top SNP for the Genomic Principal Components adjustment approach (MAF=0.25, T-risk allele). Marker rs4495304 is the top SNP in the association reported by Mizuki et al. (MAF=0.09, A-risk allele) for the GWAS of Behçet susceptibility without an. . Bottom, LD heat map based on D' values from the combined population under study including all SNPs in the 500Kb region.

Whole genome sequence in Behçet disease patients

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Unpublished data

INTRODUCTION

Behçet's disease (BD) a systemic auto-inflammatory occlusive vasculitis, is characterized by oral and genital aphthous ulcers, skin lesions, ocular inflammation and the pathognomonic pathergy test(1-3). The etiology of the disease is largely unraveled. It is, considered to be an excessive inflammatory response triggered by an (infectious) antigen in a genetically susceptible host. Prevalences vary from 70–420 per 100 000 in the Middle East (Turkey) to about 1-7 per 100 000 in Western countries. In patients of Turkish origin there is positive family history in 12% of the cases with a sibling risk ratio between 11–52 [3, 4]. The heritability of BD has been estimated to range between 20 to 60%. The strongest genetic association is with HLA-B51, it explains about 20% of the disease heritability [3, 4]. The combination of epidemiological and genetic data suggest causal involvement of both genetic and environmental factors(5).

Over the years numerous single nucleotide polymorphisms (SNP) have been found associated with BD(6-9). In 2010 the first genome-wide association study (GWAS) in BD cohorts of Turkish and Japanese origin demonstrated a range of variants in the HLA-B51 domain, but also two new association signals, the interleukin-10 (*IL10*), and the IL-23 receptor–IL-12 receptor Beta2 (*IL23R–IL12RB2*) locus(10, 11). These associations have been confirmed in an Iranian cohort(12). Another study in Algerian individuals only replicated the associations from the IL10 variants(13). GWAS in Chinese and Korean individuals reported associations of SNPs mapping to two other loci, with regions containing the transduction and activator of transcription-4 (*STAT4*) and GTPase of immune associated protein GIMAP genes, respectively. Polymorphisms in *IL10* and *IL23R–IL12RB2* were not found significantly associated in either of those studies(14,15). Most recently associations with *CCR1, STAT4* and *KLRC4* were identified by imputation analysis [14]. Kirino et al in this study identified the *IL12a* region as an area of interest but genome wide significance was not reached.

In GWAS a limited number of polymorphisms is examined, from 200.000 up to 600.000 in BD studies. The number can be increased by imputation studies. However the largest part of the genome is neglected. Whole genome sequencing now enables to look at the complete genomic code. Performing such study in a family with multiple individuals afflicted by the disease simplifies the data analyses in order to identify genetic variants so far unknown.

We therefore performed a whole genome sequence study in five family members with 3 BD patients and 2 healthy individuals.

MATERIALS AND METHODS

Study population

Five family members were included in our study (figure 1). The father and two sons were BD patients, one son and one daughter are healthy individuals.

All patients fulfilled the International Study Group (ISG) criteria of BD diagnoses(15). No exclusion criteria were applied. All subjects gave their written informed consent.

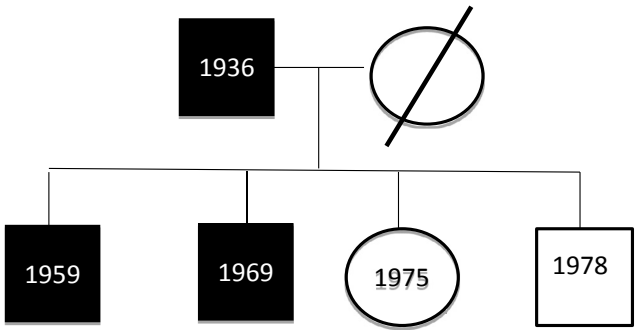

Figure 1. Family with three BD patients (black boxes) and 2 healthy individuals (white boxes)

Whole genome sequencing

Sequencing-by-ligation method from Complete Genomics (software version 2.5.0.33,

format version 2.5) as described by Drmanac et al. (2010) was used. The human genome sequencing procedures include DNA library construction, DNA Nano-Ball (DNB) generation, DNB array self-assembling, cPAL-based sequencing, imaging, image data analyses including base calling, DNB mapping, and sequence assembly. Reads were mapped to the National Center for Biotechnology Information (NCBI) reference genome, build 37. Variants were annotated using NCBI build 37 and dbSNP build 137. Data were provided as lists of sequence variants (SNPs and short indels) relative to the reference genome. Analysis of the whole genome sequencing data was performed using Complete Genomics analysis tools (cga tools version 1.8.0; http://www.completegenomics.com/sequence-data/cgatools/) and TIBCO Spotfire version 6.0.0 (build version 6.0.0.40) (http://spotfire.tibco.com/).

Data analyses

Data were analyzed using a python (2.7) script for multiple genome analysis, cga tools version 1.8.0 and Spotfire

6.0.0.40 (Tibco). Sequenced exome fraction (where weightSumSequenceCoverage >= 40x) were 0.985 (Imm4) and 0.986 (Imm5).

RESULTS

DNA isolation failed in three cases, therefore genome sequencing was performed on DNA of the affected father and of a non-affected son.

IBS (indentity by sequence) analysis showed that both samples share one allele over 99% of the genome, confirming the father and son relation.

In total 5,817,040 variants were detected in one or both family members, including single nucleotide variants (4,4681,960 and small insertions (713,715), deletions (429,173) and substitutions (205,956) (up to about 50 bp).

Of these, 410,868 variants were not present at all in any of the control samples (597 wellderly samples, healthy individuals of over 85 years).

Regarding the inheritance pattern, we searched for dominant variants in the father that were not present in the non-affected son and in the healthy controls. (Genotypes: Imm4:00|0; Imm5:01|11|1 and allowing 2 No-calls)

165,768 variants were found, of which 75,162 variants were found in genes. 821 of these variants were protein affecting variants (PAV) in 732 different genes. 346 protein affecting variants (in 322 genes) had a high (H) varScore. The top 40 list is presented in table 1.

Table 1. Top 40 list of protein affecting variants identified by whole genome sequencing.

Chromosome	Gene Variant	Chromosome	Gene Variant
chr10	DMBT1	chr9	ALDH1B1
chr1	RPTN	chr5	TTC37
chr18	DLGAP1-AS3	chr12	KCNH3
chr1	C8A	chr15	DIS3L
chr3	PFKFB4	chr11	LOC338667
chr13	FREM2	chr12	C12orf42
chr6	RNF146	chr17	FN3KRP
chr21	DNMT3L	chr5	CARD6
chr1	ITGA10	chr2	NABP1
chr22	ISX	chr22	PRR14L
chr6	PGC	chr22	CLTCL1
chr9	KIAA0020	chr3	AMIGO3
chr10	NSMCE4A	chr19	ZNF160
chr12	GLI1	chr5	PCDHGA10
chr1	MTMR11	chr1	VWA5B1
chr12	WSCD2	chr17	KIF19
chr5	CARD6	chr15	FMN1
chr19	ILVBL	chr1	SLAMF9
chr12	PLBD1	chr10	LCOR
chr2	HIBCH	chr13	NEK5

These 346 PAV could be subdivided in: 269 missense; 37 frameshift; 18 insert; 13 deletion; 6 nonsense; 2 disrupt and 1 nonsense variants.

DISCUSSION

Over the years genetic associations have been identified by a candidate gene approach as well as GWAS approach. numerous single nucleotide polymorphisms (SNP) have been found associated with BD(6-13)(14,15). In order to identify new genetic variants we performed a whole genome sequence study in a family of BD patients. An exone sequence study in a different family identified *TNFRSF9, MGEA5 and TNFAIP3* genes is possible candidates(16).

Due to a low yield in DNA we only analyzed the data of the father (case) and son (healthy). This led to a total of 346 PAV, a number too high for further analyzations. It identified interesting candidate variants that could be involved in BD, however it is not possible to focus on such number of genes. Variants identified earlier are not found in in our dataset. Therefore new patient material is collected en sequence analyses will be redone using all five family members of this unique family with such high incidence of BD.

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4

Cytokines in Beçhet's disease

Cytokine profiles in tissue

Based on

Do synovial biopsies help to support evidence for involvement of the innate immunity in the immunopathology of Behçet's disease?
Jan A.M. van Laar, Jasper H. Kappen, Paul.L.A. van Daele, P. Martin van Hagen
Arthritis Res Ther. 2009;11(2):109.

Cytokines in the colon of a patient with Behçet's disease.
Jasper H Kappen, Willem A Dik, Gemma M Dingjan, Paul LA van Daele, Herbert Hooijkaas, P Martin van Hagen, Jan AM van Laar
Arthritis Res Ther. 2009;11(4):412.

The etiology of BD is obscure, but considered a complex systemic vasculitis, caused by Th1-cytokine skewed neutrophillic and lymphohistiocytic inflammation. The innate immune response is held responsible for the inflammatory symptoms occurring in patients with BD. Activation of BD can be triggered by exogenous factors such as skin damage, introduction of bacterial components, viruses or stress resulting in inflammation[1-3].

The association of HLA-B51 with BD and hyperactive neutrophils adds to assumption that BD is an "innate disease"[4]. Other factors supporting this assumption include; prevalence of symptoms at places in close contact with the outside environment (skin, mucosa, gut), hypersensitive T-cells, TLR expression in affected cells, hyperactive neutrophillic and CD8+T-cell responses to heat shock proteins or bacterial cell wall fragments[1, 3]. A normal inflammatory innate response is rapid, non-specific and self-limiting. It contains a carefully balanced reaction between activated inflammatory cells and an exogenous trigger. In inflammatory disorders such as BD this response is disbalanced and causes excessive tissue damage.

Cytokines involved in BD are in great part pro-inflammatory (IL-1β, IL-8, IL-10, TNF-α and IFN-γ) and also associated with innate immunological patterns. Peripheral T-cells in BD patients express a predominant Th1-type cytokine pattern[5]. The rarity of BD and the lack of adequate *in* vitro or animal study models challenge to investigate immunopathological innate inflammatory patterns on the cytokine level *in vivo*. Therefore a model of easily reachable inflammatory material in BD patients with active disease is sparse.

Canete et al. describe a model of studying the immunopathology of BD by analyzing inflammatory cells, tissue and cytokines of inflamed joints from BD patients [6].

How does the study of Canete et al. add to biomechanistic studies already done in BD? First, in contrast to most of these studies analysis are performed in untreated patients. In an uncommon disease as BD, few studies in naïve patients are published. In RA for example, early and aggressive treatment is considered mandatory for optimal therapeutic outcome. It will therefore be of great therapeutic importance to appreciate possible initiating factors in BD too by testing therapy-naïve patients. This is especially important for irreversible ophthalmic disease. Second, it is of importance that representative tissue is studied. In BD most mechanistic studies are published on blood samples, and predominantly circulating cytokines or *in vitro* testing of PBMC's is done. Th1 cytokines are involved, hyperactive neutrophils and hyper reactive T-cells are held responsible for the symptoms observed[7].

But BD is an inflammatory disorder with involving tissues such as skin, joints, eyes, gut, brain, oral and genital mucosa[1, 3]. It is therefore mandatory to known that observa-

tions in the blood are comparable with bio-mechanisms on the tissue level. Mucosal tissue is predominantly involved in BD and microorganisms from the outside can easily influence observations on inflammatory cells. On the other hand, those microorganisms are also capable of stimulating an immune response in BD. Most tissue studies on the pathophysiology of BD are performed on the skin. One of the unique and disease specific features of BD is the occurrence of a positive skin pathergy reaction (SPR) varying from 30–75% of all BD patients[1, 3]. Neutrophils and later lymphohistiocytic cells occur in sterile lesions induced by a sterile needle prick[8]. Representing endothelial injury and functioning as leukocyte trafficking factors, cell adhesion molecules (ICAM-1, VCAM-1) and endothelial growth factor markers such as E-selectin, P-selectin and endoglin are linked with SPR[9]. Infiltrating cells are mainly of the HLADR+/ CD3+/CD4+/CD45RO+ type[10]. Other observations include mature dendritic cells, monocytes, (regulatory) T-cells, elevated IFN-γ, IL-12-p40 and IL-15, MIP3-α, IP-10, Mig, and iTac, and adhesion molecules[11]. Studies of ocular BD show elevated Vgamma9Vdelta2 (CD4-/CD8-) and CD8brightCD56+ T-cells that are probably different than those found infiltrating the SPR[12] . Cytokines found elevated in ocular fluid of BD patients with active uveitis are IFN-gamma, TNF-α, IL-15[13]. It must be stressed however that aqueous humour is seldomly studied, difficult approachable and is only studied in intensively and long treated BD patients [14].

In contrast to most of the inflamed organs in BD, the joint is a sterile and often involved in BD patients [1, 3]. Canete et al. use this easily accessible model that has not been studied too often in BD. Previous, more than 30 year old, studies on BD arthropathy predominantly show neutrophillic infiltration depending on the stage and treatment of the disease. A more recent study in synovial fluid (SF) of BD patients show increased chemokines (CXCL-9 and CXCL-10) related with Th1-directed inflammation[15]). The group of Canete has studied SF extensively in various other arthropathies such as SpA, RA and comparisons might be made with historical data. SF of RA, juvenile SpA, juvenile polyarthritis, and juvenile oligoarthritis compared with SpA showed merely similar CD3, 4 and 8 positive cells in all groups[16]. In the study presented it is concluded that PsA clinically resembles BD and probably also because in SF of PsA patients neutrophils are present. By showing increased intraartriculary CD15+ neutrophils, CD3+ T-cells, and perforin in SF of active BD patients as compared to PsA it can be emphasized that also in early BD the innate immunity is involved and interacts with the adaptive immunity reflected by perforin presumably secreted by CD3+Tcells. It might therefore be of interest to further study on these naïve T-cells and cytokines in sterile SF in order to relate these observations with immunological patterns already described in a-sterile tissues that are exposed to exogenous factors, or more intensively treated BD patients. These results can then be compared with various other inflammatory diseases in order to ob-

tain more insight in the immuno-etiology of BD and to develop more specific, molecular based immuno-therapy. Examples may include targeting cytokines (IL-1, IL-10, or IL6) and lymphocyte action interfering agents.

Cañete *et al* described cytokine patterns of inflamed joints from BD patients (BD)[6], we add to this an observation showing a similar cytokine profile in the colon of a BD patient with active disease, despite TNF-blocking treatment.

A 37-year old BD patient with severe colitis failed to traditional immunosuppressive treatment. Steroids were contraindicated because of a previous retinal serosal ablation, probably induced by prednisone. TNF-blockage initially successfully reduced the severity of the colitis, but relapses occurred. Variation of different TNF-blockers (etanercept, infliximab and adalimumab) could not permanently resolve the intestinal complaints. Antibodies against infliximab or adalimumab were not detected. Eventually, high dose infliximab (10mg/kg) and intravenous immunoglobulins (IVIG's) led to disease regression facilitating a hemi-colectomy. Hereafter the patient's condition improved significantly and IVIG's were terminated whilst continuing TNF-blockage.

Cytokines were evaluated in the resected colon by analyzing mRNA expression levels of cytokine genes as previously described[17]. Because of the extensive prior treatment apparently healthy colon tissue of the patient served as internal control. Intestinal inflammation was patchy and the observed difference between healthy and diseased tissue was confirmed microscopically. The mRNA expression of cytokines in diseased colon is presented relative to those of healthy colon and can be seen as a reflection of intramural cytokines (Figure 1). Comparable to Cañete *et al,* we observed a Th1 and Th17-skewed pattern (elevated IFN-γ and TNF-α, and IL-17A, respectively). We could not demonstrate elevation of IL-10, possibly because our patient was intensively treated, and colon environment is not sterile.

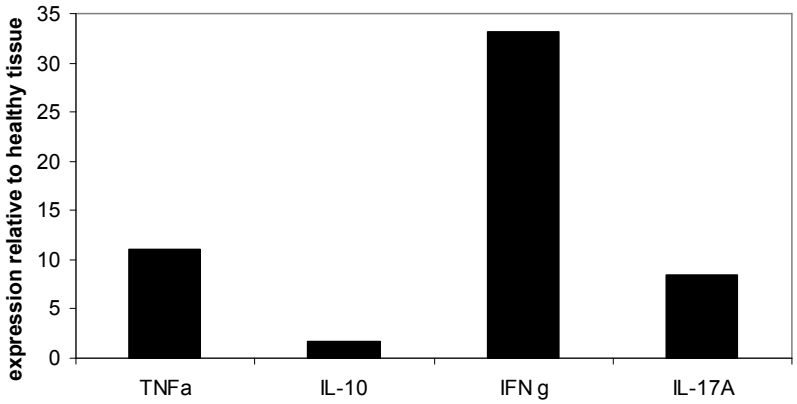

Figure 1. mRNA expression of cytokine genes in diseased relative healthy tissue.

Hence, understanding how cells and cytokines are functioning in inflamed tissues can be of great importance for therapeutic interventions[1]. In this light, one of the century's most eye-seeing examples is that by blocking TNF-α, a key cytokine in BD, disease symptoms can be effectively and rapidly blocked too[2]. Most new treatment modalities will focus on blocking cytokines in specific pathways that play a key role in immunopathophysiology of BD.

BD's immunopathology remains fascinating and highly relevant for the development of future, immune directed, therapy. Since the successful introduction of TNF-blockers it became apparent that Th1-cytokines might be key players in the immunopathology of BD, emphasizing the importance of cytokine studies[2, 3]. Our data add to Cañete *et al* that also in colonic tissue of a BD patient, IFN-gamma, TNF-alpha and IL-17A appear key cytokines, even in a treated patient. Maybe tissue cytokines reflect disease activity better than serum cytokine profiles.

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Detection of intestinal Behçet's disease by double balloon enteroscopy combined to serum cytokine profiles; improved diagnostic yield?

J.H. Kappen, P.B.F. Mensink, W.A. Dik, H. Hooijkaas, S. Lachman, P.L.A. van Daele, P.M. van Hagen, J.A.M. van Laar

Submitted

5

New treatment modalities

Mycophenolate sodium: effective treatment for therapy refractory intestinal Behçet's disease, evaluated with enteroscopy

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ABSTRACT

A 59-year old man with Behçet's disease (BD) was referred with complaints of continuous non-bloody diarrhea and abdominal pain since April 2006. On colonoscopy multiple aphthous lesions encompassed the entire colon showing a chronic merely neutrophillic infiltrate and oedema. Treatment with 30 mg prednisone and 200 mg azathioprine (AZA) was started. Attempts to lower the dose of prednisone led instantaneously to a relapse. In February 2007 symptoms worsened and mycophenolate sodium (MPS) was added, whilst tapering prednisone and AZA, yielding an immediate and sustained effect. Evaluation with a double balloon scopy four months later showed that the colonic lesions had healed. In the terminal ileum that had not been reached in the first colonoscopy, some minor ulcerative lesions could be detected. Eleven months after initiation with MPS the patient remains in good clinical condition and improved symptoms with a reduced steroid and AZA dose.

Conclusions: This case shows that MPS might be a good therapy before using biologicals or chemotherapeutics in therapy-refractory BD patients with severe ileo-colitis. The involvement of the entire gastrointestinal tract in BD and the response to the therapy can be visualized using the double balloon endoscopy.

INTRODUCTION

Although abdominal complaints may be regularly mentioned, gastrointestinal inflammation occurs infrequently (1-5 %)in the Western population of patients with Behçet's disease (BD), an idiopathic systemic vasculitis with oral and genital aphthous ulcers, skin lesions and ocular inflammation(1, 2, 3).

If present, aphthous lesions, erosions or ulcera, are most often reported in the terminal ileum and right sided colon, leading to anorexia, vomiting, diarrhoea and abdominal pain [3, 4, 5]. Mucosal lesions in other parts of the small bowel and colon are rarely reported.

Although the etiology of BD remains unknown, it is generally considered an excessive, T-cell mediated inflammatory response, possibly triggered by an (infectious) antigen in a genetically susceptible host. The inflammatory cells in the lesions are of lymphocytic and monocytic origin. Neutrophilic infiltration is present in early lesions and might indicate innate immunity involvement in the pathophysiology of BD6. This suggestion might be supported by recent findings of SNP's associated with a defective innate immunity in Crohn's disease (CD), a disease clinically similar to BD [2, 7].

Most patients with BD only need symptomatic treatment. However, patients with gastrointestinal involvement are may require more aggressive treatment5. This includes, often combined systemic anti-inflammatory or immunosuppressive drugs such as corticosteroids, azathioprine (AZA), cyclosporine and sporadically other immunosuppressives [2, 5]. The introduction of biological agents such as anti-TNF-α and interferon α has improved the therapeutic potential, also in intestinal Behcet [1, 2, 3, 8, 9]. In patients with therapy-refractory intestinal BD surgery might be the final therapeutic option [4].

Investigational drugs in BD are the mycophenolates, which are widely used in transplantation medicine and increasingly in autoimmune diseases such as CD, SLE and autoimmune rheumatic disease (ARD) [10, 11, 12]. Mycophenolate sodium (MPS) and mycophenolate mofetil (MMF) are prodrugs of mycophenolic acid (MPA), a strong inhibitor of the key enzyme monophosphate dehydrogenase (IMPDH). It suppresses both B- and T-cell proliferation, but also affects endothelial cells, including inhibition of TNF-alpha and neutrophil attachment [13].

We present the case of a patient with steroid and AZA refractory BD involving both colon and small bowel, treated with MPS and evaluated by double balloon endoscopy.

CASE

A 59-year old Caucasian man was referred from another hospital in December 2006. He complained of continuous non-bloody diarrhoea and abdominal pain since April.

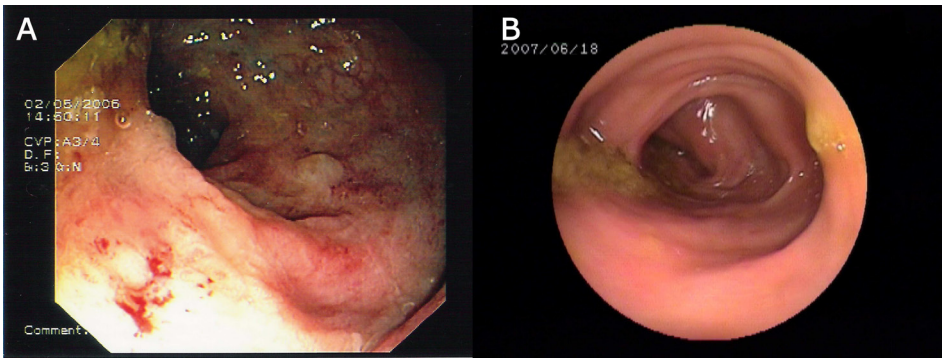
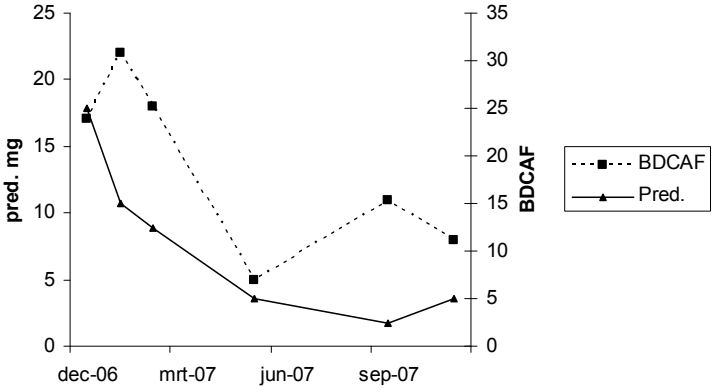


Figure 1. A: Significant colitis with aphthous lesions in the distal colon in an untreated 59 year old patient with BD. B: Distal colon of the same patient 4 months after treatment with mycophenolate sodium and low doses of azathioprine and prednisone.



Graph 1: Prednisone dose (triangles) in relation to the Behçet's disease current activity (BDCAF, squares) score in a patient with BD and gastrointestinal involvement treated with mycophenolate sodium and azathioprine (tapered swiftly from 200 mg to 50mg).

On colonoscopy multiple aphthous lesions encompassed the entire colon (Figure 1a). Biopsies showed a chronic predominantly neutrophillic infiltrate and oedema. Other symptoms were papulopustular skin lesions, orogenital ulcers and arthralgia. BD was diagnosed. Treatment with mesalazine and later 30mg prednisone combined with 200mg azathioprine (AZA) only briefly relieved the complaints of diarrhoea, abdominal pain and arthralgia. Patient was referred to our hospital. Attempts to taper the prednisone below 30 mg instantaneously led to a relapse. In February 2007 symptoms worsened and 1440 mg MPS per day was added.

The effect was immediate, both AZA and prednisone could be tapered to 25 and 2.5mg, within 2 weeks and 4 months, respectively (Figure 2).

Although some minor abdominal complaints were noted, orogenital ulcers, skin lesions and arthralgia disappeared. Four months later both colon and the entire small

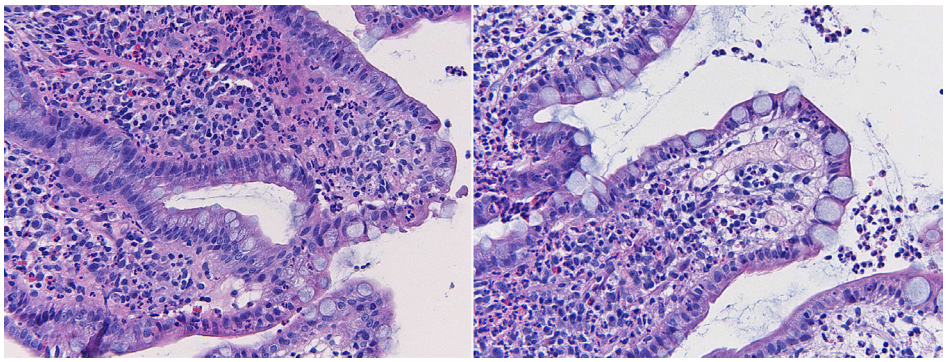


Figure 3: Limited eosinophillic and neutrophillic infiltrates in the lamina propria of the terminal ileum after successful treatment with MPS in a patient with BD and gastrointestinal involvement.

bowel were evaluated by double balloon scopy. The colonic lesions observed previously in the entire colon had totally disappeared (Figure 1b). Some minor ulcerative lesions were detected in the terminal illeum which had not been reached at the first colonoscopy. Biopsy showed a small amount of eosinophillic and neutrophillic infiltrates in the lamina propria surrounded by normal intestinal tissue (Figure 3). Eleven months after the therapy-switch the patient remains in good clinical condition (Figure 2).

DISCUSSION

This case of effective additive treatment with MPS might support a basis for the use of a new class of therapy in BD patients. The double balloon endoscopy proves a valuable tool for evaluation of the entire intestinal tract.

A variety of immunosuppressive therapy is used in BD. In our patient TNF-blocking agents were considered, but preserved for a more severe or life threatening situation [8, 9, 10]. Mycophenolates are increasingly used in the treatment of autoimmune diseases including uveitis and CD [12, 14]. Experience in BD is limited and to our knowledge there are no reports of its use in patients with gastroinestinal involvement. Adler et al. describes thirty patients with mucocutaneous BD quickly relapsing after initial response to MMF [15]. These relapses might be explained by the cessation of prednisone quickly after start of the MMF treatment. In our case tapering the other immunosuppressive therapy was monitored by the clinical condition and laboratory parameters. We therefore felt confident with the initiation of MPS in our BD patient. Up till now response lasts almost one year.

BD patients can have gastrointestinal complaints. Endoscopic evaluation was until recently restricted to the proximal and distal part of the small bowel and colon. Recent introduction of new endoscopic methods such as double balloon -or capsule endoscopy

enables to visualize the entire gastrointestinal tract. Our case shows that the small intestine can be involved even in a BD patient with minor abdominal complaints. The additional value of these techniques might be helpful in diagnosing mucosal lesions in the small bowel of BD patients with abdominal symptoms and even may change the frequency of intestinal involvement. We therefore recently have initiated a prospective case study to observe intestinal involvement in BD patients with abdominal complaints.

In conclusion, this case shows that MPS might be used up front biologicals or chemotherapeutics in BD patients with ileo-colitis, refractory to corticosteroid- or AZA-therapy Tapering of instituted immunosuppressive medication may prevent an early relapse. The involvement of the entire gastrointestinal tract in BD and the response to the therapy can be visualised by double balloon endoscopy.

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Successful long-term triple disease control by ustekinumab in a patient with Behçet's disease, psoriasis, and hidradenitis suppurativa

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INTRODUCTION

Behçet's disease (BD) is an auto-inflammatory disorder, characterized by recurrent oral aphtosis, genital ulcers, uveitis and pustular skin lesions.[1] Associated cutaneous diseases include Sweet's syndrome,[2] erythema nodosum and pyoderma gangrenosum. [3] Next to BD, both psoriasis and hidradenitis suppurativa (HS) are clear neutrophilic and IL-17-based diseases, suggesting a pathomechanistic overlap.[4-8] However, these diseases rarely co-occur.[4] Ustekinumab (anti-p40 mAb), an effective biologic treatment for psoriasis, might be effective in BD by interfering with the IL-17 signalling via IL-23 blockage. We present a 39-year-old Caucasian woman, in which the combination of BD, psoriasis and HS, was successfully treated with ustekinumab.

CASE

At the age of 5, patient developed guttate psoriasis followed by psoriasis vulgaris with severe acne vulgaris since puberty. She started smoking at the age of 13 and periodically developed inflamed and tender boils in both axilla and groins during puberty. At 35, she was diagnosed with BD according to the guidelines of the international study group on BD based on oro-genital ulcers and uveitis anterior [4]. Inquiry learned that she repeatedly developed ulcers at injection sites, highly suggestive of a pathergy reaction. This was complicated by bilateral arthritis of the distal interphalangeal joints and weight loss due to multiple intestinal ulcers at the terminal ileum. Dermatological examination revealed vulvar and multiple circumscribed punched-out vaginal scars and ulcers, and fibrotic and rope-like scarring in the groins, together with HS, Hurley stage 2. The BD-related symptoms prompted immunosuppressive treatment with diclofenac/misoprostol, ocular steroids, colchicine, intra-articular triamcinolone injection and cyclosporin, unfortunately all without with only temporarily effect. In the course of the disease she experienced an exacerbation of her psoriasis that was treated with subcutaneous injections of 45 mg ustekinumab at weeks 0, 4 and every 12 weeks thereafter. Fifty percent clinical improvement, as measured by the Psoriasis Activity and Severity Index (PASI 50), was achieved within 4 weeks, followed by PASI 75 within 3 months. Subsequently, both BD and HS skin complaints gradually decreased, and remained in complete remission for at least 36 months without adjunctive immunosuppressive treatment.

Before and 4 weeks after first injection of ustekinumab, serum was analysed using a semi-quantitative multiplex protein array to monitor changes in circulating cytokines, chemokines, and growth factors [5]. Before treatment, 64 out of 507 proteins, showed an increased, ≥ 2-fold expression compared to healthy control serum, including IL-23 and IL-12p70 (Table 1). Four weeks following the first injection of ustekinumab, 18 proteins

Table 1. Serum proteins compared to healthy controls and change induced by ustekinumab

Protein	Fold change to healthy controls (n=2)	Fold change by ustekinumab	General function
IL-23	21,60	-2,18	Th17
Ciliary neurotrophic factor (CNTF)	12,08	-1,82	Il-6 family member
Osteoprotegerin	12,05	-1,68	Serum biomarker of artritis
IL-12 p70	7,71	-1,93	Th1
CCL27	7,37	-1,63	Th1/Th17
Glut2	5,56	-2,34	Glucose metabolism
VEGF	4,03	3,33	Angiogenesis
Kininostatin	3,70	-1,61	Angiogenic inhibitor
CCL3 (MIP1α)	3,45	-1,50	Th1/Th17
TRAIL R4 (TNFRSF10D)	2,82	1,62	Marker of inflammatory DCs
IGFBP-3	2,65	1,85	Growth factor
BMP-8	2,57	1,98	Growth factor
TGF-beta 2	2,29	2,06	Growth factor
FGF Basic	2,29	1,53	Growth factor
IGFBP-6	2,24	3,27	Growth factor
Inhibin B	2,14	2,51	Endocrine function
Neurturin	2,13	2,06	Neurotrophic factor
IL-24	2,04	2,85	Th17

showed a change of more than 1.5 fold (Table 1), among which the Th1 and Th17-associated proteins IL-12p70 and IL-23 were downregulated. These findings support an important role of Th1/Th17 pathways in BD [6].

There are only two other cases describing combined occurrence of BD and HS [7]. This case is the first in which these three diseases were simultaneously present. In addition, our case is the first BD patient reported that was effectively treated with ustekinumab. The successful treatment with ustekinumab provides a valuable addition to the current therapeutic armamentarium.

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Adalimumab treatment in refractory mucocutaneous Behçet's Disease: An observational case series

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Submitted

6

Discussion

GENERAL DISCUSSION

Behçet's disease (BD) is a fascinating disorder. The epidemiology and pathophysiology of BD are widely debated, however, still need to be unravelled. This thesis encompasses translational studies to improve the insight in the demographic characteristics, genetical background, immunopathophysiology and treatment modalities of BD. Each chapter elaborates on specific segments of these subjects. In Chapter 2 it is argued that the prevalence of BD, contrary to a general assumption, doesn't decrease after migration. Also we correlate specific disease features to ethnical background. In Chapter 3 we demonstrate that some genetic variations are protective and others are associated with a higher risk on BD. We also demonstrate that it is possible to run a GWAS in a rare condition like BD in a cohort of multi-ethnic background. In Chapter 4 we show that cytokine profiles in affected tissue seem to be more relevant than serum cytokine profiles. Finally in Chapter 5 we present new treatment modalities such as TNF-α blockers, that successfully can be applied in BD patients. In this final Chapter we will discuss the results and implications of all initiated studies as well as the and suggestions for further research.

Pathophysiology

In Chapter 1 an overview on the pathophysiology of BD is presented, indicating that BD is a multifactorial auto-inflammatory vasculitis occurring in virtually all types of blood vessels, with a certain genetic predisposition, in combination with various environmental factors, leading to inflammation [1-4] (Figure 1).

The inflammation is characterized by a Th-1 and probably also Th-17 mediated immune response(4, 5). In the early phase of inflammation granulocytes and gamma-delta T-cells (Tγδ) are identified in the affected tissues(6). These are believed to be activated by environmental "triggers" [7]. Several cytokines are involved in the event mechanisms. Starting with IL6, produced by antigen presenting cells, which promotes the differentiation towards Th1 cells and IL23, produced by Tγδ cells that promotes the Th17 differentiation P40 [8, 9]. The central cytokine in the inflammatory process is tumor necrosis factor (TNF) -α, which is produced by virtually all the cells involved. TNF-α subsequently activates the production of other cytokines including IL-6, IL-8, IL-10 and IL-17.

Epidemiology

BD is a disease that is traditionally associated with countries along the former Silk Road, an ancient route of commerce from the Mediterranean Sea to the Far East (China). In Turkey occurs the highest prevalence: 20-420 cases per 100,000. In Asian countries, such as Japan, Korea, China, Iran and Saudi Arabia, prevalences vary from 13.5 to 20 cases per 100,000. In Western- European countries prevalences ranging from 1-7.5 per 100,000 are reported (figure 2) [10-12].

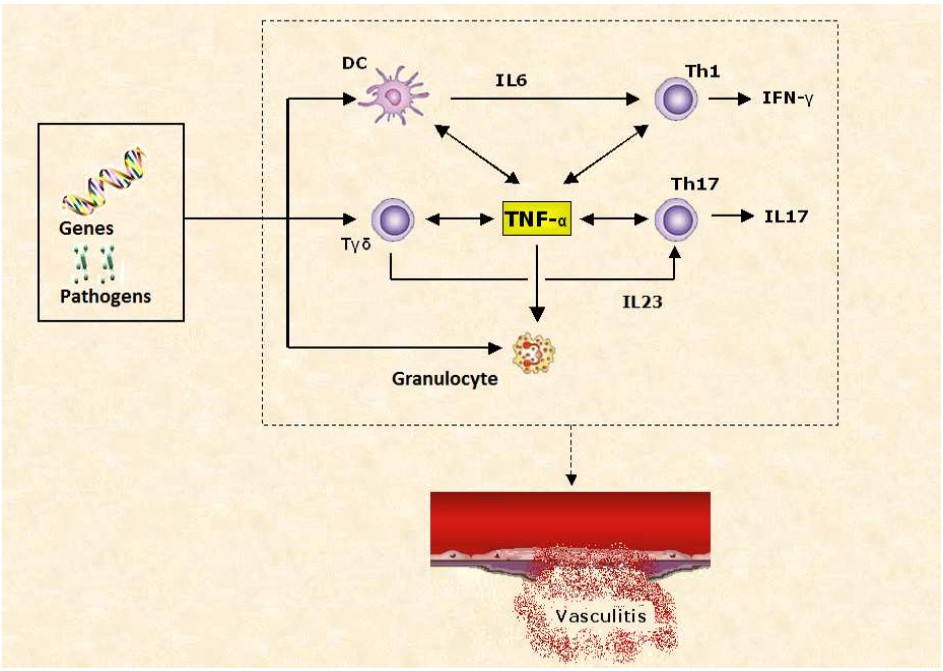

Figure 1; Immunopathophysiology of Behçet's disease
It is assumed that stimulation by pathogens of granulocytes, Tγδ and DCs, in a genetically predisposed host a cascade of inflammatory cytokines eventually lead to vascular damage and vasculitis.
Abbreviations: IL; interleukin, TNF; tumor necrosis factor, Tγδ; Tγδ subset T cells, Th; CD4 + cells Thelper, DC; dendritic cells

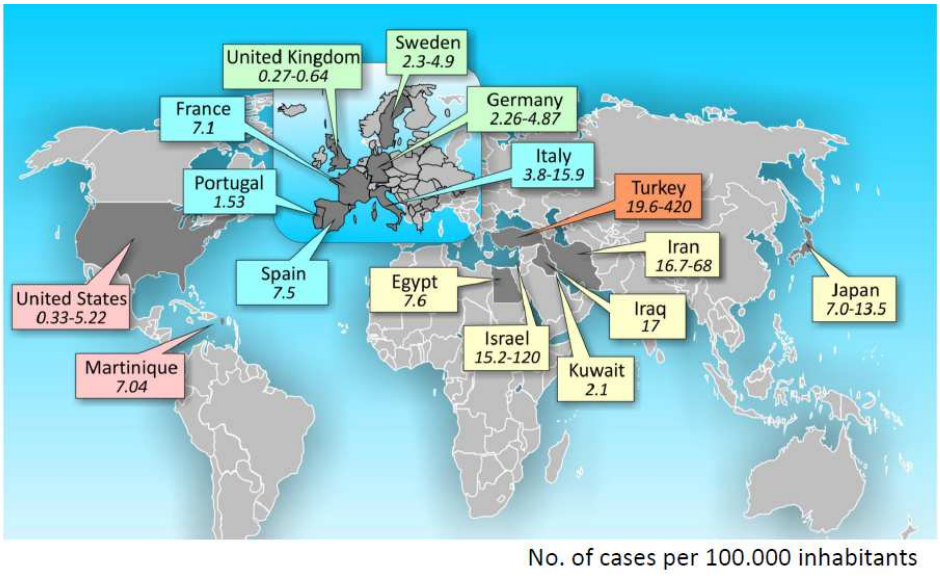

Figure 2: Prevalences of BD in different countries. Printed with permission from prof. dr. A. Mahr [12]

A remarkable phenomenon often referred to, is a decreasing prevalence of BD after migration. *Zouboulis et al.* presented that the prevalence of BD in a Turkish population decreased after migrating to Germany [13]. The data, however, could not be reproduced by the same group in another study in that was published later in 2012 [14]. A similar observation was presented in 1976 amongst Japanese immigrants on Hawaii. The prevalence of BD in Japan is 7-13.5 per 100,000 whereas the prevalence amongst the Japanese population on Hawaii is reported 0 [15]. However in 1976, about 800,000 inhabitants lived on Hawaii with 13% Japanese immigrants. It can be assumed that at least 13 BD patients of Japanese origin must be living on Hawaii. Such low numbers however, can easily be missed in epidemiological studies. We addressed these issues by identifying all BD patients treated in the Rotterdam postal area. In Chapter 2 we show that the prevalence of BD in the Rotterdam area in three ethnic groups, Dutch, Turkish and Moroccan, is 1, 72 and 35 per 100,000 respectively. This is completely in line with comparable epidemiological studies in other cohorts with similar ethic background and also with the prevalences of BD in the aforenamed countries. For example, BD amongst the Turkish population in Germany is found to be 72 per 100.000, which is similar to the reported prevalence in the European part of Turkey(14). Additionally, the fact that substantial numbers of our Turkish (up to 20%) and Moroccan patients is born in the Netherlands underlines that environmental circumstances are not decisively dictate the ability to develop BD and does not appear to be involved in the pathogenesis of BD.

The average age in our BD cohort is about forty years and does not differ amongst various ethnicities, while male/ female ratio is lower in Dutch-Caucasians as compared to Moroccans and Turks (Chapter 2) [16, 17]. These variations in male/ female ratios in different ethnic groups are comparable with observations in other cohorts [18, 19]. *Saylan et al* reported female predominance in the United States, the United Kingdom, Korea and China, whereas a male predominance was found for almost all Middle Eastern countries [20]. The cause of the female preference in Western and Asian countries remains unknown. But it is known that low male/ female ratio is often seen in auto-immune diseases. BD, however, is considered be an auto-inflammatory disease which is not associated with female predominance(21, 22). Secondly, underdiagnosing of BD in women in Middle Eastern countries can arguably be explained by the suggestion that women might visit less frequent their physician than men in these regions. Possibly female preference is present all regions, new reliable data is needed to clear this debate.

Relevant differences between ethnic groups in disease features is limited. Surprisingly we encountered a significantly higher percentage skin involvement in our cohort as compared with Germany and Morocco (Chapter 2). We expected to see more skin involvement in countries with high disease prevalence since environmental factors are considered of

great importance. Our data indicates that other factors, such as genetic susceptibility might be of more relevance than environmental factors which is supported by the observations of stable prevalences after migration as described in the first part of Chapter 2.

We also observed a significantly higher prevalence of uveitis in our Western cohort (which consists of Caucasians, Turks and Moroccans) as compared to Turkish and Moroccan cohorts (Chapter 2). Considering the rarity of BD in the Netherlands it can be argumented that BD is easily missed in patients presenting with oral ulcers. A relative underdiagnoses of the less severe cases of BD might lead to relatively higher prevalence of severe case with uveitis. Additional education on BD might minimalize this effect. This thesis can be used as a starting point for educational purposes in the future, possibly facilitated by the patients associations. An additive reason for the high percentage of uveitis found in the Erasmus MC cohort is the close cooperation between our hospital and the Rotterdam Eye Hospital hence referral of selected patients. Future research can focus on collecting a substantial amount of patient data to elucidate migrational effects on the occurrence and morbidity of BD more robustly.

Genetics

Over the years it has been widely accepted that there is a genetic component in the etiology of BD. In patients of Turkish origin there is positive family history in 12% of the cases with a sibling risk ratio between 11–52 [4, 14]. The discovery of the association with HLA-B51 has been the first breakthrough, explaining about 20% of the heritability of BD [4, 14]. Over the years numerous single nucleotide polymorphisms (SNP) have been found associated with BD(2, 23-25). The initial approach of candidate gene selection has been based on clinical similarities with other diseases. The candidate disease for this matter might be CD, both disorders share many symptoms (figure 3).

In this light, specific similar symptoms such as uveitis, arthritis, erythema nodosum and colitis with ileocecal mucosal inflammation and punched-out fissuring ulcers suggest

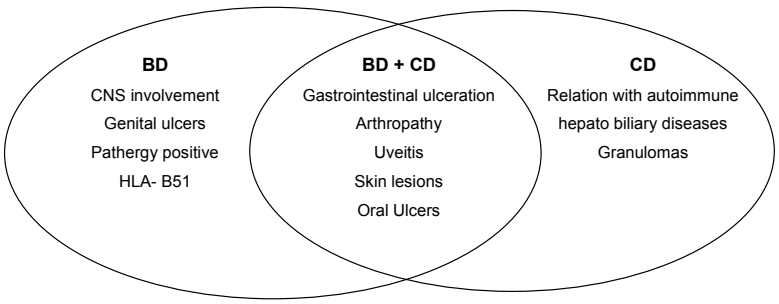

Figure 3: Symptoms of CD and BD

a possible genetic overlap between BD and CD(3, 26). Since CD has been associated with variations in NOD2 innate immunity, the NOD2/ CARD15 gene, we hypothesized that BD, considereding its association with innate immune malfunctioning, might be associated with the same variants [27, 28].

Surprisingly and contrarily at first, we determined a protective association between BD and the NOD2 variants (Chapter 3). In a protective association the variants are significantly less present in the patients as compared to the control group. Previous reports showed no association nor a protective association. A low prevalence of NOD variants in Turkish individuals however, was presented previously [29, 30]. Possibly the cohort sizes of the other studies were too limited to detect the protective association. Our observation was confirmed in 2013 by *Kirino et al.* in a targeted resequence study in Turkish and Japanese individuals [31]. Their study confirmed our results and hypotheses by demonstrating NOD2 variants are associated with a higher risk in CD, but a lower risk on BD. The exact mechanisms of this finding are unclear and have not functionally been tested. In CD both gain of function and loss of function mechanisms are proposed to explain the involvement of NOD2 variants. A loss of function might possibly lead to decreased NF-κB activation and thus reduced defensin production resulting in an increased bacterial load in the gut of CD patients and subsequently an inflammatory response with tissue damage (figure 4b) [32-34]. On top of that, NOD2 activation inhibits stimulation of Toll-like receptors by pathogen products, and as NOD2 function is impaired, TLR signaling is enhanced and generates an exaggerated inflammatory response inducing tissue damage [32, 33].

Another hypothesis is based on the observations in a second model. Here transgenic expression of the Leu1007fs variant show a gain of function, with a direct increase in NF-κB, pro-inflammatory cytokines and tissue damage (figure 4a and 4c) [32]. A potential connection between these paradoxical results lays in the kinetics of the NOD2 response. Normally, acute stimulation of human blood-derived macrophages with the NOD2 agonist, muramyl dipeptide (MDP) induces a pro-inflammatory cytokine response. By comparison, persistent treatment of cells with MDP prior to activation through TLR2 or TLR4 ligands decreases cytokine responses (tolerance), possibly through induction of IRAK-M, in support of control of TLR signaling. However, this tolerance is lost in cells from patients homozygous for the mentioned Leu1007fs homozygous patients leading to higher cytokine levels and more inflammation [35]. As gut epithelial cells, dendritic cells and macrophages are in constant contact with bacterial products of the normal gut flora it is proposed that such tolerance is a protective mechanism, loosing this tolerance leads to a failure to control responses and eventually leads to tissue damage as seen in CD [36].

The observation in this thesis that NOD2 variants decrease the risk on developing BD suggest that under continues or repetitive exogenous stimulation, NOD2 variants in BD

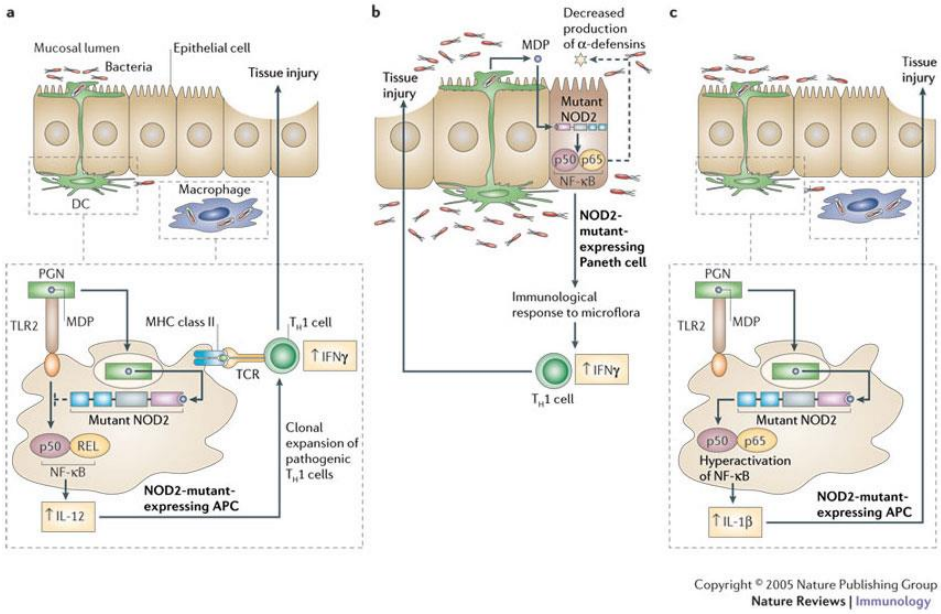

Figure 4: Possible mechanisms of Crohn's disease in patients with mutations in CARD15 [32]. *Printed with permission Strober W, Murray PJ, Kitani A, Watanabe T. Signaling pathways and molecular interactions of NOD1 and NOD2. Nature reviews Immunology. 2006;6(1):9-20.*

patients lead in some cases with a decreased production of inflammatory cytokines. Thus patients without this mutation would not be able to control their inflammatory response. This loss of function model is supported by the observation that NOD2 gene-deficient mice show reduced joint inflammation and are protected against early cartilage damage after intra-articular injection of MDP through *Streptococcus pyogenes* cell wall fragments [37].

After some reluctance it is now widely accepted that NOD2 plays a role in the pathophysiology of BD, since the complex role of NOD2 in response to bacterial challenges in different cell types more functional analyses.

As compared to the targeted candidate gene approach, Genome Wide Association Study (GWAS) involve the other spectrum of genetic evaluations in the search for disease modifying variants. In GWAS one assay compares multiple variations (up to 600.000) between patients and controls. Since GWAS results can be confounded by ancestry differences between cases and controls, potentially leading to spurious associations, novel statistical methodology such as Genomic Principal Components (GPC) and Linear Mixed Models (LMM) to correct for these for ancestry differences have been applied. These correct also for family structure and cryptic relationships [38]. In order to gain enough power in these statistical analyses, large cohorts are used to identify possible

associations. In 2010 the first genome-wide association study (GWAS) in BD cohorts of Turkish and Japanese origin demonstrated association of various variants in the known HLA-B51 domain, and two new association signals, mapping to the interleukin-10 (*IL10*), and the IL-23 receptor–IL-12 receptor Beta2 (*IL23R–IL12RB2*) locus [39, 40]. These associations have later been confirmed in an Iranian cohort [41]. Yet another study in Algerian individuals only replicated the associations from the IL10 variants [42]. GWAS in Chinese and Korean individuals reported associations of SNPs mapping to two other loci, with regions containing the signal transducer and activator of transcription 4 (*STAT4*) and GTPase of immune associated protein *GIMAP* genes, respectively. Polymorphisms in *IL10* and *IL23R–IL12RB2* are not found to be significantly associated with BD in neither of those studies [43, 44]. More recently, associations with *CCR1, STAT4* and *KLRC4* were identified by means of imputation and GWAS meta-analysis [45]. This study also identified the *IL12A* region as suggestively associated with BD, but genome wide significance (GWS) was not reached. Discrepant association results, as those observed for variants mapping to the *GIMAP* locus, might be explained by the different ethnic origin of the cohorts studied(46, 47). Therefore, studying cohorts of diverse ethnic background may be useful to understand the origin of these differences. Moreover, the inclusion of multiple ethnicities results in larger datasets (representing higher power) crucial for these analyses given the typically small effects of common genetic variants discovered by the GWAS approach [48]. Enabled by innovative novel methodology as GPC and LMM we could run a GWAS in a rare condition like BD within a unique case collection of multiethnic background (Chapter 3). We identified variants associated with BD mapping to the well-established MICA-HLA-B locus and to two regions on chromosome 6 in the *SLC22A* gene region and on chromosome 18 in an uncharacterized region. Nevertheless, all variants had relatively low MAF (2-3%) and thus their significance must be interpreted with caution. We could not find evidence for replication of the associations in an independent yet underpowered case/control set. Considering the low MAF and the very limited power of the replication further scrutiny in an expanded dataset is required to confirm these variants as real or spurious associations. Yet another GWS association with rs17810546 in the *IL12A* locus was identified by meta-analyses of our results with those reported previously in the literature, *Kirino et al.*, previously labelled this locus as suggestive for association with BD. In that study the variants did not reach GWS in the combined analysis likely due to the variant being monomorphic in the samples of Japanese origin included in the study. Variants in *CCR1-CCR3, STAT4* and *KLRC4* which were polymorphic in both Turkish and Japanese populations surpassed GWS thresholds, suggesting that the lower power likely contributed to the non-significant findings in the *IL12A* locus. All together, the common variants across this four loci identified in our study explained up to 32% of the variance in BD risk.

The role of IL12A in the pathophysiology of BD might be explained by its function. IL12 is a heterodimer composed of two chains, IL12A, also commonly known as the P35, and IL12B (or P40) [8, 9]. IL12A (P35) shares homology with other cytokines such as IL6, while IL12B (P40) is one of the chains of another heterodimer, IL23. An important function of IL12 is the promotion of the differentiation of naïve T-cells to Th1 subsets and subsequently the production of IFN-γ. BD is considered to be an Th1-cell mediated disease. IL12A variants in a gain of function manner can lead to an overexpression of Th1-cells. Differentiation to Th17 subsets is initiated by IL6 and IL23. IL6 shares homology with IL12A (P35) while one of the dimers of IL23 is IL12B (P40). Possibly IL12A (P35) variants together with yet unidentified variants in IL12A (P35) and IL12B (P40) lead to observed elevated Th17 subsets in BD(49). Moreover, involvement of the IL12 and IL23 pathway is also supported by the identification of variants in the *IL23R–IL12RB2* region in the first GWAS presented in BD [39, 40].

Some variant that have been identified in the last years are involved sensing of pathogens and other variants are more involved in the regulation of the immune system (figure 5) [50]. Potential implications of this hypotheses are that in an early phase of the pathophysiological events a defect in pathogen sensing, mainly the innate immune system must be involved. As a result the immune response is out of balance and the regulation is lost. In this phase several organs can get involved in the disease, even when the initial stimulants is already gone. The pathogenomic pathergy skin prick test would be a reflection of the early phase.

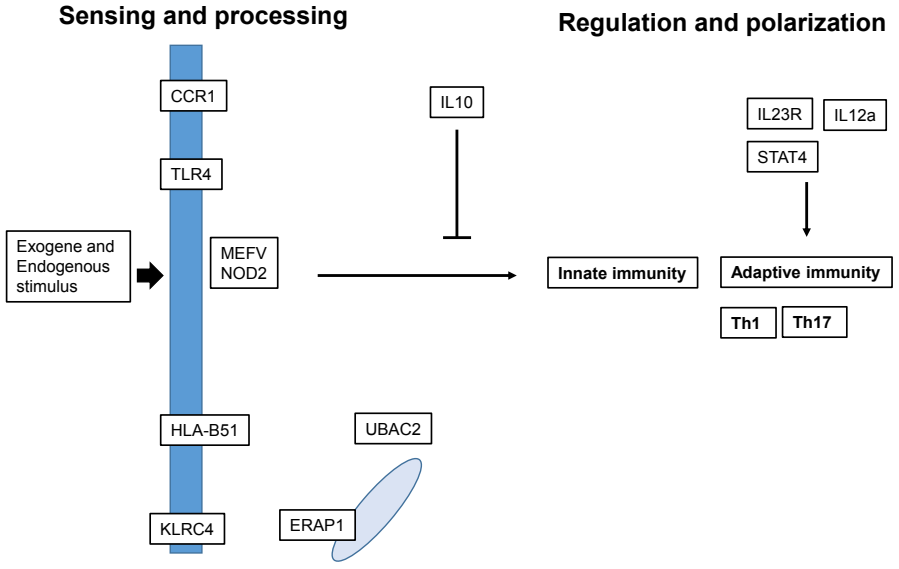

Figure 5: Genetic variants involved in the pathogeneses of BD, *adapted from Gul A. Genetics of Behcet's disease: lessons learned from genomewide association studies. Current opinion in rheumatology. 2014;26(1):56-63. [50]*

The next step to unravel the pathogenetics of BD would be a whole genome sequence study in which a complete genome is scanned for variants related to the disease. By analyzing subjects and controls from one family covariants can be filtered out. Our sequence study unfortunately had a low yield in DNA isolation in 3 of the 5 subjects (Chapter 3). The result was a list of 340 genes that possibly are involved in BD. This number is too high for further processing. Future research should therefore focus on whole genome sequencing in families to identify genes that so far could not be linked to BD. Subsequently mechanistic studies are needed to understand the exact effects of the variants such as IL12A and NOD2 and the inflammatory implications in the cytokine cascade in the disease mechanisms. In the next chapter we address the value of measuring these cytokines in BD patients.

Cytokines profiles in Behçet's disease

Apart from the genetic studies in order to identify pathways and target cytokines involved in the pathophysiology of BD we also looked for clues in cytokine levels in BD patients. In the past several cytokines have been linked to the pathophysiology of BD, such as IL12 and IFN-γ as traditional Th1 cytokines and more recently IL23 and IL17 [5, 51-53]. Also elevated serum levels of the Th2 cytokine IL10, that normally inhibits the Th1 response, are found in BD patients [54]. Last, but not least, TNF-α that appears to play a central role in many auto-immune and auto-inflammatory disease and also in BD. TNF-α is produced by virtually all cells involved and subsequently activates the production of other cytokines such as IL -1β, IL-6, IL-8, IL-10 and IL-17. Other inflammatory cells are attracted to the inflammation site and contribute to an inflammatory infiltrate.

Most studies in the pathophysiology of BD focussing on cytokine levels concentrate on serum profiles. In Chapter 4 we relate elevated IFN-γ, IL-6 and IL-10 levels with active small bowel (SB) involvement in BD. These cytokine profiles however, are not typically Th1 nor Th17 patterns as we would have expected based on the current opinion of the involvement of the Th1 and Th17 axes in the pathophysiology of BD. The increased IL-10 levels could be either part of the pathogenesis of intestinal BD as well as a physiological response on inflammation in these patients. In addition to this, although in both SB-positive and negative BD patients cytokine profiles were measured, immune modulating treatment of the B-positive group might have been more profound and therefore influenced these observations as well as the composition of the blood lymphocyte compartment in our study [55-57]. Since this study population has been limited in size with different types of immunosuppressive treatment between patients it remains difficult to draw any firm conclusions on the clinical use of cytokines and lymphocyte subsets, but IFN-γ levels might be of importance.

More interesting are the cytokine levels in affected tissue. Also in Chapter 4 we demonstrate high mRNA expressions of IFN-gamma, TNF-α and IL-17A in affected tissue

of intensively treated patients. As previously mentioned all are key cytokines in BD. In this perspective of the few data available, also in synovial fluids of naïve BD patients a similar Th1 skewed pattern has been demonstrated, it didn't report on IL17A [58]. We know that serum cytokine profiles not always seem to correlate with disease activity and suspect that serum cytokine profiles not always reflect the inflammation on tissue level. We therefore conclude that more data in cytokine profiles on tissue levels in comparison with serum levels is needed. These measurements might have implications on the development and possibly monitoring of targeted therapy in BD.

New treatment modalities

BD is a disease in which symptoms usually respond well to a wide variety of immunosuppressive therapy. Depending on the severity of the disease and involvement of vital organs immunosuppressives are titrated (table 1).

Table 1: Current immunosuppressant treatment used for Behçet's disease

Medication	Dose	Main indications
Prednisone	Local	Uveïtis Mucocutaneous involvement
	Systemic: 0.5 – 1 mg/kg	Induction treatment uveïtis Neurological involvement Refractory arthritis Gastro-intestinal ulceration
	High apoptotic dose iv: 1g/day, 1-3 times iv	Life-threatening disease, or severe neurological involvement
Colchicine	0.5 - 1.5 mg / day	Skin involvement Arthritis
Dapsone	100 - 200 mg / day	Mucocutaneous involvement Arthritis
Azathioprine	2 - 3 mg / kg / day	Uveitis
Pentoxifylline	1200 mg /day	Mucocutaneous involvement
Sulfasalazine	1- 3 g / day	Mucocutaneous involvement arthritis
Thalidomide	50 - 200 mg / day	Refractory mucocutaneous involvement
Cyclosporine	2 dd 3 - 5 mg/kg / day	Uveïtis Mucocutaneous involvement
Methotrexate	7.5 - 15 mg / week	Arthritis Uveitis (rarely)
Cyclophosphamide	750 mg / m^2 /mo IV	Life threatening involvement (vasculitis, neurological)
IFN-α-2a	9 million units /week for 3 months followed by a low maintenance dose of 3 million units /week)	Uveitis
TNF- blockers	Various doses	Uveitis Arthritis Gastro-intestinal ulceration

In Chapter 5 we also present data of a patient that was successfully treated by Myfortic (mycophenolate sodium (MPS)), we feel that MPS can be added to this list, at least for patients with intestinal BD.

Every immunosuppressant has its own, unique, point of action in the pathophysiological events in the inflammatory process in BD. New insights in this pathophysiology with the identification of novel cytokines and pathways by either genetic studies or cytokine studies can therefore be used for the development of new treatment modalities using biological agents (or biologicals). Most biologicals are exerting their effect by blocking, such as for example a key cytokine such as TNF-α [59, 60]. TNF-α blockers are increasingly used and described in refractory BD, particularly in patients with uveitis or colitis [61-66]. Evidence for efficacy of the TNF-blockers infliximab and adalimumab in BD is emerging. We add to this data of for adalimumab in refractory BD patients presented in Chapter 5. Some retrospectively studies already have demonstrated the improvement of clinical symptoms in BD patients treated with adalimumab [66, 67]. Also *c*ase-series with adalimumab report major responses of BD-related symptoms [64, 65]. Although studies with BD patients treated with infliximab involve in total 174 patients in 16 publications, limited data with adalimumab is available [64]. Therefore we followed 9 patients with therapy refractory BD that were treated with abalimumab. We observed a 100% response rate, the disappearance of oral ulcers and erythema nodosum, and the possibility to taper or terminate other anti-inflammatory drugs. Our observations are in line with other long term follow-up retrospective BD studies with adalimumab and the long term effects of this TNF-α blocker in other inflammatory diseases [65, 66, 68, 69]. Furthermore, adalimumab appears safe immunosuppressive therapy consistent with other reports of TNF-blockers in BD [60, 64, 70]. The low number of relatively minor adverse effects and its clinical activity implicate that adalimumab is as potent as its intravenous alternative, infliximab. Consensus on the indications for the use of TNF-α blockers, for example using a phenotypic approach; e.g. intestinal BD, ocular BD, as well as data when to stop the therapy is still needed. Future research might focus on these aspects helping treating physicians in the choice and the duration of TNF-α blocking therapy.

By blocking TNF-α a multi-functional cytokine is blocked, while ustekinumab (anti-P40) blocks a more specific pathway, IL23 and IL12. In our GWAS we identified IL12A variants related to BD, furthermore we observed elevated levels of IL17 on tissue level in one of our BD patients. We therefore postulated that blocking P40 might lead to better disease control. We used anti-P40 in a patient with a combination of BD an HS. It proved to be an effective treatment in this case. We feel that cytokines and pathways involved in BD as well as the result in this case support the need for exploration of the effect of anti-P40 in BD.

Only anecdotal data is present on other biologicals such as anti-IL1 (anakinra) or anti-IL6 (tociluzimab), however genetic data and cytokine data suggest a possible positive

effect by blocking the targets of these agents, namely either IL-1 or IL-6. . In the ideal world it would be most efficient if various mechanistic pathways could be blocked simultaneously, however this has so far not been exerted.

In conclusion

The pathophysiology of BD is complex, both genetic as environmental factors might play a role. In this thesis we have challenged the assumption that the prevalence of BD decreases after migration. Combined with specific apparent ethnical related disease symptoms this might accentuate the importance of the genetic compound in the pathogenesis in BD. We added proof to these increasing insights with the determination of variants of NOD2 and IL12A in patients with BD. Malfunctioning of these genes might be reflected by the cytokine patterns involved in the inflammatory cascade known in BD. The elevated levels of Th1 -as well as Th17 cytokines in in both tissue and serum of patients with active BD, described in Chapter 4 fit in this pattern and suggest that BD is more than a Th1 mediated disease. These findings can help us in the development of new treatment modalities and our understanding of the immunopathophysiology of BD. The key-cytokine in the inflammatory process of BD, TNF-α seems to play a central role (figure 6). Blocking TNF-α as described in Chapter 5 effectively down regulates the

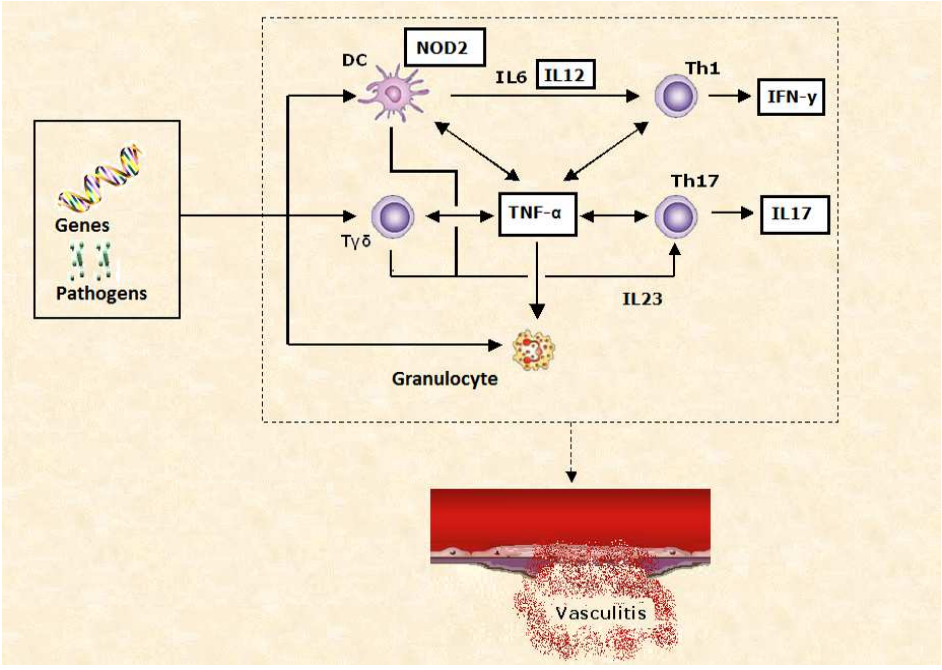

Figure 6: Pathophysiology of BD, white boxes show variants or cytokines identified in this thesis related to BD. Blocking P-40 will block Th1 promotion as well as Th17 promotion. Blocking TNF-α will lead to a broader down regulation of the inflammatory response.

inflammatory response. In case of therapy failure or intolerance to TNF-blockers new agents, such as ustekinumab (anti-P40) can secure or even enhance therapeutic efficacy via another inflammatory pathway by blocking the promotion of Th1 production by IL12 as well as blocking the promotion of IL17 production by IL23. Translational research is and will be the cornerstone in the development of new molecular designed drugs, single or combination therapies of monoclonal antibodies that might more effectively block different inflammatory pathways in order to overcome escaping mechanisms.

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7

Appendix

SUMMARY

Behçet's disease (BD) is an multifactorial auto-inflammatory vasculitis characterized by recurrent oral -and genital ulcers, uveitis, and skin lesions. The first series of patients with BD was already published in 1937 as a triad of these symptoms. Less frequently, involvement of the gastrointestinal tract, central nervous system and large vessels may occur. As in other immune mediated disorders BD is characterized by attacks rather than by a persistent inflammatory symptoms. Cases of BD cluster along the ancient Silk Road, which extends from eastern Asia to the Mediterranean basin. The reported prevalence in Turkey varies between 70 and 420 per 100.000. In Northern European countries however, the prevalence is probably less than 1 per 100.000 in Caucasians. It is believed that both genetic and environmental factors contribute to the development of the disease. Men might be more frequently and severely affected than women Susceptibility to BD is strongly associated with the presence of the HLA-B51 allele and its presence is also strongly associated with a more aggressive course. The cause of BD, however, is still unknown. Triggers such as infections, both viral and bacterial, trauma, and other stress factors may play a role in the inflammatory process.

Although the exact pathophysiology of BD remains to be elucidated, it is clear that neutrophils interacting with T-cells play an important role. Activated T-cells produce TNF-α, leading to production of other proinflammatory cytokines like IL1 and IL6. These proinflammatory cytokines stimulate migration and activation of leucocytes, thereby causing a local inflammatory response. In this thesis translational aspects of BD on areas of epidemiology, genetics, cytokines and new treatment modalities will be linked with these immuno-payhophysiological features.

In **Chapter 2** it is argued that the prevalence of BD, contrary to the general assumption, doesn't decrease after migration. Also we correlate specific disease features to ethnical background. Prevalences of BD in the Rotterdam area differed per ethnicity; 1, 71 and 39 per 100.000 for Dutch-Caucasians, Turks, and Moroccans, respectively. These figures are similar to other cohorts in countries were BD prevails. The first epidemiologic study of BD in the Netherlands initiated by the Erasmus MC demonstrates, in 110 patients no significant differences in morbidity between ethnic groups. However, uveitis and pustules were significantly more present in the EMC cohort as compared to UK, German, Turkish and Moroccan cohorts.

The heritability of BD has been estimated to range between 20 to 60%, with the strongest genetic association embracing variants in HLA-B51, explaining about 20% of the disease heritability. The combination of epidemiological and genetic data suggest causal involvement of both genetic and environmental factors. In **Chapter 3** we demonstrate that some genetic variations are protective and others are associated with a higher risk on BD. We also demonstrate that it is possible to run a GWAS in a rare condition like BD in

a cohort of multi-ethnic background. In patients of Turkish origin there is positive family history in 12% of the cases with a sibling risk ratio between 11–52 .

Over the years numerous single nucleotide polymorphisms (SNPs) have been found associated with BD. We are the first to identify a protective association with NOD2 variants. In 2010 the first genome-wide association study (GWAS) in BD cohorts of Turkish and Japanese origin demonstrated association of various variants in the known HLA-B51 domain, and two new association signals, mapping to the interleukin-10 (IL10), and the IL-23 receptor–IL-12 receptor Beta2 (IL23R–IL12RB2) locus. Other associations identified in GWAS are signal transducer and activator of transcription 4 (STAT4), GTPase of immune associated protein GIMAP genes, CCR1, STAT4 and KLRC4.

We ran a GWAS on 336 BD cases and 5,843 controls. The cases consisted of Western Europeans, Middle Eastern and Turkish individuals. Participants from the Generation R study, a multiethnic birth cohort in Rotterdam, The Netherlands were used as controls. Linear regression models were corrected for population stratification using Genomic Principal Components and Linear Mixed Modelling. Meta-analysis was performed on selected results previously published. We identified SNPs associated at genome-wide significant (GWS) level mapping to the 6p21.33 (HLA) region. In addition to this known signal two potential novel associations on chromosomes 6 and 18 were identified, yet with low minor allele frequencies. Extended meta-analysis reveal a GWS association with the IL12A variant rs17810546 on chromosome 3.

In **Chapter 4** we show that cytokine profiles in affected tissue seem to be more relevant than serum cytokine profiles. The central cytokine in the inflammatory process in BD is tumor necrosis factor (TNF) -α, which is produced by virtually all the cells involved. TNF-α subsequently activates the production of other cytokines such as interleukin (IL) -1β, IL-6, IL-8, IL-10 and IL-17. Other inflammatory cells are attracted to the inflammation site and contribute to an inflammatory infiltrate.

We demonstrate that in patients with small bowel (SB) BD IFN-γ, IL-6 and IL-10 were significantly higher in patients with SB lesions than those without. It does not reflect a typical Th1 nor Th17 cytokine profile. Elevated IL-10 could be part of the pathogenesis of intestinal BD as well as a normal response on inflammation. More interestingly are the cytokine levels in affected tissue. We present a case of an intensively treated patient with high mRNA expression of IFN-gamma, TNF-alpha and IL-17A, that appear to be key cytokines in BD. To therapeutically target such key-enzymes can be achieved by novel (molecular designed) drugs.

Finally in **Chapter 5** we present the results of such innovative treatment modalities. We start with the successful use of mycophenolate sodium up front biologicals in a BD patient with ileo-colitis, refractory to corticosteroid- or AZA-therapy. Secondly we descibe a patient with the combination of BD, psoriasis and hydradenitis suppurativa. This case is the first in which these three diseases were simultaneously present. In addition,

our case is the first BD patient reported that was effectively treated with ustekinumab. These reports add to few available data about the use of mycophenolates sodium and ustekinumab and suopply evidence to boost the armamentarium in BD.

Finally, we present data of a study of TNF-α blockage (Adalimumab). The effect of bi-weekly 40mg adalimumab in 9 patients with therapy refractory BD was studied during 6 months. The disease activity scored by the BD Current Activity Form (BDCAF) improved significantly in all patients from 5.4 to 2.4 ($p = 0.007$) within 1 month and improvement lasted the entire observation period so that 8 patients could either taper or stop concomitant therapy. Symptoms of mucocutaneous lesions, erythema nodosum and joint involvement decreased or completely disappeared. These data on patients with refractory BD treated with adalimumab provide a basis for its use in refractory mucocutaneous BD.

In **chapter 6** we discuss the implications of our results and give our view on future research. We conclude that the pathophysiology of BD is complex, both genetic as environmental factors might play a role and that translational research is be the fundament in the development of new molecular designed drugs, single or combination therapies of monoclonal antibodies that might more effectively block different inflammatory pathways in order to overcome escaping mechanisms.

NEDERLANDSE SAMENVATTING

De ziekte van Behçet (BD) is een multi-factoriele (auto)inflammatoire vascilitis die vooral voorkomt bij mensen uit landen van de Middellandse Zee gebied en langs de voormalige zijderoute naar het Verre Oosten. De symptomen bestaan uit spontane fluctuerende orale en genitale afteuze laesies, uveïtis en diverse huidaandoeningen. Bijkomende inflammatoire manifestaties kunnen zich uiten in colitis, uretritis, steriele meningitis en vaso-occlusie. In de vijfde eeuw voor Christus werden symptomen al door Hippocrates beschreven, maar pas in 1937 werd de ziekte vernoemd naar de Turkse dermatoloog Hulusi Behçet. In Turkije is ook de hoogste prevalentie: 70 tot 420 patiënten per 100.000 inwoners. In Noord- Europese landen is de prevalentie rond de 1 per 100.000 inwoners en neemt de laatste jaren toe door de veranderende bevolkingssamenstelling. De associatie met HLA-B51 positiviteit suggereert een genetische component in de etiologie. BD wordt het meest gediagnosticeerd in de derde levensdecade en de ziekte lijkt vaker voor te komen bij mannen dan bij vrouwen.

De exacte pathofysiologie van BD moet nog worden opgehelderd, de interactie tussen neutrofielen en T-cellen lijkt in ieder geval een belangrijke rol te spelen. Geactiveerde T-cellen produceren TNF-α, wat leidt tot de productie van andere pro-inflammatoire cytokines zoals IL1 en IL6. Deze pro-inflammatoire cytokines stimuleren migratie en activatie van leukocyten, waardoor een lokale ontstekingsreactie wordt veroorzaakt.

In dit proefschrift worden translationele aspecten van BD gepresenteerd op het gebied van epidemiologie, genetica, cytokines en nieuwe behandelmodaliteiten.

In **hoofdstuk 2** wordt de veronderstelling dat de prevalentie van BD afneemt na migratie ter discussie gesteld. Verder wordt de ziekte presentatie van BD in een cohort in Rotterdam beschreven. Prevalentie van BD in de regio Rotterdam verschilt per etniciteit; 1, 71 en 39 per 100.000 voor Nederlanders, Turken en Marokkanen, respectievelijk. Deze cijfers zijn gelijk aan andere cohorten uit West- Turkije en Marokko. Binnen het Erasmus MC cohort van 110 patiënten zijn geen significante verschillen in morbiditeit tussen de etnische groepen gevonden. Maar uveïtis en pustels zijn significant meer aanwezig in het EMC-cohort in vergelijking met Britse, Duitse, Turkse en Marokkaanse cohorten.

Bij patiënten van Turkse afkomst is er een positieve familiegeschiedenis in 12% en een "sibbling risk ratio"van 11-52. De erfelijkheid van BD is naar schatting tussen 20 tot 60%, met als sterkste genetische associatie met HLA-B51. In **hoofdstuk 3** laten we zien dat sommige genetische varianten beschermend en andere een risico voor de ontwikkeling van BD kunnen zijn. In de loop der jaren zijn tal van single nucleotide polymorfismen (SNP's) geassocieerd met BD. Als eerste presenteerden wij dat er ook een beschermende associatie bij BD was, namelijk met NOD2 varianten. De eerste Genome Wide Association Study (GWAS) in Turkse en Japanse BD cohorten in 2010 lieten de bekende varianten in het HLA-B51 domein zien. Daarnaast ware er twee nieuwe signalen, de eerste in inter-

leukine-10 (IL-10), en de tweede in IL-23 receptor IL-12 receptor beta2 (IL23R-IL12RB2) locus. Later zijn in GWAS studies ook relaties tussen BD en andere varianten gevonden; signal transducer and activator of transcription 4 (STAT4), GTPase of immune associated protein (GIMAP) , CCR1, STAT4 en KLRC4. Wij presenteren een GWAS met 336 BD patienten en 5843 controles. De patiënten groep bestond uit West-Europeanen, Midden-Oosten en de Turken. Het Generation R cohort, een multi-etnische geboortecohort in Rotterdam, werd gebruikt als controle groep. Genomic Principal Components en Linear Mixed Modelling zijn gebruikt voor dataverwerking. Meta-analyse werd uitgevoerd op geselecteerde resultaten eerder gepubliceerd. We identificeerden SNPs in het bekende 6p21.33 (HLA) regio, daarnaast worden twee potentiële nieuwe associaties op chromosomen 6 en 18 werden geïdentificeerd, maar met lage minor allel frequenties. Een meta-analyse laat een significante associatie met de IL12A variant rs17810546 op chromosoom 3 zien.

In **hoofdstuk 4** stellen we dat cytokines in weefsel meer relevant zijn dan cytokines in serum. Tumornecrosefactor (TNF) -α speelt een centrale rol in het ontstekingsproces in BD, het wordt door vrijwel alle betrokken cellen geproduceerd. TNF-α activeert vervolgens de productie van andere cytokinen zoals interleukine (IL) -1β, IL-6, IL-8, IL-10 en IL-17. Andere inflammatoire cellen worden aangetrokken naar het ontstoken weefsel en vormen een ontstekingsinfiltraat.

We laten zien dat bij patiënten met dunne darm betrokkenheid IFN-γ, IL-6 en IL-10 significant zijn verhoogd. Het zijn echter niet typische Th1 of Th17 profielen. Het verhoogde IL-10 kan onderdeel zijn van de pathogenese van intestinale BD maar het kan ook een normale reactie op ontsteking zijn. Aangezien het aantal patiënten in de studie beperkt is, blijft het moeilijk om definitieve conclusies te trekken over de betrokken en het klinisch gebruik van cytokines.

Relevanter lijken de cytokine niveaus in aangetast weefsel. We presenteren een casus van een intensief behandelde patiënt met hoge mRNA expressie van IFN-gamma, TNF-alfa en IL-17A.

In **hoofdstuk 5** presenteren we mogelijk nieuwe behandelingen. We laten zien dat mycophenolate sodium een toevoeging is aan de immuunsuppressiva die bij BD gebruikt kunnen worden, met name bij intestinale BD. Verder hebben wij een casus van een patiënt met BD in combinatie met psoriasis en hydradenitis suppurativa. Het is voor het eerst dat deze drie ziekten gelijktijdig worden beschreven in een patiënt. Daarnaast is het de eerste BD patiënt die effectief werd behandeld met ustekinumab (anti P40).

Tot slot, presenteren we een serie patienten die TNF-α blokkade (Adalimumab) hebben gehad. Het effect van tweewekelijkse 40 mg adalimumab bij 9 patiënten met refractaire BD therapie werd gedurende 6 maanden bestudeerd. De ziekte activiteit, gemeten met de BDCAF verbeterde significant bij alle patiënten van 5,4 (SD = 1,4) naar 2,4 (SD = 1,4; p = 0,007). De verbetering was binnen 1 maand zichtbaar en was stabiel tijdens de

gehele observatieperiode. Symptomen van mucocutane laesies, erythema nodosum en gezamenlijke betrokkenheid verminderd of geheel verdwenen.

In **hoofdstuk 6** bespreken we de implicaties van onze resultaten en geven onze visie op toekomstig onderzoek. Concluderend is de pathofysiologie van BD complex, zowel genetische als omgevingsfactoren spelen een rol. Translationeel onderzoek is het fundament voor de ontwikkeling van nieuwe moleculaire geneesmiddelen, mono- of combinatietherapieën van monoklonale antilichamen die effectief verschillende inflammatoire pathways blokkeren.

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Ook in deze beginperiode van mijn onderzoek ontmoete ik Professor Laman. Beste Jon, jij hebt samen met Jan mijn afstudeeronderzoek bedacht. Ik waardeer je enorme kennis en drive. Altijd was bereid tot antwoord of feedback als ik met lange tussenposen weer eens een vraag stelde; meestal begonnen mijn mailtjes met "Beste Jon, even een korte vraag…". Ze waren natuurlijk nooit kort. Ik vind het fantastisch dat je vandaag onderdeel bent van de commissie.

Dr Graham Wallace, we met in Portugal and a couple of months later you visited the Netherlands to discuss several possibilities of collaboration. I always admire your enthusiasm and drive as well as your incredible knowledge. We met with regular intervals at conferences and it was always a pleasure. Your support of our genetic research made our candidate approach and our GWAS study possible.

Fernando, veel geleerd, altijd ben je bereid om te helpen ook al staat je agenda dat niet altijd toe. Toch mocht ik altijd even tussendoor. De GWAS paper is de kroon op dat werk. Wat een ontzettende eer dat je plaats hebt genomen in mijn commissie.

Lisette, het begon met NOD en zie waar we nu staan. Jij een aantal Nature papers later en al lang gepromoveerd. In totaal 4 kinderen verder (jullie twee, wij twee). Ik wens je alle goeds, van harte.

Carolina, it must have been frustrating for you, trying to explain these statistics to me. I wish you all the best in the finalization of your PhD.

Paul, vaste kamergenoot van Jan. We hebben elkaar de afgelopen jaren goed leren kennen. Ik bewonder je kennis en doorzettingsvermogen, nu nog even Rotterdam lopen. Ook wij gaan elkaar hopelijk nog vaak tegen komen.

Nico, vanaf 2003 zijn we gezamenlijk betrokken bij een aantal projecten in India en zitten samen in Stichting Childtuition. In een moeilijk fase van mijn onderzoek ben jij bereid geweest om ons te steunen. Hierdoor hebben wij als eerste in een Westers land een groot genetisch onderzoek in dit ziektebeeld kunnen uitvoeren. Ik wil je daar hartelijk voor bedanken.

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En dan is er natuurlijk de afdeling longgeneeskunde in het SFG. Wat een fantastische plek om opgeleid te worden. Elke dag leer ik en elke dag lach ik.

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"ik draag je hart met me mee (ik draag het in mijn hart)
ik heb het altijd bij me (waar ik ga, daar ga jij en alles wat ik doe doe ik om jou)
Ik vrees mijn lot niet (want jij bent mijn lot)
ik wil de wereld niet (want mooi ben jij, mijn wereld, mijn ware)
en jij bent het, jij bent wat de maan altijd heeft bedoeld
en wat de zon steeds weer zal zingen dat ben jij"

E.E. Cummings

Dan wil ik eindigen met de woorden waarmee elke voordracht eindigt; More research is needed, heel graag!

BIOGRAFIE

Jasper Henk Kappen is op 29 januari 1971 in Eindhoven geboren. Hij is opgegroeid in Uden, Noord-Brabant, waar hij in 1989 zijn VWO diploma behaalde aan het Rivendell College. Ruim 6 jaar later in 1995 studeerde hij af aan de faculteit Scheikundige Technologie van de Technische Universteit Delft. Hierna is hij als technoloog en logistiek manager werkzaam geweest bij DSM om vervolgens een aantal jaar als vrijwilliger voor Artsen Zonder Grenzen (Afghanistan) en Maharogi Sewa Samiti (India) te werken. Eenmaal terug in Nederland is hij gestart met de studie geneeskunde aan het Erasmus MC in Rotterdam. Inmiddels is hij in het Sint Franciscus Gasthuis bezig met zijn specialisatie tot longarts.

Hij is getrouwd met Floor Grevink en samen hebben zij een zoon, Joris, en een dochter, Rosa.

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Behcet's disease, prevalence and symptoms in the Rotterdam area. **Kappen J.H.**, van Dijk E.H.C., Baak-Dijkstra M., Lam Tse W., van Hagen P.M., van Laar J.A.M.
Submitted

Detection of intestinal Behçet's disease by double balloon enteroscopy combined to serum cytokine profiles; improved diagnostic yield? **J.H. Kappen**, MD, MSc, P.B.F. Mensink, MD, PhD, W.A. Dik, PhD; H. Hooijkaas, PhD; S. Lachman, MD; P.L.A. van Daele MD, PhD; P.M. van Hagen, MD, PhD; J.A.M. van Laar, MD, PhD.
Submitted

Adalimumab treatment in refractory mucocutaneous Behçet's Disease: An observational case series. Diana M. Verboom, **Jasper H. Kappen**, Willem A. Dik, Marianne W. van der Ent, Paul L.A. van Daele, P. Martin van Hagen, Jan A.M. van Laar
Submitted

Nieuwe ontwikkelingen in de Medische Immunologie 2010 Chapter 16: "Ziekte van Behçet" ISBN 978-90-73436-91-6. Jan AM van Laar, P. Martin van Hagen, **Jasper H Kappen**.

PhD PORTFOLIO

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Erasmus MC department	Internal medicine, section clinical immunology
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Conferences and courses

- Second European Workshop on Immune-Mediated Inflammatory Diseases, 2007, Nurnberg, Germany
- Third European Workshop on Immune-Mediated Inflammatory Diseases, 2008, London, United Kingdom
- Fourth European Workshop on Immune-Mediated Inflammatory Diseases, 2009, Cascais, Portugal
- New Frontiers symposium, 2009, Radboud University, Nijmegen The Netherlands

Conference presentations

- 12th International Conference on Behcet's Disease, 2006, Portugal, Lisbon (Poster)
- Second European Workshop on Immune-Mediated Inflammatory Diseases, 2007, Nurnberg, Germany (Poster presentation)
- Wetenschapsdagen Interne Geneeskunde EMC, 2008, (Poster presentation, second prize)
- 13th International Conference on Behcet's Disease, 2008, Poertschach/Klagenfurt, Austria. (Oral presentation)
- Third European Workshop on Immune-Mediated Inflammatory Diseases, 2008, London, United Kingdom (Poster presentation)
- Fourth European Workshop on Immune-Mediated Inflammatory Diseases, 2009, Cascais, Portugal (Poster presentation)
- Wetenschapsdagen Interne Geneeskunde EMC, 2008, (Poster presentation)
- Minder frequent voorkomende ontstekingsziekten: state of the art, 2009, Rotterdam, Netherlands (Oral presentation)
- 14th International Conference on Behcet's Disease, 2010, London, United Kingdom (Poster presentation)
- Wetenschapsdagen Interne Geneeskunde EMC, 2011, (Poster presentation)
- 15th International Conference on Behcet's Disease, 2012, Yokohama, Japan (Oral presentation)
- 16th International Conference on Behcet's Disease, 2014, Paris, United Kingdom (Poster presentation)

Grants and funds

- 2008 and 2010 Stichting Trustfonds: funding of research
- 2008 Travel Grant Abbot
- 2009 Actelion: funding of research
- 2008 Travel Grant Baxter
- 2009 Travel Grant Baxter
- 2010 Stichting Janivo: funding of research
- 2010 Stichting Mitialto: funding of research
- 2010 dr N. Nobel: funding of research
- 2010 Travel Grant Abbot
- 2013 Travel Grant Abbvie

Teaching

- Several post-graduate lectures on Behçet's Disease
- Minor in immunology ErasmusMC, 2009 (teacher)
- Master in immunology ErasmusMC, 2010 (teacher)
- Clinical lesson Eye Hospital Rotterdam, 2009, 2010, 2011 (speaker)
- Master Student project ErasmusMC, 2011- 2012: Behcet's disease and vascular parameters (supervision)
- Student project ErasmusMC, 2011 - 2012: Aortic stiffness in patients with Behçet's disease (supervision)
- Master Student project ErasmusMC, 2012: B-cells in Behcet's disease (supervision)

Other

- Council Member - International Society for Behçet's Disease, 2014