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Immunological profiling of peripheral blood mononuclear cells and aqueous humor in Behçet disease

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Abstract

Behçhet disease (BD) is a rare, systemic, inflammatory disorder with multiorgan damage and various clinical manifestations such as oral ulcers, genital ulcers and uveitis. Pathogenesis is still unknown. BD is currently considered a rheumatic disorder with some features of both autoimmune and autoinflammatory conditions. Additionally, some authors have recently proposed to classify BD as 'MHC-I-opathy'. Despite HLA-B51 and some gene polymorphisms have been associated with BD, diagnosis is based on clinical parameters and currently laboratory tests are only of a little help.

The aims of this study were: 1) to increase the knowledge about BD pathogenesis; 2) to identify laboratory tests which can support BD diagnosis; 3) to identify potential therapeutic targets for BD treatment. In details, based on the hypothesis that impaired cytotoxicity mechanisms of circulating Natural Killer (NK), NKT and T cells might have a role in the pathogenesis of BD, the first part of the study was aimed to identify a specific profile of circulating NK, NKT and T cells able to discriminate between BD patients and healthy controls, by investigating the phenotypic characteristics, the cytotoxic potential of circulating NK and NKT cells, and by quantifying 27 plasmatic cytokines/chemokines.

The second part of the study was focused on BD-related uveitis, which may cause irreversible ocular structural changes and permanent damage in sensory retina with visual loss, if left untreated. The therapeutic armamentarium for BD uveitis is very limited.

The aim of this part of the study was to investigate differences in the mechanisms of two distinct types of endogenous uveitis (i.e. BD uveitis *versus* Vogt-Koyanagi-Harada disease, VKH-related uveitis) - to search for potential markers and therapeutic targets. Levels of pro- and anti-inflammatory cytokines were measured in aqueous humor from patients with active BD uveitis and VKH. Secondly, given the evidence about the impairment of cytotoxicity mechanisms in BD, a different distribution of NK cell subsets in the aqueous humor of patients with different types of endogenous uveitis could also be hypothesised. With this aim, frequency of NK and NKT cells in the same groups of patients has been explored.

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Grants, Publications and Awards

Grants

This work has been supported by the Italian Society of Rheumatology (Grant for Young Researchers, years 2015-2016) and by the National Association of Patients Affected with Behçet Disease (S.I.M.B.A.).

Publications

Publications derived from the clinical and laboratory activities on Behçet Disease during the years 2016-2017:

- A. Soriano, S. Croci, L. Cimino, M. Bonacini, E. Calò, A. Zerbini, M. Parmeggiani, L. Fontana, C. Salvarani.
Cytokine profiling of aqueous humor in Behçet's disease patients with active ocular involvement.
Ann Rheum Dis 2017; vol 76 (Suppl 2): 212

- A. Soriano, S. Croci, L. Cimino, L. Fontana, M. Bonacini, A. Zerbini, M. Parmeggiani, C. Salvarani.
Immunological profiling of aqueous humor of Behçet disease patients with active ocular involvement.
Clin Exp Rheumatol 2017; 34 (Suppl 102): S-154

- Bonacini M*, Soriano A*, Calò E, Zerbini A, Cimino L, Fontana L, Parmeggiani M, Salvarani C, Croci S.
Cytotoxic profile characterization of NK and NKT cells in patients with Behçet disease.
Ann Rheum Dis 2017; vol 76, (Suppl 2): 1054
*equally contributed

- *Bonacini M, *Croci S, *Soriano A, Calò E., Zerbini A, Cimino L, Muratore F, Fontana L, Parmeggiani M, Salvarani C. *Higher Frequencies of Lymphocytes Expressing the Natural Killer Group 2D Receptor and Cytotoxic Potential of NK Cells in Patients with Behçet Disease.*
Arthritis Rheumatol 2017 (ACR Suppl), in press
*equally contributed

- Soriano A, Pipitone N, Salvarani C. Cardiac Involvement in Behçet Disease. in 'Cardiac involvement in rheumatic diseases' Editors: Noel Rose, Udi Nussonovitch, Elsevier Publishing Group 2017

- Cimino L, Aldigeri R, Marchi S, Mastrofilippo V, Viscogliosi F, Coassin M, Soldani A, Savoldi L, De Fanti A, Belloni L, Zerbini A, Parmeggiani M, Chersich M, Soriano A, Salvarani C, Fontana L. *Changes in patterns of uveitis at a tertiary referral center in Northern Italy: analysis of 990 cases.* Int Ophthalmol 2017; Mar 14

- Fabiani C, Vitale A, Emmi G, Vannozzi L, Lopalco G, Guerriero S, Orlando I, Franceschini R, Bacherini D, Cimino L, Soriano A, Frediani B, Galeazzi M, Iannone F, Tosi GM, Salvarani C, Cantarini L.

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Efficacy and safety of adalimumab in Behçet disease-related uveitis: a multicenter retrospective observational study
Clin Rheumatol 2017; 36: 183-189

- Muratore F*, Pazzola G*, Soriano A*, Pipitone N, Croci S, Bonacini M, Boiardi L, Salvarani C. *Unmeet needs in the pathogenesis and treatment of vasculitides*.
Clin Rev Allergy Immunol 2017; Sep 11
*equally contributed

- Vitale A, Insalaco A, Sfriso P, Lopalco G, Emmi G, Cattalini M, Manna R, Cimaz R, Priori R, Talarico R, Gentileschi S, de Marchi G, Frassi M, Gallizzi R, Soriano A, Alessio M, Cammelli D, Maggio MC, Marcolongo R, La Torre F, Fabiani C, Colafrancesco S, Ricci F, Galozzi P, Viapiana O, Verrecchia E, Pardeo M, Cerrito L, Cavallaro E, Olivieri AN, Paolazzi G, Vitiello G, Maier A, Silvestri E, Stagnaro C, Valesini G, Mosca M, de Vita S, Tincani A, Lapadula G, Frediani B, De Benedetti F, Iannone F, Punzi L, Salvarani C, Galeazzi M, Rigante D, Cantarini L.
A snapshot on the on-label and off-label use of interleukin-1 inhibitors in Italy among rheumatogists and pediatric rheumatologists: a nationwide multi-center retrospective observational study.
Front Pharmacol 2016 Oct 24; 7:380

Awards

Dr. Soriano was awarded for the Best Research Abstract during the 4th International Congress on Controversies in Rheumatology and Autoimmunity - CORA 2017, Bologna, March 9th-11th 2017 and during the 17th International Congress on Behçet's Disease - Matera, September 15th - 17th 2016 for her work on 'Immunological profiling of aqueous humor of Behçet Disease patients with active ocular involvement'.

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Chapter 1. General Introduction and Aims

1. Introduction to Behçet Disease

Behçet's disease (BD) is a systemic inflammatory disorder whose clinical hallmark is recurrent oral and genital ulcers variably associated with eye, skin and various organ involvement. BD has a worldwide distribution and both genders can be affected.

A male preponderance in the Turkish and Arab populations has been described by some authors (N. Dilsen *et al*., 1998), while in Europe the male to female ratio is approximately the same (A. Mahr *et al*., 2008; S. Cartella *et al*., 2014; C. Pamfil *et al*., 2012; I. Olivieri *et al*., 2013; C. Salvarani *et al*., 2007). More severe course has been reported in young males with increased morbidity and mortality (I. Olivieri *et al*., 2013). BD can affect all ages, but onset is more common in the third decade of life (C. Pamfil *et al*., 2012).

BD was originally described in 1937 by a Turkish dermatologist, Hulusi Behçet (1889 – 1948), as a triad of recurrent oral and genital ulcers and iritis. A few years earlier (1930-1931), the Greek ophthalmologist Benediktos Adamantiades (1875-1962) had reported a similar case of relapsing iritis and hypopion associated with leg ulcerations and thrombophlebitis (C. Zouboulis, 2002). Therefore, some authors have proposed the alternative term of 'Adamantiades-Behçet's disease' to denote BD (K.T. Calamia *et al*., 2009; G. Cocco *et al.*, 2010).

Another term sometimes used to indicate BD is the 'Silk Route disease', pointing to the geographic distribution of such condition, which appears to have spread through the ancient 'Silk Route' over the past centuries. Historically, the Silk Road was a route of commerce that followed the eastern shores of the Mediterranean Sea, which corresponds to the 30th and 45th degrees of Northern latitudes (K.T. Calamia *et al*., 2009).

Given its protean clinical manifestations, BD has been classified within different disease frames in the past two decades.

It is currently mostly considered a systemic vasculitis, although histology is actually consistent

with periphlebitis rather than vasculitis proper because in BD lesions the inflammatory infiltrate surrounds blood vessels (mainly venules) rather than invading and destroying the vessel wall (A.M. El Asrar *et al*., 2010). Other authors consider BD a polygenic autoinflammatory disease according to some recent insights into its pathogenic mechanisms (G. Hatemi *et al*., 2008).

1.1. Epidemiology

The highest prevalence of the disease is found in the countries along the ancient Silk Route, namely in Turkey, Middle-East region, Iran, Saudi Arabia, China, Korea, Japan (11.9 - 370 per 100,000 population) (G. Cocco *et al*., 2010). In Western countries the prevalence has a wide variability, ranging between 0.12 and 7.5 per 100,000 people, with lowest prevalence rates in Northern Europe and higher ones in countries on the Mediterranean basin (K.T. Calamia *et al*., 2009) (Table 1.1.).

In the United States (US), a population-based cohort study performed in Olmsted County, Minnesota, over 45 years (1960 – 2005), showed an overall incidence of 0.38 per 100,000 and a prevalence of 5.2 per 100,000 (K.T. Calamia *et al*., 2009). These data are similar to the estimated prevalence reported in France (2.4/100,000) (A. Mahr *et al*., 2008) and Northern Italy (3.8/100,000) (C. Salvarani *et al*., 2007).

The migration of the Middle and Far East populations to the Mediterranean basin and an increased disease recognition may partially justify the increased prevalence recorded in some countries, such as Germany and Italy, in the recent years (C. Salvarani *et al*., 2007; C. Zouboulis, 1999).

Different clinical phenotypes and prognosis of BD patients according to their ethnic background have been reported. In their cohort analysis of 369 European, 350 North African and 50 Sub-Saharan African patients, Savey *et al*. observed a male preponderance in the Sub-Saharan patients, who showed more frequently cardiovascular and central nervous system involvement, with a higher mortality (L. Savey *et al*., 2014).

Interestingly, the frequency of cardiovascular involvement reached 54% in the Sub-Saharan African group, whereas the rate of immunosuppressive use was not accordingly higher. This data supports the hypothesis that lower socioeconomic conditions and suboptimal health care may influence the mortality rate (G. Hatemi *et al*., 2014). Sibley *et al*. compared clinical manifestations and activity of BD patients followed at two tertiary centres in the US (National Institute of Health and New York University), and at the Turkish tertiary referral centre of Istanbul University, Cerraphasa Medical School (C. Sibley *et al*., 2014).

American patients showed more frequently gastrointestinal and neurologic disease, they were more likely female and had longer disease duration. Eye and vascular disease frequency rates appeared similar in both US and Turkish patients.

Finally, change in disease expression over the past years has been noticed and attributed to increased awareness of the disease and greater accessibility to hospitals, together with improvements in both hygienic conditions and therapeutic strategies.

Kim *et al*. retrospectively evaluated 3674 patients divided in two decades and found a significant decline in rates of complete disease phenotypes, especially in regard to the major presenting features, such as genital ulcers, ocular involvement and skin lesions. In addition, the mean patient age increased progressively over the last three decades, together with joint, gastrointestinal and central nervous system manifestations (D. Y. Kim *et al.*, 2014).

Countries	BD Prevalence (n° of cases per 100,000 people)
Turkey	80-420
Iran	80
Japan	13.5
China	14.0
France	7.2
Germany	4.2
Italy	2.5
United States	8.6

Table 1.1. Prevalence of Behçet Disease in different geographic areas (A. Idil, 2002; N. L. Ambrose *et al.,* 2013; M. Takeuchi *et al*., 2015).

1.2. Clinical presentation and diagnostic criteria

1.2.1. Clinical presentation

Recurrent oral ulcers are one of the hallmarks of BD, often representing the first clinical sign of the disease. BD-related aphthosis is sometimes referred to as "complex aphthosis" to distinguish it from the more common simple aphthosis, which affects roughly 15% of the general population.

In contrast to simple aphthosis, complex aphthosis is characterized by frequent recurrences of ulcers, which are usually particularly painful and take longer to heal.

Moreover, in complex aphthosis genital ulcers may also coexist with oral ulcers. The most frequent sites of genital ulcers in BD are the major and minor labiae in females, and the scrotum and the shaft of the penis in males. Aphthae can be categorized as minor, major or herpetiform. Minor aphthae are usually small (< 1 cm in diameter), superficial, located on the buccal and labial mucosa; they heal within days and do not cause scarring. Major aphthous ulcers are larger (> 1 cm), deeper, persisting for weeks to months and causing scars. Herpetiform ulcers are small (medium diameter 1-3 mm); usually they manifest as a group of coalescent lesions around a larger plaque and they heal spontaneously in 1 to 4 weeks (C. B. Lynde *et al*., 2009).

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The pathergy phenomenon is nearly unique to BD. It is induced by a 20 to 22-gauge needle prick in the dermis (usually of the forearm); the test is considered positive if an erythematous papule or pustule greater than 2 mm develops within 48 hours. Skin pathergy reaction remains the most diagnostically relevant test in BD patients, although its prevalence varies according to different ethnicities.

Papulo-pustular lesions are the most common skin manifestation of BD. When papulopustular lesions occur around a hair follicle they are sometimes termed "pseudofolliculitis", whereas when they resemble acne vulgaris they are named "acneiform lesions". Erythema nodosum –like lesion is the second most common skin manifestation of BD; it affects particularly women and should be differentiated from superficial thrombophlebitis. Histology may show vasculitis, but may also be non-specific (G. Hatemi *et al*., 2013; M. Melikoglu *et al*., 2008).

Uveitis and retinal vasculitis are among the most common organ manifestations, occurring in 60%-80% of the patients. Intraocular inflammation may involve the anterior or posterior segment, or both (J. F. Arevalo *et al*., 2015).

Gastrointestinal, neurologic, and cardiovascular complications are present in variable numbers of patients. The frequency of gastrointestinal involvement has been reported as low in the Middle and Far East (2.8% in Turkey, 4% in Saudi Arabia), moderately high in China (10%) and Taiwan (32%), and higher in the United Kingdom (38-53%) and Japan (50%-60%).

Ulcers involving the gastro-intestinal tract typically appear irregular, round or oval, punched-out, deep, larger than 1 centimeter; they can be single or a few. The majority of patients with BD have ileo-cecal localization of the disease. Colonic and rectal involvement are rarer. Colonic ulcers are described as 'volcano-type' due to the nodular margins and aspects of deeply penetrating lesions at endoscopic evaluation.

Nervous system involvement, also known as 'neuro-Behçet's disease' (NBD) is observed in approximately 10-15% of the cases and may be sub-classified in two major forms: a parenchymal form, which often presents as aseptic meningoencephalitis ("neuro-Behçet" stricto sensu), or an

isolated cerebral venous sinus thrombosis often complicated by intra-cranial hypertension (S. Saip *et al*., 2014).

Cerebral (typically dural) sinus thrombosis is associated with other vascular manifestations, such as deep vein thrombosis, superficial thrombosis, and pulmonary artery aneurysms.

Vascular involvement has an estimated occurrence rate varying from 10% to 50%. Studies from Morocco, Saudi Arabia, Lebanon and Turkey reported an increased prevalence of venous lesions, as compared to Korean populations (T. Kotsis *et al*., 2011). Venous thrombosis most commonly involves popliteal and femoral veins. Thrombosis of superior or inferior vena cava, and of upper extremities' veins are also observed. Caval involvement rates vary from 2 to 13%. Thrombosis of the hepatic veins (Budd-Chiari syndrome) may occur as an extension of thrombosis from inferior vena cava.

The main features of pulmonary involvement are pulmonary artery aneurysms, arterial and venous thrombosis, pulmonary infarctions, recurrent pneumonia and pleurisy. All of them are considered rare manifestations, with an estimated prevalence of about 1%, according to different studies (F. Erkan, 1999).

1.2.2. Diagnostic criteria

The diagnosis of BD is based on a combination of clinical symptoms and signs, since there is no specific histologic, laboratory or radiologic finding. Several sets of diagnostic and classification criteria have been proposed, since the first years following the disease definition by Hulusi Behçet.

However, such criteria were not built on consensus, have not been validated and are now basically only of historical interest (F. Davatchi, 2012).

In 1990 the International Study Group on Behçet's Disease (ISGB) composed of experts from seven countries (Turkey, Iran, Japan, Tunisia, France, United Kingdom and US) presented the ISGB criteria, which soon became the most used criteria for years (ISGB, 1990).

1. Recurrent oral ulceration (aphthous or herpetiform) observed by the physician or patient recurring at least three times in one 12-month period plus two of the following:
2. Recurrent genital ulceration 3. Eye lesions: anterior uveitis, posterior uveitis, cells in the vitreous by slit lamp examination or retinal vasculitis observed by an ophthalmologist 4 Skin lesions: erythema nodosum, pseudofolliculitis, papulopustular lesions or acneiform nodules in postadolescent patients not on corticosteroids 5. Pathergy, read by a physician at 24-48 hours
Sensitivity 90%, specificity 95% - but not at onset (T. W. O'Neill *et al*., 1994)

Table 1.2. International study group for Behçet's disease (ISGB) – 1990 Criteria (*The Lancet 335 (8697):1078-80*).

It is worth noticing that in the ISGB criteria oral ulcers represent a prerequisite to classify a patient as having BD. Therefore, such criteria are by definition inapplicable to the odd BD patient with no oral ulcers (< 1-2% of the entire BD population). In addition, vascular and cardiac manifestations have not been included in the ISGB criteria because of their poor specificity. Lastly, although their authors explicitly defined the ISGB criteria as 'diagnostic criteria', they are in fact 'classification criteria'. In this regard, in a longitudinal study from Reggio Emilia group, Italy, using the specialist diagnosis as gold standard, Salvarani *et al*. showed that 87% of patients with BD fulfilled the ISGB criteria at 10 years from the diagnosis, while the percentage of patients with early BD who met the criteria was as low as 23% (Salvarani et al. *personal communication, 2012*). Therefore, the ISGB criteria should not be used to diagnose BD in the individual patient, whereas they are useful – because of their high specificity – to enrol patients in clinical trials (T. W. O'Neill *et al*., 1994).

In 2006 the new International Criteria for Behçet's Disease (ICBD) were presented; these criteria have subsequently validated in some countries, and recently revised (2010).

Ocular lesions 2 points Oral aphthosis 2 points Genital aphthosis 2 points Skin lesions 1 point Central nervous system involvement 1 point Vascular manifestations 1 point Optional: Pathergy test, when used, 1 point.
A patient scoring ≥4 points is classified as having active Behçet disease

Table 1.3. International Criteria for Behçet's Disease (ICBD) (F. Davatchi *et al*., 2010). In the validation set, ICBD had a sensitivity of 95% versus the International Study Group for Behçet Disease (ISGB) 1990 criteria 85%, and specificity 91% versus ISGB 96.0%. Pathergy test (assessed when done in >90% of patients and controls) increased sensitivity from 96% to 98.5% and slightly decreased specificity from 92.1% to 91.6% (ISGB Group, *The Lancet*, 1990; F. Davatchi *et al*., 2010; ICBD Group, *J Eur Acad Dermatol Venereol*, 2014).

Differently from the ISGB criteria, the ICBD criteria included as items both vascular and neurological manifestations. To summarize, the ISGB criteria have a very good specificity at the expense of sensitivity and accuracy, while the ICBD have shown better sensitivity (97% vs 77%), lesser specificity (97% vs 99%) and a better accuracy (97% vs 87%).

Nevertheless, the performance of the ICBD criteria needs to be validated in further different populations, therefore additional validation studies are required (F. Davatchi *et al*., 2015).

1.3. Pathogenesis of Behçet Disease

Pathogenesis of BD is still unknown. It is not clear the exact contribute of innate and adaptive immunity dysfunction nor the relevance of genetics, however several authors agree in considering combination of the three of them as pivotal in the development of the disease.

BD is currently considered a polygenic multifactorial rheumatic disorder. Differently from other rheumatic diseases, it does not have any autoimmune nor auto-inflammatory hallmark, which renders BD a clinical challenge in diagnosing and treating.

Finally, the unknown pathogenesis represents the main limitation to the therapeutic approach.

1.3.1. Genetics

Evidence for the relevance of the genetic make-up to the susceptibility of developing BD derives from both inheritance and genetic studies performed in various countries.

Familial aggregation is usually construed as evidence supporting a genetic predisposition to diseases. A significant familial clustering has been reported in 1–18% of BD patients, mostly of Turkish, Israeli and Korean origin, especially in families of probands carrying the HLA B51 allele. The familial aggregation was higher in Turks (18.2%), Koreans (15.4%), and Jews (13.2%) than in Chinese (2.6%), Japanese (2.2%), and Europeans (1%) (T. I. Kaya, 2012).

Likewise, twin concordance studies are frequently used to estimate the role of genetic factors in the pathogenesis of multifactorial diseases. In a recent twin study of Masatlioglu *et al*., the pairwise concordance rate for BD was 2/6 for monozygotic twins and 1/8 for dizygotic twins, accounting for 41% of the phenotypic variance for BD among twins (S. Masatlioglu *et al*., 2010). This data points to an interplay of genetic and as yet poorly characterized environmental factors in producing the clinical phenotype of BD.

Among the genes, the human leukocyte antigen (HLA) class I allele HLA-B51 is the genetic factor with the strongest association with BD susceptibility, independently from the different ethnicities (M. de Menthon et al., 2009). The relationship between HLA-B51 and BD was first

identified four decades ago, and then replicated in nearly every genetic study on BD (M. J. Ombrello *et al*., 2014). However, the pathogenic role of HLA-B51 is still poorly elucidated. Neutrophil hyperfunction and presentation of specific antigens to CD8+ cytotoxic lymphocytes have been proposed as possible mechanisms, but much remains to be explained, not least the fact that the HLA-B51 allele is quite common in the general population, yet BD is a rare disease. Studies looking at associations between HLA-B51 subtypes and BD in different populations have generated discordant results, de facto failing to link one or few subtypes to disease susceptibility (I. Olivieri *et al*., 2013; D.D. Demirseren *et al*., 2014).

Whether HLA-B51 modifies the clinical phenotype of BD is still debated. In this regard, HLA-B51 has been linked to male gender, ocular and skin disease, and genital ulcers, as well as to a decreased risk of gastrointestinal disease, but the respective relative risks are fairly weak (C. Maldini *et al*., 2012).

In addition to HLA-B51, some HLA-I residues have been shown to influence antigen binding and regulation of cell-mediated cytotoxicity, supporting a role for one or more pathogenic peptides in BD (M. J. Ombrello *et al*., 2014). In particular, such molecules could heighten risk of BD through the regulation of both Natural Killer (NK) cell and CD8+ cytotoxic T lymphocyte activation (M. J. Ombrello *et al*., 2014).

Other genes and loci localized outside the HLA region may also be involved in the pathophysiology of BD (M. J. Ombrello *et al*., 2014; E. F. Remmers *et al*., 2010; N. Mizuki *et al*., 2010; Y. Kirino *et al*., *Nat Genet* 2013; Y. Kirino *et al*., *PNAS* 2013).

GIMAP genes encode evolutionary conserved GTP-binding proteins that are preferentially expressed in immune cells. GIMAP has been shown to play a role in modulating peripheral T-cell function and T-cell development and selection (T. Ciucci *et al*., 2014). Lower level of GIMAP4 mRNA has been found in CD4 T cells from BD patients, which could contribute to resistance to T-cell apoptosis in BD (Y. J. Lee *et al*., 2013). However, so far, the association described in Asian populations between the GIMAP region and BD has not been replicated in Europeans (L. Ortiz-

Fernandez *et al*., 2014).

Targeted re-sequencing genetic studies also found that familial Mediterranean fever gene *MEFV* and the toll-like receptor 4 gene *TLR4* are implicated in the pathophysiology of the disease, supporting at least a partial contribution of innate immunity and autoinflammation mechanisms to its pathogenesis (Y. Kirino *et al*., *PNAS* 2013).

1.3.2. Innate and Adptive Immunity

Dysfunctions in NK, NKT and T lymphocytes have been described in patients with BD.

CD8 + CD56 + and T CD56 + γδ cells are significantly increased in BD patients as compared to healthy controls and patients with anterior idiopathic uveitis (H. G. Yu *et al*., 2004).

The number of NKT cells (CD3 + CD56 +) and NK (CD3- CD56 +) in the aqueous humor and in the peripheral blood of patients with BD was also found to be greater than other types of uveitis (H.G. Yu *et al*., 2004).

The increase in the number of NKT cells has been correlated with the inflammatory activity, suggesting that these cells may play an important role in BD immunopathogenesis (H. G. Yu *et al.*, 2004).

As for NK cells, there are several potential mechanisms that might explain their role in the pathogenesis of BD. One of these is that defects in the NK cell repertoire may allow persistent viral infections, resulting in a chronic inflammatory response, finally leading to BD.

Another is that NK cells have a lower level of inhibitory receptors or an increased level of activating receptors that could lead to impaired recognition of major histocompatibility complex (MHC) and self molecules (H. Petrushkin *et al*., 2015).

Role of NK cells in BD will be treated in details in Chapter 2 (*see Chapter 2 – Role of NK Cells in Behçet Disease – Introduction*).

In general, activation of the adaptive immune response was documented in patients with BD.

An oligoclonal expansion of T cells has been correlated with disease exacerbation, in particular of helper T lymphocytes 1 (Th1), Th22 and to a lesser extent of T helper 17 (Th17) (A. Gul, 2015). The accumulation of such T cells in inflammation sites has been reported (C. Comarmond *et al*., 2014).

In the peripheral blood, higher expansion of double negative T cells (CD3+ CD4- CD8-) was observed in patients with active disease as compared to those with inactive disease (C. Comarmond *et al*., 2014).

Some authors have shown high levels of IL-12, IL-18 and IFN-γ in active lesions of BD patients as well as high circulating levels of IL-17 and IL-23.

Th1 cell infiltrates were found in oral and genital ulcers, in the skin lesions caused by Pathergy test and in gastro-intestinal lesions (C. Comarmond *et al*., 2014).

Expression of specific transcription factors for Th1 and Th17 has been observed in patients with neuroBehçet (NBD), and Th17 /T regulatory (T reg) ratio was found to be increased in the cerebrospinal fluid (CSF) (C. Comarmond *et al*., 2014).

An increase in Th17 cells and a decrease of T reg in the peripheral blood of patients with active disease has finally been documented (C. Comarmond *et al*., 2014).

Th17 cells seem to correlate with disease activity. In particular, cells stimulated by IL-21, such as CD4 + T lymphocytes, were found to be significantly increased in the peripheral blood of BD patients.

IL-17 and IL-21-producing cells were detected in CSF, in the inflammatory infiltrates of brain parenchyma, and in cerebral blood vessels in patients with neurological involvement. The blockade of IL-21 has been proven to re-establish the homeostasis of Th17 and T reg, demonstrating how it can act upstream of the Th17 and Th1 pathways in BD (C. Comarmond *et al*., 2014).

1.4. Behçet Disease Uveitis: a vision-threatening intraocular inflammatory disease

Ocular involvement is present in around half of BD patients. This may be variable in different

series, being as high as 70% in young men and as low as 30 % among women and elderly patients (H. Yazici *et al*., 1984; E. Kural-Seyahi *et al*., 2003; I. Tugal-Tutkun *et al*., 2004). Ocular manifestations usually manifest within 5 years after the disease onset (E. Kural-Seyahi *et al*., 2003).

Bilateral involvement is frequent and reported in around 75–80 % of BD patients with eye involvement.

In a 20-year follow-up survey reported from Cerraphasa Medical Faculty, at the beginning of the disease, the ratio of bilaterality was 80 % among men and 64 % among women. At the end of the 20-year follow-up, the ratio of bilaterality increased to 87 % among men and 71 % among women (E. Kural-Seyahi *et al*., 2003).

The typical form of ocular involvement is a relapsing and remitting panuveitis and retinal vasculitis. Although initial findings commonly seem to be limited to the anterior uvea, most patients develop posterior segment findings during their disease course.

Destructive and recurrent attacks are responsible for irreversible ocular structural changes and indicate poor prognosis. Recurrent attacks especially with posterior segment and retina involvement increase the risk of permanent changes in sensory retina and cause irreversible loss of vision (Y. Ozyazgan *et al*., 2015).

The breakdown of blood-aqueous barrier is the main reason of inflammatory processes which are limited to the anterior segment and called iritis, cyclitis, and iridocyclitis in classical definition.

Aqueous flare and cells are the two inflammatory parameters of anterior chamber inflammation resulting from disruption of the blood-aqueous barrier.

Cells in the aqueous humor are seen as particles identified by backscattering light from the incoming beam with slit lamp biomicroscopy. Anterior chamber cells' movements are based on eye movements and aqueous humor dynamics (Y. Ozyazgan *et al*., 2015).

Grading systems have been developed in an effort to standardize quantification of cells and flare in the anterior chamber, based on slit lamp examination (D.A. Jabs *et al*., 2005). If the ciliary body becomes inflamed, cells can be seen behind the lens with less severe density.

Aqueous flare can be measured by laser flare photometry, which is an objective quantitative method.

The activity of anterior chamber inflammation can cause permanent damage in the anterior segment because of the inflammatory material and fibrin exudates. Severity and number of repeated inflammatory attacks involving the anterior segment determine the extent of irreversible structural changes (Y. Ozyazgan *et al*., 2015).

These inflammatory structural changes lead to irreversible loss of vision if urgent anti-inflammatory therapy is not initiated.

The prognosis of eye involvement has greatly improved over the last decades with the effective use of immunosuppressants. In a study published in 1970, 73 % of patients with ocular involvement had progressed to blindness during a mean follow-up of 3.5 years (J. G. Mamo, 1970).

Another study comparing the visual outcome of BD patients with eye involvement followed in the same centre between 1990–1994 and 2000–2004 showed that there were significantly less patients who lost useful vision and experienced fewer severe ocular complications and none of the patients became legally blind during the 2000s (A. K. Cingu, *et al*. 2012). Interestingly, patients also had milder ocular disease at referral during the 2000s.

Different combinations of immunosuppressive agents and corticosteroids have been used for the treatment of patients with posterior uveal inflammation, especially with macular involvement and retinal vasculitis. Biological agents (mainly anti-TNFα monoclonal antibodies) are added when the inflammation cannot be controlled with conventional treatment modalities.

However, the therapeutic armamentarium in BD remains limited, being the pathogenesis of BD uveitis still not completely clear.

There are a number of randomized controlled trials which guide the systemic treatment of eye involvement in BD. The first one of these was the azathioprine trial, which showed a decrease in the frequency of uveitis attacks compared to placebo and prevention of visual loss with azathioprine (H. Yazici *et al*., 1990).

Corticosteroids are used for the rapid suppression of attacks, and quick tapering is aimed due to the risk of cataracts and glaucoma with prolonged use.

In patients who continue to have attacks despite azathioprine or in patients with severe eye involvement including retinal vasculitis and macular involvement, cyclosporine may be added (Y. Ozyazgan *et al*., 2015).

Interferon-alpha (IFN-α) and anti TNFα are the most frequently used biologic agents. IFN-α has been shown to decrease the frequency of uveitis attacks and improves visual acuity in patients resistant to other immunosuppressives and corticosteroids (J.M. Durand and J. Soubeyrand, 1994; I. Kotter *et al*., 1998; I. Kotter *et al*., 2003; I. Kotter *et al.*, 2005; I. Tutgal-Tutkun *et al*., 2006; B. Bodaghi *et al*., 2007). The main factor limiting the use of IFN-α is the difficulty in tolerating this agent which causes unpleasant flu-like symptoms and depression which may be quite severe in some patients.

TNF-α antagonists, especially infliximab 5 mg/kg every 6–8 weeks, have been used with increased frequency in BD patients over the last decade (P.P. Sfikakis *et al*., 2001; S. Ohno *et al*., 2004; I. Tutgal-Tutkun *et al.*, 2005; B. Bodaghi *et al*., 2005; P.P. Sfikakis *et al*., 2007; B. Mushtaq *et al*., 2007). The rapid onset of action is an important advantage in suppressing the inflammatory attacks of BD.

1.5. Other types of endogenous uveitis: Vogt-Koyanagi-Harada Disease

Vogt-Koyanagi-Harada syndrome (VKH) is a rare multisystem disorder affecting tissues containing melanin, such as the eye, inner ear, meninges and skin, first described in 1906 (A. Greco *et al*., 2013). When affecting the eye, it is classified as an endogenous chronic bilateral panuveitis and it is included in the spectrum of conditions to consider for the differential diagnosis of ocular involvement in BD.

1.5.1. Epidemiology

Prevalence of VKH varies in different populations. It is more common in Asia, Latin America and Middle East. It appears to be relatively rare in white Caucasian individuals (A. G. O'Keefe *et al*., 2017).

VKH is the most common cause of panuveitis in India, with a prevalence of 21.08%. In Thailand, it is the most common cause of non-infectious uveitis (16%). In Saudi Arabia, prevalence of VKH is 19.4%; in China it varies from 15.9% to 16.3%. In Tunisia, VKH is the second most common cause of panuveitis with a prevalence of 15%, following BD. In Iran, VKH is the third cause of panuveitis with a frequency of 15.2%. In Japan, VKH has a prevalence ranging from 6.7% to 11%. Conversely, VKH is a rare disease in Turkey, where it accounts for only 1.2% of all uveitis cases (A. G. O'Keefe *et al*., 2017).

The age at onset is generally between 20 and 50 years. Patients older than 60 years have a lower prevalence of VHK than younger patients. Apart from Japanese population, females are generally more affected with a 2: 1 female-to-male ratio (A. G. O'Keefe *et al*., 2017).

1.5.2. Clinical manifestations

The classical clinical course of VKH includes three phases: 'prodromal' phase, 'ophthalmic' phase and 'convalescence' phase. A fourth phase, the recurrent/chronic phase, can be sometimes added to the three ones (V. M. Sakata *et al*., 2014).

The 'prodromal' phase can last from a few days to a few weeks. At this stage, clinical manifestations are mainly extra-ocular and include: headache (82%), meningism (55%), fever (18%), nausea (9%), vertigo (9%), orbital pain and hearing disorders.

The 'ophthalmic' phase follows the prodromal phase and is characterized by acute, bilateral, usually symmetrical, diffuse uveitis, with hyperemia and oedema of the optic disc and serous retinal detachment of varying size. The signs also include swelling and hyperemia of the optic nerve head and retinal oedema. Depigmentation of the tegument can also occur.

During the 'convalescence' phase, the choroidal depigmentation occurs. Also the eyebrows, eyelashes, hair and skin lose melanin, with consequent polyosi and vitiligo. This phase usually lasts for months (A. G. O'Keefe *et al*., 2017).

About two thirds of patients may develop a relapsing / chronic phase of the disease characterized by recurrent episodes of anterior uveitis (V. M. Sakata *et al*., 2014).

This phase usually develops 6-9 months after the initial presentation. Complications may include cataracts (10-42%), glaucoma (6-45%), subretinal fibrosis (8-40%) and neovascular membranes (9-14%) (V. M. Sakata *et al*., 2014).

Other organs containing melanin, such as central nervous system, ear, skin, can be affected. A good percentage of patients suffer from some form of sensory hearing loss, most often at higher frequencies, such as 4, 6, 8 and kHz (A. G. O'Keefe *et al*., 2017).

1.5.3. Diagnosis

In October 1999, during the first international VKH disease workshop, some criteria for diagnosis have been defined (R. W. Read *et al*., 2001). VKH can present itself as complete, incomplete, and probable, depending on the number of diagnostic criteria (R. W. Read *et al*., 2001).

The "complete" form of VKH requires the presence of the following five criteria: (1) no previous history of trauma or intraocular surgery prior to the beginning of uveitis; (2) no clinical or laboratory evidence of any other ocular disease; (3) bilateral ocular involvement (both early and late); (4) neurological or hearing loss signs; (5) signs on the skin.

VKH is considered "incomplete" if only criteria 1) and 3), or only 4) and 5) are present.

Finally, "probable" VKH may be considered only in the presence of ocular disease fulfilling criteria 1) and 3) (A. G. O'Keefe *et al*., 2017).

No laboratory test can be used to define the diagnosis, and no single manifestation of the disease is specific for VKH. As a matter of fact, ocular manifestations can be present in other forms of uveitis, including BD-related uveitis, sympathetic ophthalmia, posterior scleritis, intraocular

lymphoma. Retinal fluorangiography and recent advances in imaging techniques may help in achieving a correct diagnosis, in particular optical coherence tomography (OCT), which is able to assess changes in the retina and choroid.

In Japan and Europe, lumbar puncture is also performed to detect pleocytosis in the CSF. Other tests useful in the diagnostic process are green indocyanine angiography (ICG) to detect choroidal changes, and ocular ultrasound, to measure choroid thickness.

1.5.4. Pathogenesis

The etiopathogenesis of VKH is not yet clear. It has been hypothesized an autoimmune process directed against melanocytes following antigenic mimicry, maybe activated by an infectious agent in genetically predisposed individuals. This process leads to loss of melanocytes and following depigmentation (J. Y. W. Ng *et al*., 2014).

What induces the breaking of tolerance for melanocytes is not yet known. Melanocyte differentiation proteins such as tyrosinase, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2) are the most studied proteins in the pathogenesis of VKH. In pigmented cells, melanin is synthesized by tyrosine through enzymatic reactions within melanosomes. The first reactions are catalyzed by tyrosinase, which is considered the key enzyme in melanin synthesis.

It is now generally accepted that tyrosinase is the target of autoimmunity of T lymphocytes in VKH. Furthermore, it has been shown that T-specific cells for tyrosinase peptides can mediate an inflammatory response (A. G. O'Keefe *et al*., 2017).

In general, the autoimmune response against melanocytes seems to be complex and involves humoral, cellular and innate immunity (J. Y. W. Ng *et al*., 2014).

CD8 + lymphocytes, as well as the CD4 + Th17 and T regulatory cells have been implicated in pathogenesis (J. Y. W. Ng *et al*., 2014).

High IgG levels against KU-MEL-1, an antigen expressed by human melanocytes, were also found (J. Y. W. Ng *et al*., 2014). There is a molecular mimicry between some viruses and the

proteins of melanocytes, which can cause cross-reactions. Sakata *et al*. have found that the 450462 tyrosinases expressed by the melanocytes and the H glycoprotein of the Cytomegalovirus (CMV) envelope share six amino acids and that the cross-reactivity of T cells to these proteins could be involved in the disease onset (V.M. Sakata *et al*., 2014). Epstein-Barr virus DNA (EBV) was also isolated from vitreous humor of some patients with VKH (A. Greco *et al*., 2013).

In combination with environmental factors, genetic factors are of pivotal importance in the pathogenesis of the disease. Several HLA genotypes have been associated with VKH (Table 1.4).

As regards cytokines, higher serum IL-23 levels have been found in VKH patients with acute uveitis than those without active uveitis and healthy subjects (V. M. Sakata *et al*., 2014). IL-23 may increase the production of IL-17 by CD4 + T cells and is deregulated in various autoimmune diseases (J. Y. W. Ng *et al*., 2014).

Serum osteopontin (OPN) levels have been found significantly higher in patients with active disease than in patients with inactive disease and healthy controls (J. Y. W. Ng *et al*., 2014).

Levels of the inhibiting factor of macrophage migration (MIF) were also significantly higher in patients with VKH and with BD (J. Y. W. Ng *et al*., 2014).

Moreover, patients with VKH disease have elevated levels of IFN-γ in the aqueous humor and in the serum (J. Y. W. Ng *et al*., 2014).

HLA Genotypes	**Populations**
HLA-DQA1*0301	Japan
HLA-DQB1*0604	Japan
HLA-DRB1*0404	Mexico
HLA-DRB1*0405	Japan, Brasil, Korea, Saudi Arabia, Mexico

Table 1.4. Most prevalent HLA genotypes associated with VKH (J. Y. W. Ng *et al*., 2014).

1.6. General Hypotheses and Aims of the Project

The aims of this study were:

1) to increase the knowledge about BD pathogenesis;

2) to identify laboratory tests which can support BD diagnosis;

3) to identify potential therapeutic targets for BD treatment, being the therapeutic armamentarium for such disease very limited.

BD remains a rare disease in Western Europe, thus study cohorts on large populations are difficult and randomised-controlled trials on new drugs are very few.

This study has been conducted in one of the National Reference Centres for Behçet Disease and Behçet Disease Uveitis, which allowed to collect a relatively large cohort of patients affected.

Based on the hypothesis that impaired cytotoxicity mechanisms of circulating Natural Killer (NK), NKT and T cells might have a role in the pathogenesis of BD, the first part of the study was aimed to identify a specific profile of circulating NK, NKT and T cells able to discriminate between BD patients and healthy controls, by investigating the phenotypic characteristics, the cytotoxic potential of circulating NK and NKT cells, and by quantifying 27 plasmatic cytokines/chemokines.

The second part of the study was focused on BD-related uveitis, which may cause irreversible ocular structural changes and permanent damage in sensory retina with visual loss, if left untreated. Similarly for systemic BD, the therapeutic armamentarium for BD uveitis is still very limited.

The aim of this part of the study was to investigate differences in the mechanisms of two distinct types of endogenous uveitis (i.e. BD uveitis *versus* VKH-related uveitis) - to search for potential markers and therapeutic targets. Levels of pro- and anti-inflammatory cytokines were measured in aqueous humor from patients with active BD uveitis and VKH.

Secondly, given the evidence about the impairment of cytotoxicity mechanisms in BD, a different distribution of NK cell subsets in the aqueous humor of patients with different types of endogenous uveitis could also be hypothesised. With this aim, frequency of NK and NKT cells in the same groups of patients has been explored.

Alessandra Soriano

Chapter 2: Role of NK Cells in Behçet Disease

2.1. Introduction

Natural killer (NK) cells represent a heterogeneous population of innate immunity cells able to recognize cells lacking self- major histocompatibility complex of class I (MHC-I) molecule or cells which display changes in the surface self-molecules. They are characterized by the expression of specific receptors (NKRs) and surface markers such as CD16 and CD56 (D.I. Godfrey *et al*., 2000; E. Lugli *et al*., 2014).

Upon activation, NK cells release cytotoxic granules containing perforin and granzymes, leading to the destruction of cellular membrane of target cells and subsequently apoptosis (A. Mandal *et al.*, 2015).

It is known that NK cells contribute both to innate and adaptive immune responses. However, their exact functional role in the different sites of the immune response remains unclear.

Disorders in the number and functions of NK cells have been observed in several organ-specific autoimmunity models (M. A. Mieza *et al*., 1996; Y. Yanagihara *et al*., 1999; A. Kukreja *et al*., 2002).

As for the cytofluorimetric characterization, human NK cells are defined by CD3 - and CD56 + phenotype; moreover, they are commonly divided into two main subpopulations: CD56dim CD16+, and CD56bright CD16-. The CD56dim CD16+ subset comprises 90% of the circulating NK cells and mediates precociously the direct cytotoxic response. The CD56bright CD16 subset mediates the effector function by releasing IFNγ and demonstrates lower cytotoxic capacity.

NK cells also express a broad panel of receptors for MHC-1, which allow them to respond to the antigen in the absence of prior sensitization, unlike T cells. NK activity is determined by the final balance between activating and inhibitory response, and both contributing to the final control of infections.

In BD, it is known that the HLA B51 protein, encoded by the HLA-B gene of the MHC-I, represents the strongest risk factor for the development of the disease (S. Ono *et al*., 1975).

The most recent genetic studies documented the association between BD and a peptide variant of HLA-B, which independently regulates the activity of cytotoxic T lymphocytes and NK cells through HLA-E and the CD94 receptor / NKG2. These observations provide a plausible explanation for the role of MHC in conferring a risk of impairment of cytotoxic activity of NK cells in BD (M. J. Ombrello *et al*., 2013).

Natural killer T (NKT) cells represent a lymphocyte subpopulation expressing surface molecules characteristic of NK and T cells (CD3 + and CD56 + cells). Like NK cells, activated NKT cells release in the extracellular compartment pro- and anti-inflammatory cytokines/chemokines with the function of regulating the immune response (S. Joyce, 2001).

Similarly to NK cells, their activation derives from the balance of signals coming from activator and inhibitory receptors (E. Vivier and S. Ugolini, 2011; A. E. Zamora *et al*., 2015).

There are some evidence about the involvement of NK and NKT cells in the pathogenesis of BD, but with some discrepancies.

An increased frequency of circulating NK and NKT cells in BD patients compared to healthy controls (HC) has been reported (M. Treusch *et al*., 2004; N. Takeno *et al*., 2004; H. G. Yu *et al*., 2004; H. Yato *et al*., 1999). Conversely, other authors reported a decreased frequency of circulating NK and NKT cells in BD patients compared to HC (M.S. Hasan *et al*., 2017; K. Hamzaoui K. *et al*., 2006).

Yamaguchi and coll. demonstrated a significant increase in activated CD69 + circulating NK cells in patients with active disease (Y. Yamaguchi *et al*., 2010).

Prevalence of CD3-CD56bright subset on CD3-CD56dim subpopulation has been shown in subjects with active BD as compared to HC (*Hasan et al., Personal communication, International Behçet Disease Conference, Paris 2014*).

As for the discrepancies in cellular redistribution at different stages and sites of disease, it has been observed that the amount of NK CD56+ in the peripheral blood of BD patients with concomitant uveitis is higher than that found in individuals suffering from other forms of uveitis

and compared to HC (H. Yato *et al*., 1999).

NKT have been shown to be peculiarly present in the aqueous humor of subjects with BD-related uveitis, and not in the course of other forms of uveitis (H. G. Yu *et al*., 2004).

Discrepancies in the expression of NKT cells in the peripheral blood and in CSF of subjects suffering from BD with neurological involvement and in active phase have been observed, with a clear prevalence of functionally active NKTs and producing IFN-γ in CSF (K. Hamzaoui *et al*., 2006).

2.2. Hypotheses and Aims of the experiment

Based on the hypothesis that the analysis of circulating NK, NKT and T cells could increase the knowledge about the molecular mechanisms involved in the pathogenesis of BD, this part of the study aimed to identify a specific profile of circulating NK, NKT and T cells able to discriminate between BD patients and HC, by investigating the phenotypic characteristics, the cytotoxic potential of circulating NK and NKT cells, and by quantifying 27 plasmatic cytokines/chemokines.

2.3. Materials and Methods

2.3.1. Study subjects

A cohort of 38 BD patients was enrolled at the Rheumatology Division, Arcispedale Santa Maria Nuova - IRCCS, Reggio Emilia, Italy, one of the National Reference Centres for Systemic Vasculitides and Behçet Disease. All patients satisfied the International Study Group for Behçet Disease criteria (ISGB, *The Lancet*, 1990). BD activity was evaluated through Behçet Disease Activity Form (BDCAF, *University of Leeds, Vers. 06.03.2006, International Society for Behçet Disease, ISBD)*, administrated to patients during a complete clinical rheumatologic evaluation (*for BDCAF details see Supplementary Section*).

Of one subject, samples were collected in two time points in which the patient had different disease activity stages and BDCAF scores. The median age was 40 (Interquartile range; IQR: 29-50) and gender distribution was: 55% male (21/38) and 45% female (17/38).

Characteristics of BD cohort and treatment schedules are summarized in Table Supplementary S 2.2.

15 age-matched healthy controls (HC) were recruited as reference. The median age was 40 (IQR: 34-53) and gender distribution was: 33% (5/15) male and 67% female (10/15). HC did not suffer from any autoimmune disease. The median age and gender distribution were similar between the two cohorts.

The study was approved by the Local Ethics Committee (Reggio Emilia, Italy) in compliance with the Declaration of Helsinki and informed consent was obtained from all patients and HC.

2.3.2. Specimen collection

18 mL of venous blood were collected from each subject into EDTA coated tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by histopaque-1077 density gradient centrifugation (Sigma-Aldrich) and stored frozen in liquid nitrogen in 90% heat inactivated fetal bovine serum (FBS, Gibco, ThermoFisher) 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich) until use. Plasma was collected and stored at -80 °C until use.

2.3.3. Laboratory Processing

2.3.3.1. Flow cytometry

PBMCs were thawed and counted with a Fuchs-Rosenthal hemocytometer. 5 x 10^5 cells were resuspended in 100 µL Phosphate-buffered saline (PBS, Euroclone) + 1% FBS and stained for 25 minutes at 4 °C with the following antibodies: PerCP mouse anti-human CD3 (clone BW264/56),

PE anti-human CD56 (clone REA196), FITC anti-human CD16 (clone REA423), PE-Vio770^{Tm} mouse anti-human CD69 (clone FN50) and APC mouse anti-human NKG2D (clone BAT221).

Alternatively, 5 x 10^5 cells/ 100 µL PBS + 1% FBS were stained for 25 minutes at 4 °C with PerCP mouse anti-human CD3 (clone BW264/56), PE anti-human CD56 (clone REA196), PE-Vio770^{Tm} anti-human NKG2A (clone REA110), APC mouse anti-human Nkp30 (clone AF29-4D12), VioBright^{Tm} FITC mouse anti-human Nkp46 (clone 9E2) antibodies. All antibodies were purchased from Miltenyi Biotec and used as suggested by the manufacturer. After washing, PBMCs were resuspended in PBS + 1% FBS and acquired with the FACSCanto II flow cytometer (BD Biosciences), equipped with two lasers for excitation at 488 and 633 nm. Data were analysed with FACSDiva 8.0.1 software. At least 60000 lymphocytes were acquired.

2.3.3.2. Cell lines

K562 cell line, a human erythroleukemic cell line which does not express MHC class 1 molecules, was provided from Parma University, Italy and maintained in RPMI 1640 (Gibco, ThermoFisher) supplemented with 10% FBS, 100 U/mL penicillin (Euroclone) and 100 µg/mL streptomycin sulfate (Euroclone) at 37 °C, 5% CO_2.

2.3.3.3. Degranulation assay

PBMCs were thawed and counted with a Fuchs-Rosenthal hemocytometer, resuspended at a density of 2 x 10^6 cell/mL in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin sulfate. After overnight incubation at 37 °C, 5% CO_2 with or without 1 ng/mL IL-15 (Miltenyi Biotec), 5 x 10^5 PBMCs were incubated with K562 target cells, at an effector to target ratio of 5:1, in the presence of mouse anti-human CD107a APC-conjugate antibody (clone H4A3, Miltenyi Biotec) for 1 hour at 37 °C, 5% CO_2. Then, 10 µg/mL brefeldin A (Sigma-Aldrich) and 6 µg/mL monensin (Sigma-Aldrich) were added to the cells and incubation

was carried out for additional 3 hours at 37 °C, 5% CO_2. Cells were first stained with 100 µl Live/Dead Fixable Dead Cell Stain near-IR-fluorescent reactive dye at 0.1% in PBS for 15 min at room temperature and then with the antibodies against the surface antigens CD3, CD56 and CD16 for 25 minutes at 4 °C diluted in 100 µL of PBS + 1% FBS. After washing, cells were resuspended in PBS + 1% FBS and acquired with the FACSCanto II flow cytometer.

2.3.3.4. Cytokines and chemokines assay

Concentration of IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, Eotaxin, Basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF was determined in plasma of BD patients and HC by the Bio-Plex Pro Human Cytokine Grp I Panel, 27-Plex (Biorad®) following the manufacturer's instruction. Plasma was diluted four-fold in Bio-Plex Sample Diluent as recommended. Data were obtained with Bio-Plex® MAGPIX™ Multiplex Reader instrument and Bio-Plex® Manager™ software. Values extrapolated from the standard curve were considered not reliable and a concentration = 0.01 pg/ml was arbitrarily assigned (graphing on a log scale).

2.3.3.5. Statistical analysis

Statistical analyses were performed with GraphPad Prism 6 software. For comparisons between two groups non-parametric Mann-Whitney U test was used for quantitative variables, while Fisher's exact test was used for qualitative variables. Spearman test was used for correlations between two variables and receiver operating characteristic (ROC) curve was used to assess the performance of a binary classifier system. *P* values less than 0.05 (two-tailed) were considered statistically significant.

2.4. Results

2.4.1. Circulating NK, NKT and T cell percentages in BD patients and HC

Lymphocytes and monocytes were identified by forward-scatter and side-scatter. Gating strategy is shown in Fig. 2.1A. In the lymphocytes gate NK, NKT and T cells were defined as $CD3^{neg}$ $CD56^{pos}$, $CD3^{pos}$ $CD56^{pos}$ and $CD3^{pos}$ $CD56^{neg}$, respectively. No differences were found in the percentages of NK, NKT, T cells in the peripheral blood between BD patients and HC (Fig. 2.1.B). Moreover, any difference was found in the percentage of NK, NKT and T cells between BD patients with and without therapy (data not shown). The analysis of the percentage of NK $CD56^{bright}$ cells was similar between BD patients and HC.

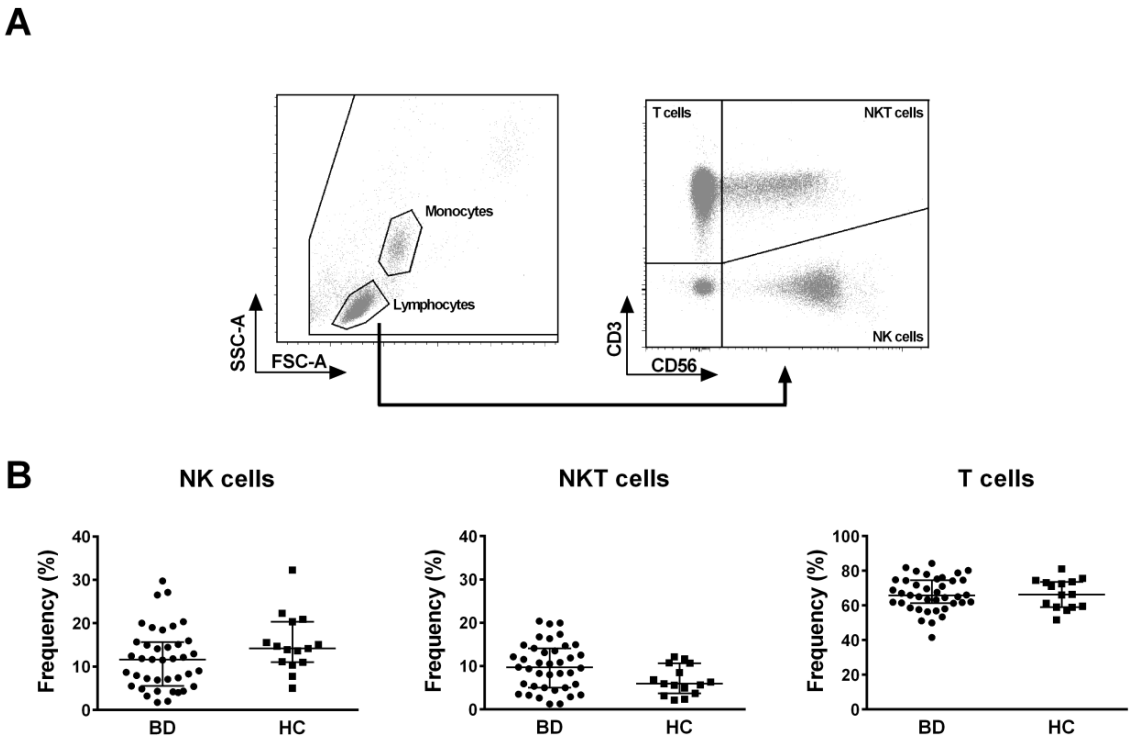

Figure 2.1. PBMCs subsets in BD patients and HC

A) Gating strategy for the detection of PBMCs subsets. A first gate was set on side-scatter area (SSC-A) *versus* forward-scatter area (FSC-A) to identify lymphocytes and monocytes (left panel). CD3 and CD56 were used to identify NK, NKT and T cells within the lymphocytes gate (right panel). **B)** Dot plot visualization of the percentage of NK, NKT and T cells in the lymphocytes gate

by flow cytometry in PBMCs from BD patients (●) and HC (■). Horizontal lines show the median ± Inter Quartile Range (IQR). Data were analysed by Mann-Whitney U test.

2.4.2. Surface markers of NK and NKT cells in BD patients and HC

In order to characterize the immunophenotype of circulating lymphocytes of BD patients, frequencies of cells expressing activator markers CD69, NKG2D, Nkp30, Nkp46 and inhibitor marker NKG2A were analysed within each lymphocytes subset compared to HC. A significantly increase of NK NKG2D^{pos}, NKT NKG2D^{pos} and T NKG2D^{pos} cells in BD patients was observed as compared to HC (Fig. 2.2.A). In particular, the median frequency of NK NKG2D^{pos} cells was 70.3% (IQR: 52.6 - 76.1 %) in BD *versus* 58.2 % (IQR: 50.1 - 63.2 %) in HC; while the median frequency of NKT NKG2D^{pos} cells was 70.4% (IQR: 61.5 - 78.6 %) in BD *versus* 58.3 % (IQR: 51.2 - 68.9 %) and the median frequency of T NKG2D^{pos} cells was 28.8 % (IQR: 24.2-37.8 %) in BD versus 23.4 % (IQR: 20.8-28.2 %) in HC.

ROC curve analysis showed that the evaluation of the percentage of NKG2D positive cells in the lymphocyte gate allowed to discriminate between BD patients and HC (Fig. 2.2.B). The better discriminating percentage was that of NKG2D^{pos} NKT cells (AUC = 0.7470; P = 0.0053) where a frequency higher than 75.5% could identify BD patients with 93.3% specificity and 38.5% sensitivity.

With the aim to identify a marker of disease activity, BD patients were classified as in active (n=23) and inactive (n=15) disease phase on the basis of physician's clinical evaluation. No difference in the percentage of NK, NKT and T cells expressing NKG2D between BD patients with active and inactive disease phase was found. Instead, the classification of BD patients based on BDCAF score revealed a direct correlation with the frequency of NK and NKT cells positive for NKG2D, while no correlation was observed whit the frequency of T cells positive for NKG2D (Fig. 2.2.C).

The following division of patients with BDCAF = 0 (n=6) and BDCAF ≥ 3 (n=33) showed a significant higher frequency of NK and NKT cells positive for NKG2D in patients with BDCAF ≥ 3 in comparison with patients with BDCAF = 0 and in comparison with HC (Fig. 2.2.D). The frequencies of NKG2D^{pos} NK and NKT cells of BD patients with BDCAF = 0 were similar to the frequencies detected in HC. No difference was observed in the frequency of T cells after the stratification in patients with BDCAF = 0 and BDCAF ≥ 3 (Fig. 2.2.D).

Concerning the frequencies of NK, NKT and T cells positive for the other inhibitor and activator surface markers, no differences were found between BD patients and HC (Fig. Supplementary 2.3). Neither the classification of BD patients as in active and inactive disease phase based on physician's clinical evaluation nor the classification based on BDCAF score nor the classification according to the concomitant presence/absence of therapy showed any difference in the percentage of NK, NKT and T cells expressing CD69, Nkp30, Nkp46 and NKG2A surface markers.

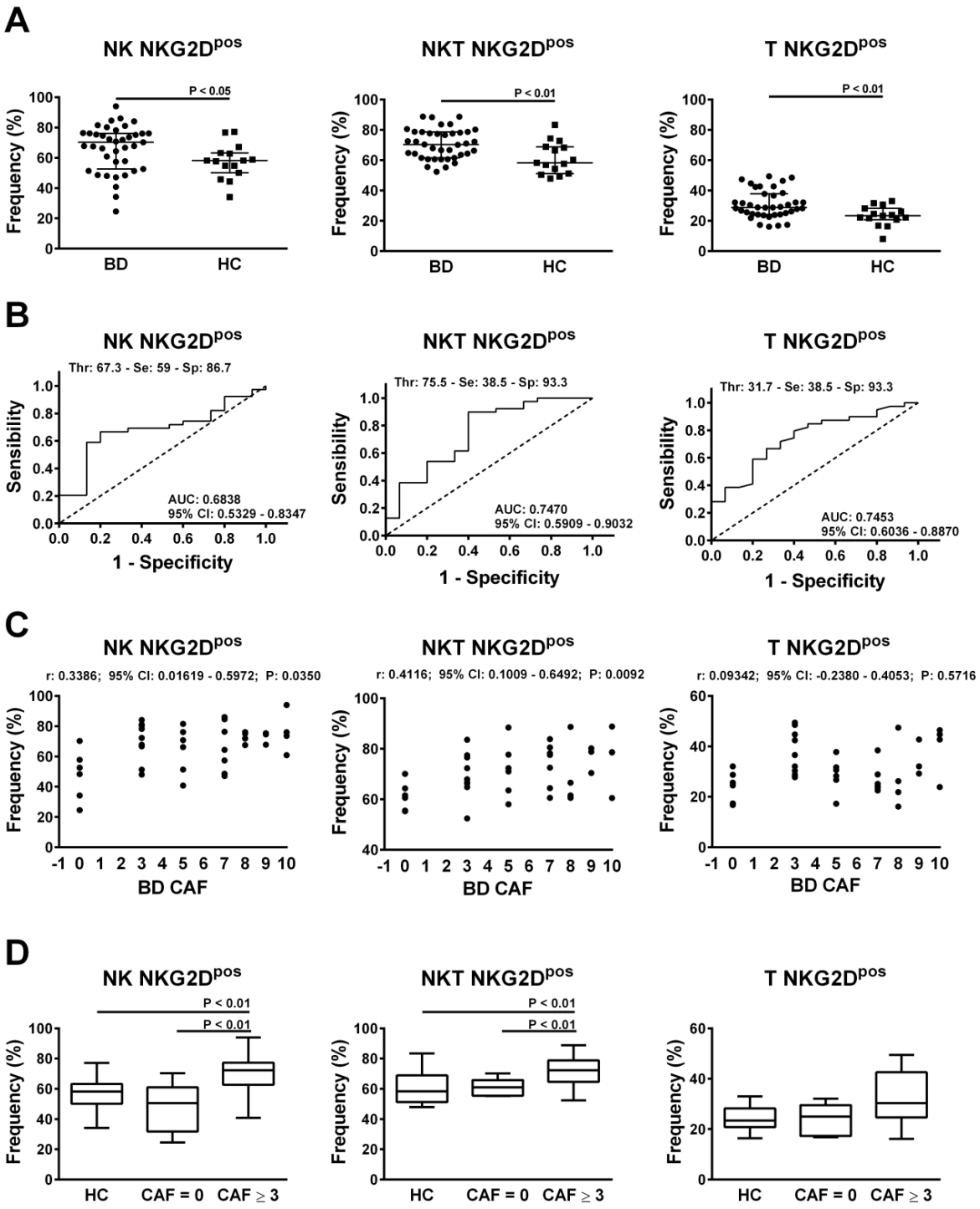

Figure 2.2. NKG2D expression in lymphocytes from BD patients and HC

A) Dot plot visualization of the percentage of NKG2D positive cells in NK, NKT and T lymphocytes gates determined by flow cytometry in PBMCs from BD patients (●) and HC (■). Horizontal lines show the median ± IQR. Data were analysed by Mann-Whitney U test. **B)** ROC

curve analysis of the percentage of NKG2D positive cells in NK, NKT and T lymphocytes gates. AUC= Area Under the Curve; CI= Confidence Interval; Thr= Threshold; Se= Sensitivity; Sp= Specificity. **C)** Dot plot visualization of the correlation between the frequencies of NK, NKT or T cells positive for NKG2D and BD CAF for each BD patients. Data were analysed by Spearman's rank order correlation test (n=39). **D)** Box plot visualization of the frequencies of NK, NKT or T cells positive for NKG2D in BD patients with BDCAF = 0 *versus* BDCAF ≥ 3 and HC. Data were analysed by Mann-Whitney U test.

2.4.3. Cytotoxic potential of NK cells

In order to study the cytotoxic potential of NK cells, PBMCs of BD patients and HC were stimulated through contact with K562 cells, which do not express MHC-1 class, and then the expression of the CD107a degranulation marker was monitored. The stimulus with K562 cells is specific for NK cells and it does not influence the cytotoxic potential of NKT cells. A significant higher frequency of NK cells positive for CD107a was induced in BD patients respect to HC: 13.43 % (IQR: 8.09-15.95 %) *versus* 9,435 % (IQR: 6.12-11.48 %), P = 0.0145 (Fig. 2.3.A). Further stimulation of PBMCs with IL-15 increased the percentages of CD107a positive cells, but no differences were found between BD patients and HC (Fig. 2.3.B).

Neither the classification of BD patients with active and inactive disease phase based on physician's clinical evaluation nor the classification based on BDCAF score showed any difference in the cytotoxic potential of NK cells either with or without IL-15 stimulation.

ROC curve analysis showed that in the absence of IL-15, a frequency of NK cells positive for CD107a higher than 12.98% could identify BD patients with 92.9 % specificity and 53.9 % sensitivity (AUC = 0.7198; P = 0.0155) (Fig. 2.3.C).

To explore whether there was a relationship between the surface marker composition of NK cells and their cytotoxic potential a Spearman's correlation test was performed. Percentages of NKG2D

positive cells directly correlated with percentages of CD107a positive cells in the gate of NK cells (Fig. 2.3.D).

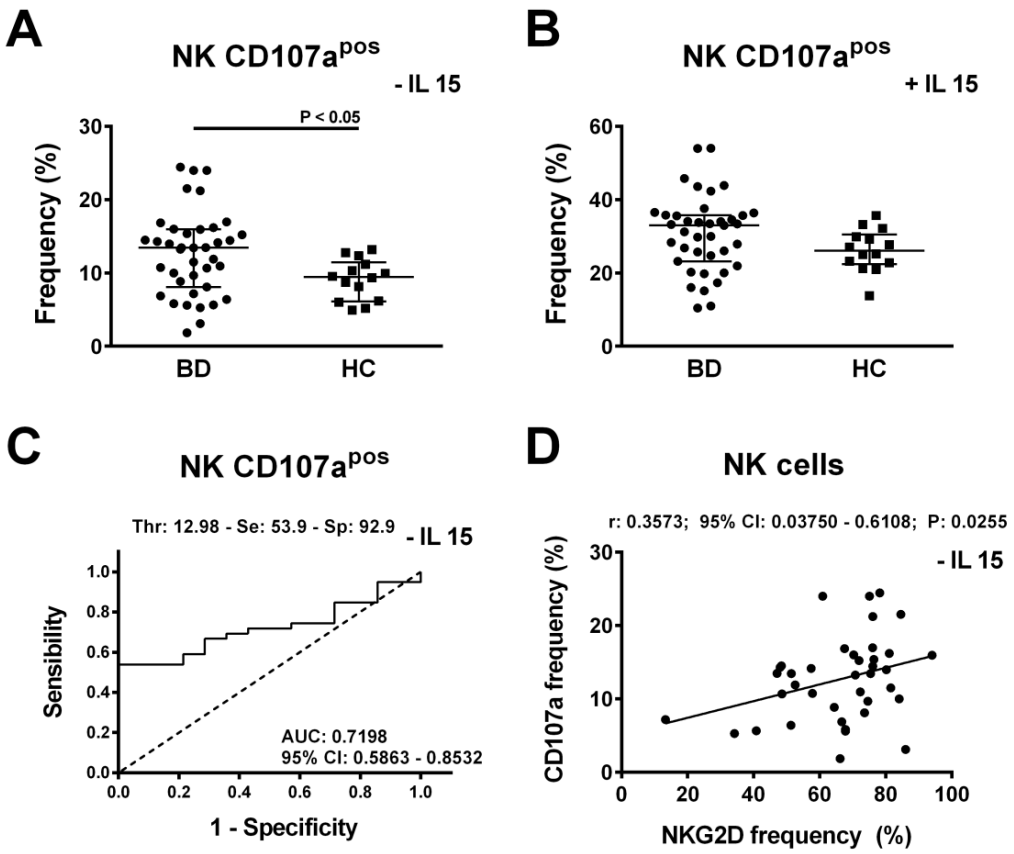

Fig. 2.3. CD107a degranulation assay of PBMCs from BD patients and HC.

Dot plot visualization of the percentage of CD107a positive cells in the NK lymphocyte gate determined by flow cytometry in PMBCs from BD patients (●) and HC (■) after stimulation with K562 in presence of 10 µg/mL brefeldin A and 6 µg/mL monensin. The assay was conducted without (**A**) or with 1ng/mL IL-15 (**B**) to maximize the stimulus. Horizontal lines show the median ± IQR. Data were analysed by Mann-Whitney U test. **C)** ROC curve analysis of the percentage of CD107a positive cells in absence of IL-15. AUC= Area Under the Curve; CI= Confidence Interval; Thr= Threshold; Se= Sensitivity; Sp= Specificity. **D)** Dot plot visualization of the correlation between the frequencies of NK cells positive for NKG2D and the frequencies of NK cells positive

for the CD107a in BD patients. Data were analysed by Spearman's rank order correlation test (n=39). r = curve slope; CI= Confidence Interval.

2.4.4. Cytokines and chemokines plasmatic levels in BD patients and HC

Multiplex analysis of cytokines and chemokines using plasma samples revealed a significantly higher concentration of IL-5, IL-10, IL-12 (p70), IL-13, IP-10 and MIP1β in BD patients in comparison to HC (Table 2.1.). GM-CSF was lower in BD patients respect to HC, but *P* value was at the significance limit (Table 2.1.). No differences were found between patients in active and inactive disease phase defined by the physician's clinical evaluation or BDCAF score.

Not all cytokines/chemokines were detected in all subjects and the difference of frequencies of detection was statistically significant between BD patients and HC in the case of IL-5 and IL-10 (Table 1). In particular, IL-5 was detected in 33/39 (77 %) of BD patients and 7/15 (47 %) of HC and IL-10 was detected in 19/39 (49 %) of BD patients and 1/15 (7 %) of HC.

	Concentration (pg/ml)			Detectable fraction		
	BD n=39	**HC** n=15	**Mann-Whitney U test P value**	**BD** n=39	**HC** n=15	**Fisher's test P value**
IL-1β	0.01 (0.01-4.46)	0.01 (0.01-0.01)	0.1416	16/39	3/15	0.2081
IL-1ra	57.28 (23.53-183.30)	30.56 (16.30-57.28)	0.0741	33/39	13/15	1
IL-2	0.01 (0.01-0.01)	n.d.	0.3205	4/39	0/15	0.5665
IL-4	1.74 (0.01-3.52)	1.04 (0.01-1.89)	0.2636	24/39	8/15	0.7582
IL-5	11.85 (4.28-26.82)	0.01 (0.01-17.55)	0.0361 *	30/39	7/15	0.0496 *
IL-6	0.01 (0.01-13.16)	0.01 (0.01-0.01)	0.0691	15/39	2/15	0.1056
IL-7	3.07 (0.01-11.76)	0.01 (0.01-0.01)	0.2486	20/39	6/15	0.5500
IL-8	5.37 (0.01-14.98)	4.79 (0.01-7.07)	0.2311	26/39	8/15	0.5302
IL-9	13.62 (3.19-46.92)	8.45 (4.78-18.06)	0.4403	33/39	13/15	1
IL-10	0.01 (0.01-26.55)	0.01 (0.01-0.01)	0.0043 **	19/39	1/15	0.0044 **
IL-12 (p70)	7.46 (0.01-27.64)	0.01 (0.01-0.01)	0.0240 *	20/39	3/15	0.0638
IL-13	8.09 (4.06-12.43)	3.04 (2.01-5.53)	0.0020 **	39/39	13/15	0.0734
IL-15	0.01 (0.01-0.01)	n.d.	0.5519	3/39	0/15	0.5519
IL-17A	8.11 (0.01-46.76)	0.01 (0.01-16.26)	0.2626	23/39	7/15	0.5434
Eotaxin	68.48 (50.44-108.90)	58.76 (49.54-96.16)	0.3758	39/39	15/15	1
Basic FGF	32.02 (8.63-68.71)	32.02 (8.63-62.66)	0.6078	32/39	14/15	0.4190
G-CSF	19.21 (9.63-59.37)	20.83 (9.42-36.80)	0.7777	33/39	13/15	1
GM-CSF	35.04 (8.12-68.53)	109.60 (19.30-162.80)	0.0436 *	32/39	15/15	1
IFN-γ	41.62 (0.01-171.4)	23.10 (0.01-66.77)	0.2424	27/39	9/15	0.5359
IP-10	573.3 (349.30-790.30)	350.50 (309.60-440.60)	0.0223 *	39/39	15/15	1
MCP-1	0.01 (0.01-35.78)	0.01 (0.01-9.79)	0.1278	18/39	4/15	0.2302
MIP-1α	3.54 (2.24-6.10)	2.70 (1.31-4.14)	0.1586	38/39	14/15	0.4822
MIP-1β	44.74 (23.67-88.38)	24.70 (18.97-32.36)	0.0090 **	39/39	15/15	1
PDGF-BB	93.77 (29.01-285.6)	76.58 (34.71-162.4)	0.6294	38/39	15/15	1
RANTES	1919 (1151-4784)	1548 (965.50-3558)	0.4374	39/39	15/15	1
TNF-α	21.39 (0.01-91.18)	12.88 (0.01-33.81)	0.1728	25/39	8/15	0.5405
VEGF	12.49 (0.01-32.35)	10.99 (0.01-23.12)	0.3400	29/39	10/15	0.7356

Table 2.1. Levels of cytokines and chemokines expressed in plasma of BD patients and HC.

List of cytokines and chemokines detected in plasma samples of BD patients and HC. The concentration is expressed in pg/ml as median (IQR). Data were analysed by Mann-Whitney U test. Fraction represents the number of subjects in which the cytokines were detectable. Data were analysed by Fisher's exact test. n.d. = not detected; * = P <0.05; ** = P <0.01.

2.5. Discussion

For the first time, an increased frequency of circulating NK, NKT and T cells positive for the activation surface marker NKG2D in BD patients in comparison to HC has been identified.

Additionally, increased cytotoxic potential of NK cells has been confirmed.

Differently from other authors observations, any difference in the frequency of circulating lymphocytes (NK, NKT and T cells) in BD patients as compared to HC was detected (K. Hamzaoui *et al*., 2006; H.G. Yu *et al*., 2004; H. Yato *et al*., 1999; M.S. Hasan *et al*., 2017; Y. Suzuki *et al*., 1992).

However, different ethnicity, clinical disease manifestations of patients' cohorts and surface markers used for lymphocyte identification could be the causes of the discrepancy of the data.

NKG2D is a homodimeric C-type lectin-like activating receptor that is expressed on almost all NK and $CD8^{pos}$ T cells and with less frequency on the surface of NKT and $CD4^{pos}$ T cells. NKG2D acts as a sensor for recognition of induced-self antigens in cells infected by pathogens or transformed cells (L. L. Lanier, 2015). In humans, eight different ligands able to bind NKG2D receptor with different affinity are known: MHC class I chain-related protein A and protein B and six HCMV UL16 binding proteins. After ligands' binding, NKG2D receptor can initiate an intracellular signal cascade that leads to NK and NKT activation (P. Spear *et al*., 2013).

Discrepancies in NKG2D expression in T and NK cells populations have been found in different autoimmune diseases. In rheumatoid arthritis, an increased percentage of NKG2D positive $CD4^{pos}$ T cells has been reported in the peripheral blood and in the synovial fluid of affected patients (V. Groh *et al*., 2003). In systemic lupus erythematosus, increased expression of T cells positive for NKG2D and decreased expression of NKG2D in NK cells were found (Z. Dai *et al*., 2009; W.X. Li *et al*., 2010; S.K. Sourour *et al*., 2017).

As for BD, some reports identified a reduction in the frequency of $NKG2D^{pos}$ cells in γδ T and T $CD8^{bright}$ subsets (G. Parlakgul *et al*., 2013; J.K. Ahn *et al*., 2006).

However, to the best of current knowledge, this is the first study that describes an increase of NKG2D positive cells in circulating NK, NKT and T populations.

It could be speculated that determining frequency of circulating NK, NKT and T cells positive for NKG2D could be useful as a laboratory test to discriminate between BD patients and HC with high degree of specificity, but low sensitivity.

Additionally, classification of patients based on the BDCAF score correlated with the frequency of NKG2D^{pos} NK and NKT cells in the peripheral blood of BD patients. Interestingly, BD patients with BDCAF = 0 had a NKG2D profile similar to HC, while BD patients with BDCAF $\geq$ 3 had a higher frequency of NKG2D^{pos} NK and NKT cells, suggesting that only patients with BDCAF = 0 might be considered inactive/in remission phase.

As a result to the increased frequencies of circulating NK and NKT cells positive for NKG2D in the peripheral blood, the immune system of BD patients might be more prone to respond to stress signals when exposed to tissue cells.

Recently, Hasan and coll. reported an increased NK cells cytotoxicity in the peripheral blood of BD patients (M.S. Hasan *et al.*, 2017). They used phorbol-12-myristate-13-acetate (PMA) and ionomycin to activate NK cells and evaluated cytotoxic potential by monitoring the frequency of NK cells positive for CD107a surface marker.

In this study, frequency of NK cells positive for the CD107a surface marker was similarly evaluated, but following stimulation by the contact with cells missing MHC-I class molecules (K562 cells). Differently from PMA and ionomycin stimulus, that is a stimulus that better reflects the physiologic activation of NK cells. In these experimental conditions, frequency of NK cells of BD patients positive for CD107a was higher than in HC.

Direct correlation between frequency of NK cells positive for NKG2D and frequency of NK cells positive for CD107a was found, together with higher cytotoxic potential of NK cells of BD patients as compared to HC, when activated by a physiological stimulus.

All cytokines/chemokines we found increased in plasma of BD patients have been shown to be linked with NK and NKT activity, based on literature data.

IL-5, IL-10 and IL-13 can be released by activated NK and NKT cells (Y. Wu *et al*., 2017; D.I. Godfrey *et al*., 2000).

IL-12 is produced by dendritic cells, macrophages and neutrophils and is a crucial factor for the secretion of IFN-γ by NK and NKT cells (Y. Wu *et al*., 2017; E.C. Reilly *et al*., 2011).

Furthermore, IL-12 can enhance the cytotoxic activity of NK cells and can induce extravasation of activated lymphocytes into tissue (Y. Wu *et al*., 2017; M.J. Robertson,1999).

MIP-1β, also known as CCL4, has a double role: on one hand, it acts as a NK cell chemoattractant signal produced by dendritic cells; on the other hand, it is produced by activated NK cells (M. J. Robertson, 2002).

IP-10, also known as CXCL10, is produced by dendritic cells and can attract NK cells in the site of inflammation (M. J. Robertson, 2002). High circulating levels of IP-10, reported also in other autoimmune diseases such as systemic lupus erythematosus, Kawasaki disease and autoimmune hepatitis, suggest a key role for this factor in the inflammatory 'milieau' of different autoimmune rheumatic disorders (K.O. Kong, 2009; T.M. Ko *et al.*, 2015; K. Nishiotj *et al*., 2001).

Increased levels of IL-10, IL-12 (p70) and IL-13 in plasma of BD patients as compared to HC have been previously reported by other authors, while data regarding IL-5, IP-10 and MIP-1β are reported here for the first time (N. Akdeniz *et al*., 2004; B.C. Aridogan *et al*., 2003; K. Hamzaoui *et al*., 2002).

As regards IL-13 and MIP-1β, it is important to highlight that the transcription of these factors is directly under control of NKG2D pathway (Y.H. Chang *et al*., 2013). Therefore, high circulating levels of IL-13 and MIP-1β in BD patients might be downstream related to increased activation of NKG2D pathway in NK, NKT and T cells as compared to HC.

The limits of this part of the study concern the small number of patients and the fact that 74% of them were on treatment. However, it has to be considered that BD is a rare disease and this study is monocentric and explorative.

In spite of these limits, the strength of the study is that this study cohort represents a real-life cohort of patients, clinically evaluated by experts physicians in a reference centre and well characterized in terms of active/inactive disease phase, independently of therapeutic schedules.

It might be speculated that increased frequency of NKG2D positive NK, NKT and T cells and the increased NK cytotoxicity probably represent a pathogenic hit of BD.

Taken together, these data support the hypothesis that through an increased frequency of NKG2D activator receptor on the cells surface, NK and NKT cells of BD patients could be more prone to respond to stress signals when exposed on tissue cells, leading to cyclic auto-inflammation.

Concerning the clinical practice, monitoring both the frequencies of NK and NKT cells positive for NKG2D and of NK cells positive for CD107a after activation with K562 cells, could help the clinician to identify BD patients and/or to confirm disease activity state during the follow-up.

Chapter 3: Immunological profiling of aqueous humor in Behçet's disease patients with active ocular involvement.

Introduction

Ocular involvement is one of the most disabling complications of BD, a vision-threatening intraocular inflammatory disease causing blindness if left untreated. The typical form of ocular involvement is a relapsing and remitting panuveitis and retinal vasculitis.

Destructive and recurrent attacks, especially with posterior segment and retina involvement, may cause irreversible ocular structural changes and permanent damage in sensory retina.

The risk of irreversible damage to ocular tissue, which may result in loss of vision, warrants early and intensive treatment, especially in patients at high risk such as young men who tend to follow an aggressive disease course.

The management strategy involves local and systemic measures including immunosuppressants, corticosteroids, and biologic agents (mainly anti-TNFα) to rapidly suppress inflammation.

The prognosis of eye involvement has greatly improved over the last decades with the effective use of immunosuppressives.

However, therapeutic armamentarium is still limited and pathogenic mechanisms of BD-related uveitis remain unclear.

Further insights into the pathogenesis of BD-related uveitis are strongly needed in order to expand the therapeutic spectrum and to improve long-term prognosis in the case of ocular involvement.

3.2. Current evidence on immunological profiling of aqueous humor in Behçet Disease uveitis

Data on magnitude and patterns of cytokine response in BD-related uveitis are scarce. This is mainly due to technical difficulties in obtaining aqueous humor from anterior chamber through limbic paracentesis in the course of active disease.

In BD, such a procedure might be related to the development of pathergy phenomenon, which

can worsen the ocular picture (*see also Chapter 1, § 1.2.1. Clinical presentation*).

The first report on cytokine profiles in the aqueous humor from specific clinical entities of endogenous uveitis, including VKH and BD, dates back to 2011.

El Asrar and coll. for the first time analysed cytokine concentrations in aqueous humor from patients with active uveitis associated with specific diagnoses: VKH, BD – related uveitis and HLA B27 – related uveitis (A. M. El Asrar *et al*., 2011). Authors analysed 13 patients with VKH, 10 patients with BD – related uveitis and 7 patients with HLA B-27 –related uveitis.

Their findings suggested that both Th17 and Th1 subsets might be involved in endogenous uveitis immunopathogenesis.

In details, IL-17 levels in the aqueous humor from patients with all types of uveitis were higher than those in the aqueous humor from normal controls. Furthermore, IL-17 levels in the aqueous humor from patients with uveitis significantly correlated with clinical disease activity (A. M. El Asrar *et al*., 2011). Additionally, increased levels of IFN-γ in the aqueous humor from patients with BD, VKH disease, and HLA-B27-associated uveitis, supported the involvement of both Th1 and Th17 subsets in endogenous uveitis immunopathogenesis rather than an exclusive role of one of these subsets.

IFN-γ-driven immune responses seemed to be more potent in BD as compared to VKH disease and HLA-B27-associated uveitis (A. M. El Asrar *et al*., 2011). These results were consistent with those of Ahn and coll. who reported that levels of IFN-γ in the aqueous humor were significantly higher in patients with BD than in uveitis patients without BD (J.K. Ahn *et al*., 2006).

In a previous study, El Asrar and coll. demonstrated that levels of Th1 chemoattractant IP-10/CXCL10, induced by IFN-γ, in the aqueous humor were significantly higher in patients with BD than in patients with VKH disease (A.M. El Asrar *et al*., 2004).

Therefore, they speculated that Th1-type immune responses are more potent in patients with BD compared with patients with VKH disease and patients with HLA-B27-associated uveitis.

3.3. Hypothesis and Aims of the experiment

In the recent years it became clear that successful therapeutic strategies in BD uveitis might rely on a better characterization of immune response, in order to design effective treatment for each patient, on an individual basis (A. M. El Asrar *et al*., 2011).

The aim of this part of the study was to investigate differences in the mechanism of two distinct types of uveitis (BD and VKH-related uveitis) - to search for potential markers and therapeutic targets - by determining cytokine profiles.

Levels of pro- and anti-inflammatory cytokines were measured in aqueous humor from patients with active BD uveitis and VKH.

Secondly, given the recent evidence about the impairment of cytotoxicity mechanisms in BD, a different distribution of NK cell subsets in the aqueous humor of patients with different types of endogenous uveitis could be hypothesised. With this aim, frequency of NK and NKT cells in the same groups of patients has been explored.

3.4. Materials and Methods

This is an experimental, exploratory, monocentric, independent study, conducted at the Rheumatology Division, Arcispedale Santa Maria Nuova - IRCCS, Reggio Emilia, Italy, in cooperation with the Ocular Immunology Unit, Arcispedale Santa Maria Nuova - IRCCS, Reggio Emilia, Italy, one of the National Reference Centres for Behçet Disease Uveitis.

This study has been supported by the National Society of Rheumatology (2015/2016 Selected Grant for Young Researchers), and by the National Association of Patients Affected With Behçet Disease (S.I.M.B.A.).

3.4.1. Study subjects

Study population included:

1) patients with BD-related uveitis, in active disease phase;

2) patients with VKH, in active phase;

3) healthy, Caucasian, selected subjects of the same age and sex undergoing phacoemulsification intervention for cataract, not affected with any other concomitant inflammatory / infectious disease nor with prior history of uveitis.

A cohort of 9 BD patients, 10 VKH patients, and 10 HC recruited as a reference, was enrolled. All BD patients satisfied the International Study Group for Behçet Disease criteria (ISGB, *The Lancet*, 1990). BD activity was evaluated through Behçet Disease Activity Form (BDCAF, University of Leeds, Vers. 06.03.2006, International Society for Behçet Disease, ISBD), administrated to patients during a complete clinical rheumatologic and ophthalmologic evaluation.

Diagnosis of VKH was based on the Revised International Diagnostic Criteria (R. W. Read *et al*., 2001).

The median age for BD cohort was: 29 (Interquartile range; IQR: 26-33) and gender distribution was: 66.6% male (6/9) and 33.3% female (3/9).

The median age for VKH cohort was: 47 (Interquartile range; IQR: 36-63) and gender distribution was male (5/10) and female (5/10).

The median age for HC cohort was: 41 (Interquartile range; IQR: 30-54) and gender distribution was male (4/10) and female (6/10)

Patients were examined with indirect ophthalmoscopy, slit-lamp biomicroscopy and fluorescein angiography. They were considered as having active uveitis at ophthalmologic evaluation in the case of:

- presence of ≥ 2 cells in the anterior chamber (Hogan scale) (M. J. Hogan *et al*., 1959);
- presence of vitreitis 2+ (Nussenblatt scale) (R.B. Nussenblatt, 1990);
- presence of clinically significant macular edema, supported by ocular tomography (OCT);
- presence of clinically significant papillitis (for patients with VKH) and / or inflammatory retinal exudative lifting;
- presence of active retinal vasculitis with 'photo fundus'.

The study was approved by the Local Ethics Committee (Reggio Emilia, Italy) in compliance with the Declaration of Helsinki and informed consent was obtained from all patients and HC.

3.4.2. Specimen Collection

3.4.2.1. Samples of aqueous humour

Samples of aqueous humour (100-200 µl) were obtained from the anterior chamber through limbic paracentesis with the use of 27-gauge needle attached to a tuberculine syringe after the application of topical local anesthetic oxybuprocaine hydrochloride 0.4% (Benoxinato – Alfa Intes Pharmaceuticals, Ltd, Italy). The procedure was conducted under surgical microscope.

Ethylenediaminetetraacetic acid (EDTA) was added at 2 mM to prevent cell aggregation. Cell concentration was determined by manual counting in a Neubauer hemocytometer. Aqueous humor samples were centrifuged at 400 x g for 8 minutes. Cell pellets were analyzed by flow cytometry while supernatants were frozen at -80 °C for cytokine profiling.

3.4.3. Laboratory processing

3.4.3.1. Flow cytometry

Cells were suspended in 100 µL Phosphate-buffered saline (PBS, Euroclone) + 1% FBS and stained for 25 minutes at 4 °C with PerCP mouse anti-human CD3 (clone BW264/56) and PE anti-human CD56 (clone REA196). Antibodies were purchased from Miltenyi Biotec and used as suggested by the manufacturer.

After washing, cells were suspended in PBS + 1% FBS and acquired with the FACSCanto II flow cytometer (BD Biosciences), equipped with two lasers for excitation at 488 and 633 nm. Data were analyzed with FACSDiva 8.0.1 software. Data were considered reliable if at least 100 lymphocytes were acquired, given the low cellularity of some aqueous humor samples.

3.4.3.2. Cytokine profiling

Concentration of IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, Eotaxin, Basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF was determined in aqueous humor samples by the Bio-Plex Pro Human Cytokine Group I Panel, 27-Plex (Biorad®) following the manufacturer's instruction. Cell-free aqueous humor samples were centrifuged at 10000 x g for 10 minutes at 4°C then were diluted four-fold in Bio-Plex Sample Diluent as recommended.

Moreover, Bovine Serum Albumin (BSA) at 0.5 % was added to the samples as recommended for samples which should not contain albumin. Data were obtained with Bio-Plex® MAGPIX™ Multiplex Reader instrument and analyzed with Bio-Plex® Manager™ software. Values extrapolated from the standard curve were considered not reliable and a concentration = 0.01 pg/ml was arbitrarily assigned (graphing on a log scale).

3.4.3.3. Statistical analysis

Statistical analyses were performed with GraphPad Prism 6 software. For comparisons between two groups non-parametric Mann-Whitney U test was used for quantitative variables, while Fisher's exact test was used for qualitative variables. *P* values less than 0.05 (two-tailed) were considered statistically significant.

3.5. Results

3.5.1. Cytokines and chemokines levels in aqueous humor of BD, VKH patients and HC

Multiplex analysis of cytokines and chemokines using aqueous humor samples revealed a significantly higher concentration of IL-2, IL-6, IL-8, IL-10, IL-13, IP-10 in BD and VKH patients as compared to HC. In particular, a 3000-fold increase in IL-6 levels was found in the aqueous humor of patients with BD as compared to HC.

Minor 'fold changes' were observed in the case of IL-1β, IL-1RA, IL-4, IFN-γ, TNF-α, G-CSF, Eotaxin, MIP-1α, PDGF- β (Fig. 3.1.). No statistical significance was found in the case of IL-5, IL-7, IL-9, IL-12, IL-15, IL-17, FGF- β, MCP-1, MIP-1 β, Rantes and VEGF levels between BD, VKH and HC (data not shown).

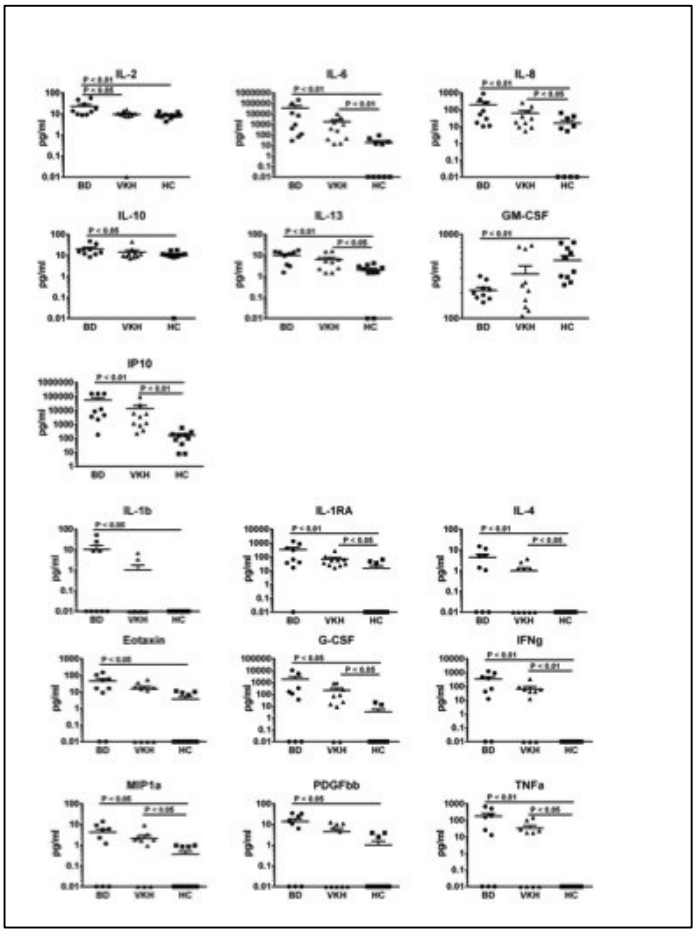

Figure 3.1. Levels of cytokines and chemokines expressed in aqueous humor of patients with BD, VKH and HC (dot plot visualization). The concentration has been determined in pg/ml. Data were analysed by Mann-Whitney U test. When cytokines were not detected a value = 0.01 was arbitrarily assigned (graphing on a log scale).

3.5.2. NK, NKT and T cell percentages in BD and VKH patients

Lymphocytes and monocytes were identified by forward-scatter and side-scatter. Aqueous humor from patients with VKH and BD was analysed. In the lymphocytes gate NK, NKT and T cells were defined as $CD3^{neg}$ $CD56^{pos}$, $CD3^{pos}$ $CD56^{pos}$ and $CD3^{pos}$ $CD56^{neg}$, respectively.

The frequency of NKT (CD3+ CD56+) cells was higher in BD patients as compared to VKH, while that of NK (CD56+ $CD3^{neg}$) and T cells ($CD56^{neg}$ CD3+) was similar. Finally, no difference was found between NKT and NK subsets in terms of proportion of CD16+ cells in both BD and VKH groups.

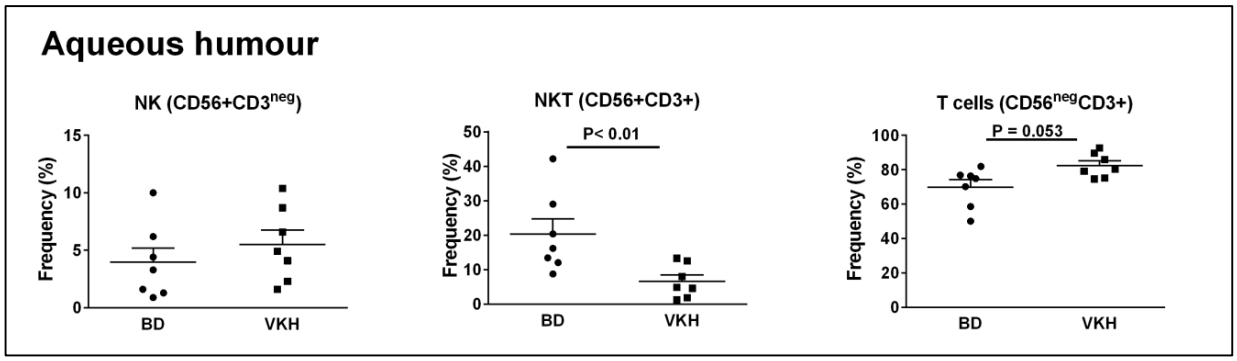

Fig. 3.2. **NK, NKT and T cell percentages in BD and VKH patients**

Dot plot visualization of the percentage of NK, NKT and T cells in the lymphocytes gate by flow cytometry in AH from BD and VKH patients. Horizontal lines show the median ± Inter Quartile Range (IQR). Data were analysed by Mann Whitney U test.

Discussion

In this part of the study, cytokines profiles of aqueous humor of patients with BD and VKH-related uveitis during active disease have been analysed, by comparing them with that from HC population, to search for novel biomarkers of specific endogenous uveitis entities.

Data from El Asrar and coll. about a marked increase in aqueous humor levels of IL-1β, IFN-γ, TNF-α, compared to HC have been confirmed; however, cytokines levels in aqueous humor from BD patients did not differ significantly as compared to VKH (A.M. El Asrar *et al*., 2011).

In their previous study, El Asrar and coll. additionally demonstrated that levels of the Th1 chemoattractant IP-10 (also known as CXCL10), induced by IFN-γ, in the aqueous humor were significantly higher in patients with BD than in patients with VKH disease (A. M. El Asrar *et al*., 2004). Thus, they eventually assumed a more potent Th1 polarization in BD.

In this study, higher levels of IP-10, IFN-γ, TNF-α were detected in aqueous humor from BD patients as compared to VKH ones, however such a difference did not reach the statistical significance.

It is well known that TNF-α, a proinflammatory cytokine, plays a central role in both the induction and maintenance of inflammation in autoimmune disorders (P. Vassalli, 1992). In the present study, TNF-α levels in the aqueous humor of uveitis were significantly enhanced compared to controls and were higher (although with no statistically significance) in BD patients as compared to VKH.

These findings also support the clinical evidence regarding the favorable effect of anti-TNF-α therapy for refractory BD-related uveitis (A.M. El Asrar *et al*., 2005).

Regarding new potential therapeutic targets for BD-related uveitis treatment, it is noteworthy that cytokine profiling of aqueous humor in BD patients as compared to VKH and HC in our study showed remarkably high levels of IL-6 in BD patients during active disease.

IL-6 is a pleiotropic cytokine, with a multiplicity of functions that specializes in the crosstalk between stromal cells and immune cells and ferries information between injured tissues and the immune system (T. Kishimoto, 2011).

Endothelial cells, vascular smooth muscle cells (VSMCs) and fibroblasts can release IL-6, but it can also derive from lymphocytes and macrophages, enabling positive feed-forward looping in the amplification of inflammatory reactions.

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Deriving from vascular cells, including endothelial cells and VSMCs, IL-6 seems to be an important connector between injured vascular walls and immune cells.

Besides its systemic effect (activating hepatocytes), IL-6 also shapes the local cytokine environment and orchestrates the patterning of immune reactions.

Of particular importance is the role of IL-6 as a polarizing cytokine, guiding the differentiation of T cells into selected functional lineages. Specifically, IL-6 is critically involved in promoting the differentiation of Th17 lineage, first described in 2005 (C.T. Weaver *et al*., 2006; E. Bettelli *et al*., 2007; Z. Chen *et al*., 2007).

Initially, it was believed that a combination of IL-1β, IL-6 and IL-23 was sufficient to direct the differentiation of a T cell into a Th17 cell. It is now clear that naive T cells do not express IL-1R and IL-23R, but these receptors are upregulated when the cells are exposed to TGF-β and IL-6 or IL-21 (L.E. Harrington *et al*., 2006; M.S. Maddur *et al*., 2012). This mechanism assigns a key role to IL-6 in directing T-cell responses.

Targeting IL-6 for non-infectious uveitis has gaining attention in the recent years, however no randomised controlled trials are available in the case of BD-related uveitis (P. Lin, 2015) nor analogous results have been found in the literature on aqueous humor from BD patients. It has to be highlighted the careful evaluation of patients with active uveitis performed during the entire duration of this study, in order to obtain samples with adequate cellularity. Evidence of a favourable effect of anti-IL-6 therapy in BD came from rheumatologic experience rather than ophthalmologic one, especially in refractory BD cases and in neuroBehçet disease (O. Addimanda *et al*., 2013).

Such findings need to be validated in larger cohorts, but might suggest a rationale in treating BD-related uveitis with anti-IL-6 therapy.

Differently from El Asrar' study, IL-17 levels did not differ in BD, VKH e HC aqueous humor samples. This is in line with an earlier paper by Luger and coll. who showed, by using the animal model of experimental autoimmune uveitis (EAU), that conditions of disease induction affect either a dominant Th17 or Th1 effector category (D. Luger *et al*., 2008).

Several other studies demonstrated the involvement of IL-17 and IL-17-producing Th17 cells in the pathogenesis of EAU (S. Hohki *et al*., 2010; T. Yoshimura *et al*, 2007; R. Zhang *et al*., 2009).

Hohki et al. showed increased frequency of Th17 cells rather than Th1 cells in the early stage and increased Th1 cells in the late stage of EAU (S. Hohki *et al*., 2010).

In contrast, Yoshimura et al. demonstrated the differential requirement of the two responses – Th1 at the early induction phase and Th17 at the later maintenance phase of EAU (T. Yoshimura *et al*, 2007).

As regards NK, NKT and T cell percentages in BD and VKH aqueous humor, frequency of NKT (CD3+ CD56+) cells was higher in BD patients as compared to VKH, while that of NK (CD56+ $CD3^{neg}$) and T cells ($CD56^{neg}$ CD3+) was similar. No difference was found between NKT and NK subsets in terms of proportion of CD16+ cells in both BD and VKH groups. These results are in line with previous evidence (H. Yu *et al*., 2004) and do not support a specific role of NK in BD-related active uveitis. However, this is an explorative study and results need to be validated in largest cohorts even in this case.

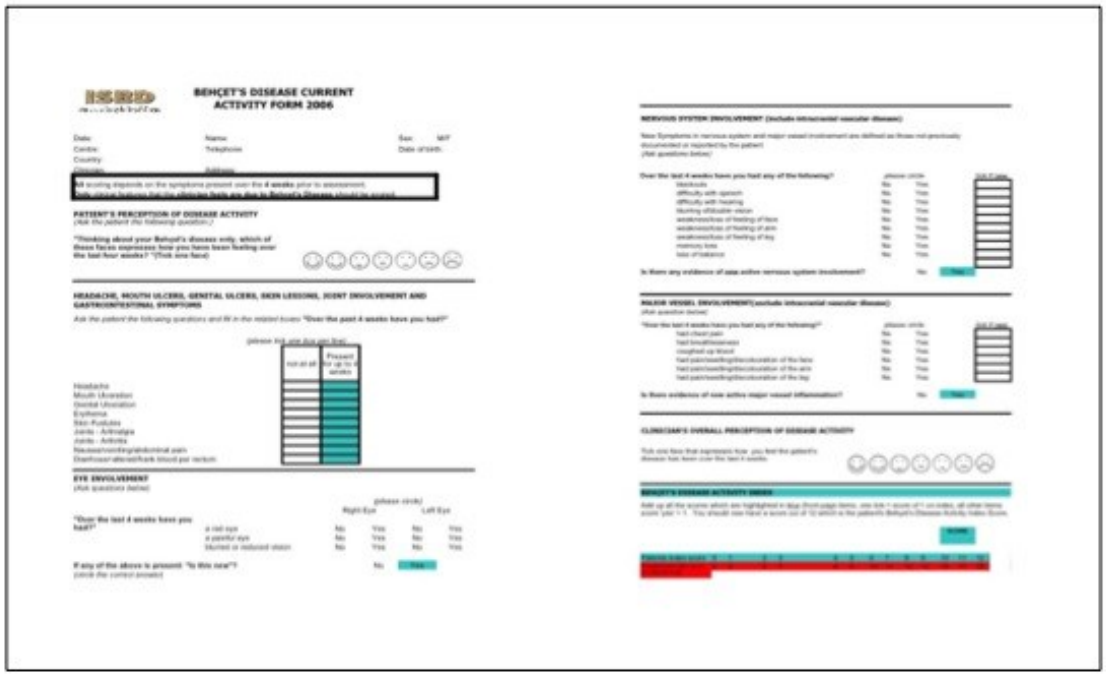
Appendices (Supplementary)

S. 2.1. Behçet Disease Current Activity Form.

It was produced for the International Scientific Committee on Behçet Disease with the participation of investigators in five countries. It was created in 1999 and then revised. It is based on data from 524 persons. The questions have been honed down to the minimum number (14), which will reliably give a unidimensional scale and each is dichotomized.

Bhakta BB, Brennan P, James TE, Chamberlain MA, Noble BA, Silman AJ. Behcet's disease: evaluation of a new instrument to measure clinical activity. Rheumatology (Oxford) 1999;38:728-733.

Lawton G, Bhakta BB, Chamberlain MA, Tennant A. The Behcet's disease activity index. Rheumatology (Oxford) 2004;43:73-78.



Transformed index score (on interval scale; see the red line in the BDCAF) was considered for statistical analyses.

S. 2.2. Characteristics of BD patients' cohort

	District involvement						Disease activity	
	Mucocutaneous	**Ocular**	**Gastrointestinal**	**Musculoskeletal**	**Nervous system**	**Vascular**	**BDCAF score**	**Therapy**
Pt#1	X	X					10	None
Pt#2	X	X					10	Steroid
Pt#3	X	X					8	Steroid + DNA synthesis inhibitor
Pt#4	X		X	X	X		8	Steroid
Pt#5	X					X	7	Cell cycle inhibitor
Pt#6		X					3	Steroid + TNFα inhibitor
Pt#7		X					8	DNA synthesis inhibitor
Pt#8	X	X					7	Steroid
Pt#9	X	X				X	7	None
Pt#10	X	X					3	Transcription inhibitor
Pt#11		X					5	IFNα
Pt#12	X	X		X			10	Steroid
Pt#13	X	X		X			9	None
Pt#14	X					X	7	Cell cycle inhibitor
Pt#15		X					3	None
Pt#16	X	X		X			5	Steroid
Pt#17	X	X					7	None
Pt#18	X			X			9	None
Pt#19	X			X		X	9	Steroid + Cell cycle inhibitor + Transcription inhibitor
Pt#20	X	X					0	Cell cycle inhibitor
Pt#21	X					X	0	Transcription inhibitor
Pt#22	X		X	X		X	3	DNA synthesis inhibitor + TNFα inhibitor
Pt#23	X						5	Cell cycle inhibitor
Pt#24	X	X			X	X	3	DNA synthesis inhibitor
Pt#25	X		X	X			10	Steroid + DNA synthesis

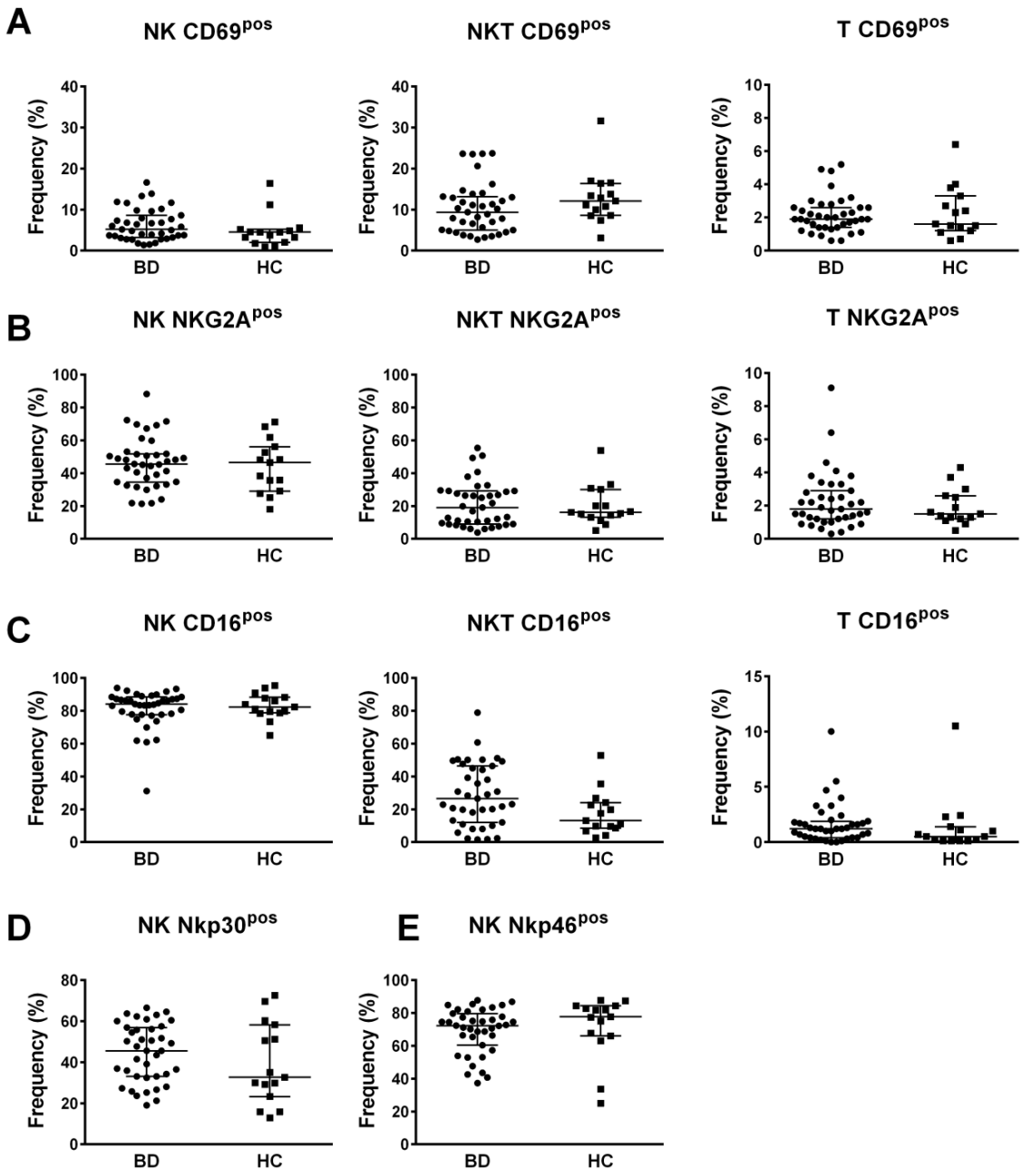
								inhibitor
Pt#26	X			X			7	None
Pt#27	X	X		X	X	X	3	Steroid + Cell cycle inhibitor
Pt#28	X	X			X		0	TNFα inhibitor
Pt#29	X		X	X		X	8	None
Pt#30		X					0	None
Pt#31	X	X		X		X	5	DNA synthesis inhibitor
Pt#32	X	X		X		X	3	Steroid + TNFα inhibitor
Pt#33	X				X		0	Steroid + DNA synthesis inhibitor
Pt#34	X	X		X		X	7	DNA synthesis inhibitor + TNFα inhibitor
Pt#35	X			X			3	None
Pt#36	X			X		X	0	DNA synthesis inhibitor + Cell cycle inhibitor
Pt#37	X				X	X	5	Steroid + Cell cycle inhibitor
Pt#38	X	X		X			5	Steroid + DNA synthesis inhibitor + TNFα inhibitor

DNA synthesis inhibitor = Mycophenolate mofetil, Azathioprine, Methotrexate;
Cell cycle inhibitor = Colchicine, Cyclophosphamide;
Transcription inhibitor = Ciclosporin;
TNFα inhibitor = Adalimumab, Infliximab.

The district involvement is referred to the historical clinical features of the disease for each patient, while the BDCAF score is referred to the disease activity at the moment of blood sample collection.

S. 2.3. Profile of markers of activation and inhibition

Dot plot visualization of the percentage of CD69 (**A**), NKG2A (**B**), CD16 (**C**), Nkp30 (**D**) and Nkp46 (**E**) positive cells in NK, NKT and T lymphocyte gates determined by flow cytometry in PMBCs from BD patients (●) and HC (■). Horizontal lines show the median ± IQR. Data were analysed by Mann-Whitney U test.



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