An Exploration of Non-Antineutrophil Cytoplasmic Antibodies Serum Biomarkers in Systemic Vasculitis : An Investigation of Behçet's Disease

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Avec la permission de l'école doctorale de l'UPMC (ED394), la thèse a été rédigée en anglais (américain), ma langue de recherche et de publication usuelle.

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1. Introduction

Behçet's Disease (BD) is an inflammatory disorder of unknown etiology characterized by recurrent attacks of oral aphthous ulcers, genital sores, and ocular lesions (triple-symptom complex).[1,2,3] These manifestations are usually self-limiting except for ocular lesions, which may lead to permanent impaired vision.[3,4] Recurrent relapsing episodes of clinical manifestations represent a hallmark of BD and are unpredictable in frequency, severity and duration.[5] Less frequent, although more severe, acute manifestations may additionally involve the vasa vasorum and corresponding larger vessels, the central nervous system, and the gastrointestinal tract.[6,7,8,9] BD has a heterogeneous onset and is associated with significant morbidity and an elevated mortality rate.[10,11]

Hypotheses and observations concerning the specific pathophysiological mechanisms of BD, a non-antineutrophil cytoplasmic antibodies (ANCA) inflammatory vasculitis, are multiple. Consequently, this study presents an exhaustive review of the disease in an attempt to recap the immunopathological pathways suggested by extant literature, and provides a detailed summary of the clinical aspects of, and treatment options for the disease. Moreover, a quantitative statistical analysis of 20 protein signatures proposes biomarkers specific for BD and provides a predictive model for identifying patients with BD with a high level of sensitivity and specificity. The objective is to provide clinicians and researchers with both a qualitative and a quantitative approach for interpreting and elucidating the physiopathology of BD, and assess the disease relative to other vasculitides and autoimmune syndromes.

BD is classified as a systemic vasculitis associated with potentially severe clinical symptoms, particularly in males with early age onset.[12,13] Currently, there exists no specific diagnostic tool or serum biomarker to identify and quantify the severity of BD; prognosis is difficult to ascertain.[1,3,14] Consequently, providing an objective clinical tool for measuring the onset and severity of the disease remains a major goal.[15,16]

Treatment options are unspecific and aim to relieve symptoms and control disease progression and severity.[1,12,17] Disease management usually includes systemic anti-inflammatory and / or immunosuppressant and / or biologic therapies.[11,18]

Onset of clinical manifestations of BD in both sexes usually starts in the third and fourth decades of life.[2,19] BD in male patients is marginally more common than in females and results in a poorer clinical prognosis due to a higher intensity of inflammatory activity, especially with regards to ophthalmological, cardiac, and neurological lesions.[13] As in most other autoimmune diseases, BD progression is generally more acute in patients with an early onset age.[11,20,21]

Prevalence of BD worldwide ranges between 1 / 1,000 and 1 / 10,000 with a significant presence in Asian countries north of the equator from the Mediterranean to East Asia (between 30° and 40° North), hence the term Silk Road Disease.[1,19] Turkey has reported the highest prevalence ranging from 80-420 / 100,000.[22] Disease occurrence in other countries such as Saudi Arabia, Iran, Korea, China, and Japan, oscillates between 13.5 and 85 / 100,000 with the latter possessing the highest reported incidence in East Asia.[15,19] Occurrence is far lower in Western countries ranging from 0.12 to 0.64 / 100,000.[1] The specific role of genetic and environmental factors in BD remains unclear.[2,10,20]

The major histocompatibility complex (MHC) antigen HLA-B51 allele is more prevalent among patients with BD from the Silk Road areas (up to 81%) relative to Caucasian patients in Western countries (13%).[1,19,23,24] Additional genes from the MHC locus have been investigated, including several Tumor Necrosis Factor (TNF) genes and the MHC class I related gene (MICA); yet their exact involvement remains undetermined as these genes may possibly be activated by a HLA-B51-induced inflammatory cascade.[10,23,24] Investigations of BD pathogenesis have targeted other non-MHC genes, namely interleukin 1 (IL-1), coagulation factor V, intercellular adhesion molecule 1 (ICAM-1), and endothelial nitric oxide synthase (eNOS).[6,21] No conclusive results were reached.[10,21] Various microbes were proposed as actors in the pathogenesis of BD, notably *Streptococcus sanguis* and herpes simplex virus type 1; none were confirmed as direct protagonists in the disease.[25,26,27]

Currently there exists no laboratory markers specific for BD diagnosis and prognosis is consequently difficult to ascertain.[14,15,28] Blood tests serve to detect systemic inflammation, through increased erythrocyte sedimentation rate (ESR), elevated C-reactive protein (CRP), leukocytosis (and eosinophilia), and thrombocytopenia, mostly during the active phases of the disease.[29,30,31,32] Consequently, BD in remission is difficult to gauge.[17,26,27] Studies indicate that the serum concentrations of various cytokines including TNFα and interferon gamma (IFNγ) are routinely elevated in BD.[33,34,35] The specificity of these cytokines, used

individually, remains low.[33] Conversely to other rheumatic diseases, autoantibodies, such as antinuclear, rheumatoid factor, cryoglobulinemia, and antineutrophil cytoplasmic antibodies (ANCA) have not been detected in BD, hence the classification of the disease as a non-ANCA vasculitis.[1]

As BD does not have any pathognomonic symptoms or exclusive laboratory findings, a differential diagnosis is made on the basis of benchmarks proposed by the International Criteria for Behçet's Disease (2013) that provide excellent specificity, but low sensitivity.[15,16] Identifying BD thus depends intrinsically on an exclusionary process that rests on eliminating possible diagnoses that mimic BD symptoms.[1,3]

The gold standard for diagnosis of BD remains biopsy.[36] It corroborates BD-specific histological tissue inflammation patterns.[37] However, given the nature of the surgical procedure, a biopsy may fail to retrieve samples of inflamed tissue rendering a definite histopathological verdict statistically unreliable, especially when the latter does not correspond with clinical observations.[36] This makes identifying novel serum biomarkers all the more imperative for reliably supplementing differential diagnoses and consequently selecting the most appropriate treatment.[1,11,12]

Research has demonstrated that a Th1-type polarization of immune response occurs in BD, its intensity remaining correlated with disease activity.[38] Gamma delta T lymphocytes (γδ) play a role in the immune response to infections and in autoimmunity by recognizing bacteria-derived and autologous antigens.[17,27,35] Patients with BD have increased numbers of γδ T cells in circulation and in mucosal lesions.[25] This is corroborated by the fact that γδ T lymphocytes have an activated phenotype in BD (they express activation markers, such as CD25, CD29 and CD69) and produce inflammatory cytokines, including IFN-γ, TNFα and IL-8.[35,39] A culture of γδ T lymphocytes from BD patients proliferates in response to mycobacterial HSP-derived peptides and to proteins from microorganisms in oral ulcers.[25,27] In addition, γδ T lymphocytes proliferate in the presence of IL-12.[28] Serum levels of IL-12 are increased in BD, suggesting a role of IL-12 in Th1-type polarization.[34] As antigen-presenting cells (APCs) produce IL-12, it is likely that they are also involved in the Th1-type polarization in BD.[27,28] They also produce IL-18, which was shown to increase neutrophil functions.[28] Neutrophils are hyperactive in BD, with increased chemotaxis, phagocytosis, superoxide production and myeloperoxidase expression, and produce several cytokines, including IL-12.[6,34,40] The precise mechanism of neutrophils hyperactivity is not known, however, T cells

are fundamental in their activation.[6] It is currently assumed that complex interactions between T cells, neutrophils and APCs are involved in the immune pathogenesis of BD as hypersensitivity of T cells and APCs to different types of antigens results in cytokines being produced.[41] This leads to neutrophil hyperactivation that contributes to the stimulation of Th1 cells.[6] IL-21 has also been proposed as a key modulator of TGF-β signaling in BD, leading to the reciprocal differentiation of T cells into Th17 cells.[42]

Consequently, this study attempts to isolate biomarkers that would identify patients with BD via a quantitative determination of proteins assumed to be involved in the pathogenesis of the disease by comparing BD patients with both healthy donors and CA patients with non-autoimmune vasculitis.[14,43]

2. NOD Model of Autoimmunity

Autoimmune diseases are serious immunological disorder that involves the alteration of tolerance mechanisms leading to the generation of immune responses against self-antigens, with potentially devastating clinical outcomes. Viable experimental models are essential in elucidating the etiology of those mechanisms that lead to autoimmunity. To better understand the processes underlying these diseases as a group, laboratory models such as the NOD mouse model exhibiting type-1 diabetes (T1D) provide a coherent framework highlighting the major pathways of autoimmunity in general. Behçet disease is thought to develop subsequent to an autoimmune response, which, in turn may be triggered by innate and adaptive immunity. As such, a presentation of the NOD model is necessary before examining the specific pathogenesis of autoinflammation in BD.[40,44] The fact that the NOD mouse harbors multiple defects in tolerance pathways, and the resulting spontaneous development of autoimmunity, has led to the evolution of this strain as a natural experimental model for autoimmune / autoinflammation disease in general, and BD in particular.[45]

T1D is generally preceded by the infiltration of mononuclear leukocytes and a subsequent invasion of islets, causing severe insulitis.[46,47] Mononuclear infiltrates demonstrated heterogeneous composition, including $CD4^+$ T helper (Th) cells, $CD8^+$ cytotoxic T cells, natural killer (NK) cells, B cells, dendritic cells and macrophages.[44,45] Subsequently, $CD4^+$ and $CD8^+$ T cells were identified as the key protagonists in NOD disease.[48,49,50] Most importantly for this study is the fact that NOD mice are susceptible to a variety of autoimmune diseases besides T1D that share clinical manifestations with BD.[51,52,53] Hence, NOD mouse exhibits similarity to humans with T1D, whereby autoimmunity targets multiple organs that are not restricted to the endocrine system, and from which we can extrapolate parallels with BD pathogenesis.

The genetic susceptibility of NOD mouse to T1D is controlled by multiple loci, notably the major histocompatibility complex (MHC) locus. NOD mice express a unique MHC haplotype, $H\text{-}2^{g7}$, which was found to be strongly associated with disease susceptibility.[54,55,56,57] Studies suggest the involvement of MHC in distorting thymic selection, affecting Th1 versus Th2 responses and the stimulation of regulatory cells.[58,59] As the MHC locus includes other genes implicated in autoimmune responses, polymorphisms in such genes probably contribute to T1D and BD susceptibility.[60,61]

A majority of studies highlight the role of T cells in the development and progression of autoimmune diseases. These include reduced numbers of natural killer (NK) cell activity, defective macrophages and NK T cells (NKT), deficient $CD4^+CD25^+$ regulatory T cells (Tregs) along with other abnormalities.[62,63] NK cell-mediated cytotoxicity was shown to be attenuated in NOD mice versus other strains.[64,65,66,67,68] These characteristics have been observed in human autoimmune diseases in general.

B cells have similarly been implicated in the development of T1D. B cell deficiency triggered by antibody treatment prevented the onset of insulitis and diabetes in NOD mice.[69,70] B cells may produce autoantibodies or may contribute to the selection or activation of autoreactive T cells by acting as antigen-presenting cells (APCs).[71,72] Research on human autoimmune disease pathways indicated similar results.

T cells appear to be the key regulators of T1D. It was suggested that both $CD4^+$ and $CD8^+$ T cells are involved in the onset and progression of the disease.[73] $CD4^+$ T cells directly mediate T1D.[74] $CD8^+$ T cells have also been implicated in the effector function; antibody therapy targeting the autoreactive population of $CD8^+$ T cells reduced the development of T1D.[75,76]

The antigen specificity of T cells in diabetic NOD mice has been thoroughly investigated. T cells recognize antigens produced in pancreatic islets, including insulin, GAD, insulinoma-associated protein 2 (IA2) and heat shock protein 60 (HSP60).[77] Defects in these same tolerance pathways have been observed in the tissues of multiple human autoimmune diseases.[78]

Expansion and trafficking of T cells is thought to play a key role in the progression of T1D. Researchers believe that the priming of naive T cells takes place in secondary lymphoid organs where tissue dendritic cells presenting antigens have gained access.[79] Subsequently, activated T cells change their surface receptors and migrate into nonlymphoid tissues where their antigen is located. Priming events in NOD mice were shown to occur in pancreatic lymph nodes, whose ablation by the age of three weeks resulted in the protection of mice against diabetes.[80] Evidence points to the involvement of $CD11c^+$, $CD11b^+$, and $CD8\alpha^-$ dendritic cells in antigen presentation in NOD mice.[81]

T cell numbers are well regulated in normal hosts. In T1D mice however, naive T cells undergo expansion referred to as homeostatic proliferation upon transfer to lymphopenic hosts,

whereby they express cell surface markers and produce cytokines.[82] It is speculated that the lymphodeficiency of spontaneous autoimmune diabetes contributes to T1D disease pathogenesis.[83]

Studies indicate that cell trafficking play an important role in the development and progression of autoimmunity in general and T1D in particular.[84] Cytokines included monocyte chemoattractant protein 1 (MCP1), MCP3, MCP5, macrophage inflammatory protein 1α (MIP1α), MIP1β, CCR5, and IFN-inducible protein 10 (IP-10).[85] Lymphotoxin (LT), a cytokine essential for the formation of splenic lymphoid follicles, is also involved in the pathogenesis of T1D.[86]

It is hence believed that central tolerance pathways are altered in NOD mice. Given the important role of T cells in the pathogenesis of T1D, the defect in thymic development and selection is deemed a likely disease contributor.[59,87,88,89] In this context, investigations of the Aire gene demonstrated its role in the ectopic expression of many self-antigens in medullary thymic epithelial cells.[90,91,92,93,94] Whether this tolerance pathway is abnormal in human autoimmune diseases has yet to be determined.

Studies have also investigated the role of GAD65, an autoantigen originally identified in humans with autoimmune diabetes.[95] Early during disease onset, autoantibody and T cell reactivity to GAD65 were observed in both human subjects and NOD mice; injection of GAD65 into the thymus prevented the onset of T1D.[96,97,98,99,100,101] Thus, GAD65 is expressed in the thymus of NOD mice, and T cells reactive to this autoantigen undergo negative selection.[102,103,104,105,106]

Peripheral tolerance mechanisms control autoreactive T cells that escape thymic selection, and thus maintain self-tolerance initiated in the thymus and prevent autoimmune diseases.[107,108] Knockout mice for genes involved in peripheral tolerance, including IL-2, Fas and CTLA4, were shown to promote autoimmune disease.[109,110]

Co-stimulation of naive T cells likewise plays an important role in preventing autoreactivity. Activation of the TCR CD28 on naive T cells involves binding of its ligands B71 (CD80) and / or B72 (CD86) that are upregulated on APCs.[111,112] TCR activation also results in several events that prevent human autoimmune responses: these include upregulation of antiapoptotic factors in stimulated T cells, stabilization of IL-2 mRNA, and production of effective immunological synapse.[111,112,113] It was demonstrated that CD28 / B7 co-stimulation

affects Th1 / Th2 cell differentiation. CD28 knocking out activated Th1 responses to autoantigens in NOD mice.[114] Alternatively, Th2 differentiation from naive T cells is presumed to depend on CD28 / B7 co-stimulation.[115,116] Therefore, the absence of Th2 regulatory cytokines may be the reason behind development of T1D. Moreover, CD28 was found to be required for the upregulation of CTLA4, which is involved in disease progression.[117] Anti-CTLA4 treatment was found to accelerate insulitis and diabetes in NOD mice.[118,119] As a consequence, polymorphism in the CTLA4 gene was correlated with autoimmune susceptibility in NOD mice and in humans.[60]

Another costimulatory pathway involves ICOS, expressed on T cells, and its ligand ICOSL, expressed on both lymphoid and nonlymphoid tissues.[120] This pathway was shown to enhance IL-10 production and regulate Th2 responses.[120,121] Treatment with anti-ICOS accelerated diabetes in NOD mice, and ICOS gene polymorphism was associated with disease susceptibility.[122,123] Other costimulatory pathways, such as the CD40 / CD154 and OX40 / OX40L have additionally been investigated. Treatment with anti-CD40L mAb inhibited insulitis and diabetes when administered to juvenile mice.[124] Thus, the CD40 / CD154 pathway plays a role in $CD4^+$ T cell activation in human autoinflammatory responses.[125,126]

It is generally assumed that the progression of T1D is the result of Th1 responses and that Th2 responses prevent disease development in NOD mice.[59,127] Th1 cells are known to produce proinflammatory cytokines, such as IFNγ and TNFα, and to stimulate cell-mediated immune responses and the production of opsonizing IgG antibodies. On the other hand, Th2 cells produce anti-inflammatory cytokines, such as IL-4, IL-5 and IL-10, and induce selected humoral responses.[128] It was shown that administration of cytokines that promote Th1 responses, such as IL-12, accelerate T1D in NOD mice.[129] Alternatively, the incidence of diabetes was significantly inhibited upon blocking Th1 responses.[130] Th1 / Th2 balance, as discussed earlier, appears to be controlled by the CD28 / B7 co-stimulation pathway. Th1 / Th2 polarization is also affected by other molecules, such as Tim3.[131] Targeting Tim3 by Tim3-Ig fusion protein or anti-Tim3 mAb was shown to accelerate diabetes in NOD mice. Inhibition of STAT4, which controls the IFNγ signalosome, prevented T1D.[132] In contrast, disruption of STAT6, which plays an important role in IL-4 signaling, enhanced disease development.[133] Moreover, it was demonstrated that APCs in NOD mice produce cytokines that enhance Th1 responses.[134,135] NKT cells also influence Th cell differentiation. When stimulated by CDd1-expressing APCs, NKT cells produced cytokines that induce the differentiation of Th2 cells.[136,137] NKT cell counts in NOD mice were shown to be significantly low compared to

other mouse strains.[138,139] Restoring normal numbers of these cells prevented the development of diabetes in NOD mice and in several human autoimmune pathologies.[138,139,140,141,142]

Tregs or suppressor T cells were reported in T1D rodents.[143,144] Research has shown that the adoptive transfer of $CD4^+CD25^+$ Tregs in mice reversed thymectomy-induced autoimmunity.[145,146] Tregs were found to express several surface markers, including CD25, CTLA4 and GITR. Studies also demonstrated the importance of the transcription factor FoxP3 in the generation of Tregs. FoxP3 deficiency prevented the production of Tregs and resulted in the development of autoimmune disease, including diabetes, in mice.[147,148] Treatment with Tregs from FoxP3-sufficient donors protected those mice from autoimmunity.[149] Moreover, FoxP3 transduction of naive $CD4^+$ T cells resulted in the acquisition of the Treg phenotype.[150] Thus, $CD4^+CD25^+$ Tregs represent an essential component of peripheral tolerance, whereby autoreactivity to self-antigens is suppressed. Furthermore, it was shown that $CD4^+CD25^+CD62L^+$ Tregs, which are derived from the thymus, exhibited the highest suppressive activity in transfer models.[63,151] Essentially, $CD4^+CD25^+$ Tregs and $CD4^+CD62L^+$ Tregs were thought to control T1D.[152] Elimination of CD28 led to the depletion of Tregs by altering the thymic development and other peripheral homeostatic mechanisms, which resulted in enhancing the development of diabetes in NOD mice.[153,154] The reactivity of Tregs was shown to be dependent on TGF-β, whose ability to induce FoxP3 expression has been extensively documented.[155,156]

It was thus hypothesized that Tregs are generated in the thymus upon exposure of thymocytes to self-antigens. This notion was supported by the fact that self-antigen presentation in the thymus produces large numbers of Tregs.[157,158] Antigen specificity of Tregs was reported to play an important role in their suppressive activity.[159]

Taken together, the evidence mentioned above presents NOD mouse as an important model for studying autoimmune disease and provides an insight into the mechanisms underlying human autoimmune development and progression.

Autoimmune disorders that target particular organs or tissues are thus classified as local or organ-specific, and those that target antigens that are not tissue specific are termed systemic or non-organ specific. Criteria for classifying a disease as autoimmune were first established by the German-American immunologist Ernst Witebsky and colleagues in 1957 (Witebsky's postulates) and were later modified in 1994. Several diseases, including vasculitis, are known

to have an autoimmune origin and the NOD model has contributed to their understanding. It is largely believed that BD follows comparable immune pathways, as is discussed in the following chapter.

3. Autoinflammation in Behçet's Disease

To date, the etiology of BD remains unknown. The disease is thought to develop subsequent to an autoimmune response, which, in turn may be triggered by innate and adaptive immunity influences in response to infectious, or other environmental agents in genetically susceptible individuals.[1,20,160,161]

Four criteria support evidence of a genetic pull on the susceptibility to BD: peculiar geographical distribution, familial aggregation, correlation with class I HLA antigens, and polymorphisms in genes that control immune responses.[17,23]

As previously discussed, BD mainly occurs in countries of the Old Silk Route, amongst which Turkey shows the highest prevalence of the disease. This may suggest the intervention of a genetic background in disease development, especially that, and as noted earlier, Turkish immigrants in Germany had significantly higher disease prevalence than native Germans.

Although many cases of BD are sporadic, diagnostic criteria have indicated an occasional positive family history for the disease.[23] Isolated manifestations of BD were noted in first-degree relatives of patients. These include recurrent oral ulcers, genital ulcers or a positive skin pathergy test.[20] The pattern of disease inheritance does not follow Mendelian rules, as reported by multi-case family studies.[20] Nevertheless, genetic anticipation, whereby children of affected parents exhibit an earlier disease onset, was noted in certain cases.[20]

In order to demonstrate familial aggregation, a parameter referred to as sibling recurrence risk ratio and denoted by λs was used.[20] This ratio is obtained by dividing the risk of developing BD among siblings of an affected patient by the risk of developing the disease among the general population.[20] Thus, a significant variation from unity indicates familial aggregation, which supports the notion of genetic predisposition for a disease.[20]

Gul *et al.* (2000) conducted a study to investigate λs in Turkey in an attempt to assess the contribution of genetic factors to the pathogenesis of BD.[20] The study group included 170 unrelated patients diagnosed according to the criteria of the International Study Group (ISG) for BD. Patients included both males (n=98) and females (n=72) and were between 16 and 66 years old. The first phase of the study involved interviewing patients with a detailed questionnaire in order to identify possible relatives with BD. Of the 170 index patients, 18%

had family members, mainly siblings, with BD. Among patients with juvenile onset, 33% reported positive family history, compared to 17% of patients with adult onset. The second phase involved selection of the immediately older sibling or, if not available, the immediately younger sibling as the second sibling for the evaluation. Patients who had no siblings were eliminated. A telephone interview was done with the siblings of index patients, and using a standard questionnaire, the presence of disease manifestations was determined. These manifestations included recurrent oral ulcers for a minimum of three times in a 12 month period, recurrent genital ulcers, uveitis or eye disease causing blurred vision and redness, and skin lesions, such as painful red nodules in the legs or frequent spots or acne-like lesions in arms and legs. Siblings with recurrent oral ulcers represented 13% of total siblings, and were invited for further examination. Those with BD represented 4% of total siblings. In order to calculate λs, data from three previous studies on the prevalence of BD in Turkey were used. The λs value was found to be 11.4 – 52.5. The results of this study demonstrate high λs value and familial aggregation, which suggests a genetic predisposition for BD.

Reports also reviewed the contribution of genetic factors to the pathogenesis of BD.[2,19,20] As mentioned prior, among the evidence supporting genetic predisposition for BD is the association with class I HLA antigens, which includes HLA-A, HLA-B and HLA-C.

Several studies reported a close association between HLA-B5 and BD development.[23] These studies demonstrated that HLA-B5 is heterogeneous in composition and includes at least HLA-B51 and HLA-B52.[23] Within the MHC locus, HLA-B51 and HLA-B5701 were reported to be involved in BD, the former being the most strongly associated with disease pathogenesis, most notably along the Old Silk Route.[1,2,17,20] Additionally, a preliminary study by Ohno and colleagues in 1978 narrated a high frequency of HLA-B51 among BD patients compared to normal controls, unlike HLA-B52 which did not show a significant difference.[23] Likewise, the occurrence of HLA-B51 allele was significantly higher among BD patients (55%) compared to normal subjects (10-15%) in Japan.[2] The HLA-B51 allele was reported to have a higher prevalence among BD patients along the Old Silk Route (up to 81% of Asian patients) compared to patients living in Western countries (13%).[2] Hence, HLA-B51 is thought to represent the strongest risk factor for BD along the Silk Route.[19] The risk of disease development associated with HLA-B51 allele, relative to that among non-carriers was reported to be 6.7 in Japan, relative to a low of 1.3 in the United States.[2] HLA-B51 allele was also reported to occur more commonly among patients with posterior uveitis or progressive central nervous system disease, and was thus associated with disease severity.[2] For instance, visual

acuity below 0.01 was described in 51% of patients with HLA-B51 and in 31% of non-carrier patients.[2] Moreover, the frequency of HLA-B51 allele in patients with central nervous system involvement was found to be 55% in one study and 84% in another.[10]

Ohno *et al.* (1982) performed another study to assess the frequency of class I HLA antigens in BD patients compared to healthy subjects, including both males and females.[23] Peripheral blood lymphocytes were typed for eight HLA-A antigens, 16 HLA-B antigens and four HLA-C antigens, in addition to the split antigens of HLA-B5. The results did not show any significant differences in HLA-A and HLA-C frequencies between BD patients and healthy controls. Among the HLA-B antigens, HLA-B5 was found to occur in 61% of BD patients versus 31% of control subjects, and was thus significantly associated with the development of BD. In addition, HLA-B5 was more frequent amongst complete-type patients rather than incomplete-type patients and in male patients rather than in female patients. HLA-B5 was also more common amongst patients with ocular lesions compared to those without such lesions, and did not affect visual prognosis in males, unlike females, suggesting the involvement of other genetic factors in BD. Similarly, using separate groups of patients and healthy subjects, the frequency of HLA-B51 was significantly higher in BD patients (62%) compared to control subjects (21%). However, HLA-B52 did not show significant difference, suggesting that HLA-B51, but not HLA-B52, was strongly associated with BD, especially since HLA-B51 was found to occur in 89% of patients with HLA-B5. Individuals with HLA-B51 were reported to have a six fold risk of developing BD relative to individuals who did not have this antigen.

Previous findings by Yazici *et al.* (1977) also demonstrated a role for the HLA-B5 antigen in BD.[23] Nineteen patients of Turkish caucasian origin, including males and females, were selected for the study. Healthy controls, including laboratory technicians, medical students, house staff and faculty members, were also used. HLA typing revealed a significant association between HLA-B5 and BD. The HLA-B5 antigen was present in 84% of patients and only in 27% of healthy subjects. However, no significant difference between patients and controls was noted for the other HLA antigens tested.

As mentioned before, various studies establish a significant association between HLA-B5 and its split antigen HLA-B51 on one hand, and the susceptibility to BD on the other. However, the strength of this genetic association greatly differed between studies, and the reported risk increase varied from 1.3 to 16.[10] De Menthon *et al.* (2009) suggested that this variability may be due to small sample sizes, ethnic differences between populations of interest,

and / or clinical subtypes of BD.[10] As such, De Menthon *et al.* conducted a systemic review of literature and a meta-analysis of case-control studies investigating genetic association between HLA-B51 / B5 and BD, in an attempt to assess the true risk increase for the development of BD associated with the presence of HLA-B51 / B5, and to identify the factors that mitigate this risk. Case-control studies were utilized in which association between genotypes of class I HLA and BD were identified both electronically and manually. The electronic search was performed on the PubMed Medline database and was restricted to the period extending between January 1, 1973 and December 31, 2007. Reference lists of retrieved articles were used for manual search for studies, which also involved review articles on BD, textbooks, conference proceedings and communication with experts. Both full publications and abstracts were included in the study, and no language restriction was imposed. Two investigators independently extracted data from the collected studies, using a specific questionnaire. They were assisted by native or fluent speakers for data collection from articles written in foreign languages. The extracted data included: authors, journal, publication year, type and language, location of the study population, allele genotyped (HLA-B5 and / or HLA-B51), genotyping method, numbers of cases and controls, definition or classification criteria used for BD, sample description (BD and control) and numbers and / or percentages of HLA-B51 / B5-positive cases and controls. Out of 411 electronically-identified and 37 manually-identified studies, only 149 studies were eligible for inclusion in the meta-analysis. Seventy-one studies were further excluded due to the presence of duplicate information or overlapping samples (case and / or control). Consequently, 78 studies published between 1975 and 2007 and investigating 80 different populations from various geographical locations (Eastern Asia, Middle East / North Africa, Southern Europe, Northern / Eastern Europe and North America) with a total of 4,800 cases and 16,289 controls were included in the meta-analysis. The number of cases did not include immigrant patients, patients with possible / probable BD, or familial cases. The odds ratios (ORs) for BD development in HLA-B51 / B5-positive individuals exceeded one for all 80 case-control data sets, ranging between 1.18 and 34.62. The pooled OR for BD susceptibility in HLA-B51 / B5 carriers was 5.78. Furthermore, the risk for developing BD was increased by a factor of 5.9 in HLA-B51 carriers. Those results suggested that HLA-B51 / B5 was a susceptibility allele, supporting previous data. Moreover, the random-effects pooled prevalence of HLA-B51 / B5 was 57.2% in BD cases and 18.1% in healthy controls. Notably, the pooled prevalence of HLA-B51 / B5 in cases and controls from North / Eastern Europe was 1.4-1.9 fold lower than those from Eastern Asia, the Middle East / North Africa and Southern Europe. The pooled rate of HLA-B51 / B5-positive BD cases thus varied with geographic location. However, allele-

associated risk increase for the development of BD was relatively similar among the different ethnic populations. In addition, population-attributable risks (PAR) were calculated to assess the contribution of HLA-B51 / B5 to BD development. PAR were 44.4% for Eastern Asia, 49.4% for the Middle East / North Africa, 52.2% for Southern Europe, and 31.7% for Northern / Eastern Europe. The study concluded that HLA-B51 / B5 accounts for 32%-52% of BD cases across the different geographic locations. This meta-analysis further demonstrated other covariables: sex distribution, and epistatic or environmental risk determinants.

Verity *et al.* (1999) reviewed the worldwide distribution of HLA-B51 in healthy individuals.[19] This was accomplished by compiling data from Medline in addition to International Histocompatibility Workshops. Populations were sorted into five categories based on HLA-B51 prevalence, which ranged from 0% to greater than 15%. Twenty-two different ethnic populations were found to have a high HLA-B51 prevalence (>10%). These populations were mainly distributed north of the equator and were found to cover ancient trading routes, spanning Western Europe and Japan; 77% of populations with high HLA-B51 prevalence were located between latitudes 30° and 45° north. Interestingly, countries with high prevalence of BD were reported to exhibit similar patterns of distribution. On the other hand, most of the 13 populations located south of the equator had a low prevalence of HLA-B51. Only one population, representing Indians in the upper Amazon region, was found to have a high prevalence of HLA-B51. Likewise, low prevalence of BD was reported in countries lying south of the equator. Finally, European countries located above 45° north, where BD is uncommon, had a low prevalence of HLA-B51. For instance, in Sweden (60° north), where prevalence of BD is 1.18 / 100,000, the frequency of HLA-B51 in the general population is 3%. Thus, latitude and disease prevalence are significantly correlated and reflect the geographical distribution of HLA-B51.

The geographical distribution of BD is thought to be due to a genetic risk factor that was spread by ancient traveling traders. As such, it was hypothesized that the predominant distribution of HLA-B51 and BD along the Old Silk Route is due to ancient trade roads. However, several observations challenged this hypothesis. First, the trading route is not as broad as the region with high BD and HLA-B51 prevalence. Second, HLA-B51 and BD prevalence are not high in maritime regions. And third, a high prevalence of HLA-B51 with no BD cases was reported among certain Amerindian tribes who migrated into North American (however, this absence of BD cases among Alaskan Eskimo and Canadian Inuit populations may be explained by the lack of disease awareness in physicians treating these populations,

leading to underreporting of the disease; alternatively, the absence of BD may suggest the contribution of other genetic risk factors or environmental triggers that could be lacking among Amerindians).

Association of BD with other genes within the MHC locus, such as MHC class I chain-related gene A (MICA) and tumor necrosis factor (TNF) gene, was also reported.[1,17,19] High frequency of MICA-A6 allele was narrated among BD patients from Japan, Korea and Iran, in addition to other countries.[17] Furthermore, TNFα gene promoter polymorphism has shown significant association with BD in Turkish and UK Caucasian patients.[17] However, involvement of those genes was attributed to a linkage disequilibrium with HLA-B51 gene.[1,2,17,19,20] Thus, the involvement of those genes may be independent of the absence of HLA-B51.[19]

Other non-HLA susceptibility loci for BD were identified as well.[162] These include IL-1, coagulation factor V, intercellular adhesion molecule 1 (ICAM-1), endothelial nitric oxide synthetase (eNOS) and Mediterranean fever gene (MEFV), in addition to others.[1]

Mizuki and colleagues (2010) performed a genome-wide association study (GWAS) for BD in Japanese populations, whereby 612 cases and 740 controls were genotyped using 500,568 SNPs.[162] The area most significantly associated with BD was HLA-B. Furthermore, the independent contribution of HLA-A and HLA-B to the risk of BD was demonstrated. Association with BD was also noted outside the HLA complex. Two non-HLA regions, interleukin10 (IL-10) and the intergenic region located between IL-23R and IL-12RB2 revealed a strong association with the disease. Associations at the IL-10 and IL-23R – IL-12RB2 loci were further analyzed by fine mapping. The most significant association for IL-23R – IL-12RB2 loci was found in the intergenic region between IL-23R and IL-12RB2. Weak associations were found in IL-23R and IL-12RB2. In addition, the promoter region of IL-10 exhibited the most significant association for the IL-10 locus. This was in line with other studies that identified IL-23R and IL-12RB2 as susceptible loci for several immune diseases, including inflammatory bowel disease, psoriasis, psoriatic arthritis and ankylosing spondylitis (IL-23R encodes a subunit of the IL-23 receptor). IL-23 is a heterodimeric pro-inflammatory cytokine that is known to stimulate Th17 cell proliferation and to increase pro-inflammatory cytokine production. IL-12RB2 encodes a chain of IL-12 receptor. IL-12 is essential for Th1 cell responses, T cell and NK cell cytotoxicity, and IFNγ production. IL-12RB2 was reported to influence IL-12 binding, IL-12-mediated signaling, and Th1 cell differentiation. In addition,

SNPs in the promoter region of IL-10 were shown to be associated with several other diseases besides BD. IL-10 is an anti-inflammatory cytokine that is known to inhibit inflammatory cytokine production: it also suppresses the co-stimulatory activity of macrophages for T cell and NK cell activation. The three SNPs exhibiting the most significant association (rs1495965 in IL-23R – IL-12RB2 and rs1800871 – rs1800872 in IL-10) were assessed for replication in both Turkish and Korean cohorts. rs1495965 in IL-23R – IL-12RB2 showed significant association with BD in the Turkish, but not in the Korean, cohort. On the other hand, significant associations for rs1800872 and rs1800871 in IL-10 were noted in both cohorts.

Interestingly, comparable results were obtained by another group conducting a similar study on a Turkish population.[22] Remmers *et al.* (2010) performed a GWAS of 311,459 autosomal SNPs in 1,215 BD cases and 1,278 healthy controls.[22] HLA-B typing was performed on 1,190 cases and 1,257 controls in order to assess the contribution of HLA-B51 to BD. HLA-B51 variant was found to occur in 59.1% of cases and 29.3% of controls. Among the genotyped SNPs, HLA-B51 showed the most significant association with the disease. The haplotype containing the HLA-B51 variant was found to occur at a frequency of 0.321 in cases and 0.144 in controls. In contrast, the identical SNP haplotype lacking HLA-B51 exhibited equal frequencies (0.04) among cases and controls. This was explained by the fact that HLA-B51 variant was required for disease association in the HLA-B region. Furthermore, HLA-A was found to be strongly associated with the disease. Notably, this association was independent of HLA-B51.

In addition, this study demonstrated significant associations for non-MHC SNPs, one located in the promoter region of CPLX1 and the other in the first intron of IL-10. Genotyping additional SNPs in these regions has shown that the most significantly associated SNP was in the promoter region of CPLX1. CPLX1 encodes complexin 1, which is known to regulate exocytosis. For IL-10, the promoter region, in addition to the first, second, and third introns of the gene, had significantly associated SNPs. Fine-mapping the IL-23 – IL-12RB2 region suggested that disease association was further linked to IL-23R.

Association with IL-10, IL-23R–IL-12RB2 and CPLX1 was also investigated by Remmers *et al.* (2010) using five case-control sets from Turkey, the Middle East, Europe and Asia. The disease-associated IL-10 SNP previously identified in the Turkish population of the study was significantly replicated in the Middle Eastern Arab and Greek collections, but was of nominal significance in the UK / European descent collection. Similar results were obtained for

IL-23R – IL-12RB2 and CPLX1 SNPs in the replication collections of the Turkish discovery collection. These associations did not show sufficient significance. This was explained by the small sample size and / or by the absence of severe disease manifestations with which these markers are associated.

Remmers *et al.* (2010) also suggested that IL-10 variants leading to a reduced IL-10 expression may present a risk factor for BD.[22] Studies have linked reduction in IL-10 levels to inflammation. In addition, variants in IL-10 were associated with several diseases, including ulcerative colitis, T1D, systemic lupus erythematosus and severe juvenile rheumatoid arthritis. However, the IL-10 variant associated with ulcerative colitis, T1D and systemic lupus erythematosus was not found to be associated with BD in this study. Moreover, IL-10 variants involved in BD were not associated with these three diseases.

Several hypotheses were formulated in an attempt to explain the etiology of BD.[2,17,19,25,27] Early Hippocratic descriptions suggest that the disease may have been endemic in ancient Greece, thus raising the possibility that both genetic and environmental factors may have contributed to disease development. Furthermore, immigrants from endemic regions were reported to have an intermediate risk for disease development in low prevalence areas, thus also supporting the role of environment in the disease. This remains to be confirmed.

Infectious etiology was first hypothesized by both Behçet and Adamantiades and was thought to be implicated in the initiation of BD as well as relapses. One hypothesis suggests that the development of BD may be due to a viral infection; viral etiology has been hypothesized since the disease was first described.[27] It was suggested that viruses could be isolated from ocular and nervous tissues of BD patients; however, attempts to verify these claims were repeatedly unsuccessful. Comparison of antibody titers from patients with BD, and healthy controls did not show significant differences in serum hemagglutinating or complement fixing antibodies to measles, mumps V and S, rubella, adenovirus, herpes simplex virus (HSV), influenza, parainfluenza, respiratory syncytial virus, or psittacosis. Nevertheless, the hypothesis of viral etiology was supported by a study that reported the inability of HSV to replicate in mitogen-treated mononuclear cells isolated from BD patients. Subsequently, in situ hybridization using labeled viral DNA probes was performed on blood mononuclear cells in order to detect complementary RNA. Hybridization with HSV1 DNA was significantly greater in mononuclear cells from 10 out of 20 BD patients than in those from healthy controls.[44,45,46] However, relatively few patients exhibited significant hybridization with HSV2 DNA in their

mononuclear cells. This suggests the presence and transcription of at least part of the HSV1 genome in the peripheral blood mononuclear cells of patients with BD (exhibiting ocular, arthritic, and aphthous symptoms). These findings were subsequently confirmed by DNA – DNA hybridization methods. Further studies involving the use of polymerase chain reaction (PCR) demonstrated the presence of a 211-bp HSV1 DNA fragment in peripheral blood leukocytes, but not in oral ulcers, of certain BD patients.[20] In addition, anti-HSV1 antibodies were detected in the sera of a significantly higher number of BD patients compared to normal controls. Immune complexes with HSV1 antigen were also reported to occur in BD patients. Inoculation of ICR mice with HSV1 induced BD-like symptoms including ulcers, uveitis and arthritis.[1,20]

In addition to HSV, several other viruses were proposed as protagonists in the etiology of BD. These include hepatitis C virus, parvovirus, varicella zoster virus, human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), human herpes viruses 6 (HHV6) and 7 (HHV7).[25,26,27] Nevertheless, up-to-date, the literature does not unequivocally support their involvement.

Another hypothesis that attempts to elucidate the etiology of BD suggests the implication of bacteria, notably streptococci, the most commonly investigated microorganism, in the disease pathogenesis. This hypothesis was supported by several clinical observations in BD patients presenting frequent tonsillitis, dental caries and periodontal infections. Antibacterial therapy usually led to improved mucocutaneous and arthritic symptoms.[26,27] Streptococcal antigens induced hypersensitivity responses in skin and mononuclear cells and stimulated the development of clinical manifestations in BD patients but not in healthy controls, thus supporting the postulate implicating streptococci. Oral microbial flora was also thought to be involved in the initiation of BD, since oral ulcerations manifest themselves early during disease development.

Hirohata *et al.* (1992) hypothesized that *Streptococcus sanguis*, involved in recurrent oral ulcers, dental caries and periodontal infections, shared antigenic determinants with oral epithelial cells and tissues given that the serotypes of the bacterium were isolated from lesions of BD patients. Debris of *S. sanguis* are thought to be released from infectious foci in the oral cavity and become immunoreactive in lesions of patients.[26] Moreover, adhesion of *S. sanguis* to buccal epithelial cells, which takes place early during infections, was found to be heightened in BD patients. In addition, antibodies to *S. sanguis* were detected in sera of BD patients. The

study revealed that antigens derived from *S. sanguis* were able to stimulate the production of IL-6 and IFNγ by T cells in BD patients.[26] The study included 17 patients with BD, 13 patients with other rheumatic diseases and 13 healthy volunteers with no signs or symptoms of BD or any other diseases. Peripheral blood mononuclear cells were obtained from BD patients, disease controls and healthy controls, and subsequently, monocytes and T cells were purified. Soluble antigen extracts were obtained from both *S. sanguis* and *Escherichia coli*. This extract contained high amounts of endotoxin in *E. coli* and proteins, sugars, RNA, phosphorus in addition to trace amounts of endotoxin in *S. sanguis*. Antigens from *S. sanguis* significantly enhanced IL-6 production by T cells from BD patients but not from healthy or disease controls. It was thus suggested that the observed effect of *S. sanguis* antigens might be specific for BD. Further experiments revealed that monocytes are essential for *S. sanguis*-mediated stimulation of T cells from BD patients. However, IL-6 production in monocytes alone was not enhanced by *S. sanguis* antigens, possibly due to their negligible endotoxin content, and antigen processing by monocytes was not required for T cell stimulation. This suggests that T cell – monocyte interactions whereby *S. sanguis* antigens bind to, but are not necessarily processed by monocytes, are involved in *S. sanguis*-mediated stimulation of T cells. In addition, both *S. sanguis* and *E. coli* antigens significantly enhanced IFNγ production by T cells from BD patients but not from healthy controls, suggesting that bacterial antigens other than those of streptococci can stimulate T cells from BD patients. Soluble antigen extract from *S. sanguis* was also found to increase γδ T cells in short-term T cell cultures and to stimulate the secretion of proinflammatory mediators (CXCL8 and TNFα). Lastly, IL-10 responses were noted in patients with BD upon stimulation with *S. sanguis*.

Although many studies focused on the role of *S. sanguis* in the etiology of BD, various species of streptococcus were additionally proposed: *S. salivarius*, *S. mitis* and *S. mutans*, along with others.[26] Saliva samples and subgingival plaque from BD patients were found to predominantly contain *S. mitis* and *S. salivarius*. High levels of *S. mutans* were reported in saliva of most BD patients and were found to be associated with elevated numbers of oral ulcers, increased scores of dental and periodontal indices, and a higher clinical severity score.[26] In addition, *S. mutans* was found to enhance IL-12, IFNγ and TNFα levels, monocyte function and Th1 responses.[26]

Given the involvement of several streptococcus species in autoimmune disease pathogenesis, it was proposed that these common antigenic determinants account for the observed effects. Indeed, streptococci may be directly implicated in disease development or

may harbor antigens that cross-react with host oral mucosa, leading to adverse immune reactions.

Early findings supporting the autoimmune hypothesis of BD reported T cell responses to oral mucosal homogenates. These responses were thought to be triggered by mucosal epithelial cells or by cross-reactive microbial antigens. Subsequent studies demonstrated significant homology between the microbial 65 kDa heat shock protein (HSP) and the human mitochondrial 60 kDa HSP.[27] Thus, it was postulated that the pathogenesis of BD might be due to an autoimmune response to endogenous HSP.[27]

Involvement of HSPs in the pathogenesis of BD has been thoroughly investigated.[17,25,27] HSPs, also referred to as stress proteins, are intracellular immunoreactive proteins found in virtually all uni- and multicellular organisms, and are named according to their molecular weight. HSPs are involved in the folding and unfolding of other proteins and are synthesized in response to a variety of adverse stimuli, including elevated temperatures, anoxia, heavy metal ions, hydrogen peroxide, and viral infections. HSP65, initially identified in mycobacteria, was reported to exist in both gram-positive and gram-negative bacteria.[26,27] As mentioned before, HSP65 bears a high degree of homology with the human mitochondrial HSP60. Thus, HSPs were proposed to be involved in autoimmune diseases. This hypothesis was investigated by various studies. Heat-killed *Mycobacterium tuberculosis* was reported to induce adjuvant arthritis in rats.[1,20,27] HSP-primed T cell clones were isolated from these rats and were capable of disease transfer into naive animals, thus providing evidence for the implication of HSP in the development of adjuvant arthritis.[1,20,27] The amino acid residues 180-188 of HSP65 were identified as the reactive T cell epitope. The responsiveness of T cells to HSP65 in NOD mice provided robust evidence supporting involvement of HSPs in autoimmune disease induction.[27,50,51,54] Other studies also reported a role for human HSP60 in the development of autoimmune diabetes in this model and demonstrated that the isolation of T cell clones were responsive to the 437-460 peptide within human HSP60.[27] These studies also suggested the involvement of mycobacterial HSP65 in rheumatoid arthritis. This was corroborated by the isolation of HSP65-sensitized T cell clones from the synovial fluid of patients. However, unlike adjuvant arthritis, the T cell epitope that lies between residues 180 and 188 of mycobacterial HSP was not conserved in human HSP.[27] This epitope could sensitize T cells only in patients with juvenile rheumatoid arthritis. Subsequent studies identified other T cell epitopes in rheumatoid arthritis patients: the 241-255 and the 251-265 peptides.[27] In addition, a T cell epitope including residues 2-13 of mycobacterial HSP was identified in patients with reactive

arthritis.[10,17,20] Moreover, T cell clones isolated from synovial fluid of those patients exhibited responsiveness to both mycobacterial and human HSP.[27]

Studies have demonstrated the existence of HSP65 in streptococcus species presumed to be involved in the development of BD, including *S. sanguis*, *S. pyogenes*, *S. faecalis* and *S. salivarius*.[26,51] In addition, mycobacterial HSP65 was shown to cross-react with the *S. sanguis* strains and was able to increase γδ T cells in BD patients, but not in controls. These studies detected IgA and IgG antibodies to both the mycobacterial 65 kDa HSP and the human mitochondrial 60 kDa HSP in the sera of BD patients.[26] B cell epitopes were identified as peptides 111-125 and 311-326 in mycobacterial HSP and their human counterparts 136-150 and 336-351 peptides, respectively. These epitopes overlapped with previously identified T cell epitopes. As microbial and human HSPs share significant homology, cross-reactivity was further demonstrated using polyclonal and monoclonal antibodies.[26,51]

In order to identify T cell epitopes in the 65 kDa HSP, overlapping synthetic peptides derived from mycobacterial sequences of HSP65 were used to stimulate T cells. Four peptides were shown to induce significant lymphoproliferative responses in BD patients compared to patients with related disease (recurrent oral ulcers), unrelated disease and healthy controls.[26] The peptides 111-125, 154-172, 219-233 and 311-325 exhibited significantly higher frequency of sensitized lymphocytes in BD patients compared to other diseases and healthy controls. Comparative studies revealed similar lymphoproliferative T cell responses to human peptides when compared to mycobacterial peptides.[27,47,111,159]

Collectively, these results demonstrate the presence of four overlapping B and T cell epitopes in mycobacterial HSP and its human homologue for BD.[27,47,111,159] Interestingly, none of these epitopes overlaps with those mapped for adjuvant arthritis, reactive arthritis, or rheumatoid arthritis.

The pathogenicity of mycobacterial and human HSP peptides was also tested in Lewis rats.[27,51,52] Two mycobacterial peptides in addition to their corresponding human peptides were shown to induce uveitis in these rats. Mononuclear infiltrates in the ciliary body and the iris and, to a lesser extent in photoreceptors, were also demonstrated.[27,51,52]

Due to the high abundance of both gram-negative and gram-positive bacteria in oral mucosa of BD patients, HSPs may be implicated in the etiology of this disease, especially since oral ulceration was the earliest and the most conserved clinical manifestation of BD.

Streptococci or their products may invade the lamina propria through the sore mucosa and may be carried by macrophages to elicit immune responses.[26,27]

In addition to HSPs, other streptococcal antigens are thought to be implicated in the etiology of BD.[26,27] A T cell hyper-reactivity to superantigens was proposed. Superantigens, which are known to activate T cells regardless of antigenic TCR specificity, significantly enhanced the production of IL-6 and IFNγ by T cells from BD patients.[26,27] Moreover, lipoteichoic acid, a conserved toxin found in the cell walls of gram-positive bacteria, was capable of inducing clinical manifestations of BD in rat models.[26,27] Lipoteichoic acid was also found to stimulate the secretion of CXCL8 by peripheral blood mononuclear cells of BD patients. In addition, serum levels of IgG antibodies against lipoteichoic acid were significantly higher in patients with active BD. Finally, Th1 responses were induced by αB-crystalline protein, a small stress protein, and BES1 peptide 373-385, a streptococcal antigen, in BD patients.[26,27]

It is worth mentioning that microbial factors other than viruses and bacteria were also proposed as possible etiological agents for BD. High serum antibodies against yeast *Saccharomyces cerevisiae* were reported in BD patients with gastrointestinal involvement.[69,96,97] In a separate study, IgG and IgA antibodies against *S. cerevisiae* were shown to be higher in BD patients.[69,96,97] However, no significant correlation between these antibodies and clinical manifestations or disease course was noted.

To date, no evidence supports the role of a specific microorganism in the etiology of BD. The widely acceptable theory for the role of infectious agents is that microorganisms share antigenic determinants with human proteins that lead to cross-reaction, thus favoring an autoimmune response. This remains to be conclusively validated.

Another hypothesis describing the etiology of autoimmunity suggests the involvement of cytokines along with other mediators in autoinflammatory diseases such as BD. Peripheral blood of BD patients contained IFNγ and IL-12, suggesting the involvement of Th1 cells in disease pathogenesis.[17] In addition, elevated IL-1, IL-6, IL-18, TNFα and chemokine levels are thought to account for the activation of innate and adaptive immune responses in BD.[20] Neutrophils of BD patients were hyperactive, as shown by their increased phagocytosis and superoxide production, enhanced chemotaxis, and elevated production of lysosomal enzymes.[2,6,17,20] In addition, lymphocytes of BD patients showed abnormal function.[20] Clonal

expansion of autoreactive T cells specific for HSP60 peptides was also noted in BD patients.[2] Moreover, γδ T cells were also increased in blood and mucosal lesions of BD patients.[1] It was therefore suggested that interactions between T cells, neutrophils, and APCs contribute to the pathogenesis of BD.[1,163]

Link *et al.* (1994) utilized in situ hybridization to detect the expression of IFNγ, IL-4 and TGF-β mRNA in blood and cerebrospinal fluid mononuclear cells of patients with multiple sclerosis, a chronic inflammatory demyelinating disease of the central nervous system.[33] Multiple sclerosis is used to study neuro-BD as both pathologies are presumed to share etiological characteristics. Compared to healthy controls, multiple sclerosis patients had a higher numbers of cells expressing each of the three cytokines, with those expressing IL-4 mRNA being the highest in number. This suggests the implication of the IFNγ and the IL-4 producing Th1 and Th2 cells, respectively, in multiple sclerosis.[31,33] Furthermore, the number of cytokine expressing cells was significantly higher in the cerebrospinal fluid than in the peripheral blood of multiple sclerosis patients. A positive correlation was found between the cerebrospinal fluid and blood of multiple sclerosis patients for the number of cells expressing TGF-β, IL-4 and IFNγ.[33] They proposed that TGF-β plays a role in reducing inflammatory reactions in autoimmune diseases. The number of TGF-β expressing cells was significantly higher in blood of patients with no or slight disability compared to those with moderate or severe disability. However, the number of cells expressing IFNγ mRNA was significantly lower in both the blood serum and cerebrospinal fluid of patients with no or little disability.

A similar study by Matusevicius *et al.* (1999) attempted to detect the expression of IL-17 mRNA in blood and cerebrospinal fluid mononuclear cells of multiple sclerosis patients.[31] The number of cells expressing IL-17 mRNA was significantly higher in the cerebrospinal fluid relative to the blood of multiple sclerosis patients. In contrast, such a difference was not seen in aseptic meningoencephalitis disease controls. Furthermore, comparison of IL-17 expressing cell numbers in the cerebrospinal fluid showed significantly higher counts in multiple sclerosis patients compared to healthy controls. Similarly, blood of multiple sclerosis patients exhibited higher numbers of IL-17 expressing mononuclear cells relative to healthy controls: 38% of multiple sclerosis patients and 35% of disease controls had high numbers of IL-17 expressing blood mononuclear cells compared to only 5% of healthy controls. The numbers of blood mononuclear cells expressing IL-17 mRNA was significantly higher in patients during exacerbation than during remission of multiple sclerosis.

To further identify IL-17 expressing and producing cells in brains of multiple sclerosis patients, a study was conducted by Tzartos *et al.* (2008) using immunostaining and in situ hybridization.[32] Tissues obtained from multiple sclerosis patients included acute, chronic active and inactive lesions, and normal-appearing white matter. Expression of IL-17 mRNA was found to be higher in active areas of acute lesions and in the borders of chronic active lesions compared to inactive areas of chronic active lesions and normal-appearing white matter. IL-17 mRNA expression was detected in T cells, astrocytes and oligodendrocytes. The level of IL-17 expression within all the identified cell types was higher in active areas of lesions (acute lesions and active borders of chronic active lesions) compared to inactive areas and control tissues. Even though IL-17 protein production was detected in T cells, astrocytes and oligodendrocytes, it was not identified in the microglia of active lesion tissues. Astrocytes in normal-appearing white matter exhibited higher IL-17 immunoreactivity than control brains, whereas astrocytic processes, but not cell bodies, were IL-17-positive in inactive lesions. T cell densities were significantly higher, especially in perivascular areas of acute lesions, and active borders of chronic active lesions, compared to normal-appearing white matter and control tissues. IL-17$^+$ T cells exhibited higher densities within acute lesions, and active borders of chronic active lesions, compared to inactive areas of chronic active lesions, inactive lesions, normal-appearing white matter, and control tissues. These findings suggest the implication of IL-17 in the initiation and persistence of lesions. In addition, most of T cells were IL-17-positive in acute lesions and active areas of chronic active lesions, unlike inactive areas of chronic active lesions and inactive lesions. IL-17-positive T cells included both CD4$^+$ and CD8$^+$ T cells in active lesions of multiple sclerosis. The frequencies of CD4$^+$IL-17$^+$ and CD8$^+$IL-17$^+$ T cells were significantly higher in acute lesions and in active borders of chronic active lesions relative to inactive areas of chronic active lesions and inactive lesions. Results of this study strongly suggest the implication of IL-17, produced by T cells as well as glial cells, in the pathogenesis of multiple sclerosis and by extension neuro-BD.

Weaver *et al.* (2007) extensively reviewed the IL-17 family of cytokines and its contribution to innate and adaptive immunity.[28] Adaptive immunity is known to enhance the body's defense mechanisms, and is characterized by antigen specificity and memory, differently from innate immunity. Pathogen-activated cells of the innate immune system produce cytokines that drive the differentiation of naive T cells into effector T cells that produce distinct profiles of cytokines. In addition to the commonly identified Th1 and Th2 cells, the lineage of effector CD4$^+$ T cells termed Th17 was noted in this study. While Th1 and Th2 cells are known to be

involved in defense against intracellular pathogens and parasitic helminthes, respectively, Th17 cells appear to have evolved to enhance protection against extracellular bacteria and some fungi. However, IL-17 production is not restricted to Th17 cells. Certain IL-17 family members were also produced by Th2 cells, $CD8^+$ T cells, γδ T cells, natural killer (NK) cells, and granulocytes. IL-17 was surmised to play a key role in adaptive immunity and in mediating autoinflammatory responses. Th17 cells were thus confirmed as the primary effectors in several autoimmune disorders that were previously attributed to Th1 cells.

$CD4^+$ T cells play a key role in immunity by providing help to other cells of the immune system, both innate and adaptive. Early studies recognized two classes of $CD4^+$ T cells: those that promote humoral immunity (class switching by B cells) and those that promote cell-mediated immunity (macrophage activation).[85] Further studies have led to the development of the Th1 – Th2 hypothesis that suggested that $CD4^+$ T cell subsets produce reciprocal patterns of immunity (delayed-type hypersensitivity / cell-mediated immunity by Th1, and allergic / humoral immunity by Th2) through the production of distinct profiles of cytokines.[85] These cytokines, in addition, promote the development of their respective cell subset while inhibiting the development of others. Consequently, induction of one type of response inhibits the development of the other.

Th1 cells were identified as producers of IFNγ, which is involved in macrophage activation, eradication of intracellular pathogens, and induction of IgG2a production by B cells.[85] On the other hand, Th2 cells were identified as producers of IL-4 and IL-5, which are involved in IgG1 and IgE class switching and eosinophil recruitment.[85] Subsequently, Th2 cells were shown to produce IL-13, which is implicated in IgE class switching and mucosal activation.[85]

Various studies have demonstrated that Th1 and Th2 require antigen-induced differentiation of naive T cell precursors.[69,78,111] In addition, IL-4 was shown to mediate the development of Th2. On the other hand, Th1 development was shown to be dependent on IFNγ and the cofactor IL-12. Although Th1 and Th2 cells can produce IFNγ and IL-4, respectively, it remains unclear which cells initiate effector T cell differentiation in primary versus secondary responses.

The IL-17 cytokine family includes six members: the founding member, IL-17 (also referred to as IL-17A), IL-17B, IL-17C, IL-17D (or IL-27), IL-17E (or IL-25) and IL-17F. At

least three members are produced by T cells and exhibit pro-inflammatory effects. IL-17 is thought to bridge innate and adaptive immunity by regulating components of the innate immune system, including TGF-β and IL-1 family members.[155,156,165]

Investigating the crystal structure of IL-17F has shown homology to the cysteine knot family of proteins that harbor an unusual pattern of intra-chain disulfide bonds.[155,156,165] The four cysteine residues involved in the formation of the cysteine knot in IL-17F are conserved in all IL-17 family members and across species. It was suggested that members of the IL-17 family exist as homodimers that, with the exception of IL-17B, are stabilized by inter-chain disulfide bonds. Surface features were found to be preserved amongst all IL-17 family members.[165]

The IL-17 receptors constitute a unique family of cytokine receptors that includes five members, IL-17R being the founding member (also referred to as IL-17RA).[155,156,165] These receptors are transmembrane proteins that may also be produced in secreted forms via alternative splicing. Functional details of the IL-17 receptor family have not been fully elucidated. It remains unclear whether members of the IL-17 receptor family can interact with other components for signal transduction. Little is known about the signaling pathway induced by IL-17 family members. Recent evidence suggests that signaling via IL-17R may involve MAP kinases or NF-κB.[165]

As pointed out earlier, production of IL-17 family members is not restricted to Th17 cells. Other $CD4^+$ effector T cells, $CD8^+$ T cells, γδ T cells, natural killer (NK) cells and granulocytes represent sources for IL-17 as well.[165]

IL-17A and IL-17F induce the expression of various cytokines and chemokines by different cells. Target cells include epithelial cells, endothelial cells, fibroblasts, osteoblasts and monocytes / macrophages. IL-17A and IL-17F induce the expression of colony-stimulating factors (GM-CSF and G-CSF), CXC chemokines (CXCL8, CXCL1 and CXCL10), metalloproteinases, and IL-6.[26] Thus, IL-17A and IL-17F recruit and activate neutrophils. IL-17E (IL-25) on the other hand, induces the expression of CC chemokines (CCL5 and CCL11), both of which are involved in the recruitment of eosinophils. IL-17E also induces the expression of Th2 cytokines (IL-4, IL-5 and IL-13) and influences basophilic responses.

Th17 lineage has been associated with multiple autoimmune disorders: research indicates that Th17 cells emerge as a distinct subset of effector $CD4^+$ T cells, characterized by a

novel cytokine profile different than that of Th1 or Th2.[114] Th17 cells were reported to produce IL-17A, IL-17F, IL-6, TNFα and GM-CSF, but neither IL-4 nor IFNγ. TGF-β was identified as a critical factor for the commitment of Th17. IL-6 also contributes to this sequence cells by inhibiting TGF-β-induced production of $FoxP3^+$ Tregs and by promoting the generation of Th17 instead.[128] Thus, commitment to Th17, Th1 or Th2 lineages is induced by TGF-β and IL-6, IFNγ or IL-4, respectively. TNFα and IL-1β are thought to increase the frequency of Th17, but are nonessential cofactors. In addition, IL-23 was reported to stimulate the production of IL-17A and IL-17F by memory $CD4^+$ T cells.[154,162] Indeed, IL-23 is not required for Th17 commitment and early IL-17 production, but is vital for expanding and maintaining committed Th17 and / or extending their function. IL-23R is upregulated by TGF-β and IL-6. Furthermore, IL-23 was found to be associated with autoimmunity in murine experimental models.[26,155,162] A positive correlation between IL-23, IL-17-producing effector T cells and disease development was reported.[162] In contrast, IL-12, IFNγ-producing Th1 cells and disease development have shown a negative correlation.[162] Thus, the development of IL-17-producing effector T cells stimulated by IL-23 is thought to be involved in autoimmune inflammation. Experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis, was both inhibited and treated upon administration of neutralizing IL-23 antibodies.[23,162] However, treatment with IL-17A antibodies produced relatively modest effects on CNS inflammation.[22,162] Studies suggested that IL-17F, also produced by Th17, might be producing compensatory effects in the absence of IL-17A, or that IL-23 could have other effects that are independent of IL-17 induction.[22,162] IL-23 was also shown to induce the expression of IL-17A in non-T cell populations and to activate the innate immune system.[162]

Research further established that Th17 differentiation from naive $CD4^+$ T cell precursors was independent of Th1 or Th2 differentiation.[114] Th1- and Th2-polarized cells failed to respond to IL-23. IFNγ and IL-4, which are involved in the development of Th1 and Th2 cells, respectively, were found to inhibit the development of Th17. The blocking of IFNγ and IL-4 was essential for inducing Th17 development. Thus, Th17 cells develop from a lineage that is distinct from, and antagonized by, that of Th1 and Th2 cells.[114]

Master regulators, transcription factors essential for the development of different $CD4^+$ T cell lineages were also identified in autoimmune diseases.[63,158,159] T-bet, GATA3 and FoxP3 are known to specify Rh1, Th2 and Treg development, respectively. The orphan nuclear receptor, retinoic orphan receptor (ROR) γt was identified as the master regulator of Th17 development.[63,158,159] RORγt expression was reported to be induced by IL-6 and TGF-β. Unlike

T-bet and GATA3, RORγt is a nuclear receptor whose ligand is yet unidentified; it remains unknown whether or not activation of RORγt is ligand-dependent. Furthermore, RORγt may directly induce IL-17 transcription or may act indirectly by inducing or suppressing other factors. A response element for RORγt was identified in an evolutionarily-conserved region of IL-17A promoter, suggesting a direct role for RORγt in IL-17 transcription.[63,158,159]

IL-27 (IL-17D) was reported to negatively influence the development of Th17 cells.[28,165,166] Although few studies addressed its sources, it was suggested that activated dendritic cells and macrophages generate IL-27 as well as IL-12 and IL-23.[166] One of the mechanisms through which IL-27 suppresses Th17 development is competition with IL-6 for receptor binding. It is assumed that IL-6 and IL-27 act antagonistically, in part by affecting IL-23R. Antagonism is thought to occur by direct action on naive T cells, rather than Tregs. Furthermore, IL-27 is thought to act on naive T cells via a STAT1-dependent mechanism to suppress the development of Th17 cells.[28,165,166]

IL-25 (IL-17E), another member of the IL-17 cytokine family, is produced by Th2 cells. IL-25 plays an important role in host defense against parasitic helminthes. It was suggested that the protective role of IL-25 may be due to the induction of Th2 responses and suppression of Th1 or Th17 responses.[128]

Several investigations examined the factors promoting the development of Th17 cells. In a study conducted by Yang *et al.* in 2007, IL-6 knockout mice exhibited significant reduction in Th17 cells compared to wild type mice, suggesting that IL-6 is required for Th17 cell differentiation *in vivo*. [164] In addition, IL-6 induced the development of Th17 cells *in vitro*, and was synergized by IL-23, which, when utilized alone, did not induce Th17 cell differentiation. IL-6 upregulated the expression of IL-23R in activated T cells, which indicates that IL-6 is required for inducing IL-23 responsiveness.[164] Hyperactivation of STAT3 in naive $CD4^+$ T cells enhanced Th17 cell differentiation, which was further augmented in the presence of IL-6 and IL-23. Overactivation of STAT3 upregulated IL-23R and IL-17 while suppressing Th1-specific IL-12Rβ2 and IFNγ. STAT3 also downregulated the expression of T-bet and GATA3, the transcription regulators of Th1 and Th2 cells, respectively, but upregulated RORγt, the Th17-specific regulator.[164] Consequently, the expression of IL-17, IL-23R and RORγt was impaired in STAT3-deficient naive $CD4^+$ T cells under Th17 polarizing conditions, indicating a role for STAT3 in Th17 cell differentiation. In contrast, increased expression of T-bet and FoxP3 was noted, suggesting that STAT3 inhibits the differentiation of Th1 and

Tregs.[164] Similarly, STAT3 appeared to be important for Th17 cell differentiation *in vivo*. Collectively, the results of this study suggest a synergistic effect for IL-6 and IL-23 in Th17 cell differentiation.[164] Accordingly, STAT3 was deemed essential and sufficient for cytokine-mediated Th17 cell differentiation as an upstream regulator of RORγt.

Ivanov *et al.* (2006) also reported the involvement of RORγt in the differentiation of Th17 cells.[165] Their findings suggested that RORγt was necessary for the generation of Th17 cells *in vivo* and that IL-6 was essential for the expression of RORγt, and thus Th17 cell differentiation. To further confirm the requirement for RORγt in Th17 cell differentiation, naive $CD4^+$ splenic T cells were purified from both wild type and RORγt-deficient mice and were cultured under different polarizing conditions. Cotreatment with IL-6 and TGF-β in the presence of neutralizing antibodies against IFNγ and IL-4 significantly induced the differentiation of Th17 cells from naive $CD4^+$ T cells of wild type mice compared to RORγt-deficient mice, indicating that RORγt was required for the generation of Th17 cells *in vitro*. Furthermore, IL-6 / TGF-β cotreatment induced the expression of RORγt and IL-17 mRNA in a temporal manner that suggested transcriptional regulation of IL-17 by RORγt. Thus, RORγt was important for cytokine-induced Th17 cell differentiation.[165] Moreover, forced expression of RORγt in the absence of exogenous polarizing cytokines induced the expression of IL-17 mRNA in naive $CD4^+$ T cells. Thus, RORγt was considered sufficient to induce differentiation of Th17 cells. In order to investigate the role of RORγt in Th17-mediated autoimmune diseases, EAE was induced in wild type and RORγt-deficient mice. Reduced disease susceptibility was noted in mice deficient in RORγt, suggesting the requirement of RORγt in Th17-mediated autoimmune diseases.[165] RORγt-deficient mice exhibited reduction in numbers of Th17 cells, which was accompanied by decreased levels of Th17, but not Rh1, cytokines and chemokines during disease, further supporting the implication of Th17 cells, rather than Th1 cells, in EAE.[165]

In addition to RORγt, the transcription factor interferon-regulatory factor 4 (IRF4) was reported as a key regulator of Th17 cell differentiation by Brustle *et al* (2007).[166] Naive $CD4^+$ T cells were purified from IRF4-deficient mice and were primed *in vitro*. Subsequently, cells were cultured under Th1 (IL-12 and anti-IL-4), Th2 (IL-4 and anti-IFNγ), or Th17 differentiation conditions (anti-IFNγ, anti-IL-4, TGF-β, IL-6, IL-1β, TNF, and IL-23). IRF4 deficiency inhibited the development of Th2 and Th17, but not Th1 cell differentiation. Treatment of wild-type Th cells with IRF4-specific siRNA inhibited Th17 cell development.[165] These results suggest that IRF4 is essential for the development of Th17 cells. Given the

previously reported involvement of Th17 cells in EAE, this study further investigated the role of IRF4 for the induction of this disease. IRF4-deficient mice were completely resistant to EAE induction, unlike RORγt-deficient mice that were previously reported to exhibit partial resistance by Ivanov *et al.* (2006).[165] It was subsequently demonstrated that failure to develop EAE in IRF4-deficient mice was due to the lack of IL-17-producing $CD4^+$ Th cells. In order to examine the relationship between IRF4 and RORγt, the expression of RORγt by IRF4-deficient Th cells cultured under Th17-inducing conditions was observed. The expression of RORγt was significantly reduced in the absence of IRF4 and was associated with impaired Th17 cell differentiation.

The activity of IRF4 itself seemed to be controlled by IRF4-binding protein (IBP), as revealed by Chen *et al.* (2008).[167] In this study, IBP-deficient mice developed rheumatoid arthritis-like joint disease and large-vessel vasculitis. IBP-deficient T cells exhibited enhanced responsiveness to low levels of stimulation, increased expression of RORγt, and enhanced IRF4-mediated production of IL-17 and IL-21. IBP was detected in the nucleus as well as the cytoplasm of $CD4^+$ T cells and was found to physically interact with IRF4 in the nucleus. Nuclear IBP was found to exert its effects by sequestering IRF4, thereby preventing it from transcribing its target genes. Thus, IBP appeared to play an important role in preventing autoimmunity.

In view of the role IL-2 plays in Treg development and the reciprocal TGF-β-mediated regulation of Tregs and Th17 cells, Laurence *et al.* (2007) investigated the potential role of IL-2 in Th17 cell differentiation.[168] Under Th17 polarizing conditions (IL-6, TGF-β, anti-IFNγ and anti-IL-4), naive $CD4^+$ T cells showed marked differentiation into IL-17- rather than IFNγ-producing cells *in vitro*, consistent with previous reports. Treatment with IL-2 under Th17 polarizing conditions significantly reduced the proportion of IL-17-producing cells. Furthermore, blockade of endogenous IL-2 in cultures of activated T cells increased the proportion of IL-17-producing cells, an effect which was reversed upon treatment with exogenous IL-2. Notably, the increase in the proportion of IL-17-producing cells was coupled to a decrease in $FoxP3^+$ cells following IL-2 blockade. This suggests that IL-2 expands Tregs while restricting Th17 cell development *in vitro*, consistent with reciprocal regulation of these lineages. Similarly, IL-2 inhibited the development of Th17 cells *in vivo*. To further examine the role of IL-2 in Th17 cell development, $CD4^+$ T cells from IL-2-deficient or wild-type mice were stimulated under Th17 conditions *in vitro*. IL-2-deficient T cells showed a significant increase in the percentage of IL-17-producing cells compared to wild-type cells. Addition of

IL-2 reduced the percentage of IL-17-producing cells in wild-type cells, and normalized IL-17 production by IL-2-deficient cells. Furthermore, IL-17 was not detectable in the sera of wild-type mice and was significantly increased in IL-2-deficient mice. This suggests that IL-2 inhibits IL-17 production. This study moreover investigated the role of STAT3 in Th17 cell differentiation. Reduction of STAT3 amounts in $CD4^+$ T cells resulted in significant reduction of IL-17-producing cells. STAT3-deficient $CD4^+$ T cells failed to upregulate the expression of RORγt under Th17 conditions. These results confirm the role of STAT3 in Th17 cell development. Moreover, the addition of IL-2 to $CD4^+$ T cells under Th17 conditions reduced the expression of RORγt, suggesting that IL-2 may inhibit Th17 cell differentiation by inhibiting the expression of RORγt. Furthermore, the signaling pathway of IL-2 was altered by deleting STAT5. STAT5-deficient $CD4^+$ T cells produced IL-17-producing cells under Th0 conditions. Likewise, STAT5-deficient mice had elevated serum levels of IL-17. These results suggest that IL-2 also inhibits Th17 cell differentiation via STAT5 signaling.

Other studies reported a role for Th1 and Th17 cells in the pathogenesis of autoimmune diseases. Deng *et al.* (2010) reported the implication of Th1 and Th17 responses in giant cell arteritis (GCA), a systemic vasculitis that commonly involves large and medium arteries of the head.[38] Peripheral blood mononuclear cells (PBMC) were obtained from GCA patients and healthy donors and were subsequently analyzed for frequencies of Rh1, Th17 and $FoxP3^+$ $CD4^+$ Tregs. The frequency of IL-17-producing Th17 cells among $CD4^+$ T cells ranged from 1.1% to 5.3% in GCA patients and from 0.03% to 0.59% in healthy controls. Furthermore, IFNγ-producing Th1 cells represented 20.6% of $CD4^+$ T cells in GCA patients and only 11.8% in controls. In contrast, the frequency of $FoxP3^+$ $CD4^+$ Tregs among $CD3^+$ T cells did not show significant difference between patients and controls. Thus, expansion of Th1 and Th17 cells was associated with disease in GCA patients. These observations were consistent with the plasma IL-17 and IFNγ protein levels that were significantly elevated in patients when compared to controls. Quantification of transcripts in temporal artery samples showed high levels of IL-17, IFNγ and FoxP3 expression in GCA specimens compared to control GCA-free specimens. Immunostaining of temporal arteries indicated marked infiltration of $CD3^+$, $IL\text{-}17^+$ and $IFN\gamma^+$ T cells within all layers of GCA specimens. In contrast, control artery samples were negative for $IL\text{-}17^+$ and $IFN\gamma^+$ T cells. This study further investigated cytokines that contribute to Th1 (IL-12) and Th17 (IL-1β, IL-6 and IL-23) development. Monocytes from GCA patients induced the differentiation of Th1 and Th17 cells from naive $CD4^+$ T cells. Th17 cell differentiation was suppressed upon treatment with neutralizing antibodies against IL-1β, IL-6

and IL-23, indicating the importance of these cytokines for Th17 differentiation in GCA. Protein levels of IL-1β, IL-6 and IL-12 and transcript levels of IL-1β, IL-6, IL-23 and IL-12 were significantly elevated in both plasma and circulating monocytes of GCA patients. The expression levels of Th1- and Th2-promoting cytokines were significantly upregulated in tissues from GCA patients.

Amadi-Obi *et al.* (2007) investigated the implication of Th17 cells in two human inflammatory diseases, namely uveitis and scleritis, and confirmed their findings in experimental autoimmune uveoretinitis (EAU), a mouse model of human uveitis.[34] As highlighted earlier, uveitis is commonly associated with BD and may progress to blindness. PBMCs isolated from patients with inactive scleritis and healthy controls expressed IL-17, which was significantly upregulated in the PBMCs of scleritis and uveitis patients, suggesting that Th17 cells mediate inflammation in these cohorts. In order to further validate the involvement of Th17 cells in the pathogenesis of uveitis, EAU was experimentally induced in mice. PBMCs, lymph nodes, and retinal RNA was extracted from both mice with EAU and control mice. Real-time quantitative RT-PCR analysis showed temporal pattern of IL-17 expression in EAU mice. IL-17 expression reached its peak levels in PBMCs and lymph nodes seven days post-immunization. In contrast, peak levels of IL-17 mRNA were detected in the retina at day 14 and were significantly reduced upon disease resolution. The observed temporal expression of IL-17 was consistent with the migration of Th17 cells from secondary lymphoid organs to the retina in EAU. Moreover, the clinical score of EAU mice was significantly reduced upon treatment with IL-17 antibodies prior to EAU priming, thus validating the involvement of Th17 cells in uveitis. IL-17 upregulated TNFα in retinal cells, suggesting that Th17 cells may contribute to the pathogenesis of EAU via TNFα. A larger quantity of Th17 cells compared to Th1 cells were detected in the retina of EAU mice during the early stages of the disease. However, the number of Th1 cells significantly increased upon resolution of the disease, contrary to Th17 cells which showed a concomitant decrease. Thus the increase in the number of Th17 was correlated with EAU pathogenicity. Furthermore, IL-17 expression in the PBMCs of healthy controls and patients with scleritis and uveitis was upregulated by IL-2 treatment. Treatment with IFNγ was found to downregulate the expression of IL-17 in IL-2-induced PBMCs of uveitis and scleritis patients. On the other hand, IL-27 mRNA expression was detected in the retina of healthy and EAU mice and was upregulated in EAU mice, suggesting the implication of IL-27 in uveitis. The expression of IL-27 mRNA in the retina of EAU mice was temporally correlated with disease progression, as noted prior with IL-17.

Therefore, IL-27 inhibited the proliferation of uveitogenic T cells that mediate EAU and reduced IL-2 and IL-17 secretion, suggesting that IL-27 inhibits IL-17 expression by regulating the expression of IL-2.

Kebir *et al.* (2007) reported that Th17 cells were also involved in CNS inflammation.[169] In an attempt to assess the adeptness of T cell migration across the blood-brain barrier (compared to what was previously reported for Th1 cells), Th1 and Th17 cells were generated from peripheral blood $CD4^+$ lymphocytes via IL-12 and IL-23 treatment, respectively. Using an *in vitro* model of the human blood-brain barrier, Th17 cells showed more efficient migration than Th1 or freshly isolated $CD4^+$ lymphocytes. Using this *in vivo* mouse model, the study demonstrated that both Th1 and Th17 cells migrate into the CNS upon peripheral priming and expansion. To confirm the human *in vitro* and mouse *in vivo* observations, brain sections of multiple sclerosis patients were subjected to immunostaining, and showed considerable numbers of IL-17- or IL-22-positive $CD45RO^+$ cells in lesions, compared to normal-appearing white matter and non-inflamed brain tissues. These results suggest the implication of Th17 in the formation of multiple sclerosis lesions. Further experiments were conducted to test the hypothesis that Th17 cells express cytolytic enzymes (perforin, granzyme A and B) that mediate their encephalitogenic activity. Granzyme B was expressed in 22.5% of IL-17-producing $CD4^+CD45RO^+$ cells, 17.3% of IL-22-producing cells and 60% of cells that coexpressed IL-17 and IL-22. Granzyme B^+ Th17 cells showed significant cytolytic activity compared to inactivated T cells. Receptors for IL-17 and IL-22 were detected on blood-brain barrier endothelial cells and on CNS vessels within heavily infiltrated multiple sclerosis lesions, in contrast to normal subjects. Further investigations demonstrated the functionality of IL-17 and IL-22 receptors on blood-brain barrier endothelial cells that exhibited enhanced permeability subsequent to IL-17 or IL-22 treatment. These Th17 cytokines, in addition, induced the secretion of MCP1 and the expression of IL-6 and CXCL8 by blood-brain barrier endothelial cells and promoted the transmigration of ex-vivo $CD4^+$ lymphocytes.

Based on previous studies reporting the involvement of Th17 cells in the pathogenesis of rheumatoid arthritis, Hirota *et al.* (2007) investigated the expression of cell-specific cell surface molecules in Th17 cells that may contribute to their migration into inflamed joints, a common clinical manifestation of BD.[170] The SKG mouse strain, which is known to spontaneously develop T cell-mediated autoimmune arthritis, was used as an animal model for human rheumatoid arthritis. Most of Th17 cells in SKG mice expressed CC chemokine receptor 6 (CCR6). Interestingly, Th1 cells did not express CCR6. Th17 cell differentiation was induced

in vitro by forced expression of RORγt, a transcription factor previously reported to mediate differentiation of naive $CD4^+$ T cells into Th17 cells, or alternatively by TCR stimulation in the presence of IL-6 and TGF-β. RORγt induced the differentiation of Th17 cells that coexpressed IL-17 and CCR6. However, for Th17 cells to express CCR6, IL-6 / TGF-β-cotreatment of naive $CD4^+$ T cells required the presence of IL-1 and the neutralization of IFNγ and IL-4, which are known to induce Th1 and Th2 cell differentiation, respectively. To investigate the possibility that inflamed synovial tissue produces CCR6 ligand (CCL20) to recruit arthritogenic Th17 cells, *in vitro* cultures were prepared from SKG mice. Synoviocytes spontaneously produced CCL20, which was augmented upon treatment with IL-1β, IL-17 or TNFα and inhibited by IFNγ or IL-4. In addition to synoviocytes, $CCR6^+$Th 17 cells produced CCL20. $CCR6^+CD4^+$ T cells purified from SKG mice significantly expressed CCL20 mRNA compared to $CCR6^-$ $CD4^+$ T cells. Th17 cells induced *in vitro* by TCR stimulation in the presence of IL-6 and TGF-β produced CCL20. Subsequently, *in vitro* migration assays showed preferential recruitment of Th17 cells, but not Th1 cells, in response to CCL20. These results indicate that both synoviocytes and $CCR6^+$Th 17 cells secrete CCL20 to recruit more $CCR6^+$Th 17 cells to inflamed joints. The study demonstrated that CCR6 was required for the development of Th17 cell-mediated autoimmune arthritis. IL-17 and CCL20 were also detected in synovial fluid of RA patients, and human Th17 cells expressed CCR6, unlike Th1 or Th2 cells. This suggests that Th17 cells express both CCR6 and CCL20, which are required for Th17 cell trafficking and initial destructive autoimmune reactions in joints. Synoviocytes were thus reported to recruit Th17 cells through CCL20 production.

Reboldi *et al.* (2009) also investigated the role of CCR6 in Th17 cell-mediated CNS pathology using EAE mice.[43] Most CCR6-knockout mice failed to develop EAE and did not show leukocyte infiltration in the CNS. Naive $CD4^+$ T cells from CCR6-knockout mice differentiated under Th1, Th2, or Th17-polarizing conditions *in vitro*. Thus, CCR6 was not required for the differentiation of naive $CD4^+$ T cells. Likewise, CCR6 was not required for the priming and differentiation of Th17 cells *in vivo*. Th17 and Rh1 cells were primed in the lymph nodes of CCR6-knockout mice and were entered the circulation. They were not able to reach the CNS and induce EAE. Conversely, the expression of CCR6 on transferred T cells was significant and sufficient for the development of EAE in CCR6-knockout mice. It was revealed that CCR6 was required for early, but not late migration of Th17 cells into the CNS. In addition, CCL20 (CCR6 ligand) was constitutively expressed in the epithelial cells of the choroid plexus in both mice and humans. These results suggest that the CCR6 / CCL20-

mediated entry of Th17 cells into the CNS through the choroid plexus was essential for the induction of EAE.

Engelhardt *et al.* (2001) and Ransohoff *et al.* (2003) addressed the role of the choroid plexus in CNS inflammation and discussed the paths for leukocyte entry into the CNS.[30,171] The blood-brain and blood-CSF barriers are formed at the level of microvascular endothelial cells and the choroid plexus epithelial cells, respectively. These barriers provide bidirectional control over the movement of a wide range of molecules. As such, the inflammatory and immune responses in the CNS are limited and are different from those occurring in other internal organs.[30,171] While normal CNS was found to contain few leukocytes, autoimmune diseases involving the CNS were characterized by heavy leukocyte recruitment both into the CNS parenchyma and the cerebrospinal fluid. T cells were the predominant leukocytes in the CSF of healthy humans, unlike blood serum. In addition, the $CD4^+$ / $CD8^+$ T cell ratio was increased in the CSF compared to peripheral blood. The animal model of multiple sclerosis, EAE, provides a better understanding of the mechanisms underlying leukocyte migration into the CNS. Ultrastructural alterations of the choroid plexus, mainly at the level of the epithelium, were evident during EAE and increased with disease severity.[30,171] Leukocyte recruitment into the choroid plexus occurred during EAE, but was not involved in disease pathogenesis, unlike CNS infections. Thus, choroiditis was thought to occur as a secondary event. Additionally, autoreactive $CD4^+$ T cells that target CNS myelin proteins are involved in the pathogenesis of EAE. These cells are activated outside the CNS and cause inflammation and demyelination upon migration to the CNS. T cells were also observed entering healthy central nervous systems.[30,171] These models confirmed that lymphocytes enter the CNS across the blood-brain barrier. During EAE, endothelial cells of cerebral vessels upregulate the adhesion molecules: intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) were found to mediate adhesion of inflammatory cells to inflamed cerebral vessels via their respective ligands LFA-1 (αLβ2-integrin) and the α4integrins (α4β1 and α4β7) *in vitro*. Similarly, ICAM-1 and VCAM-1, as well as MAdCAM1, were also upregulated on the choroid plexus epithelial cells in EAE and mediated leukocyte adhesion to inflamed choroid plexus, thus implicating the blood-CSF barrier of the choroid plexus in CNS inflammation. The expression of adhesion molecules on the choroid plexus epithelial cells was induced by cytokine (IL-1 or TNFα) or LPS treatment *in vitro*.[30,171]

In addition, expression of MHC molecules was shown to be induced on the choroid plexus epithelium during CNS inflammation *in vivo*, thus suggesting the involvement of the

choroid plexus in antigen presentation and activation of intraventricular lymphocytes.[30,171] Thus, adhesion molecules may play a role in co-stimulation or interactions between APCs and T cells during T cell activation. It was suggested that the antigens may be derived from the periphery or may be of intraventricular origin. In addition to adhesion molecules and MHC antigens, the cytokines IL-1, IL-6 and TNFα were induced in the choroid plexus epithelial cells upon lipopolysaccharide (LPS) stimulation *in vivo*. The choroid plexus epithelial cells were responsive to cytokines, as demonstrated by the presence of cytokine receptors on their surface.[30,171] Matrix metalloproteinases (MMPs), proteolytic enzymes involved in remodeling of the extracellular matrix, were shown to mediate leukocyte recruitment to the CNS, and their high CSF levels correlated with disease activity in multiple sclerosis. Cyclooxygenase 2 (COX-2), involved in prostaglandin synthesis, was also induced in the choroid plexus epithelium upon peripheral LPS administration.[30,171] Taken together, these findings suggest the involvement of the choroid plexus in CNS inflammation.

Three distinct routes were described for leukocyte migration into the CNS. In the first route, characteristic of normal physiological conditions, leukocytes move from the blood into the CSF across the choroid plexus. The second route involves leukocyte migration from blood to the subarachnoid space. In the third route, leukocytes move from the blood into the parenchymal perivascular spaces. In this case, leukocytes cross the blood-brain barrier and the basal lamina at the endothelial layer.[23,24]

In a study by Lee *et al.* (2008), IL-17 was shown to have differential effects on chemokines in HT-29 colonic epithelial cells.[35] IL-17 significantly inhibited TNFα-induced expression of CXCL10, CXCL11 and CCL5 in HT-29 cells. This suggested that the increase in IL-17-producing cells at inflammatory sites was accompanied by a reduced recruitment of Th1 cells as a result of the suppression of the expression of the chemoattractive chemokines CXCL10, CXCL11 and CCL5. In contrast, IL-17 strongly synergized the TNFα-induced expression of CXCL8, CXCL1 and CCL20. Thus, accumulation of IL-17-producing cells at inflamed sites further recruits IL-17-producing Th17 cells in addition to Tregs by upregulating their chemoattractive chemokine CCL20. IL-17 exerted its effects on chemokine expression at the transcriptional or posttranscriptional levels, as shown by a luciferase reporter gene assay. Subsequently, a chemotaxis assay was performed on human T cells using conditioned media of HT-29 cells treated with TNFα and / or IL-17. TNFα induced the production of chemokines that were chemoattractive to Th17 cells. The observed effect of TNFα was synergized by IL-17. In addition, TNFα resulted in transient activation of ERK and p38 MAPK, an effect

synergized and prolonged by IL-17 cotreatment. TNFα / IL-17 cotreatment resulted in persistent nuclear localization of ERK. The effects of IL-17 on chemokine (CXCL8 and CXCL10) expression were shown to be partially mediated by the ERK and p38 MAPK signaling pathways. The study also demonstrated that TNFα / IL-17 cotreatment induced phosphorylation of EGFR, suggesting that IL-17 regulates chemokine (CXCL8 and CXCL10) expression partially through EGFR transactivation.

Geri *et al.* (2011) reported the first evidence for the involvement of IL-21 in BD.[29] The frequency of Tregs, Th17 and Th1 cells was examined in the peripheral blood of active BD patients, BD patients in remission, and healthy controls. Active BD patients exhibited significantly higher frequency of Th17 cells compared to patients in remission and healthy controls. Th1 cells were significantly increased in active BD patients compared to patients in remission, but did not significantly differ from healthy controls. Moreover, the frequency of Tregs was significantly decreased in BD patients with active disease and in remission compared to healthy controls. A similar pattern was noted for activated Tregs. The addition of serum from active BD patients increased the frequency of Th17 and Th1 cells and decreased FoxP3 expression in $CD4^+$ T cells purified from healthy controls. In order to investigate the mechanism that induces Th17 cell differentiation and Treg suppression, serum levels of Th17-promoting cytokines were measured. IL-21 was significantly increased in the serum of active BD patients compared to patients in remission and healthy controls. However, levels of the other IL-17A-promoting cytokines (IL-1β, IL-6, TGF-β and IL-23) did not show significant difference between the study groups. IL-21-producing $CD4^+$ T cells were also significantly expanded in the peripheral blood of patients with active disease compared to those in remission and healthy controls, and were positively correlated with Th17 cells and negatively correlated with Tregs. Additionally, IL-21, IL-17A and IFNγ were significantly increased in the cerebrospinal fluid of active BD patients with CNS involvement. Immunohistochemical analysis of brain tissues showed marked expression of IL-21, IL-17A, CXCL8 (chemoattractant for polymorphonuclear leukocytes) and CCL20 (chemoattractant for Th17 cells) in the choroid plexus of active BD patients with CNS involvement. Collectively, these results suggest that IL-21 induces the differentiation of Th17 cells and the production of Th17 and polymorphonuclear leukocyte chemoattractants in the CNS lesions of BD patients. IL-21 was thus identified as playing a role in T cell homeostasis of BD. Treatment of purified $CD4^+$ T cells with IL-21 increased Th17 and Th1 cell frequencies and decreased FoxP3 expression. Conversely, an IL-21 blockade decreased the proportion of Th17 and Th1 cells and increased $FoxP3^+$ Tregs. This

suggested that IL-21 contributes to the pathogenesis of BD by disrupting the balance between Th17 cells and Tregs.

A study by Nurieva *et al.* (2007) previously demonstrated that IL-21 induced Th17 cell differentiation whilst inhibiting the expression of FoxP3.[172] They revealed that the expression of IL-17 and IL-21 mRNA was significantly increased in Th17 cells compared to other effector cells. Contrary to previously reported descriptions of IL-17, the study reported that IL-21 expression appeared to be induced by IL-6 alone, rather than by the synergistic effects of IL-6 and TGF-β. As IL-6-deficient cells did not produce IL-21, they suggested that IL-6 was both necessary and sufficient for the induction of IL-21. They also proposed that IL-21 upregulated its own expression, indicating an autocrine regulation. IL-21 expression was furthermore confirmed to be regulated by STAT3, contrary to IL-17 expression, which was reported to be regulated by RORγt. STAT3-deficient Th cells failed to produce IL-21 mRNA, which were normally expressed in RORγ-deficient Th cells. IL-21 also appeared to play an important role in Th17 cell differentiation. Activated naïve Th cells differentiated into Th17 cells in the presence of IL-21. This effect of IL-21 was synergized by the addition of TGF-β. Combined treatment enhanced the generation of Th17 cells and inhibited FoxP3 expression. Furthermore, IL-23 was found to potentiate the effect of IL-21 / TGF-β cotreatment. IL-21 upregulated the expression of IL-23R, RORγ, and Th17 cytokines and inhibited the expression of T-bet and FoxP3. These results indicate that IL-21 selectively regulates Th17 differentiation *in vitro*. IL-21-mediated Th17 cell differentiation appeared to be dependent on STAT3. Naïve Th cells from STAT3-deficient mice differentiated in the presence of IL-21 and TGF-β exhibited a reduced expression of IL-17 and other Th17 genes (RORγ, IL-23R, IL-21 and IL-22) and an increased expression of FoxP3, compared to wild-type cells. A similar pattern of IL-17 and FoxP3 expression was observed in RORγ-deficient Th cells, indicating that IL-21-mediated Th17 cell differentiation was also dependent on RORγ. IL-21 deficiency impaired the generation of Th17 cells *in vivo* and protected mice against EAE.

The results of this study suggest that IL-21 was induced in T cells by IL-6 and was necessary and sufficient for inducing Th17 cell differentiation via STAT3-dependent upregulation of RORγ. Thus, IL-21 acts in an autocrine manner, similar to the action of IFNγ in Th1 cells and IL-4 in Th2 cells. IL-21 appears not only to regulate the generation of Th17 cells, but also to modulate the balance between Th17 cells and Tregs, as reported by Fantini *et al.* (2007) in an experimental model of colitis.[42] IL-21 treatment reduced TGF-β-mediated expression of FoxP3 in $CD4^+$ T cells, which was accompanied by the loss of suppressive

function of Tregs, and preferentially induced the expansion of FoxP3$^-$ T cells. In addition, TGF-β / IL-21 cotreatment significantly increased the expression of IL-17 and RORγt. CD4$^+$ T cells treated with TGF-β and IL-21 failed to suppress colitis. In fact, autocrine IL-21 expression suppressed the induction of Tregs and induced the generation of Th17 cells.

Consistent with these findings, IL-21 was found to suppress the development of Tregs in a study by Bucher *et al.* (2009).[173] Treatment with anti-IL-21 antibodies significantly prolonged the survival of mice with graft-versus-host disease (GVHD), suggesting a role for IL-21 in GVHD. In addition, an IL-21 blockade resulted in the development and infiltration of FoxP3$^+$ Tregs during GVHD. Subsequently, it was demonstrated that IL-21 blockade attenuates GVHD through the induction of FoxP3$^+$ Tregs.

Vollmer *et al.* (2005) described the differential effects for IL-21 during the initiation and progression of EAE.[174] Treatment of mice with IL-21 prior to EAE induction significantly increased disease severity. In contrast, administration of IL-21 during EAE progression had less effect. IL-21 did not affect antigen-specific T cell proliferation, but rather augmented autoreactive Th1 cells upon pretreatment. IL-21 also showed differential effects on IFNγ production by NK cells, which was enhanced upon pretreatment. This may explain the heightened commitment of Th1 cells when mice were treated with IL-21 before EAE induction. The differential effects of IL-21 on EAE were attributed to its differing actions on NK cells. Furthermore, IL-21 pretreatment significantly increased the levels of circulating IgG and IgG2b antibodies, thus demonstrating an effect for IL-21 on autoantibody production.

Effects of IL-21 on the differentiation of B cells and generation of plasma cells were reported by Ozaki *et al.* (2004).[175] IL-21 was found to enhance Ig production, isotype switching and production of plasma cells. Habib *et al.* (2002) also reported the requirement of the γc chain for IL-21R signaling and for IL-21-induced activation of the JAK / STAT pathway.[176] The study also demonstrated that γc-associated JAK3 tyrosine kinase was an important mediator of γc-dependent IL-21 signaling.

Eastaff-Leung *et al.* (2010) reported similar findings in the peripheral blood of IBD patients to those observed in BD patients concerning the altered ratio of Tregs / Th17 cells.[39] Whereas Tregs and Th17 cells showed equivalent numbers in controls, IBD patients exhibited a decrease in Tregs and an increase in Th17 cells, and thus a disrupted Tregs / Th17 cell ratio in favor of Th17 cells. The expression of FoxP3, IL-17A, IL-1β and IL-6 was significantly higher

in the intestinal mucosa of IBD patients than in that of controls. Thus, the increase in Th17 cells was attributed to the cytokine environment of the intestinal mucosa. The increased FoxP3 expression in intestinal mucosa of IBD patients suggested active recruitment of Tregs for suppression of a proinflammatory response. The cytokine environment of the intestinal mucosa hence favored the generation of Th17 cells and inhibited the suppressive function of Tregs.

Immune tolerance towards self-antigens is initiated in the thymus and involves clonal deletion of autoreactive T lymphocytes. Peripheral exposure to extrathymic self-antigens as well as nonpathogenic foreign substances also plays a role in immune tolerance. One proposed mechanism for peripheral tolerance involves active immune suppression mediated by T cells. In 1997, Groux *et al.* reported the generation of a unique $CD4^+$ T cell subset that displayed immunoregulatory properties in BD patients.[177] Mouse ovalbumin-specific naïve $CD4^+$ T cells stimulated with splenic APCs and ovalbumin peptide in the presence of IL-10 displayed a cytokine profile (elevated levels of IL-10 and IL-5, and low levels of IL-2 and IL-4) that was distinct from that of Th0, Th1 or Th2 cells, and exhibited low proliferation in response to antigenic stimulation. These IL-10-induced cells, now known as Tregs, were designated T regulatory cells 1 (Tr1). The antigen-specific Tr1 cells were generated *in vitro* from naïve $CD4^+$ T cells of humans as well. They further demonstrated that antigen-specific activation of Tr1 inhibited the antigen-specific proliferation of other T cells, and thus suppressed antigen-specific T cell responses, in both mice and humans. This was explained by the cytokine profile of Tr1 cells that included high levels of the immunosuppressive cytokine IL-10 and low levels of the T-cell growth-promoting cytokines IL-2 and IL-4. They reported that Tr1 cells prevented the development of IBD, a T-cell-mediated disease, in mice.

Miyara *et al.* (2009) further identified three subpopulations of Tregs.[178] Human peripheral blood lymphocytes exhibited three distinct subpopulations of $FoxP3^+CD4^+$ T cells: two subpopulations, designated resting Tregs (rTregs), harbored a fully functional FoxP3 gene, hardly secreted cytokines and potently suppressed proliferation, and one population, designated activated Tregs (aTregs), expressed the FoxP3 gene to a lesser extent, secreted cytokines and did not suppress proliferation. The three subpopulations of $FoxP3^+CD4^+$ T cells exhibited different patterns of gene expression, as shown by a DNA microarray analysis. Notably, one of the populations contained cells with Th17 cell potential. Moreover, rTregs were not anergic and were able to proliferate upon TCR stimulation. They acquired aTregs phenotype and could exert suppression during and after proliferation and conversion to aTregs. After proliferation and exertion of suppression, aTregs died. The aTregs suppressed the proliferation of rTregs,

thus exerting a negative feedback. The results of this study revealed that the conversion of rTregs into aTregs occurred *in vivo*. There was a decrease in the proportion of aTregs and an increase in the proportion of rTregs in active systemic lupus erythematosus, an autoimmune disease.

Matzinger (2002) proposed that immune responses in situations similar to BD develop from a continuous autoimmune cascade as a result of signals emitted by injured host-cells.[160] This 'danger model' reaction inferred that the immune reaction overreacts to external stimuli. T cells and other antigen-presenting cells take hold of a process that feeds on itself in cases of a favorable genetic terrain. The 'pattern-recognition theory' consigns the injury by a 'non-self' entity to a permanent aggression, activating an uncontrolled adaptive response. As mentioned before, heat shock proteins, given their resemblance with pathogenic proteins, were proposed in this context (HSP60).[160]

Medzhitov (2002) proposed in this context that autoimmune disease such as BD may be an autoantigen-derived autoinflammatory disease.[161] This adaptive reaction to external triggers persists in situations of permanent pathogenic presence via autoantigen-activated dendritic T or B cells. This might help to explain why both anti-microbial agents and immunosuppressant therapies are to some extent effective in autoimmune diseases in general and BD in particular.[179] The depiction of Toll-like receptors (TLRs) as innate structures that detect and react to permanent proteins in infectious agents additionally suggested mechanisms by which the body's reaction to 'non-self' develops into a state of autoinflammation.[161,180] This may play a role in BD for several reasons: either the loss of built-in regulation of TLRs or the erroneous recognition of self-proteins by TLRs.[160] Cohen (2014) proposed that TLRs and the receptors IL-1, IL-18 and IL-33 play a crucial role in these circumstances: a state of hyperactivation becomes permanent, which brings about inflammatory tissue damage as observed in BD.[181]

Several reports illustrated histopathological findings in BD; in particular those involving neuro-BD.[8,37] In 1997, Hadfield *et al.* reported a case of neuro-BD that failed to respond to combined immunosuppressive treatment.[8] The patient exhibited waxing and waning neurological deficits and died three years after the initial diagnosis. Histopathological observations at the level of both the bowel and the central nervous system were performed. Diffuse mucosal necrosis, along with extensive intraluminal transudative fluid, was reported in the small and the large bowel. Extravasation of red blood cells was noted in some regions, suggesting occlusion with recanalization. No areas of perforation were noted. Diffuse venous

thrombosis and focal myonecrosis were observed; however, no vasculitis was diagnosed. Diffuse and patchy pneumatosis with dilation foci in the distal ileum and large bowel were found, suggesting pseudo-obstruction. On the other hand, gross examination of the brain showed minimal hydrocephalus and an arteriovenous malformation that involved the central aspect of the right temporal lobe. Patchy soft areas of discoloration without frank cavitation were observed in the basal ganglia, the thalamus, and the right cerebellar hemisphere. Atrophy was also detected in the medullary pyramids. Microscopic brain examination showed patchy areas of encephalitis at several locations within the neuraxis, mainly in the basal ganglia, right internal capsule, right inferior temporal gyrus, right hippocampus, and right cerebellar hemisphere. These sites exhibited rarefied or necrotic and cystic parenchyma, along with high infiltrations by mononuclear and polymorphonuclear leukocytes. The center of lesions showed complete destruction of myelin and axons, while demyelination with relative preservation of myelin was observed at the margins. Dense polymorphonuclear infiltration was observed around arterioles, and was mainly composed of eosinophils. Macrophages were also found, but were more abundant around venules which, unlike arterioles, exhibited sparse acute inflammatory cells in infiltrates that also contained lymphocytes. Fibrinoid necrosis, disruption of the elastica, intimal hyperplasia, microaneurysm formation and thrombosis were not detected. Areas surrounding the lesions exhibited edema, astrocytosis and macrophage infiltrates around and inside venules. In addition, scattered microglial nodules were noted. The white matter showed patchy demyelinated pale areas. Meningeal involvement was minimal and did not include polymorphonuclear leukocytes, but rather scattered lymphocytes. Vascular malformation was also observed in the meninges. Ultrastructural examination, as well as cultures and special stains, demonstrated the absence of viruses and microorganisms. In situ hybridization for HSV1, EBV and CMV was negative. The study inferred that the observed focal necrotic brain lesions might be due to acute inflammation, as demonstrated by the presence of neutrophilic and eosinophilic infiltrates in perivascular spaces and brain parenchyma, rather than due to vasculitis and thrombosis, which were absent. Furthermore, viral, fungal, bacterial and parasitic causes were excluded as shown by the negative tests for viruses and microorganisms.

In 2006, Arai *et al.* also reported an autopsy case of neuro-BD.[37] The total clinical course of the patient was three years. Four hours after death, autopsy was performed. The brain was fixed in buffered formalin, and slices were dehydrated, embedded in paraffin and sectioned. Sections were subsequently stained or subjected to immunohistochemistry. Gross

examination of the brain showed cerebral edema with flattened whole gyri, and symmetrical cerebral convexities with no clear herniations. Leptomeningeal thickening was absent except at the base of the brain around the arterial circle of Willis. Atrophy of the cranial nerves was also absent. Brain vessels did not exhibit thrombosis. No subdural or subarachnoidal hemorrhages were observed. Cerebral sections showed necrotic lesions in the left caudate nucleus and putamen and foci of brownish softening in the thalamus and hypothalamus. Cerebral white matter showed diffuse pale softening. The lateral ventricles exhibited symmetrical narrowing. Pseudohypertrophy of the bilateral inferior olivary nuclei was observed in the brain stem. Spots of brownish lesions were found in the midbrain near the medulla oblongata. However, no changes were noted at the level of the cerebellum. Microscopic cerebral examination showed tissue destruction in the cerebral lesions (left caudate nucleus and putamen) in addition to infiltration by mononuclear cells, including foamy macrophages and lymphocytes, and proliferation of astrocytes and microglia. Perivascular lymphocytic infiltration was observed and was significant around necrotic lesions but remained otherwise mild in the cerebrum. Remarkably, no fibrinoid necrosis or thrombosis was observed in these vessels. Diffuse myelin pallor was observed in the cerebral white matter and was thought to be due to severe brain edema rather than due to demyelination. The subarachnoid space of the cerebral base exhibited neutrophilic infiltration. The midbrain, pons and medulla oblongata showed meningoencephalitis upon microscopic examination of the brain stem. Leptomeninges on the ventral side and in the Virchow-Robin spaces showed dense inflammatory infiltrates that were either constituted of neutrophils or foamy macrophages and reactive astrocytes, thus demonstrating acute and chronic inflammation. Staining for microbes was negative. Secondary degeneration was observed in the pyramidal tracts of pons and in the medulla oblongata. In addition, loss of myelin sheaths and axons with foamy macrophages and reactive astrocytes were seen. Similar observations were noted in the optic nerve. Enlargement of neurons and vacuolar degeneration accompanied by gliosis were observed in the bilaterally pseudohypertrophic inferior olivary nuclei. Microscopic examination of the cerebellum showed small-scattered foci of non-bacterial or non-fungal acute inflammation. These foci consisted of neutrophilic infiltrates and cell debris with perivasculitis in their center. Fibrinoid necrosis, thrombosis and endothelial degeneration were absent in these vessels. Similar observations were noted in subarachnoidal spaces. The presence of lesions with neutrophilic inflammation surrounding vessels in brain parenchyma was explained by acute perivasculitis rather than vasculitis due to the absence of fibrinoid necrosis, thrombosis and endothelial degeneration.

Destruction of brain tissues and subsequent gliosis were thought to be the result of neutrophilic perivascular inflammation that eventually resulted in neurological dysfunction.

Another recent study by Hirohata (2008) reported histological examination of brain biopsies or autopsies from three patients with different types of neuro-BD: acute neuro-BD, chronic progressive neuro-BD, and neuro-BD in a long-term remission.[36] Brain tissues were fixed in formaldehyde and embedded in paraffin prior to sectioning. Sections were subsequently stained or subjected to immunohistological staining for B cells, T cells and monocytes. Histopathological examination of the patient with acute neuro-BD showed infiltrates of mononuclear and, to a less extent, polymorphonuclear leukocytes around small vessels in brain parenchyma. As shown by immunohistological examination, mononuclear infiltrates consisted mainly of $CD45RO^+$ T cells, and foam cells were composed of $CD68^+$ monocytes and very few $CD20^+$ B cells. Foam cells were presumed to be activated macrophages involved in the phagocytosis of damaged white matter. Apoptotic neurons, some of which were binucleated, were observed in inflammatory lesions. A role for proinflammatory mediators secreted by infiltrating T cells and monocytes in inducing apoptosis and binucleation of neurons was suggested. Similar observations were noted in the patient who died of progressive chronic neuro-BD, who, in addition, displayed disseminated foci of demyelination and gliosis in the hippocampus, pons and internal capsule. Examination of the patient with clinically inactive neuro-BD and who died of myocardial infarction revealed atrophy of the basal pons with formation of cystic or moth-eaten lesions. The lesions consisted of isomorphic gliosis and included viable neurons. It was suggested that the lesions resulted from repeated inflammatory attacks. In addition, infiltrates consisting of $CD45RO^+$ T cells and $CD68^+$ monocytes were observed around small vessels, suggesting the occurrence of minor inflammatory attacks during the remission stage.

Kobayashi *et al.* (2000) conducted an immunohistochemical study in an attempt to investigate the immunopathological mechanism of arterial lesions in vasculo-BD.[6] This study also included comparison of findings to those of Takayasu's Arteritis and inflammatory aneurysm, which, along with vasculo-BD, are inflammatory diseases of the aorta, mainly involving the tunica media and tunica adventitia. Specimens from six BD patients, four Takayasu's Arteritis patients and seven patients with inflammatory aneurysm were obtained at surgery and / or at autopsy. Paraffin-embedded tissue sections were stained or subjected to immunohistochemistry. Arterial lesions observed in BD specimens included abdominal aortic aneurysm, subclavian aneurysm, and iliac aneurysm. All lesions were of saccular type except

for one case of ruptured aneurysm. Cases with active arteritis exhibited lymphocytic and neutrophilic infiltrates mainly in the media and adventitia. However, cases with chronic arteritis showed scattered lymphoplasmacytic infiltrates and fibrous tissue. Inflammation around the vasa vasorum was mainly observed in active arteritis, and neutrophils were more abundant in BD compared to Takayasu's Arteritis or inflammatory aneurysm. Disruption of the internal elastic lamina was not as severe as in Takayasu's disease or in inflammatory aneurysm. Lymphocytic infiltrates in the media and adventitia mainly constituted of $CD3^+$ T cells in vasculo-BD. $CD20^+$ B cells were also present, and $CD8^+$ T cells were less prevalent than $CD4^+$ T cells. In addition, $CD68^+$ macrophages and monocytes were observed and were mainly abundant in the adventitia. The density of vasa vasorum was significantly higher in specimens from vasculo-BD than in those of Takayasu's disease or inflammatory aneurysm. In the BD samples, endothelial cells of the vasa vasorum in the media and adventitia expressed HLA-DR, unlike cases of Takayasu's Arteritis and inflammatory aneurysm. In addition, endothelial cells as well as infiltrating lymphocytes and neutrophils around the vasa vasorum expressed IL-1α and TNF-β. Staining for CMV and in situ hybridization for EBV were negative.

Collectively, these studies provide considerable insight as to the mechanisms and pathways by which autoinflammation cascades contribute to the pathogenesis of BD, and the ensuing histopathological outcomes.

4. Systemic Vasculitides

Vasculitis refers to a heterogeneous group of disorders that are characterized by an inflammatory destruction of blood vessels, regardless of type, size, and location. Vasculitis is primarily due to an immunological continuous feedback loop resulting in cytokine production, leukocyte migration and subsequent acute tissue damage, and is typical of an uncontrolled inflammatory cascade generally observed in other autoimmune diseases. Even though vasculitis is contingent to an immunological response, the actual cytokine trigger mechanisms remain poorly understood. It is assumed that these mechanisms grossly resemble those observed in similar autoimmune deficiency syndromes as revealed in the NOD model and human autoinflammatory diseases.

Different approaches have been undertaken for classifying vasculitides. One such approach implicates the underlying cause of the disease. For example, syphilitic aortitis would be considered an infectious vasculitis. Given that the causes and mechanisms of most vasculitides have not yet been distinctly mapped, the validity of this approach is limited. Another commonly used approach that focuses on the location of the affected blood vessel for classification. According to the International Statistical Classification of Diseases and Related Health Problems (ICD) – 10th revision (1992), cutaneous leukocytoclastic vasculitis is classified under the "L" category of skin conditions.[41,44,45,46,47] However, multi-organ systemic vasculitis disease renders this model obsolescent.

The most commonly used system in clinical practice for the classification of vasculitides remains the caliber of the affected blood vessels. For example, large vessel vasculitis affects large blood vessels and includes Takayasu's Arteritis, which involves the aorta and its main branches as well as the pulmonary arteries, along with others. Cerebral vasculitis is a form of medium vessel vasculitis that affects arteries of the brain and, occasionally, the spinal cord. On the other hand, Granulomatous Polyangiitis (GPA, formerly Wegener's Granulomatosis) is classified as a small vessel vasculitis and is known to involve arterioles and venules of many organs, most notably the kidneys, the lungs and the nose. BD, however, is a systemic form of vasculitis, and affects blood vessels of almost all types and sizes, including arteries and veins, both small and large. Given that BD impacts various types of vessels and expresses clinical manifestations at various body locations such as the eyes, mouth, skin and genitals, its classification is difficult using the aforementioned approaches.

Another difficulty in classifying systemic vasculitides stems from the fact that many conditions have vasculitis as an accompanying or atypical co-symptom. This includes rheumatoid arthritis, systemic lupus erythematosus, petechia, purpura, dermatomyositis, lymphomas and chronic hepatitis C (CHC) for example. Therefore, it is additionally imperative to be able to differentiate between vasculitides and vasculitis-inducing adjunct diseases.

Until recently, and in conjunction to a differential diagnosis, generic systemic inflammation laboratory tests of blood serum were the main investigative tools for patients with difficult to classify vasculitides. Active disease generally exhibited inflammation, detected in serum via an increased erythrocyte sedimentation rate (ESR), an elevated C-reactive protein (CRP), and anemia and leukocytosis – notably eosinophilia. A definite diagnosis of vasculitis was generally confirmed pursuant to a biopsy of the tissues affected when they were in reachable tissues such as the skin, sinuses, lung, nerve, or kidney for example. The biopsy confirmed blood vessel tissue histopathological damage and pattern.

Significant breakthroughs occurred respectively in 1982 and 1985 when Davies *et al.* and Woude *et al.* identified the presence of anti-neutrophil cytoplasmic antibodies (ANCA), mainly of the IgG type, against antigens in the cytoplasm of neutrophil granulocytes and monocytes in segmental necrotizing glomerulonephritis and in GPA.[182,183] Vasculitis-associated systemic diseases became classifiable by the presence or lack of these distinct biomarkers retrieved from serum. Consequently, vasculitides today are catalogued according to: those associated with ANCA biomarkers, and those not associated with ANCA biomarkers. In vasculitis, ANCA biomarkers are further divided into two main sub-groups, c-ANCA and p-ANCA, based on the main target antigen they link to: c-ANCA, (classical) or cytoplasmic antineutrophil cytoplasmic antibodies targeting the proteinase-3 antigen (PRO3); and p-ANCA, or peri-nuclear (protoplasmic-staining) antineutrophil cytoplasmic antibodies targeting the myeloperoxidase antigen (MPO). A third distinct ANCA, x-ANCA, is observed in several inflammatory diseases, but is not prevalent in vasculitis.[182,183]

For non-ANCA-associated vasculitides, clinical diagnosis, general inflammatory laboratory tests and biopsy remain the main tools for detection and differentiation. Such vasculitides include Behçet's disease, Mixed Cryoglobulinemia, Takayasu’s Arteritis, Giant Cell Arteritis and Sjögren Syndrome, in addition to others. No breakthrough has been made in identifying a biomarker (or a weighted combination of several biomarkers) specific to non-

ANCA-associated vasculitides. Diagnosis for non-ANCA-associated vasculitides is thus intrinsically contingent on a differential diagnosis conducted by an expert specialist.

5. Behçet's Disease

BD, also known as the Silk Road Disease, is a rare systemic vasculitis disorder of unknown etiology.[2,17] While chronic inflammatory disorders are persistent in nature, BD is characterized by recurrent attacks of acute inflammation.[2] Recurrent oral aphthous ulcers, genital ulcers, skin lesions, and ocular injuries are the most common manifestations that characterize BD, and are all self-limiting in time except for ocular lesions, which ordinarily lead to permanent impaired vision.[2,17] Relapsing episodes of clinical manifestations represent a hallmark of BD; frequency and duration are unpredictable.[2,17] Other less frequent, yet life-threatening, manifestations involve the central nervous system (CNS), the main large vessels and the gastrointestinal tract.[2,17] BD is known to have a heterogeneous onset and is associated with significant morbidity and high mortality.[1]

The first description of an illness that shared the clinical manifestations of BD was reported by Hippocrates in the 5th century BC, in the "Third Book of Endemic Diseases"; another early description of similar symptoms was reported by the Chinese physician Zhong-Jing Zhang in approximately 200 AD.[1,19] However, until the 18th century, no further descriptions of patients with manifestations similar to those of BD were reported.[1,19] In 1908, a triad of symptoms involving urogenital ulceration and iritis was reported by Bluthe *et al.*; subsequently, Planner and Remenovsky (1923) followed by Shigeta (1924) reported similar observations.[19] However, these symptoms were thought to be caused by tuberculosis or syphilis, until they were proposed by Hulusi Behçet, a Turkish dermatologist, as a separate disease entity in 1937.[19]

Born in Istanbul in 1889, Hulusi Behçet graduated from the Gulhane Military Medical School in 1910 and completed his specialization in dermatology and syphilology in 1914.[19] In 1924, Behçet first documented the clinical examination of a patient with recurrent aphthous stomatitis, genital ulcers, erythema nodosa and visual disturbances.[19] Behçet subsequently documented two other similar cases in 1930 and 1936, and proposed in 1937 that the three observed major signs (recurrent oral aphtae, genital ulcerations, and hypopyon uveitis) constituted an independent clinical syndrome.[1,19] Behçet published his findings in both German and French journals in 1937 and 1938, respectively.[1] Later in 1939-1940, he started using the term "triple-symptom complex" to refer to the triad of observed clinical signs.[1] After Behçet's descriptions, similar reports from different regions followed.[2] The eponym "Behçet" was first

used by Jansen, a Danish physician, in 1941 and subsequently become the norm.[1] In 1947, the disease was recognized in the International Congress of Dermatology in Geneva, and was formally named as "Morbus Behçet" as proposed by Zurich Medical Faculty professor Mischner.[1,19] Worth mentioning is that Benedictos Adamantiades reported a similar case in 1931, thus the disease is sometimes referred to as Adamantiades-BD.[1,19] However, "Behçet's Disease" remains the preferable name as recommended by the International Associations and Societies of Behçet.[1]

BD is now recognized worldwide as a systemic vasculitis that, in addition to the triple-symptom complex, has neurological, gastrointestinal and cardiovascular involvements.[1,19] Due to its high prevalence in countries that coincide with the Old Silk Route, an ancient trade route that extends between the Mediterranean, Middle East and Far East, the disease was designated by Ohno as the "Silk Road Disease" in 1982.[1,19]

Reports reviewing the epidemiology of BD confirm that the disease mainly occurs along the Old Silk Route between latitudes 30° and 45° north in an area extending from the Mediterranean basin to eastern Asia and including Turkey, Iran, Iraq, Israel, India, Korea, China and Japan, with a prevalence ranging between 1 / 10,000 and 1 / 1,000.[1,2,17] This peculiar geographical distribution of BD may be explained by the contribution of itinerant traders in spreading a genetic risk factor.[19] Among the Old Silk Road countries, Turkey was reported to have the highest disease prevalence ranging from 80-420 cases per 100,000 depending on the geographical location.[1,20] A cost-analysis study conducted in Turkey reported that BD imposes a significant economic burden both directly and indirectly.[19,23] Following Turkey, Japan has the high reported prevalence of 7-8.5 / 10,000. Disease prevalence in Asian countries, including Japan, Korea, China, Iran and Saudi Arabia, was found to range from 13.5 to 20 cases per 100,000.[1,20] Disease prevalence is lower in Western countries and ranges from 0.12 to 0.64 / 100,000, with 0.64 cases per 100,000 in the United Kingdom and 0.12-0.33 cases per 100,000 in the United States.[184] In addition to a higher prevalence, BD was found to exhibit more severe manifestations, including ocular, vascular and neurological inflammation, in Old Silk Road countries versus Western countries. Interestingly, immigrants from countries with high prevalence tend to have a lower probability of developing the disease in relatively low prevalence regions; for instance, disease prevalence among Turkish individuals in Germany was found to be 21 / 100,000, which is lower than that of Turkey but higher than that of the German population (0.42-0.55 / 100,000) at large.[19,184]

Whereas BD primarily affects males in the Middle East, it is more recurrent amongst females in Japan and Korea. However, the disease appears to be associated with worse clinical manifestations in males in all regions combined.[19] Higher risk of ocular, neurological and cardiovascular involvement was noted amongst male individuals who generally exhibit an earlier disease onset and worse prognosis with regards to blindness and mortality.[17]

BD rarely develops before puberty or after the age of 50. Typically, it occurs between the 3rd and the 4th decades of age. Onset age was correlated with disease severity. It was reported that earlier onset is associated with more severe clinical manifestations. However, studies from Korea and Turkey suggest the absence of a link between late disease onset (40 years of age or above) and mild clinical evolution.[17]

Familial aggregation of BD was reported to occur in 1-18% of patients, most notably in Turkish, Israeli, and Korean patients.[20] In addition, the incidence of familial aggregation was found to be higher in juvenile patients with BD. The frequency of the disease within families is 2-5%, except in the Middle East where it increases up to 10-15%. However, the frequency of concordance among twins remains unsettled. One study reported concordance for the disease among a pair of monozygotic twins, while a separate study reported discordance between two other pairs.[20,22]

Although no studies reported disease prevalence in a particular socio-economic group, a study was conducted in Turkey in order to assess the economic impact of BD patients. Compared to ankylosing spondylitis and inflammatory bowel disease patients, BD patients were observed to have lower monthly income, wealth score and education, and higher unemployment.[19,23]

Collectively, the epidemiological data presented above suggest the involvement of both genetic and environmental factors in the development of BD.

6. Clinical Features of Behçet's Disease

A systemic disease, BD may affect almost all vascularized body systems and is characterized by episodes of relapses and remissions leading to sequelae.[1,11,12,185] Although several clinical manifestations are associated with BD, the triple-system complex of oral and genital aphthae and uveitis first described by Behçet in 1937 generally illustrates disease pattern. Clinical manifestations in children (<16 years) resemble those of adults.[20,21] Children exhibit more frequent perianal aphthosis and arthralgia, less frequent genital ulcers and vascular involvement, and a more severe course of uveitis.[1,186] Over the past few years, modern treatment strategies, involving immunosuppressant therapy and the use of aggressive approaches have led to improvements in the prognosis of severe forms of BD.[4,187] Prognosis for the disease is usually reserved, especially when ocular, cardiovascular, neurological, and / or gastrointestinal manifestations appear.[5,185,188]

6.1. Oral Ulcers

Recurrent oral ulcers represent the earliest disease manifestation in 47-86% of patients.[188] It may take years for the other symptoms to appear afterwards, and oral ulcers are observed in all patients during their clinical course. Lesions resemble common oral aphthous ulcers, but are more painful and wider. They have disciform appearance with round and sharp erythematous border, covered with a grayish-white pseudomembrane or a central yellowish fibrinous base and grow rapidly from a flat ulcer to a deep sore.[1] They may occur as single ulcers or in crops and heal with little scarring.[1,2] Oral ulcers most commonly affect the gingival and buccal mucosa, tongue and lips, yet may also appear in the soft and hard palates, pharynx and tonsils.[1] In certain cases, oral ulcers derive from buccal trauma. Minor ulcers (<1 cm in diameter) heal without scarring in 4-14 days whereas major ulcers (>1 cm in diameter) are more painful and heal with scarring in 2-6 weeks. Herpetiform ulcers occur in recurrent crops of small sores that are 0.2-0.3 cm in diameter, are painful and may coalesce. Treatment is usually symptomatic and prognosis of oral ulcerations is favorable.[2,188]

6.2. Genital Ulcers

Genital ulcers develop in 57-93% of patients.[5] They are painful and morphologically resemble oral ulcers, but are larger, deeper, have more irregular margins and heal with white or

pigmented scars.[188] Male genital lesions most commonly involve the scrotum and usually leave a scar that will help with the diagnosis retrospectively. They may also affect the epididymis; penile lesions are less frequent.[1] In females, vulvar, vaginal and cervical lesions are especially common.[2] Rarely, deep vaginal lesions may perforate the bladder resulting in fistulae.[5,188] Both males and females may develop perineal, perianal and groin lesions.[188] In cases of fistulae and internal lesions, prognosis is unfavorable when the infectious risk is inadequately evaluated.[1,2]

6.3. Ocular Manifestations

Ocular disease, involving the retina and the uvea, occurs in 30-70% of BD patients and is associated with high morbidity.[4] It is the primary cause of blindness in approximately 25% of patients despite aggressive corticosteroid treatment.[4] Ocular symptoms occur more frequently in males and are associated with disease severity, even though prognosis is improving with the use of aggressive immunosuppressant therapy.[1,2] They usually occur two-three years after the onset of oral or genital ulcerations but remain the first disease manifestation in 10-20% of patients.[1] Typically, ocular disease is a chronic relapsing bilateral non-granulomatous uveitis that may involve the anterior segment, the posterior segment, or both (panuveitis).[185,186] The latter is associated with a worse prognosis and is more common among males.[1] Ocular disease is characterized by the formation of hypopyon: a visible layer of pus in the anterior chamber observed in approximately one-third of patients.[2] Episodes of anterior uveitis subside spontaneously yet repeated attacks result in irreversible structural deformities.[7,186] Ocular inflammation also includes iridocyclitis, keratitis, episcleritis, scleritis, vitritis, vitreous hemorrhage, retinal vasculitis, retinal vein occlusion, retinal neovascularization, and optic neuritis.[4]

Symptoms include blurred vision, photophobia, lacrimation, floaters, hyperemia and periorbital or global pain.[1,2] Recurrent inflammatory attacks are associated with secondary complications such as posterior and peripheral anterior synechia, iris atrophy, cataracts resulting from inflammation or treatment, secondary glaucoma (occasionally neovascular), atrophic retina, optic atrophy, macular edema, macular degeneration, retinal veins occlusion, sheathed vessels, chorioretinal scars and proliferative vitreoretinopathy and phthisis bulbi.[1,4] Prognosis is correlated with frequency, severity of ocular inflammation, and extent of lesions, and remains in many cases unfavorable.[4]

6.4. Cutaneous Manifestations

Skin involvement affects 38-99% of BD patients.[1,11] Cutaneous manifestations commonly include papulopustular (28-96%) and acne-like lesions.[5,20] Wounds exhibit a wide distribution affecting the face, limbs, trunk and buttocks.[1] Skin lesions are characterized by thrombosis and vasculitis.[11] Early lesions exhibit leukocytoclastic vasculitis or neutrophilic vascular reactions whereas mature lesions are characterized by lymphocytic vasculitis.[5]

Erythema nodosum lesions occur in 15-78% of patients, mainly in females and in the lower limbs.[1,2] These lesions are painful, may form ulceration and usually heal leaving residual pigmentation. Cutaneous ulcers are rare and only affect 3% of BD patients.[1] They resemble aphthous ulcers, are recurrent, and typically heal with scarring. They appear in the neck, breast, axillae, inguinal region, legs and interdigital skin of the feet.[188] Prognosis for most cutaneous lesions in BD is usually favorable.[1]

6.5. Cardiovascular Manifestations

Behçet's disease may affect blood vessels of different sizes and types, including arteries and veins as well as the heart organ.[189] Cardiovascular features were reported to affect 7-49% of patients, more frequently males.[1,190] They occur approximately 3-16 years after the onset of BD.[13]

Vascular BD commonly affects veins causing recurrent superficial thrombophlebitis and deep venous thrombosis in 30-40% of patients.[7,191,192] Thromboses of the superior and inferior vena cava (0.2-9% of patients), dural sinuses and supra-hepatic veins (2-3.2% of patients), and pulmonary arterial aneurysms (1% of patients) may also occur and are associated with poor prognoses.[1,189,192] Occlusion and aneurysms of major arteries commonly lead to bleeding, infarction, organ failure and restricted movements of arms and legs.[2] Rupture of aneurysms may be fatal. At the level of lungs, thrombosis, aneurysm, and arteriobronchial fistula cause recurrent episodes of dyspnea, cough, chest pain and hemoptysis.[2]

Cardiac involvement includes pericarditis, myocarditis, endocarditis, mitral valve prolapse, valve lesions, intracardiac thrombosis, endomyocardial fibrosis, myocardiopathy, and coronary artery lesions, and is the result of systemic vascular involvement.[29,190,193] The prognosis in these cases is unfavorable with frequent recurrences.[1,193] The highest direct

mortality rate in cardiovascular involvement was attributed to large vessel vasculitis as a result of sudden death by aneurysm rupture or thrombosis (9.8% of patients in one study in Turkey).[21,190,194]

6.6. Neurological Manifestations

Neurological involvement in BD (neuro-BD) occurs in 5-10% of patients and is more frequent in males.[7,8,37] It usually occurs around five years after the onset of the disease and is associated with long-term morbidity and mortality.[1,195] Neurological disease affects the CNS more frequently than the peripheral nervous system.[196,197] Headache syndromes represent the most common neurological symptom and occur in 70% of patients.[7,198]

Neuro-BD may be parenchymal (80% of patients), non-parenchymal, or mixed brain disease.[1,7] Parenchymal brain disease affects the brainstem and / or basal ganglia and is correlated with a particularly poor prognosis.[7,199] Non-parenchymal brain disease is characterized by dural sinus thrombosis, arterial vasculitis, and aseptic meningitis, and comprises the most devastating symptom category of BD.[7,37]

Most parenchymal neuro-BD cases present as meningoencephalitis (75%) that exhibit subacute onset and are associated with exacerbation of systemic manifestations.[195,196] Flare-ups peak within a few days and may last for periods of weeks. Brainstem involvement, including ophtalmoparesis, cranial neuropathy, and cerebellar or pyramidal dysfunction, has additionally been reported.[1,7,197,200] Cerebral or spinal cord involvement was observed in association with subcortical dementia, accompanied by ataxia.[195,199] Cerebral hemispheric involvement, including encephalopathy, hemiparesis, hemisensory loss, seizures and dysphasia, and mental changes, including cognitive dysfunction and psychosis, were observed as well.[7,8,201] Spinal cord involvement, manifested by pyramidal signs in the limbs, sensory level perturbance and sphincter dysfunction, has also been reported.[7,201,198] Other less common clinical symptoms involving the CNS were reported as well such as: stroke, epilepsy, brain tumor-like neuro-BD, movement disorders, acute meningeal syndrome, optic neuropathy, spinal cord involvement and asymptomatic and subclinical neurological involvement.[195,200,202]

Non-parenchymal neuro-BD, also referred to as vasculo-BD or angio-BD, involves the main vascular structures of the CNS.[197] Clinical syndromes include vascular disorders,

intracranial hypertension, and intracranial aneurysms.[203] More rarely, mixed parenchymal and non-parenchymal disease were reported with limited collected data.[203]

Classically, meningitis or meningoencephalitis, neurological deficits including motor disturbances, and brainstem symptoms and psychiatric symptoms including personality changes develop in patients with neuro-BD.[2,7] These symptoms are associated with disease exacerbations and gradually cause irreversible disability.[2] At late stages, dementia develops in approximately one-third of patients. As such, psychiatric or cognitive symptoms may represent the first manifestation of neuro-BD.[2,204] Cognitive impairment in neuro-BD patients includes poor memory, attention and motivation, in addition to personality changes.[204] Psychosomatic symptoms, including anxiety and depression, are the most common psychiatric symptoms in BD and physicians must remain particularly attentive to other causes of psychiatric morbidity; however, these manifestations are rarely a result of direct CNS involvement. Prognosis for neuro-BD, in all its forms, is unfavorable.[5,7,198]

6.7. Articular Manifestations

Articular involvement occurs in 45-60% of patients and includes either monoarthritis or polyarthritis.[205] Articular disease includes arthralgia, arthritis and synovitis.[205] Non-erosive, non-deforming oligoarthralgia commonly involving the knees, ankles, elbows, and wrists is the most frequent manifestation.[1,2] Neutrophilic and mononuclear cell infiltrates in the synovium and small-vessel lesions with thrombosis typically characterize articular disease.[2] Destructive changes rarely occur in patients with articular involvement.[206] As anti-inflammatory treatment is generally effective, prognosis is generally favorable.[207]

6.8. Gastrointestinal Manifestations

Gastrointestinal involvement occurs in 3-26% of patients and varies among different populations.[9,208] It is much more frequent in Japan than in the Middle East and the Mediterranean region.[1,19,11] Mucosal inflammation and ulceration occur throughout the gastrointestinal tract and are generally located in the ileocaecal region.[9,208] The esophagus, ascending colon, and transverse colon are less frequently involved.[2] Clinical symptoms include anorexia, vomiting, dyspepsia, diarrhea, melena, abdominal pain and, less frequently, perforation requiring surgical intervention.[207,209] Prognosis is unfavorable as gastrointestinal involvement is typically acute and chronic.

7. Diagnosis of Behçet's Disease

A Behçet's disease diagnosis is typically confirmed by elimination of other disease scenarios, even when a triple-symptom complex is evident. As there exists no targeted diagnostic test for BD, diagnosis of clinical symptoms is challenging, especially when symptoms are non-concomitant. Investigating the clinical history of patients helps exclude other conditions during diagnosis of BD. These conditions include Reiter's syndrome, sarcoidosis, Stevens-Johnson syndrome, familial Mediterranean fever, multiple sclerosis, systemic lupus erythematosus, mixed connective tissue diseases, celiac disease, inflammatory bowel disease (Crohn's disease and ulcerative colitis), Herpes Simplex virus infection, syphilis, Sweet's syndrome, Vogt-Koyanagi-Harada syndrome, bullous skin disorders, erythema multiforme, recurrent aphthous stomatitis, seronegative arthropathies, and Easle's disease.[1] For instance, differentiation between the gastrointestinal involvement in BD and inflammatory bowel disease is often difficult due to the similarity in the extraintestinal symptoms (oral ulceration, erythema nodosum, uveitis and arthritis). In addition, intestinal ulceration in BD patients is indistinguishable from that in patients with ulcerative colitis. Likewise, neurological involvement in BD patients may be misdiagnosed as multiple sclerosis. Distinguishing features, such as granuloma formation in patients with Crohn's disease and negative pathergy test in patients with inflammatory bowel disease, may help with the differential diagnosis. HLA typing and measurement of serum IgD levels, which are known to be elevated in patients with active BD, may also be helpful.[2]

In the absence of a universally accepted diagnostic test, detection of BD has centered on the identification of a number of the typical clinical features associated with the disease. Over the years, five major independent sets of criteria were proposed for the diagnosis of BD, each characterized by its own clinical features and number and nature of criteria that should be met in order for the diagnosis to be positive.[3,33] Notably, all the different sets of diagnostic criteria shared the three major symptoms initially described by Behçet as a separate clinical entity (oral ulceration, genital ulceration, and eye lesions).[1]

At present, there are no or few findings specific for BD.[1] Elevated levels of inflammatory markers may be associated with the disease. These include C-reactive protein, erythrocyte sedimentation rate, peripheral leukocyte and platelet counts and serum cytokines (TNFα, IFNγ, IL-1β, IL-6 and IL-8). Moderate anemia of chronic disease may also be present;

however, autoantibodies, such as antinuclear antibodies and rheumatoid factor, are usually absent.[1]

According to Mason and Barnes (1969), diagnosis of BD was positive when three major criteria or two major and two minor criteria were present.[210] Major criteria included oral ulceration, genital ulceration, eye lesions (uveitis, corneal ulceration, retrobulbar neuritis) and skin lesions (pustules, ulceration, erythema nodosum, erythema multiforme). Minor criteria included gastrointestinal lesions, thrombophlebitis, cardiovascular lesions, arthritis, CNS lesions, and family history.

Another set of diagnostic criteria was proposed by BD Research Committee of Japan in 1972, and was subjected to revision in 1987 and 2003.[211,212] These diagnostic criteria classified the disease as complete, incomplete and suspected. Complete disease was considered when four major criteria were present, incomplete disease when three major criteria, two major and two minor criteria or typical recurrent ocular symptom along with one major or two minor criteria were present, and suspected disease when one or two major criteria were present. Major criteria comprised recurrent aphthous ulceration of the oral mucous membrane, skin lesions (subcutaneous thrombophlebitis, folliculitis, acne-like lesions, cutaneous hypersensitivity), eye lesions (iridocyclitis, chorioretinitis, retino-uveitis, definite history of chorioretinitis or retino-uveitis) and genital ulceration. Minor criteria involved arthritis without deformity or ankylosis, gastrointestinal lesions characterized by ileocaecal ulcers, epididymitis, vascular lesions, and CNS symptoms.

In 1974, O'Duffy considered positive diagnosis of BD when oral or genital ulceration along with two other criteria and vasculitis in tissue biopsies were present.[213] Major criteria included oral ulceration, genital ulceration, uveitis and dermal vasculitis (erythema nodosum). Minor criteria included arthritis, CNS involvement, colitis, phlebitis and large vessel arteritis.

Subsequent diagnostic criteria included a positive pathergy test. Zhang (1980) considered complete disease when three major or two major and two minor criteria were present and incomplete disease when patients exhibited two major, or one major and two minor criteria.[214] Major criteria included oral ulceration, genital ulceration and uveitis. Minor criteria included skin (erythema nodosum, erythema multiforme, pathergy), arthritis, vasculitis (thrombophlebitis, arteritis, aneurysm), pulmonary (hemoptysis, lung infiltration, interstitial fibrosis), gastrointestinal lesions (ulceration, bleeding, perforation), renal (renal damage,

ulceration of bladder, hematuria, epididymitis) and neurological features. In 1986, Dilsen *et al.* also proposed a set of diagnostic criteria that included a pathergy test.[215]

More recently, an International Study Group (ISG) for BD, which was founded during the Fourth International Conference on BD (1985) in London, compared the performance of the previously defined sets of benchmarks and established a new internationally approved set of diagnostic criteria for BD. The ISG included proponents of four of the five previously described sets of diagnostic criteria in an attempt to agree on one set to be quoted in all future work, and thus enable comparison between studies and promote collaborative research. These criteria, published in 1990 (and updated in 2013) provided simpler means for diagnosis of BD, and unlike the previously defined sets of criteria, excluded rare and subjective features and showed more specificity with little or no loss of sensitivity.[2,16] The new set of diagnostic criteria proposed by the ISG imposed the presence of recurrent oral ulceration, in addition to any two of recurrent genital ulceration, eye lesions, skin lesions or positive pathergy test. Recurrent oral ulceration included minor aphthous, major aphthous or herpetiform ulceration observed by a physician or patient and recurring at least three times in a 12-month period. Recurrent genital ulceration included aphthous ulceration or scarring observed by physician or patient. Eye lesions included anterior uveitis, posterior uveitis or cells in vitreous on slit lamp examination, or retinal vasculitis, and observed by an ophthalmologist. Skin lesions included erythema nodosum observed by the physician or patient, pseudofolliculitis or papulopustular lesions, or acneiform nodules observed by a physician in post-adolescent patients not receiving corticosteroid therapy. Finally, positive pathergy test was to be read by a physician at 24-48 hours.

Likewise, there is no diagnostic test for neuro-BD, and thus diagnosis of neurological involvement in BD is based on clinical aspects.[198] However, unlike BD, no criteria were established for the diagnosis of neuro-BD. In such cases, analysis of blood work, and CSF and MRI examination provide nonspecific tools for the diagnosis of neurological involvement.[7,198]

Measurement of disease activity is essential for the assessment of disease progression and for proper clinical management. Several forms were proposed for the assessment of the clinical activity of BD.[1] These include the Iranian BD Dynamic Activity Measure, the European BD Current Activity Form, and the standard proposed by the BD Research Committee of Japan.

In 2004, Lawton *et al.* conducted a study in an attempt to define a set of clinical features that can be used as a standard index for measurement of BD activity, and thus provide international means for monitoring disease progression, clinical management, and evaluating the efficacy of therapeutic interventions.[15] Five countries participated in this study, namely China, Korea, Iraq, Turkey and UK. Between 1995 and 2002, 542 BD Current Activity Forms were completed. Using the Rasch method, the study analyzed fourteen items, previously defined to form an index of disease activity. These items included the presence or absence of arthralgia, arthritis, diarrhea, erythema nodosum, eye inflammation, genital ulcers, headaches, mouth ulcers, nausea / vomiting, new CNS involvement, new major vessel inflammation and pustules over the last four weeks prior to the clinic visit, in addition to a Likert scale. The latter is represented by "smiley" faces ranging from very bad to very good and describes how the patient or the clinician felt the disease activity had been over the last four weeks. It was inferred that the BD Current Activity Form provided a convenient tool that may be used to develop an overall score for disease activity, that can serve as an index in clinical trials involving therapeutic interventions.[14,15]

8. Treatment of Behçet's Disease

As no curative solution is currently available, treatment of BD attempts to relieve symptoms, resolve inflammation, limit tissue damage, reduce recurrence frequency and severity, and avoid life-threatening complications.[1] Choice of treatment depends on the combinations of clinical symptoms and the severity of organ involvement, with priority given to treatment of ocular, gastrointestinal, CNS, and cardiovascular manifestations.[17]

8.1 EULAR Guidelines

Mendes *et al.* (2009) summarized the recommendations of the European League Against Rheumatism (EULAR) for the management of BD.[1] The recommendations combined current evidence from clinical trials and were developed by a multidisciplinary expert committee. The committee included specialists from six European countries plus Tunisia and Korea and consisted of nine rheumatologists, three ophthalmologists, one internist, one dermatologist, and one neurologist, in addition to a patient representative.

Nine recommendations were developed for the treatment of BD in its different aspects (eye involvement, refractory eye involvement, major vessel disease, anticoagulation, gastrointestinal involvement, joint involvement, neurological involvement, cyclosporine A neurotoxicity, and mucocutaneous involvement). The recommendations target all doctors and surgeons who are involved in the treatment of BD and are summarized in ***Table 1*** below.

Table 1: EULAR General Treatment Recommendations for the Treatment of Behçet's Disease[1,18]

I.	Any patient with BD and inflammatory eye disease affecting the posterior segment should be on a treatment regime that includes azathioprine and systemic corticosteroids;
II.	If the patient has severe eye disease defined as >2 lines of drop in visual acuity on a 10 / 10 scale and / or retinal disease (retinal vasculitis or macular involvement), it is recommended that either cyclosporine A or infliximab be used in combination with azathioprine and corticosteroids. Alternatively IFN-α with or without corticosteroids could be used instead;
III.	There is no firm evidence to guide the management of major vessel disease in BD. For the management of acute deep vein thrombosis in BD, immunosuppressive agents such as corticosteroids, azathioprine, cyclophosphamide or cyclosporine A are recommended. For the management of pulmonary and peripheral arterial aneurysms, cyclophosphamide and corticosteroids are recommended;
IV.	Similarly, there are no controlled data on, or evidence of benefit from uncontrolled experience with anticoagulants, antiplatelet or anti-fibrinolytic agents in the management of deep vein thrombosis or for the use of anticoagulation for the arterial lesions of BD;
V.	There is no evidence-based treatment that can be recommended for the management of gastrointestinal involvement of BD. Agents such as sulfasalazine, corticosteroids, azathioprine, TNFα antagonists and thalidomide should be tried first before surgery, except in emergencies;

VI. In most patients with BD, arthritis can be managed with colchicine;
VII. There are no controlled data to guide the management of CNS involvement in BD. For parenchymal involvement, agents to be tried may include corticosteroids, IFN-α, azathioprine, cyclophosphamide, methotrexate and TNFα antagonists. For dural sinus thrombosis, corticosteroids are recommended;
VIII. Cyclosporine A should not be used in BD patients with central nervous system involvement unless necessary for intraocular inflammation;
IX. The decision to treat skin and mucosa involvement will depend on the perceived severity by the doctor and the patient. Mucocutaneous involvement should be treated according to the dominant or codominant lesions present. Topical measures (i.e. local corticosteroids) should be the first line of treatment for isolated oral and genital ulcers. Acne-like lesions are usually of cosmetic concern only. Thus, topical measures as used in acne vulgaris are sufficient. Colchicine should be preferred when the dominant lesion is erythema nodosum. Leg ulcers in BD might have different causes. Treatment should be planned accordingly. Azathioprine, IFN-α and TNFα antagonists may be considered in resistant cases.

It should be noted that differences in health care systems and in the economic status of different countries, as well as the side effects engendered by therapies, were all taken into consideration while developing the recommendations.

8.2. Therapies

A variety of approaches were undertaken for the treatment of BD, including anti-inflammatory and immunosuppressive therapies.[11,216] However, long-term treatment was associated with significant adverse effects and none of the treatment strategies had healing outcomes.[11]

The choice of the treatment depends on the clinical manifestations and severity of involvement.[1,2] Priority is given to the treatment of gastrointestinal and CNS involvement and large-vessel lesions, which necessitate the use of high corticosteroid doses and / or immunosuppressants, and sometimes surgical intervention.[2] ***Table 2*** below summarizes commonly used therapies with a focus on organ involvement.

Table 2: Characteristic Therapies for Behçet's Disease

Treatment	Characteristic clinical manifestation focus	Immunosuppressive mechanisms of action
Apremilast	Mucocutaneous	Phosphodiesterase-4 inhibitor
Anti-Tumor Necrosis Factor-α	Ocular Neurological Cardiovascular Gastrointestinal	Neutralize biological activity of TNFα
Azathioprine	Articular Ocular Cardiovascular Mucocutaneous	Inhibit synthesis of DNA and RNA and proliferation of T and B lymphocytes
Colchicine	Articular Mucocutaneous Ocular Neurological Cardiovascular Gastrointestinal	Inhibit neutrophil function
Corticosteroids	Articular Mucocutaneous Ocular Neurological Cardiovascular Gastrointestinal	Inhibit neutrophil function General immunosuppressive activity
Cyclosporine A	Neurological Ocular	Inhibit lymphocyte function, inhibit activation and recruitment of T lymphocytes
Dapsone	Mucocutaneous	Antibacterial agent
Interferon-α	Ocular Neurological Cardiovascular Gastrointestinal	Antiviral activity
Methotrexate	Mucocutaneous Neurological Ocular Articular	Inhibit synthesis of DNA, RNA, and thymidylates
Pentoxyfilline	Mucocutaneous	Inhibit synthesis of cytokines
Sulfasalazine	Articular Gastrointestinal	General immunosuppressive activity
Thalidomide	Mucocutaneous Gastrointestinal	Unidentified immunomodulatory activity

Corticosteroids are used for treatment of ocular disease, mucocutaneous disease, gastrointestinal, neurological, and cardiovascular involvement and are thought to inhibit neutrophil function.[1,2] Treatment is initiated with high doses, which depending on the clinical

manifestations, are tapered over several weeks.[1] Corticosteroids are administered topically (for treatment of ocular and mucocutaneous disease), as periocular injections (for treating ocular disease) and / or as systemic therapy (oral prednisolone or intravenous methylprednisolone).[1] Corticosteroid drops are prescribed for attacks of anterior uveitis.[2] Topical injection of corticosteroids, sometimes combined with systemic administration, is used for the treatment of acute attacks of posterior uveitis.[2] Systemic corticosteroids are administered for erythema nodosum cases that are refractory to colchicine treatment.[2]

Although corticosteroids have potent anti-inflammatory effects, they are often used in combination with other treatments due to their inability to prevent relapses.[1] Combined treatment also provides means for reducing administered doses of corticosteroids.

Severe side effects were reported with corticosteroid treatment. These include increased intraocular pressure, cataract, gastrointestinal ulceration, hypertension, diabetes mellitus, electrolyte abnormalities, osteoporosis, decreased resistance to infections, and Cushingoid appearance.[1]

A study has shown that low-dose depot corticosteroids failed to treat genital ulcers, oral lesions, folliculitis and arthritis, but were effective in treating erythema nodosum in female patients.[1]

Colchicine is a plant alkaloid that exerts its anti-inflammatory effects by inhibiting neutrophil migration by interfering with microtubule formation. It has shown efficacy in treating uveitis (anterior and posterior), and cutaneous and articular involvement in BD.[1,2] Associated side effects include nausea, vomiting, diarrhea and abdominal pain. It may also rarely cause alopecia and bone marrow suppression. Thus, blood counts should be closely monitored in patients on colchicine treatment.

Alkylating agents are known to interfere with DNA replication, thus inhibiting lymphocyte proliferation and functioning. They are used in combination with corticosteroids for treatment of refractory eye disease and CNS involvement.[1] Due to their dose-dependent side effects, they are used in cases that are refractory to other treatments. A study on BD patients with refractory uveitis has shown improvement in ocular involvement in two-thirds of cases and a reduction in the number of attacks upon short-term chlorambucil treatment.[1]

Treatment with alkylating agents is associated with side effects, such as bone marrow suppression, hepatotoxicity, secondary malignancies, and infertility.

Current treatment strategies recommend the use of cyclophosphamide for cases involving neurological and cardiovascular disease.[1]

Methotrexate, a folate analogue, has shown efficacy in the treatment of CNS involvement, anterior uveitis, and severe mucocutaneous disease.[1] As a DNA synthesis inhibitor, side effects are usually severe, notably affecting the liver and bone marrow organs.

Calcineurin inhibitors impair the activation and recruitment of T lymphocytes. They are useful for treating most features associated with BD, mainly eye disease refractory to colchicine, corticosteroids, azathioprine, and cyclophosphamide; cyclosporine A is the most commonly used therapy in this instance.[2] Treatment with cyclosporine A in combination with corticosteroids has a corticosteroid-sparing effect, and thus allows the use of reduced doses of the latter.[1]

Due to their cytostatic effects, relapses may occur upon reduction or discontinuation of calcineurin inhibitors.[1]

Side effects associated with calcineurin inhibitors include neurotoxicity, hepatotoxicity, nephrotoxicity, hypertension, hirsutism, paraestesia, gastrointestinal manifestations, and gingival hyperplasia. However, most of these symptoms subside upon discontinuation of the drug, and in some cases require the intervention of corticosteroids.[2] Cyclosporine A treatment may cause irreversible CNS disability, and is thus contraindicated in patients with neurological involvement.[2]

Azathioprine inhibits DNA and RNA synthesis. It is a pro-drug that is converted to 6-mercaptopurine, which is subsequently transformed into 6-thioguanine nucleotides. These proteins inhibit purine ring synthesis and subsequently the synthesis of DNA and RNA. In addition, azathioprine interferes with the proliferation of T and B lymphocytes.

Azathioprine was shown to reduce the incidence, frequency and severity of eye disease, to exert a positive effect on arthritic and mucocutaneous lesions, and to improve the long-term prognosis of BD.[1]

Side effects of azathioprine include gastrointestinal intolerance with anorexia, nausea and vomiting, bone marrow suppression, infection, and an elevated risk of leukemia.[1]

Thalidomide is a cyclic derivative of glutamic acid and is known to exert immunomodulatory effects, by reducing TNF production and activity, and impairing neutrophil migration.

Thalidomide has shown efficacy in the treatment of oral and genital ulcers, papulopustular skin lesions, neurological and gastrointestinal involvement.[1]

Side effects associated with thalidomide treatment include teratogenicity, peripheral neuropathy, sedation, dizziness, headache, nausea, and weight gain.

Sulfasalazine is used for treating gastrointestinal involvement, and may also be used for treating arthritis in BD patients.[1,2] It is known for its anti-inflammatory and immunosuppressive effects, including inhibition of prostaglandin and leukotriene synthesis, free radical scavenging, immunosuppressive activity, inhibition of white blood cell adhesion and function, and inhibition of cytokine synthesis.

Dapsone was shown to be effective in treating oral, genital and cutaneous lesions.[1] It was reported to have anti-infectious and anti-inflammatory properties.

Pentoxyfilline was shown to inhibit the synthesis of cytokines, such as TNFα, and is used in the treatment of oral and genital ulcers.[1]

IFN-α is a cytokine that has immunomodulatory effects. It was shown to decrease the number of circulating γδ T cells, to increase the expression of HLA-1 on peripheral monocytes of BD patients, and to inhibit the adhesion of T cells to endothelial cells *in vitro*.[1]

Due to its antiviral activity against HSV1, IFN-α has been used to treat BD.[1]

IFN-α has shown efficacy in treating ocular lesions, neurological and vascular involvement, and reducing the frequency of arthritis, genital and papulopustular lesions, and the duration of oral ulcers.[217]

Side effects associated with IFN-α treatment include a flu-like illness upon initiation of the treatment, leucopenia, thrombocytopenia, alopecia, pruritus, depression, and autoantibody

production.[1] Nevertheless, IFN-α is well tolerated and side effects usually improve or disappear upon dose reduction.[217]

TNFα is a pro-inflammatory cytokine that is responsible for maintenance of inflammation. BD is thought to be mediated by Th1 cytokines such as TNF.[185] Several findings demonstrated the involvement of TNFα in the pathogenesis of BD.[1] Levels of TNFα were increased in aqueous humor and serum of BD patients with uveitis.[1] Furthermore, TNFα producing cells, as well as circulating TNF and soluble TNF receptors, were increased during the active phase of the disease.[185] Another evidence implicating TNFα in BD stemmed from the involvement of this cytokine in experimental models of uveitis.[1] Thus, inhibiting TNFα has emerged as a strategy for the treatment of BD.

Aprelimast is a phosphodiesterase-4 inhibitor that produces a similar result to biologic agents by preventing the production of TNFα by rheumatoid synovial cells. It is primarily used to treat mucocutaneous symptoms and has the advantage of oral administration.[1]

Biologic agents on the other hand require intravenous administration. Infliximab reduces the frequency of uveitis attacks, is effective in treatment of refractory macular edema, and in improving visual acuity, most notably in cases resistant to combination therapy with azathioprine, cyclosporine and corticosteroids, and has a corticosteroid-sparing effect. In addition, it was reported to be effective in treatment of refractory mucocutaneous, gastrointestinal and CNS involvement as well as arthritis (in addition to one case of pulmonary aneurysm with life-threatening hemoptysis).[1]

Etanercept was reported to be additionally effective in the treatment of mucocutaneous lesions and arthritis.[1]

Adalimumab was shown to maintain remission in patients with uveitis and stable visual acuities after being switched from infliximab.[1] Furthermore, adalimumab has shown superior efficacy in patients with refractory disease, including uveitis, CNS involvement, colitis, severe oral lesions, and arthritis.[1]

Sfikakis *et al.* (2007) reviewed published reports on the use of infliximab and etanercept therapy in BD.[12] Subsequently, they summarized the discussion of a panel meeting held in May 2006 in an attempt to assess the efficacy of anti-TNFα agents and to establish recommendations for the use of such agents in BD patients.[11] Infliximab was administered at doses of 3, 5 or 10

mg / kg as a single to four infusions given within a six month period to patients with severe mucocutaneous manifestations, severe gastrointestinal involvement, or sight-threatening ocular relapse. Patients exhibited rapid improvement of long-lasting urogenital ulcerations within two weeks of infliximab infusion. Likewise, gastrointestinal and extraintestinal symptoms improved or completely resolved within one to two weeks following infliximab administration. In addition, a single infusion of infliximab at 5 mg / kg resulted in remission of ocular inflammation within 24 hours and complete suppression with improvement in visual acuity within seven days of administration in patients with panuveitis. Oral ulcers and oligoarthritis accompanying ocular relapse were resolved within two and four days of infliximab administration, respectively. No adverse effects for infliximab were observed in any of the patients. Furthermore, the healing effects of infliximab showed persistence post-treatment for several weeks in many patients.

The main safety considerations included infections (notably tuberculosis), demyelinating disease, malignancies, and congestive heart failure.[11,12] Side effects associated with anti-TNFα therapy include upper respiratory tract infection, headache, autoantibody production, infusion reaction, rash, eczema, contact dermatitis, and pruritus.[1] Both short- and long-term administration of infliximab were well tolerated in almost all BD patients.[11,12] Infliximab treatment resulted in mild side effects and did not necessitate a discontinuation of therapy.[11,12] However, two patients developed psoriasis, two developed tuberculosis, and one developed vitreous hemorrhage.[11,12] In some cases, infliximab treatment induced the production of autoantibodies with no apparent clinical impact.[11,12]

Recommendations for optimal use of anti-TNFα therapy suggest a number of criteria for identification of patients eligible for anti-TNFα treatment of BD: "(1) a definite diagnosis of BD; (2) presence of active disease, including objective signs of inflammation; (3) previous failure of drugs that have a documented efficacy in controlling BD manifestations, combined or not, with low dose corticosteroids; (4) presence of contraindications or intolerance to these conventional regimens; (5) absence of contraindications to anti-TNFα treatment".[11,12]

No studies compared the long-term efficacy of available anti-TNFα agents. However, it is believed that infliximab has an advantage over etanercept in treating severe BD.[11,12] Without a doubt, the rapid results obtained with infliximab, which are especially critical in cases of sight-threatening ocular disease, give it an advantage over etanercept.[11,12] Whether or not

infliximab represents a better choice than etanercept in non-ocular manifestations require further testing.

As mentioned prior, evidence suggests the involvement of IL-17 and IL-21 in the pathogenesis of BD as the level of these cytokines was correlated with disease.[29,31,35] Treatments that aim to target these cytokines may constitute a promising target for novel therapy in the future. In this context, IL-21 blocking agents (neutralizing antibodies or receptors) may be recommended for patients with severe and / or refractive BD, given that IL-21 blockade restored the altered balance between Th17 cells and Tregs, which is known to influence disease pathogenesis in BD.[29,31,35]

9. Prognosis of Behçet's Disease

BD is characterized by episodes of relapses and remissions, has a variable course and is associated with significant morbidity and mortality. Disease prognosis depends on the clinical involvement. Morbidity and disability are predominantly associated with loss of visual acuity, vascular lesions, and neurological involvement.[1]

Male patients typically have a more severe disease outlook, an earlier onset, and a more widespread vascular involvement compared to females, and subsequently a higher mortality rate.[1,17] Disease prognosis was also linked to geographical location. Eastern and Mediterranean regions are correlated with more severe disease.[1]

The highest mortality rate was reported in Turkey (9.8%) and was attributed to large vessel vasculitis, which is known to cause sudden death by aneurysm rupture or thrombosis, notably when in combination with Budd-Chiari Syndrome.[1]

Over the past few years, modern treatment strategies, involving immunosuppressant therapy, and the use of aggressive approaches have led to improvement in the prognosis of BD. The risk of vision loss in Turkish male patients was reduced in 1990 compared to 1980.[17] As disease prognosis improves, the rate of mortality has decreased over time. For instance, disease prevalence among uveitis patients (23.2% in 1981-1983 vs. 5.8% in 1999-2001), disease severity, frequency of ocular attacks and loss of visual acuity were all reduced in the Japanese population over a period of 10 years.[17] The observed changes were attributed to environmental changes rather than genetic background, which was considered to be constant during the study period.[17]

Neurological involvement represents one of the most serious causes of long-term morbidity and mortality in BD.[1,7] Parenchymal brain disease is more common and is associated with worse prognosis.[1] Poor prognosis involves progressive disease course, frequent relapses and residual neurological impairments in remission.[7,198] Retrospective series from 10-15 years ago reported a mean of 20-30% of patients with residual neurological impairments and an elevated ten-year mortality rate of 10%.[7] Moreover, one-third of patients had single episodes, one-third had repeated relapses with remission, and one-third underwent a progressive disease course with accrual of neurological impairments.[7,198] A primarily progressive disease course was also observed in one series.[7,198]

Most patients with acute parenchymal inflammatory episode recover well following steroid therapy; patients with brainstem and spinal cord lesions recover less well, and those with abnormal CSF indices have the worst prognosis.[7] On the other hand, patients with silent neurological involvement tend to develop clinically evident manifestations, but have a lower risk of impairment.[7]

Patients with venous sinus thrombosis and intracranial hypertension present a good recovery when given suitable and prompt therapies, and have a lower risk of recurrence.[7]

Oral ulcers heal without scarring, unlike genital ulcers that usually heal with white or pigmented scars.[1]

Erythema nodosum lesions do not ulcer and may heal spontaneously leaving residual pigmentation. Cutaneous ulcers, on the other hand, commonly heal leaving a scar.[1]

Ocular disease has a more severe clinical course in men than in women, and in children than in adults.[1] It is associated with significant morbidity and causes blindness in 25% of patients with ocular lesions despite therapeutic intervention, although prognosis is improving with the use of modern immunosuppressant therapy and of more aggressive treatment strategies.[1] Recurrent attacks of inflammation lead to secondary complications, including posterior and / or peripheral anterior synechia, iris atrophy, cataract resulting from inflammation and / or medication, secondary glaucoma (sometimes neovascular), atrophic retina, optic atrophy, macular edema, macular degeneration, retinal veins occlusion, sheathed vessels, chorioretinal scars and / or proliferative vitreoretinopathy and phthisis bulbi.[1] Episodes of anterior uveitis are known to subside spontaneously; however, recurrent attacks lead to irreversible structural changes, including deformity of the iris and secondary glaucoma.[2]

In patients with vascular disease, thromboses of the vena cavas, of dural sinuses and of supra-hepatic veins and pulmonary arterial aneurysms are associated with poor prognosis.[1] Occlusion of major veins and arteries and aneurysms often cause bleeding, infarction, organ failure and restricted movement of the arms and legs.[2] The rupture of aneurysms may be fatal.[2] In addition, vascular lesions in the lungs (thrombosis, aneurysm and arteriobronchial fistula) cause recurrent episodes of dyspnea, cough, chest pain and hemoptysis.[2]

Other clinical manifestations such as articular involvement and gastrointestinal involvement seldom result in a poor general prognosis, although they still generate a negative impact on quality of life.[2]

10. Study Cohort

This study provides a template for the statistical analysis of cohorts that deal with the identification of biomarkers in diseases with covariate clinical manifestations such as activity and inactivity (remission) as in BD. The intent is to present a biostatistical protocol that thoroughly investigates the data and may be utilized in similar studies (***Supplementary Figure 1***).

The protocol hinges on four types of analyses: descriptive, bivariate, regressions, and confirmatory tools. They are discussed in detail in the methodology chapter. Other analyses evidently remain available in the arsenal of biostatistics in general, however, they habitually require an advanced level of expertise in programming.

Descriptive statistics verify the distribution and the normality of the continuous variables (covariates). Q-Q plots graphically illustrate the distribution of the data set and the summary tables display the details regarding the observations.

The bivariate analysis serves to examine the distribution of the covariates in relationship to a disease activity status, usually active and inactive (in remission), relative to the control groups (healthy donors or other diseases). Box plots graphically depict the results. In cases of extreme values and skewed data, log-transformations are applied in order to improve the normality of the data variables and increase the possibility of conclusive outcomes. The means of the variables and the activity groups are assessed using analyses of variance. These tests assume that the data follows a normal distribution and that the sample size is large enough to allow parametric tests (***Supplementary Figure 1: "parametric"***). In cases when the data is not normally distributed, nonparametric tests are utilized, with or without corrective enhancements (***Supplementary Figure 1: "nonparametric"***).

Prior to running logistic regression models on the results obtained in the bivariate analyses, a Generalized Additive Models (GAM) and a Principle Component Analysis (PCA) may be used to determine the log-linearity of the covariates. If the assumption of linearity holds then the covariates are analyzed as continuous, if not then the covariates are dichotomized based on a cut-off that is either confirmed by literature or clinically meaningful; only in exceptional cases should the cut-off be the median as it alters the results. This allows for the

smoothing of data in cases where it is not normally distributed; it also clearly defines covariate biomarker patterns.

Should the data be normally distributed, a Pearson correlation matrix is used to assess the correlation between covariates. In situations of nonparametric data, a Spearman correlation matrix is used to rank the correlation between covariates. If two or more covariates are highly correlated, careful consideration should be given before their inclusion in a regression model.

All variables that are significantly associated in the bivariate analysis are entered into various types of regression models to determine the ability of the covariates to predict outcomes. This includes a range of options from linear to multivariate models. It is important at this stage to define the p-value to be used as it either restricts or expands the scope of the regression analyses. Model accuracy should be carefully estimated using sensitivity and specificity of the disease to ensure proper results. Bootstrapping adjusts the model for overfitting. Results are noted and prediction models, when possible, are constructed for further assessment.

10.1. Methodology

A total of 213 subjects including 22 patients with active BD status (BDA) and 46 patients with inactive BD status (BDI), 47 healthy donors (HD), and 98 coronary angiography patients (CA) were consecutively enrolled in this study. All BD patients fulfilled the disease criteria as defined by the International Study Group for BD.[1,2] This encompassed an established diagnosis of oral ulcerations (at least three times in last year) together with any two of the following symptoms: recurrent genital ulceration (aphthous ulceration with scarring); eye lesions (such as uveitis or retinal vasculitis); skin lesions (erythema nodosum, pseudofolliculitis or papulopustular lesions, acneiform nodules in post-adolescent patients not on corticosteroid therapy); and / or a positive pathergy test (read by a physician at 24-48 hours post-incision). Criteria for exclusion from the study included BD patients whose disease was equivocal and healthy donors who were pregnant at the time, or with a history of substance abuse or other illnesses. Disease activity was defined according to International Criteria for Behçet's Disease (2013).[1] BD was defined as active (BDA) when two or more of the following clinical manifestations were detected: recurrent oral ulcerations; recurrent genital ulceration; ocular lesions; and / or skin lesions. All other BD patients were defined as inactive (BDI). Healthy

donors and coronary angiography patients were analyzed as controls, the latter exhibiting atherothrombotic disease. All enrolled subjects gave informed consent before participation.

Quantitative determination of 20 biomarkers was performed in accordance with the manufacturer protocol (Merck Sharp Dohme): Caldesmon, Clusterin, Calponin 1 (CNN1), C-Reactive Protein (CRP), Growth-Regulated Protein-α CXCL-1 (GROα), Intercellular Adhesion Molecule 1 (ICAM-1), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interferon Gamma-Induced Protein 10 CXCL10 (IP-10), Interferon-Inducible T-Cell Alpha Chemoattractant-CXCL11 (I-TAC), Monocyte Chemoattractant Protein 1 CCL2 (MCP1), Monokine Induced By Gamma Interferon CXCL9 (MIG), Macrophage Inflammatory Protein 1-α (MIP1α), Serum Amyloid A (SAA), E-Selectin (SELE), P-Selectin (SELP), Soluble Intercellular Adhesion Molecule 3 (sICAM-3), Thrombomodulin (THBD), Vascular Cell Adhesion Molecule 1 (VCAM-1), and Vascular Endothelial Growth Factor (VEGF).

These biomarkers were chosen as they represent either markers of vascular wall layer involvement (intima, media, and adventitia) or markers of different autoinflammatory / autoimmunological processes that are integral to endothelial and smooth muscle injury as observed in clinical vasculitis. Biomarkers for inflammatory markers are: CRP, IL-6, and IL-8. Biomarkers for endothelial markers are: SELE, SELP, THBD, VCAM-1, and VEGF. Biomarkers for smooth muscle injury are: Caldesmon and CNN1. The remaining biomarkers, Clusterin, GROα, ICAM-1, IP-10, I-TAC, MCP1, MIG, and MIP1α, are presumed to play a combination of roles in vascular injury.

Whole blood was harvested from patients in serum separator tubes (SSTs) and after 30 minutes was centrifuged, aliquoted, and directly frozen at -80°C. Three homemade internal quality controls at different concentrations (low, medium, and high) were prepared by spiking the industrial lyophilized serum Medidrug Basis-line S (Medichem) with the provided calibrator. Upon analysis, serum samples were thawed on ice and were analyzed according to the manufacturer's (Merck Sharp Dohme) protocol for immunoassay using the following panels: V-PLEX Plus Vascular Injury Panel 1, V-PLEX Plus Vascular Injury Panel 2, and Human Pro-Inflammatory 9-Plex.

All kits followed the same protocol: (1) Addition of sample: the plate was washed three times with at least 150 µL of wash buffer per well. 25 µL of diluted sample were added per well. The plate was sealed with an adhesive plate seal and incubated with shaking for 2 hours at

room temperature; (2) Addition of detection antibody solution: the plate was washed three times with at least 150 µL of wash buffer per well. 25 µL of detection antibody solution was added to each well. The plate was sealed with an adhesive plate seal and incubated with shaking for 1 hour at room temperature; (3) Reading of results: the plate was washed three times with at least 150 µL of wash buffer per well. 150 µL of 1X Read Buffer T was added to each well. The plate was immediately analyzed on the corresponding manufacturer reader (Merck Sharp Dohme).

Laboratory personnel who collected samples and processed the assay were blinded with regards to the clinical data associated with this study.

The objective was to identify with statistical certainty biomarkers that significantly differentiated between: Behçet's disease – all status (BD) vs. healthy donors (HD); Behçet's disease – all status (BD) vs. coronary angiography patients (CA); Behçet's disease – all status (BD) vs. healthy donors in combination with coronary angiography patients (HD + CA); and active BD patients (BDA) vs. inactive BD patients (BDI).

Descriptive statistics of the data was performed to verify the distribution and the normality of the population and the biomarkers. Data were described as the mean ± standard deviation. The minimum and maximum data points were defined, as well as the median, and the lower and upper quartiles. All entries with missing observations were removed from the dataset. A bivariate analysis was performed to examine the distribution of individual biomarkers in relationship to four set disease variables: BD patients with active disease (BDA), BD patients with inactive disease (BDI), healthy donors (HD), and coronary angiography patients (CA). The purpose was to identify biomarkers that revealed significant differences between BD in its active and inactive forms, and the non-BD controls: HD and CA. Given the absence of a normal distribution, two nonparametric analyses of variance (ANOVA) were conducted to assess the association between individual biomarkers and the four set disease variables (BDA, BDI, CA, and HD).

A Kruskal-Wallis test was performed to test the difference in biomarker levels in each of the four set disease variables (p-value <0.05). The biomarkers that were significant in the Kruskal-Wallis test were retained and tested using a Mann-Whitney test (p-value <0.05) for pairwise combinations of the four set disease variables resulting in six possible disease pairwise combinations: BDA vs. HD, BDI vs. HD, BDA vs. CA, BDI vs. CA, BDA vs. BDI, and HD vs.

CA. The purpose was to identify biomarkers that show significance as covariates that differentiate between the six disease pairwise combinations. A Bonferroni correction for pairwise comparison between the medians of each of the four set disease variables was applied to counteract for the problem of multiple comparisons.

The linearity assumption for the biomarkers identified in the Mann-Whitney with Bonferroni correction test was examined using the Generalized Additive Models (GAM). When linearity did not hold for any single biomarker, the data were dichotomized at the median level. The significance between different groups was tested using an exact and then univariate logistic regression models, and unadjusted odds ratio and their 95% confidence interval were reported; a p-value smaller than 0.05 was considered to be statistically significant.

In order to identify pairwise correlations between biomarkers, a Spearman rank correlation coefficient test was performed. Covariate biomarkers with a p-value <0.01 and <0.05 were considered to be statistically significant in a 2-tailed model. A Pearson product-moment correlation coefficient test was also performed. A table summarizing the pairwise correlations per disease combination (BDA vs. HD, BDI vs. HD, BDA vs. CA, BDI vs. CA, BDA vs. BDI, and HD vs. CA) was generated. An attempt to confirm the linear correlation coefficient of all biomarkers was made using a Principle Component Analysis (PCA).

All biomarkers that were significantly associated with the four set disease activity variables (BDA, BDI, HD, and CA) in the bivariate analysis were entered into logistic regression model with manual and stepwise selection to determine the ability of the covariates to predict Behçet's Disease, in either active or inactive form (p-value <0.05). Bootstrap was applied to the models. A minimum of two hundred iterations was performed in order to estimate the slope coefficients of the model; this corrected for overfitting. In case no result was obtained, the biomarkers that were significantly associated with the disease activity variables (BDA, BDI, CA, and HD) were entered into a multivariate regression model and the process of validation of the predictive and discriminatory power of the model was repeated.

When no statistically significant predictive model was obtained, a pooling of data from comparable disease variables was attempted. This amplified the data by decreasing the number of the set disease variables from four (BDA, BDI, HD, and CA) to three [BD (BDA+BDI), HD, and CA]. Hypothesis testing was performed to identify those biomarkers that possess the same distribution across the disease variables before the pooling was completed. Only those

biomarkers that did not reject the null hypothesis were retained and logistic and multivariate regression models were attempted in order to obtain a predictive model for BD with the pooled data. The data used in the multivariate regressions was normalized using a natural log when non-transformed data did not yield significant results.

10.2. Results

The descriptive statistics of the study population are presented in ***Table 3***.

Table 3: Descriptive Statistics of Cohort

	Total	Total %	FEMALE	FEMALE %	MALE	MALE %	AGE MEAN	AGE MIN	AGE MAX	AGE STDEV
	213	**100%**	**79**	**37.09%**	**134**	**62.91%**	**49.88**	**16**	**89**	**18.64**
BEHÇET'S DISEASE	68	31.92%	25	36.76%	43	63.24%	37.71	16	67	10.96
CORONARY ANGIOGRAPHY PATIENTS	98	46.01%	30	30.61%	68	69.39%	66.31	44	89	12.06
HEALTHY DONORS	47	22.07%	24	51.06%	23	48.94%	33.26	22	48	6.55

	CATEGORY	Total	FEMALE	FEMALE %	MALE	MALE %	AGE MEAN	AGE MIN	AGE MAX	AGE STDEV
BEHÇET'S DISEASE	ACTIVE	22	8	36.36%	14	63.64%	37.36	19	67	10.843
	INACTIVE	46	17	36.96%	29	63.04%	37.87	16	67	11.129

% = Percentage

MIN = Minimum

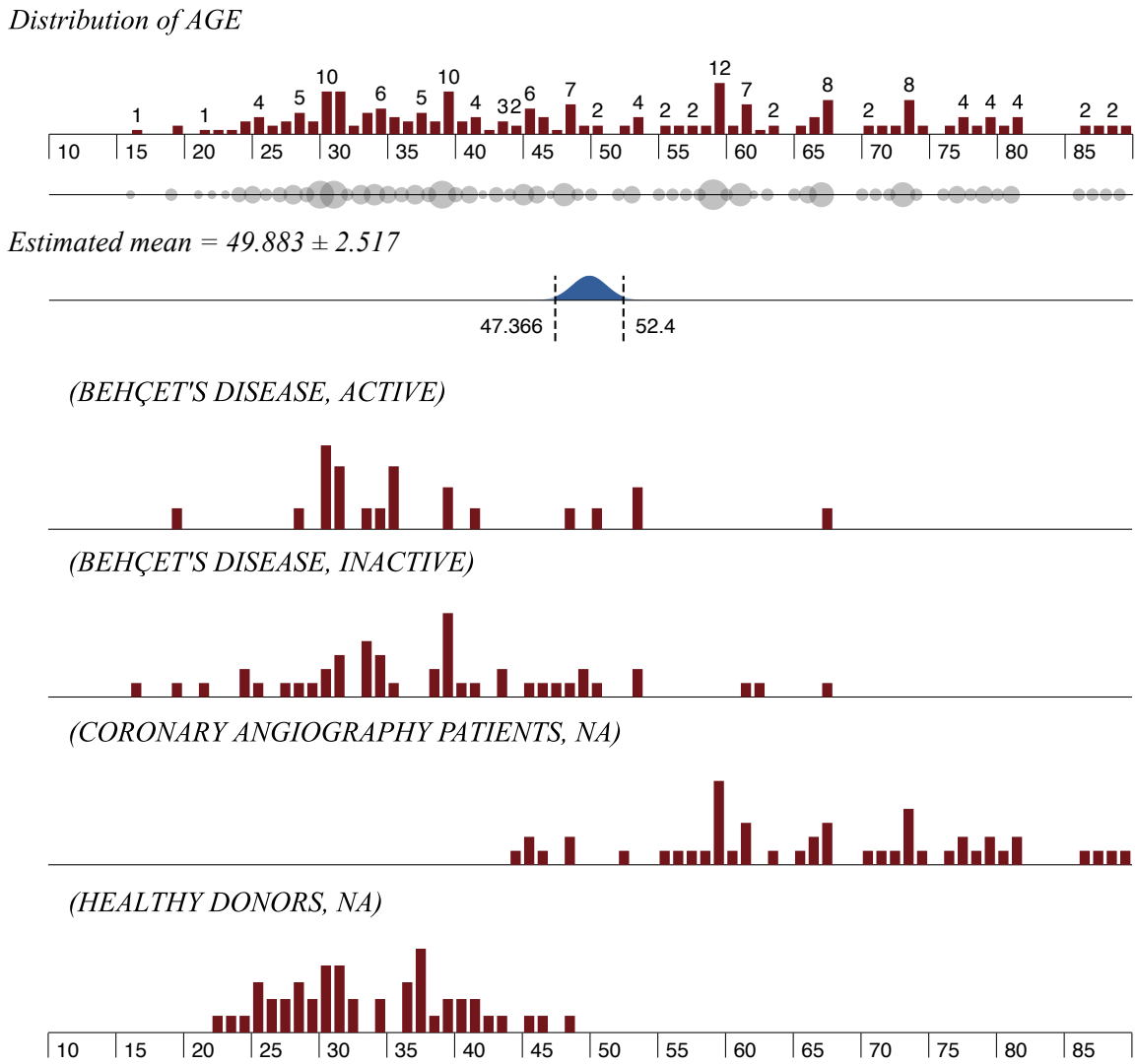
MAX = Maximum

STDEV = Standard Deviation ±

As may be observed, only 22 patients presented BD in active form and 46 in inactive form. This small sample size may impact the validity of the statistical analysis (n=22 BDA and n=46 BDI / n=213 Total).

The age distribution of the study population is presented in ***Figure 1***.

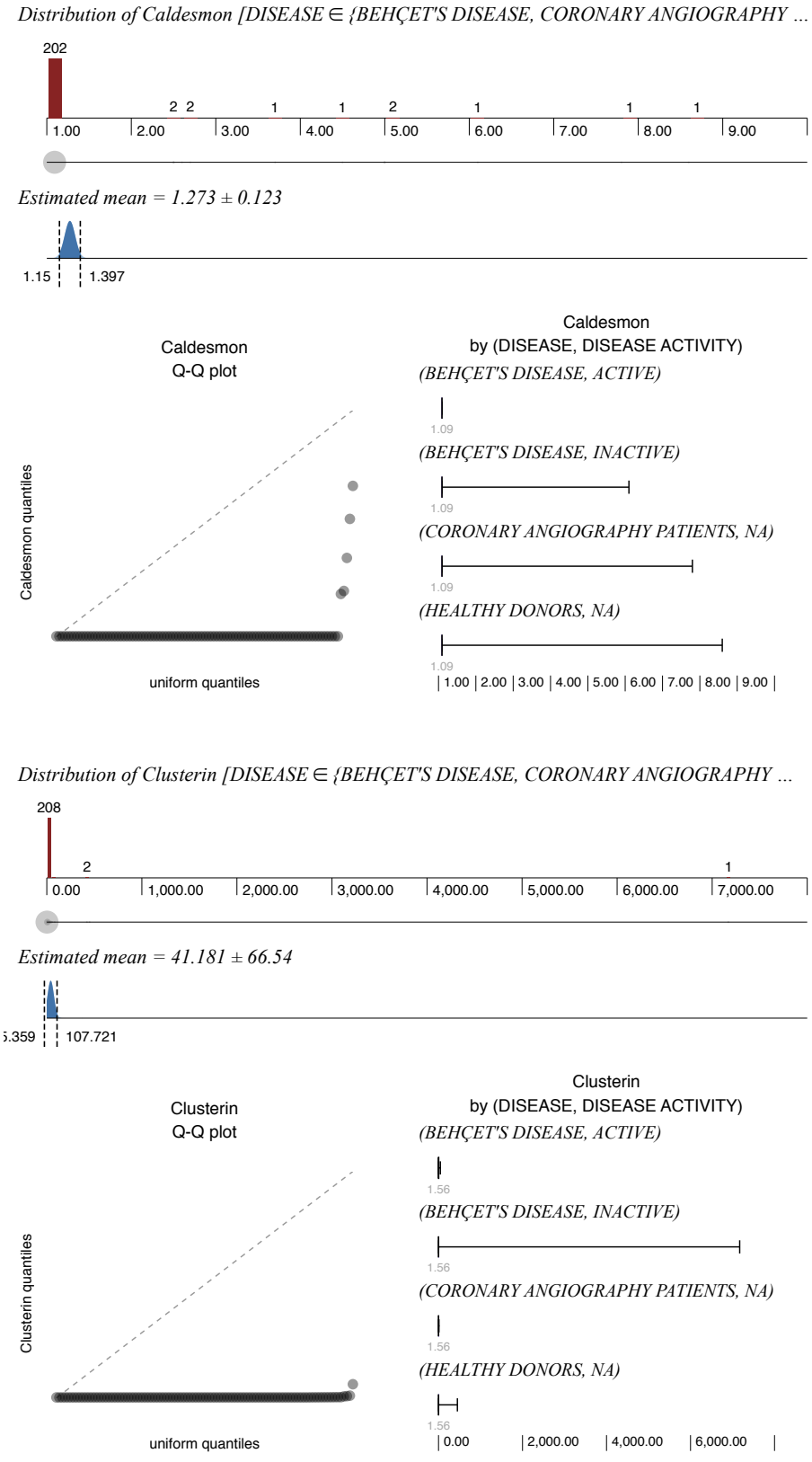
Figure 1: Age Distributions of Cohort



While there is concordance between the age distribution of BDA, BDI, and HD, the coronary angiography (CA) group is on average 28 years older than the other groups. This may impact the comparative results as older patients may present different inflammatory signatures.

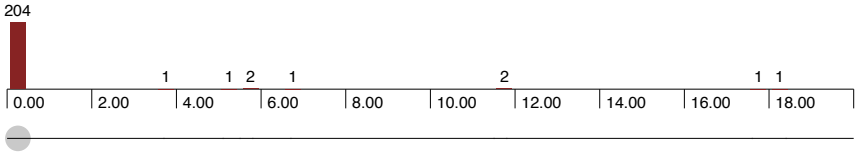
The general characteristics of serum biomarker levels of the study population, including frequency, uniformity, and distribution by disease category and activity are graphically illustrated in ***Figure 2***.

Figure 2: Frequency, uniformity, and distribution of individual biomarkers tested*

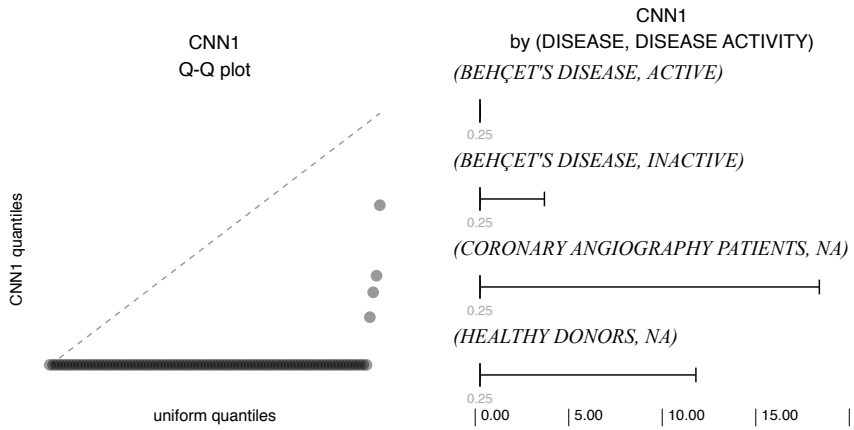


* Interleukin 6 (IL-6) and Interleukin 8 (IL-8) are abbreviated INTLK6 and INTLK8 respectively in the database of this study. Box plots graphically represent the distribution of the data: the ends mark the maximum and minimum values, and the median separates the second (yellow) and third (purple) quartiles represented by colored squares.

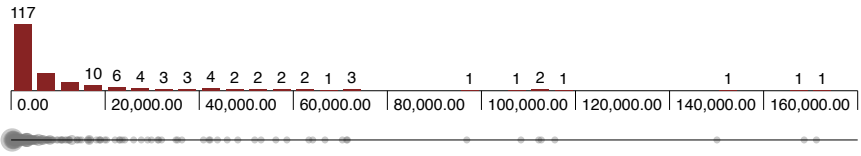
Distribution of CNN1 [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



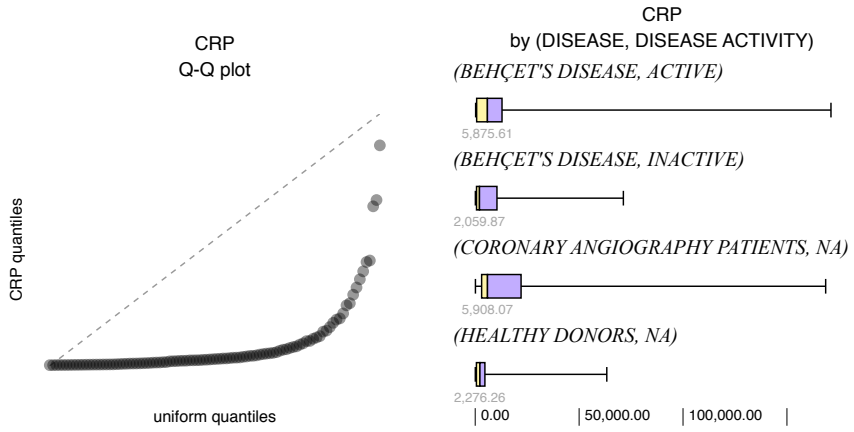
Estimated mean = 0.644 ± 0.292



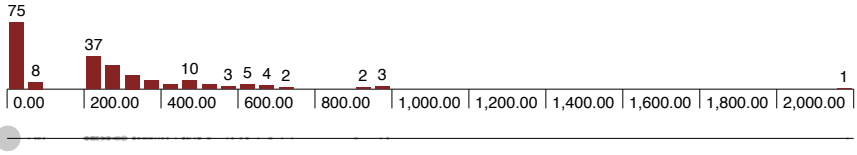
Distribution of CRP [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY PATIENTS, ...



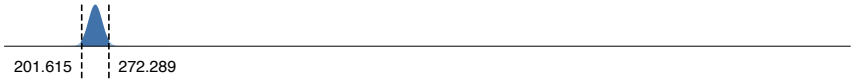
Estimated mean = 14,882.976 ± 3,769.775

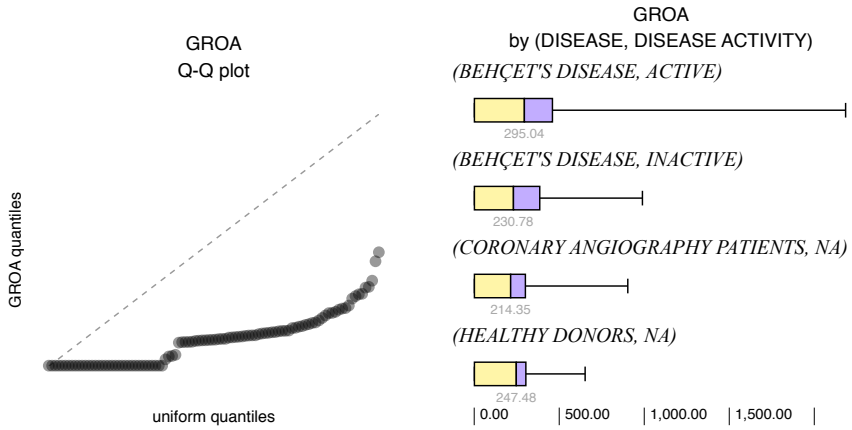


Distribution of GROA [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

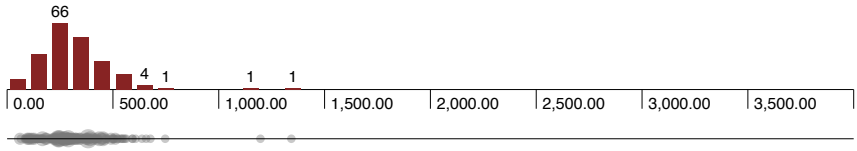


Estimated mean = 236.952 ± 35.337

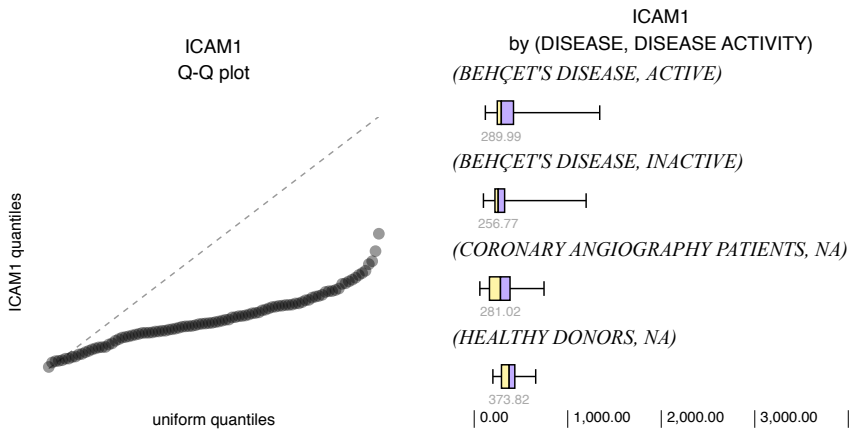




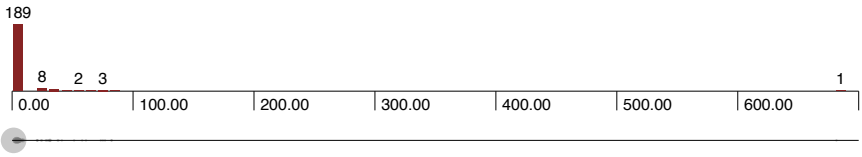
Distribution of ICAM1 [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



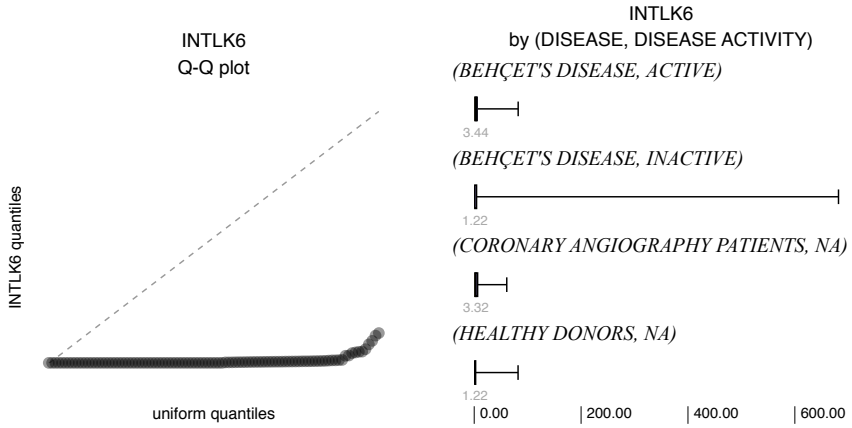
Estimated mean = 317.318 ± 22.038



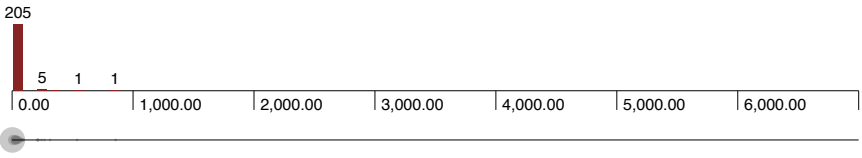
Distribution of INTLK6 [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



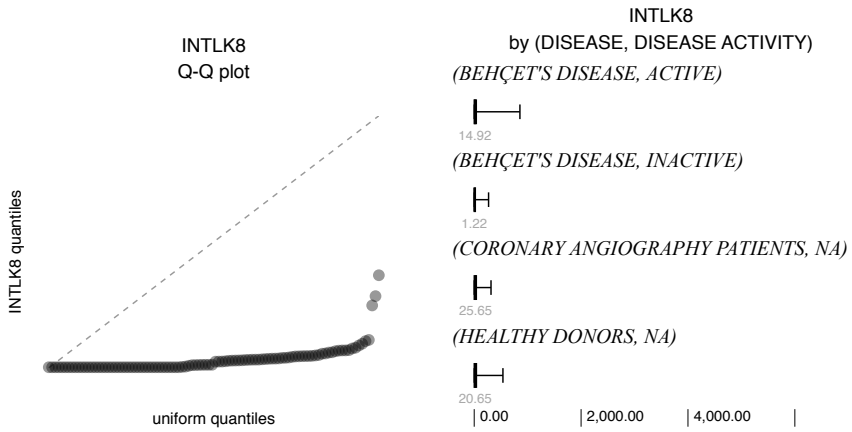
Estimated mean = 10.301 ± 6.546



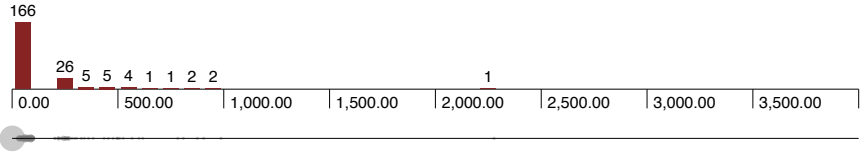
Distribution of INTLK8 [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



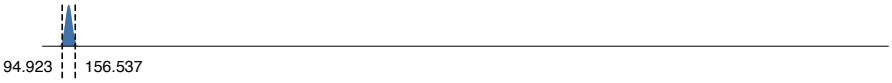
Estimated mean = 33.54 ± 10.755

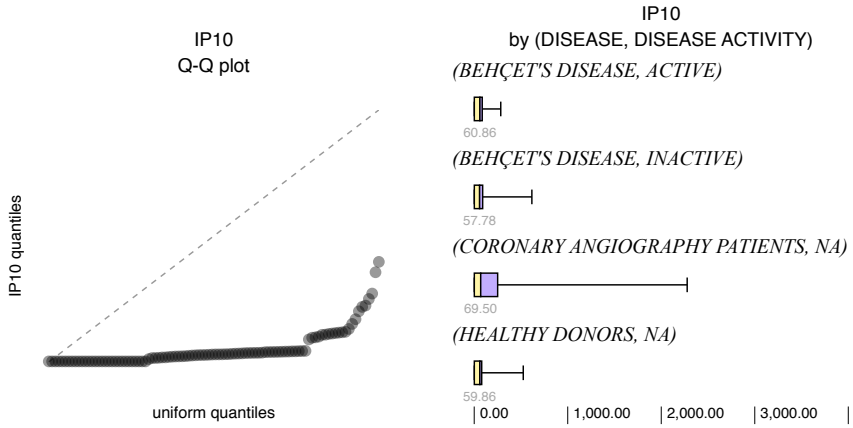


Distribution of IP10 [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY PATIENTS, ..

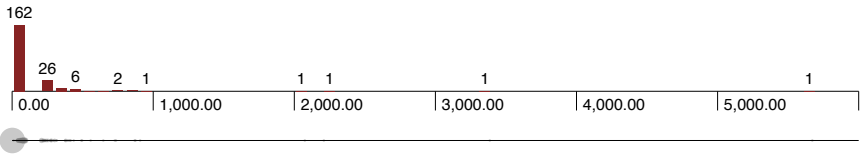


Estimated mean = 125.73 ± 30.807

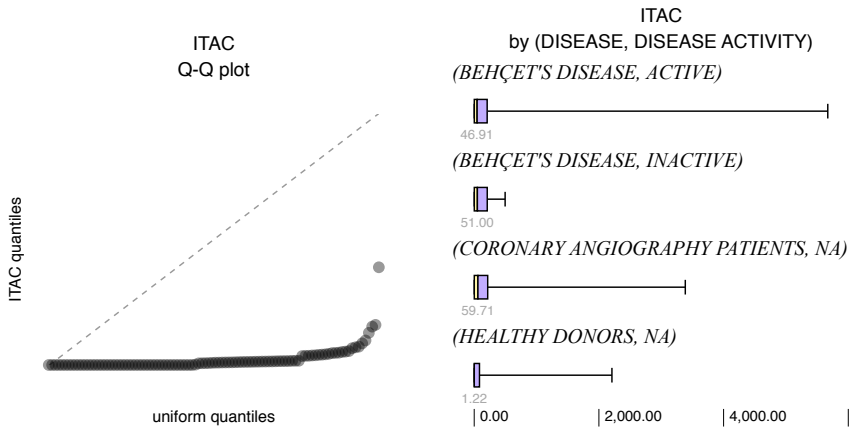




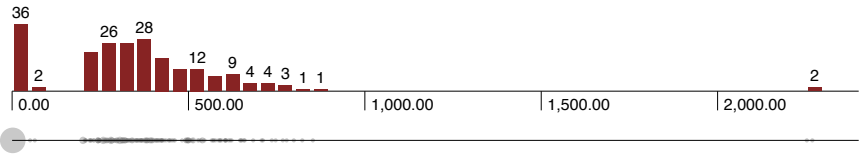
Distribution of ITAC [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY PATIENTS, ..



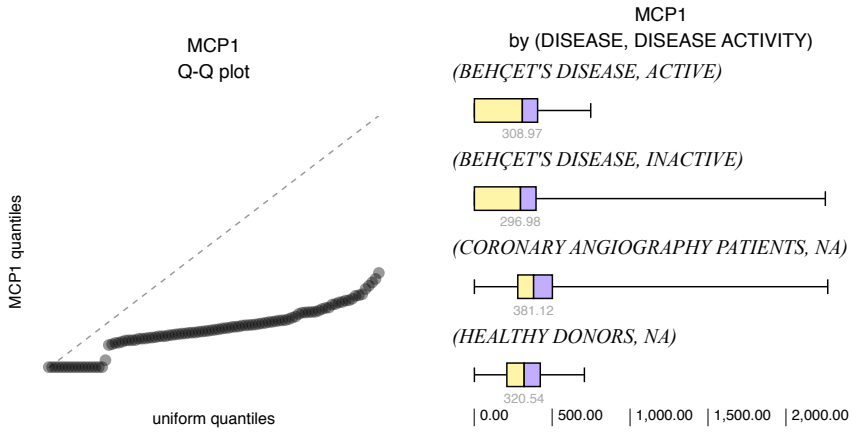
Estimated mean = 164.839 ± 68.904



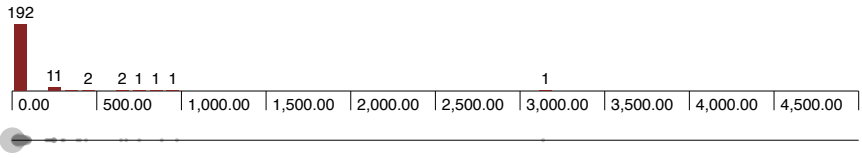
Distribution of MCP1 [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



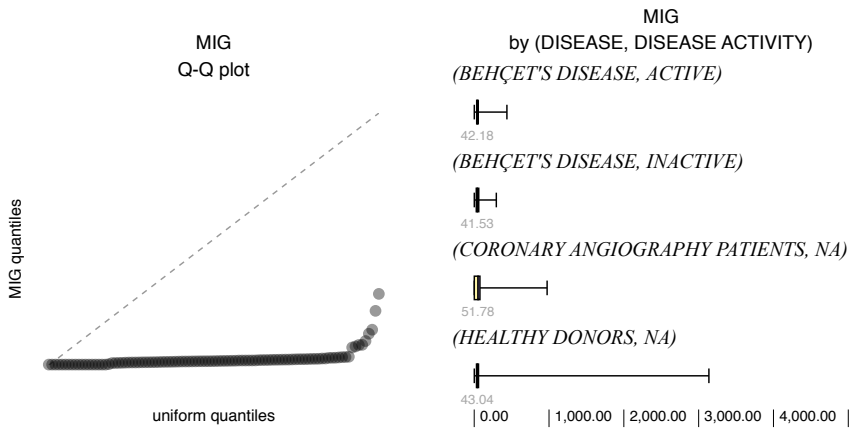
Estimated mean = 352.166 ± 37.228



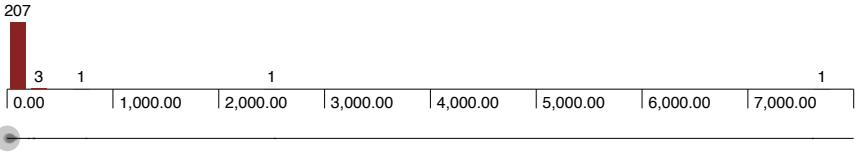
Distribution of MIG [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY PATIENTS, ...



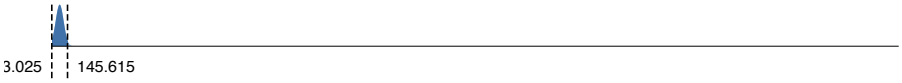
Estimated mean = 89.186 ± 33.427

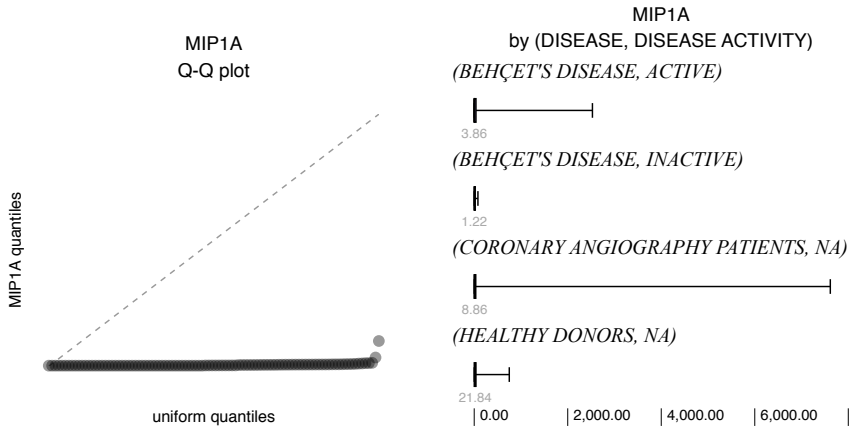


Distribution of MIP1A [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

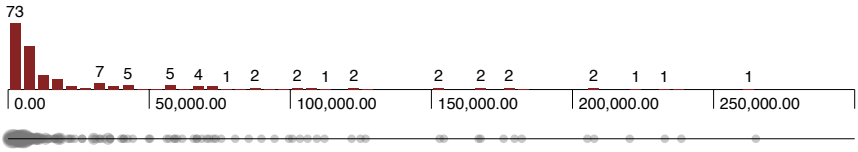


Estimated mean = 71.295 ± 74.32

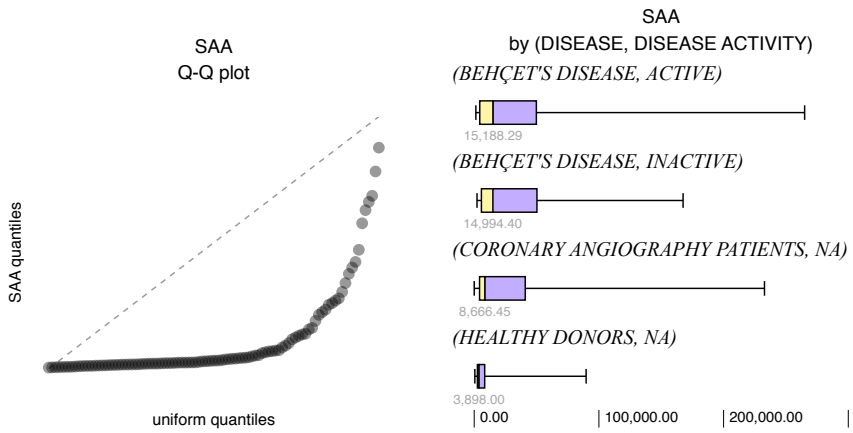




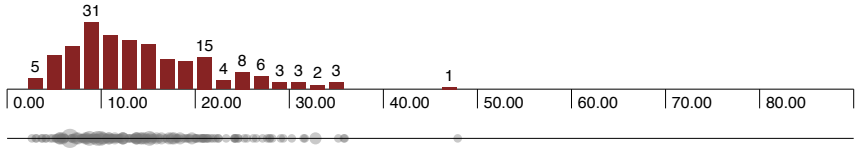
Distribution of SAA [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY PATIENTS, ...



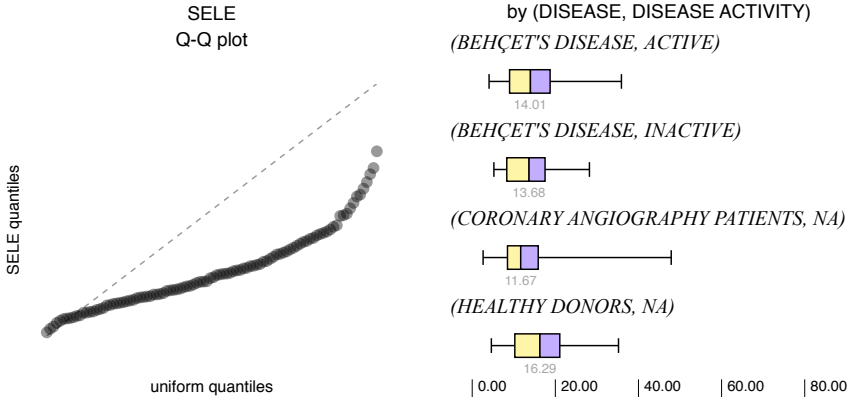
Estimated mean = 31,095.81 ± 6,914.007



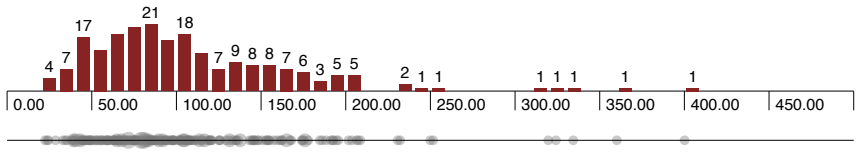
Distribution of SELE [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



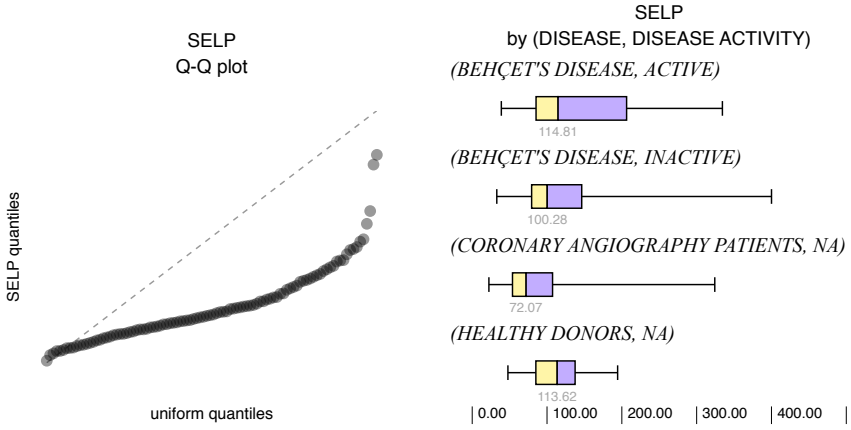
Estimated mean = 14.366 ± 1.018



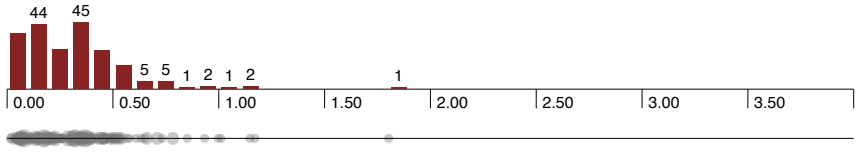
Distribution of SELP [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



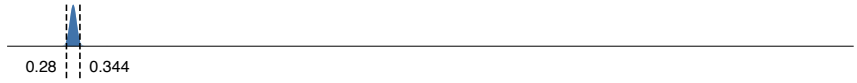
Estimated mean = 108.085 ± 8.241

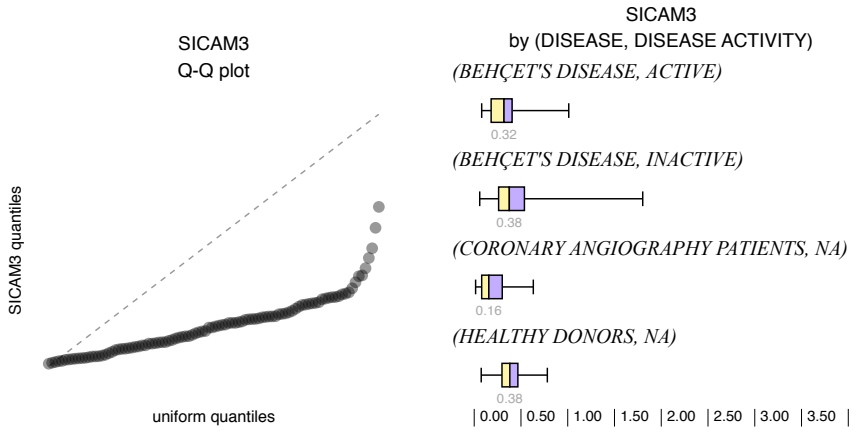


Distribution of SICAM3 [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

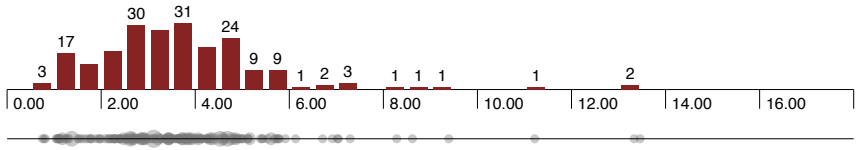


Estimated mean = 0.312 ± 0.032

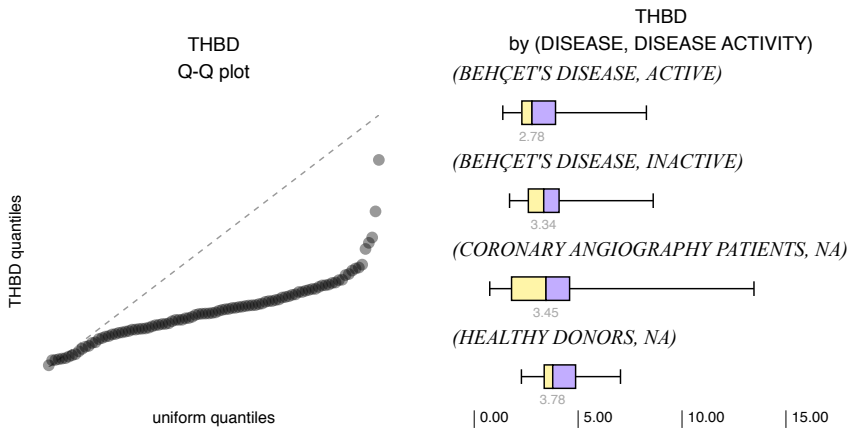




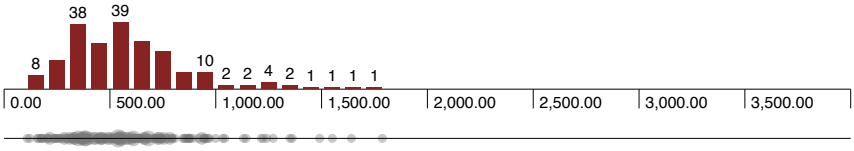
Distribution of THBD [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



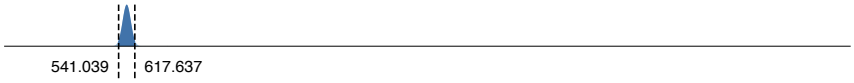
Estimated mean = 3.656 ± 0.248

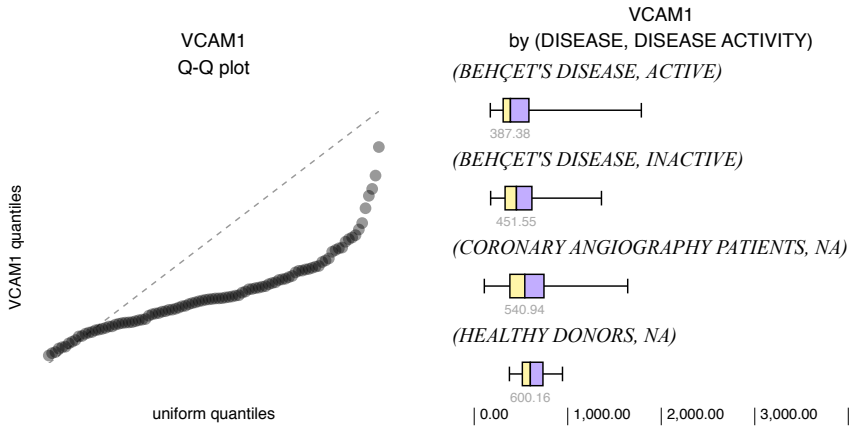


Distribution of VCAM1 [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

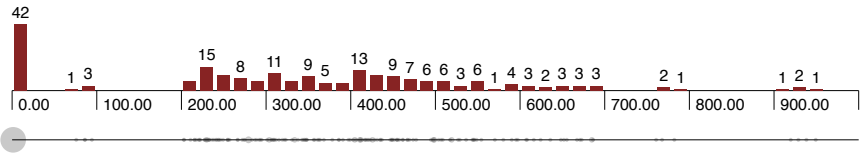


Estimated mean = 579.338 ± 38.299

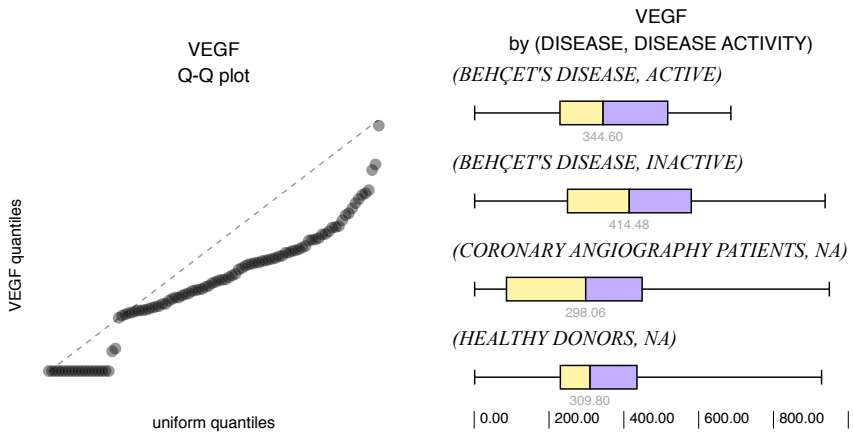




Distribution of VEGF [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



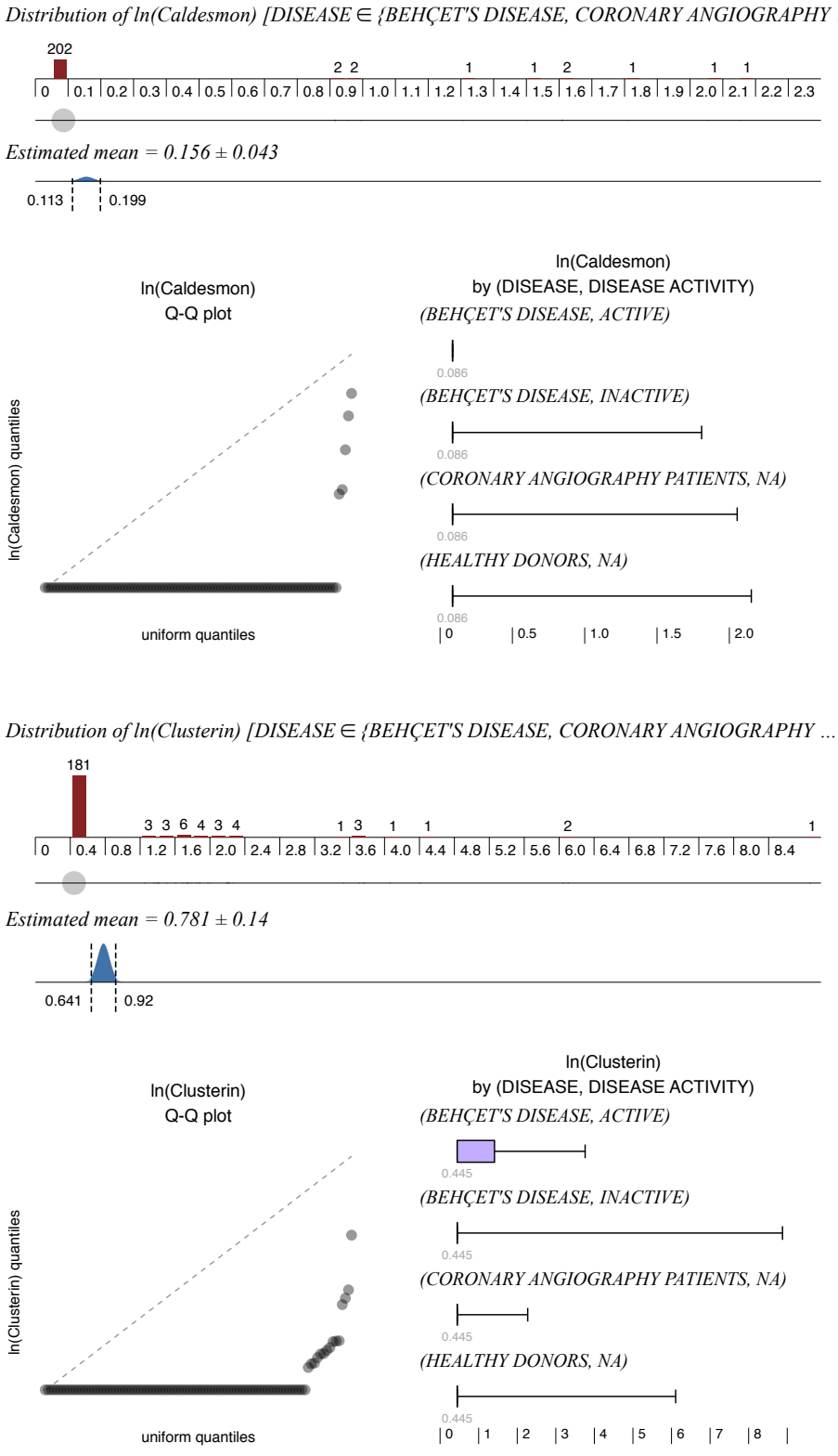
Estimated mean = 327.778 ± 29.623



Except for SELE, SELP, and VEGF, the uniform distribution of the biomarkers is extremely skewed, with outliers in many cases. As a result, the distribution overlap between the four set disease variables (BDA, BDI, HD, and CA) did not allow for any biomarker tested to demonstrate a significant difference. Consequently, normalization was employed.

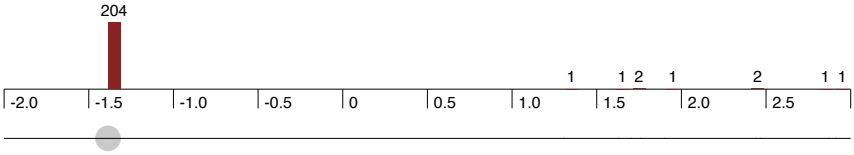
The general characteristics of serum biomarker levels after transformation using a natural log of the study population, including frequency, uniformity, and distribution by disease category are graphically illustrated in ***Figure 3***.

Figure 3: Frequency, uniformity, and distribution of individual biomarkers tested (natural log-transformed data)[*]

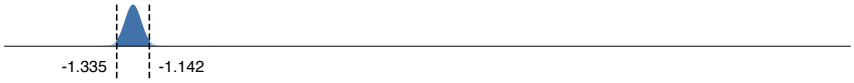


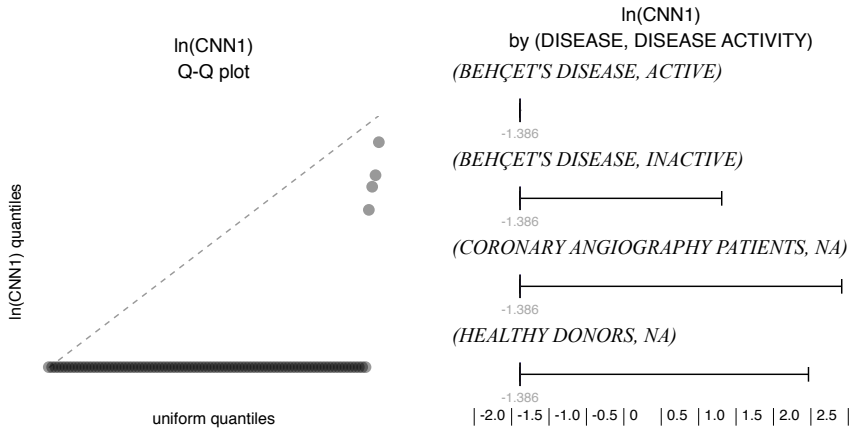
[*] Interleukin 6 (IL-6) and Interleukin 8 (IL-8) are abbreviated INTLK6 and INTLK8 respectively in the database of this study. Box plots graphically represent the distribution of the data: the ends mark the maximum and minimum values, and the median separates the second (yellow) and third (purple) quartiles represented by colored squares.

Distribution of ln(CNN1) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

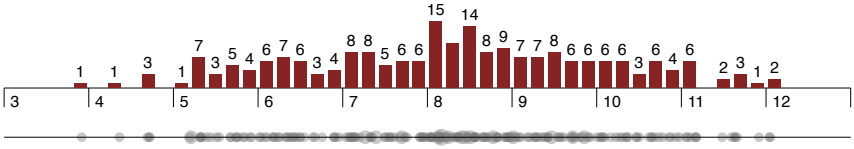


Estimated mean = -1.239 ± 0.096

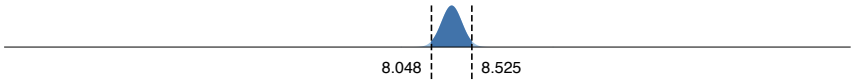


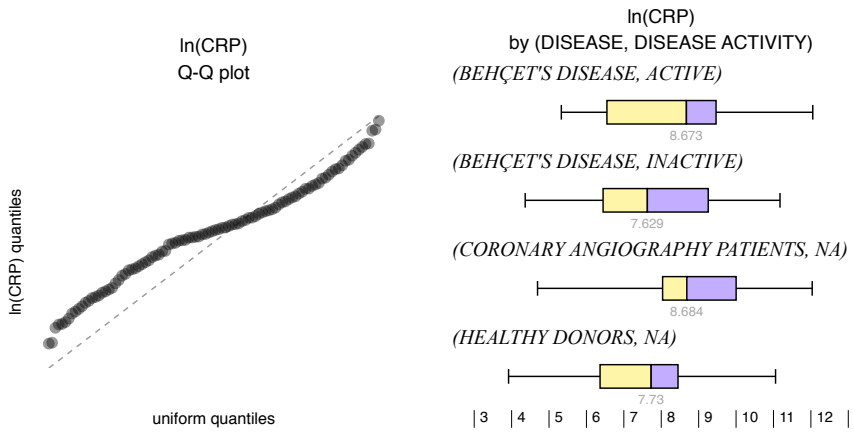


Distribution of ln(CRP) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

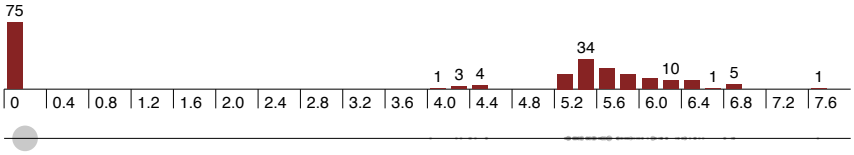


Estimated mean = 8.287 ± 0.239

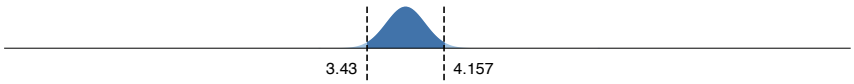


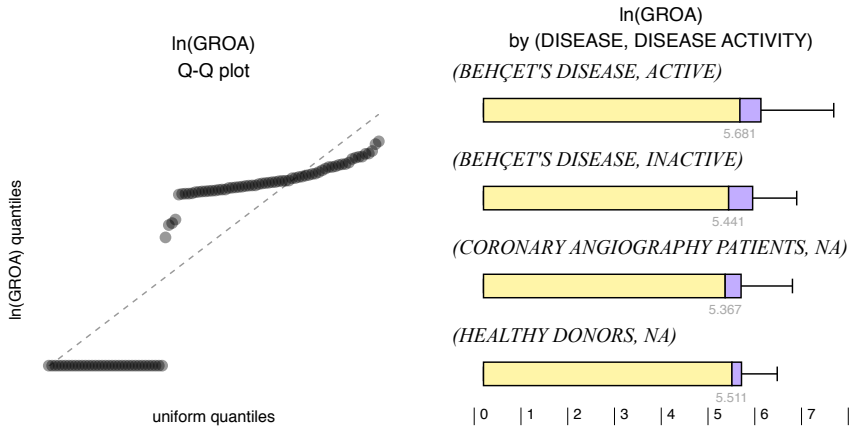


Distribution of ln(GROA) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

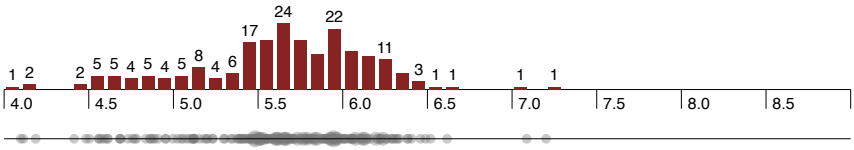


Estimated mean = 3.794 ± 0.364

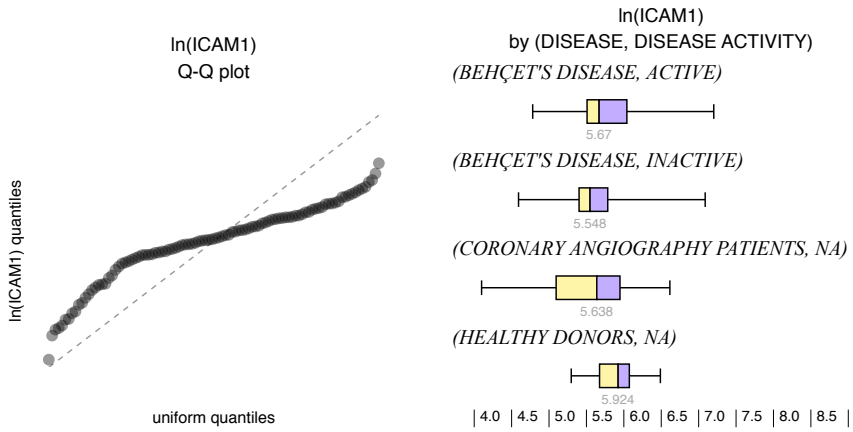




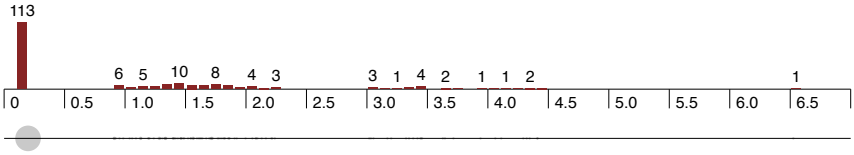
Distribution of ln(ICAM1) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



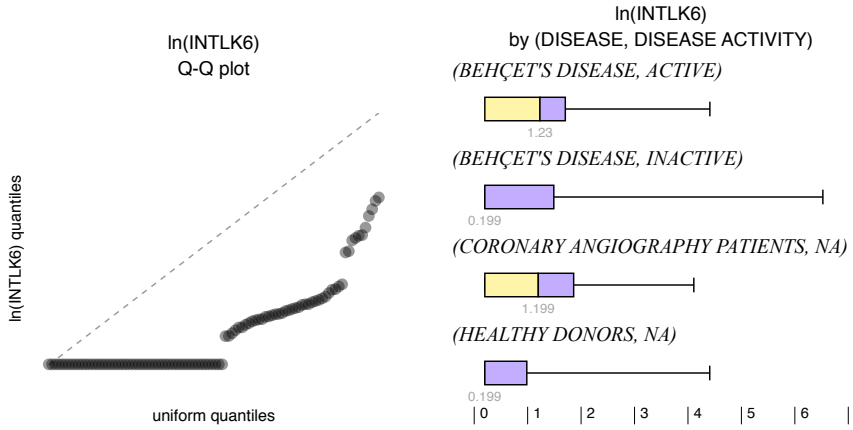
Estimated mean = 5.635 ± 0.07



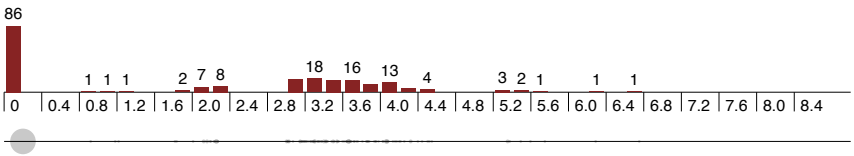
Distribution of ln(INTLK6) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



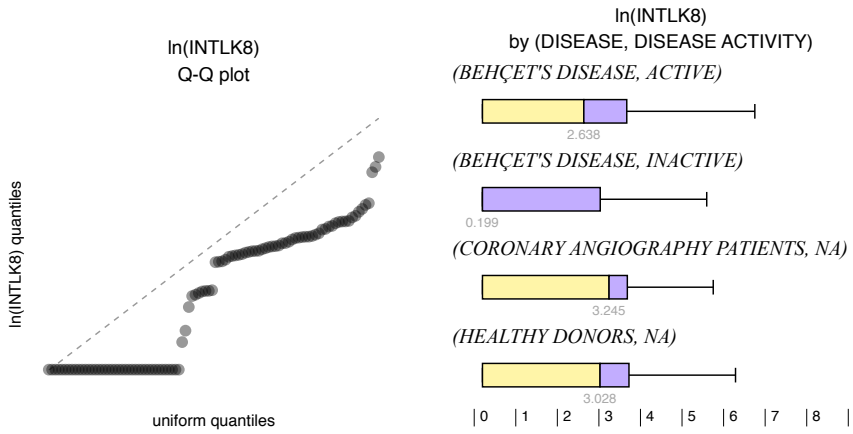
Estimated mean = 1.08 ± 0.161



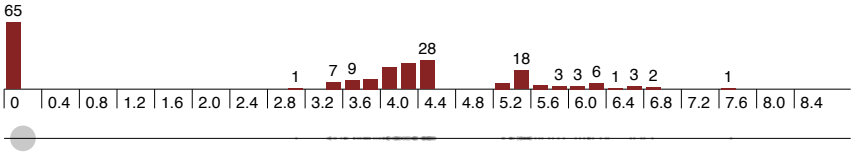
Distribution of ln(INTLK8) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



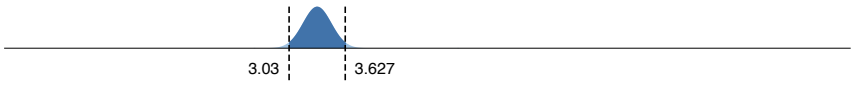
Estimated mean = 2.166 ± 0.239

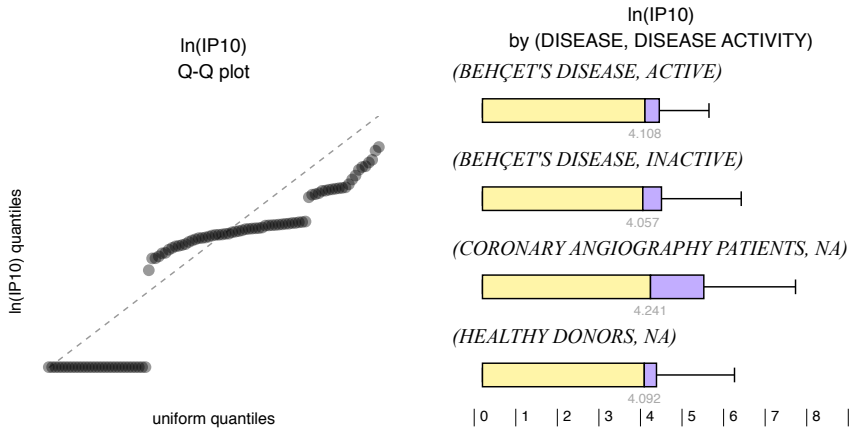


Distribution of ln(IP10) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

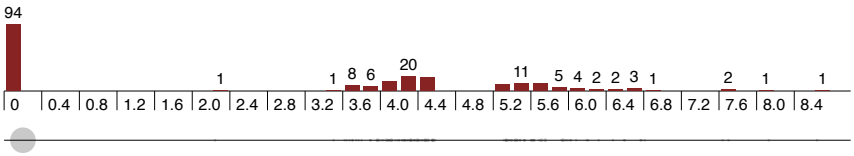


Estimated mean = 3.328 ± 0.298

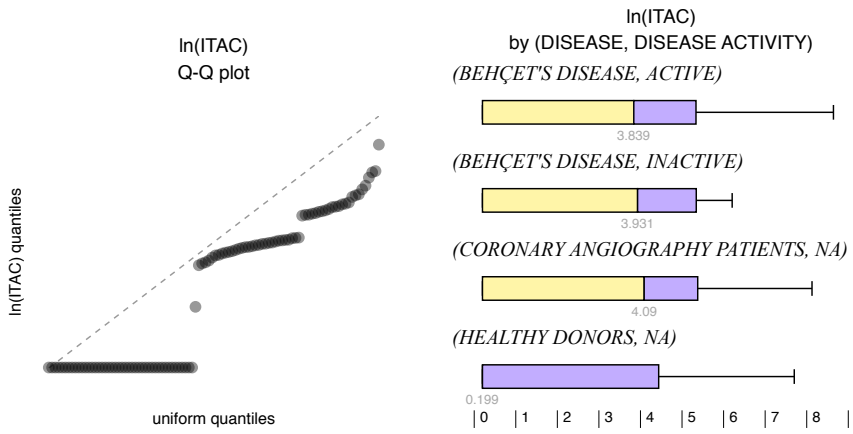




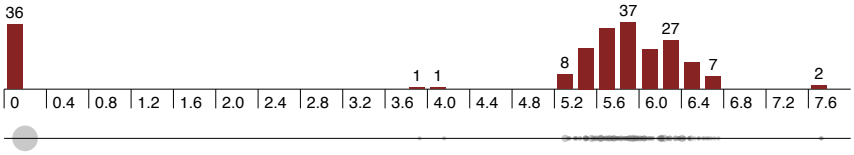
Distribution of ln(ITAC) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



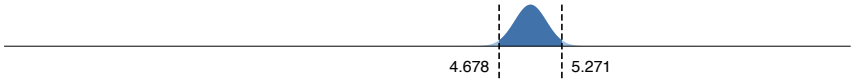
Estimated mean = 2.849 ± 0.336

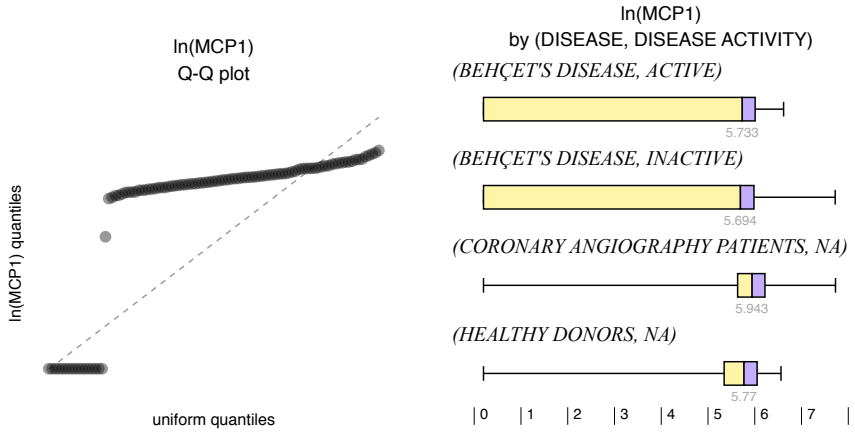


Distribution of ln(MCP1) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

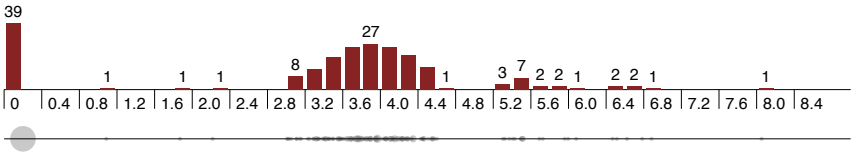


Estimated mean = 4.974 ± 0.297

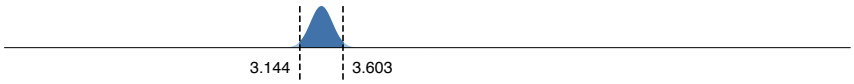


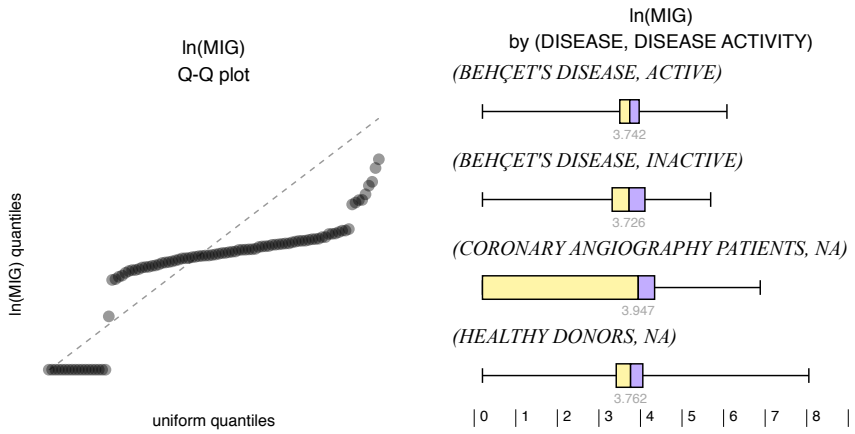


Distribution of ln(MIG) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

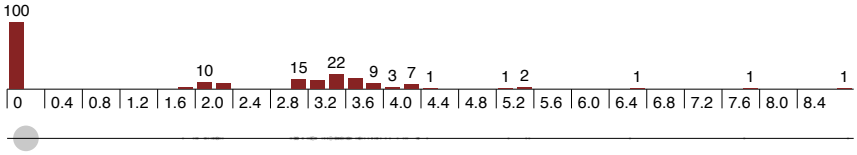


Estimated mean = 3.373 ± 0.229

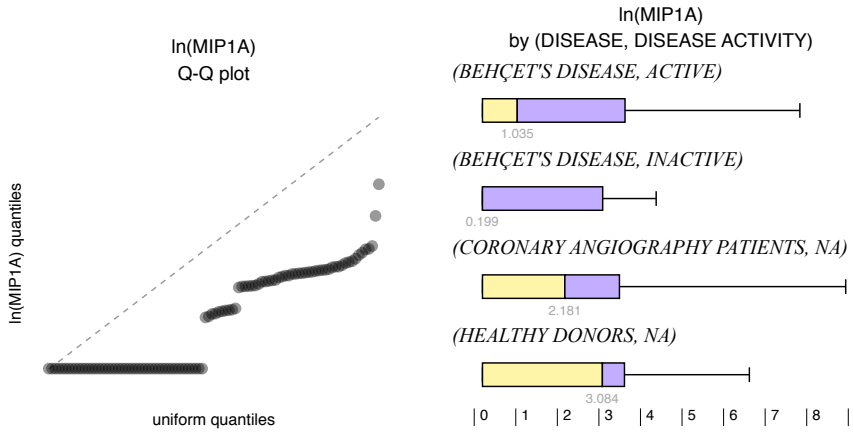




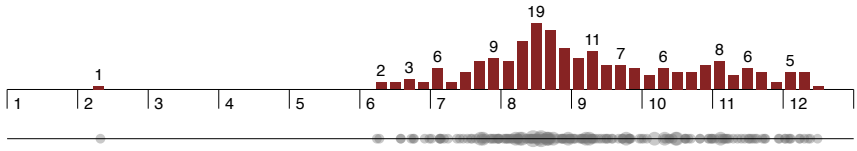
Distribution of ln(MIP1A) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



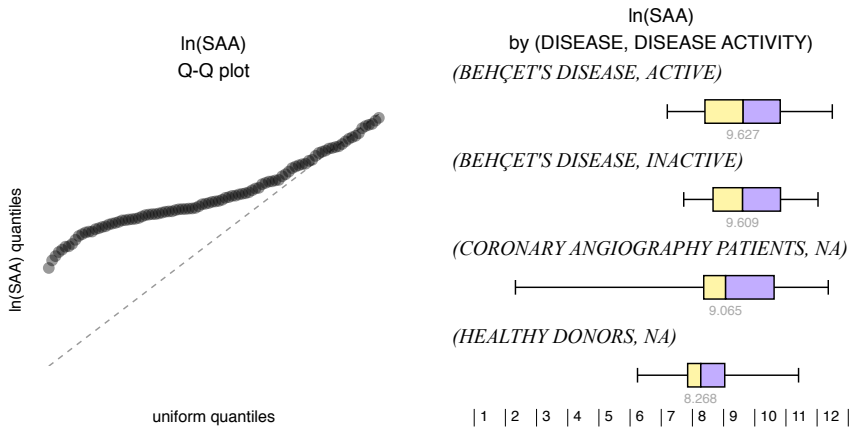
Estimated mean = 1.933 ± 0.243



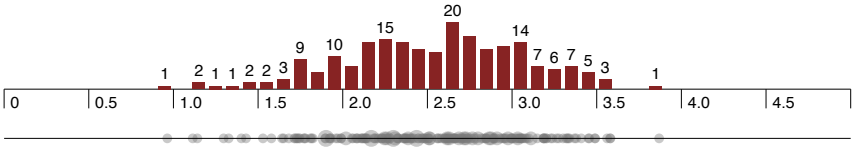
Distribution of ln(SAA) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



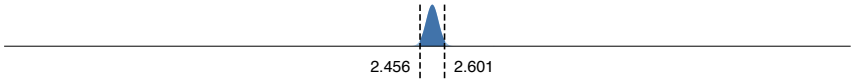
Estimated mean = 9.245 ± 0.208

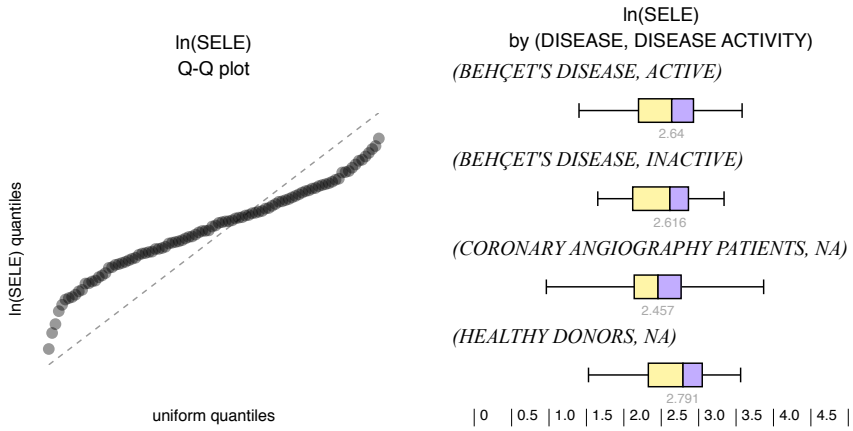


Distribution of ln(SELE) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

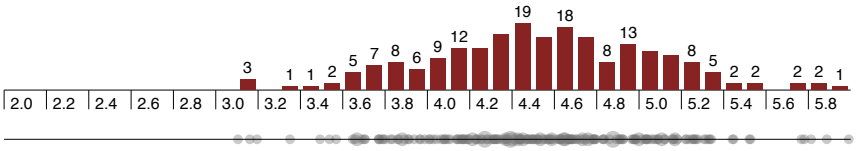


Estimated mean = 2.529 ± 0.072

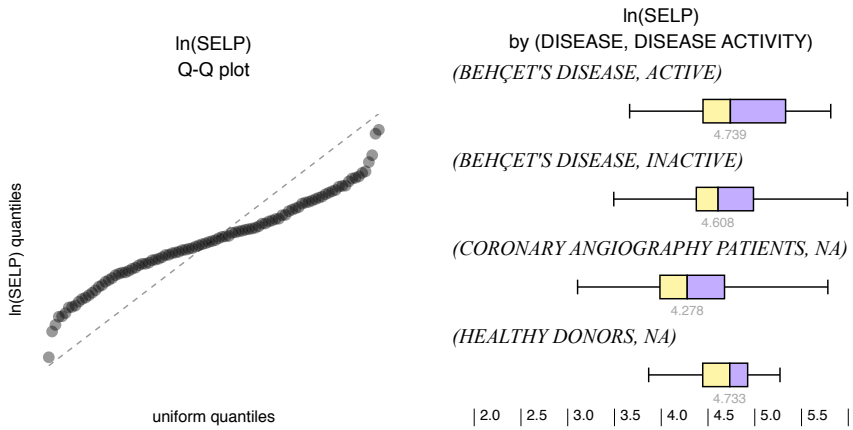




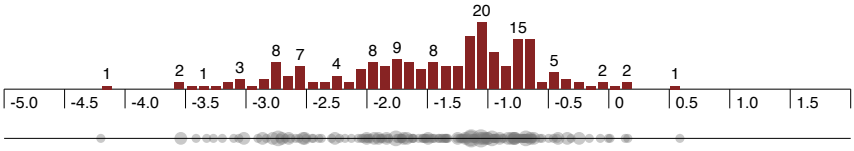
Distribution of ln(SELP) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



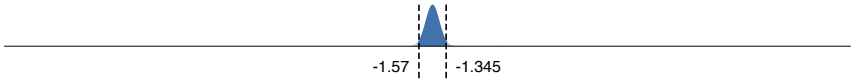
Estimated mean = 4.543 ± 0.072

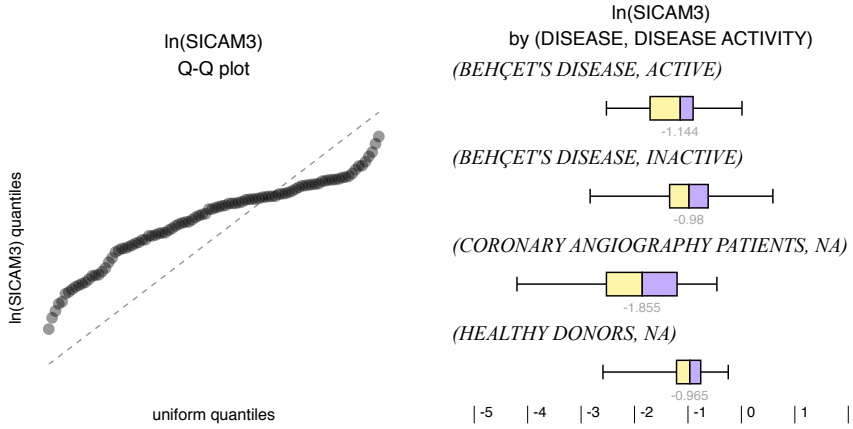


Distribution of ln(SICAM3) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

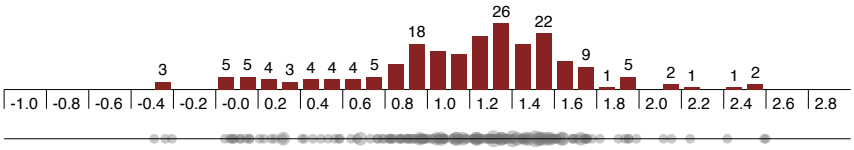


Estimated mean = -1.458 ± 0.112

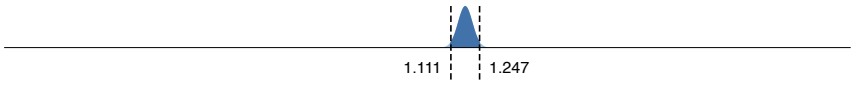


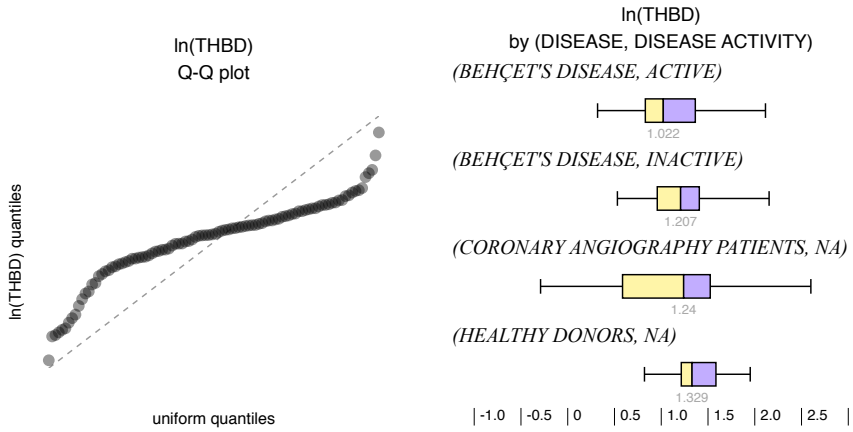


Distribution of ln(THBD) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

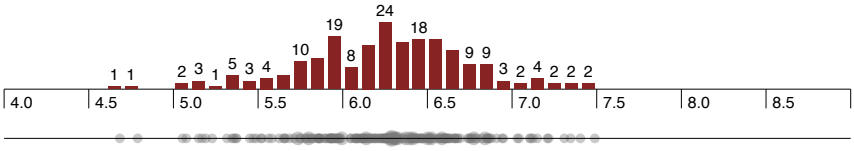


Estimated mean = 1.179 ± 0.068

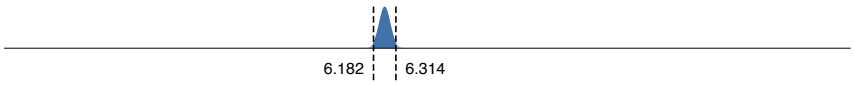


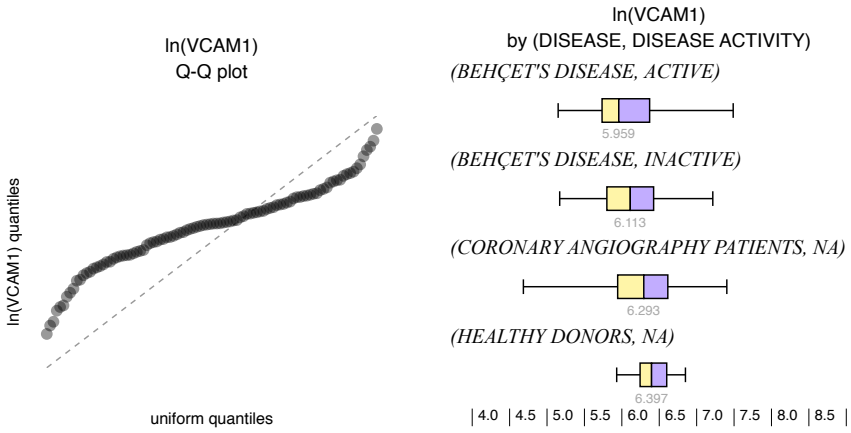


Distribution of ln(VCAM1) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...

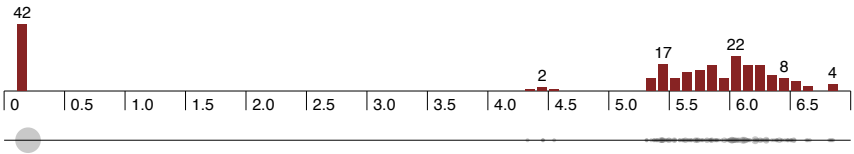


Estimated mean = 6.248 ± 0.066

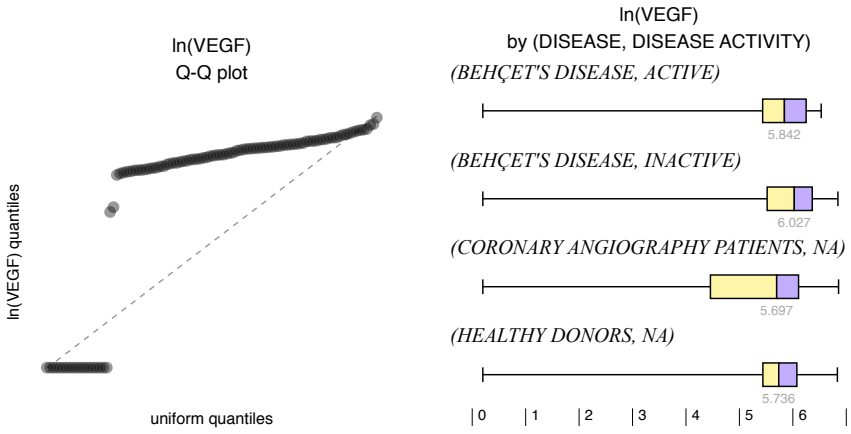




Distribution of ln(VEGF) [DISEASE ∈ {BEHÇET'S DISEASE, CORONARY ANGIOGRAPHY ...



Estimated mean = 4.797 ± 0.313



The application of a natural log corrected for the uniformity of the biomarkers CRP, ICAM-1, SAA, SELE, SELP, sICAM-3, THBD, VCAM-1. All others remained extremely skewed. Again, the distribution overlap between the four set disease variables (BDA, BDI, HD, and CA) did for not allow any biomarker tested to demonstrate a significant difference.

The Kruskal-Wallis test on non-transformed data revealed that the biomarkers Caldesmon, Clusterin, CRP, ICAM-1, IL-6, IL-8, MCP1, SAA, SELE, SELP, sICAM-3, THBD, and VCAM-1 segregated between the four set variables of disease: BDA, BDI, CA, and HD. The biomarkers CNN1, GROα, IP-10, I-TAC, MIG, MIP1α, and VEGF did not yield a result that would allow segregating between BD patients, coronary angiography patients, and healthy donors due to an overlap of results across all four of the set disease variables (BDA, BDI, HD, and CA). ***Table 4*** illustrates the results.

Table 4: Kruskal-Wallis test results for significant differences*

Yellow indicates significant differences

BDA, BDI, HD, and CA

	Caldesmon	Clusterin	CNN1	CRP	GROA	ICAM1	INTLK6	INTLK8	IP10	ITAC
Chi-Square	11.335	12.024	2.357	18.571	4.591	17.043	14.324	13.805	2.856	3.291
df	3	3	3	3	3	3	3	3	3	3
Asymp. Sig.	.010	.007	.502	.000	.204	.001	.002	.003	.414	.349

	MCP1	MIG	MIP1A	SAA	SELE	SELP	SICAM3	THBD	VCAM1	VEGF
Chi-Square	9.909	1.095	4.702	24.251	9.602	24.289	60.282	9.233	16.729	7.753
df	3	3	3	3	3	3	3	3	3	3
Asymp. Sig.	.019	.778	.195	.000	.022	.000	.000	.026	.001	.051

Chi-Square = Chi-Squared test
df = Degrees of freedom
Asymp. Sig. = Asymptotic significance

Biomarkers: Caldesmon, Clusterin, Calponin 1 (CNN1), C-Reactive Protein (CRP), Growth-Regulated Protein-α CXCL-1 (GROα), Intercellular Adhesion Molecule 1 (ICAM-1), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interferon Gamma-Induced Protein 10 CXCL10 (IP-10), Interferon-Inducible T-Cell Alpha Chemoattractant-CXCL11 (I-TAC), Monocyte Chemoattractant Protein 1 CCL2 (MCP1), Monokine Induced By Gamma Interferon CXCL9 (MIG), Macrophage Inflammatory Protein 1-α (MIP1α), Serum Amyloid A (SAA), E-Selectin (SELE), P-Selectin (SELP), Soluble Intercellular Adhesion Molecule 3 (sICAM-3), Thrombomodulin (THBD), Vascular Cell Adhesion Molecule 1 (VCAM-1), and Vascular Endothelial Growth Factor (VEGF).

The 13 biomarkers that revealed significant differences in the Kruskal-Wallis test were retained for a Mann-Whitney test with Bonferroni correction. A Holm-Bonferroni correction was also attempted and produced analogous results to Bonferroni and was thus considered as redundant. These results indicated that, after pairwise comparison (p-value <0.05) between the

* Interleukin 6 (IL-6) and Interleukin 8 (IL-8) are abbreviated INTLK6 and INTLK8 respectively in the database of this study.

four set disease variables (BDA, BDI, CA, and HD), in six possible combinations (BDA vs. HD, BDI vs. HD, BDA vs. CA, BDI vs. CA, BDA vs. BDI, and HD vs. CA), several biomarkers were able to differentiate between the disease categories. ***Table 5*** summarizes the results.

Table 5: Summary of Mann-Whitney with Bonferroni correction test results for significant differences by disease activity

Yellow indicates significant differences
Red indicates no significant differences

	BDA	BDI	HD	CA
BDA			**SAA, THBD, VCAM1**	**Clusterin, SELP, SICAM3**
BDI			**ICAM1, SAA, VCAM1**	**Caldesmon, Clusterin, CRP, IL-8, SELP, SICAM3**
HD				**Clusterin, CRP, ICAM1, IL-6, SAA, SELE, SELP, SICAM3**
CA				

BDA = Behçet's disease active
BDI = Behçet's disease inactive
HD = Healthy donors
CA = Coronary angiography patients

Biomarkers: Caldesmon, Clusterin, C-Reactive Protein (CRP), Intercellular Adhesion Molecule 1 (ICAM-1), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Serum Amyloid A (SAA), E-Selectin (SELE), P-Selectin (SELP), Soluble Intercellular Adhesion Molecule 3 (sICAM-3), Thrombomodulin (THBD), and Vascular Cell Adhesion Molecule 1 (VCAM-1).

No pairwise comparison was able to differentiate between BD patients with active (BDA) and inactive (BDI) disease conditions. The pairwise comparison between BDA vs. HD yielded significance for the biomarkers SAA, THBD, and VCAM-1. The pairwise comparison between BDI vs. HD yielded significance for the biomarkers ICAM-1, SAA, and VCAM-1. The pairwise comparison between BDA vs. CA yielded significance for the biomarkers Clusterin, SELP, and sICAM-3. The pairwise comparison between BDI vs. CA yielded

significance for the biomarkers Caldesmon, Clusterin, CRP, IL-8, SELP, and sICAM-3. The pairwise comparison between HD vs. CA yielded significance for the biomarkers Clusterin, CRP, ICAM-1, IL-6, SAA, SELE, SELP, and sICAM-3.

These results confirm the implication of several biomarkers such as ICAM-1, SAA, THBD, and VCAM-1 in the signature of BD patients relative to healthy donors.

Table 6 displays the individual results for each combination.

Table 6: Mann-Whitney with Bonferroni correction test results by disease activity*

Yellow indicates significant differences
Pink indicates significance after Bonferroni correction
NB: Only the biomarkers that were significant in the Kruskal-Wallis test were examined below
Bonferroni **α** = 0.008333333

BDA vs. HD
Test Statistics[a]

	Caldesmon	Clusterin	CRP	ICAM1	INTLK6	INTLK8	MCP1	SAA	SELE	SELP	SICAM3	THBD	VCAM1
Mann-Whitney U	473.000	490.000	383.000	370.000	364.000	499.000	480.000	267.000	449.000	468.000	346.500	283.000	259.000
Wilcoxon W	726.000	1618.000	1511.000	623.000	1492.000	1627.000	733.000	1395.000	702.000	1596.000	599.500	536.000	512.000
Z	-1.399	-.470	-1.725	-1.893	-2.289	-.239	-.478	-3.219	-.876	-.631	-2.196	-3.013	-3.322
Asymp. Sig. (2-tailed)	.162	.638	.084	.058	.022	.811	.632	.001	.381	.528	.028	.003	.001

[a] Grouping Variable: Disease Activity

BDA vs. CA
Test Statistics[a]

	Caldesmon	Clusterin	CRP	ICAM1	INTLK6	INTLK8	MCP1	SAA	SELE	SELP	SICAM3	THBD	VCAM1
Mann-Whitney U	1067.000	845.500	985.000	918.000	1065.000	1037.000	784.000	971.000	904.000	637.000	632.500	940.500	763.000
Wilcoxon W	1320.000	5696.500	1238.000	5769.000	1318.000	1290.000	1037.000	5822.000	5755.000	5488.000	5483.500	1193.500	1016.000
Z	-.474	-3.029	-.631	-1.085	-.092	-.282	-1.996	-.726	-1.180	-2.991	-3.022	-.933	-2.136
Asymp. Sig. (2-tailed)	.636	.002	.528	.278	.927	.778	.046	.468	.238	.003	.003	.351	.033

[a] Grouping Variable: Disease Activity

BDI vs. HD
Test Statistics[a]

	Caldesmon	Clusterin	CRP	ICAM1	INTLK6	INTLK8	MCP1	SAA	SELE	SELP	SICAM3	THBD	VCAM1
Mann-Whitney U	1030.500	1074.000	975.000	588.000	958.000	785.000	994.000	464.000	866.000	1003.000	1020.000	760.000	607.000
Wilcoxon W	2158.500	2202.000	2103.000	1669.000	2086.000	1866.000	2075.000	1592.000	1947.000	2084.000	2148.000	1841.000	1688.000
Z	-.722	-.075	-.815	-3.788	-1.139	-2.462	-.672	-4.741	-1.652	-.599	-.469	-2.467	-3.642
Asymp. Sig. (2-tailed)	.471	.940	.415	.000	.255	.014	.502	.000	.099	.549	.639	.014	.000

[a] Grouping Variable: Disease Activity

BDI vs CA
Test Statistics[a]

	Caldesmon	Clusterin	CRP	ICAM1	INTLK6	INTLK8	MCP1	SAA	SELE	SELP	SICAM3	THBD	VCAM1
Mann-Whitney U	1986.000	1893.000	1633.000	2243.000	1778.000	1413.000	1646.000	1950.000	1980.000	1493.000	894.000	2246.500	1721.000
Wilcoxon W	6837.000	6744.000	2714.000	3324.000	2859.000	2494.000	2727.000	6801.000	6831.000	6344.000	5745.000	3327.500	2802.000
Z	-3.081	-2.835	-2.661	-.047	-2.162	-3.734	-2.610	-1.303	-1.174	-3.261	-5.827	-.032	-2.284
Asymp. Sig. (2-tailed)	.002	.005	.008	.962	.031	.000	.009	.193	.240	.001	.000	.974	.022

[a] Grouping Variable: Disease Activity

BDA vs. BDI
Test Statistics[a]

	Caldesmon	Clusterin	CRP	ICAM1	INTLK6	INTLK8	MCP1	SAA	SELE	SELP	SICAM3	THBD	VCAM1
Mann-Whitney U	440.000	480.500	428.000	400.000	419.000	337.500	497.000	484.000	480.000	442.000	364.500	410.000	470.000
Wilcoxon W	693.000	1561.500	1509.000	1481.000	1500.000	1418.500	1578.000	737.000	1561.000	1523.000	617.500	663.000	723.000
Z	-1.759	-.450	-1.023	-1.390	-1.266	-2.393	-.119	-.288	-.341	-.839	-1.855	-1.258	-.472
Asymp. Sig. (2-tailed)	.079	.653	.307	.165	.205	.017	.905	.773	.733	.401	.064	.208	.637

[a] Grouping Variable: Disease Activity

HD vs. CA
Test Statistics[a]

	Caldesmon	Clusterin	CRP	ICAM1	INTLK6	INTLK8	MCP1	SAA	SELE	SELP	SICAM3	THBD	VCAM1
Mann-Whitney U	2131.500	1947.000	1308.000	1470.000	1529.000	2169.000	1828.000	1417.000	1578.000	1326.000	741.500	1822.500	1989.000
Wilcoxon W	6982.500	6798.000	2436.000	6321.000	2657.000	3297.000	2956.000	2545.000	6429.000	6177.000	5592.500	6673.500	6840.000
Z	-2.292	-2.765	-4.203	-3.519	-3.511	-.578	-2.008	-3.743	-3.063	-4.127	-6.596	-2.030	-1.326
Asymp. Sig. (2-tailed)	.022	.006	.000	.000	.000	.563	.045	.000	.002	.000	.000	.042	.185

Mann-Whitney U = Mann–Whitney–Wilcoxon test
Wilcoxon W = Nonparametric Wilcoxon signed-rank test
Z = z-test
Asymp. Sig. (2-tailed) = Pearson Chi-squared value (2-sided)

Biomarkers: Caldesmon, Clusterin, Calponin 1 (CNN1), C-Reactive Protein (CRP), Growth-Regulated Protein-α CXCL-1 (GROα), Intercellular Adhesion Molecule 1 (ICAM-1), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interferon Gamma-Induced Protein 10 CXCL10 (IP-10), Interferon-Inducible T-Cell Alpha Chemoattractant-CXCL11 (I-TAC), Monocyte Chemoattractant Protein 1 CCL2 (MCP1), Monokine Induced By Gamma Interferon CXCL9 (MIG), Macrophage Inflammatory Protein 1-α (MIP1α), Serum Amyloid A (SAA), E-Selectin (SELE), P-Selectin (SELP), Soluble Intercellular Adhesion Molecule 3 (sICAM-3), Thrombomodulin (THBD), Vascular Cell Adhesion Molecule 1 (VCAM-1), and Vascular Endothelial Growth Factor (VEGF).

* Interleukin 6 (IL-6) and Interleukin 8 (IL-8) are abbreviated INTLK6 and INTLK8 respectively in the database of this study.

Prior to testing for the magnitude of association between each biomarker and disease status, the linearity assumption for each biomarker across the six disease pairwise combinations was assessed using a Generalized Additive Models (GAM). No significant conclusions from the Akaike information criterion (AIC) were obtained. The GAM test failed to identify a recognizable pattern when comparing BD and HD, and BD and CA. The ensuing regression models at a 95% confidence interval (CI) (p-value <0.05) generated no exploitable results. The wide range of the AIC results demonstrated that the quality of the regression models was weak, due to the non-uniformly distributed data. ***Table 7*** displays the test results for BDA vs. HD.

Table 7: Generalized Additive Models test results: example of BDA vs. HD

BDA vs. HD

Parametric coefficients:	**Estimate**	**Std. Error**	**z value**	**p-value**	
Intercept	**1.358**	**0.5448**	**2.493**	**0.0127**	*

	edf	**Ref. df**	**Chi. sq**	**p-value**	
LnSAA	**1.027**	**1.054**	**20.913**	**6.41E-06**	***
LnTHBD	**1**	**1**	**6.079**	**1.37E-02**	*
LnVCAM1	**2.733**	**3.442**	**18.283**	**0.00072**	***

R-squared adjusted = 0.57
Deviance explained = 54.3%
Un-Biased Risk Estimator = -0.28122
Scale esimate = 1
Count = 115

Significance: *** 0.001
** 0.01
* 0.05

Abbreviations:
Chi. Sq = Chi-squared test
edf = Effective degrees of freedo
Estimate = Estimation
Ref. df = Reference degrees of freedom
Std. Error = Standard deviation
p-value = Significance
z-value = Standard score

Akaike information criterion (AIC)
GAM test of various combinations in BDA vs. HD

	df	**AIC**
Variation 1	**7.742678**	**74.82633**
Variation 2	**22.647714**	**92.81457**
Variation 3	**6.061195**	**81.57629**
Variation 4	**5.759761**	**82.65975**
Variation 5	**4.642003**	**88.05278**
Variation 6	**8.166527**	**93.00779**
Variation 7	**5.02268**	**117.23678**

Biomarkers: Serum Amyloid A (SAA), Thrombomodulin (THBD), and Vascular Cell Adhesion Molecule 1 (VCAM-1).

The Spearman rank correlation coefficient test is shown in ***Table 8***. The tests identified covariate biomarkers and a p-value smaller than 0.01 and 0.05 was considered to be statistically significant in a 2-tailed model.

Table 8: Biomarker correlation results from the Spearman rank correlation coefficient test*

Yellow indicates significant correlation

		Caldesmon	Clusterin	CNN1	CRP	GROA	ICAM1	INTLK6	INTLK8	IP10	ITAC	MCP1	MIG	MIP1A	SAA	SELE	SELP	SICAM3	THBD	VCAM1	VEGF
Caldesmon	Cor. Coef.	1.000	-.025	.386◆◆	.145	.092	.174	-.113	-.078	-.301◆	.027	-.046	-.154	.053	.169	-.039	.108	.288◆	.281◆	.198	.055
	Sig. (2-tailed)		.838	.001	.239	.457	.155	.357	.527	.013	.826	.709	.209	.667	.167	.754	.383	.017	.020	.105	.657
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
Clusterin	Cor. Coef.	-.025	1.000	.280◆	.096	.027	-.062	.045	-.191	.003	-.085	.092	.059	-.253◆	.031	.052	.000	.225	.204	.113	.096
	Sig. (2-tailed)	.838		.021	.434	.825	.616	.714	.120	.979	.488	.455	.631	.037	.799	.671	.998	.066	.095	.360	.435
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
CNN1	Cor. Coef.	.386◆◆	.280◆	1.000	-.047	-.060	-.078	.073	-.108	-.148	.166	.166	.128	-.100	-.078	.016	.171	.184	.152	.072	.109
	Sig. (2-tailed)	.001	.021		.705	.625	.528	.557	.381	.227	.176	.175	.300	.416	.528	.900	.163	.134	.214	.562	.376
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
CRP	Cor. Coef.	.145	.096	-.047	1.000	.164	.441◆◆	.302◆	.006	-.015	.009	-.302◆	.138	.014	.789◆◆	.322◆◆	.122	.133	.320◆◆	.430◆◆	.001
	Sig. (2-tailed)	.239	.434	.705		.181	.000	.012	.964	.906	.940	.012	.263	.910	.000	.007	.322	.279	.008	.000	.995
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
GROA	Cor. Coef.	.092	.027	-.060	.164	1.000	.283◆	.113	.049	.161	.110	.010	.140	.181	.147	.155	.065	.160	.180	.188	.050
	Sig. (2-tailed)	.457	.825	.625	.181		.019	.357	.690	.189	.374	.937	.254	.140	.232	.207	.599	.193	.142	.126	.687
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
ICAM1	Cor. Coef.	.174	-.062	-.078	.441◆◆	.283◆	1.000	.277◆	.064	.098	-.023	.018	.190	.097	.394◆◆	.285◆	.043	-.072	.272◆	.642◆◆	.245◆
	Sig. (2-tailed)	.155	.616	.528	.000	.019		.022	.604	.427	.855	.885	.121	.431	.001	.018	.730	.561	.025	.000	.044
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
INTLK6	Cor. Coef.	-.113	.045	.073	.302◆	.113	.277◆	1.000	.292◆	.007	.013	.182	.239◆	.149	.323◆◆	.173	.190	-.147	.018	.261◆	.139
	Sig. (2-tailed)	.357	.714	.557	.012	.357	.022		.016	.954	.914	.138	.049	.226	.007	.158	.121	.230	.886	.032	.260
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
INTLK8	Cor. Coef.	-.078	-.191	-.108	.006	.049	.064	.292◆	1.000	-.107	-.205	-.027	-.111	.144	-.054	-.051	.068	-.259◆	-.091	.001	-.098
	Sig. (2-tailed)	.527	.120	.381	.964	.690	.604	.016		.384	.094	.828	.369	.242	.660	.683	.581	.033	.463	.996	.427
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
IP10	Cor. Coef.	-.301◆	.003	-.148	-.015	.161	.098	.007	-.107	1.000	.075	.154	.104	-.181	.111	.275◆	-.214	.080	-.002	.056	.030
	Sig. (2-tailed)	.013	.979	.227	.906	.189	.427	.954	.384		.542	.209	.399	.139	.367	.023	.079	.516	.986	.652	.809
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
ITAC	Cor. Coef.	.027	-.085	.166	.009	.110	-.023	.013	-.205	.075	1.000	-.122	.165	-.080	.059	.323◆◆	.090	.110	.102	-.050	-.005
	Sig. (2-tailed)	.826	.488	.176	.940	.374	.855	.914	.094	.542		.323	.179	.516	.635	.007	.465	.371	.410	.688	.968
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
MCP1	Cor. Coef.	-.046	.092	.166	-.302◆	.010	.018	.182	-.027	.154	-.122	1.000	.218	-.014	-.205	-.217	.107	-.037	-.122	-.034	.187
	Sig. (2-tailed)	.709	.455	.175	.012	.937	.885	.138	.828	.209	.323		.074	.908	.094	.076	.384	.766	.322	.786	.128
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
MIG	Cor. Coef.	-.154	.059	.128	.138	.140	.190	.239◆	-.111	.104	.165	.218	1.000	.034	.221	.215	-.106	-.049	-.036	.162	.101
	Sig. (2-tailed)	.209	.631	.300	.263	.254	.121	.049	.369	.399	.179	.074		.784	.070	.079	.390	.693	.770	.188	.414
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
MIP1A	Cor. Coef.	.053	-.253◆	-.100	.014	.181	.097	.149	.144	-.181	-.080	-.014	.034	1.000	-.042	.033	.370◆◆	-.040	.175	.071	-.019
	Sig. (2-tailed)	.667	.037	.416	.910	.140	.431	.226	.242	.139	.516	.908	.784		.736	.791	.002	.744	.155	.567	.880
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
SAA	Cor. Coef.	.169	.031	-.078	.789◆◆	.147	.394◆◆	.323◆◆	-.054	.111	.059	-.205	.221	-.042	1.000	.462◆◆	.044	.206	.325◆◆	.459◆◆	-.031
	Sig. (2-tailed)	.167	.799	.528	.000	.232	.001	.007	.660	.367	.635	.094	.070	.736		.000	.719	.092	.007	.000	.800
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
SELE	Cor. Coef.	-.039	.052	.016	.322◆◆	.155	.285◆	.173	-.051	.275◆	.323◆◆	-.217	.215	.033	.462◆◆	1.000	.032	.235	.397◆◆	.227	.038
	Sig. (2-tailed)	.754	.671	.900	.007	.207	.018	.158	.683	.023	.007	.076	.079	.791	.000		.798	.053	.001	.063	.760
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
SELP	Cor. Coef.	.108	.000	.171	.122	.065	.043	.190	.068	-.214	.090	.107	-.106	.370◆◆	.044	.032	1.000	.141	.402◆◆	.173	.186
	Sig. (2-tailed)	.383	.998	.163	.322	.599	.730	.121	.581	.079	.465	.384	.390	.002	.719	.798		.251	.001	.158	.128
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
SICAM3	Cor. Coef.	.288◆	.225	.184	.133	.160	-.072	-.147	-.259◆	.080	.110	-.037	-.049	-.040	.206	.235	.141	1.000	.597◆◆	.025	.226
	Sig. (2-tailed)	.017	.066	.134	.279	.193	.561	.230	.033	.516	.371	.766	.693	.744	.092	.053	.251		.000	.842	.064
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
THBD	Cor. Coef.	.281◆	.204	.152	.320◆◆	.180	.272◆	.018	-.091	-.002	.102	-.122	-.036	.175	.325◆◆	.397◆◆	.402◆◆	.597◆◆	1.000	.415◆◆	.201
	Sig. (2-tailed)	.020	.095	.214	.008	.142	.025	.886	.463	.986	.410	.322	.770	.155	.007	.001	.001	.000		.000	.101
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
VCAM1	Cor. Coef.	.198	.113	.072	.430◆◆	.188	.642◆◆	.261◆	.001	.056	-.050	-.034	.162	.071	.459◆◆	.227	.173	.025	.415◆◆	1.000	.125
	Sig. (2-tailed)	.105	.360	.562	.000	.126	.000	.032	.996	.652	.688	.786	.188	.567	.000	.063	.158	.842	.000		.310
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68
VEGF	Cor. Coef.	.055	.096	.109	.001	.050	.245◆	.139	-.098	.030	-.005	.187	.101	-.019	-.031	.038	.186	.226	.201	.125	1.000
	Sig. (2-tailed)	.657	.435	.376	.995	.687	.044	.260	.427	.809	.968	.128	.414	.880	.800	.760	.128	.064	.101	.310	
	N	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68	68

◆◆ Correlation is significant at the 0.01 level (2-tailed)
◆ Correlation is significant at the 0.05 level (2-tailed)
Cor. coef. = Correlation coefficient
Sig. (2-tailed) = Significance (2-tailed)
N = Count

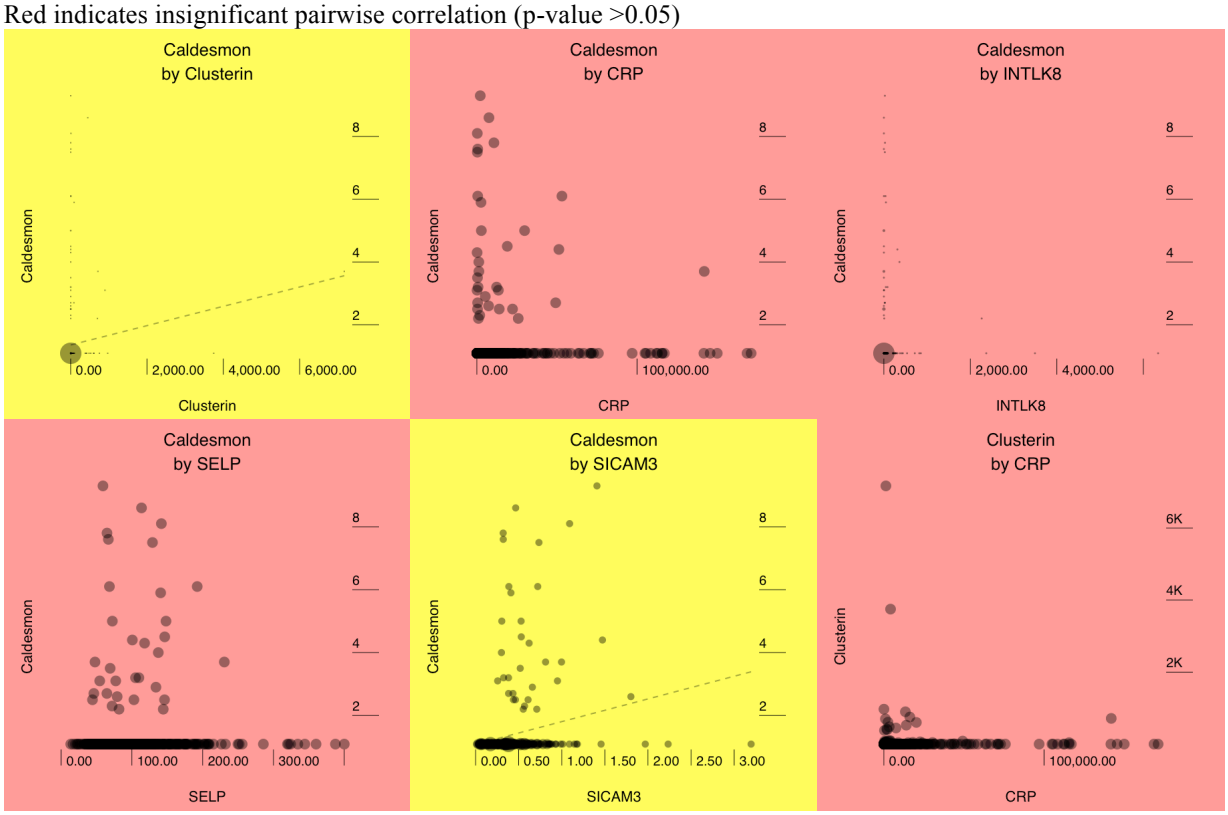
Biomarkers: Caldesmon, Clusterin, Calponin 1 (CNN1), C-Reactive Protein (CRP), Growth-Regulated Protein-α CXCL-1 (GROα), Intercellular Adhesion Molecule 1 (ICAM-1), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interferon Gamma-Induced Protein 10 CXCL10 (IP-10), Interferon-Inducible T-Cell Alpha Chemoattractant-CXCL11 (I-TAC), Monocyte Chemoattractant Protein 1 CCL2 (MCP1), Monokine Induced By Gamma Interferon CXCL9 (MIG), Macrophage Inflammatory Protein 1-α (MIP1α), Serum Amyloid A (SAA), E-Selectin (SELE), P-Selectin (SELP), Soluble Intercellular Adhesion Molecule 3 (sICAM-3), Thrombomodulin (THBD), Vascular Cell Adhesion Molecule 1 (VCAM-1), and Vascular Endothelial Growth Factor (VEGF).

* Interleukin 6 (IL-6) and Interleukin 8 (IL-8) are abbreviated INTLK6 and INTLK8 respectively in the database of this study.

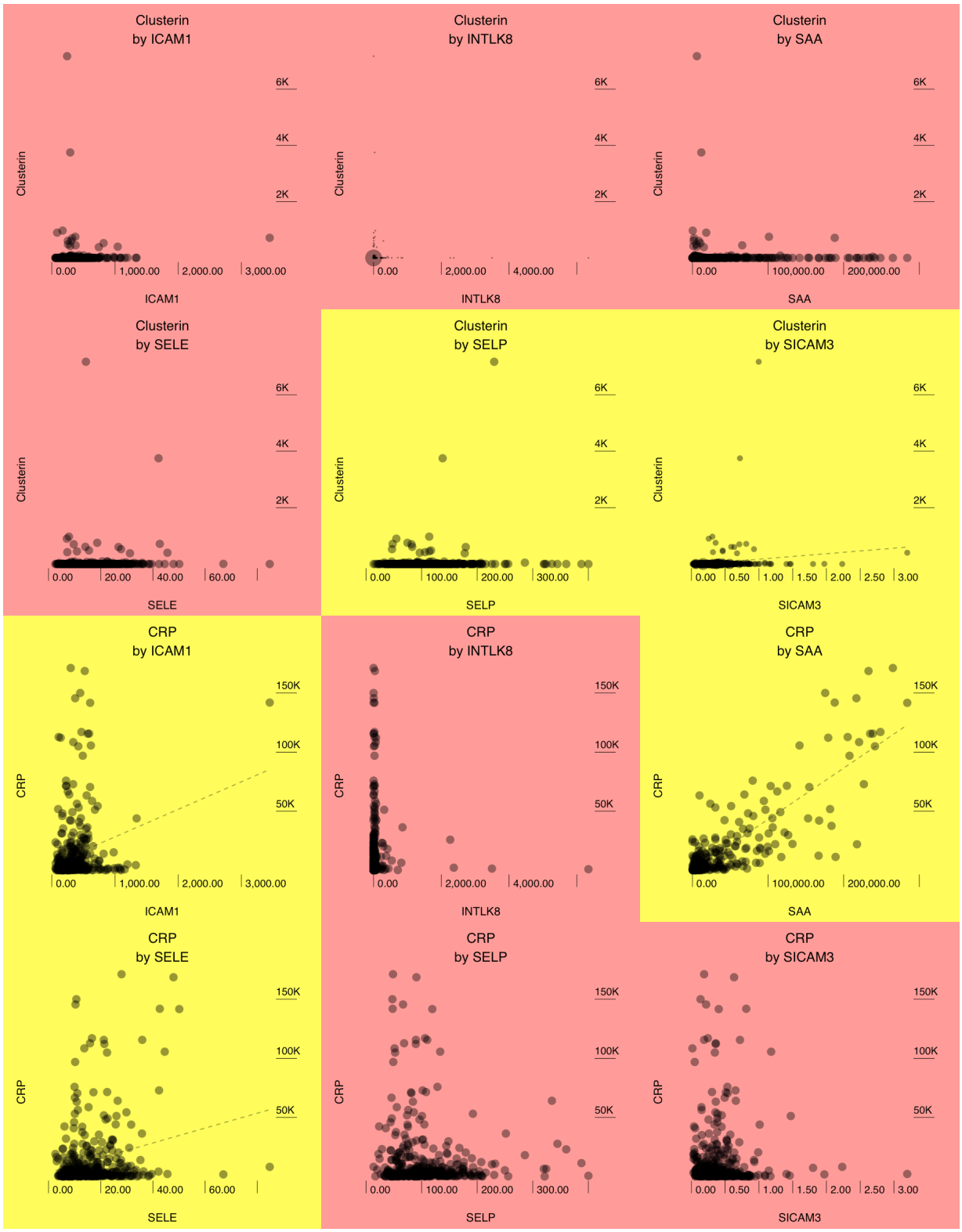
The Spearman test results were compared to the biomarkers identified as significant in the Mann-Whitney test with Bonferroni correction in each of the six disease pairwise combinations. The Spearman test corroborated the results obtained in the Mann-Whitney test. Seventeen out of the 34 possible combinations of biomarker pairs had a significant correlation coefficient: Caldesmon by Clusterin, Caldesmon by sICAM-3, Clusterin by SELP, Clusterin by sICAM-3, CRP by ICAM-1, CRP by SAA, CRP by SELE, ICAM-1 by SAA, ICAM-1 by SELE, ICAM-1 by VCAM-1, IL-8 by SELP, SAA by SELE, SAA by THBD, SAA by VCAM-1, SELE by SELP, SELE by sICAM-3, and SELP by sICAM-3. The pairwise correlations are graphically illustrated in ***Figure 4***.

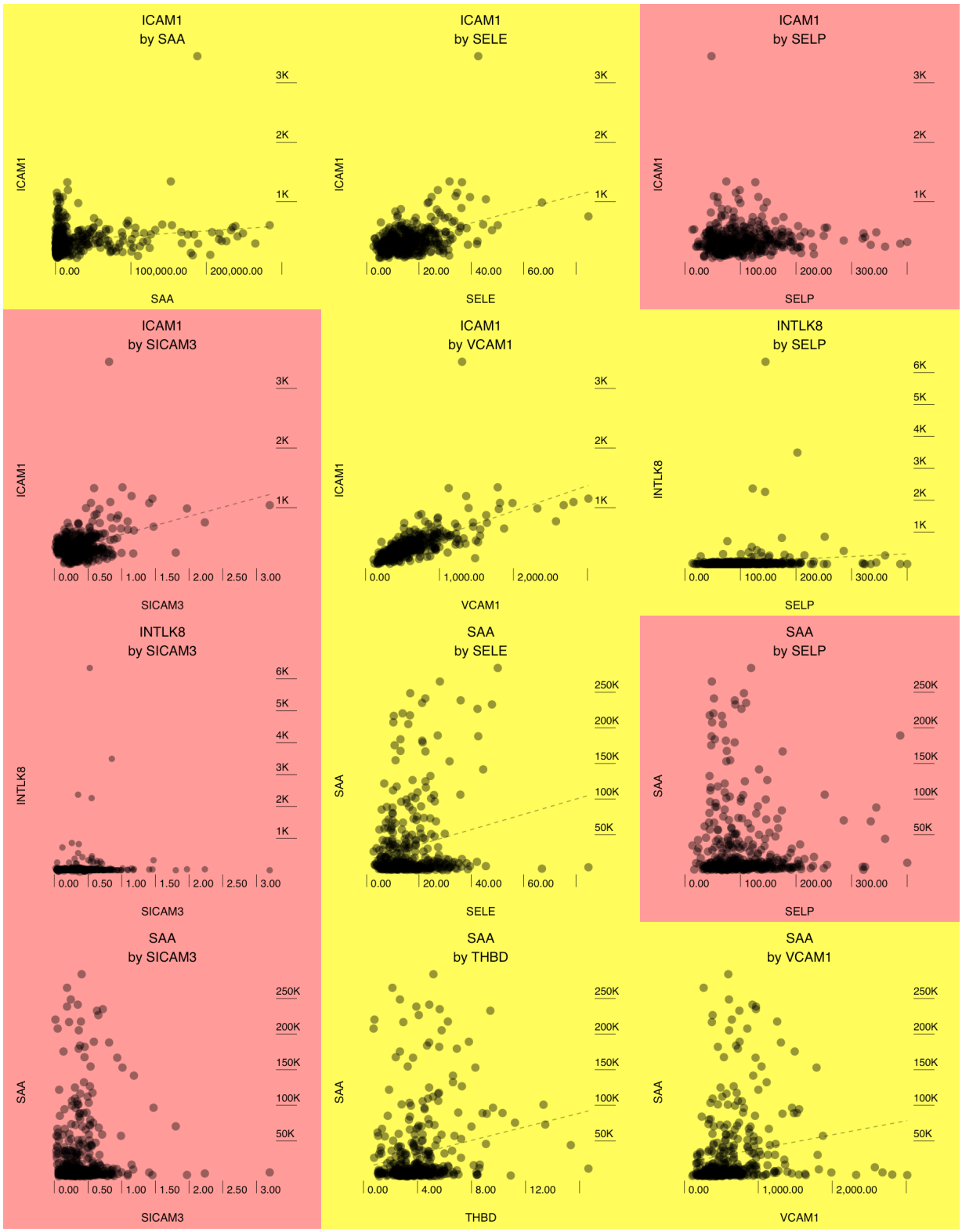
Figure 4: 2-axis graphic illustration of pairwise correlations of biomarkers retained after the Mann-Whitney test with Bonferroni correction*

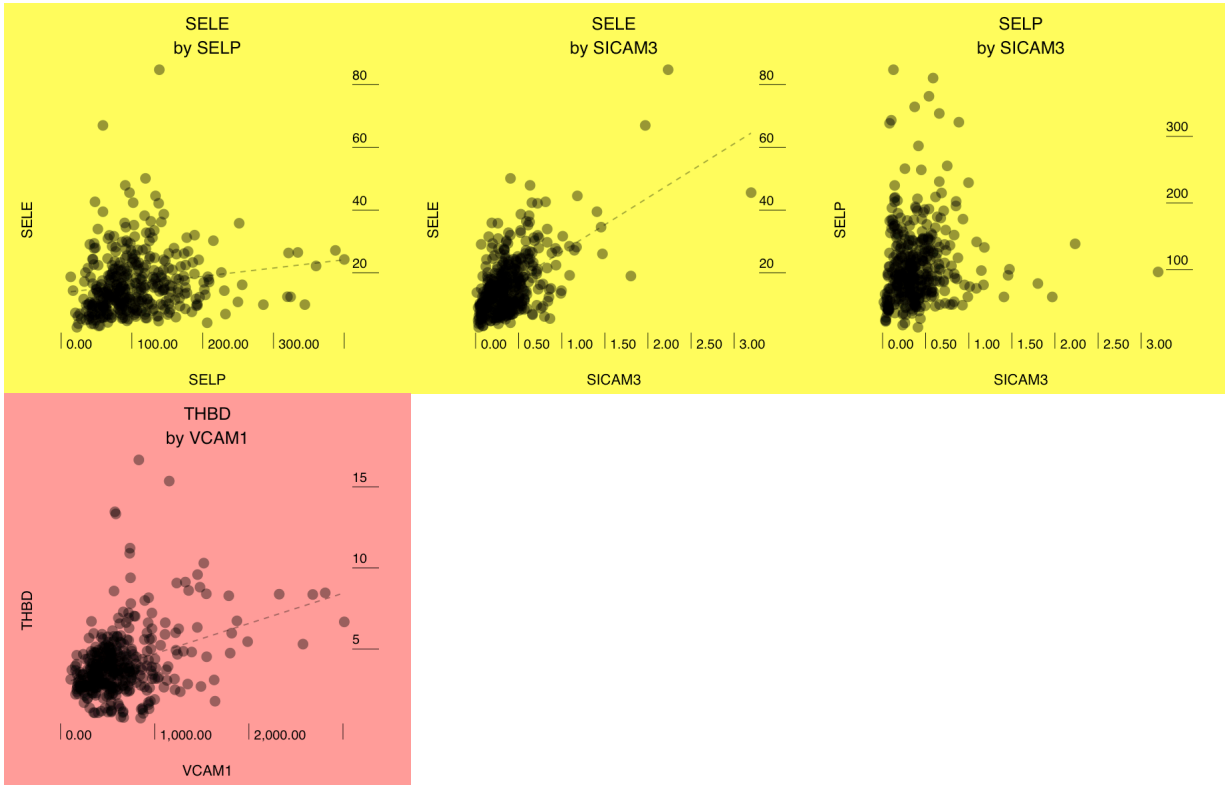
Yellow indicates significant pairwise correlation (p-value <0.05)
Red indicates insignificant pairwise correlation (p-value >0.05)



* Interleukin 6 (IL-6) and Interleukin 8 (IL-8) are abbreviated INTLK6 and INTLK8 respectively in the database of this study.


Clusterin
by ICAM1
Clusterin
6K
4K
2K
0.00
1,000.00
2,000.00
3,000.00
ICAM1
Clusterin
by INTLK8
Clusterin
6K
4K
2K
0.00
2,000.00
4,000.00
INTLK8
Clusterin
by SAA
Clusterin
6K
4K
2K
0.00
100,000.00
200,000.00
SAA
Clusterin
by SELE
Clusterin
6K
4K
2K
0.00
20.00
40.00
60.00
SELE
Clusterin
by SELP
Clusterin
6K
4K
2K
0.00
100.00
200.00
300.00
SELP
Clusterin
by SICAM3
Clusterin
6K
4K
2K
0.00
0.50
1.00
1.50
2.00
2.50
3.00
SICAM3
CRP
by ICAM1
CRP
150K
100K
50K
0.00
1,000.00
2,000.00
3,000.00
ICAM1
CRP
by INTLK8
CRP
150K
100K
50K
0.00
2,000.00
4,000.00
INTLK8
CRP
by SAA
CRP
150K
100K
50K
0.00
100,000.00
200,000.00
SAA
CRP
by SELE
CRP
150K
100K
50K
0.00
20.00
40.00
60.00
SELE
CRP
by SELP
CRP
150K
100K
50K
0.00
100.00
200.00
300.00
SELP
CRP
by SICAM3
CRP
150K
100K
50K
0.00
0.50
1.00
1.50
2.00
2.50
3.00
SICAM3


ICAM1
by SAA
ICAM1
SAA
ICAM1
by SELE
ICAM1
SELE
ICAM1
by SELP
ICAM1
SELP
ICAM1
by SICAM3
ICAM1
SICAM3
ICAM1
by VCAM1
ICAM1
VCAM1
INTLK8
by SELP
INTLK8
SELP
INTLK8
by SICAM3
INTLK8
SICAM3
SAA
by SELE
SAA
SELE
SAA
by SELP
SAA
SELP
SAA
by SICAM3
SAA
SICAM3
SAA
by THBD
SAA
THBD
SAA
by VCAM1
SAA
VCAM1



A Pearson product-moment correlation coefficient test was considered inconclusive, as normality could not be assumed for the data.

A PCA using natural log-transformed data was attempted in order to identify the correlation between biomarkers and the four set disease variables. No particular cluster of covariate biomarkers was detected. This test was particularly unconvincing as it failed to segregate between any of the four set disease variables. The results are graphically illustrated in ***Figure 5***.

Figure 5: Individuals Factor Map of biomarkers (Principal Component Analysis – 8 components extracted)

Given the large amount of results generated, only those correlations pertaining to the development of a predictive model were retained. Despite various attempts, logistic (p-value <0.05) and multivariate (p-value <0.2) regression models failed to correlate between the four set disease variables of BDA, BDI, CA, and HD. In all circumstances, individual combinations failed after bootstrap and the predictive power was moderate at best.

Failure to achieve results may be explained by the fact that the dataset was heterogeneous, skewed, and contained multiple outliers; this conceivably is due to the small sample size of patients with either active (n=22) or inactive (n=46) Behçet's disease. Consequently, a pooling of BDA and BDI data were applied using normalized data (natural log). A Hypothesis test was performed to identify those biomarkers that possess the same distribution across the disease variables BDA and BDI so that they may be combined. All of the biomarkers were retained except for IL-8 as its distribution was not comparable across BDA and BDI. Regardless, IL-8 is not of particular interest, as it did not segregate between BD and HD in any case. ***Table 9*** displays the results.

Table 9: Hypothesis test to identify biomarkers that may be pooled when combining BDA and BDI patient data*

Orange indicates rejection of null hypothesis

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Caldesmon is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.079	Retain the null hypothesis.
2	The distribution of Clusterin is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.653	Retain the null hypothesis.
3	The distribution of CNN1 is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.489	Retain the null hypothesis.
4	The distribution of CRP is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.307	Retain the null hypothesis.
5	The distribution of GROA is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.400	Retain the null hypothesis.
6	The distribution of ICAM1 is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.165	Retain the null hypothesis.
7	The distribution of INTLK6 is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.205	Retain the null hypothesis.
8	The distribution of INTLK8 is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.017	Reject the null hypothesis.
9	The distribution of IP10 is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.739	Retain the null hypothesis.
10	The distribution of ITAC is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.902	Retain the null hypothesis.
11	The distribution of MCP1 is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.905	Retain the null hypothesis.
12	The distribution of MIG is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.623	Retain the null hypothesis.
13	The distribution of MIP1A is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.322	Retain the null hypothesis.
14	The distribution of SAA is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.773	Retain the null hypothesis.
15	The distribution of SELE is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.733	Retain the null hypothesis.
16	The distribution of SELP is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.401	Retain the null hypothesis.
17	The distribution of SICAM3 is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.064	Retain the null hypothesis.
18	The distribution of THBD is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.208	Retain the null hypothesis.
19	The distribution of VCAM1 is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.637	Retain the null hypothesis.
20	The distribution of VEGF is the same across categories of DiseaseAct.	Independent-Samples Mann-Whitney U Test	.454	Retain the null hypothesis.

Asymptotic significances are displayed (significance level is 0.05)

* Interleukin 6 (IL-6) and Interleukin 8 (IL-8) are abbreviated INTLK6 and INTLK8 respectively in the database of this study.

Various attempts at logistic regressions failed. No significant results were obtained. Consequently, a regression model of binary response was attempted. The multivariate regressions for the disease set variables BD (BDA+BDI), HD, and CA yielded meaningful predictive models at a 95% CI that demonstrated both specificity and sensitivity. After various iterations, the models were enhanced to a 99% CI for all combinations.

Table 10 summarizes the results of the regression for BD (BDA+BDI) vs. HD.

Table 10: Multivariate regression test results for biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from HD

✓	Explanatory variable	Coefficient	Std. error	Visualization	Z-score	p-value	Significance	99% Confidence Interval
☐	ln(Caldesmon)	-	-		-	-		-
☐	ln(Clusterin)	-	-		-	-		-
☐	ln(CNN1)	-	-		-	-		-
☑	ln(CRP)	0.36	(0.125)		2.873	0.004		(0.037, 0.683)
☐	ln(GROA)	-	-		-	-		-
☑	ln(ICAM1)	1.107	(0.221)		5.021	<0.001		(0.539, 1.675)
☐	ln(INTLK6)	-	-		-	-		-
☐	ln(INTLK8)	-	-		-	-		-
☐	ln(IP10)	-	-		-	-		-
☐	ln(ITAC)	-	-		-	-		-
☐	ln(MCP1)	-	-		-	-		-
☐	ln(MIG)	-	-		-	-		-
☐	ln(MIP1A)	-	-		-	-		-
☑	ln(SAA)	-1.042	(0.199)		-5.237	<0.001		(-1.554, -0.529)
☐	ln(SELE)	-	-		-	-		-
☐	ln(SELP)	-	-		-	-		-
☐	ln(SICAM3)	-	-		-	-		-
☐	ln(THBD)	-	-		-	-		-
☐	ln(VCAM1)	-	-		-	-		-
☐	ln(VEGF)	-	-		-	-		-
☐	(constant)	0	(0)		-	-		(0, 0)

Coefficient = Constant
Std. error = Standard deviation
z-score: Standard score
p-value: Significance
99% Confidence Interval: Probability

Biomarkers: C-Reactive Protein (CRP), Intercellular Adhesion Molecule 1 (ICAM-1), and Serum Amyloid A (SAA).

The equation for calculating the score regression coefficient is:

$$z = 0.36 * \{\ln[CRP(ng/mL)] = 8\}$$
$$+ 1.107 * \{\ln[ICAM1(ng/mL)] = 4\}$$
$$+ -1.1042 * \{\ln[SAA(ng/mL)] = 13\}$$

The distribution of individual biomarkers is detailed in ***Figure 6***.

Figure 6: Estimated coefficient distributions for individual biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from HD

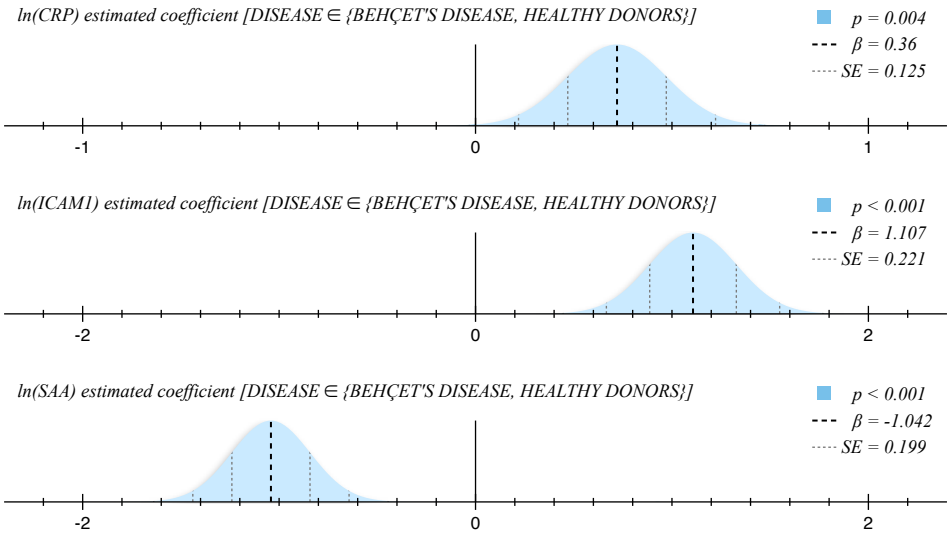


Figure 7 displays the residual distribution for each biomarker on a 2-axis plot.

Figure 7: Ordered probit residual 2-axis plots for individual biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from HD

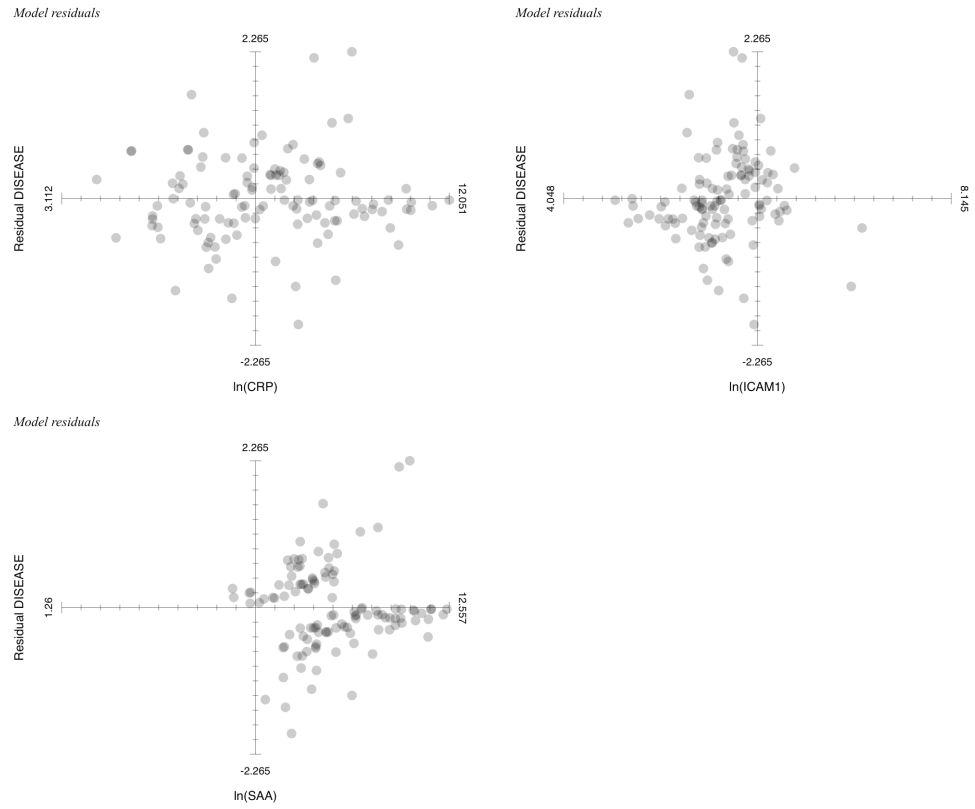
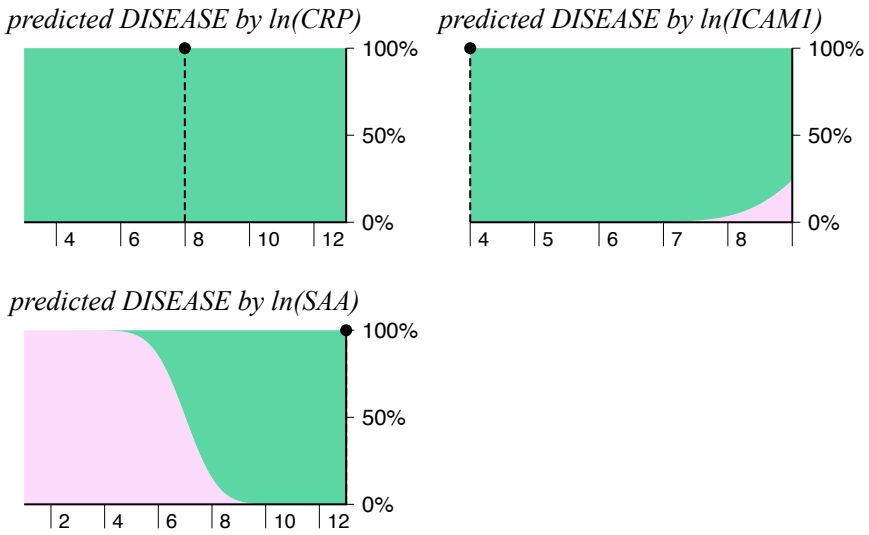


Figure 8 illustrates the discriminatory power of the Area Under the Curve for each individual biomarker.

Figure 8: Discriminatory power as illustrated by the Area Under the Curve for individual biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from HD



Similarly, a predictive model (99% CI) was constructed that could segregate between BD (BDA+BDI) vs. CA. ***Table 11*** summarizes the results of the regression for BD (BDA+BDI) vs. CA.

Table 11: Multivariate regression test results for biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from CA

✓	Explanatory variable	Coefficient	Std. error	Visualization	Z-score	p-value	Significance	99% Confidence Interval
☐	ln(Caldesmon)	-	-		-	-		-
☑	ln(Clusterin)	-0.553	(0.191)		-2.898	0.004		(-1.044, -0.061)
☑	ln(CNN1)	0.633	(0.174)		3.631	<0.001		(0.184, 1.083)
☑	ln(CRP)	0.358	(0.121)		2.956	0.003		(0.046, 0.67)
☐	ln(GROA)	-	-		-	-		-
☑	ln(ICAM1)	-1.54	(0.323)		-4.771	<0.001		(-2.372, -0.709)
☐	ln(INTLK6)	-	-		-	-		-
☐	ln(INTLK8)	-	-		-	-		-
☐	ln(IP10)	-	-		-	-		-
☐	ln(ITAC)	-	-		-	-		-
☐	ln(MCP1)	-	-		-	-		-
☐	ln(MIG)	-	-		-	-		-
☐	ln(MIP1A)	-	-		-	-		-
☑	ln(SAA)	-0.421	(0.133)		-3.158	0.002		(-0.764, -0.078)
☐	ln(SELE)	-	-		-	-		-
☐	ln(SELP)	-	-		-	-		-
☑	ln(SICAM3)	-1.437	(0.285)		-5.041	<0.001		(-2.171, -0.703)
☑	ln(THBD)	1.168	(0.327)		3.571	<0.001		(0.325, 2.01)
☑	ln(VCAM1)	1.194	(0.343)		3.479	<0.001		(0.31, 2.078)
☐	ln(VEGF)	-	-		-	-		-
☐	(constant)	0	(0)		-	-		(0, 0)

Coefficient = Constant
Std. error = Standard deviation
z-score: Standard score
p-value: Significance
99% Confidence Interval: Probability

Biomarkers: Clusterin, Calponin 1 (CNN1), C-Reactive Protein (CRP), Intercellular Adhesion Molecule 1 (ICAM-1), Serum Amyloid A (SAA), Soluble Intercellular Adhesion Molecule 3 (sICAM-3), Thrombomodulin (THBD), and Vascular Cell Adhesion Molecule 1 (VCAM-1).

The equation for calculating the score regression coefficient is:

$$
\begin{aligned}
z = & -0.553 * \{\ln[Clusterin(ng/mL)] = 0\} \\
& + 0.633 * \{\ln[CNN1(ng/mL)] = -2\} \\
& + 0.358 * \{\ln[CRP(ng/mL)] = 8\} \\
& + -1.54 * \{\ln[ICAM1(ng/mL)] = 4\} \\
& + -0.421 * \{\ln[SAA(ng/mL)] = 13\} \\
& + -1.437 * \{\ln[SICAM3(ng/mL)] = 0\} \\
& + 1.168 * \{\ln[THBD(ng/mL)] = 1\} \\
& + 1.194 * \{\ln[VCAM1(ng/mL)] = 4\}
\end{aligned}
$$

The distribution of individual biomarkers is detailed in ***Figure 9***.

Figure 9: Estimated coefficient distributions for individual biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from CA

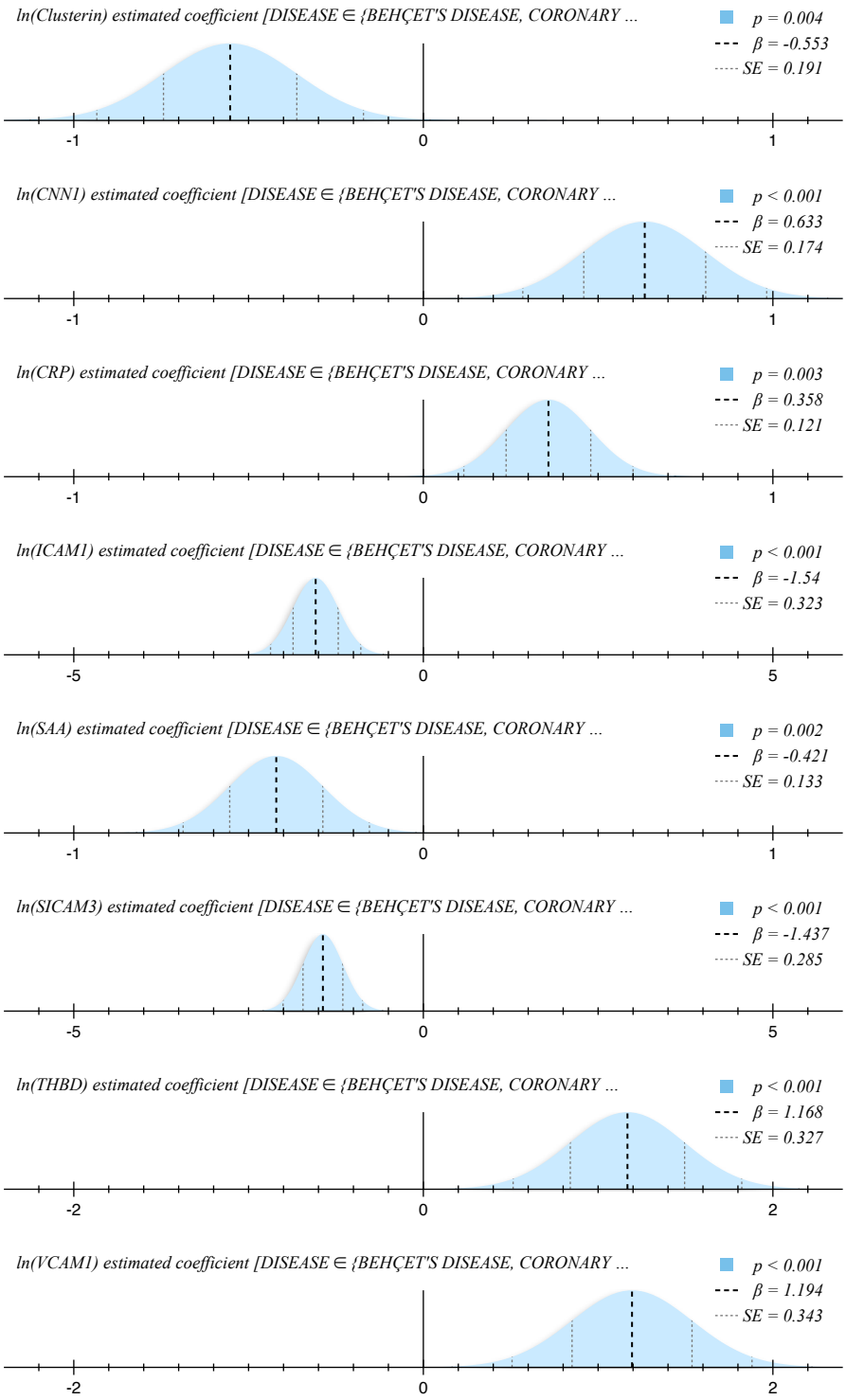


Figure 10 displays the residual distribution for each biomarker on a 2-axis plot.

Figure 10: Ordered probit residual 2-axis plots for individual biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from CA

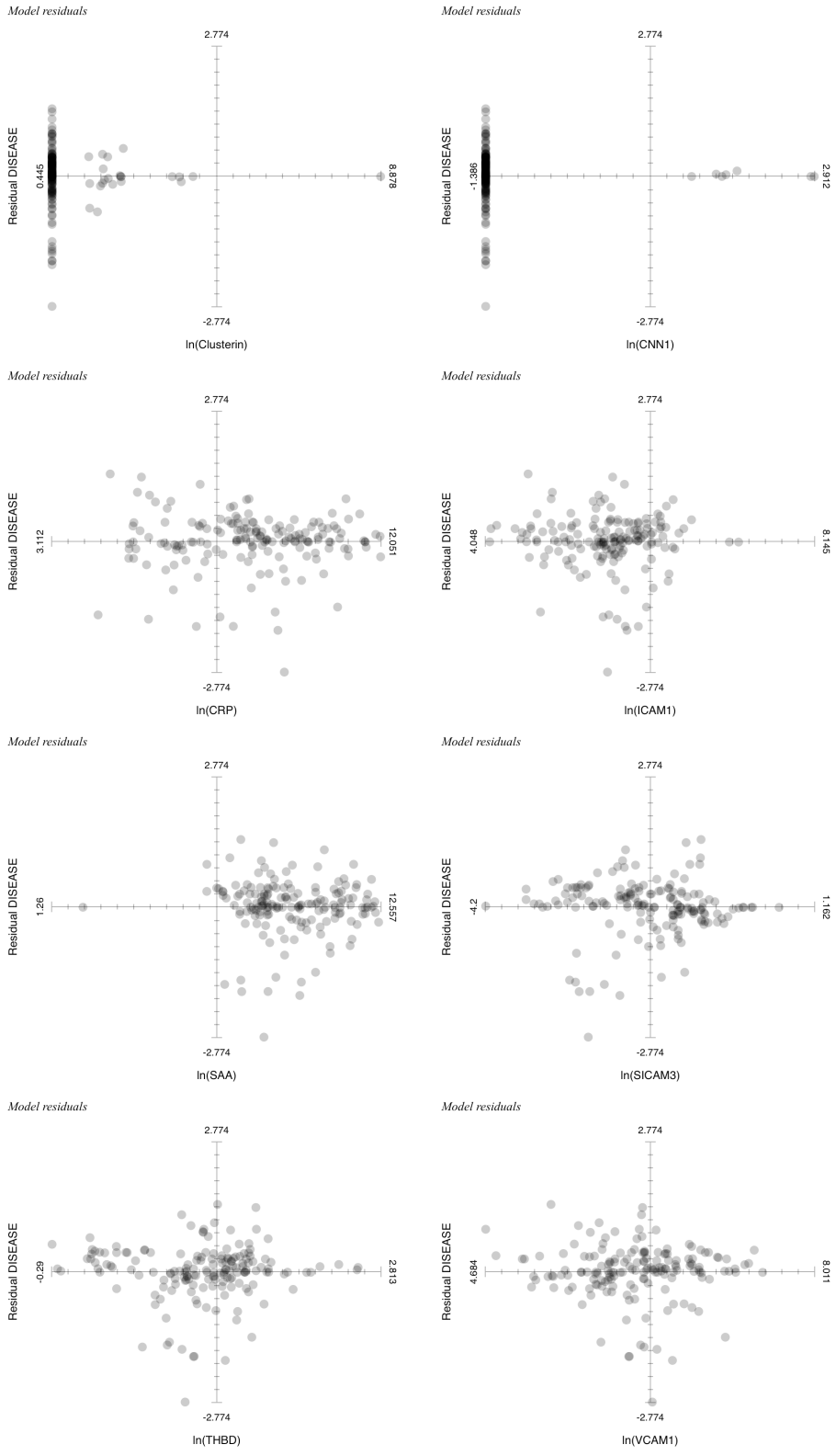
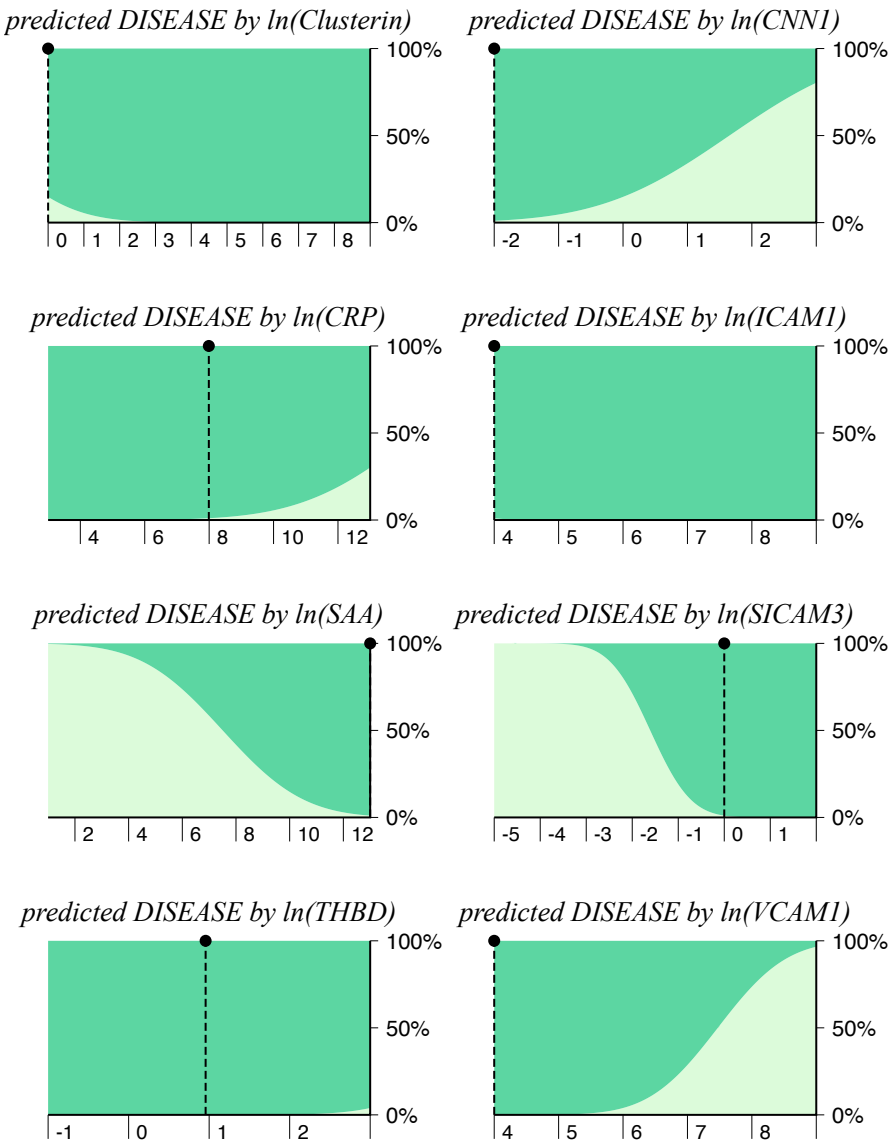


Figure 11 illustrates the discriminatory power of the Area Under the Curve for each individual biomarker.

Figure 11: Discriminatory power as illustrated by the Area Under the Curve for individual biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from CA



Finally, ***Table 12*** summarizes the results of the regression for BD (BDA+BDI) vs. CA and HD.

Table 12: Multivariate regression test results for biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from both CA and HD

✓	Explanatory variable	Coefficient	Std. error	Visualization	Z-score	p-value	Significance	99% Confidence Interval
☐	ln(Caldesmon)	-	-		-	-		-
☐	ln(Clusterin)	-	-		-	-		-
☐	ln(CNN1)	-	-		-	-		-
☐	ln(CRP)	-	-		-	-		-
☐	ln(GROA)	-	-		-	-		-
☐	ln(ICAM1)	-	-		-	-		-
☐	ln(INTLK6)	-	-		-	-		-
☐	ln(INTLK8)	-	-		-	-		-
☐	ln(IP10)	-	-		-	-		-
☐	ln(ITAC)	-	-		-	-		-
☐	ln(MCP1)	-	-		-	-		-
☐	ln(MIG)	-	-		-	-		-
☐	ln(MIP1A)	-	-		-	-		-
☑	ln(SAA)	-0.391	(0.064)		-6.11	<0.001		(-0.555, -0.226)
☐	ln(SELE)	-	-		-	-		-
☑	ln(SELP)	-0.51	(0.134)		-3.794	<0.001		(-0.856, -0.164)
☐	ln(SICAM3)	-	-		-	-		-
☑	ln(THBD)	0.614	(0.17)		3.605	<0.001		(0.175, 1.052)
☑	ln(VCAM1)	0.925	(0.138)		6.722	<0.001		(0.571, 1.279)
☐	ln(VEGF)	-	-		-	-		-
☐	(constant)	0	(0)		-	-		(0, 0)

Coefficient = Constant
Std. error = Standard deviation
z-score: Standard score
p-value: Significance
99% Confidence Interval: Probability

Biomarkers: Serum Amyloid A (SAA), P-Selectin (SELP), Soluble Intercellular Adhesion Molecule 3 (sICAM-3), Thrombomodulin (THBD), and Vascular Cell Adhesion Molecule 1 (VCAM-1)

The equation for calculating the score regression coefficient is:

$$z = -0.391 * \{\ln[SAA(ng/mL)] = 13\}$$
$$+ -0.51 * \{\ln[SELP(ng/mL)] = 6\}$$
$$+ 0.614 * \{\ln[THBD(ng/mL)] = -1\}$$
$$+ 0.925 * \{\ln[VCAM1(ng/mL)] = 4\}$$

The distribution of individual biomarkers is detailed in ***Figure 12***.

Figure 12: Estimated coefficient distributions for individual biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from both CA and HD

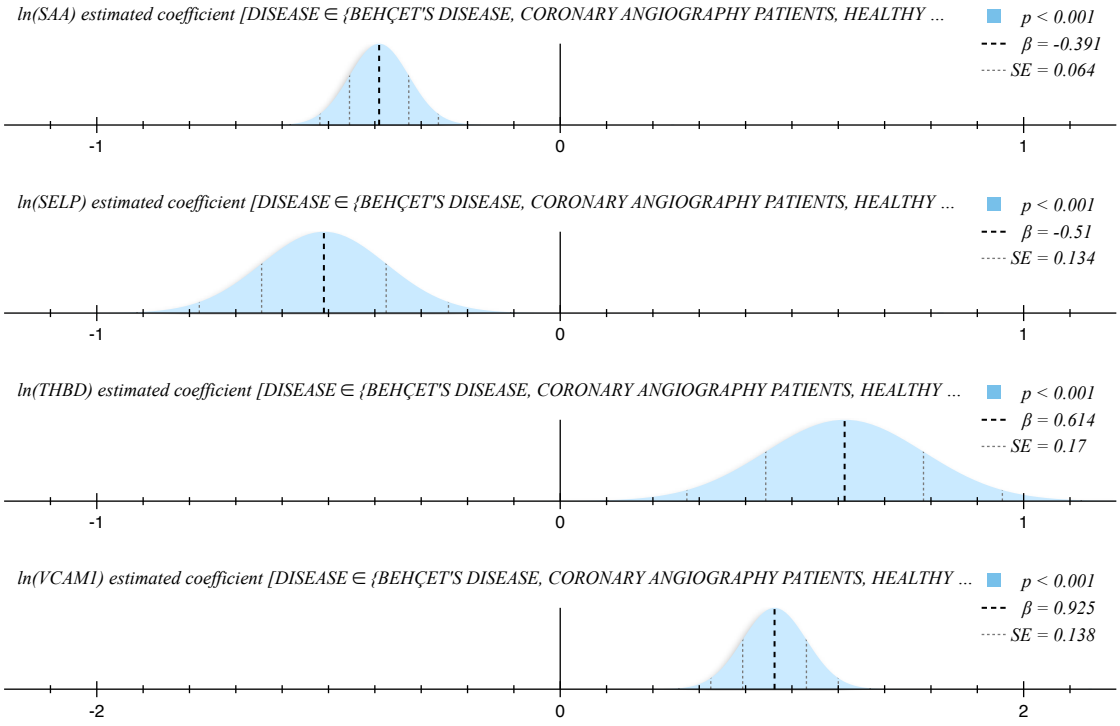


Figure 13 displays the residual distribution for each biomarker on a 2-axis plot.

Figure 13: Ordered probit residual 2-axis plots for individual biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from both CA and HD

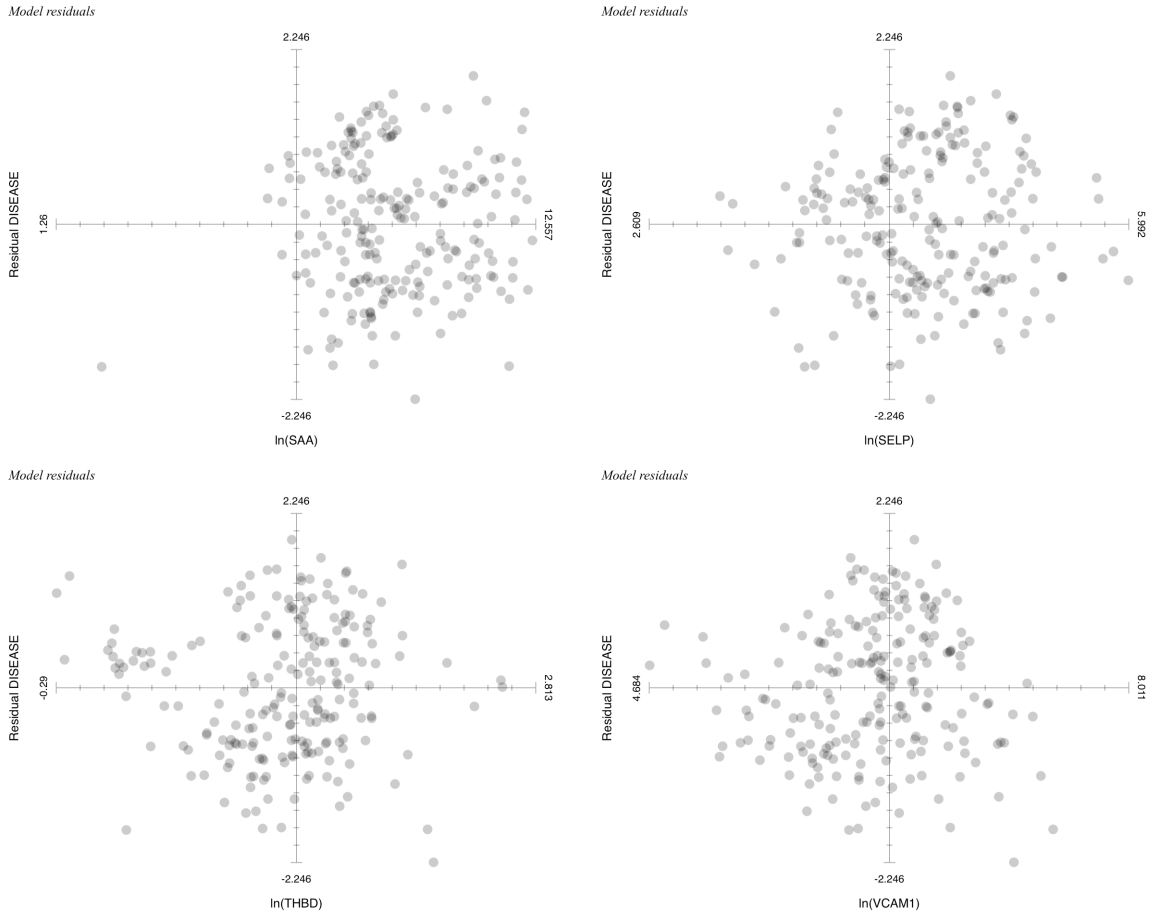
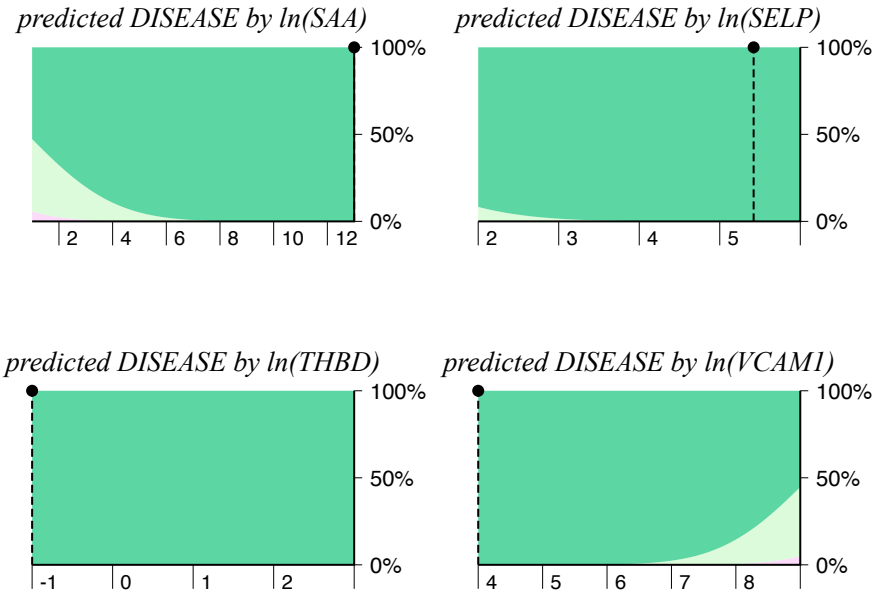


Figure 14 illustrates the discriminatory power of the Area Under the Curve for each individual biomarker.

Figure 14: Discriminatory power as illustrated by the Area Under the Curve for individual biomarkers that were significant at a 99% confidence interval in distinguishing BD (BDA+BDI) from both CA and HD



It should be noted that in the aforementioned regression formulas, when the multiplier is for a particular variable is negative, the corresponding biomarker moves in an inverse relationship with the other listed biomarkers. This is the case with: SAA in BD (BDA+BDI) vs. HD; ICAM-1, SAA and sICAM-3 in BD (BDA+BDI) vs. CA; and SAA and SELP in BD (BDA+BDI) vs. HD and CA (HD+CA). This is of particular importance in any future research that endeavors to decipher the etiology of the disease.

10.3. Discussion

There is considerable experimental evidence supporting the role of T cells, neutrophils, and cytokines in the pathogenesis of BD.[218,219] Various biomarkers are presumed to mediate the sequence of events leading to autoinflammation.[220,221] Tissue injury as observed in BD histopathology is a vital consequence of this chemokine-induced cascade.[222] Identifying the role of individual and / or groups of biomarkers is consequently primordial for isolating potential serum signatures for Behçet's disease as well as understanding its pathogenesis.[218] In this context, a comparison with both healthy donors and coronary angiography patients was performed in an attempt to assess serum biomarkers that segregated patients with BD from HD, and CA respectively.

The statistical protocol utilized employed a set of standard tools to analyze this study's dataset; however, the cohort data were atypical as they were not homoscedastic. This is to be expected with small sample sizes that deal with multifaceted covariances. Autoimmune vasculitis in general, and Behçet's disease in particular, are rare occurrences; assembling a cohort with a sufficiently large database is challenging in the best of scenarios. Biostatistics, like other analytical sciences, presents limits in such circumstances; below a certain threshold, the validity of mathematical concepts is undermined. The presence of heteroscedasticity is the primary concern when using analyses of variance; it invalidates statistical tests of significance that assume that the data are uniformly distributed and uncorrelated.

The same holds true with regression analyses. In the presence of dependent variables, as was the case with the biomarkers tested, model functions that are built on nonlinear combinations of parameters lose validity. Presuming that nonparametric regression may solve the problem is erroneous as the predictor of the model does not take a predetermined form, but rather is built from information directly derived from non-uniform data (even after log transformation). Limits are quickly attained in this case as nonparametric regression is truly effective only with larger sample sizes than those of this study: the data defines the model shape as well as the model estimates. It is thus seemingly difficult to arrive to solid and applicable clinical results without using the advanced multivariate analysis proposed in the statistical protocol; purely descriptive outcomes, although an essential step, are of little value in developing applied models.

Nevertheless, the analyses of variance utilized remained an excellent mechanism for exposing the covariate biomarkers of this study. Contrary to large cohort events, the statistical process for small cohorts is one of elimination of insignificant biomarkers rather than the confirmation of significant ones. This was the main function of the Kruskal-Wallis and Mann-Whitney tests. However, this technique of elimination reaches its applicative limits rapidly. Biomarkers, given the complexity of the immune organ, are rarely univariate protagonists. They are influenced by, and in turn influence, multiple other biomarkers, in multiple steps and with distinctive recurring effects.[223]

Thus, as expected, the regression analysis with backward selection did not yield a viable outcome in this case, even when the p-value was augmented to <0.2. This was due to the assumption that the biomarker signatures of BDA and BDI were truly representative of independent disease states. It became evident that this assumption was a handicap as no result was obtained that could validate it. It is for this reason that BDA and BDI were pooled after verification for all biomarkers that did not reject the null hypothesis and hence could be combined together (IL-8 was eliminated). Not only did this increase the count, but it also removed a preconceived clinical (and yet to be proven biological) notion that these two states, using the biomarkers tested in this study, are in fact separate disease states.

The choice of employing manual regression on log-transformed data, followed by an automated stepwise confirmation gave more leeway in achieving predictive models as the data was fitted (input) in a series of successive approximations. The ranking effect obtained by the combined natural log-transferred data facilitated this process. The assumptions were amended to fit the multivariate models that support the constraints of the dataset. Given the heteroscedasticity of the data discussed previously, as confirmed by both the GAM and PCA tests, it was henceforth assumed that: (i) the profile of the response surface is undetermined; (ii) the profile of the response of one biomarker may depend on multiple other biomarkers; and (iii) the profile may be constructed using not only a quantitative (logit) function (which achieved no results), but also a binary (probit) one (with standard error corrections).

This was certainly more resource consuming. However, it allowed a freer hand in the interpretation of the data. Simply put, only a process of exploration, rather than automated linear analysis, could lead to valid regression equations given the particularities of the dataset of this study. A quantile function using probability units allowed for the modeling of complex

interactions between biomarkers and for the assimilation of numerous covariances affecting their interactions. A search for significant differences in covariant biomarkers was attempted and models that took into consideration these variances were constructed manually, through trial and error.

Encouragingly, the biomarkers singled out in this investigation were identified in prior research, either alone or in combination, as inflammatory proteins in vascular autoinflammatory diseases.[223-246] This validates, at least in this particular case, the approach of the statistical protocol utilized. This study hence both confirms prior observations and provides new physiopathological evidence for BD.[224]

With regards to the differentiation between BD and HD, the specific role of biomarkers ICAM-1, SAA, THBD, and VCAM-1 in the etiology of the disease remains to be precisely clarified. The current understanding of autoimmune diseases only begins to explain the mechanisms by which these biomarkers influence recurring tissue inflammation in BD. Given their differentiating power in identifying BD relative to HD, future research should focus on understanding the underlying mechanisms of these biomarkers, individually or in combination, in this particular disease.

Existing evidence indicates that ICAM-1 is implicated in leukocyte activation and migration.[225] A member of the immunoglobulin superfamily that binds to lymphocyte function-associated antigen 1 and macrophage 1, ICAM-1 is stimulated by inflammatory cytokines such as IFNγ, IL-1 and TNFα.[225,226] It is involved in the activation of autoreactive Th1 but not Th2 cells and it plays an important role in down-regulating autoimmune inflammation, notably in the central nervous system.[225] ICAM-1 is known to be a cell surface molecule that delivers a second signal to activate T cells.[225] It may be presumed that this biomarker plays a similar role in the etiology of BD.

The role of SAA in autoimmunity has also been notably documented. It remains one of the most prominent positive acute phase reactants to interleukin 1 when released by activated macrophages.[227,228] It was reported that its concentration increases to up to 1,000 folds in sera in cases of inflammation, and notably in vasculitis.[227,229] As such, it may be surmised that its mechanisms of action are the same in BD. High levels of SAA also correlate with different diseases and symptoms including rheumatic disease activity, secondary amyloidosis, familial

Mediterranean fever (FMF), cancer and AIDS. It is additionally considered a good predictor of progression from a non-inflammatory thrombotic condition to an inflammatory one.[227,229]

The biomarker THBD is a trans-membrane glycoprotein expressed on the surface of all vascular endothelial cells. Research indicates that its expression ensures a rapid and localized hemostatic and inflammatory response to injury.[230,231] The presence of TNFα as a pro-inflammatory biomarker provided the first evidence of a link between THBD and inflammation. This may be described by the endocytosis of THBD in the presence of TNFα that promotes inflammation and coagulation.[230] Interestingly enough, THBD suppresses the expression of ICAM-1. This may explain why this study detected THBD in active BD (BDA) and not in inactive BD (BDI); conversely, ICAM-1 was present in inactive BD (BDI) and not in active BD (BDA).

As a member of the immunoglobulin (Ig) superfamily, VCAM-1 acts as ligands for leukocytes to facilitate their entry into inflamed tissues.[232] Along with ICAM-1, VCAM-1 was proven to reinforce the binding of circulating cells to cerebral endothelium and to contribute to the overall increased state of adherence of immune cells in the tissues of autoimmune mice. Its increased expression correlates generally with inflammation and disease activity and it may be surmised that its functions in BD are similar.[232,233,234]

With regards to the differentiation between BD and CA, the biomarkers Caldesmon, Clusterin, CRP, ICAM-1, IL-8, SELP, and sICAM-3 may play a role in explaining the etiology of autoimmune vasculitis relative to non-autoimmune vasculitis. There currently is very little evidence as to the specific role of these biomarkers in BD in general, and as to why they would segregate with non-autoimmune vascular disease such as atherothrombosis. The complexity of the functions of these biomarkers makes forming a conclusion in this aspect difficult.

Research indicates that Caldesmon is expressed by smooth muscle cells and is a specific marker of inflammation of soft tissue in the skin, especially in cases of tumors.[235] Caldesmon was defined as a specific endothelial molecule, and may be associated with the presence or absence of tumor-infiltrating lymphocytes.[236] Likewise, Clusterin plays a bidirectional function in the regulation of other pro-inflammatory cytokines such as TNFα and IL-6.[237,238,239] CRP in blood serum serves as reliable marker of disease activity in various vasculitides.[240,241] It is known as a marker of acute and chronic inflammation and trauma and

participates in host defense.[241,242] It also binds to autoantigens and promotes clearance of apoptotic cells. It activates the chemical complement cascade, enhances phagocytosis and binds to Fc receptors such as Fcγ that lead to cytokine production including IL-1 and IL-10.[240,241,242] It also interacts with a variety of mediators and receptors leading to pro- and anti-inflammatory activities under different conditions of cell activation, tissue deposition, and ligand availability.[242] The exact role of CRP in differentiating between autoimmune and non-autoimmune disease remains to be elucidated. Interleukin 8 exhibits chemotactic activities against T-lymphocytes, basophils and neutrophils.[222] It induces neutrophils to release lysosomal enzymes and increases expression of Mac1 and CR1. IL-8 plays a causative role in acute inflammation by recruiting and activating neutrophils and its production occurs ordinarily in the presence of inflammatory stimuli such as LPS, IL-1 and TNFα.[222,243,244] In inflammation, P-selectin was observed to be expressed by exocytosis on the cell surfaces of activated platelets and stimulated endothelial cells.[245] As such, its role in inflammation was repeatedly observed.[246]

There are two main limitations to this study. First, the sample size was small, which may limit the diagnostic applicability of the biomarkers tested. Second, the statistical validity of the tests applied is dependent on the cogency of the small sample size, which may reduce their impact, as these models are better suited for larger cohort datasets with uncorrelated variables.

Despite these limitations, this investigation reproduced complementary results to those obtained by others on vasculitis and confirmed with statistical reliability multiple biomarker signatures for BD.[218] These results present new potential clinical applications for the disease and provide avenues for future research. Larger, prospective studies are needed to confirm the proteins identified in this study as reliable biomarkers for BD.

11. Conclusion

A quantitative statistical analysis of 20 biomarker signatures proposed biomarkers specific for BD and postulated predictive models for identifying patients with BD relative to healthy donors and coronary angiography patients with a high level of sensitivity and specificity. The purpose was to provide clinicians and researchers with both a qualitative and a quantitative approach for interpreting and elucidating the physiopathology of BD, and assess the disease relative to other vasculitides using a standard statistical protocol.

Bivariate analysis identified four biomarkers that were able to differentiate between BD patients and healthy donors: ICAM-1, SAA, THBD, and VCAM-1. No outcome was obtained that would allow differentiating BDA from BDI. This contributes to our understanding of the pathogenesis of BD. The predictive model (99% CI) that segregates with high sensitivity and specificity between BD (active and inactive combined) and HD using the biomarkers CRP, ICAM-1, and SAA should be confirmed by further research and may serve as the basis for a prospective serum biomarker signatures specific to classifying BD patients.

Bivariate analysis also identified six biomarkers that were able to differentiate between BD patients and coronary angiography patients: Caldesmon, Clusterin, CRP, IL-8, SELP, and sICAM-3. A predictive model (99% CI) that segregates BD (active and inactive combined) from CA used the biomarkers Clusterin, CNN1, CRP, ICAM-1, SAA, sICAM-3, THBD, and VCAM-1. This complements the extant literature on autoimmune pathogenesis in vasculitis.

In conclusion, the bivariate and multivariate analyses that segregate between BD, HD, and CA represent a significant avenue for identifying the mechanisms that lead to vasculitis. The statistical protocol devised for this study was conclusive and should be exploited again with similar datasets. The validity of the results depends on the replicability of the proposed statistical protocol.

This investigation reproduces complementary results to those obtained by other studies on vasculitis diseases in general, and BD in particular; it confirms existing theoretical constructs and proposes new potential clinical applications. Results of the present study should be extended to an in-depth analysis of the specificity of the biomarker combinations that would differentiate BD from other non-ANCA systemic vasculitides.

Acknowledgement

We would like to thank all participating members of the DIVI group of the Safe-T/IMI European Study, and particularly the Firalis laboratory that performed all dosages for biomarkers, without whom this study would have not been possible.

Supplementary Figure 1: Proposed statistical methodology for vasculitis cohort analysis

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Une exploration de biomarqueurs sériques non-anticorps anti-cytoplasme des polynucléaires neutrophiles des vascularites systémiques: une étude de la maladie de Behçet

Résumé:
Les hypothèses retraçant les mécanismes physiopathologiques de la maladie de Behçet (MB), une vascularite inflammatoire non liée aux anticorps anti-cytoplasme des polynucléaires neutrophiles (ANCA), sont multiples. Cette étude propose une compilation exhaustive des mécanismes immunopathologiques décrits dans la littérature contemporaine, et fournit un résumé détaillé des aspects cliniques de la maladie et de ses différents traitements. Cette étude inclut également une analyse statistique de 20 signatures de protéines proposées comme biomarqueurs potentiels de la MB. Vingt-deux patients avec une MB active (MBA) et 46 patients avec une MB inactive (MBI), répondant aux critères de *l'International Criteria for Behçet Disease* (2013), ainsi que 47 donneurs sains (DR) et 98 patients subissant une angiographie coronaire (AC) ont fourni des échantillons de sérums pour une étude de dosage multiplex. Les résultats indiquent que les protéines sériques ICAM-1, SAA, THBD, et VCAM-1 jouent un rôle essentiel dans la différenciation entre les patients MB et les DR. De même, Caldesmon, Clusterin, CRP, IL-8, SELP et SICMA3 permettent un tri entre les patients MB et AC. Les modèles de signatures des biomarqueurs proposés dans cette étude et qui séparent entre les patients atteints par la MB, les DR et / ou les AC, représentent une nouvelle piste pour le développement de tests sériques pour la MB, avec une sensibilité et une spécificité élevées. Ceci peut éventuellement compléter les outils de diagnostic clinique établis. Ces résultats apportent une contribution significative à l'interprétation actuelle de la pathogénie de la MB en tant que vascularite auto-immune non-ANCA. Cette enquête fournit un bilan à la fois qualitatif et quantitatif aux cliniciens et aux chercheurs dans ce domaine.

Mots clés: Mots-clés: Behçet, biomarqueur, cytokine, vascularite, Th17, IL-17

An Exploration of Non-Antineutrophil Cytoplasmic Antibodies Serum Biomarkers in Systemic Vasculitis: An Investigation of Behçet's Disease

Abstract:
Hypotheses concerning the specific pathophysiological mechanisms of Behçet's Disease (BD), a non-antineutrophil cytoplasmic antibodies (ANCA) inflammatory vasculitis, are numerous. This study offers an exhaustive review of the disease in an attempt to recap the immunopathological pathways described by extant literature, and provides a detailed summary of the clinical aspects of, and treatment options for the disease. In addition, this investigation completed a statistical analysis of 20 protein signatures that were proposed as potential biomarkers for BD. Twenty-two patients with active BD (BDA) and 46 patients with inactive BD (BDI) fulfilling the International Criteria for Behçet's Disease, 47 healthy donors (HD), and 98 coronary angiography patients (CA) provided serum samples for a multiplex assay study. Findings indicate that serum proteins ICAM-1, SAA, THBD, and VCAM-1 play a significant role in differentiating BD patients from HD. Likewise, Caldesmon, Clusterin, CRP, IL-8, SELP, and sICAM-3 segregate between BD and CA. The biomarker predictive models proposed in this study that segregate between BD, HD, and / or CA represent a significant avenue for the development of sera testing specific to BD with a high level of sensitivity and specificity. This may serve as a supplement to established clinical diagnostic tools. These results represent a noteworthy complement to the current interpretation of the pathogenesis of BD as an autoimmune non-ANCA vasculitis. This investigation provides expert clinicians and researchers with both qualitative and quantitative outcomes.

Keywords: Behçet, biomarker, cytokine, vasculitis, Th17, IL-17