



Barts and The London
School of Medicine and Dentistry

PhD Thesis

**The expression of Suppressor of Cytokine Signalling
(SOCS), JAK-STAT signalling pathway and cytokine
profile in Behçet's Disease**

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*This work is dedicated
to my dear mother, my beloved husband
and my darling children.*

DECLARATION

“Except for the help listed in the Acknowledgments, the contents of this PhD thesis are entirely my own work. This work has not been submitted, in part or in full, for a degree or diploma of this or any other University or Examining Board.”

Signed by candidate:

Name of candidate: Mojgan Hamedi

Month and Year of Submission: August 2013

ABSTRACT

Abstract: Behçet's disease (BD) is a chronic, multi systemic, recurrent vasculitis disease of unknown aetiology. The clinical manifestations are composed of relapsing episodes of recurrent oral ulcers, uveitis, skin lesions and genital ulcers along with musculoskeletal and neurological involvement. Pro-inflammatory cytokines are a key feature of the disease but the triggers for their induction are not well understood and/or controversial. Many cytokines (including IFN γ , IL-12, IL-23, IL-10 and IL-6) activate the JAK-STAT signalling pathway which is negatively regulated by Suppressor of Cytokine Signalling (SOCS) proteins. Therefore, it was hypothesised that SOCS proteins may be dysregulated in BD.

The expression of SOCS 1-3 mRNA and protein was studied in peripheral blood mononuclear cells (PBMCs), Neutrophils and buccal mucosal cells (BMC) of BD patients and compared with healthy controls (HC) and recurrent aphthous stomatitis (RAS) patients. SOCS 1 and 3 were significantly upregulated in PBMCs of BD patients compared with HC ($p=0.0149$; $p=0.0007$) and there were subtle differences between expression in relapsed and symptom free BD (quiet BD). SOCS1 and SOCS 3 also significantly upregulated in BMC from oral ulcers of BD compared with HC (both at $p=0.0001$). Cytokines were examined in serum, saliva and culture supernatants from stimulated PBMCs. IL-6 were significantly upregulated in the saliva of relapsed BD patients compared with HC ($p=0.0104$) and the capacity for IL-10 secretion from BD was compromised. Phosphorylation of STATs, transcription factors ROR γ t, T-bet and 48 protein kinases were investigated using a novel PhosphFlow method and by microarray analysis. STATs were upregulated in BD and seven novel kinase proteins showed differential phosphorylation in BD.

Conclusion: SOCS 1-3 expression has changed in BD patients with differences in PBMC and Neutrophil expression between the SOCS proteins. Phosphorylation of STATs and several kinases show up-regulation in BD and seven kinases with altered phosphorylation states in BD were identified as novel targets for future investigation.

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ABBREVIATIONS

A

ACTB	Actin, beta
AEC	3-Amino-9-ethylcarbazole
AMP	Adenosine monophosphate
ANOVA	Analyses of variance
APC	Antigen presenting cells
AS	Ankylosing spondylitis

B

B2M	Beta-2 microglobulin
BCE	Behcet's Centre of Excellence
BCIP	5-bromo-4-chloro-3'-indolyphosphate
BD	Behçet's Disease
BD-RE	BD-relapsed
BD-MA	BD mouth active
BMBB	Buccal Mucosal Brush Biopsies
bp	Base pair
BSA	Bovine Serum Albumin

C

cDNA	Complementary DNA
CI	Confidence Interval
CIS	Cytokine Inducible SH2 containing protein
Ct	Threshold Cycle
CSC	Cytokine secreting cells
CSF	Cerebrospinal fluid

D

DMF	Dimethylformamide
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DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide
E	
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eHSP	Extracellular Heat shock protein
ELISPOT	Enzyme-linked immunosorbent spot
F	
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FERM	Four-point-one, Ezrin, Radixin, Moesin
FOXP3	Forkhead box P3
G	
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA3	Trans-acting T-cell-specific transcription factor GATA-3
GP 130	Glycoprotein 130
GWAS	genome wide association study
H	
HEK	Human embryonic kidney
HLA	Human leukocyte Antigen
HRP	Horseradish peroxidase
HSP	Heat shock protein
HSV	Herpes simplex virus
I	
IBD	Irritable bowel disease
IFN	Interferon
IL	Interleukin
ISG	International Study Group

ITREG	Inducible T regulatory
J	
JAK	Janus Kinase
JH	Janus homology
K	
KIR	Kinase inhibitory region
L	
LREC	City Local Research Ethical Committee
LPS	Lipopolysaccharide
M	
MICA	MHC class I chain-related molecules A
MHC	Major Histo compatibility
mRNA	messenger RNA
MBAA	multiplex bead array assay
MFI	Mean Fluorescence Intensity
N	
NKG2D`	Natural-killer group 2, member D
NKT	Natural Killer T cell
O	
P	
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PVDF	Polyvinylidene fluoride

Q

QPCR Quantitative real time polymerase chain reaction

R

RAS Recurrent aphthous stomatitis

RBC Red blood cell

RE Relapsed

ROR RAR-related orphan receptor gamma

ROU Recurrent oral ulceration

S

SOCS Suppressor of Cytokine signalling

SLE Systemic lupus erythematosis

SNP single nucleotide polymorphism

T

T-bet T-box expressed in T cells

TBS Tris-buffered saline

TCR T cell receptor

TGF T cell growth factor

Th T helper

TNF Tumour necrosis factor

TLR Toll like receptor

TTBS Tween, Tris Buffered saline

TYK2 tyrosine kinase 2

U

UBC Ubiquitin C

V, W, X, Y, Z

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CHAPTER 1

LITERATURE REVIEW

Chapter 1: Literature review

1.1 Behçet's disease

Behçet's disease (BD) is a chronic, multi systemic, recurrent vasculitis disease of unknown aetiology. The first description of this disease in medical literature goes back to the 5th century BCE, by Hippocrates (460–377 BC). He described an endemic condition with painful watery eyes of a chronic character, aphthous ulcers and sores in the mouth, genital sores and fluxes and large pustules in his Epidemion, book 3, case 7 (Feigenbaum, 1956). In more modern times, Benediktos Adamantiades, a Greek ophthalmologist (1875-1962) reported a case of relapsing iritis with hypopyon, scrotal ulcers, oral aphthous and sterile arthritis of both knees and brought these three symptoms together as signs of a single disease (Adamantiades, 1931). Therefore, some references still call this syndrome, "Adamantiades' syndrome" or "Adamantiades-Behçet syndrome. However, the syndrome was internationally recognised as Behçet Disease and named after Hulusi Behçet, a Turkish dermatologist (1889 -1948), who described the condition as a new disease in 1937, after many years following up his three patients suffering from recurrent oral and genital ulcers, eye and skin diseases (Verity *et al.*, 2003).

1.2 BD Diagnosis

Diagnosis of BD is based on clinical features since there is no diagnostic laboratory test so far. There was a variety of diagnostic criteria prior to 1990 which made it difficult to compare and analyse the results from different studies. In 1990, the International Study Group (ISG) proposed a unique diagnostic criterion based on a collaborative study with

914 Behçet's disease patients, from 12 centres in 7 countries with controls from the same centres [International Study Group-ISG- for Behçet's Disease, (1990)]. According to the proposal of the ISG, diagnosis of BD requires the presence of oral ulceration plus any two of genital ulceration, typical defined eye lesions, typical defined skin lesions or a positive pathergy test in the absence of other clinical explanation (Table 1.1).

Main Criteria:	
Recurrent oral ulceration	Minor aphthous, major aphthous, or herpetiform ulceration observed by physician or patient, which recurred at least 3 times in one 12 month period
Plus two of the following:	
Recurrent genital ulceration	Aphthous ulceration or scarring, observed by physician or patient
Eye lesions	Anterior uveitis, posterior uveitis, or cells in the vitreous humour on slit lamp examination; or retinal vasculitis observed by ophthalmologist
Skin lesions	Erythema nodosum observed by physician or patient, pseudofolliculitis, or papulopustular lesions; or acneiform nodules observed by physician in post-adolescent patients not on corticosteroids
Positive pathergy test	Read by physician at 24–48 hours performed with oblique insertion of a 20-gauge or smaller needle under sterile conditions

Table 1.1: International Study Group Criteria for diagnosis of Behçet's disease.

1.3 BD Epidemiology

BD is a rare disease and mostly affects inhabitants along the ancient Silk Road from mediterranean countries to the Middle East and Far East regions located between latitudes 30° and 45° north (Verity *et al.*, 2003). While Turkey with 80-420 patients per

100,000 has the highest prevalence in the world, regions like northern parts of Europe and Asia, Africa and Australia has the least frequency (Azizlerli *et al.*, 2003, Altenburg *et al.*, 2006, Zouboulis and Kaklamanis, 2003, Deuter *et al.*, 2008). BD is a rare disease in the UK with a frequency of 1-2 in 100,000 people (Zouboulis and Kaklamanis, 2003). The ratio of male to female of BD varies with ethnic origin and the average age of disease onset is 30. BD is very rare in children. Kari 2001 studied the clinical spectrum of BD in childhood in 10 UK cases with an age range from 1.2-10 years where their clinical features were similar to that of adults (Kari *et al.*, 2001).

1.4 Clinical Symptoms of BD

The clinical manifestations of BD show a very wide spectrum and can be observed in any organ of the body with unpredictable courses of remissions and exacerbations. However, the main symptoms of BD are composed of relapsing episodes of recurrent oral ulcers, uveitis, skin lesions and genital ulcers. It can also affect musculoskeletal, vascular, gastrointestinal and nervous system. Clinical features of BD follow several patterns based on ethnic, geographical and individual diversities (Al-Otaibi *et al.*, 2005, Krause *et al.*, 2001). For example while gastrointestinal symptoms are hardly seen in Turkish and Saudi Arabian patients, they occur in 33% of Thai and 25% of Spanish patients (al-Dalaan *et al.*, 1994, Al-Otaibi *et al.*, 2005, Chen and Chang, 2001, Gürler *et al.*, 1997).

The symptoms usually appear less severe and recur at longer intervals after the fourth decade (Bardak, 1999, Yazici *et al.*, 1984). Mortality also decreases remarkably with the passage of time. The age of onset and the gender also can affect the prognosis of the

disease as male patients with early onset tend to have poorer prognosis (Kural-Seyahi *et al.*, 2003).

1.4.1 Recurrent oral ulcerations

Recurrent oral ulcers are the first symptom of up to 86.5% of all patients with BD (Al-Otaibi *et al.*, 2005) (Figure 1.1). These lesions are similar to recurrent aphthous stomatitis (RAS) and cannot be differentiated from them, however they are more numerous, frequent, larger and more painful. All three types of RAS: minor aphthous, major aphthous and herpetiform ulceration can be seen in BD.

1.4.2 Genital ulcers

Genital ulcers occur in 60 to 65% of cases, less common than oral ulcers and hardly seen as the first symptoms of BD (Krause *et al.*, 1999). In men the ulcers are located mostly on the scrotum and in women on the vulva, major and minor labia, cervix, and vagina and heal in a few days, leaving scars (Al-Otaibi *et al.*, 2005). The ulcers can also exist in the oesophagus, stomach, small intestine and perianal area and sometimes cause perforation (Saadoun *et al.*, 2012).



Figure1. 1: Oral Ulceration in BD (Royal London Hospital with patient consent).



Figure 1.2: Skin Lesions in BD (Royal London Hospital with patient consent).

1.4.3 Cutaneous manifestations

Cutaneous features of BD mostly occur in the form of papulo-pustular lesions and erythema nodosum-like lesions (Figure 1.2b). Papulo-pustular lesions are found in 40–70% of the patients. This acne like lesions appears simultaneously on the skin of the back, face and chest and change from papules form to pustules in 24-48 hours. Erythema nodosum-like lesions are painful purplish nodules which mostly occur on the lower extremities (Al-Otaibi *et al.*, 2005).

1.4.4 Pathergy phenomenon

A Pathergy phenomenon is a non-specific hyper-reactivity of the skin in response to trauma from any intercutaneous injection or needle prick in BD. This condition was described for the first time by Blobner in 1937 and reviewed by others later (Al-Otaibi *et al.*, 2005). Pathergy tests are performed by pricking the skin with a sterile needle or intradermal injection of normal saline which cause formation of 1- 2mm papule. The papule may remain unchanged or transform in to a 1-5mm pustule containing sterile pus at its centre within 24-48 hours and disappear after 4-5 days. In spite of vast investigations in immune histopathology of the test, the aetiology is still unknown. A positive pathergy test is one of the diagnostic criteria for BD but it has some limitations. The test is more specific in some populations around the ancient Silk Road and much less in other regions. Furthermore, the pathergy phenomenon is not specific to BD and has been reported in other conditions such as pyoderma gangrenosum and Sweet's syndrome and even has been positive in some healthy controls. The other limitation that decreases the efficacy of the test is problems with standardising the procedure itself and the assessment method (Varol *et al.*, 2010)

1.4.5 Ocular manifestations

A variety of eye lesions have been reported in BD such as anterior and posterior uveitis, cataract, glaucoma, vitritis, retinitis and panuveitis. Ocular involvement is severe, frequent (43%-65% of patients), often bilateral and can compromise rapidly the visual function. It is the most common cause of morbidity in BD since it may lead to blindness if not treated within five years (Al-Otaibi *et al.*, 2005).

1.4.6 Vascular manifestations

Vasculitis is one of the basic pathologic conditions of BD with an estimated range of 2-46% which can affect any size and type of vessels including both arteries and veins (Ko *et al.*, 2000) (Fig1.3). The vascular complications may include thrombophlebitis, deep vein thrombosis, arterial obstruction and aneurysms. Venous involvement is more common (14-39%) than arterial involvement but the later has greater morbidity since it may lead to aneurysm or arterial occlusion (Saadoun *et al.*, 2012). One of the most frequent venous involvements is deep vein thrombophlebitis. It occurs mostly in the lower extremities, superior vena cava, the inferior vena cava and the upper extremities respectively (Ko *et al.*, 2000). Pulmonary artery aneurysm is also seen in the main pulmonary arteries or their branches in BD and is a sign of poor prognosis (Ceylan *et al.*, 2010).

1.4.7 Neurological Involvement

The neurological complications vary from 2.2% to 40% of cases and mostly affect the central nervous system (CNS). The most common clinical presentations of CNS comprise bilateral pyramidal signs, headache, mental disorders (memory defects, disinhibition and apathy), hemiparesis, sphincter dysfunction, brain stem findings and

the pyramido-cerebellar syndrome. Other neurological symptoms such as, meningitis, seizures and hemiplegia have been reported (Akman-Demir *et al.*, 1999).

1.4.8 Other clinical implications

Gastrointestinal (GI) involvement causes abdominal pain, nausea, vomiting, diarrhoea and constipation in these patients. The most common form of extra oral involvement of GI in BD is mucosal ulcers which mostly affect the ileocecal area and colon. The GI manifestations are recurrent even after surgical treatment (Bayraktar *et al.*, 2000). Renal involvement in the form of glomerulonephritis, amyloidosis, renal vascular involvement and interstitial nephritis were observed in BD. However, considerable variation in frequency of renal involvement from 0-55% has been reported (Akpolat *et al.*, 2003). Cardiac symptoms such as mitral valve prolapse and dilatation of the proximal aorta were observed in 50% and 30% of patients with BD, respectively (Morelli *et al.*, 1997). Regarding musculoskeletal disease, mono-arthritis is the most common form of observed arthritis in BD. Knees, ankles and wrists were the most commonly involved joints in these patients. The arthritis in BD is usually temporary and does not leave any deformity (Yurdakul *et al.*, 1983).

1.5 Aetiopathogenesis

The aetiopathogenesis of BD remains unknown but an autoimmune reaction triggered by an infectious or environmental agent in a genetically susceptible individual is the most widely accepted hypothesis (Figure 1.3).

1.5.1 Genetic susceptibilities

There are some strong evidences to support genetic predisposition in BD such as: exceptional geographic distribution, strong association of some MHC genes with 40-

80% of BD patients, positive family history up to 12% of non-Caucasoid patients and the results of several extensive genome wide association studies (GWAS) (Al-Otaibi *et al.*, 2005, Krause *et al.*, 2001) (Table 1.2).

The major histocompatibility complex (MHC) is a large cluster of genes which resides on short arm of chromosome 6 and is divided into class I and II based on their function. MHC complex genes are one of the most comprehensively studied regions in the human genome because of their important role in autoimmune, infectious and

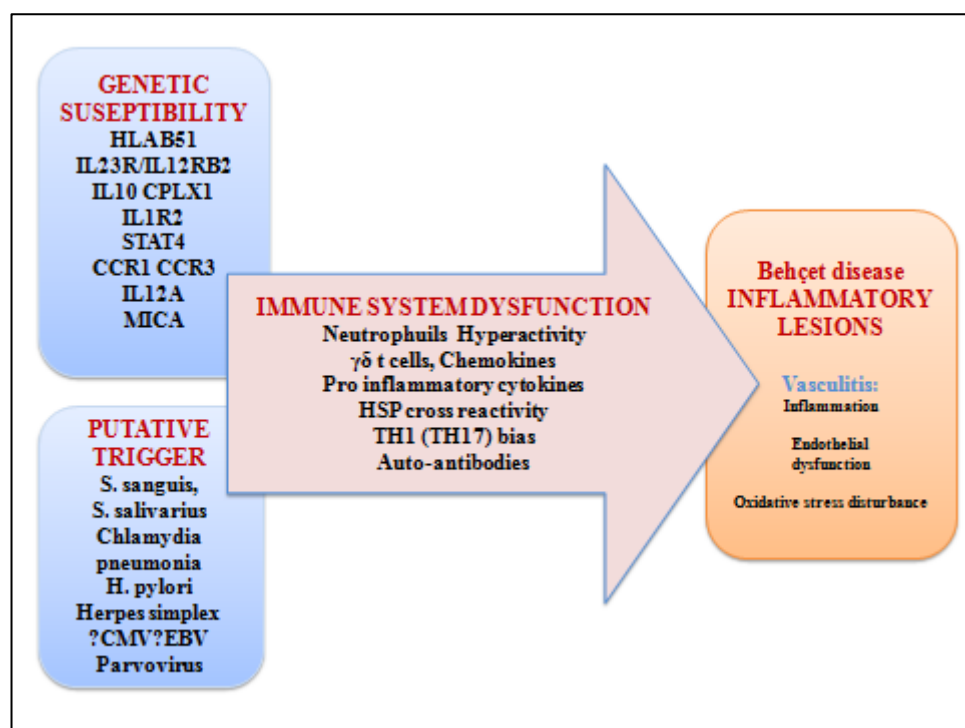


Figure 1.3: Overview of the aetiopathogenesis of Behçet's Disease.

inflammatory diseases and also in cancer and transplantation. The class I and II MHC genes encode human leukocyte antigens (HLAs) which are present on individual tissue types and also perform a fundamental role in antigen presentation. The highly polygenic and polymorphic characteristics of these genes lead to effective and rapid immune response but at the same time many of these polymorphisms are associated with susceptibility to several infectious diseases. Furthermore, defects in MHC genes may

cause autoimmune disorders such as multiple sclerosis, arthritis, diabetes, and inflammatory bowel disease.

HLA-B51 is the most studied gene and by far the strongest associated genetic factor to BD. To confirm the genetic effect of the HLA-B5 or HLA-B51 (HLA-B51/B5) allele on the risk of developing of BD a systematic review and meta-analysis of case-control genetic association study was performed (de Menthon *et al.*, 2009). Data on 4,800 patients with BD and 16,289 controls was extracted from 78 independent studies published between 1975-2007. The results showed a strong association between BD and HLA-B51/B5 (Odds ratio=5.78, 95% Confidence Interval(CI) 5.00-6.67) and its consistency through different ethnicities (de Menthon *et al.*, 2009).

A recent GWAS study on 311,459 autosomal single nucleotide polymorphisms (SNPs) in 1,215 BD cases and 1,278 healthy controls from Turkey again strongly confirmed the association of BD with HLA-B51 and also established associations with the interleukin10 (IL10) variant and a variant located between the *IL23 receptor* (IL23R) and *IL12 receptor β 2* (IL12RB2) genes (Table 1-2). IL23 is a pro-inflammatory cytokine which stimulates T helper cell proliferation and increases the production of inflammatory cytokines such as IL1, IL6, IL17, and TNF α . IL10 is a potent suppressor of inflammatory cytokines such as IL1, IL6, IL12, TNF α and IFN γ which inhibits T cell and NK cell activation (Mizuki *et al.*, 2010, Remmers *et al.*, 2010).

Another GWAS study carried out with 320,438 SNPs on 612 BD patients and 740 healthy controls in Japan found the same results. In a very recent systematic review and meta-analyses of observational studies (Maldini *et al.*, 2012) the relationships between BD clinical features and HLA-B51/B5 was investigated. Their results indicated HLA-

B51/B5 carriage predominates in males and the association with moderately higher frequency of genital ulcers, ocular and skin involvements and less frequency with gastrointestinal manifestations. However, the genetic contribution of HLA-B51 was responsible for less than 20% of the genetic risk; therefore, other possible genetic factors should be considered (Remmers *et al.*, 2010).

Gene	Protein	Odds ratio
HLA B51	Human leukocyte antigen B 51	3.49 (95% CI* 2.95–4.12)
IL23R/IL12RB2	Interleukin 23 receptor/interleukin 12 receptor beta 2	1.28 (95% CI 1.18–1.39)
IL10	Interleukin 10	1.45 (95% CI 1.34–1.58)
CPLX1	Complexin-1	1.16 95% CI 1.07–1.27
IL1R2	Interleukin 1 receptor type II	1.28 95% CI 1.14–1.44
STAT4	Signal transducer and activator of transcription 4	1.27 95% CI 1.13–1.42
CCR1	Chemokine (C–C motif) receptor 1	1.40 95% CI 1.18–1.66
CCR3	Chemokine (C–C motif) receptor 3	1.29 95% CI 1.15–1.46
IL12A	Interleukin 12 alpha	1.63 95% CI 1.30–2.03
MICA	MHC class I chain-related A	1.69 95% CI 1.43–1.99

Table 1.2: Key genetic susceptibility genes linked to Behçet's disease. Adopted from (Pineton de Chambrun *et al.*, 2012). *CI: Confidence Interval

MICA (MHC class I related chain A) and MICB are genes in the MHC class I region, which are polymorphic, do not appear to present peptides or associate with beta-2-microglobulins. These proteins show homology with classical HLA molecules, but they

are not expressed on normal circulating lymphocytes and have a selective tissue distribution, mostly in epithelial cells, endothelial cells and fibroblasts and act as ligands for cells expressing a common activator natural killer-cell receptor (NKG2D).

Several studies suggested an association between BD and the MICA allele; however, MICA and HLA-B51 are strongly associated together because they are in linkage disequilibrium (Hughes *et al.*, 2005). The recent GWAS by (Remmers *et al.*, 2010) did not confirm any independent association between MICA and BD. Other reported genes in MHC region which may associate independently with BD are HLA-A26, HLA-B15, HLA-B5701 (Piga and Mathieu, 2011). In spite of large studies, genetic susceptibilities are not completely able to justify the aetiology of BD since the healthy control group in some studies had a high carriage rate (18%) of HLA-B51 too (de Menthon *et al.*, 2009).

1.5.2 Infection

Antigenic stimuli like Herpes simplex virus (HSV), *Streptococcus sanguis* and microbial Heat Shock Proteins (HSPs) have been also implicated in the aetiology of BD (Lehner, 1997, Lehner *et al.*, 1991) which may act as an infective triggering factor through a molecular mimicry with tissue antigens such as human heat shock protein 60 and /or 70.

1.5.2.1 Herpes simplex virus (HSV)

Initially, in 1937, Behçet proposed a viral aetiology with recognizing intra and extra nuclear inclusions in aphthous and hypopyon samples (Al-Otaibi *et al.*, 2005). Early studies claimed finding HSV-1 DNA in peripheral blood lymphocytes and monocytes and elevated circulating antibodies to HSV-1 patients with BD. HSV DNA has been also identified in the genital and intestinal ulcers of patients with BD (Lee *et al.*, 1996).

However, they could not find any trace of HSV-1 DNA in oral ulcers of BD (Studd *et al.*, 1991). This hypothesis is still controversial since HSV antibody usually exists in healthy controls as well and the data on effectiveness of anti-viral therapy is rare (Direskeneli, 2001).

1.5.2.2 *Streptococcus sanguis*

Investigations carried out on oral microbial flora have become a matter of interest because oral ulcers are the first symptom of up to 86.5% of all patients with BD. Streptococci especially *Streptococcus sanguis* are one of the most studied bacteria in pathogenesis of BD and several studies have supported this hypothesis. The serum antibody titres against oral streptococci strains 113-20, 114-23, and 118-1 isolated from BD patient have shown to be significantly higher than control groups (Yokota *et al.*, 1992). Furthermore, the uncommon oral *S. sanguinis* serotypes are significantly increased in BD patients compared with healthy and disease controls including Vogt-Harada disease, sarcoidosis and herpes simplex infection (Kaneko *et al.*, 2008). The strong delayed type cutaneous hypersensitivity reactions in BD patient in comparison to healthy and disease controls have been observed following skin tests against streptococcal antigens (Mizushima, 1989). In a study by (Isogai, 2003), BD-like symptoms were elicited in experimental gnotobiotic mice after inoculating *S. sanguinis* from BD patients in their traumatised oral tissue.

1.5.3 Immunopathogenic aspects of BD

The key immunological features of BD are alterations in cytokine levels, increased activity of $\gamma\delta$ T cells, elevated level of Th1 and Th17 cytokines, increased T- and B-cell responses to heat-shock proteins (HSP) and neutrophil hyperactivity. There is also some evidence for autoimmune nature of BD including inflammatory responses to some auto-

antigens and the perpetuation of this abnormal immune response even in the absence of ongoing infection.

1.5.3.1 Heat-shock proteins (HSPs)

Heat-shock proteins (HSPs) are the most abundant and highly conserved soluble intracellular proteins present in all cells in a variety of intracellular locations. Under stress conditions such as high temperatures, toxins, oxidative conditions and glucose deprivation, their levels can increase by three fold. They play an important role as molecular chaperones in folding and unfolding of proteins, assembly of multi subunit complexes and a variety of other functions including degradation of proteins, thermo tolerance and buffering the expression of mutations (Srivastava, 2002). Therefore, they protect cells from severe damage and premature death by preventing denaturation and degradation of cellular proteins. HSPs can be classified into families based on their molecular weight such as HSP60, HSP70 and HSP90.

Extracellular HSP (eHSP)

Following stress conditions, HSPs not only activate inside the cells, but also they have been found outside the cells participating in signalling pathways and priming cells of the immune system (De Maio, 2011) (Figure 1.3). The detection of HSP70, HSP 90, HSP 27 and HSP 60 outside the cells has been reported (De Maio, 2011). The exact mechanism by which HSP is secreted into the extracellular environment is not fully understood but it has been suggested that HSP can leave the cell by two mechanisms: passive (secondary to cell death and lysis) and active (a physiological secretory process) (Asea, 2005).

The HSP 70 family are one of the most studied classes of HSPs. Recent studies have shown that eHSP70 might act as a cytokine and initiate the innate and adaptive immune

responses by binding non-specifically to receptors such as CD14, TLR2 and 4, CD36 and 40. It can cause translocation of NF- κ B and induce a strong proinflammatory response. In addition, it may specifically bind to an antigenic peptide and receptors such as CD91 and LOX1 and present the antigenic peptide to MHC class I and II (Asea, 2008, Asea *et al.*, 2000, Basu *et al.*, 2001, Binder *et al.*, 2000, Delneste *et al.*, 2002, Draude *et al.*, 1999).

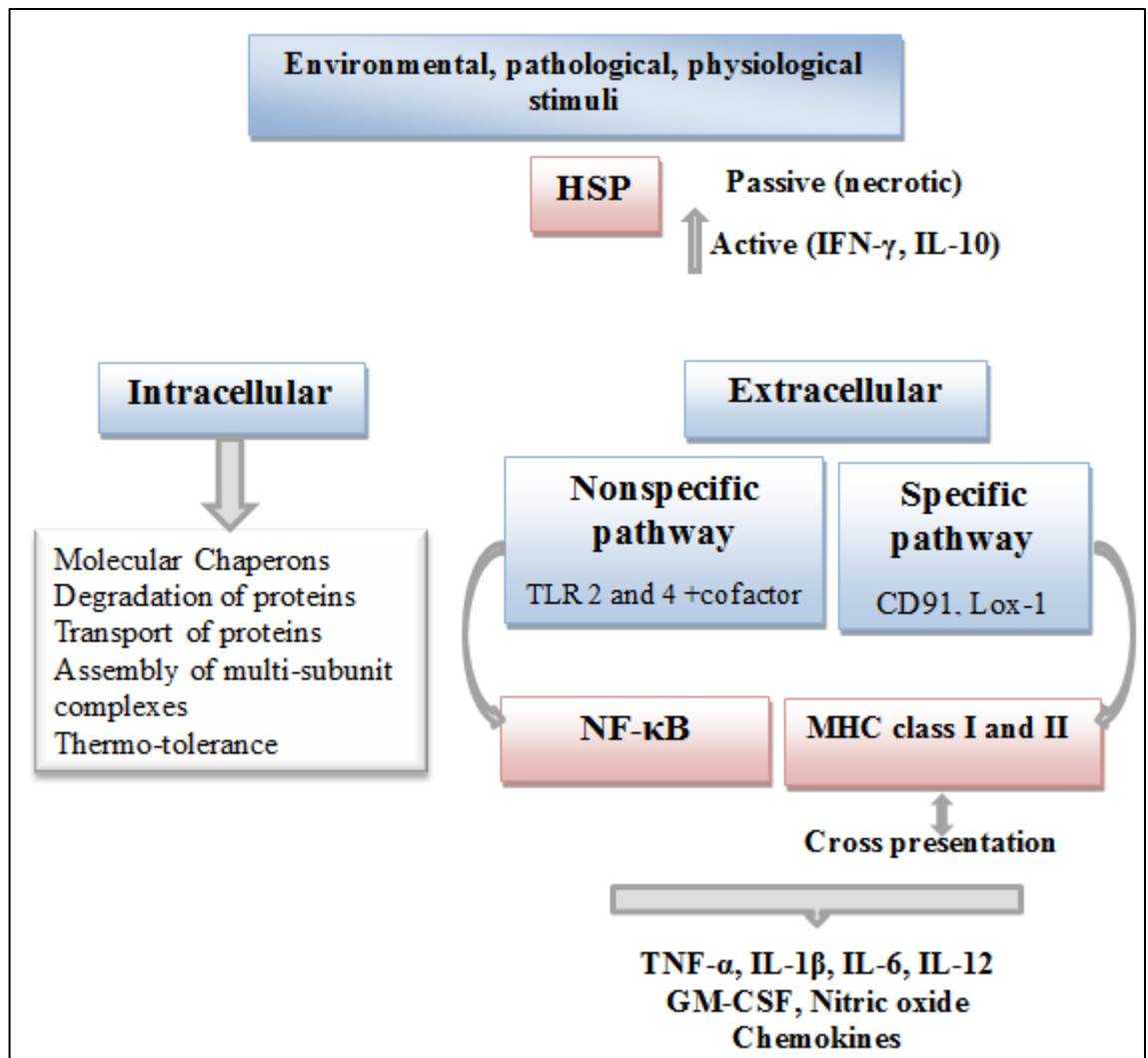


Figure 1.4: Dual functions of HSPs following stress conditions

HSP and BD

It is widely reported that microbial HSPs could be involved in the pathogenesis of BD through cross-reactivity with human HSP which induce proliferation of autoreactive T cells, specific to human HSP60 and result in an autoimmune reaction and tissue destruction (Lehner, 1999, Pineton de Chambrun *et al.*, 2012). HSP peptides from bacteria including *S.sanguis* have been found in the sera of BD patients (Isogai, 2003). Significant sequence homology of about 60% exists between human HSP60 and microbial HSP65. Autoantibodies against HSP65 and HSP60 have been reported in BD patients. The upregulation of HSP 60/65 expression in mucocutaneous lesions of BD patient and increased T- and B-cell responses against mycobacterial and human 60/65 kDa HSP in BD patients have been reported (Ergun *et al.*, 2001).

Both subcutaneous injection of HSP peptide 336-51 with adjuvant and oral and nasal administration without adjuvant are able to induce experimental autoimmune uveitis in rats (Phipps *et al.*, 2003). In a clinical trial on 8 BD patients, following oral administration of p336–351-CTB (peptide conjugated to the mucosal adjuvant, recombinant cholera toxin B subunit), with gradual withdrawal of the immunosuppressive drugs, no relapse of uveitis was seen in 5 of the patients (Stanford *et al.*, 2004).

(Birtas-Atesoglu *et al.*, 2008), reported a significant upregulation of both free human HSP70 and anti-HSP70 antibodies in patients with BD. It was concluded that HSP70 may mediated innate and adaptive immune responses and participate in proinflammatory cytokine activation and tissue destruction in BD.

Although currently HSP is the most studied putative triggering factor in pathogenesis of BD, the implication of some other autoantigens have been considered too. Significant

upregulation of autoantibodies against $\alpha\beta$ -crystalline have been reported. This is a small stress protein that has been found in cerebral spinal fluid (CSF) and serum of BD patients with neurological involvement (Pineton de Chambrun *et al.*, 2012). Furthermore, increased immune response to retinal S antigen in BD uveitis has been observed (Ermakova *et al.*, 2003).

1.5.3.2 $\gamma\delta$ T lymphocytes

$\gamma\delta$ T lymphocytes comprise approximately 5% of T cells population and are highly abundant in mucosal tissues. This subset of T cells is defined by their T cell receptors (TCR) which are constituted by a γ and a δ chain as opposed to the α and β chains of the majority of T cells. $\gamma\delta$ TCRs contain a broad specific spectrum of ligand recognition including bacterial phospho antigens, non-classical MHC-I molecules and unprocessed proteins (Ferreira, 2013). $\gamma\delta$ T cells can act as antigen-presenting cells linking innate and adaptive immunity, produce a diverse range of cytokines, exert cytotoxic effector function and regulates $\alpha\beta$ T cells (Al-Otaibi *et al.*, 2005, Ferreira, 2013).

$\gamma\delta$ T cells play important roles in mucosal immunity as the first line of host defence. Since many common manifestations of BD occur in mucosal regions, $\gamma\delta$ T lymphocytes have been subject to several investigations in BD. Accumulation of cytotoxic $\gamma\delta$ T cells in the sites of inflammation including, broncho alveolar lavage CSF and PBMCs of BD patient has been reported (Hamzaoui *et al.*, 1994). Moreover, significant $\gamma\delta$ T cell proliferative responses have been elicited following stimulation with HSP peptides in active BD patients (Hasan *et al.*, 1996). Freysdottir *et al.*, 1999 reported significant elevated numbers of activated $\gamma\delta$ T cells in PBMCs of BD patients compared to RAS and healthy controls. They also observed polyclonal rather than oligoclonal activation

of the $\gamma\delta$ T cells and suggested BD may have been caused by different antigenic stimuli or responses which are responsible for the wide clinical heterogeneity of the disease.

Infliximab, an anti-TNF α monoclonal antibody used for treatment of BD patients, was able to suppress *in vivo* and *in vitro* $\gamma\delta$ T cells expansion, activation and cytotoxic activity. This mechanism may explain its effectiveness in BD patient (Pineton de Chambrun *et al.*, 2012). Overall, $\gamma\delta$ T cells seem to play a role in pathogenesis of BD but more studies need to clarify the exact mechanism.

1.5.3.3 Neutrophil hyperactivity

Neutrophils are the most abundant leucocytes which play a crucial role in innate immune response. They are the first immune cells appear in the site of inflammation following infection or trauma and function as phagocytes. Inappropriate activation of neutrophils leads to tissue damage and inflammation progression. The implication of neutrophils in pathogenesis of Behçet's disease has been reported by several studies. (Eksioglu-Demiralp *et al.*, 2001) showed neutrophil hyperactivity in BD by presenting data on increased expression level of surface markers such as CD11a, CD10, and CD14 associated with activated neutrophils. Additionally, the upregulation of reactive oxygen species (ROS) in BD have been observed (Yazici *et al.*, 2004). Histopathological analysis of BD lesions also indicated the vascular infiltration of neutrophils (Kobayashi *et al.*, 2000).

The exact mechanism of neutrophil activation is still unknown. However, the raised level of IL-6, IL-8, IL-18 and TNF- α may cause the retaining of a "primed" state of neutrophils which may be accountable.

1.5.3.4 Cytokines and BD

Cytokines are small, secreted, signalling proteins which are produced in response to antigens by variety of cell types including macrophages/monocytes, dendritic cells, lymphocytes, neutrophils, endothelial cells and fibroblasts. They are usually produced temporarily and are potent even at picomolar concentrations. They interact with specific cell surface receptors to increase or decrease expression of membrane proteins, proliferation and secretion of effector molecules (Abbas AK, 2003). Cytokines interact in a network by inducing each other, transmodeling cell surface receptors, and by synergic, additive, or antagonistic interactions on cell function (Balkwill and Burke, 1989). Appropriate cytokine production leads to protective immunity while inappropriate cytokine production causes tissue destruction and disease progression. Therefore, the cytokine network plays an important role in the onset, progression and tissue damage in inflammatory diseases. They are regulators of host responses to infection, immune responses, inflammation, and trauma. Some cytokines act to make disease worse (proinflammatory), whereas others serve to reduce inflammation and promote healing (anti-inflammatory). Considerable attention has also been given to blocking cytokines, which are harmful to the host, particularly during overwhelming infection. The study of cytokines implicated in the pathogenesis of BD provides important information on finding diagnostic or activity markers and new therapies. Cytokines involved in pathogenesis of BD can be categorized into proinflammatory cytokines and chemokines, Th1 type, Th2 type and Th17 type cytokines.

1.5.3.4.1 Pro-inflammatory cytokines

Pro-inflammatory cytokines, including IL-1 α , IL-1 β , IL-6 and TNF- α , play major roles in innate and adaptive immune responses and promote inflammatory reactions in target tissue. They are produced early following tissue injury or in response to microorganisms

and their products. Several studies have confirmed the elevated levels of IL-1 α , IL-1R, and IL-1 β in the serum of patients with BD (Zhou *et al.*, 2012, Düzgün *et al.*, 2005). However, there were no significant differences in the serum levels of these cytokines and their inhibitors between active and inactive patients. Significant association of IL-1A -889 polymorphism with increased susceptibility for BD has been reported in a study done on 132 BD patients and 105 healthy in Turkey (Karasneh *et al.*, 2003).

TNF- α is one of the most studied cytokines in BD. The role of TNF- α in BD pathogenesis is supported by several studies confirming its therapeutic effectiveness in BD. A significantly higher serum level of TNF- α and its soluble receptor (TNFR1) were observed in patients with active BD in comparison to controls (Düzgün *et al.*, 2005). Besides, high levels of TNF- α were found in the aqueous humor from patients with uveitis (Santos Lacomba *et al.*, 2001). In another study, (Ahmad *et al.*, 2003), association of TNF-1031C allele with susceptibility to BD in Caucasoid patients was observed. A recent meta-analysis study of TNF polymorphisms showed association of -1031C, -238A and the -857T promoter polymorphisms with BD in various ethnic groups (Touma *et al.*, 2010).

The association of IL-6 with disease activity in Neuro-BD has been verified by several studies (Zhou *et al.*, 2012). In a study done on Neuro BD patients, a significant elevated level of IL-6 in CSF was found. It suggested IL-6 as a disease activity marker along with CSF cell count and protein levels (Akman-Demir *et al.*, 2008, Borhani Haghighi *et al.*, 2009).

1.5.3.4.2 Th1 type cytokines

Th1 cells are responsible for directing cell mediated immunity by releasing cytokines such as interferon- γ (IFN- γ) and IL-12. Most of the studies are in favour of Th1 polarization of the immune response in Behçet's disease (Frassanito *et al.*, 1999, Zhou *et al.*, 2012). Ben Ahmed *et al.*, 2004 reported the significant upregulation of IL-12 and IFN- γ mRNA expression level in BD mucocutaneous lesions compared with controls. In a study by (Ahn *et al.*, 2006), cytokine profiles in the aqueous humour of BD patients with uveitis were investigated. They observed higher level of IFN- γ in BD uveitis in contrast to higher concentrations of IL-4 in patients without Behçet's uveitis, suggesting a strong Th1 polarization in Behçet's uveitis (Ahn *et al.*, 2006). The recent GWAS studies also revealed the association of IL23R-IL12RB2 variants with BD (Mizuki *et al.*, 2010, Remmers *et al.*, 2010). However, some studies reported higher serum level of IL-4, IL-10 and IL-13 and lower level of IL-12 and IFN- γ in active BD patients compared to inactive BD, recurrent aphthous stomatitis, and healthy controls. They concluded a complex cytokine profile Th1/Th2 cell type in BD (Raziuddin *et al.*, 1998, Aridogan *et al.*, 2003). IL-18 promotes a Th1 response by stimulating the secretion of IL-12 and INF- γ and suppression of IL-10. Therefore, the elevated level of IL-18 in the serum of BD patient supported the hypothesis of a cell mediated immunity nature in BD (Musabak *et al.*, 2006, Oztas *et al.*, 2005).

1.5.3.4.3 Th2 type cytokines

Th2 cells are responsible for promoting humoral immunity by producing IL-4, IL-5, IL-13 and IL-10. IL-10 is a potent anti-inflammatory cytokine with an inhibitory role on Th-1cytokines, proinflammatory cytokines, antigen presenting cells and B-cell proliferation which results in humoral immunity promotion. IL-10 is the most well addressed cytokine among this group in BD. Despite several studies on Th1 polarisation

of BD, the study by (Ben Ahmed *et al.*, 2004) revealed increase level of IL10 and unchanged IL-4 level. The association of BD with IL-10 gene was confirmed and validated in different GWAS studies (Mizuki *et al.*, 2010, Remmers *et al.*, 2010, Wallace *et al.*, 2007, Xavier *et al.*, 2012). Remmers (2010) also reported the association of IL-10 variant (the rs1518111 A allele) with reduction in both its mRNA and protein level expression in BD. Overall, there is no data regarding the possible role of IL-4, IL-5 and IL-13 in BD.

1.5.3.4.4 Th17 type cytokines

Th17 cells are a subset of T cells characterized by the secretion of IL-17. They have been implicated in the pathogenesis of a variety of immune mediated diseases such as inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis and periodontal disease. Th-17 cells are differentiated from naïve CD4⁺ T cells in the presence of TGF- β and IL-6 *in mouse model studies* (Bettelli *et al.*, 2006, Mangan *et al.*, 2006, Veldhoen *et al.*, 2006). Tumour necrosis factor- α (TNF- α) and IL-1 β can also induce mouse Th17 differentiation, but only in the presence of TGF- β and IL-6 (Nakae *et al.*, 2007, Sutton *et al.*, 2006, Veldhoen *et al.*, 2006). Besides, since IL-23 receptors are only expressed on activated T cells, they are unable to induce a Th17 differentiation of naïve T cells but can induce upregulation of IL-17 in memory T cells. In humans IL-6 and IL-23 induce a small amount of IL-17 alone and greatly enhance Th17 differentiation in the presence of IL-1 β (Acosta-Rodriguez *et al.*, 2007, Wilson *et al.*, 2007).

IL-17 is a proinflammatory cytokine which induces the production of IL-1, TNF - α , IL-6 and IL-8. Interestingly, besides Th-17 cells, IL-17 can also be produced by CD81⁺ T cells, $\gamma\delta$ T cells, natural killer T (NKT) cells, neutrophils, and eosinophils. IL-17 increases chemokine CXCL8/IL-8 production resulting in neutrophil influx to the site of

inflammation. Th17 cells express the chemokine receptor CCR6, which mediates homing to skin and mucosal tissues and has been shown to play an important role in recruitment of pathogenic T cells in many inflammatory diseases associated with IL-17 such as psoriasis, inflammatory bowel diseases, allergic asthma and rheumatoid arthritis (Hirota *et al.*, 2007, Lundy *et al.*, 2005, Ruth *et al.*, 2003, Teraki *et al.*, 2004).

In BD patients, upregulation of neutrophil activation associated with increased CXCL8 (IL-8) production have been reported (Kurauchi *et al.*, 2005). Besides, elevated levels of IL-6, IL-1 β , IL-8 and TNF- α which are involved in differentiation of naïve CD4⁺ T cells to TH-17, have been observed (Akman-Demir *et al.*, 2008, Fossiez *et al.*, 1996). In a study on BD patients with active uveitis the upregulation of IL-23, IL-17, IFN- γ have been shown in addition to the increased frequency of IL-17 and IFN- γ producing cells in PBMCs (Chi *et al.*, 2008). The elevated serum levels of IL-17A in active BD patients have been observed suggesting the important role of IL-17 in acute attacks of the disease (Ekinci *et al.*, 2010).

In a recent study on Behçet's uveitis and folliculitis, in addition to showing the importance of Th-17 cells, the significant increase in the Th17/Th1 ratio in these patients compared to healthy controls have been reported (Kim *et al.*, 2010). Moreover, Chi (2008) presented data on the significant elevated levels of IL-23p19 mRNA expression, IL-23, IL-17, and IFN- γ in BD patients with active uveitis. They also reported increased frequencies of IL-17-producing and IFN- γ -producing T cells in PBMCs of active BD patients with uveitis. Additional evidence for the possible role of IL17 in the pathogenesis of BD has arisen from GWAS studies on IL-23 which reported the strong association of IL-23 receptor (IL-23R) genes with BD (Mizuki *et al.*, 2010, Remmers *et al.*, 2010). Further studies also validated the strong relationship between

polymorphisms of *IL23R* and *IL17* and BD (Jiang *et al.*, 2010, Kim *et al.*, 2012). On the other hand, an investigation of BD patients with gastrointestinal involvement, a Th1 pattern, not a Th17, has been reported (Ferrante *et al.*, 2010).

1.5.3.4.5 Chemokines

Chemokines are small cytokines (approximately 8-10 kilodaltons in size) which are well known for their chemotactic function in immune system. They cause attraction of monocytes and dendritic cells (DCs) to the sites of inflammation, directing them to lymphatic vessels, leading to T cells and APCs through lymphatic drainage organs. They also play a role in T-cell homing. Furthermore, several evidences suggest some chemokines such as CCL2 and CCL3 are involved in direction of T cell differentiation (Luther and Cyster, 2001). IL-8, also known as neutrophil chemotactic factor, is the most studied chemokine in BD. (Gür-Toy *et al.*, 2005) found a correlation between high serum levels of IL-8 and the increased number of clinically involved organs and suggested IL-8 as a disease serologic activity marker. Besides (Durmazlar *et al.*, 2009) observed that the elevated levels of IL-8 in BD is more prominent in patients with vascular involvement than other involved organs. It concluded that serum IL-8 levels may be considered as a maker for vascular involvement. However, the study on German and Turkish cohort did not find any correlation between susceptibility to BD and polymorphism in IL-8 and IL-8R genes (Zhou *et al.*, 2012).

1.6 Treatment

The main goal in effective treatment of BD is to prevent irreversible damage to the vital organs particularly in high risk patients such as young men and to prevent exacerbations of mucocutaneous and joint involvement. These do not usually cause serious harm but affect patients quality of life (Hatemi *et al.*, 2008). In 2008, a multidisciplinary expert committee developed evidence-based European League Against Rheumatism (EULAR)

guidance for the management of Behçet's disease and suggested nine recommendations for the management of different aspects of BD (Hatemi *et al.*, 2008)(Table 1.3). However, the differences in health care systems, the economic status of different countries and patient preferences should be taken into consideration (Hatemi *et al.*, 2008).

NO.	Recommendation
1	Any patient with BD and inflammatory eye disease affecting the posterior segment should be on a treatment regime that includes azathioprine and systemic corticosteroids.
2	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.
3	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.
4	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.
5	There is no evidence-based treatment that can be recommended for the management of gastrointestinal involvement of BD. Agents such as sulfasalazine, corticosteroids, azathioprine, TNF α antagonists and thalidomide should be tried first before surgery, except in emergencies.
6	In most patients with BD, arthritis can be managed with colchicine.
7	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 TNF α antagonists. For dural sinus thrombosis corticosteroids are recommended.
8	Cyclosporine A should not be used in BD patients with central nervous system involvement unless necessary for intraocular inflammation.
9	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 co dominant lesions present.
	Topical measures (ie, 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 TNF α antagonists may be considered in resistant cases.

Table 1.3: Nine recommendations on Behçet disease management by (Hatemi *et al.*, 2008).

1.7 JAK-STAT (Janus kinase signal transducers and activators of transcription) pathway

The majority of cytokines, expressing type I and type II cytokine receptors, mediate their responses through activation of the JAK/STAT signalling pathway. This pathway was uncovered for the first time through a study on transcriptional activation in response to interferons (Darnell *et al.*, 1994). The JAK-STAT pathway comprises JAK (Janus kinase) and STAT (signal transducers and activators of transcription) (Figure 1.5). The JAK-STAT pathway plays an important role in mediating the effect of most cytokines in immunoregulation, host defence and immunopathology (Darnell *et al.*, 1994, Leonard and O'Shea, 1998, O'Shea and Murray, 2008).

JAKs constitute a family of non-receptor, intracellular tyrosine kinases including JAK1, JAK2, JAK3 and TYK2 which are associated with intracellular domain of cytokine receptors. They selectively phosphorylate and activate STATs. JAKs are protein kinases of 120-140 KDa and constitute seven regions of a high homology called Janus homology domains 1 to 7 (JH1-7). JH1 is the kinase domain, containing two tyrosine molecules which are phosphorylated following stimulation by a ligand. JH2 is the pseudo-kinase domain which lacks enzymatic activity but is required for JH1 catalytic activity. JH3-JH4 region shares some similarity with Src-homology-2 (SH2) domains. JH4-JH7 region constitutes a FERM domain (Four-point-one, Ezrin, Radixin, Moesin) which mediate protein-protein interactions. The JH6 and JH7 domains mediate the binding of JAKs to receptors (Kisseleva *et al.*, 2002, Shuai and Liu, 2003).

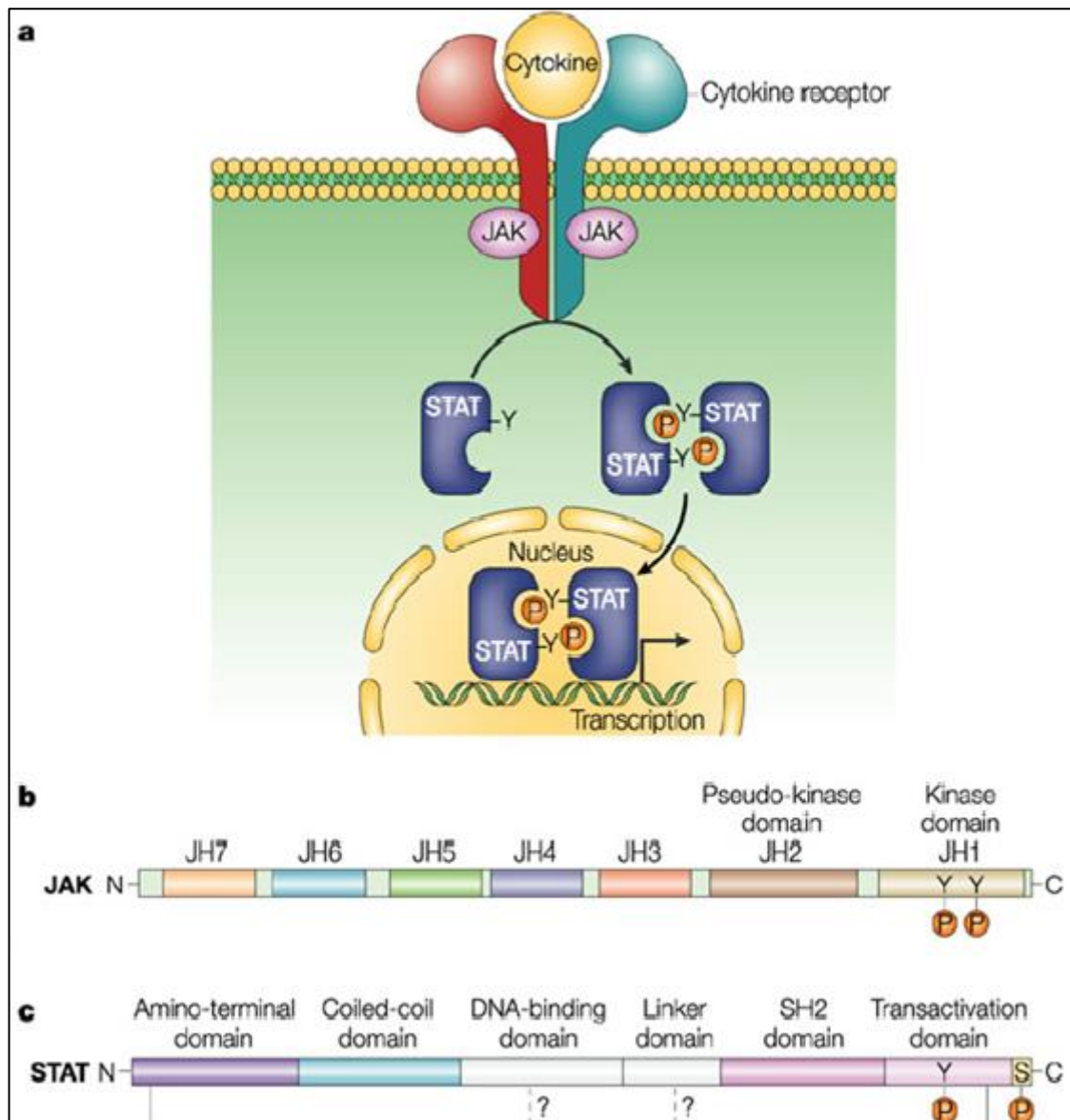


Figure 1.5: JAK-STAT pathway. a) JAK-STAT signalling pathway. b) The domain structure of JAKs. c) The domain structure of STATs (Adapted from Shuai and Liu; 2003).

STATs are a family of transcription factors hidden in the cytoplasm and consist of seven structurally and functionally related proteins: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. All STATs family members constitute six conserved domains: N-terminal domain, Coiled-coil domain, DNA-binding domain, Linker domain which connects the DNA-binding domain with the SH2/dimerization domain, SH2 domain and tyrosine activation motif which are involved in the activation and

dimerization of STATs, and transcriptional activation domain (Kisseleva *et al.*, 2002, Shuai and Liu, 2003) (Figure 1.5).

Generally, JAK-STAT pathway signalling comprises three components: Cytokine receptor, JAK protein and STATs. Binding a cytokine to its receptor induces conformational changes of the receptor chains and subsequent activation of the JAK family of kinases which are constitutively associated with the receptor. Then, the activated JAKs phosphorylate specific tyrosine motifs on the cytoplasmic tails of the receptor which act as docking sites to recruit and phosphorylate STATs through their SH2 domains. Activated STATs then dimerize and are subsequently released from the receptor and translocate to the nucleus where they activate gene transcription (Kisseleva *et al.*, 2002, Shuai and Liu, 2003) (Figure 1.5).

The human genetic studies have suggested several evidences to support the implication of this pathway in human diseases. The association of polymorphisms of genes encoding different parts of signalling pathways of cytokines such as IL-12A, IL-6 and IL-23 with autoimmune disease has been presented in Figure 1.6. The implication of STAT3, which is also activated by IL-6 and its receptors, in multiple sclerosis, asthma, rheumatoid arthritis and cardiovascular diseases has been reported (O'Shea and Plenge, 2012). The association of several genes in IL-12 pathway (IL12A, IL-12R and STAT4) with primary biliary cirrhosis, systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis, celiac disease and Behçet has been reported by several GWAS studies (O'Shea and Plenge, 2012). Furthermore, polymorphisms of the genes encoding both subunits of IL-23 (IL23A and IL12B), JAK2, TYK2, and STAT3 have all been linked to autoimmune diseases such as psoriatic arthritis, Grave's disease, Crohn's disease and multiple sclerosis (O'Shea and Plenge, 2012).

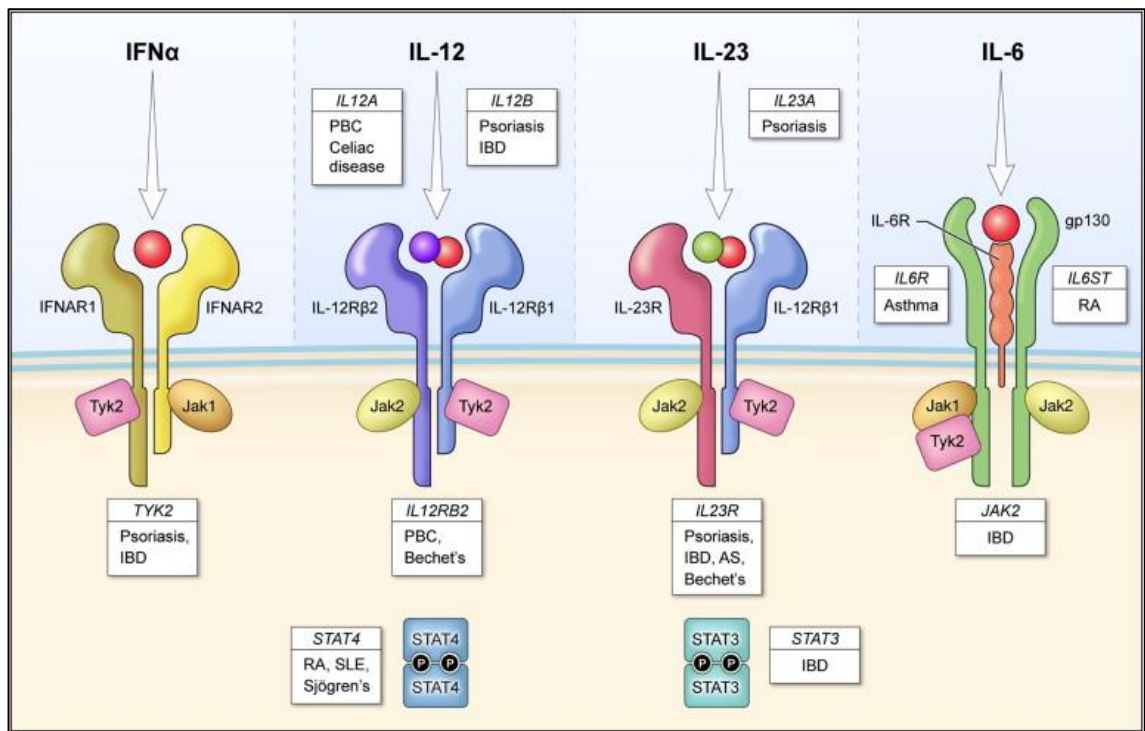


Figure 1.6: GWAS study results on association of genetic factors links of JAKs-STATs related cytokine signalling with human diseases. Adapted from (O'Shea and Plenge, 2012). AS, ankylosing spondylitis; IBD, inflammatory bowel disease; PBC, primary biliary cirrhosis; SLE, systemic lupus erythematosus.

Therefore, cytokine signalling pathways are strongly regulated to avoid excessive immune responses and pathogenic consequences in the host. So far, three distinct negative regulation mechanisms have been found. These include protein tyrosine phosphatases inhibitors such as SHP-1 and CD45, the protein inhibitors of activated STATs (PIAS) and the suppressor of cytokine signalling (SOCS) proteins (Krebs and Hilton, 2001).

1.8 Suppressor of Cytokine Signalling (SOCS) proteins

One of the mechanisms which negatively regulate cytokine signalling pathways is a family of intracellular proteins called SOCS proteins. They regulate cytokine signalling by inactivation of the Janus kinase (JAK), blocking access of the STAT proteins to receptor binding sites, and ubiquitination of signalling proteins. SOCS proteins are also involved in a variety of other biological processes such as regulation of macrophage and dendritic-cell activation and T-cell development, differentiation and function. Several lines of research supported the implication of SOCS dysregulation in diseases of the immune system. Nevertheless, SOCS proteins may be activated by other stimulants such as lipopolysaccharide (LPS), isoproterenol, statins and cyclic AMP (cAMP). Therefore, they play a role in many other immunological processes (Yoshimura *et al.*, 2007) .

1.8.1 The structure and function of SOCS proteins

SOCS family comprises eight members: CIS (Cytokine Inducible SH2 containing protein) and SOCS1–SOCS7. They contain three defined regions: a central SH2 domain, an amino-terminal domain of variable length and divergent sequence and a carboxy-terminal 40-amino-acid module called SOCS box (Yoshimura *et al.*, 2007)(Fig. 1.6).

The SOCS proteins bind to their targets by the central SH2 domain. The SH2 domain of SOCS1 directly binds to the activation loop of JAKs while the SH2 domains of CIS, SOCS2, and SOCS3 bind to phosphorylated tyrosine residues of stimulated cytokine receptors (Tamiya *et al.*, 2011). Moreover, SOCS1 bind directly to the type I IFN receptor (IFNAR) and the IFN- γ receptor (IFNGR).

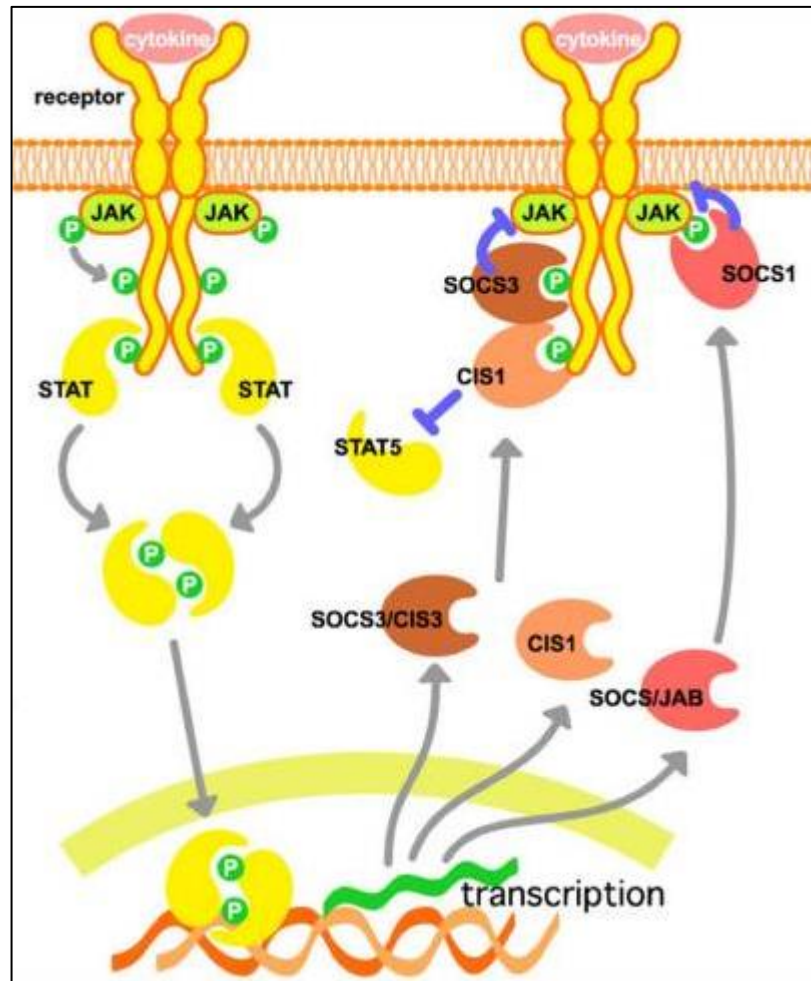


Figure 1.7: SOCS function adapted from (Yoshimura *et al.*, 2012).

The SOCS box recruits the ubiquitin-transferase system for proteasomal degradation. The SOCS box interacts with elongin B and elongin C, cullin-5 and RING-box-2 (RBX2) to recruit E2 ubiquitin transferase 8. It has been postulated that SOCS-box-containing molecules such as CIS–SOCS-family proteins, possibly act as E3 ubiquitin ligases and mediate proteins degradation through their N-terminal regions (Yoshimura *et al.*, 2007).

SOCS1 and SOCS3 are strong inhibitors of JAKs as their structure contains an extra region called kinase inhibitory region (KIR). KIR acts as a pseudo substrate and enables

SOCS1 and 3 to inhibit the tyrosine kinase activity of JAK directly. SOCS1 mediates its inhibitory function on JAKs by binding to the activation loop of its catalytic domain using both KIR and SH2 domains (Yoshimura *et al.*, 2007). Most of the receptors which are activated by STAT3 are specifically inhibited by SOCS3. SOCS3 also inhibits the IL-12-induced STAT4 activation pathway (Yoshimura *et al.*, 2007).

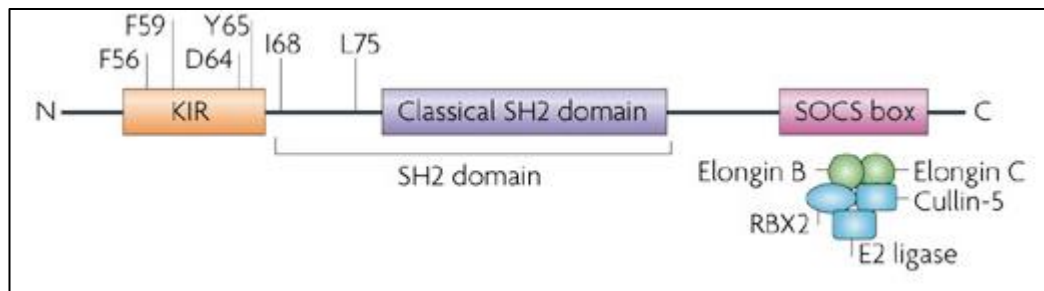


Figure 1.8: SOCS Structure. Adapted from (Yoshimura *et al.*, 2007).

Cytokine-inducible SH2 (CIS) is the first member of SOCS family which was identified by Yoshimura *et al.*, (1995). *In vivo* studies on CIS transgenic mice suggested CIS as a specific inhibitor of the STAT5 pathway (Matsumoto *et al.*, 1999). SOCS1 was first identified by three research groups with different approaches (Yoshimura *et al.*, 2012).

1.8.2 The role of SOCS proteins in innate immunity

The regulation of innate immunity following activation of TLRs by pathogens is crucial as any excessive responses cause damage to host tissue. Since TLR ligands, such as LPS are potent inducers of SOCS, it has been postulated that SOCS1 and SOCS3 proteins negatively regulate TLR signalling pathways (Naka *et al.*, 2005). Several studies have reported upregulation of pro-inflammatory cytokines such as TNF, IL-12 and IFN γ , and nitric oxide in response to TLR of macrophages, DCs and fibroblasts of SOCS1-deficient mice (Nakagawa *et al.*, 2002, Yoshimura *et al.*, 2007). Moreover, it

has been revealed that SOCS1-deficient mice are oversensitive to LPS-induced shock, causing a surge in inflammatory cytokines such as tumour-necrosis factor (TNF) and IL-12 production, conversely, introducing SOCS-1 inhibited NF-kappa β and STAT1 activation in macrophages (Nakagawa *et al.*, 2002, Yoshimura *et al.*, 2007).

Regarding the mechanism, some studies support the possible direct effect of SOCS1 on some components of TLR–NF- κ B (nuclear factor- κ B) signalling pathway. It has been suggested that SOCS1 may bind to p65 subunit of NF- κ B or MyD88-adaptor-like protein (MAL), providing for their ubiquitination and degradation (Yoshimura *et al.*, 2007).

Contrary to SOCS1, the role of SOCS3 in innate immunity is more complicated. SOCS3 might play an important role in regulation of opposing activity of IL-6 and IL-10 in macrophages following stimulation with LPS but there are some contradictory reports.

SOCS3 inhibited IL-6 signalling through binding to the IL-6R subunit gp130 (Tyr759) (Yoshimura *et al.*, 2007). Several studies reported the inhibitory function of SOCS3 on IL-6 but not IL-10 activated by STAT3 in primary human macrophages (Niemand *et al.*, 2003, Yasukawa *et al.*, 2003). It has been suggested that, when SOCS3 action is blocked, macrophages appear to be hypersensitive to the anti-inflammatory properties of IL-6, therefore, SOCS3 shows a critical role in inhibiting IL-6 signals and inducing immune responses to control for example in *T.gondii* infection (Whitmarsh *et al.*, 2011). In contrast, mice lacking SOCS3 developed severe histopathological symptoms such as increased numbers of neutrophils in the inflamed synovium, bone marrow, peripheral blood, and spleen during experimental arthritis, due to elevated levels of IL-6 (Wong *et al.*, 2006).

1.8.3 Role of STATs and SOCS proteins in Th-cell differentiation

Naïve CD4⁺ T cells, following interaction with their cognate antigen, can differentiate into at least four major lineages of T helper (Th) cells Th1, Th2, Th17 and inducible T regulatory (iTreg) cells. They are defined based on their distinct cytokine production profiles and their functions. Particular cytokines environment and transcription factors are key elements in determining CD4 T cell fates and effector cytokine production (Figure1.9).

Th1 cells, which are developed to increase immune responses to intracellular pathogens, are characterized by IFN- γ production, a potent activator of cell-mediated immunity. Th1 polarization is mainly induced by IL-12 and IFN- γ through STAT4 and STAT1/T-bet pathway (Figure1.9).

Th2 cells, which evolved to eliminate parasitic infections by production of IL-4, IL-5, and IL-13, are potent activators of B-cell and humoral responses. Th2 polarization is mainly induced by IL-4 through STAT6/GATA-3 path. Any dysregulation in Th1 responses to self-antigens or commensal flora can give rise to tissue destruction and chronic inflammation, while dysregulation in Th2 responses lead to allergy and asthma.

SOCS1 and SOCS3 play an important role in Th1 and Th2 cell differentiation. SOCS1 has been shown as a negative regulator of IL-12, IFN- γ and STAT 4 signalling pathway through acting as a pseudo-substrate for JACK2 (Eyles *et al.*, 2002). Therefore, as predicted, the removal of SOCS1 in CD4⁺ T cells enhances IFN- γ and STAT4 and subsequently Th1 cells production, whereas overexpression inhibits it (Eyles *et al.*, 2002) (Figure1.9). Moreover SOCS1 deletion leads to IFN- γ and IL-12 production and increased Th1 cell population (Fujimoto *et al.*, 2002).

The reported data on the role of SOCS3 in Th1 and Th2 cell differentiation is complex. SOCS-3 prevents IL-12-induced STAT4 activation by binding to STAT4 docking site, Tyr-800 on the IL-12 receptor β 2 chain (Yamamoto *et al.*, 2003). It seems that deletion of SOCS3 in T cells, suppresses both Th1 and Th2 cell responses and it has been suggested that this inhibition might not be the result of a direct effect on the differentiation of Th1 and Th2 cells but it is because of the increased levels of the immunosuppressive cytokines TGF- β and IL-10 (Kinjyo *et al.*, 2006).

Th17 cells are characterized by IL-17A, IL-17F, IL-22, and IL-21 secretion and play crucial roles in autoimmunity and inflammation in response to extracellular bacterial and fungal pathogens particularly at mucosal surfaces. The Th17 differentiation is primarily driven by IL-6, IL-23 and TGF- β through the STAT3/ ROR γ t pathway. Involvement of two signalling pathways: TGF- β -SMAD and JAK-STAT signalling, induces distinct Th17 and Treg cell lineages from Th1 and Th2 cells. It has been reported that IL-6, IL-21 and IL-23 induce ROR γ t through STAT3 signalling pathway. Moreover, it has been demonstrated that while SOCS3 inhibits STAT3 signalling, its blocking causes increased production of Th17 cells (Palmer and Restifo, 2009). Unlike SOCS3, the deletion of SOCS1 results in decreased Th17 cell production, and the reducing of Th17-mediated diseases (Tanaka *et al.*, 2008).

Regulatory T (Treg) cells (Tregs) are defined by the expression of CD4, CD25 and play important role in maintaining peripheral tolerance, preventing autoimmune diseases such as type 1 diabetes, limiting chronic inflammatory diseases such as asthma and inflammatory bowel disease (IBD) and anti-tumour immunity (Vignali *et al.*, 2008). Forkhead box P3 (FOXP3) is key transcription factor, for development, maintenance and function of Treg cells.

Treg polarization is primarily driven by IL-2 and TGF- β through STAT5 /Foxp3 pathway (Figure 1.9). The role of SOCS family in Treg cell differentiation has not been well established. It has been suggested that SOCS1 and SOCS3 might prevent the differentiation of Treg cells, but more investigation is required (Palmer and Restifo, 2009)(Figure 1.9).

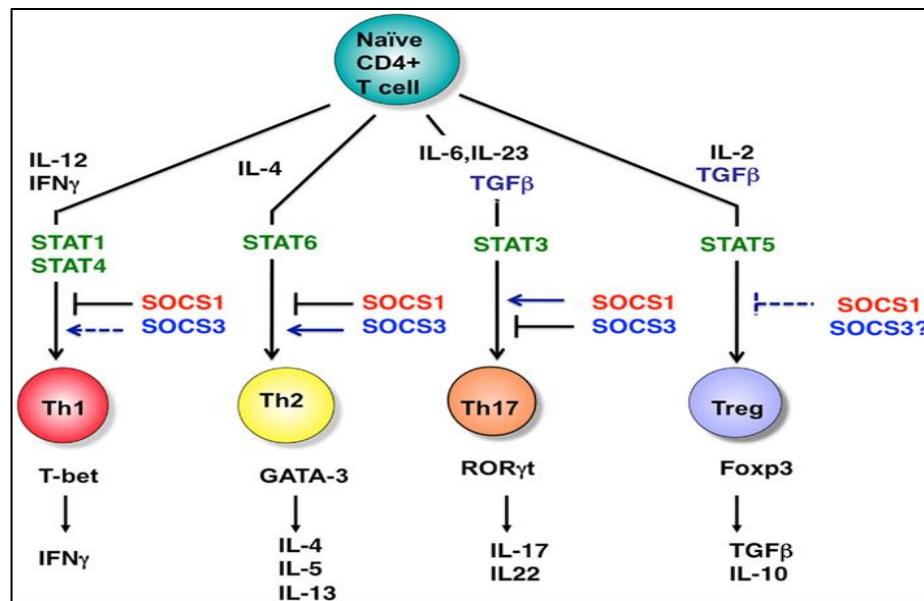


Figure 1.9: Possible roles for SOCS proteins in T cell differentiation. Adopted from (Yoshimura *et al.*, 2012).

The role of Treg cells in BD has been investigated by a few studies. In a study on Ocular BD, the percentages of Treg cells in CD4+Tcells from BD with ocular complications were significantly decreased before the ocular attack compared with those after ocular attack and normal controls (Nanke *et al.*, 2008). In a recent study, the percentage of CD4+CD25+FOXP3+Treg in active patients was found lower than clinically inactive patients and healthy controls. Furthermore, there was no difference between the percentage of CD4+CD25+Treg in active and inactive patients and healthy controls (Gündüz *et al.*, 2013).

1.8.4 SOCS and Behçet's disease

Cytokines play a pathologic role in BD. Several cytokines implicated in BD such as IFN γ , IL-12, IL-23, IL-10 and IL-6 exerts their effect through activation of JAK-STAT signalling pathway which is negatively regulated by SOCS proteins. SOCS proteins are also involved in a variety of other biological processes such as regulation of macrophage and dendritic-cell activation and T-cell development, differentiation and function. Therefore, the implication of SOCS dysregulation in diseases of immune system such as BD should be considered.

In vivo studies with gene-disrupted or transgenic mice have supported the crucial role of SOCS proteins in regulating immune and inflammatory responses. The studies of SOCS1- deficient mice have revealed the significant role of SOCS1 in innate immunity and pathogenesis of inflammatory and autoimmune diseases (Fujimoto *et al.*, 2004). Mice deficient in SOCS1 signalling develop severe skin and eye lesions through the recruitment of Th1 and Th17 cells into these non-lymphoid tissues (Yu *et al.*, 2008). In another study, the role of SOCS3 in keratinocyte function was investigated using keratinocyte-specific SOCS3 gene deficient mice which developed a severe form of skin inflammation (Uto-Konomi *et al.*, 2012). Their results show that skin homeostasis is strictly regulated by the IL-6-STAT3-SOCS3 axis. Eye and skin lesions are also characteristic of Behçet's disease. Therefore, a dysfunction of SOCS proteins may associate with the development of inflammatory diseases including BD.

At present, data regarding the expression and possible implication of SOCS proteins in Behçet's disease are very limited. In a recent study, the expression of Th17 related cytokines and their signalling molecules were investigated in PBMC and skin lesions of patients with BD (Shimizu *et al.*, 2012). They found a significant rise in TGF β receptor type 1, IL-12 receptor β 2 and SOCS1 following stimulation of PBMCs. However, levels

of SOCS in unstimulated cells were not measured. SOCS3 positively regulates TLR4 signalling by feedback inhibition of TGF- β /Smad3 signalling (Liu *et al.*, 2008) and has been shown to influence Th17 cells.

1.9 Phosphorylation and Protein Kinases

Protein phosphorylation is the most ubiquitous post-translational modification that occurs in biology and plays an important role in most signalling processes. It can be regarded as a binary switch where addition or removal of phosphate groups will either turn on or turn off specific reactions. Identification of site-specific phosphorylated substrates is fundamental for understanding the molecular mechanisms of many signal transduction pathways. Analysing the phosphorylation profiles of protein kinase enzymes and their protein substrates is essential for understanding how cells recognise and respond to their environment and any changes that occur within the environment.

The human genome contains about 500 protein kinase genes and they constitute about 2% of all human genes. Up to 30% of all human proteins may be modified by kinase activity, and kinases are known to regulate the majority of cellular pathways, especially those involved in signal transduction (Manning *et al.*, 2002). Analysis of kinase activity in disease is a vast area of investigation and is beyond the scope of this literature review. In recent years some investigation has been carried out into kinase activity in Behçet's disease and the Suzuki groups have reported that excessive expression of Txk, a member of the Tec family of tyrosine kinases contributes to the excessive Th1 cytokine production in BD (Nagafuchi *et al.*, 2005). Other Kinases investigated have include Rho kinase (Oguz *et al.*, 2012) and Gas6 (Gheita *et al.*, 2012). While JAK2 and STAT3 polymorphisms have been reported in the Han Chinese population (Hu *et al.*, 2012). In this thesis the investigation of kinase activity has been focused on the Th1 and Th17 profiles of cytokine signalling in an attempt to try to understand the role of SOCS

and the signalling molecules that might contribute to the overall pro-inflammatory profile which is the signature of the disease.

1.10 Hypothesis

Since increased production of pro-inflammatory cytokines are a key feature of BD, it is important to try to understand if this is a result of the response to pathogens or if it might be due to dysregulation of the mechanisms that control the immune response.

Several cytokines implicated in BD such as IFN γ , IL-12, IL-23, IL-10 and IL-6 exerts their effects through activation of the JAK-STAT signalling pathways which are negatively regulated by SOCS proteins. In this respect, any dysregulation in this balance can affect cellular responsiveness to cytokines and cause subsequent pathological effects. At present there is limited data regarding expression of SOCS family in BD. Therefore this project was designed to investigate the expression of SOCS1-3 and to test the hypothesis that SOCS proteins are dysregulated in Behçet's Disease.

1.11 Aims

Aims of this project were to:

- Investigate expression of SOCS1 and SOCS3 in PBMCs, Neutrophils and Buccal mucosal cells of patients with BD.
- Investigate Th1, Th2 and Th17 cytokines profile in BD.
- Investigate expression of phosphorylation status of STAT1, STAT2, STAT3, STAT5 and the transcription factors t-bet and ROR γ t in BD.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2: Materials and Methods

2.1 Ethics

This project was carried out as part of the “Immunoregulation at the Mucosal Barrier Programme” and has ethical approval of the East London and the City Local Research Ethical Committee (LREC), Ref P/03/122 (Appendix 1). Healthy control samples were obtained from members of staff and the patient cohort was recruited from the outpatient departments at the Royal London Hospital, The Behcet’s Centre of Excellence (BCE) at the Bart’s and the London NHS Trust and St. Thomas’ Hospital with informed consent (Patient and Healthy Volunteer invitations Appendix 2 and 3, Consent forms Appendix 4). Ethical approval was obtained for all sites. All samples were anonymised, coded and logged according to ethical guidelines. The samples were stored in a locked freezer at -80°C and all patient data was entered onto a secure non-networked computer database that was password protected.

2.2 Subjects

Behçet’s Disease (BD) patients were diagnosed according to the International Study Group for Behçet’s Disease (Lancet 1990).

Recurrent aphthous stomatitis (RAS) patients were used as disease controls and most of the ulcers in these volunteers were of the minor type. These were compared with healthy control volunteers (HC). The criteria for HCs were age 18 or older and self-identified as healthy (Table 2.1). A small number of patients with oro-gential ulceration unrelated to BD were also recruited and used in limited studies (N=3). Demographic and clinical features of the investigated patients and controls are presented in Table 2.1.

The demographics for the patients and controls used for each experiment are described in the relevant sections of the results Chapters 3, 4 and 5.

Demographic and Clinical Features of the Investigated Patients and controls		
Study Period	2009-2013	n=119
Mean Age \pm SD, years	44.47 \pm 13.6(17-78)	
Gender	%	
Male	31.1	
Female	69.9	
Race or Ethnicity		
White British	23.5	
Irish	3.4	
Turkish	2.5	
Bangladeshi	2.5	
Other	9.2 Missing data for the rest	
Clinical Manifestations		
Oral Ulcers	40.8	
Genital Ulcers	13.3	
Eye Lesions	10.7	
Skin Lesions	25.3	
Joint involvement	47.6	
Encephalitis	12	
Vascular involvement	3.4	
Relapse	23.6	
Treatment		
Prednisolone	39.7	
Azathioprine	38.1	
CellCept*	4	
Cyclosporin A	2.4	
Colchicine	45.2	
Aza+Colch+Pred	7.1	
Pred+Aza	7.1	
Pred+Colch	11.4	
Aza+Colch	9.5	
Controls	Healthy controls(n=20)	RAS(n=10)
Mean Age \pm SD, years	37.27 \pm 11.8(19-61)	43.5 \pm 14.6(30-61)
Female	60%	70%
Male	40%	30%

Table 3.1: Demographic and clinical features of the investigated patients and controls

*CellCept is trade name for Mycophenolic acid

Pred: Prednisolon Aza: Azathioprine Colch: Colchicine

All the BD study population were examined for evidence of activity in the eyes, mouth, skin, joints, and were graded as being relapsed (BD-RE) or quiet (BD-Q).

The patients were assessed in each clinic based on two questionnaires: clinical disease activity form (Appendix 5), patient perception of disease activity questionnaire (Appendix 6).

BD patients were classified based on the overall disease activity, following a consultant-led assessment, into the following categories:

- 1) Relapsed (BD-RE): Severe disease activity in more than two of the BD symptoms despite treatment.
- 2) Quiet (BD-Q): Disease activity in fewer than three of the BD symptoms

2.3 Sample preparation

Blood, Buccal Mucosal Brush Biopsies (BMBB) and saliva samples were collected from the 4 subject groups: For BD and RAS patient samples were collected during quiet and active/relapsed periods of disease.

2.3.1 Blood Samples

The un-clotted blood samples (EDTA tube) and clot activator tubes were transferred immediately to the Laboratory (for samples from London Royal Hospital and BCE) and within a few hours for samples from Guys and St Thomas' Hospital.

2.3.1.1 Separation of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) and plasma were isolated from blood samples via density gradient centrifugation using Ficoll-Paque™ Plus according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). The EDTA blood

was diluted 1:1 with Dulbecco's Phosphate Buffered Saline (PBS-Sigma-Aldrich Company Ltd, Dorset, UK) and layered on to FICOLL-PAQUE™ PLUS and centrifuged at room temperature for 35 minutes at 1700 rpm with brake off (in order to prevent disturbance of the gradient). The PBMCs appeared as a “fluffy” interface between the top layer (diluted plasma) and the lower layer (Ficoll). Red cells and Neutrophils sedimented through the Ficoll. The diluted plasma (1:2) was pipetted off into sterile containers, labelled and frozen at -80°C until further use.

Following two washing steps with PBS at 1200 rpm for 10 minutes with the brake on, the PBMCs were resuspended in RPMI 1640 containing 2mM vol/vol L-glutamine, 200 units/ml Penicillin and 200 units/ml Streptomycin (Lonza, Slough, UK). Viable cells were enumerated using the Trypan Blue exclusion method according to the manufacturer's protocol (Sigma Aldrich, UK). The cells were resuspended in 0.4% Trypan Blue and counted under low magnification using a haemocytometer. The cells were either resuspended at 25×10^6 cells /ml and frozen in 10% dimethyl sulfoxide (DMSO-Sigma Aldrich, UK) in 90% v/v foetal calf serum (FCS: PAA Biologics) or immediately used in different assays.

2.3.1.2 Serum

Serum samples were separated from clot activator tubes by centrifuging at room temperature for 6 min at 3300 rpm, the supernatant was collected, aliquoted and stored at -80°C until further use.

2.3.1.3 Isolation of Neutrophils

After isolation of PBMCs and plasma using Ficoll density-gradient centrifugation, the remaining ficoll was removed as much as possible from the red cell pellet by pipete.

The pellet was resuspended in red blood cell (RBC) lysis buffer [8.26 g ammonium chloride (NH₄Cl), 1 g potassium bicarbonate (KHCO₃) and 0.037 g EDTA in 1 litre double distilled water (ddH₂O), filter-sterilized] and incubated on ice for 10 minutes and then centrifuged at 500× g for 10 minutes. After two washing steps with 2% FCS in PBS at 1200 rpm for 10 minutes with the brake on (in order to pellet the cells), the cells were counted and resuspended at required numbers in appropriate lysis buffer and stored at -80°C until further use.

2.3.2 Saliva Samples

Saliva was collected in 20ml universal tubes and centrifuged at 3500× g, 15minutes, at 4 °C. The supernatant was aliquoted and frozen at -80 °C.

2.3.3 Buccal mucosal brush biopsies (BMBB)

The brush biopsies were taken from both ulcerated and non-ulcerated sites of oral mucosa of BD patients, RAS and HC. This procedure was carried out using 10 strokes of a nylon bristle cytology brush (Flow gene, UK) with 360° rotation from each side of buccal oral mucosa followed by dipping up and down in a 1.5ml eppendorf tube containing 500µl RLT lysis buffer (QIAGEN Ltd, Manchester, UK) (Fedele, 2009). The samples stored in -80C until subsequent RNA isolation was carried out.

2.4 Methods

2.4.1 Western blot Analysis

Western blotting was carried out to evaluate protein expression levels of SOCS1 and 3 in the four studied groups: HC, RAS, BD quiet and BD relapsed. Proteins from

Neutrophils and PBMCs were extracted using a modification of the method described by Bussmeyer *et al* 2010. The cells were resuspended at 10^6 /40 μ l in lysis buffer containing 20 mM HEPES [pH 7.9], 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% [vol/vol] Nonidet P-40, 20% [vol/vol] glycerol, 1 mM dithiothreitol, 1mM Na₃VO₄, 50mM NaF, 1mM PMSF, 1 μ g/ml Aprotinin and 20% glycerol (all from Sigma-Aldrich, UK) and incubated on ice for 30 minutes. The NP-40 in this lysis buffer enables extraction of cytoplasmic, membrane-bound, or whole cell extracts. To obtain higher extraction yields, the lysate was disrupted using a Bioruptor sonicator (Diagenode, Cambridge, UK) on a medium intensity setting, pulsed for 10sec ON, 10 sec REST, for 60 seconds in total. This step was followed by centrifugation at 12,000 g for 20 min at 4°C. The supernatant was collected and the protein concentration was quantified using a colorimetric assay, BioRad DC (detergent compatible) Protein assay (Life science; Hertfordshire, UK) according to the manufacturer's instructions. To perform the assay, 5 dilutions of a Bovine Serum Albumin (BSA) standard protein from 0.2-3 mg/ml were made (Sigma Aldrich, UK). 5 μ l of standards and samples were added to a 96 well microtiter plate (Nunc, UK), 25 μ l reagent A and 200 μ l reagents B, respectively were added. After 15 minutes incubation at room temperature, the intensity of the colour was measured at 750nm on a spectrophotometric plate reader (Fluo Star OPTIMA; BMG Labtech, Germany). A standard curve was generated using a four parameter logistic (4-PL) curve-fit (Microsoft Office Excel) for each set of samples assayed. The protein concentrations of the samples under investigation were calculated using the standard curve.

For Western blotting purpose, 15 μ g of Lysate was mixed with Nu PAGE 4 times concentrated (4x) sample buffer and 10 times concentrated (10x) reducing agent (Invitrogen; Life Science, Paisley, UK). The samples were denatured by boiling for 5

min at 95°C and separated on NuPAGE® Novex® 12% Bis-Tris Gels (1.0-mm thick, 12-well) (Invitrogen, UK) at 120V for 90 minutes and transferred to a Polyvinylidene fluoride (PVDF) membrane (Immobilon; Sigma-Aldrich, UK) at 200 mA for 1 hour at 4°C. The membrane was blocked in a blocking buffer containing 5% wt/vol dried milk in 0.05% vol/vol Tween 20 in Tris-Buffered Saline (9 g Sodium chloride, 100 ml 1M Tris Base PH 7.5, diluted to 1000 ml with distilled water: TTBS) (Sigma-Aldrich, UK) for 1 hour at room temperature. The membranes were then incubated overnight at 4°C in the presence of the following primary antibodies:

0.5µg/ml Monoclonal mouse Antihuman SOCS-3 antibody (R&D Systems; Abingdon, UK), 1µg/ml Rabbit polyclonal Anti-SOCS1 antibody (Abcam3691, Cambridge, UK), specific for amino acids 199-211 of human SOCS 1, 1µg/ml Rabbit polyclonal Anti-SOCS2 antibody (Abcam 92847, UK) specific for the C-terminus of SOCS2 protein from Human and 0.5µg/ml was used. To assure equal sample loading mouse monoclonal anti GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) antibody (Abcam9484,UK) was chosen as loading control based on literature review and previous experiments in our department (Seoudi *et al.*, 2013, Payvandi *et al.*, 2004, Li *et al.*, 2003). The membrane was washed three times for 10 minutes with TTBS, then probed with the secondary antibody at 1:1000 dilution of 10µg/ml stock solution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody or goat anti-Rabbit IgG antibody (Thermo Scientific, Northumberland, UK).

Following another 3, 10 minute washing steps, the protein bands were detected using a chemiluminescence system (Amersham ECL, GE Healthcare) according to the manufacturer's instructions. Therefore, the membranes were incubated in ECL

Detection Reagents for 4 minutes to produce chemiluminescent signals corresponding to the protein bands and then were developed on a radiographic film.

To compare relative protein expression between samples, the pixel densities of each band were quantified by densitometry using a scanner (Epson Stylus SX100, UK) and Image J software (<http://rsbweb.nih.gov/ij/index.html>). To normalize the data, each target band was compared to the loading control and fold changes were calculated by dividing the value of the target proteins to the value of loading control x100 and their results divided by the value of positive control to compare samples in different gels.

2.4.2 The Enzyme-linked immunosorbent spot (ELISPOT) assay

In order to enumerate Cytokine (IL-17, IL-6 and IL-23)-secreting cells in isolated PBMCs from HC, RAS, BD quiet and BD relapsed, ELISPOT analysis was performed.

The following cytokine ELISPOT assay kits: Human Interleukin-23 (MABTECH, Sweden), ELISPOT Human IL-17 and IL-6 (R&D system; Abingdon, UK) were used according to the manufacturer's instructions

To perform the assays first the 96-well PVDF membrane ELISPOT plates (Millipore, Watford, UK) were treated with 15µl /well of 35% ethanol for 1min and washed twice with PBS. Using PVDF membrane treated with ethanol, resulted in a more efficient binding of capture antibodies and having a greater number and better quality of spots. The plate was then coated with 100 µl /well capture antibody diluted in filtered PBS and incubated overnight at 4°C. After removal of coating antibody and washing 3x with 200ul/well PBS, the membrane was blocked with 200 µl /well complete Medium (RPMI-1640, 10%FCS, 1% Penicillin/Streptomycin/L-Glutamine) for 1 hour at 37° C. PBMCs were diluted in culture medium containing an appropriate stimulus: for

antigenic stimulus 10^6 cells/ml stimulated with $20\mu\text{g/ml}$ Human HSP70_{HEK} 293(Human embryonic kidney) (supplied by Dr Britta Eis-Vespa, Hanover, Germany) and for polyclonal stimulus 5×10^5 cells/ml stimulated with 50ng/ml LPS(Lipopolysaccharide) and $10\mu\text{g/ml}$ phytohaemagglutinin (PHA)(Sigma Aldrich, UK). Following aspiration of the blocking buffer, the cells were brought into wells in triplicate at $100\mu\text{l/well}$. Unstimulated cells acted as a negative control and sterile culture media as a background control. Following incubation for 24 at 37°C , $5\% \text{CO}_2$, they were emptied, washed with 0.05% Tween20 (Sigma Aldrich, UK) in PBS and probed with Biotinylated monoclonal antibody overnight at 4°C . To detect the spots two methods of spot development were compared: ELISPOT Blue colour Module (R&D SYSTEMS, UK) and the AEC (3-Amino-9-ethylcarbazole) coloured substrate system. In the ELISPOT Blue colour Module, the plates were incubated with Streptavidin-AP for 2 hours at room temperature. After incubation, the wells were washed 3 times with 0.05% Tween20 in PBS and stained with $100\mu\text{l/well}$ BCIP/NBT solution. Spots were visible after 20-30 minutes incubation in the dark at room temperature.

In the AEC substrate system, the wells were incubated with Streptavidin-HRP (Sigma – Aldrich) for 1hour at 37°C . After washing, a freshly prepared AEC substrate solution [1 tablet of AEC in 2.5 ml of dimethylformamide (DMF) plus 47.5 ml of 50 mM acetate buffer (pH 5.0), 25 ml of fresh $30\% \text{(v/v)}$ hydrogen peroxide] was added to develop the spots. The wells were rinsed with tap water to stop colour development and dried overnight.

Spots were counted by use of an immunospot image analyser, Wallac Victor 1420 multilabel counter (PerkinElmer, Cambridge, UK). All determinations were made in triplicate. The mean values calculated and the results presented as the number of cytokine secreting cells in 10^6 PBMCs.

2.4.3 Human Phospho-Kinase Array

Expression of phosphorylated proteins in PBMCs was assessed by using the Proteome Profiler Human Phospho-Kinase Array kit (R&D Systems, Abingdon, United Kingdom) according to the manufacturer's instructions. This pilot experiment was carried out on PBMCs from 3HC, 3 Active-minor-RAS, 3 BD-Quiet and 3 BD-Relapsed blood samples were separated using Ficoll density-gradient centrifugation. Isolated cells were resuspended in Lysis buffer 6, included in the kit at 1×10^7 cells/mL and gently rocked for 30 minutes at 4°C. Then the cell suspension was centrifuged at 14,000 x g for 5 minutes and the supernatant collected. The protein concentration of the supernatant was quantified using BioRad DC Protein assay (Life science, UK) according to the manufacturer's instructions. All the samples were kept at -80 °C until further use.

Each Human Phospho-Kinase Array kit contains eight nitrocellulose membranes in four pairs which capture and control antibodies of 46 kinase phosphorylation sites printed in duplicate on the membranes. Part A membranes contain 28 antibodies and part B 18 antibodies. All the buffers and reagents were included in the kit. To perform the assay the nitrocellulose membranes were incubated in blocking buffer for 1 hour at room temperature. Samples were loaded, at 1mg/ml protein concentration, onto the membranes and incubated over night at 4°C. Following washing steps, the membranes was incubated first in specific detection buffers for 2 hours and then incubated in diluted Streptavidin-HRP for 30 minutes at room temperature on a rocking platform with 3 washing steps in between. Finally chemiluminescent detection reagents were applied to produce images following development on x-ray films, which detect signals in each capture spot corresponding to the amount of phosphorylated protein sites.

Pixel densities of each spot were quantified by densitometry using scanner (Epson Stylus SX100, UK) and Image J software (<http://rsbweb.nih.gov/ij/index.html>). All the signal values were exported to a spread sheet file in Microsoft Excel. The average signal (pixel density) of the pair of duplicate spots for each phosphorylated kinase protein were calculated and subtracted from averaged background signal. To compare between different membranes, all the values on each membrane normalised to the Reference Spot value $\times 100$ according to the manufacturer's instructions.

2.4.4 mRNA expression analyses

The quality and quantity of the mRNA was evaluated throughout the study.

2.4.4.1 Total RNA purification and quantification

In order to investigate the mRNA expression of SOCS1-3 in PBMCs, Neutrophils and BMBB of BD patients in compare to HC and RAS, total RNA was extracted and reverse-transcribed to cDNA before proceeding to quantitative real time polymerase chain reaction (qPCR). Total RNAs were isolated from the cells using RNeasy MiniKit (QIAGEN, Manchester, UK) according to the manufacturer's instructions. Briefly 5×10^6 cells/ ml were lysed in RLT lysis buffer included in the kit and homogenized by vortexing for 5 minutes. In order to provide appropriate binding conditions, 1 volume of 70% ethanol was added to the cell lysate and applied to the RNeasy Mini spin column. All the contaminants were washed away by three washing steps using buffer RPE and centrifugation steps. Total RNA was eluted by addition of 30-50ul RNase-free water to the RNeasy spin column membrane. The concentration of RNA was determined by measuring the absorbance at 260nm using NanoDrop Nd-1000 Spectrophotometer (Labtech, UK).

Finally to remove any contaminating DNA, all RNA preparations were treated by Ambion® TURBO DNA-free™ DNase kit (Applied Biosystems, Paisley, UK) according to the manufacturer's instructions. The kit has been designed in addition to remove any contaminating DNA from RNA samples, inactivate DNase and remove any divalent cations from the sample. All the buffers and reagents were included in the kit. In a 50 µl Turbo DNase digestion, 5 µl of 10×TUBRO Buffer, 8 µl Nuclease-free H₂O, 1 µl TURBO DNase (2U /10µg RNA) were added to 36 µl of RNA sample and loaded into the Veriti® Thermal Cycler (Applied Biosystems, UK) and run at 37°C for 30minutes. After completing the run, 5 µl of DNase Inactivation Reagent was added to each 50 µl sample and incubated for 5 minutes at room temperature with occasional vortexing. After centrifuging at 10,000×g for 1.5 minutes, the clear supernatant was collected and stored in -80°C until further use.

2.4.4.2 cDNA Generation

Total RNA up to 2µg was reverse transcribed in a reaction volume of 20µl using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK) according to the manufacturer's instructions. 10µl of 2× RT Master Mix contains 10× RT Buffer, 10× random primers, 25×dNTP mix (100Mm), MultiScribe™ Reverse Transcriptase, RNase Inhibitor and Nuclease-free H₂O (all included in the kit) was added to 10µl of the total RNA and loaded into the Veriti® Thermal Cycler (Applied Biosystems, UK) and run at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and cooled down to 4°C. The resulting cDNA was then stored at -20°C until further use.

2.4.4.3 Identification and validation of suitable endogenous reference genes

In order to identify the most suitable reference genes for normalizing data in gene expression studies, geNorm kit (Primer Design, Southampton, UK) was used according to the manufacturer's instructions. geNorm is a fast, established and standardized tool which is precisely designed and validated and has been used widely in many other studies (Etschmann *et al.*, 2006). Six commonly used candidate reference genes (Table 2.2) were chosen and the mRNA expression level of these genes were measured on 24

Gene Symbol	Gene Name	Biological Process	Cellular Component
ACTB	actin, beta	involve in cell motility, structure, and integrity	contractile apparatus and one of the two nonmuscle cytoskeletal actins
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	enzyme in catalyzing the sixth step of glycolysis	
UBC	ubiquitin C	Ubiquitination , protein degradation, DNA repair, cell cycle regulation, kinase modification, endocytosis, and regulation of other cell signaling pathways	
B2M	beta-2-microglobulin	antigen processing and presentation of peptide antigen via MHC class I immune response	Golgi membrane extracellular region plasma membrane early endosome membrane MHC class I protein complex
YWHAZ	Homo sapiens phospholipase A2	mediate signal transduction by binding to phosphoserine-containing proteins	
18S rRNA	18S ribosomal RNA	protein synthesis	component of the ribosome

Table 2.2: Annotation of candidate reference genes

PBMC and 24 Neutrophil samples from the four study groups: HC, RAS, BD: Relapsed (RE) and Quiet (Q) by qPCR and the data were analysed using gNorm software (qbasePLUS) which was included in the kit. Using qbasePLUS permits fully automated calculations with easy and fast generation and interpretation of statistical results (Hellemans *et al.*, 2007).

2.4.4.4 Primer Design

Specific cDNA sequences were obtained from the public GenBank sequence database of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>), and primers were designed and ordered using Universal ProbeLibrary Assay Design Centre (Roche Applied Science, <http://www.rocheappliedscience.com/sis/rtpcr/upl/ezhome.html> Table 2.2).

Targets	Sequence		Amplicon length (bp)	T _m *
	Forward	Reverse		
SOCS1	5'- CCCCTGGTTGTTGTAGCAG -3'	5'- GTAGGAGGTGCGAGTTCAGG-3'	62	82
SOCS2	5'- GGAGCTCGGTCAGACAGG -3'	5'-GTTCCCTTCTGGTGCTCTTTT-3'	84	79
SOCS3	5'- AGACTTCGATTCGGGACCA -3'	5'- AACTTGCTGTGGGTGACCA-3'	128	93
B2M	5'- TTCTGGCCTGGAGGCTATC -3'	5'-TCAGGAAATTTGACTTTCCATTC-3'	86	81
GAPDH	5'-AGCCACATCGCTCAGACAC-3'	5'- GCCCAATACGACCAAATCC -3'	66	82
ACTB	5'- CCAACCGCGAGAAGATGA-3'	5'- CCAGAGGCGTACAGGGATAG-3'	97	83
UBC	5'-AGGCAAAGATCCAAGATAAGGA-3'	5'-GGACCAAGTGCAGAGTGGAC -3'	132	85

Table 2.2: The gene sequences of primers used in RT-PCR.

*The specific amplicon melting temperature (T_m) was determined by direct observation in its melting curve

2.4.4.5 Reverse transcription polymerase chain reaction (RT-PCR)

In order to perform amplification and detection of our target cDNA, real-time PCRs were performed in the LightCycler 480 system (Roche Applied Science, West Sussex,

UK) using LightCycler 480 Sybr Green I Master mix (Roche Applied Science, UK) which contains Fast Start Taq DNA Polymerase and DNA double-strand-specific SYBR Green I dye for product detection and characterization. In 10 μ l reaction, 2 μ l of cDNA was added to 5 μ l of 2 \times Sybr Green I Master mix, 1 μ l of 5 μ M F/R primer and 3 μ l of dH₂O and was run in a PCR amplification starting with denaturation step (95°C for 5min) followed by 45 cycles of amplification step (melting: 95°C for 10 seconds (s), primer annealing: 60°C for 6s and extension: 72°C for 6s) and finally melting analysis consists of melting (95°C for 30s), cooling/annealing (65°C for 30s), gradual heating with continuous acquisition (65°C to 99°C) and cooling /termination (40 °C for 5s). Results were analysed with LightCycler® 480 Software, Version 1.5 (Roche Applied Science, UK) using Advanced Relative Quantification method. In this method, quantification was carried out using standard curves and the mean target gene expression values from duplicate samples were normalized by dividing them by the mean values obtained for house-keeping genes.

2.4.4.6 Standard curve generation

Performing a standard curve is necessary for absolute quantification of each gene. The mRNA gene copy number of an unknown sample was calculated based on the standard curve of that gene. Therefore, to prepare the standard curve for each of our genes one of the PBMC samples was selected and incubated with 10 μ g/ml phycohaemagglutinin (PHA) for 3 days and then stimulated with human IFN γ for 1 hour. RNA was extracted and converted to cDNA (as described in 2.7.2, 3). A 50 μ l normal PCR reaction contains 5 μ l of cDNA, 25 μ l of 2 \times Sybr Green I Master mix, 7 μ l of 5 μ M F/R primer and 15 μ l of dH₂O, was performed using LightCycler 480 Sybr Green I.

The PCR product purified using MinElute PCR Purification Kit (QIAGEN, Manchester, UK) according to the manufacturer's instructions. The kit has been designed to perform a fast clean-up of up to 10 µg of DNA samples from enzymatic reactions which may interfere with subsequent downstream applications. 5 volumes (250µl) of Buffer PB were added to 1 volume (50 µl) of the PCR sample and mixed. To bind DNA, the sample was applied to the QIA-quick column and centrifuged for 1 minute at 13,000 rpm. Buffer PB allows the efficient binding of single or double-stranded PCR products as small as 100 bp and the quantitative (99.5%) removal of primers up to 40 nucleotides to the silica membrane of QIAquick column. Any impurities such as salts and enzymes were washed away by adding 0.75 ml of ethanol-containing Buffer PE to the QIA-quick column and centrifuged for 1 minute. An additional centrifugation for 1 min was carried out to remove any residual Buffer PE. To elute DNA, 20 µl Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of the membrane and the column was centrifuged for 1 minute. The concentration of DNA was determined by measuring the absorbance at 260nm using NanoDrop Nd-1000 Spectrophotometer.

To generate a standard curve, the total number of DNA copies in the remaining 18 µl (2 µl used for NanoDrop) was calculated based on the Avogadro's Constant: 1mole= 6.02×10^{23} molecules and serial dilutions (one to five) of the standard from 10^7 to 10^3 were made. The threshold cycle (CT values) were used to calculate and plot a linear regression line by plotting the logarithm of template concentration (X-axis) against the corresponding threshold cycle (Y-axis) using LightCycler® 480 quantification software, Version 1.5 (Roche Applied Science, UK).

2.4.5 Cytokine secretion assays

2.4.5.1 PBMC cell culture

PBMCs from HC, BD relapsed (BD-R) and quiet (BD-Q) samples were separated using Ficoll density-gradient centrifugation (1700 rpm for 35min) as described in section 2.3.1.1. After washing with PBS, isolated cells were resuspended in completed medium: RPMI 1640 (Lonza, UK) supplemented with 1% Penicillin/Streptomycin solution (P/S) and 10% Fetal Calf Serum (FCS), as previously described and incubated at a density of 2×10^5 /well. The cells were stimulated with HSP70 (10 $\mu\text{g/ml}$), HSP27(200 $\mu\text{g/ml}$; Enzo Life Sciences, UK) and 10 $\mu\text{g/ml}$ phycohaemagglutinin (PHA: Sigma-Aldrich UK) incubated for 24 and 48 hours at 37°C, 5% CO₂ and 100% humidity. Following incubation the non-adherent cells were collected and centrifuged at 800g for 10 minutes. The supernatant was collected and frozen for further cytokine assays.

2.4.5.2 Multiplex cytokine quantification assay

The levels of Th1/Th2 cytokines plus IL-17A in serum, saliva and cell culture supernatants from stimulated PBMCs were quantified in a multiplex bead array assay (MBAA) using Ready-to-Use FlowCytomix Multiplex (eBioscience, Bender MedSystems[®], Ireland UK) according to the manufacturer's instructions.

Test procedure was performed using FACS tubes (Beckton-Dickenson, UK). Two series of standard curves in 1:7 serial dilutions from 0-10,000 pg/ml for IL-17A and IL-8 and 0-20,000 pg/ml for the remaining cytokines were generated. To perform the assay first, 25 μl of Standard Mixture dilutions 1 to 7, samples and assay buffer (as a negative control) were added to designated tubes. Then 25 μl of Bead Mixture and 50 μl of Biotin-Conjugate Mixture contain all cytokines in equal volumes were added to all test tubes including blanks and incubated in the dark at room temperature for 2 hours on a

shaker at 500 rpm. Biotin conjugated secondary antibody mixture bound to the analytes captured by the first antibodies coated by the beads. The tubes were washed 3 times with 1 ml of Assay Buffer and the beads were pelleted at 200 x g for 5 minutes. 50 µl of Streptavidin- Phycoerythrin (PE) Solution was added to all tubes and incubated at room temperature for 2 hours while protected from light with an aluminium foil. After another 3 washing steps, 500 µl assay buffer was added to each tube and samples were analysed on a flow cytometer.

To collect data the beads were run on a FACS Calibur flow cytometer (BD FACS Canto II analyser). Further quantification and analysis of data from flow cytometer were performed using the FlowCytomix Pro 2.4 software (eBioscience, Bender MedSystems, UK). The minimum detectable concentrations using this kit was 1.6 pg/ml for IFN- γ , 4.2 pg/ml for IL-1 β , 16.4 pg/ml for IL-2, 20.8 pg/ml for IL-4, 1.6 pg/ml for IL-5, 1.6 pg/ml for IL-6, 1.5 pg/ml for IL-9, 1.2 pg/ml for IL-10, 1.9 pg/ml for IL-12 p70, 1.5 pg/ml for IL-13, 4.5 pg/ml for IL-17A, , 2.5 pg/ml for IL-22, and 43.3 pg/ml for TNF- α .

2.4.6 Phospho-specific flow cytometry

Isolated PBMCs from HCs (n=7), BD-Q (n=8) and BD-RE (n=8) were investigated to measure the phosphorylation status of Signal transducer and activator of transcription proteins, STAT1, STAT2, STAT3, STAT5 and the transcription factors Tbet and ROR γ t in subpopulations of PBMCs (CD4 Cells) following activation using a Phospho-specific flow cytometry method (BD™ PhosphFlow, BD Biosciences). This method enables the simultaneous analysis of multiple intracellular phosphoprotein and cell surface markers in discrete subpopulations of cells. This was a pilot study on which a power calculation was not carried out.

To perform the assay, the PBMCs were isolated using Ficol density gradient, resuspended at 10×10^6 cells/ml in PBS. The assay was performed in 2 stages: first

stimulation-fixation and second permeabilization. In the first stage, 500µl (5×10^6 cells) of cell suspension in a FACS tube were stimulated with 1.8×10^8 units/mg Interferon- α (IFN- α) (PeproTech, London, UK) for 15min at 37°C using water bath. An unstimulated control sample of cells was set up in parallel. After stimulation, the cells were fixed immediately at equal volumes of 4% paraformaldehyde solution and incubated at 37°C for 10 minutes. Following incubation, fixed cells immediately were transferred to nunc cryovial tube and frozen at -80°C until further use.

Intra cellular staining reagents	
Antibody	Supplier
Anti-Human/Mouse T-bet PerCPCy5.5	BD Biosciences (UK, Oxford)
Alexa Fluor® 647 Mouse IgG1 κ Isotype Control	BD Biosciences (UK, Oxford)
PerCP-Cy TM 5.5 Mouse IgG2a κ Isotype Control	BD Biosciences (UK, Oxford)
Alexa Fluor® 647 Mouse IgG2a, κ Isotype Control	BD Biosciences (UK, Oxford)
PerCP-Cy TM 5.5 Mouse anti-Stat3 (pY705)	BD Biosciences (UK, Oxford)
Alexa Fluor® 647 Mouse Anti-Stat5 (pY694)	BD Biosciences (UK, Oxford)
Alexa Fluor® 647 Mouse Anti-Stat1 (pY701)	BD Biosciences (UK, Oxford)
Pacific Blue TM Mouse Anti-Human CD3	BD Biosciences (UK, Oxford)
FITC-AntiCD4	Biolegend (London, UK)
PerCP/Cy5.5 Mouse IgG1 κ Isotype Control	Biolegend (London, UK)

Table 2.3: Antibodies used for PhosphoFlow experiments

In the second stage, the fixed cells were thawed at 37°C in a water bath, and immediately washed with FACS buffer (2% Fetal calf serum, 1 mM EDTA, 0.1% sodium azide in PBS) and centrifuged at 400g for 10 minutes. The supernatant was

removed and 50 μ l of residual volume was left. After disrupting the pellet by vortexing, the cells were permeabilized by adding 1ml permeabilization buffer (70% methanol pre chilled in -200C) vortexed again and incubated for 30minutes on ice. Then cells were washed 3 times by adding 3ml FACS buffer and centrifuging at 600g for 6 minutes. At the final wash, the cells were resuspended in FACS buffer at 10×10^6 cells /ml. To stain the cells, 100 μ l of cell suspension was added to all tubes with appropriate volume of antibody combinations (Table 2.3 and 2.4).

Fluorochrome	FITC	PerCP-Cy	Alexa Fluor 647	Pacific Blue
Group1	Anti-CD4 10 μ l	IgG1 Isotype Control 3 μ l	IgG2a Isotype Control 5 μ l	Anti-CD3 5 μ l
Group2	Anti-iCD4 10 μ l	Anti-tbet 3 μ l	Anti-Stat1 5 μ l	Anti-CD3 5 μ l
Group3	Anti-CD4 10 μ l	IgG2a Isotype Control 5 μ l	IgG1 Isotype Control 3 μ l	Anti-iCD3 5 μ l
Group4	Anti-CD4 10 μ l	Anti-Stat3 5 μ l	Anti-STAT5 5 μ l	Anti-CD3 5 μ l

Table 2.4: Combination of BD PhosphoFlow antibodies.

First 1 full drop (approximately 60 μ l) of the BD™ CompBeads Negative Control (FBS) that have no binding capacity, and 1 drop of the BD™ CompBeads Anti-Mouse Ig, κ beads which binds light chain-bearing immunoglobulin as a positive control to each tube and vortex. These controls provided distinct positive and negative

(background fluorescence) stained populations to optimize fluorescence compensation settings for multicolour flow cytometric analyses.

In order to confirm the specificity of primary antibody binding and to rule out non-specific Fc receptor binding or other cellular protein interactions, isotype control antibodies in parallel with target antibodies were used. After adding the appropriate antibodies, the mixture was incubated for 60 minutes while protected from the light. Following another washing step, cells were resuspended in 500µl of FACS buffer prior to flow cytometric analysis (BD FACS Canto II analyser Flow cytometry).

2.5 Statistical analysis

GraphPad StatMate 2 software was used to evaluate sample size and power. (GraphPad Software, Inc. USA). The Statistical analysis was performed using GraphPad Prism 5 GraphPad Software, Inc. USA). Analyses of variance (ANOVA), non-parametric Mann–Whitney U-test and Kruskal-Wallis test were used. Significant was set at $P < 0.05$.

CHAPTER 3

SOCS EXPRESSION

Chapter 3: SOCS Expression

In order to identify the factors that may cause dysregulation of cytokines in BD, PBMC and neutrophils were subjected to analysis of SOCS1-3 mRNA and protein expression using RT-PCR and western blot respectively. The mRNA expression of SOCS1 and 3 in buccal mucosal brush biopsies (BMBB) were also investigated.

3.1 mRNA expressions of SOCS1-3 in BD

3.1.1 Identification and validation of suitable endogenous reference genes

In order to verify the most suitable reference genes for normalizing data in gene expression studies, 6 possible candidates: ACTB, GAPDH, UBC, B2M, YWHAZ and 18S rRNA were selected for examination following a literature review (Zhang *et al.*, 2005, Stamova *et al.*, 2009).

The expression level of these genes was measured in 24 PBMC and 24 Neutrophil samples from 4 study groups: HC, RAS, and BD: Relapsed (RE) and Quiet (Q) by qPCR and the data were analysed using qbasePLUS software (Primer Design, Southampton, UK). The results showed the average expression stability value (M) of reference genes at each step during stepwise exclusion of the least stably expressed reference (Appendix 7). The least stable gene is located at the left and in this respect they were ranked based on increasing expression stability; therefore the most stable genes are seen on the right. The gNorm analysis showed that B2M, BACT and UBC in neutrophils and B2M, BACT and GAPDH in PBMCs are the most stably expressed genes for normalization in our study on Behçet's disease. For buccal mucosal cells GAPDH was determined to be the most appropriate based on previous experiments in our department (Seoudi *et al.*, 2013).

3.1.2 mRNA expressions of SOCS1-3 in PBMCs & Neutrophils

The mRNA expressions of SOCS1-3 were quantified in 54 PBMCs and neutrophil samples including BD-All (n=28), BD-Q(n=18), BD-RE(n=10), HC (n=16), and RAS (n=10). The demographic and clinical features of the study groups are presented in Table 3.1. Of the 28 investigated BD patients, 7 were male and 21 were female.

Demographic and Clinical Features of the Investigated Patients(n=28)		
Mean Age \pm SD, years	37.27 \pm 11.8(19-61)	
Gender	n	%
Male	7	25
Female	21	75
Clinical Features		
Oral Ulcers	16	57.1
Genital Ulcers	4	14.3
Eye Lesions	1	3.6
Skin Lesions	9	32.1
Joint involvement	12	42.9
Encephalitis	2	7.9
Vascular involvement	2	7.9
CNS	3	10.7
Relapse d	10	35.7
Treatment		
Prednisolone	5	17.9
Azathioprine	8	28.6
CellCept	1	3.6
Colchicine	9	32.1
	Healthy controls(n=16)	RAS(n=10)
Mean Age \pm SD, years	37.27 \pm 11.8(19-61)	43.5 \pm 14.6(30-61)
Male	8(50%)	3(30%)
Female	8(50%)	7(70%)

Table 3.1: Demographic and Clinical characteristics of gene expression studied groups.

The most frequent clinical symptoms were oral ulcers (57.1%), joint involvement (42.9%), and skin lesions (32.1%). From the disease activity view point, 10 patients (35.7%) were clinically diagnosed as fully relapsed (i.e. activity in 3 or more systems). Treatment information of 16 patients was available (Table 3.1) and 3 patients were not on any immunosuppressant medication at the time of sample collection. The data on relative mRNA expression from BD patients were compared with HC and RAS and also with disease activity in BD: quiet and relapsed, using ANOVA and the Mann–Whitney U-test

3.1.3 SOCS1 is upregulated in PBMCs from BD patients

The mRNA expression levels of SOCS1 in PBMCs and neutrophils were shown to exhibit a different pattern in BD patients compared to HC. As can be seen from Figure 3.1, SOCS1 expression has increased in both PBMC and neutrophils in BD patients compared to healthy controls. This difference was not significant in neutrophils (ANOVA: $P=0.9353$; Figure 3.1a) despite the mean level in HC being lower than that of BD-Q and the combined values of all BD (HC= 0.4272 compared with BD-Q = 0.6127 and All BD = 0.1709). However, PBMC displayed a significant difference between study groups (ANOVA: $P=0.0274$; Figure 3.1b). The SOCS1 level in BD patients was higher than both HC and RAS, $p=0.0149$ and $p=0.0325$ respectively, in PBMC. Moreover, both the quiet and relapsed subgroups showed significantly higher levels of SOCS1 at $p=0.0343$ and $p=0.0427$ respectively (Figure 3.1b). No significant differences were found between quiet and relapsed patients. Comparison of RAS with both BD-RE and BD-Q patients just failed to reach significance and was probably a reflection of the small number of RAS samples available ($n=3$; $p=0.0528$ and 0.0669 respectively).

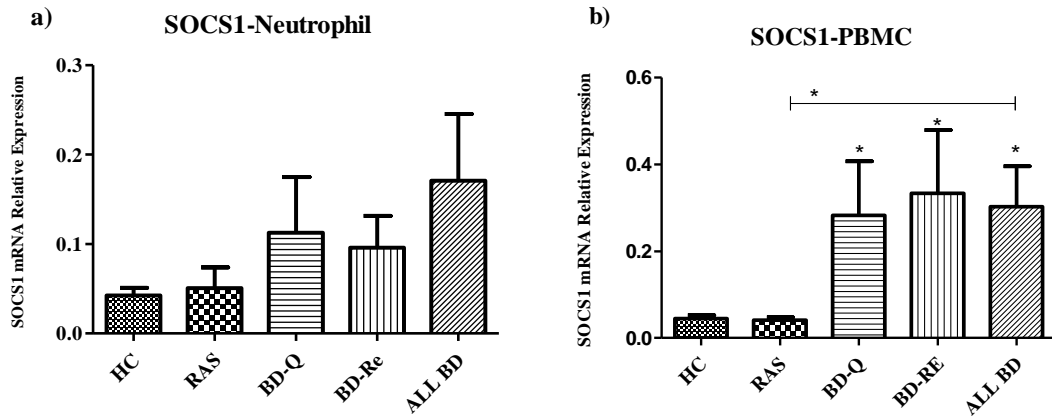


Figure 3.1: SOCS1 expressions in PBMCs and neutrophils. mRNA expressions of SOCS1 were quantified by RT-PCR and the results were normalized against the expression levels of B2M, BACT and GAPDH. The mean mRNA relative expressions of SOCS1 were compared between HC, RAS, and BD patients with relapsed or quiet BD using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM. * $P < 0.05$

3.1.4 SOCS 3 is highly expressed in the Neutrophils of quiet BD

The expression of SOCS 3 mRNA showed significant upregulation in both PBMCs and Neutrophils. As can be seen, both display marked differences in SOCS3 expression (Figure 3.2a; ANOVA: $P = 0.0024$ and Figure 3.2b; $P = 0.0031$). The most striking result to emerge from this graph is the very high level of expression in BD-Q. From the scale of the y axis it can be observed that there is about a 10 fold difference in expression in Neutrophils compared to PBMCs. There is significant upregulation of both PBMC and neutrophil SOCS3 when all BD patients are grouped together and compared to HC at $p = 0.0007$ and $p = 0.0015$ respectively. In addition, both PBMC and neutrophils demonstrated increased expression of SOCS3 in RAS in comparison with HC at $p = 0.0307$ $p = 0.0149$. There was no significant difference within BD subgroups, relapsed and quiet. From the data in Figure 3.2a, it is apparent that in neutrophils both quiet and relapsed patients display marked higher expression of SOCS3 compared to HCs at $p = 0.0008$ and $p = 0.0439$ respectively (Figure 3.2a). Similarly, the same rise was seen in

PBMCs at $p=0.0066$ and $p=0.0055$ respectively (Figure 3.2b). However unlike SOCS1, the ratio of quiet to relapsed SOCS3 level in neutrophils and PBMCs is reversed. While in neutrophils, the mean levels of SOCS3 (1.385 ± 0.1914) in quiet patients were higher than relapsed (0.4099 ± 0.0422), in PBMCs the mean level of relapsed (0.04053 ± 0.02377) is higher than quiet patients (0.0263 ± 0.009).

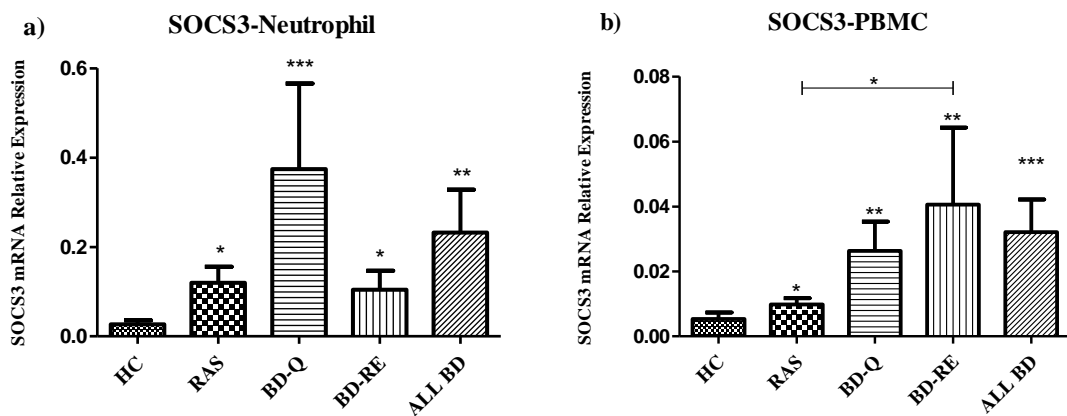


Figure 3.2: SOCS3 expressions in PBMCs and neutrophils. mRNA expressions of SOCS3 were quantified by RT-PCR and the results were normalized against the expression levels of B2M, BACT and GAPDH. The mean mRNA relative expressions of SOCS3 were compared between HC, RAS, and BD patients with relapsed or quiet BD using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

3.1.5 SOCS2 is upregulated in the PBMCs of relapsed BD

SOCS2 expression showed significant differences between HC and the BD subgroups in Neutrophils (Figure 3.3a; ANOVA $P=0.946$). Similarly, as can be seen from Figure 3.3b, BD relapsed patients display a higher mean level of SOCS2 mRNA expression in PBMCs than HC, but this just fails to reach significance (Mann-Whitney; $p=0.0587$), despite an unpaired t test giving a significant difference of $p=0.0166$ and F test for variance of <0.0001 . There was an interesting difference between RAS and BD-RE

($p=0.0133$). The mean levels of SOCS expression in BD-Q and RE were somewhat different with BD-RE having the higher levels but the comparison did not reach significance ($p= 0.0616$). Since SOCS2 is thought to have a somewhat different function than SOCS 1 and 3 this may be reflected in these data.

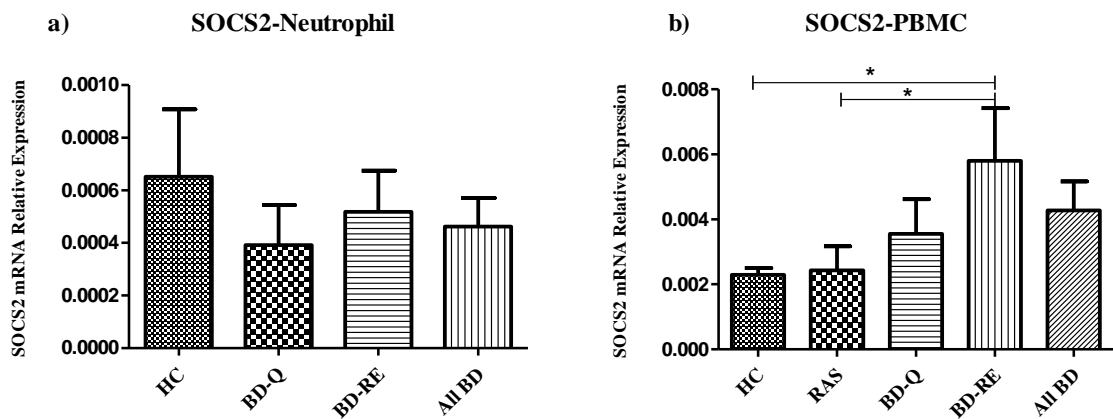


Figure 3.3: SOCS2 expressions in PBMCs and neutrophils. mRNA expressions of SOCS2 were quantified by RT-PCR and the results were normalized against the expression levels of B2M, BACT and GAPDH. The mean mRNA relative expressions of SOCS2 were compared between HC, RAS, and BD patients with relapsed or quiet BD using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM. * $p<0.05$

3.2 Western blot analysis of SOCS3 and SOCS1 in Neutrophils and PBMC

In order to investigate post transcriptional regulation of SOCS, the protein expression of SOCS1 and 3 was analysed in PBMC and neutrophil samples in BD ($n=22$) and HC ($n=11$ PBMCs, $n=7$ Neutrophils) using the western blot method. The demographic and clinical features of investigated BD patients are presented in Table 3.2.

Of the 22 investigated BD patients, 8 were male and 14 were female. The most frequent

Demographic and Clinical Features of the Investigated Patients (n=22)		
Mean Age \pm SD	40.68 \pm 15.3	
Gender	n	%
Male	8	36.4
Female	14	63.6
Clinical Features		
Oral Ulcers	9	40.9
Genital Ulcers	1	4.5
Eye Lesions	2	9.1
Skin Lesions	5	22.7
Joint involvement	17	77.3
Encephalitis		
Vascular involvement	1	4.5
Gastrointestinal	1	4.5
Relapsed	4	18.2
Treatment		
Prednisolone	7	31.8
Azathioprine	9	40.9
Colchicine	6	27.3

Table 3.2: Demographic and Clinical characteristics of investigated patient in protein expression experiments.

clinical symptoms were joint involvement (77.3%), oral ulcers (40.9%), CNS (27.3%) and skin lesions with 22.7%. 4 patients (18.2%) were clinically diagnosed as relapsed. The data on SOCS1 and SOCS3 relative pixel density of BD patients were compared with those of HCs and also with disease activity: quiet and relapsed using ANOVA and Mann–Whitney U-test (Figures 3.4 and 3.5). SOCS2 was not abundantly present in all samples since was it difficult to detect by western blot in unstimulated samples. SOCS2 could only be reliably detected following *in vitro* stimulation. Therefore the focus of

the experiments was on SOCS1 and 3. SOCS1 and SOCS3 bands were detected around 30kDa (Figure 3.4c and 3.5c).

SOCS1 and 3 protein expression results in PBMCs were summarized in Figure 3.4. While for SOCS3, strong bands were detected, SOCS1 bands were faint in most of samples (Figure 3.4c). As can be observed from Figure 3.6a, SOCS1 protein expression in PBMC did not display any significant difference between investigated groups (ANOVA $P=0.9631$). However, mean relative pixel density in SOCS1 has shown a

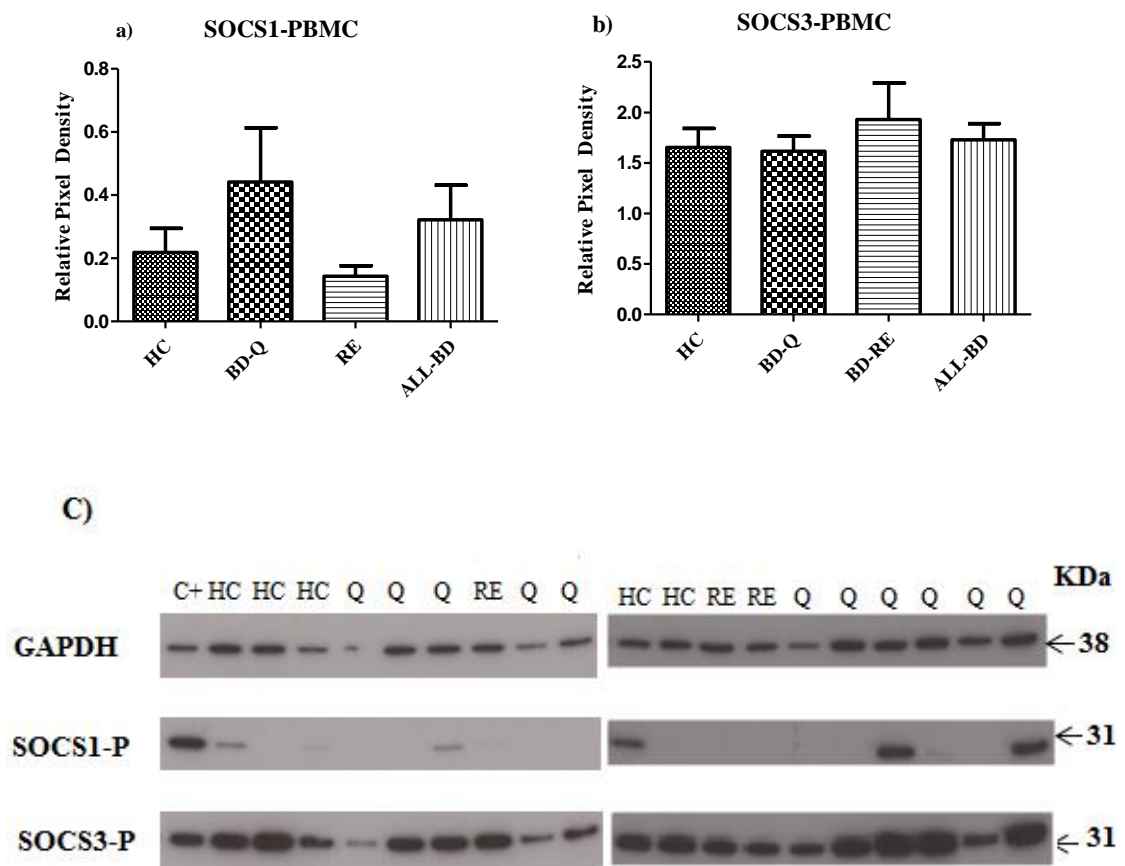


Figure 3.4: SOCS1 and SOCS3 protein expressions in PBMCs. Relative protein expression of SOCS1 and 3 were quantified by western blot. The results were normalized against GAPDH as a loading control and between membranes by a positive control. The mean relative pixel density of SOCS1 and SOCS3 were compared between HC and BD patients with relapsed or quiet BD using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM of HC (n=8), BD-Q (n=9), RE (n=6) for SOCS1 (a) and HC (n=11), BD-Q (n=9), RE (n=5) for SOCS3(c).

100% increase in BD quiet and a 50% reduction in BD-RE when they were compared with HCs but these differences were not significant ($p=0.743$ and $p=0.4278$ respectively). No noticeable difference between investigated groups was seen in SOCS3 protein expression by PBMCs (Figure 3.4b; ANOVA $P=0.9481$).

SOCS1 protein is highest in the Neutrophils of relapsed BD

The protein expression level of SOCS1 in neutrophils was shown to express a markedly different pattern in BD patients compared to HC (ANOVA, $P=0.0308$). As can be seen from Figure 3.5a, SOCS1 protein expression was increased significantly in BD relapsed compared to HCs at ($p=0.0317$) in neutrophils. The most striking observation from the graphs in Figure 3.5 is the significant difference between BD relapsed and quiet of both SOCS1 and SOCS3 but in slightly different ways. While SOCS1 is upregulated in relapsed compared to quiet ($p=0.0338$), SOCS3 has shown a significant reduction in relapsed compared to quiet at $p=0.0393$. There was no difference in SOCS 3 protein expression, between HCs when compared to BD in neutrophils. Overall, the expression of SOCS1 and 3 has increased in BD patient compared to healthy controls both in messenger and protein level; however, there were some differences in the ratio of quiet to relapsed patients even between neutrophils and PBMCs.

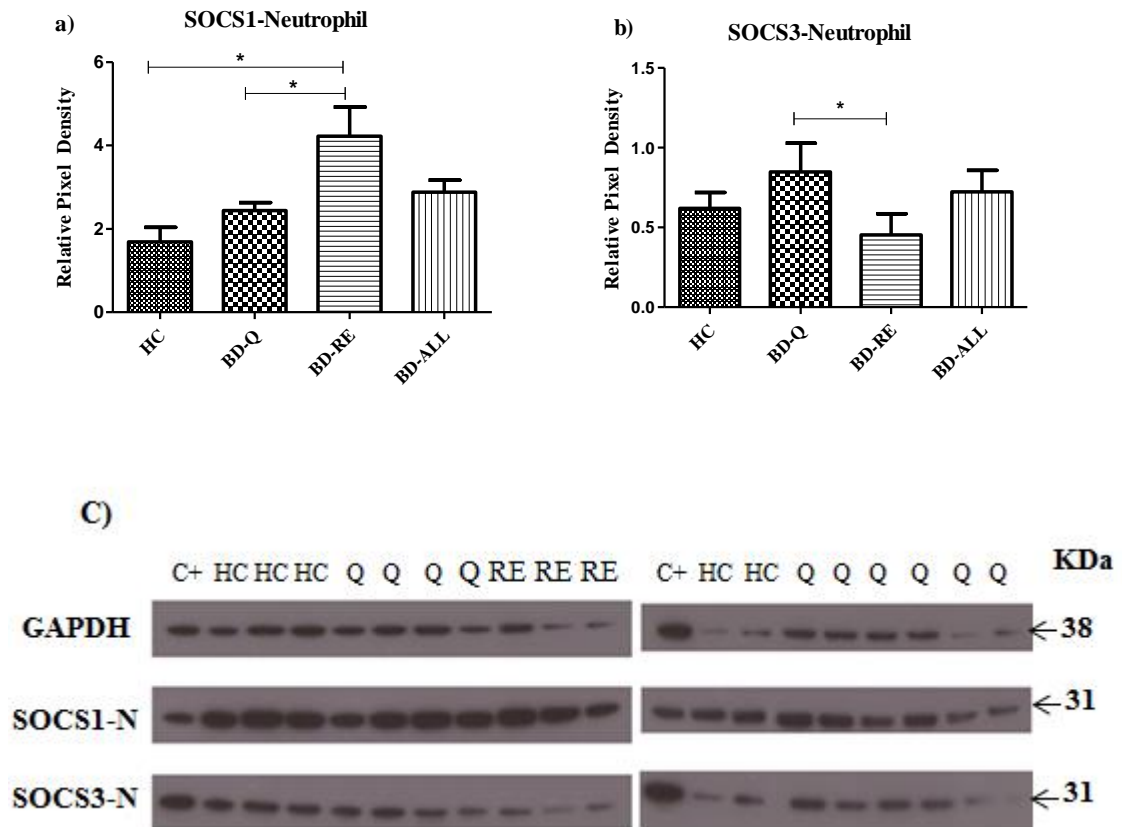


Figure 3.5: SOCS1 and 3 protein expression in neutrophils. Relative protein expression of SOCS1 and 3 were quantified by western blot. The results were normalized against GAPDH as a loading control and between membranes by positive control. The mean relative pixel density of SOCS1 and SOCS3 were compared between HC and BD patients with relapsed or quiet BD using one-way ANOVA and Mann–Whitney U-test (a and b). Bars represent mean values \pm SEM of HC (n=.5), BD-Q (n=12), RE (n=4) for SOCS1 (a) and HC (n=.7), BD-Q (n=13), RE (n=6) for SOCS3(c). * $p < 0.05$

3.3 mRNA expressions of SOCS1-3 in Buccal Mucosal Cells (BMC)

Considering the importance of oral ulcers in Behcet’s disease, SOCS1 and 3 expressions were investigated in buccal mucosal cells (BMC). The samples were taken from ulcer and non-ulcer sites of buccal oral mucosa by brush biopsy (BMBB: Chapter 2 section 2). The mRNA expressions of SOCS1 and 3 were quantified in 34 BMC samples including BD (n=17 pairs of matched ulcer and non-ulcer samples) and HC (n=17). The demographics and clinical features of investigated patients are presented in Table 3.3.

Demographic and Clinical Features of the Investigated Patients(n=17)		
Mean Age \pm SD	35(25-57)	
Gender	n	%
Male	5	33.3
Female	10	66.7
Clinical Features		
Oral Ulcers	17	100
Genital Ulcers	3	18.8
Eye Lesions	1	6.3
Skin Lesions	3	18.8
Joint involvement	12	75
Encephalitis	2	12.5
CNS	5	31.3
Relapsed	7	41.2
Treatment		
Prednisolone	2	12.5
Azathioprine	3	18.8
CELLCEPT	1	6.3
Colchicine	7	41.2

Table 3.3: Demographic and Clinical characteristics of investigated patients with Behcet's disease.

Of the 17 investigated BD patients, 66.7% female and 33.3% were male. All the patients were selected based on having active oral ulcers. Around 41% were diagnosed as fully relapsed. The most frequent clinical symptoms after oral ulcers were joint (75%), and CNS (31.3%) involvements. The treatment information of 15 patients was available and one patient did not have any medication. Half of the patients were prescribed colchicine alone or in combination with prednisolone or azathioprine. The data on relative mRNA expression of ulcerated areas were compared with non-ulcerated

areas and HCs also comparing BD disease activity: quiet and relapsed using ANOVA and Mann–Whitney U-test.

SOCS 1 and 3 are increased in ulcerated mucosa

The mRNA expression levels of SOCS1 and SOCS3 in matched ulcer and non-ulcer sites of BD patients and buccal mucosal cells from healthy controls display very significant differences (Figure 3.6a: ANOVA, $P=0.0001$; Figure 3.6b: ANOVA, $P=0.0001$). As shown in Figure 3.6a, SOCS1 has increased significantly in ulcer sites compared to non-ulcer sites and also compared to non-ulcerated healthy controls ($p=0.0114$ and $p=0.0001$ respectively). Although, the mean expression level of SOCS1 in non-ulcer sites (0.2243 ± 0.08457) was higher than HCs (0.05750 ± 0.01147) it failed to reach significance ($p=0.4282$). Similarly, SOCS3 expression followed the same pattern as SOCS1. As can be seen from Figure 3.6b, SOCS3 has also increased significantly in ulcer sites compared to non-ulcer sites and healthy control samples ($p=0.0004$ and $p=0.0001$ respectively). The mean expression level of SOCS3 in non-ulcer sites (0.08460 ± 0.05601) were higher than HCs (0.0215 ± 0.007025) but similar to SOCS3, it failed to reach significance ($p=0.7305$).

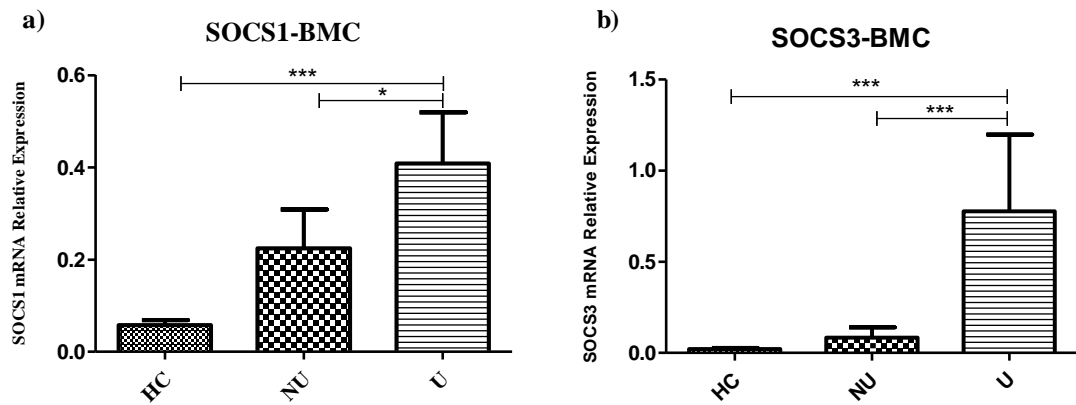


Figure 3.6: SOCS1 and SOCS3 expressions in buccal mucosal cells from brush biopsies. mRNA expressions of SOCS1 and SOCS3 were quantified by RT-PCR and the results were normalized against the expression levels of GAPDH. The mean mRNA relative expressions of SOCS1 and 3 were compared between HC, Ulcers (U) and Non-Ulcer (NU) sites of BD patients using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Further analysis of SOCS1 and 3 expressions in BMC, comparing quiet and relapsed patients is presented in Figure 3.7. As can be seen from Figure 3.7a and 3.7b, as predictable, there are no noticeable differences in both SOCS1 and 3 in non-ulcer sites comparing relapsed with quiet patients ($p=0.4396$, $p=0.4634$ respectively), although the errors are large. There is a significant upregulation of SOCS3 in HCs in comparison to non-ulcer sites of quiet patients (Figure 3.7b: $p=0.042$). Conversely, in ulcer sites, the relapsed patients displays higher relative mean expression of SOCS1 (0.5876 ± 0.2597) than in quiet pa

tients (0.2452 ± 0.506). In the same way, SOCS3 was higher in relapsed patients (1.433 ± 1.004) than in quiet BD (0.36 ± 0.101 ; Figure 3.7d) but both failed to reach significance. However, both relapsed and quiet patients display very significant increase of both SOCS1 and SOCS3 in ulcer sites compared to HCs (Figures 3.7c and 3.7d). In ulcer sites both quiet and relapsed cases show a significant increase of SOCS1 at $p=0.0008$ and

p=0.0063 respectively (Figure3.7C). Similarly, SOCS3 presents the same trend at p=0.0047 and p=0.0063) respectively (Figure3.7d).

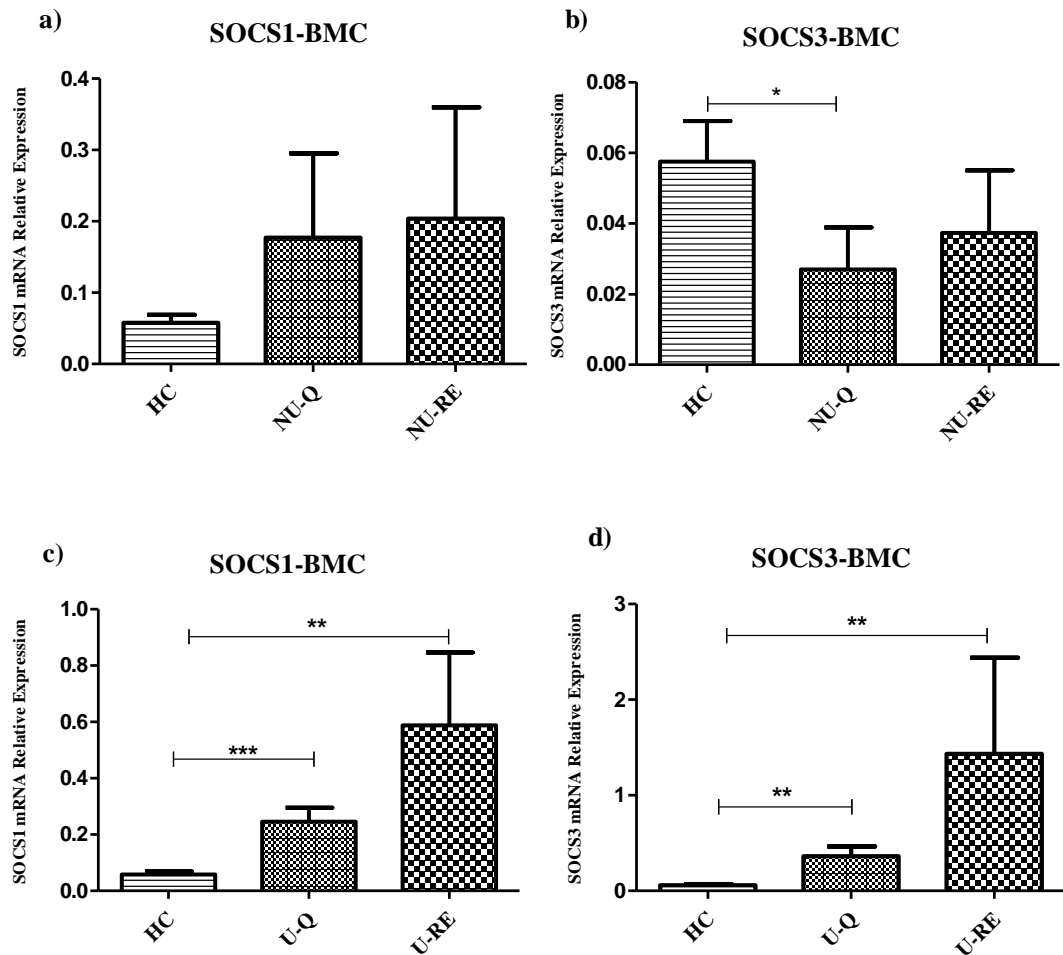


Figure 3.7: SOCS1 and SOCS3 expressions in buccal mucosal cells from brush biopsies. mRNA expressions of SOCS1 and SOCS3 were quantified by RT-PCR and the results were normalized against the expression levels of GAPDH. The mean mRNA relative expressions of SOCS1 and 3 in relapsed patient were compared to quiet ones in non-ulcer sites(a and b) and ulcer sites(c and d) using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

BD patients frequently present with only oral ulceration and in this respect are regarded as not relapsed. However, samples from ulcer sites of these “quiet” patients revealed

interesting differences. Figure 3.8 demonstrates further analysis of SOCS1 and 3, comparing ulcer and non-ulcer sites in which relapsed and quiet patients were distinctly different. As can be seen, there is a significant upregulation of SOCS1 and SOCS3 in ulcer sites of quiet patients compared to their non-ulcer sites at $p=0.0117$ and $p=0.0031$ respectively (Figure 3.8a and 3.8b). Regarding relapsed patients, SOCS3 was also significantly increased in ulcer sites compared to non-ulcer sites at $p=0.177$ (Figure 3.8d). The mean level of relative expression of SOCS1 in ulcer sites (0.5876 ± 0.259) was higher than non-ulcer sites (0.2035 ± 0.156) but it failed to reach significance ($p=0.202$; Figure 3.8C).

The relative expression of both SOCS 1 and SOCS3 is much higher in the relapsed patients compared with the “quiet” patients and there was also a striking difference between ulcer and non-ulcer sites. This might suggest that the local environment is critical for SOCS expression and might further suggest that the expression in the Neutrophils and lymphoid compartment may not reflect activity in the mucosal tissue. Conversely, the activity in the local mucosal tissue might be dependent on the type of cell infiltrating the mucosa and whether these cells arrive with their SOCS proteins up- or down regulated or are dependent on the signals from the local environment to influence their expression of SOCS proteins.

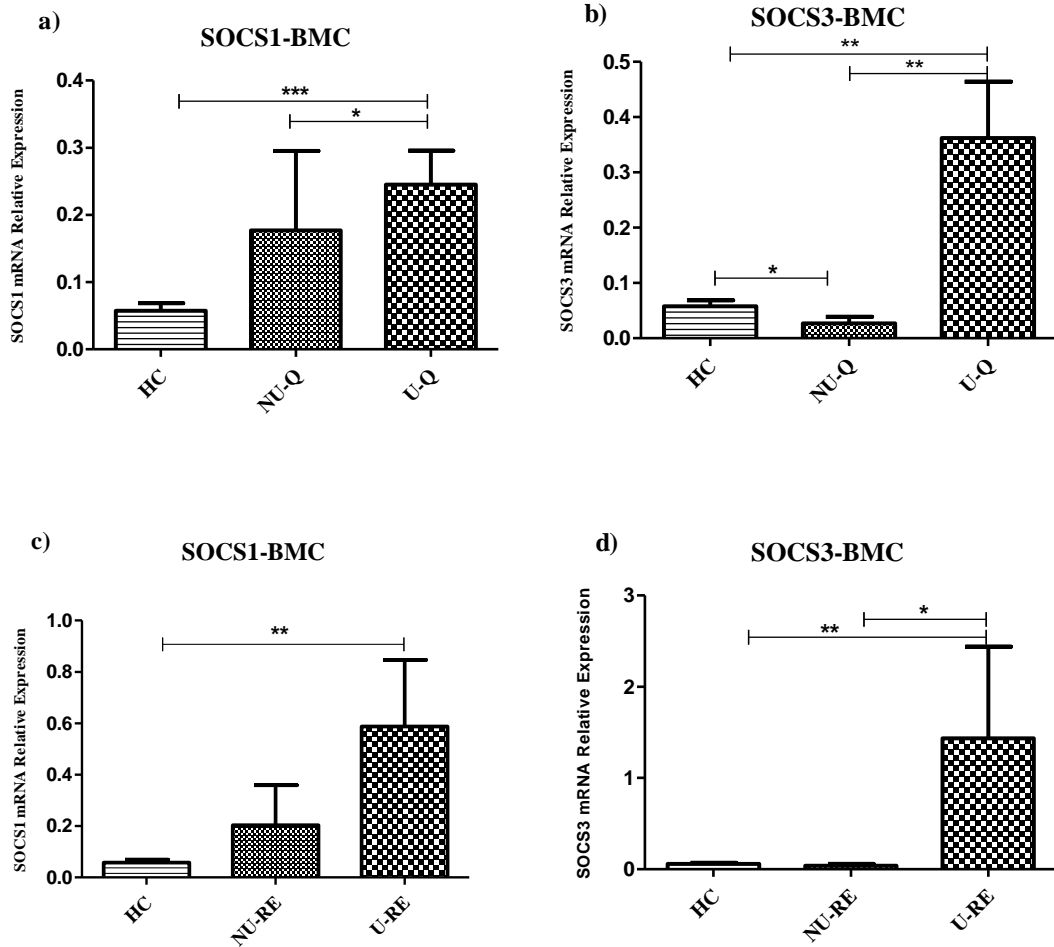


Figure 3.8: SOCS1 and SOCS3 expressions in buccal mucosal cells from brush biopsies. mRNA expressions of SOCS1 and SOCS3 were quantified by RT-PCR and the results were normalized against the expression levels of GAPDH. The mean mRNA relative expressions of SOCS1 and 3 in non-ulcer sites and ulcer sites were compared in quiet (a and b) and relapsed(c and d) patients using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

CHAPTER 4

CYTOKINE

PROFILE IN A UK COHORT OF

BD PATIENTS

Chapter 4: Cytokine profile in a UK cohort of BD patients

4.1 Serum and saliva cytokine profiles in Behçet's disease

Cytokines are thought to contribute to the pathogenesis of BD and their levels in the serum or saliva might provide diagnostic or activity markers for the disease. Therefore the levels of 12 cytokines representative of the Th1, Th2 and the Th17 pathways were quantified in a multiplex bead analysis detection system as described in Chapter 2 section 2. Serum and saliva of patients with BD are compared with healthy controls (HC), recurrent aphthous stomatitis (RAS) and patients with Oro-Genital Ulceration (OGU).

4.1.1 Serum cytokine profile

The 12 cytokines were quantified in 79 serum samples including BD (n=58), HC (n=12), OGU (n=3) and RAS (n=6). Of the 58 investigated BD patients, 15 were male and 43 female. The most frequent clinical symptoms were oral ulcers (40.7%), joint disease (32.2%) and skin lesions (20.3%). From the disease activity viewpoint, 16 patients (27.1%) were considered as fully relapsed. Treatment information of 47 patients was available (Table 4.1). The data on the levels of detectable cytokines from BD patients were compared with HC, OGU, and RAS and also with disease activity in BD (quiet and relapsed) using ANOVA and Mann–Whitney U-test (Figures 4.1 and 4.2).

As can be summarised from Table 4.2, the most abundant cytokines in the serum of BD patients were IL-8 (detected in 56 out of 58 patient sample), TNF- α (39 /58), IL-1 β (23 /58) and IL-4(21/58) respectively. IL-6 was undetectable (only 2 out of 79), also IL-5 and TNF- β was found only in a few samples. The results showed that IL-8, IFN- γ ,

Demographic and Clinical Features of the Investigated Patients (n=58)		
Mean Age \pm SD, years	42.34 \pm 12.6 (22-78)	
Gender	n	%
Male	15	25.4
Female	43	74.6
Clinical Features		
Oral Ulcers	24	40.7
Genital Ulcers	9	15.3
Eye Lesions	5	8.5
Skin Lesions	12	20.3
Joint involvement	19	32.2
Encephalitis	3	5.1
Vascular involvement	3	5.1
Relapsed*	16	27.1
Treatment		
Prednisolone	4	6.8
Cyclosporin A	1	1.7
CellCept	1	1.7
Colchicine	11	18.6
Azathioprine	7	11.9
Aza+Colch	9	15.3
Pred+Colch	3	5.1
Cyc+Col	1	1.7
Aza+Cyc	2	3.4
Pred+Aza	5	8.5
Pre+Aza+Col	2	3.4

Table 4.1: Demographic characteristics, clinical features and immunosuppressive drug treatment of investigated BD patients at the time of serum collection. * Where relapsed is defined as disease activity in at least 3 tissues: See Chapter 2 section 2.

TNF- α and IL-1 β were raised in BD patients compared to HC but the differences were not statistically significant. The results revealed that mean serum level of relapsed cases were higher than quiet cases for IL-12p70 (12.54 vs 7.3pg/ml) and IL-10 (5.7 vs 3.4pg/ml) but lower than quiet for IL-17A (6.1 vs 14.9pg/ml), IL-2 (8.4 vs 22.4pg/ml) and IL-8 (140 vs 1659pg/ml) . However the differences did not reach statistical significance except for IL-8 (P=0.0406) (Figures 4.1 and 4.2). Since many samples gave

undetectable values in the method the data were re-analysed comparing those samples which gave positive results (Table 4.2 and Fig 4.3). However, no significant differences were observed and it was concluded that the very large range of values obtained from both the patients and the controls made comparisons very difficult. This has been observed before by Curnow *et al* (2008).

Cytokine	Range (pg/ml)	Number of positive samples (range pg/ml)			
		BD n=58	RAS n=6	HC n=12	OGU n=3
IL-12p70	0-20,000	7/58 (0-166)	1/6	1/12	0/3
IFN-γ	0-20,000	17/58 (0-234)	2/6	5/12	0/3
IL-17A	0-10,000	15/58 (0-164)	2/6	2/12	0/3
IL-2	0-20,000	8/58 (0-258)	4/6	6/12	1/3
IL-10	0-20,000	16/58 (0-53)	3/6	4/12	1/3
IL-8	0-10,000	56/58 (0-15295)	6/6	12/12	3/3
IL-6	0-20,000	1/58 (0-1.15)	0/6	1/12	0/3
IL-4	0-20,000	21/58 (0-106)	1/6	3/12	1/3
IL-5	0-20,000	1/58 (0-34.6)	1/6	2/12	0/3
IL-1β	0-20,000	23/58 (0-240)	2/6	4/12	2/3
TNF-α	0-20,000	39/58 (0-837)	3/6	9/12	0/3
TNF-β	0-20,000	3/58 (0-862)	6/6	2/12	0/3

Table 4.2: The number of serum samples with detectable cytokine levels measured by the FlowCytomix method.

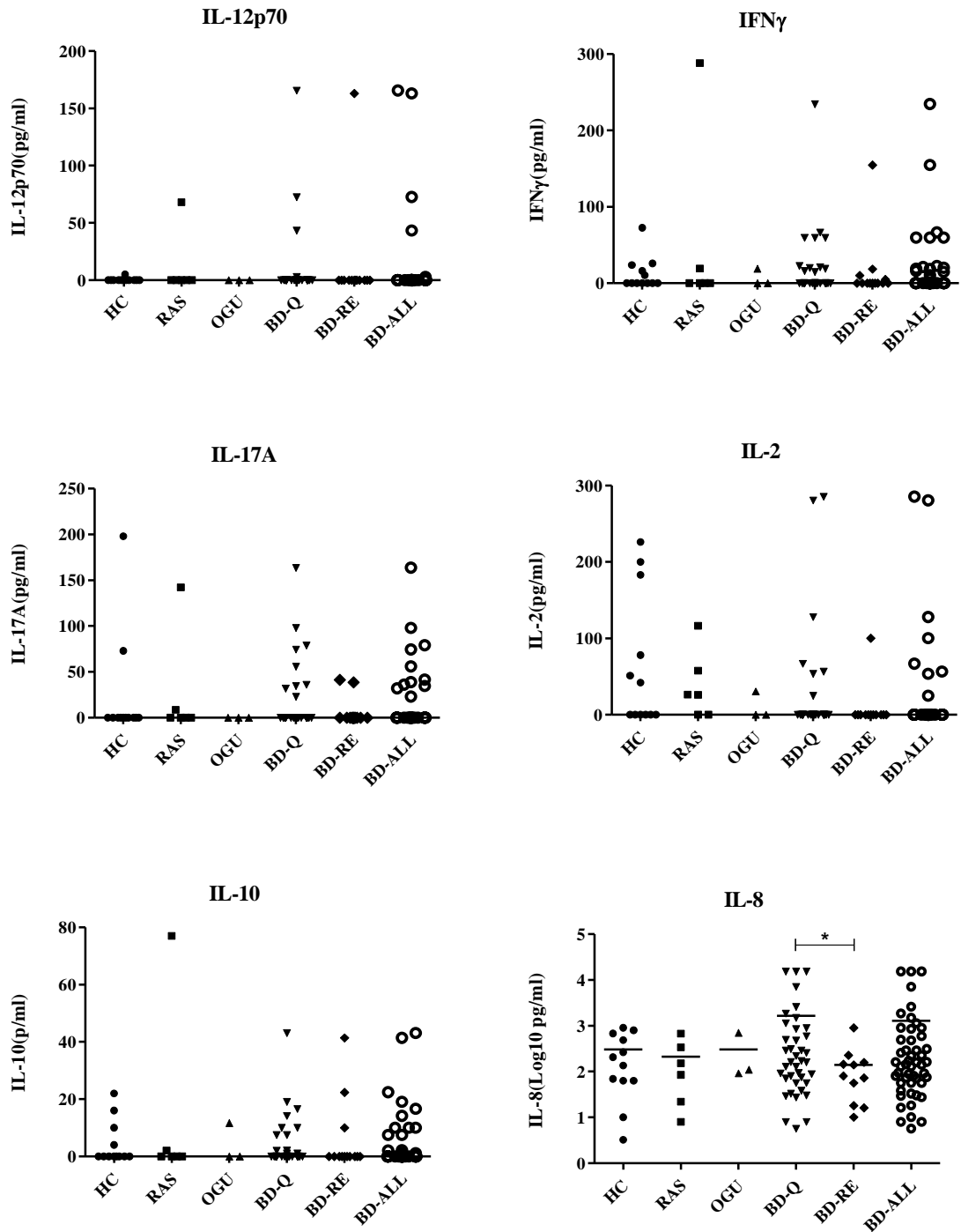


Figure 4.1: Serum levels of IL-12p70, IFN- γ , IL-17A, IL-2, IL-10 and IL-8 were measured by multiplex bead analysis. The mean concentration (pg/ml) of each individual cytokine were compared between HC, RAS, OGU and BD patients with Relapsed or quiet BD using one-way ANOVA and Mann–Whitney U-test. Horizontal lines represent the mean cytokine levels (pg/ml) in each group. There was no significant difference between study groups except for IL-8 BD-Q which is significantly higher than BD-R ($P=0.0406$). * $p<0.05$

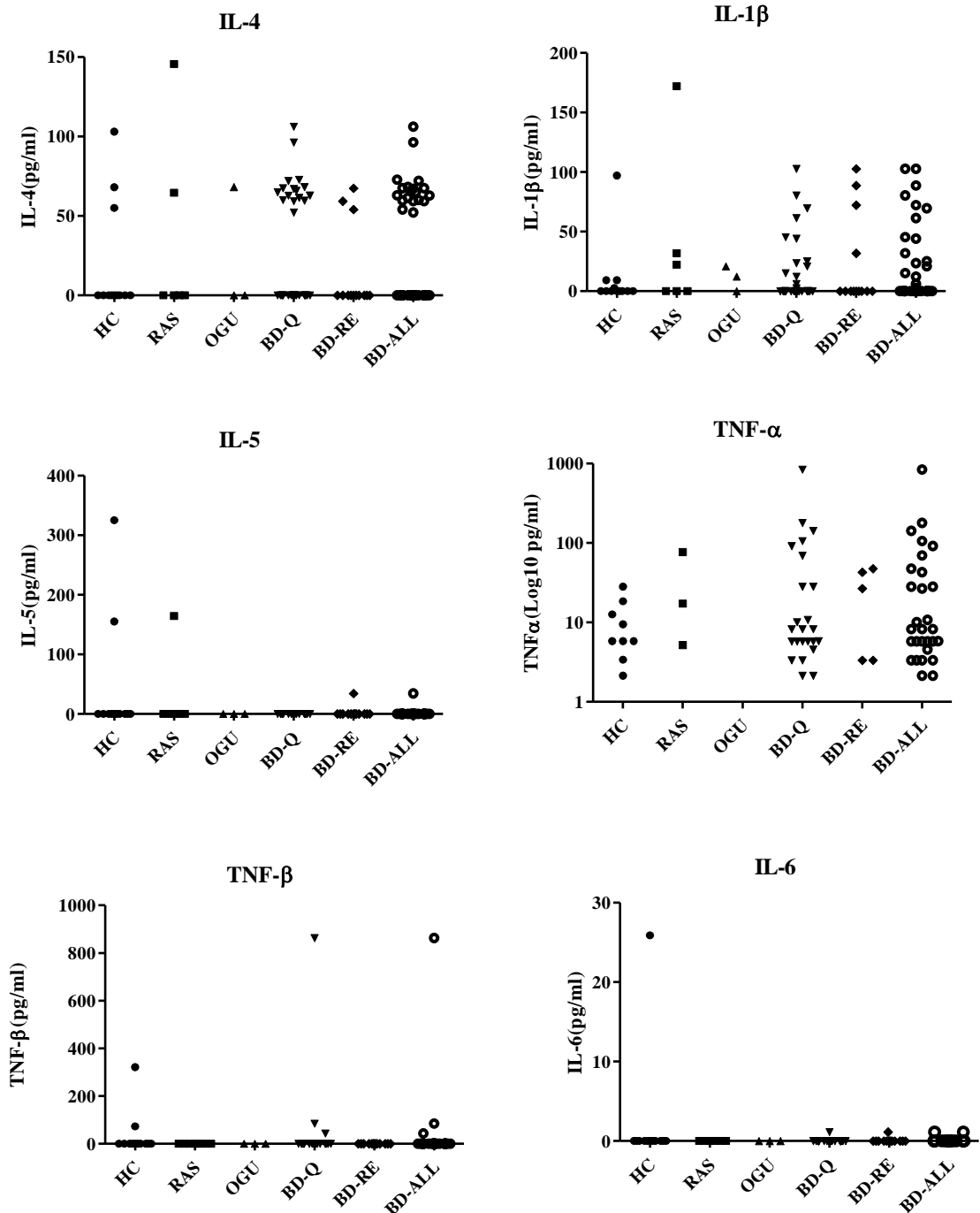


Figure 4.2: Serum cytokine levels (pg/ml) of IL-4, IL- β , IL-5, TNF α , TNF- β and IL-6 were measured by multiplex bead analysis. The mean concentration (pg/ml) of each individual cytokine were compared between HC, RAS, OGU and BD patients with Relapsed or quiet BD using one-way ANOVA and Mann–Whitney U-test. There was no significant difference between study groups.

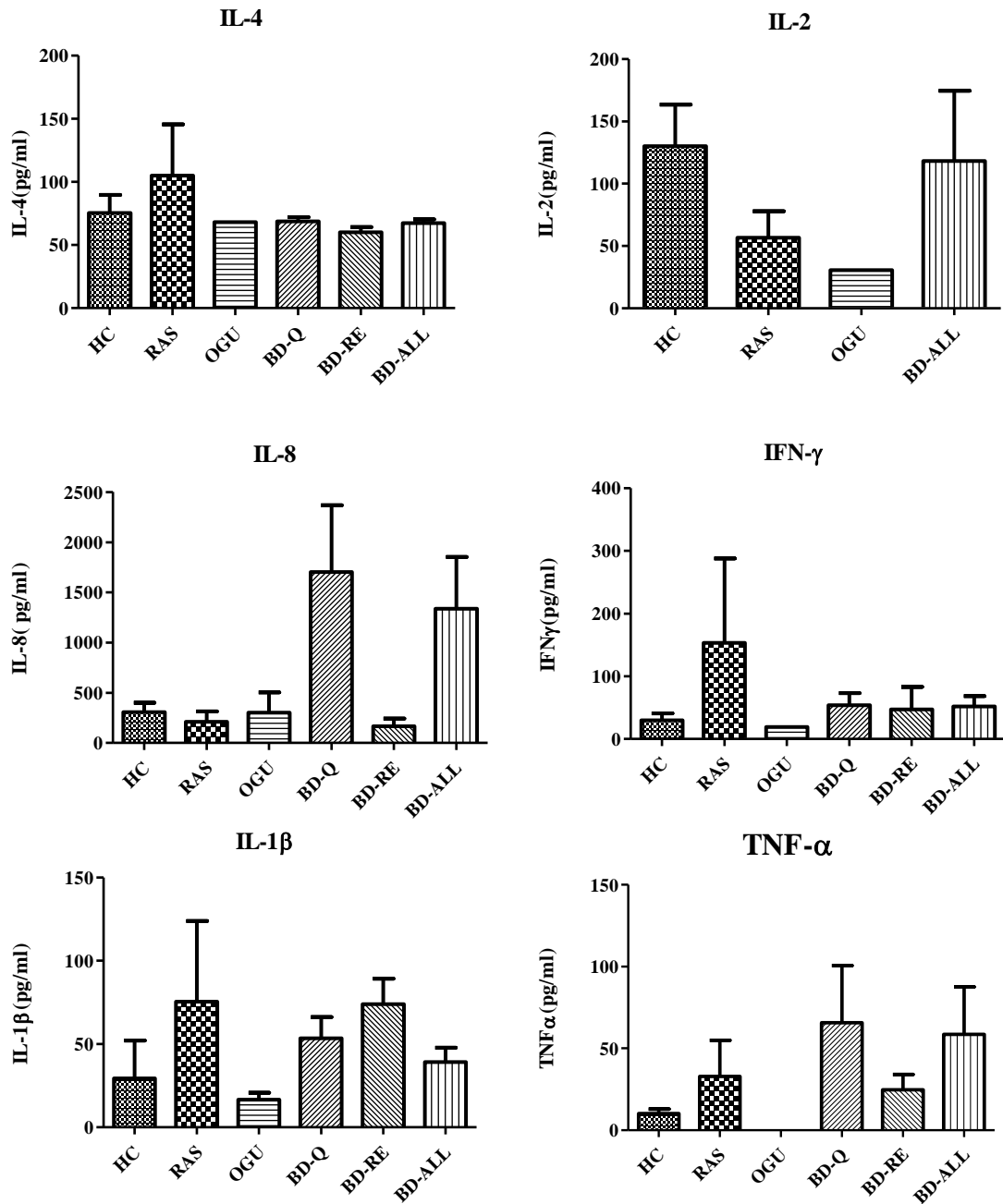


Figure 4.3: Detectable serum levels of IL-4, IL-2, IL-8, IFN- γ IL- β and TNF α , (pg/ml).The mean concentration (pg/ml) of each individual cytokine were compared between HC, RAS, OGU and BD patients with Relapsed or quiet BD using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM.

In order to evaluate whether the immunosuppressive medication course prescribed to BD patients affected their cytokine profile, the serum levels of IL-8 and TNF- α , which were the most abundant cytokines in our investigated samples, were selected and

divided into different groups according to drug treatment: BD patients with only Prednisolone or Colchicine (Pred or Col), BD patients with Prednisolone or Colchicine in combination with other immunosuppressant drugs (Pred+ or Col+) (Figure 4.4). Unfortunately, due to the nature of presentation at our clinics, hardly any patients could be found who were not already taking medication when entering the study. The results showed no significant differences; therefore Prednisolone or Colchicine did not appear to affect IL-8 and TNF- α serum level in these patients.

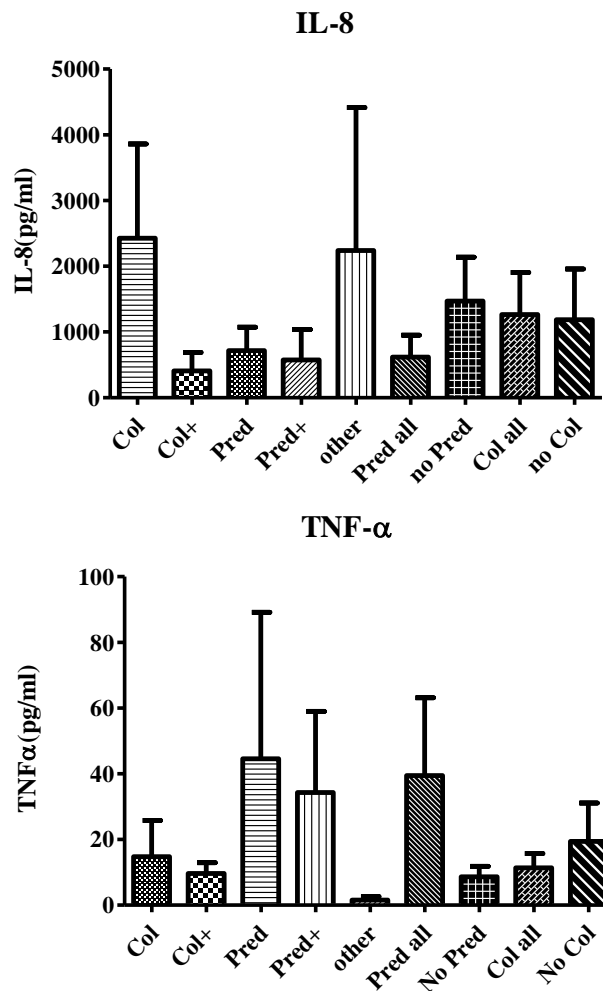


Figure 4.4: Serum levels of IL-8 and TNF- α (pg/ml) in BD patients with prednisolone alone (Pred, n=4) or colchicine alone (Col, n=11) treatment, prednisolone or colchicine plus another

immunosuppressant drug (Pred+ n=9, Col+ n=15) and any other treatments (other, n=7) at the time of sample collection. Bars represent mean values \pm SEM in each study group.

4.1.2 Matched Serum and Saliva cytokine profile

The levels of the 12 cytokines were also quantified in 37 matched serum and saliva samples from HC (n=10), RAS (n=7), BD (n=20) using a multiplex bead analysis detection system (Table 4.3).

Demographic and Clinical Features of the Investigated Patients(n=20)		
Mean Age \pm SD, years	37.30 \pm 9.25 (22-53)	
Gender	N	%
Male	6	30
Female	14	70
Clinical Features		
Oral Ulcers	9	45
Genital Ulcers	4	20
Eye Lesions	3	15
Skin Lesions	3	15
Joint involvement	10	50
Encephalitis	2	10
Vascular involvement	1	5
CNS	2	10
Relapsed	13	65
Treatment		
Prednisolone	7	35
Pred+	3	15
Colchicine	4	20
Azathioprine	3	15
No Treatment	2	10

Table 4.3: Demographic characteristics, clinical features and immunosuppressive drug treatment of investigated BD patients at the time of serum collection.

Of the 20 BD patients investigated 30% were male and 70% were female. The most frequent clinical symptoms were joint involvement (50%) oral ulcers (45%) and genital ulcers (20%) (Table 4.3) 65% of patients were considered as fully relapsed. The mean cytokine levels (pg/ml) from BD patients in either relapsed or quiet phases of disease were compared with HC and RAS using ANOVA and Mann–Whitney U-test. BD groups were further subdivided into those patients with active oral ulcers compared with orally quiet patients.

The most abundant cytokines in the serum of BD patients were IL-8(detected in 17 out of 20 BD samples), IL-2 (11/20), TNF- α (10/20) and IL-10(10/20). Interestingly, cytokines seemed to be more abundant in the saliva, with 17 out of 20 samples containing IL-8, IL-10, IL-1 β and TNF- β .

The results, as shown in Figures 4.5,-4.8 suggest that the salivary proinflammatory cytokine profile is quite different from serum. Very little IL-1 β was detected in HC or RAS serum and only 5 out of 20 BD patients had measurable levels. However, it was interesting to note that the range in serum and saliva were very different. Maximum values obtained in a serum sample from a BD-Q sample was 399.0pg/ml, whereas in saliva the range for all groups was up to 4000.0pg/ml with one HC sample lying outside the axis range (not shown) at >8000.0pg/ml. Similarly IL-6 was not detected in serum (except for one BD-Q patient; Fig 4.5) but was detected in all groups of saliva but at a maximum of 724pg/ml in one BD-relapsed sample. TNF- α and IL-6 were higher in BD patients compared to HCs but only IL-6 reached significant increase in BD-RE compared with HC at $p=0.0283$. Further statistical analysis revealed that IL-6 had increased significantly in patients with oral ulcers compared to HCs regardless of overall disease activity($p=0.0498$). IL-6 levels were also significantly higher in

relapsed BD versus quiet BD ($p=0.0079$). IL-6 was higher in the saliva of patients with ulcers than without ($p=0.0269$). TNF- α levels were higher in BD relapsed and patients with oral ulcers compared with HCs but these just failed to reach significance ($p=0.0586$ and $p=0.0602$ respectively).

Serum and saliva cytokine levels of Th1 cytokines IL-12P70, IFN- γ and IL-2 did not reach any significant differences among the study groups (Figure 4.6). Saliva IFN- γ was detected in only 13 out of 37 samples and was the least abundant cytokine in saliva. From the data in Figure 4.7, it is apparent that Th2 cytokines IL-4 and IL-5 were detected only in very few serum samples and in saliva no significant differences were found between investigated groups.

The results obtained for IL-17A, IL-8 and TNF- β are presented in Figure 4.8. As can be seen, while IL-17A and TNF- β were detected only in a few of BD serum samples, they were quite abundant in BD saliva samples; however, there were no significant differences between investigated groups. IL-8 was plentiful in both serum and saliva nevertheless no changes were observed in comparing healthy controls to BD cases.

Matched Serum & Saliva Cytokine Profile in BD

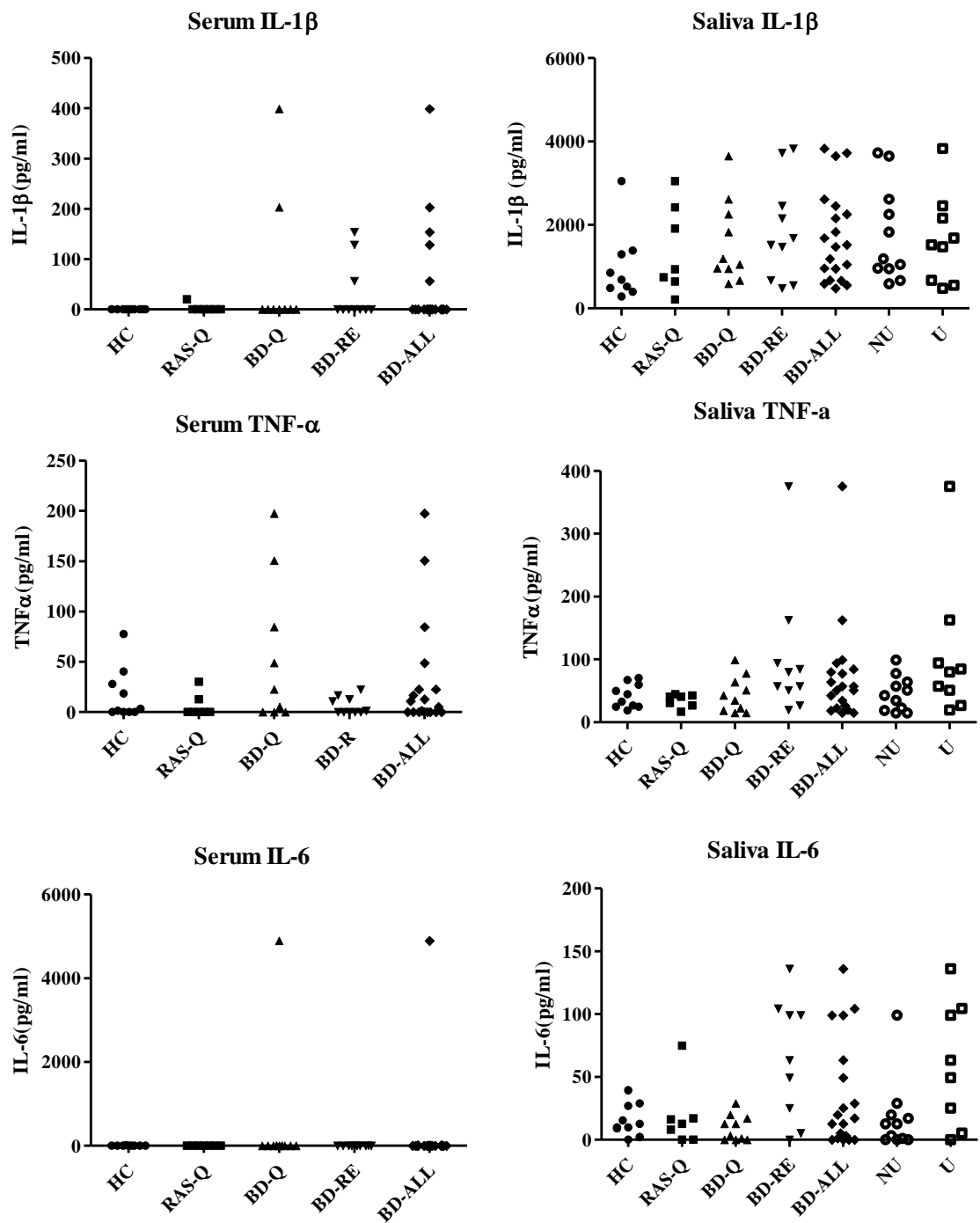


Figure 4.5: Comparison between serum and saliva cytokine levels (pg/ml) of IL- β , TNF α , and IL-6 in investigated groups: HC, RAS, BD patients in relapsed or quiet state and BD with oral ulcer (U) and without oral ulcer (NU) using multiplex bead analysis assay.

Matched Serum & Saliva Cytokine Profile in BD

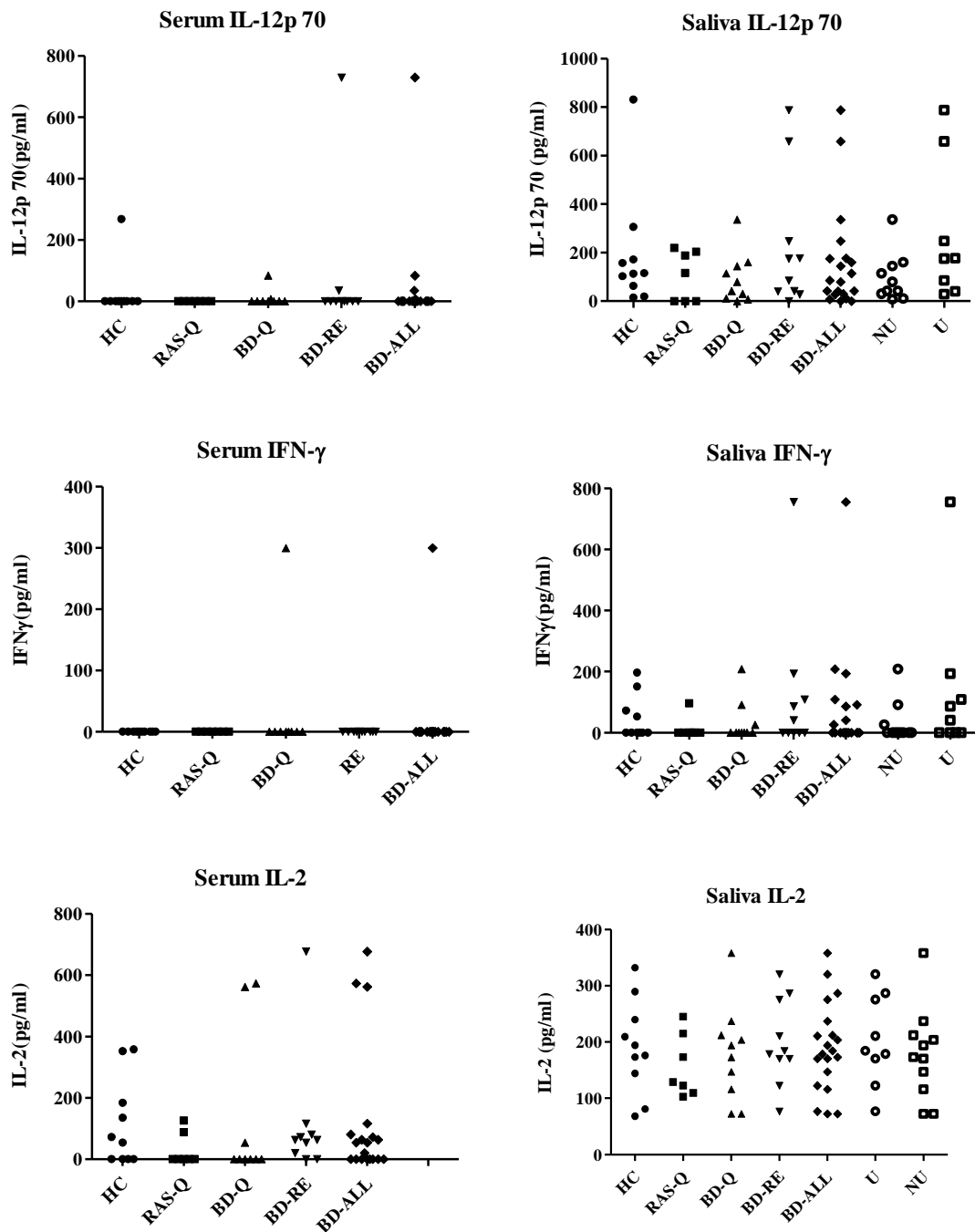


Figure 4.6: Comparison between serum and saliva cytokine levels (pg/ml) of IL-12p70, IFN γ , and IL-2 in investigated groups: HC, RAS, BD patients in relapsed or quiet state and BD with oral ulcer (U) and without oral ulcer (NU) using multiplex bead analysis assay.

Matched Serum & Saliva Cytokine Profile in BD

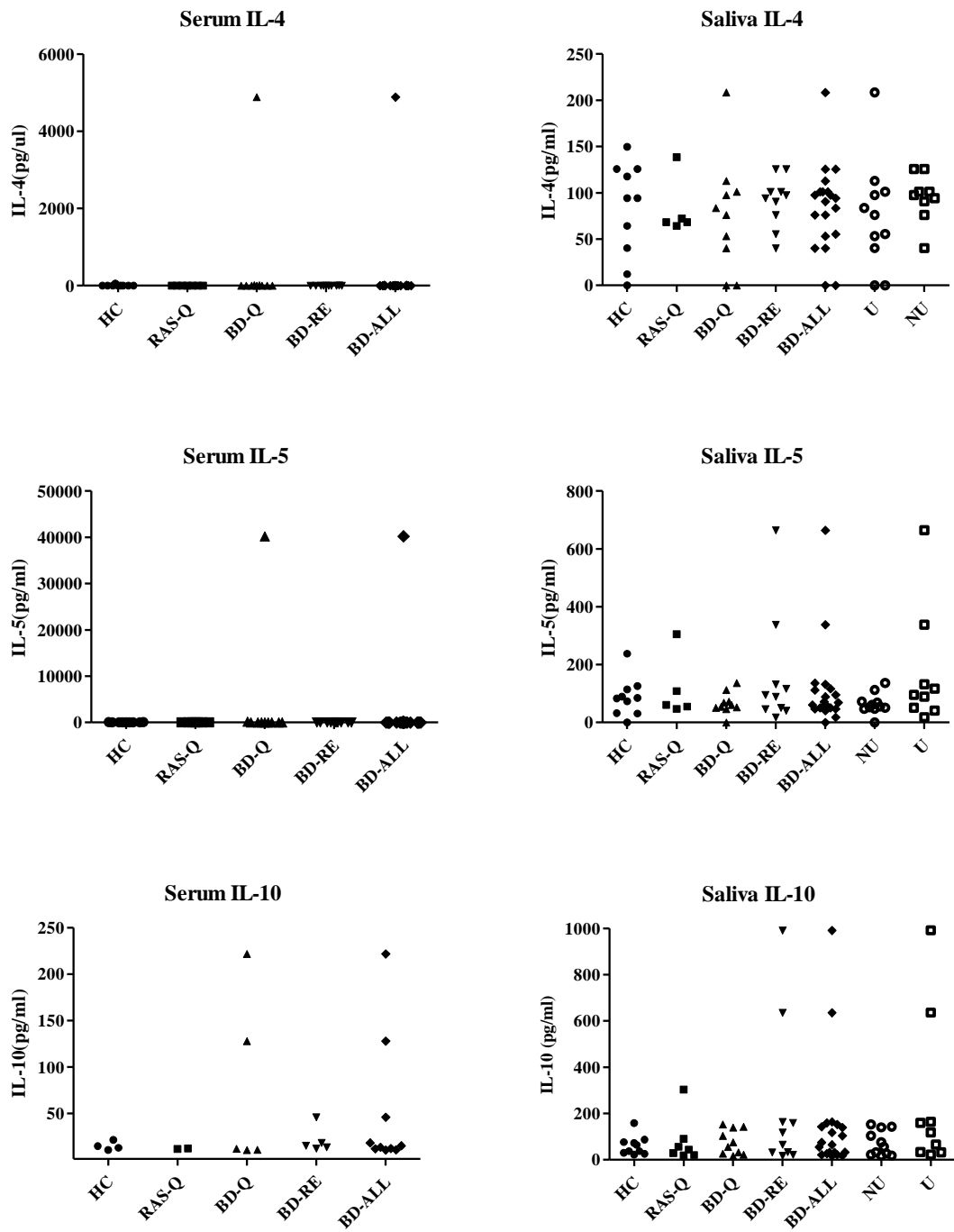


Figure 4.7: Comparison between serum and saliva cytokine levels (pg/ml) of IL-4, IL-5, and IL-10 in investigated groups: HC, RAS, BD patients in relapsed or quiet state and BD with oral ulcer (U) and without oral ulcer (NU) using multiplex bead analysis assay.

Matched Serum & Saliva Cytokine Profile in BD

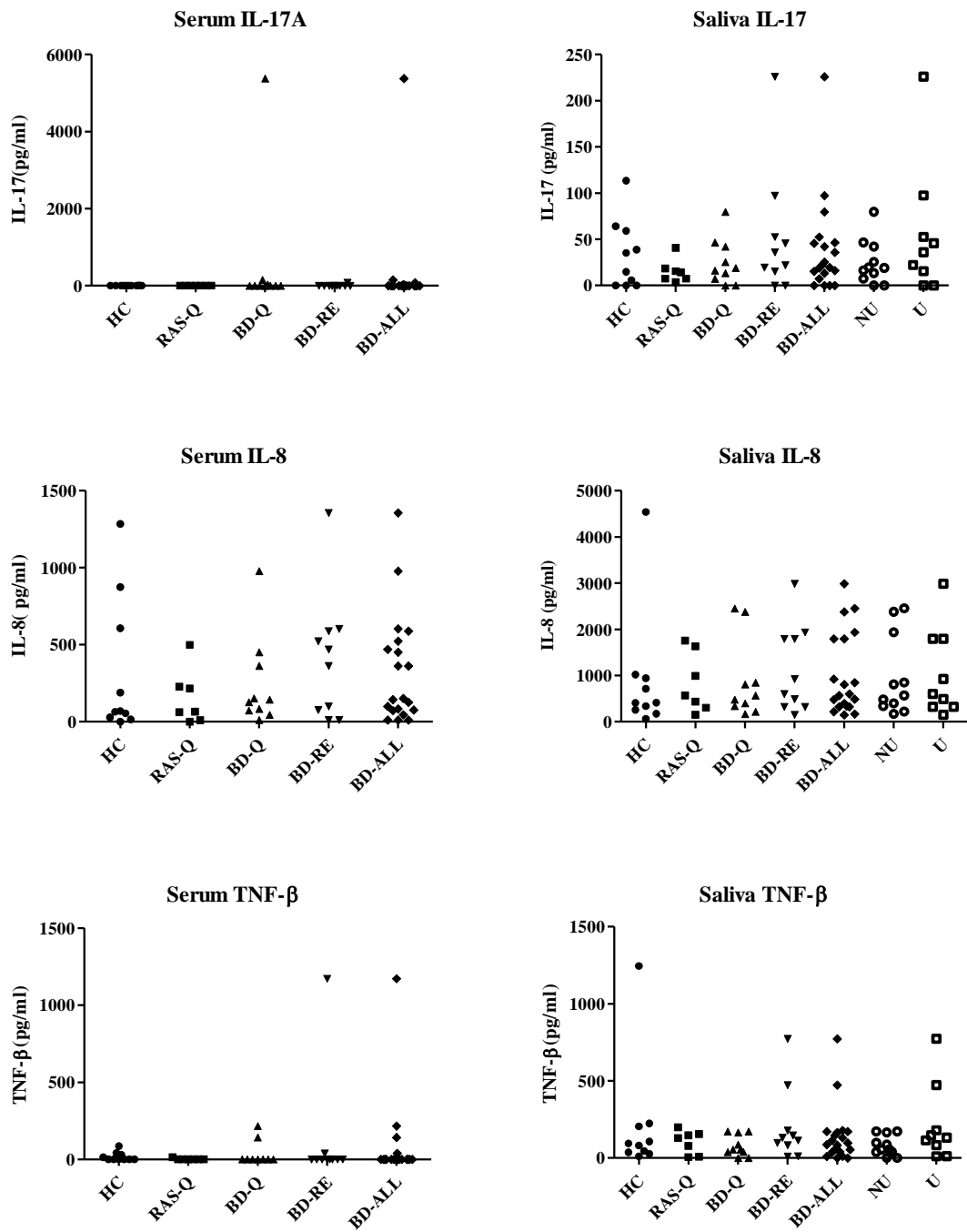


Figure 4.8: Comparison between serum and saliva cytokine levels (pg/ml) of IL-17, IL-8 and TNF- β in investigated groups: HC, RAS, BD patients in relapsed or quiet state and BD with oral ulcer (U) and without oral ulcer (NU) using multiplex bead analysis assay.

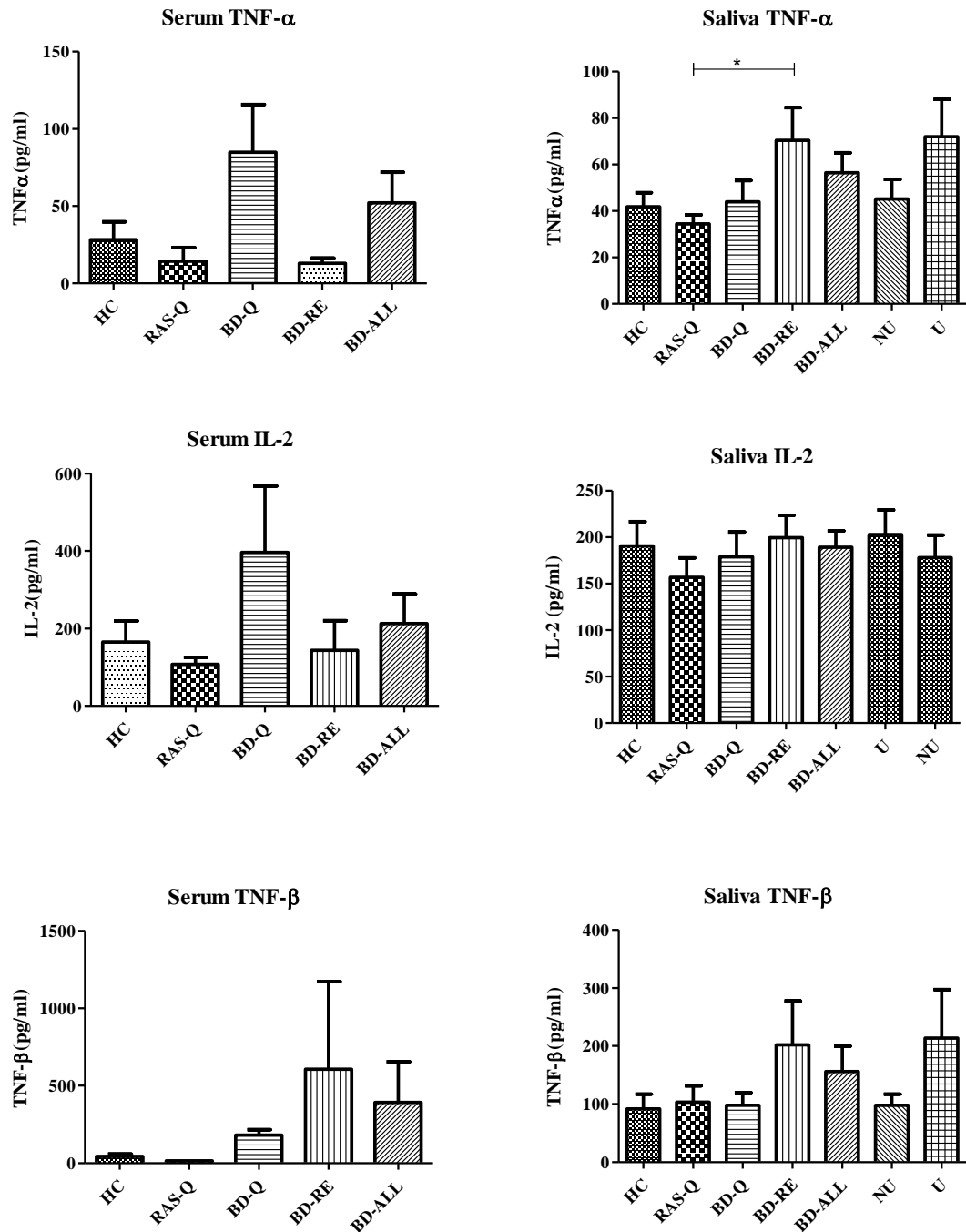


Figure 4.9: Detectable serum and saliva cytokine levels (pg/ml) of TNF- α , IL-2 and TNF- β . The mean concentration (pg/ml) of each individual cytokine were compared between HC, RAS, and BD patients with relapsed or quiet BD and BD with oral ulcer (U) and BD without oral ulcer (NU) using one-way ANOVA and Mann-Whitney U-test. Bars represent mean values \pm SEM of all samples in each investigated group. * $p < 0.05$

This revealed a difference in the amount of TNF α when RAS and BD-relapsed groups were compared (Fig3.9: p=0.0386) which was not apparent when all values were included. The limitation of the assay methodology will be discussed.

Similarly, analysis of Salivary IL-6 (Fig 4.10) revealed significant differences between HC and BD-RE (p=0.0104) and also HC and patients with ulcers (p=0.0183). Levels of IL-6 were greater in relapsed patients compared with quiet patients (p=0.0080). Patients with ulcers also appear to produce a great deal more IL-6 than those without ulcers (p=0.0272). RAS patients had lower levels of IL-6 than BD-RE but this failed to reach significance (p=0.0517). There were significant differences in the variances (F test) in many of the group comparisons (data not shown).

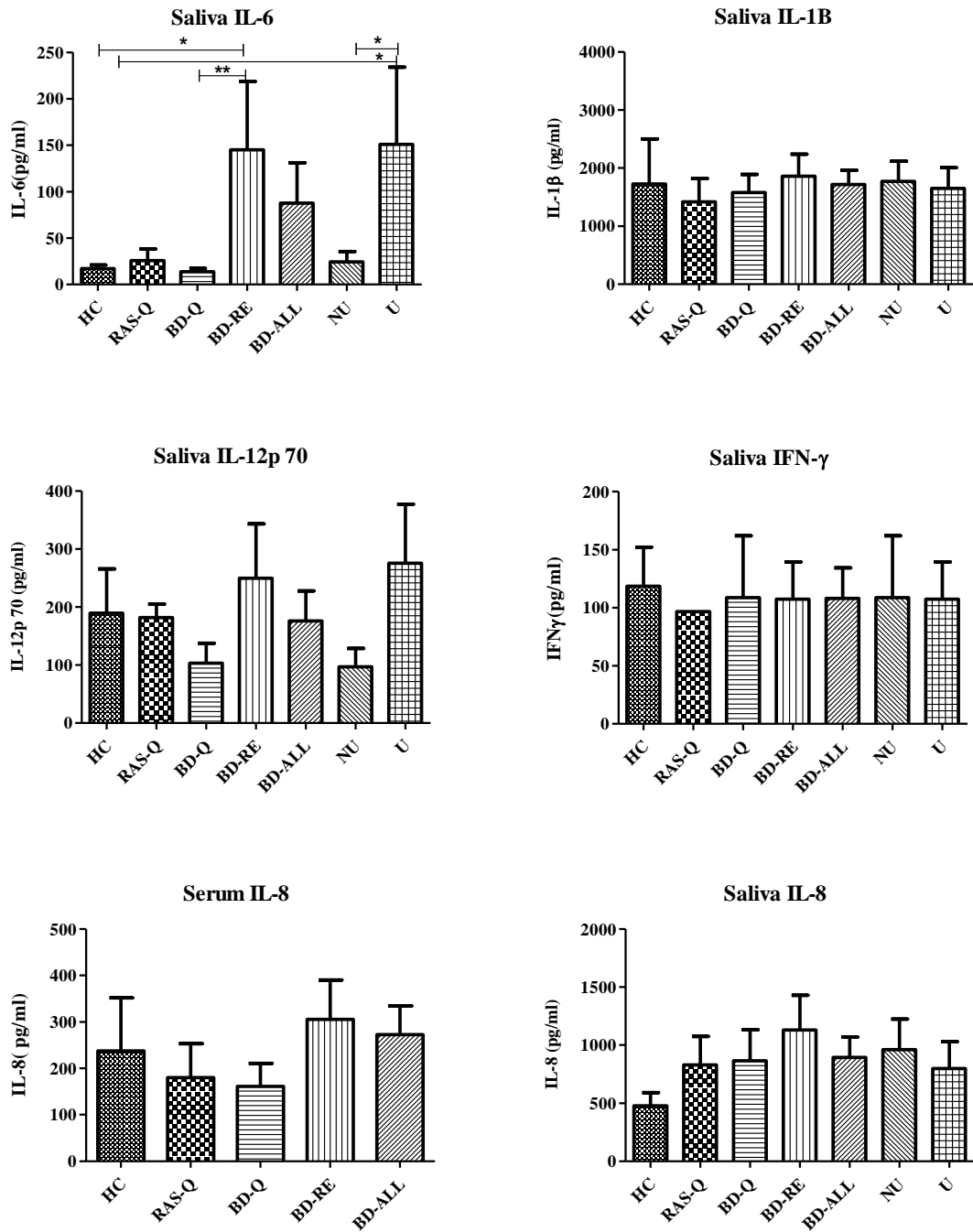


Figure 4.10: Detectable saliva cytokine levels (pg/ml) of IL-6, IL-1 β , IL-12p70, IFN- γ , and saliva and serum level of IL-8. The mean concentration (pg/ml) of each individual cytokine were compared between HC, RAS, and BD patients with Relapsed or quiet BD and BD with oral ulcer (U) and BD without oral ulcer (NU) using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM of all samples in each investigated group.

* $p < 0.05$, ** $p < 0.01$

4.1.3 Cytokine secretion profile following stimulation of PBMCs with HSP70

In order to understand the possible role of HSP70 in aetiology of BD, PBMCs of HCs (n=10), BD-RE (n=5) and BD-Q (n=8) were stimulated with 10µg/ml Human HSP70 for 24hours, and the cytokine secretion profile in supernatant was quantified using multiplex bead analysis detection system (see Chapter 2 section 2.4.5.1). Demographic characteristics and clinical features of investigated patients are summarized in Table 4.4.

Demographic and Clinical Features of the Investigated Patients(n=13)		
Mean Age ±SD	40.5±13.7(19-63)	
Gender	n	%
Male	5	38.5
Female	8	61.5
Clinical Features		
Oral Ulcers	9	69.2
Genital Ulcers	3	23.1
Eye Lesions	2	15.4
Skin Lesions	7	53.8
Joint involvement	8	61.5
Encephalitis	2	15.4
CNS	2	15.4
Relapsed	2	15.4
Treatment		
Prednisolone	4	30.8
Azathioprine	5	38.5
Colchicine	5	38.5
No treatment	1	7.7

Table 4.4: Demographic characteristics, clinical features and immunosuppressive drug treatment of investigated BD patients at the time of Blood collection.

The data on the levels of detectable cytokines were compared amongst HC, BD quiet without any activity (QQ), BD quiet with activity in mouth (Qa) and BD relapsed (RE)

using one-way ANOVA and Mann–Whitney U-test. Wilcoxon matched pairs test was used to compare the levels of cytokines in unstimulated compared to stimulated PBMCs. IFN- γ , IL-17, IL-4, IL-5 and TNF- β levels fell outside of the detection range and are not presented.

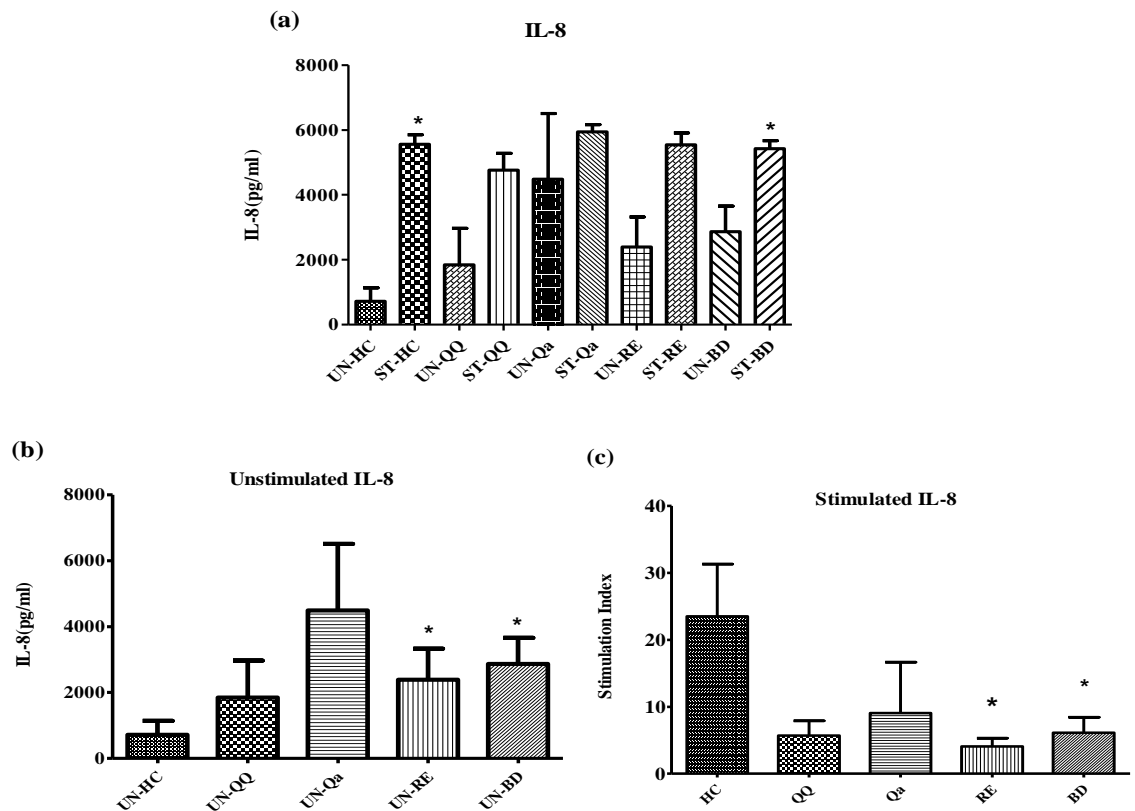


Figure 4.11: PBMC cell cultures stimulation with 10 μ g/ml HSP70 for up to 24hours. The level of cytokines in supernatant was quantified using multiplex bead analysis assay. The results are expressed as pg/ml (a and b) or stimulation index (Mean concentration of IL-8 in stimulated cells divided by unstimulated).

(c). Bars represent mean values \pm SEM of all samples in each investigated group. * $p < 0.05$

Following stimulation with Human HSP 70, IL-8 levels in both HCs and BD samples, significantly up regulated at $p=0.0313$ and $p=0.0171$ respectively but in RE failed to reach significance at just $p=0.0556$ (Figure 4.11a). Spontaneous secretion of IL-8 was

measured in unstimulated cells (ANOVA $P=0.0966$), and cultures of BD-RE cells produced more IL-8 than HC ($p=0.0303$). Overall Combined BD compared to HC was also significant ($p=0.0201$). Following stimulation with Human HSP70 all cultures were induced to secrete IL-8 (ANOVA: $P= 0.3996$; Fig 4.11 a). However, if the stimulation index (SI: Fig 4.11c) was compared this was much lower in the BD patients than in the HC, suggesting that the already high levels of secretion of IL-8 in these cultures could not be further stimulated with Human HSP70. BD or RE displayed a significant decrease in comparison to HC ($p=0.0201$ and $p=0.0303$ respectively: Figure 4.11 b and c).

Secretion of IL-10 was also investigated (Figure 4.12). There was no significant differences in the spontaneous secretion of IL-10 in unstimulated cultures (Fig 4.12b, ANOVA: $P=0.4597$). Following HSP70 stimulation, IL-10 levels significantly increased in HCs and BD (Fig 3.12 a, $p=0.0313$ and $p=0.0156$ respectively). However, the levels in the healthy controls were much higher than those in any of the stimulated BD groups following stimulation. Mean levels in the healthy controls were 1197 ± 442.5 pg/ml, whereas the mean level in the BD groups only reached 166.8 ± 79.89 pg/ml in the relapsed patients. Measurement of the stimulation index again suggested that BD cells were less able to respond to stimulation by Human HSP70.

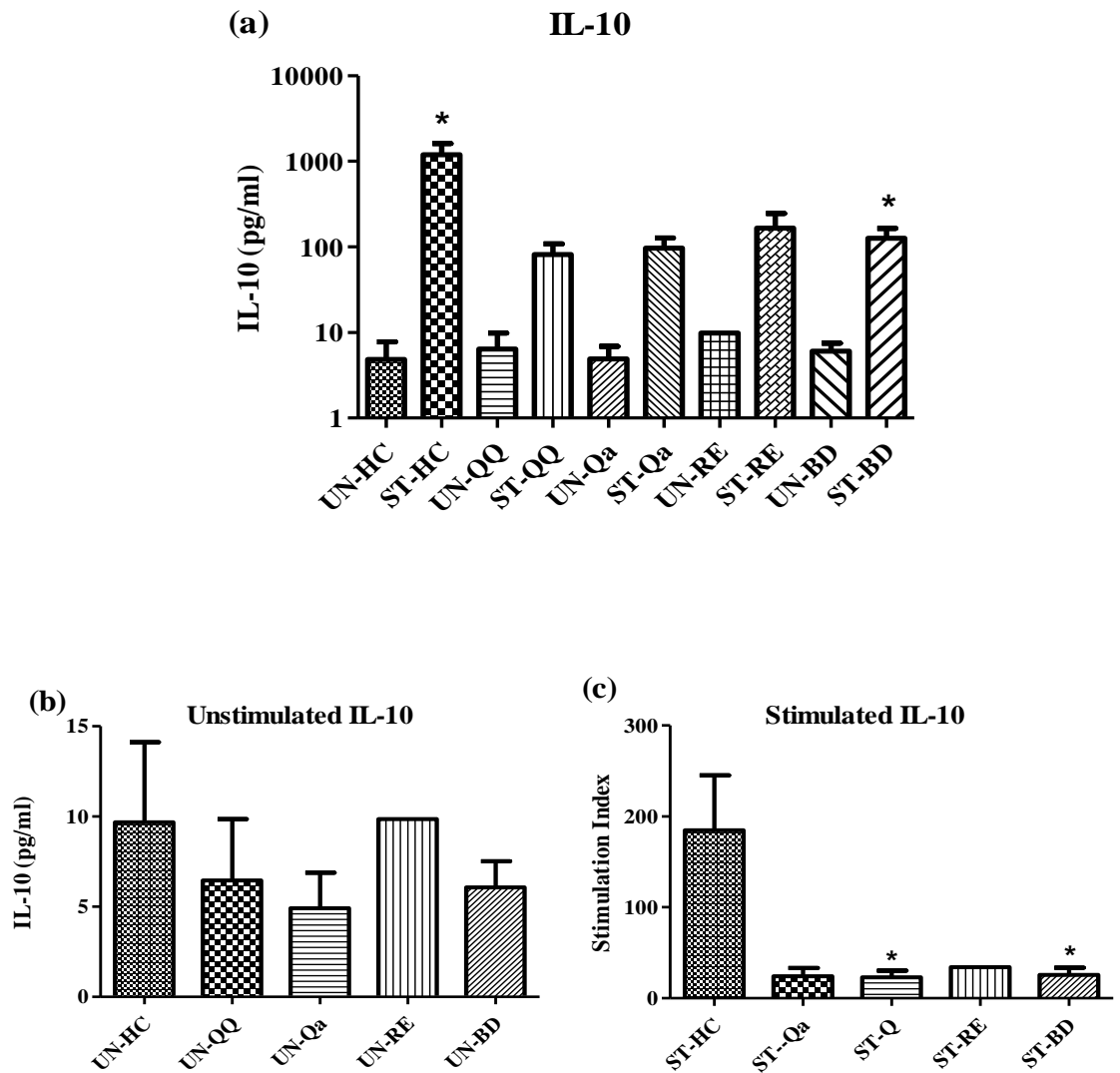


Figure 4.12: PBMC cell cultures stimulation with 10µg/ml HSP70 up to 24hours. The level of IL-10(pg/ml) in supernatant was quantified using multiplex bead analysis assay. The results for stimulated IL-10 expressed as stimulation index. Bars represent mean values ± SEM of all samples in each investigated group. * $p < 0.05$,

4.1.4 ELISPOT Results

The Possible role of Th17 cells in pathogenesis of BD has been subjected in several studies. The significant elevated levels of IL-23p19 mRNA expression, IL-23, IL-17, and IFN- γ in BD patients with active uveitis has been reported (Chi *et al.*, 2008). In GWAS studies, the strong association of IL-23 receptor (IL-23R) genes with BD has been reported (Mizuki *et al.*, 2010, Remmers *et al.*, 2010). Moreover, the strong relationship between polymorphisms of *IL23R* and *IL17* and BD has been reported (Jiang *et al.*, 2010, Kim *et al.*, 2012). Therefore, Secretion of the key cytokines of Th17 cells in PBMCs was investigated using ELISPOT analysis. This technique is very useful in that it enables enumeration of cells actively secreting cytokines but has the disadvantage of using quite large numbers of cells. IL6, IL17 and IL12p70/23 capture antibodies were used to coat PVDF plates and the cells were either left unstimulated or stimulated for 24 hours with either PHA or Human HSP70 (HEK70). The results were expressed as cytokine secreting cells (CSC)/10⁶ PBMCs.

4.1.4.1 IL-6 secreting cells

There was some spontaneous secretion of IL-6 in 1 out of the six samples tested (80 CSC/10⁶). There was some secretion following stimulation with HSP70, with two samples giving 115 and 291 CSC/10⁶ PBMCs respectively. Stimulation with PHA induced very high numbers of IL-6 secreting cells in 2 out of 5 of the samples tested (1000 and 2000 CSC). However in one of the samples the density of the spots was too great to count and in two other samples the background made counting difficult and these samples were scored as zero (Fig 4.13 a). In the BD samples there was also some spontaneous secretion ranging from 15 CSC to 77 CSC/10⁶ PBMC. Stimulation with either HEK or PHA induced further secretion but while this failed to reach significance with HEK (n=27, Wilcoxon p =0.0937), it was highly significant with PHA stimulation

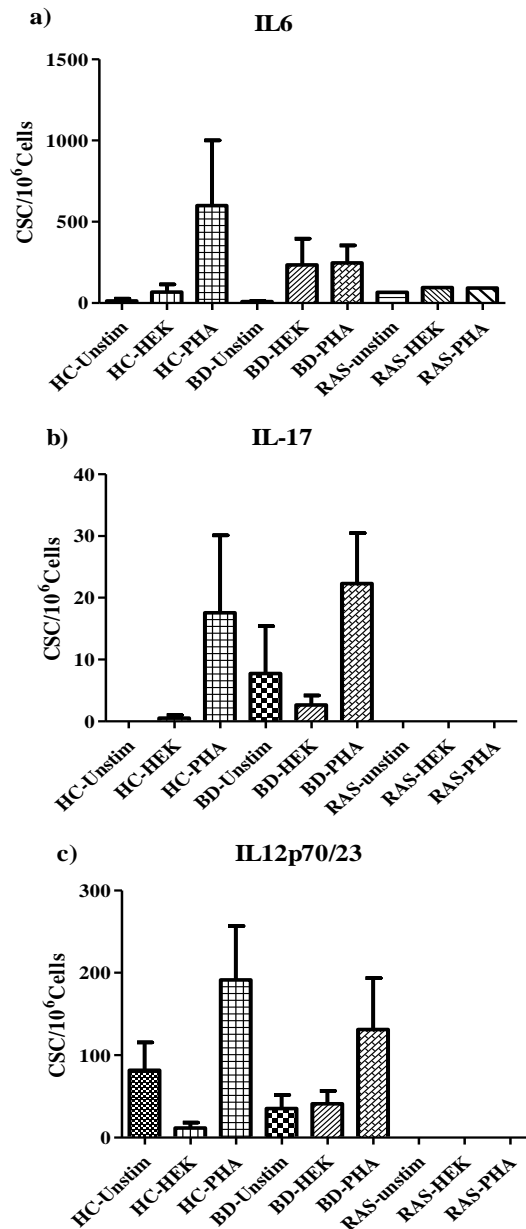


Figure 4.13: Cytokines secreting cells in PBMCs. The results are expressed as Cytokine Secreting Cells (CSC)/10⁶ PBMCs. Healthy controls (n=6), BD (n=17 for IL-17 and n=27 for IL-6 and IL-12p70/23) were stimulated with PHA or Human HSP70 (HEK) and compared with unstimulated cells.

(n=27, p= 0.005). Only two samples were able to be tested in the RAS group and the range of CSC in unstimulated cells gave 54 and 77 CSC, while HEK stimulation resulted in 82 and 102 CSC and PHA values were 104 and 83 respectively. Because of the wide range of CSC values obtained, there was no significant difference between HC

and BD in either unstimulated cultures or cells stimulated with HEK or PHA (Mann-Whitney $p=0.2838$, $p=0.8212$ and $p=0.9363$ respectively).

4.1.4.2 IL-17 secreting cells:

Only 1 BD patient out of 17 tested gave spontaneous secretion of IL-17 (130 CSC/ 10^6 PBMCs). Stimulation with HSP70 (HEK) or PHA showed no significant difference between HC and BD. There was a significant difference between both unstimulated cells and those either stimulated with HEK or PHA (Wilcoxon, $p=0.002$ and $p=0.0093$ respectively) demonstrating that the cells were capable of producing this cytokine (Fig 4.13b).

4.1.4.3 IL-12p70/23 secreting cells

There were no significant differences between unstimulated cells and HEK stimulated ones in both HCs and BD (Wilcoxon $p=0.156$ and $p=0.5$ respectively) while following stimulation with PHA significant increase in IL-12p70/23 secreting cells was seen (Wilcoxon $P=0.05$ and $P=0.03$ respectively)(Fig 4.13c). Furthermore there were no significant differences between HCs and BD both in spontaneous secretion of IL-12p70/23 (Mann-Whitney, $p=0.188$) and following stimulation with HSP70 (HEK) or PHA (Mann-Whitney, $p=0.88$, $p=0.151$ respectively).

Taken together, these results indicate that the BD cells were less able to respond to stimulation by HSP70(HEK). Furthermore, there was no significant differences in the number of IL6, IL17 and IL12p70/23 secreting cells, between controls and BD both before and following stimulation

CHAPTER 5
STAT PHOSPHORYLATION
PROFILE

Chapter 5: STAT Phosphorylation profile

5.1 Human Phospho-Kinase Array

In order to analyse the phosphorylation profiles of kinases and their protein substrates in BD, the relative levels of protein phosphorylation of the PBMCs from BD patients (n=6) in comparison to HCs (n=3) and RAS (n=3) were quantified using Human Phospho-Kinase Array in a pilot study (Figure5.1). The demographic and clinical features of the investigated patients are presented in Table 5.1. Of the 6 studied patients three were fully relapsed, one was fully quiet and the other two were generally quiet but one had activity in eye and the other in mouth, joints and skin. All the patients were on immunosuppressant treatments (Table 5.1).

Patient	Sex	Age	Active sites	Relapsed	Treatment
P1	Male	50	All quiet	No	Azathioprine
P2	Male	47	Eye	No	Prednisolone Azathioprine
P3	Male	57	Mouth-Joints-Skin	No	Prednisolone Colchicine
P4	Female	51	Mouth-Genital-Joints	Yes	Prednisolone CellCept
P5	Male	52	Mouth-Eye-Joints-Encephalitis	Yes	Prednisolone Colchicine
P6	Female	27	Mouth-Genital-Joints	Yes	Preinisolone Colchicine

Table 5.1: Demographic and Clinical characteristics of investigated BD patients

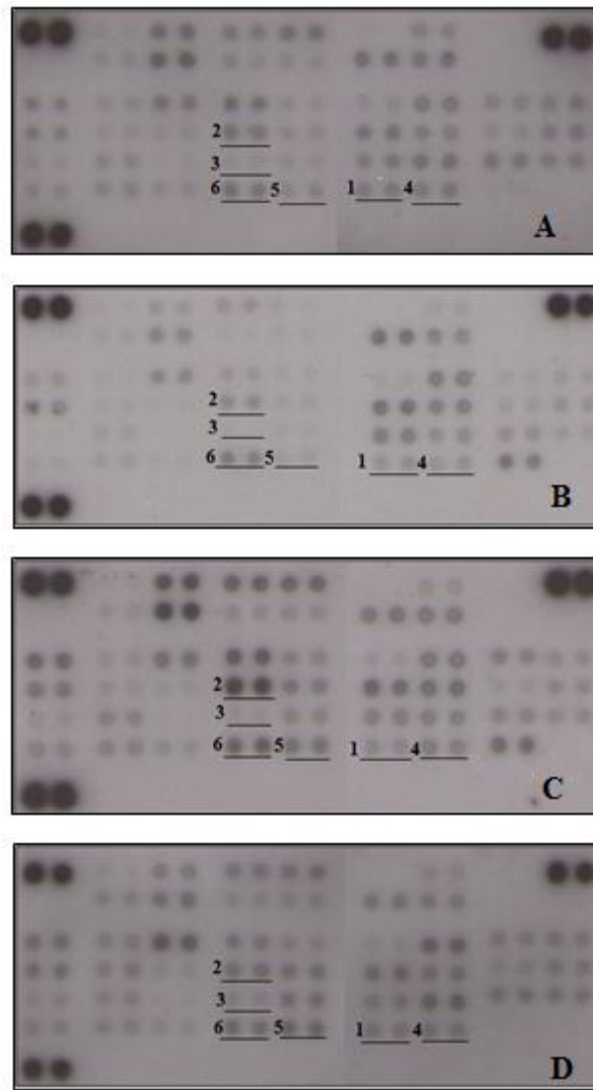


Figure 5.1: Multiplex detection of phosphorylated proteins in cell lysates from HCs (A), RAS-active (B), BD-quiet(C) and BD-relapsed (D) by Human Phospho-Kinase Array.

1: STAT1 2: STAT2 3:STAT3 4: STAT4 5: STAT5 6:STAT6

The data on the relative levels of protein phosphorylation from BD patients were compared with HC and RAS and also with disease activity in BD, quiet and relapsed, using ANOVA and Mann–Whitney U-test (Figures 5.2-5.8). As can be seen from Figures 5.2, with the exception of STAT 6, all the STATs displayed a noticeable difference in BD patients compared to healthy controls. The mean relative pixel density of STAT6 in BD (15.26 ± 3.009) were greater than HCs (8.63 ± 0.6714) but failed to reach significance ($p=0.3810$). Interestingly STAT 6 is associated with Th2 differentiation which is not the classical profile observed in Behçet’s patients. STAT1, STAT2 and

STAT4 were significantly increased in BD compared to HCs at $p=0.0476$, $p=0.0467$ and $p=0.0238$ respectively. Increased phosphorylation of STAT 1 and 4 would be consistent with activation of a Th1 differentiation pathway, while STAT 2 is associated with the defence against virus. STAT3 has also shown a marked difference between study groups (ANOVA, $P=0.0213$). Significantly higher expression of STAT3 was observed in BD compared to both HCs and RAS samples each at $p= 0.0238$. This would be consistent with a Th17 profile. It was interesting to observe that in this respect RAS behaved in a similar manner to HC. All the STAT5 proteins: STAT 5a, STAT 5b and STAT 5a/b displayed significant rise in BD patients compared to HCs all at $p=0.0476$ (Figure 5.2). STAT5 proteins are associated with Tregs.

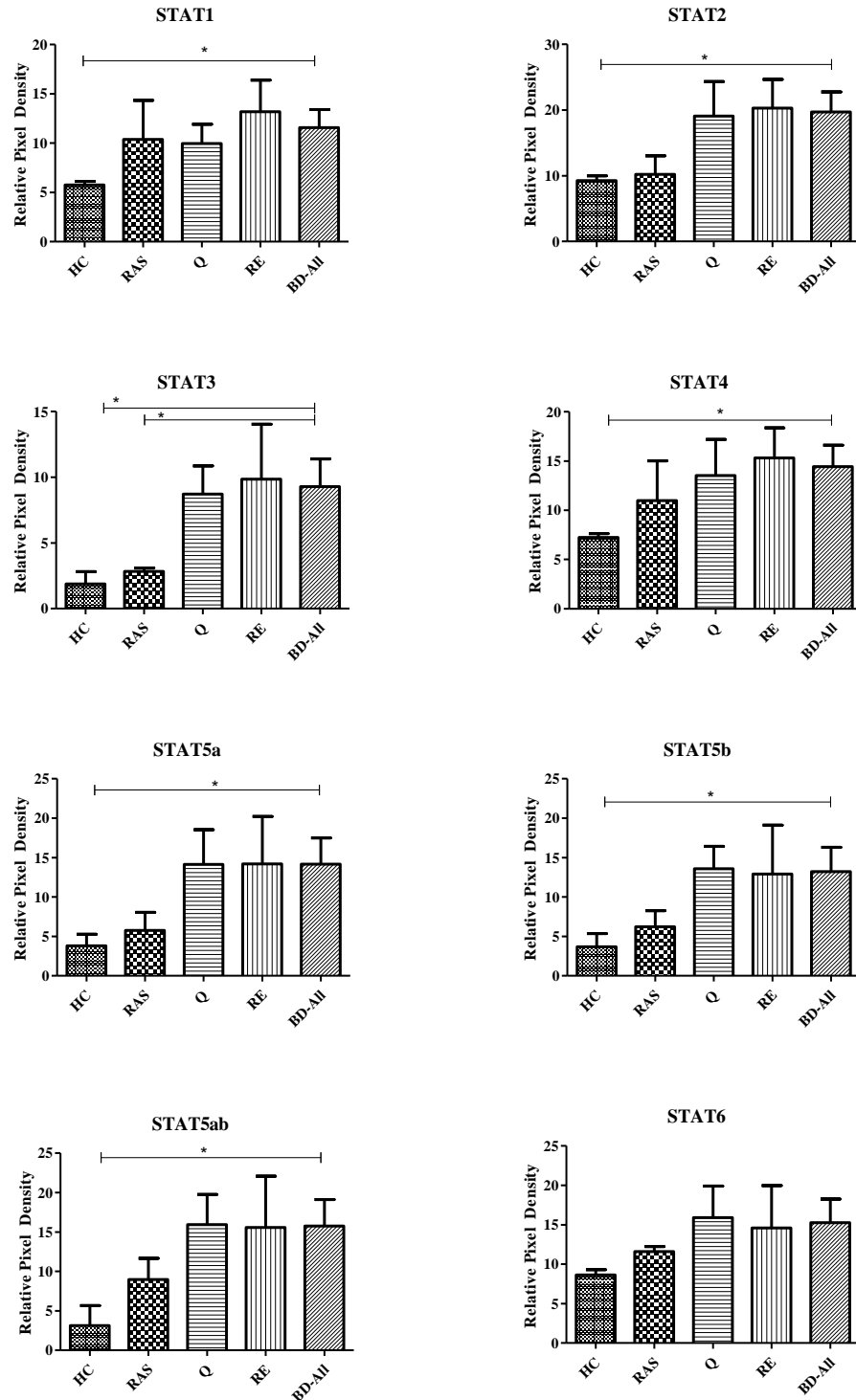


Figure 5.2: The relative expression of phosphorylated proteins, STAT1, STAT2, STAT3, STAT4, STAT5 and STAT6 in PBMC were quantified by Human Phospho-Kinase Array. The mean relative pixel density was compared between HC, RAS, and BD patients with relapsed or quiet BD using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$

Although our main interest was the STAT proteins in this project, using the Proteome Profiler Human Phospho-Kinase Array, we were able to look at a total of 43 kinase phosphorylation sites and have identified 7 new, and as far as we are aware, previously unidentified kinase phosphorylation sites which may be altered in Behçet's disease compared to HC and RAS. As can be seen from Figure 5.4, AMPKa1 and CRBE revealed significant increase in BD compared to HCs at $p=0.0476$ and $p=0.0238$ respectively. Similarly, Yes showed the same trend at $p=0.0238$. Another interesting marker Fyn, displayed very remarkable differences between the studied groups (ANOVA, $p=0.0021$). Fyn were significantly higher in BD patients compared to HC ($p=0.0126$). Further analysis of Fyn relative pixel density also showed a significant increase in both relapsed and quiet compared to HC at $p=0.0048$ and $p=0.0298$ respectively. Fgr and FAK are another two protein tyrosine kinases which show a considerable differences in BD (ANOVA, $P=0.0218$ and $P=0.0342$ respectively). Fgr was significantly increased in BD patients compared to HCs and RAS at $p=0.0238$. Similarly, FAK showed a significant rise in BD compared to HCs at $p=0.0238$ and to RAS at $p=0.0476$ and finally RASK123 also showed significant rise in BD compared with HCs at $p=0.0238$.

Further investigations are needed since the sample size was quite small. The significance of the changes in these phosphor kinase proteins will be discussed in Chapter 6. Graphs of the remaining phosphor-kinase activities where no significant differences were observed are presented in Appendix 8.

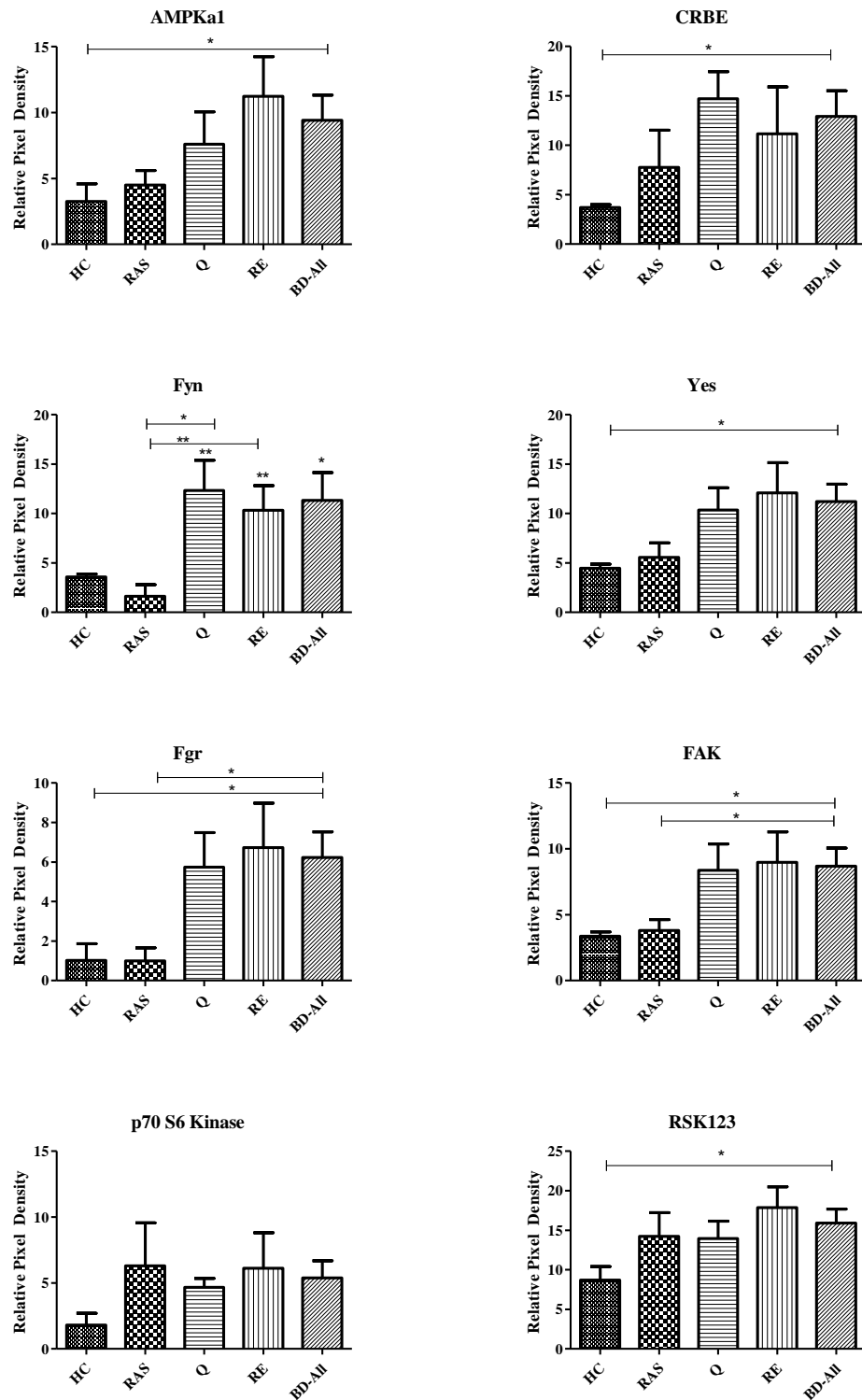


Figure 5.3: The relative expression of 8 phosphorylated proteins, in PBMC were quantified by Human Phospho-Kinase Array. The mean relative pixel density was compared between HC, RAS, and BD patients with relapsed or quiet BD using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM of three independent experiments. * $p<0.05$, ** $p<0.01$

Overall, using the Human Phospho-Kinase Array, the relative levels of 46 kinase phosphorylation sites in BD patients compared to HCs and RAS volunteers were able to be detected simultaneously. STAT1, STAT2, STAT3, STAT4 and STAT5 all displayed significant increase in BD patient compared to HCs. Furthermore, AMPKa1, CRBE, YES, FYN, Fgr, FAK and RASK123 showed a difference in BD and can be considered as new targets in BD for future investigations. To our knowledge these proteins have not been investigated in BD to date.

5.2 Phospho-specific flowcytometry results

In order to investigate key transcription factors in the differentiation of Th1 and Th17 cell signalling pathways a novel technique which uses Flow cytometry to measure STATs and their transcription factors in specific T cell subsets was used. Using this method allowed us to identify the subset of T cells expressing the transcription factors. The transcription factor ROR γ t is associated with Th17 differentiation and is closely linked with STAT3, while T-bet controls Th1 differentiation and is associated with STAT1 and 4. Phospho-specific flowcytometry was used to investigate phosphorylation status of STAT1, STAT2, STAT3, STAT5 and the transcription factors t-bet and ROR γ t in CD4⁺ T Cells isolated from PBMCs following activation with IFN- α for 15min at 37°C. This method enables the investigation of specific T cell subsets as compared with the phosphor array method which utilised PBMCs.

The investigated samples include HCs (n=7), BD-Q (n=8) and BD-RE (n=8). The demographic and clinical features of investigated BD patients are presented in Table 5.2. Of the 15 investigated BD patients, 5 male and 10 were female. The most frequent clinical symptoms were joint involvement (68.8%), CNS (62.5%) oral ulcers (50%) and genital ulcers with 24%. 8 patients (50%) clinically diagnosed as relapsed.

Demographic and Clinical Features of the Investigated Patients(n=15)		
Mean Age \pm SD	41.60 \pm 15.8(17-74)	
Gender	n	%
Male	5	31.3
Female	10	62.5
Clinical Features		
Oral Ulcers	8	50
Genital Ulcers	4	24
Eye Lesions	2	12.5
Skin Lesions	3	18.8
Joint involvement	11	68.8
Encephalitis	1	6.3
CNS(Headache)	10	62.5
Vascular	1	6.3
Relapse	8	50
Treatment		
Prednisolone	4	25
Azathioprine	5	31.8
Colchicine	3	18.8
CELLCEPT	1	6.3

Table 5.4: Demographic characteristics, clinical features and immunosuppressive drug treatment of investigated BD patients at the time of serum collection for Phospho-specific flow cytometry.

PBMCs were either stimulated with IFN α or left unstimulated for 15 minutes after which they were fixed and permeabilized and stained with antibodies specific for the STATs and transcription factors as described in Chapter 2 section 2.4.6. The cells were counter stained with CD4 antibody and the cells were gated in the FACS machine on the CD4⁺ population. Flow cytometry was carried out using a FACS canto II (BD biosciences, UK) and was later analysed with the Flow-Jo software (BD biosciences, UK). A representative gating strategy is shown in Figure 5.4. The gate was first set for total lymphocytes (Figure 5.4.a) and then gated onto the population of CD3⁺ CD4⁺ cells

(Figure 5.4b). The difference in the overlay represents the isotype control in blue and the STAT5 staining in red.

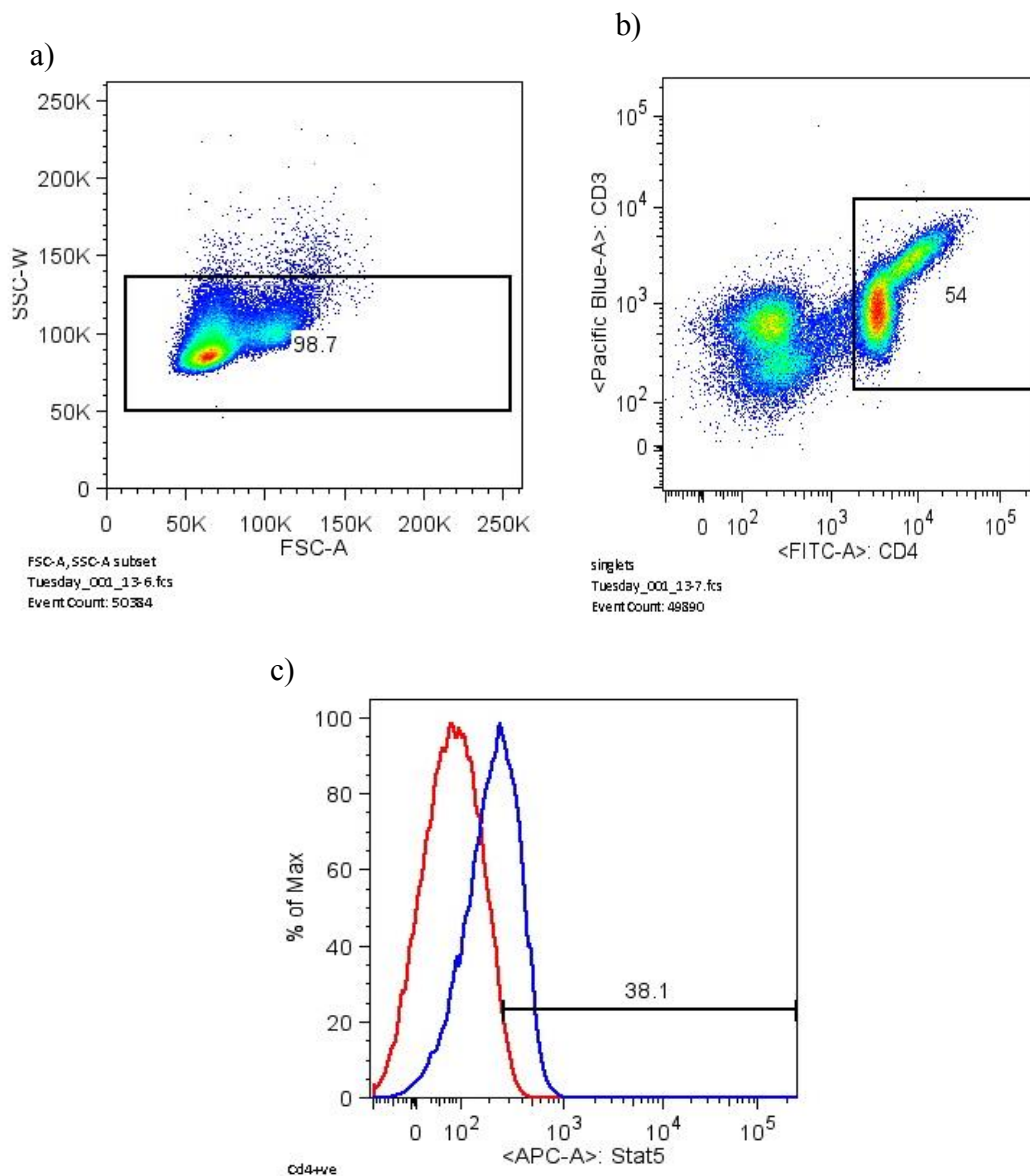


Figure 5.4: Representative FACS gating strategy: A) Forward and side scattered of total PBMCs. B) Gate position for CD3⁺CD4⁺ cell population. C) Overlay of Isotype control with anti phosphoSTAT5 antibody.

The results were initially expressed as median fluorescent intensity or as a percentage of maximum fluorescence (Figure 5.5). As can be seen in the case of the STAT proteins there was increased expression following stimulation. However, for T-bet there was a

decrease in expression following stimulation in BD-Q, and patients with mouth activity

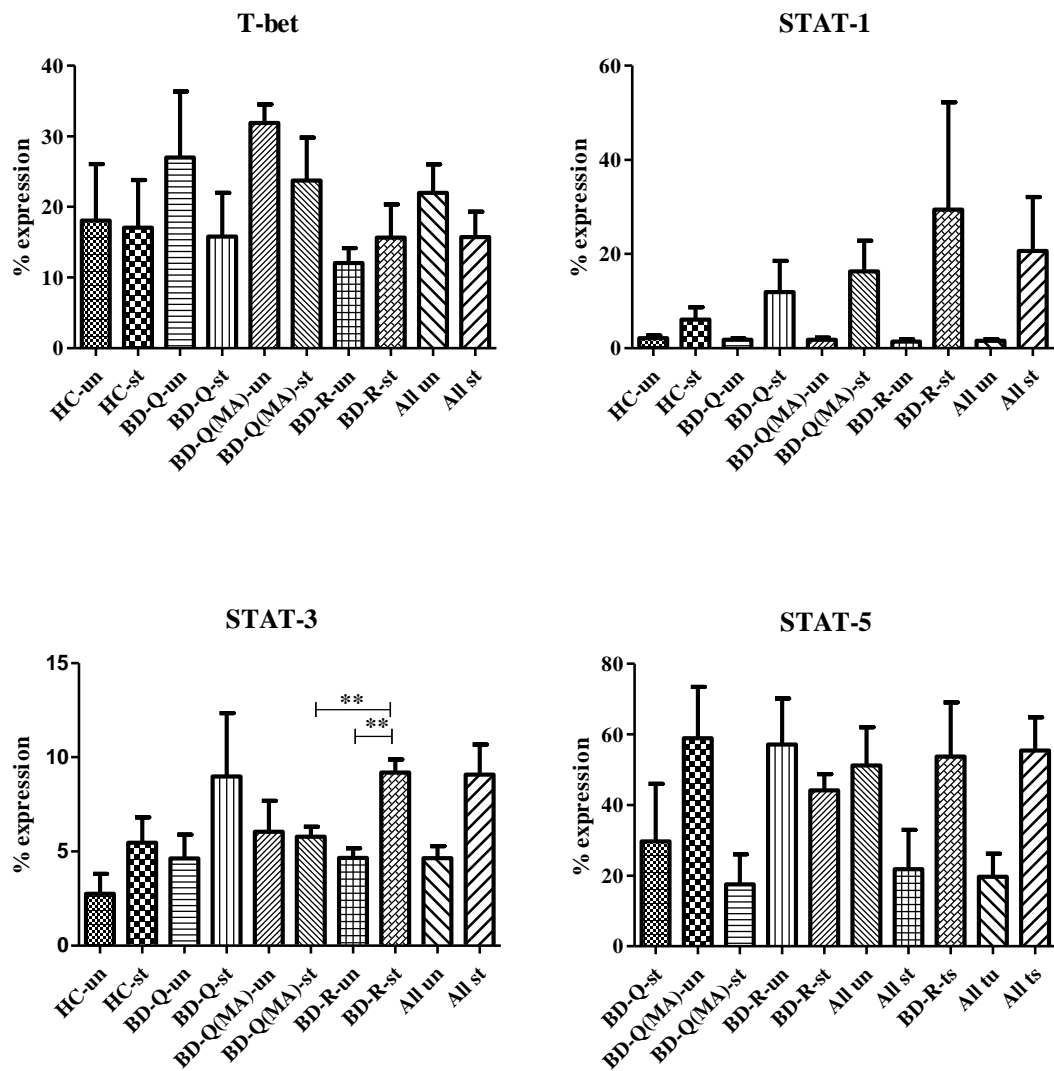


Figure 5.5: Percentage expression of STATs and T-bet following stimulation with IFN α . The results were analysed using ANOVA and paired t tests to examine the differences following stimulation. Bars represent mean values \pm SEM of all samples in each investigated group.

* $p < 0.05$, ** $p < 0.01$

only [BD-Q (MA)], however the difference just failed to reach significance ($p=0.0651$).

A comparison of the levels of STAT3 in patients who were mouth active only with fully relapsed patients revealed a highly significant difference ($p=0.0286$). Investigation of STAT 5 showed an increase in the expression following stimulation but only BD-

relapsed patients showed differences which were close to statistical significance ($p=0.0550$).

Because the percentage expression was highly variable the results were difficult to interpret and therefore, stimulation index (SI) was used to interrogate the results. Stimulation index (SI) i.e. MFI (Mean Fluorescence Intensity) of the stimulated cells divided by the unstimulated cells from BD patients were compared with HCs and also with disease activity: quiet and relapsed using ANOVA and Mann–Whitney U-test (Figures 5.6-5.8). Two separate sets of experiments were carried out.

In the first series of experiments [E1(Experiment 1): Fig 5.6] phosphorylated STAT1, 3 and 5 along with transcription factor T-bet were examined in HC compared with quiet and fully relapsed BD as well as patients with mouth activity only [BD-Q(MA)]. As can be seen, pSTAT1 displayed higher expression in BD than HCs. The mean stimulation index of pSTAT1 in BD-RE (13.77 ± 8.1) was greater than BD-Q (9.917 ± 7) and HCs (2.764 ± 1.3) but they failed to reach significance. There were no noticeable differences in pSTAT 3 and pSTAT 5 between study groups (ANOVA, $P=0.9684$ and $P= 0.9958$ respectively). The mean stimulation index of pSTAT5 was lower in BD-RE (6.805 ± 3.6) compared to HCs (9.641 ± 8.6) but it failed to reach significance. The most remarkable result of this experiment was increased level of T-bet in BD-RE compared to BD-Q at $p=0.0571$, however this just failed to reach significance.

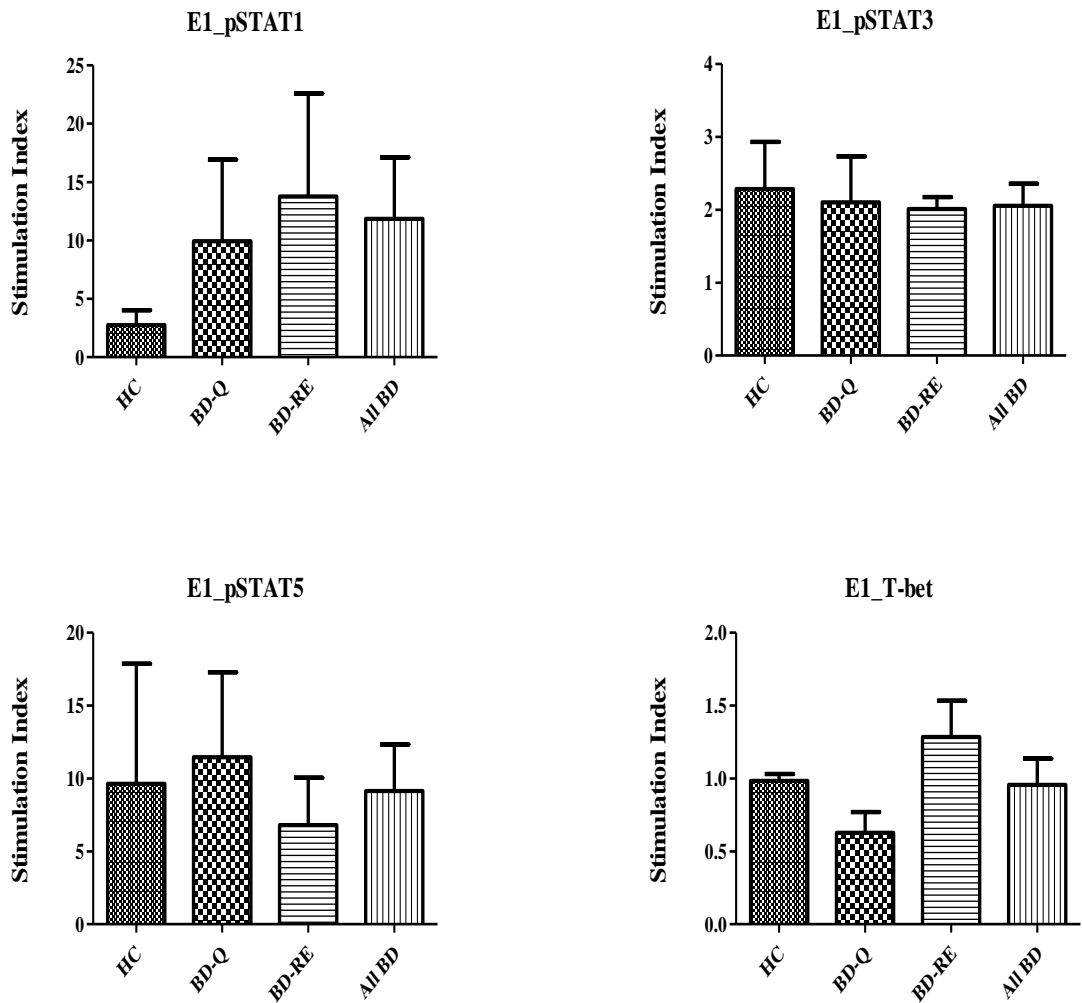


Figure 5.6: Expression of pSTAT1, pSTAT3, pSTAT5 and T- bet in CD4+ T cells. PBMCs were stimulated with 1.8×10^8 units/mg IFN- α for 15min at 37°C. Expression of pSTAT1, pSTAT3, pSTAT5 and t- bet in CD4+ T cells were investigated using Phospho-specific flow cytometry method. Mean stimulation index from HCs were compared to BD-Q and BD-RE using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM of all samples in each investigated group.

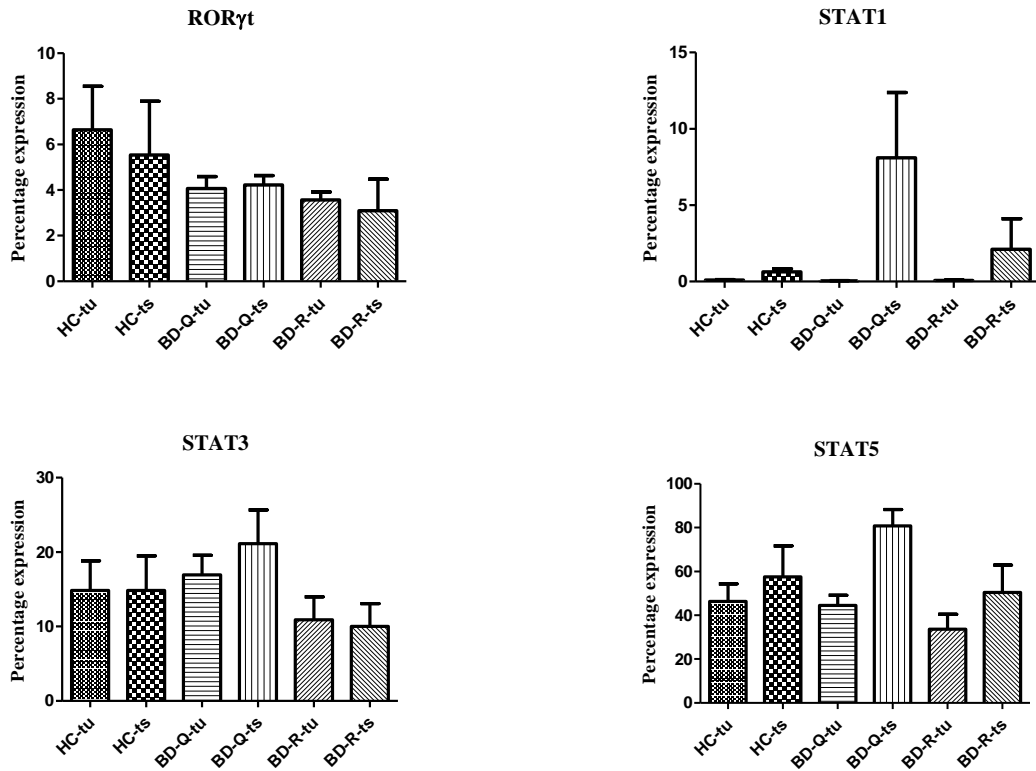


Figure 5.7: Percentage expression of ROR γ t, STAT1, 3 and 5 following stimulation with IFN α . The results were analysed using ANOVA and paired t tests to examine the differences following stimulation. Bars represent mean values \pm SEM of all samples in each investigated group.

tu: T cell unstimulated, ts: T cell stimulated with IFN α

In a second series of experiments [E2 (Experiment2): Fig 5.8] the comparison of phosphorylated STAT1, 3 and 5 along with transcription factor ROR γ t were shown. pSTAT1 results confirmed E1 experiment and show higher level in BD than HCs at $p=0.6828$. Again there were no significant difference in pSTAT3 and pSTAT5 between investigated groups (ANOVA, $P=0.5293$ and $P=0.2913$) respectively. ROR γ t slightly increased in BD (0.9391 ± 0.2) compared to HCs (0.7232 ± 0.17) but it failed to reach significance.

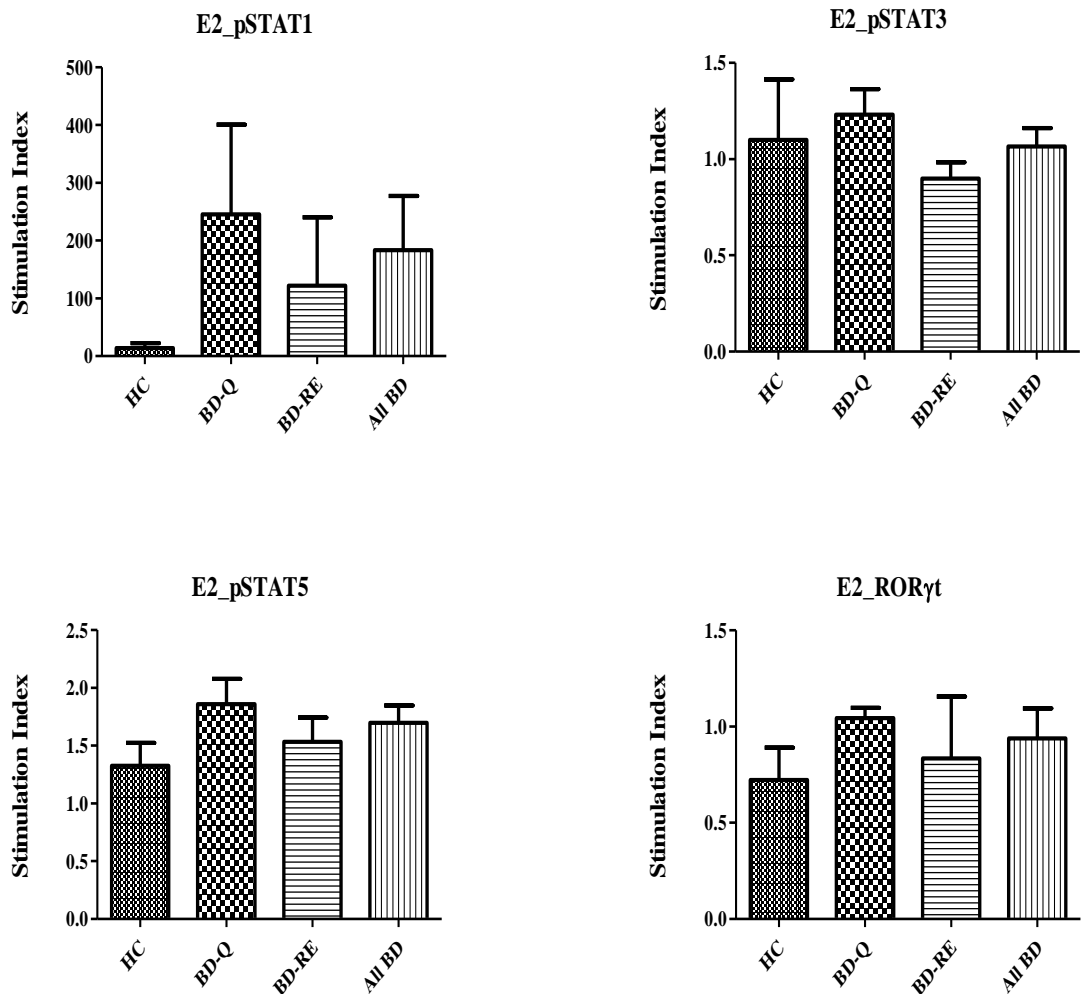


Figure 5.8: Expression of pSTAT1, pSTAT3, pSTAT5 and ROR γ t in CD4⁺ T cells. PBMCs were stimulated with 1.8×10^8 units/mg IFN- α for 15min at 37°C. Expression of pSTAT1, pSTAT3, pSTAT5 and ROR γ t in CD4⁺ T cells were investigated using Phospho-specific flow cytometry method. Mean stimulation index from HCs were compared to BD-Q and BD-RE using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM of all samples in each investigated group.

The data for pSTAT 1, pSTAT3 and pSTAT5 in experiments 1 and 2 were combined to increase sample number and their results are shown in Figure 5.9 and 5.10. Similar overall conclusion can be drawn from the results.

The percentage expression is shown in Fig 5.9 and it can be seen that STAT1 and STAT5 show significant upregulation on stimulation with IFN α . STAT 3 did not show any change following stimulation.

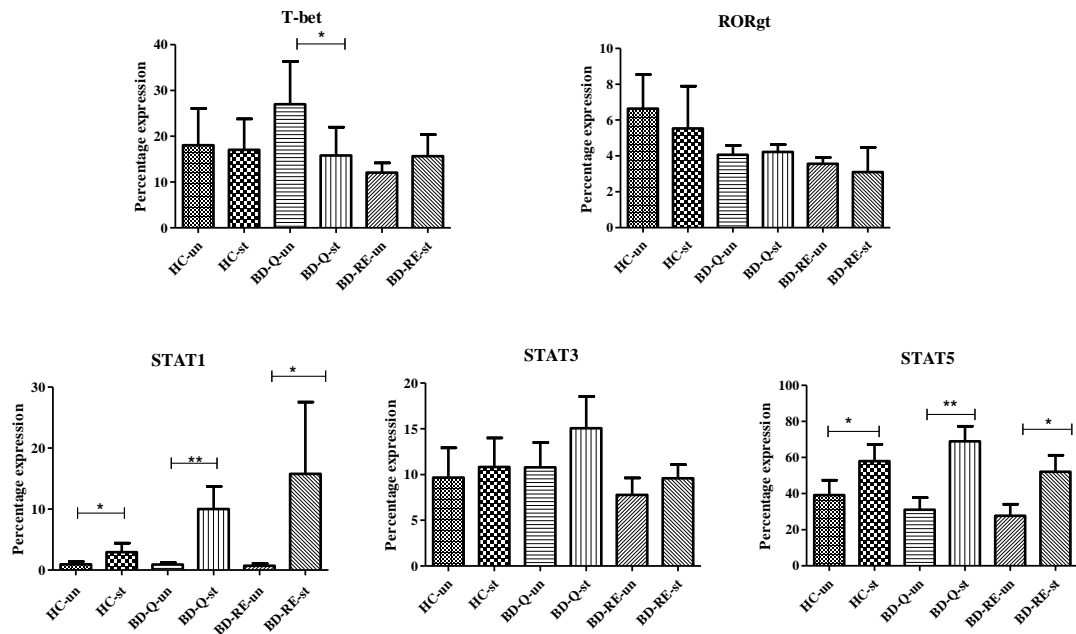


Figure 5.9: Percentage expression of T-Bet, ROR γ t and phospho-STATs 1, 3 and 5 in PBMCs following stimulation with IFN α . ANOVA and paired t tests were used to analyse the data.

* $p < 0.05$, ** $p < 0.01$

st: stimulated with IFN α , un: unstimulated

The data was combined from the two series of experiments for STATs 1, 3 and 5 and the data is presented in Fig 5.10.

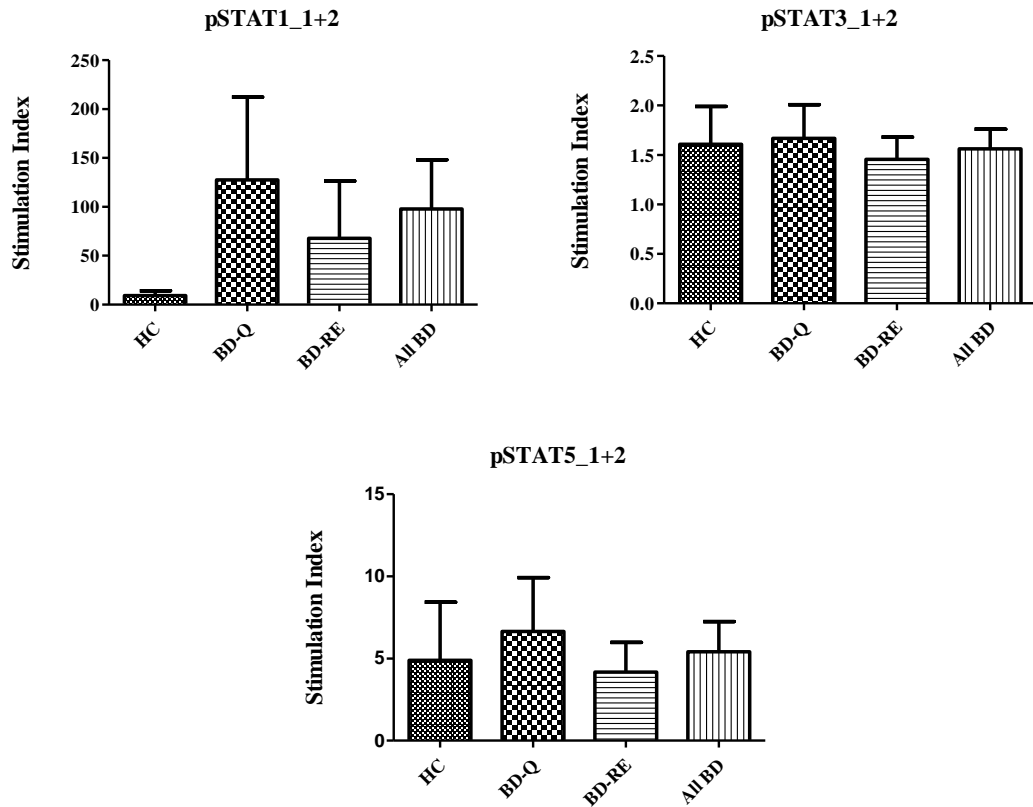


Figure 5.10: Expression of pSTAT1, pSTAT3 and pSTAT5 in CD4+ T cells were investigated using Phospho-specific flow cytometry method. PBMCs were stimulated with 1.8×10^8 units/mg IFN- α for 15min at 37°C. Mean stimulation index from HCs were compared to BD-Q and BD-RE using one-way ANOVA and Mann–Whitney U-test. Bars represent mean values \pm SEM of all samples in each investigated group. $*p < 0.05$

CHAPTER 6

DISCUSSION

Chapter 6 Discussion

6.1 SOCS expression and Behçet's disease

Although the aetiopathogenesis of Behçet's disease is still unknown, immune dysfunction seems to be involved. It is generally accepted that cytokines play a key pathogenic role in BD. Several cytokines associated with BD such as IFN γ , IL-12, IL-23, IL-10 and IL-6 have their effect through activation of JAK-STAT signalling pathway. This pathway is tightly regulated by SOCS proteins to avoid excessive immune responses and pathogenic consequences in the host. In this respect, any dysregulation in this balance can affect cellular responsiveness to cytokines and cause subsequent pathological effects.

The implication of SOCS protein in human disease has been reported in several studies. Higher levels of SOCS3 mRNA and SOCS1 SNPs in T cells have been associated with asthma pathogenesis. These SNPs in SOCS1 are associated with increased SOCS1 mRNA levels compared to healthy controls have been reported in the skin of patients with atopic dermatitis (Harada *et al.*, 2007). The studies of mice deficient in SOCS1 signalling develop severe skin and eye lesions through the recruitment of Th1 and Th17 cells into these non-lymphoid tissues (Yu *et al.*, 2008). SOCS3 gene deficient mice developed a severe form of skin inflammation (Uto-Konomi *et al.*, 2012). Some of the characteristic of Behçet's disease is Eye and skin lesions. On our knowledge, SOCS protein expression has not been investigated in neutrophils and oral ulcers of BD patients to date. Therefore, SOCS expression in PBMCs, neutrophils and buccal mucosal cells of patient with BD were investigated in this project. Our results showed altered level of mRNA expression of SOCS in BD. In PBMCs, SOCS1 message was significantly higher in BD compared to HC and RAS. Both quiet and relapsed

subgroups of BD separately showed significantly higher mean levels of SOCS1 but no significant differences were observed between quiet and relapsed patients. Similarly, neutrophils displayed higher levels of SOCS1 in BD patients, but the results failed to reach significance. For SOCS3, a significant upregulation in both PBMC and neutrophils of BD patients and also RAS compared to HC was seen. There was no significant difference in SOCS3 between relapsed and quiet patients. For SOCS2 mRNA expression in PBMCs, BD relapsed patient displays a significant higher level than healthy controls and RAS. Surprisingly, SOCS2 level in neutrophils did not follow the same trend and hardly any changes between study groups were seen.

Because BD patients frequently present with oral ulceration as the first symptom of their disease and ulcers are a dominant feature throughout the disease, special attention was given to the oral mucosa. mRNA expression of SOCS1 and 3 were also investigated in buccal mucosal cells (BMC) to compare matched ulcer and non-ulcer sites of BD patient. SOCS1 and SOCS3 have increased significantly in ulcer sites compared to non-ulcer sites and also compared to non-ulcerated healthy controls. The mean expression level of SOCS1 and 3 in non-ulcer sites were higher than HCs but it failed to reach significance. There were no significant differences of both SOCS1 and 3 in non-ulcer sites comparing relapsed patients with quiet ones. This suggests that the non-ulcer sites are comparable to normal tissue in healthy controls. In ulcer sites, the relapsed patient displayed higher relative mean expression of SOCS1 and SOCS3 but both failed to reach significance. There is a significant upregulation of SOCS1 and SOCS3 in ulcer sites of quiet patients compared to their non-ulcer sites. Regarding relapsed patients, both SOCS1 and SOCS3 were also significantly increased in ulcer sites compared to non-ulcer sites.

All these results showed a trend of higher levels of SOCS1 and SOCS3, especially in fully relapsed patients and in active oral ulcers which suggest that this might be the results of increased level of cytokines in BD and that upregulation of the negative regulators of cytokine signalling attempts to dampen down cytokine activation.

In order to investigate post transcriptional regulation of SOCS1 and 3 in PBMCs and neutrophils from BD patients, western blot analysis was performed. SOCS2 protein was not detected in western blot. Generally SOCS2, in both qPCR and western blot was of low abundance and difficult to detect (data not shown). Thus, stimulation of cell cultures was needed to be able to detect them. SOCS1 protein expression displayed an increase in BD quiet compared to HCs, however the reverse was observed in BD-RE, which was decreased compared to HCs. No noticeable differences in SOCS3 protein expression between investigated groups was seen in PBMCs.

Regarding the SOCS molecule and its function, broad studies have been recorded but very limited investigation was available on SOCS expression in pathological conditions, although, overexpression of SOCS proteins has been detected in other inflammatory and autoimmune diseases. Increased levels of SOCS3 in the intestinal mucosa of patients with Crohn's disease has been reported (Suzuki *et al.*, 2001). Like Behcet's this is a disease of the mucosa and Crohn's patients also exhibit oral manifestaions which might suggest a common pathway if SOCS dysregulation in mucosal tissue undergoing pathological changes due to overt inflammation. Moreover, the level of SOCS1 and SOCS3 mRNA expression was significantly elevated in PBMCs from rheumatoid arthritis (RA) patients when compared with healthy volunteers (Isomäki *et al.*, 2007). In a study on Th17 related cytokines and their signalling pathways in BD, significant upregulation of TGF- β receptor type1, IL-12 receptor β 2 and SOCS1 in PBMCs

following 24h PHA stimulation was observed (Shimizu *et al.*, 2012). Significant upregulation of SOCS1 in PBMC in this project is in agreement with the Shimizu (2012) study; although they used PBMCs following stimulation with PHA while our experiments were done on unstimulated PBMC. The results of this project are also close to a similar study on the expression of SOCS in rheumatoid arthritis (Isomäki *et al.*, 2007), where significant upregulation of SOCS1 and SOCS3 were also found in inflammatory lesions in rheumatoid arthritis.

There is controversy in interpreting these results. As discussed earlier, increased proinflammatory cytokines such as IL-6, TNF- α , IL-12 and IL-15 in BD have been reported. High level of these cytokines could lead to an increase of SOCS proteins which inhibit cytokine signalling and eventually suppress the damaging effect of proinflammatory cytokines. On the other hand, SOCS proteins are also implicated in signalling pathways of anti-inflammatory cytokines such as IL-10, thus high level of SOCS might suppress their function and promote more inflammation.

There are some considerations which should be taken into account when interpreting data in SOCS studies. First SOCS proteins play a variety of roles in many other immunological processes. SOCS proteins along with STATs molecules have an important role in Th1 and Th2 cell differentiation. SOCS1 has been shown as a negative regulator of IL-12, IFN- γ and STAT 4 signalling pathway (Eyles *et al.*, 2002). SOCS3 deletion in T cells, indirectly suppresses both Th1 and Th2 cell responses, as a result of enhancing the levels of immunosuppressive cytokines TGF- β and IL-10 (Kinjyo *et al.*, 2006). Inhibition of STAT3 signalling by SOCS3 induce Th17 cells production (Palmer and Restifo, 2009). On the other hand, SOCS1 deletion results in decreased level of Th17 cells and reducing Th17-mediated diseases (Tanaka *et al.*,

2008). The role of SOCS proteins in innate immunity especially regarding TLRs was subjected to many investigations. TLRs signalling pathway should be controlled tightly since its uncontrolled activation leads to immunological and inflammatory disease. It has been shown that SOCS1 and SOCS3 proteins negatively regulate TLR signalling pathways (Naka *et al.*, 2005). In a recent study from our group, splice variants of both TLR 2 and 4 have been shown to be increased in BD patients which may have an effect on their ability to respond to microbial antigens (Seoudi *et al.*, 2013).

Secondly, considering other factors which might affect regulation of the JAK-STAT pathway, such as mechanisms required for regulating SOCS protein levels should be considered. There are limited studies about SOCS protein regulation, itself, in the literature. MicroRNAs (miRNAs) are small non-coding RNAs which act by binding to complementary sequences on target mRNAs and cause silencing of gene expression (Collins *et al.*, 2013). In a recent study, the augmentation of JAK STAT signal transduction by miR-19a through control of SOCS3 expression has been shown (Collins *et al.*, 2013). It has been suggested that miR-155 mediated SOCS1 suppression increases the JAK-STAT signaling required for Treg/Th17 cell differentiation, and also is associated with enhancing IL-12 production in DCs (Collins *et al.*, 2013).

Thirdly, cytokine signalling pathways are not only controlled by SOCS proteins, but are also controlled by other molecules and negative regulation pathways. These include protein tyrosine phosphatase inhibitors such as SHP-1 and CD45 and the protein inhibitors of activated STATs (PIAS) (Krebs and Hilton, 2001). PIAS are a family of proteins which interact with many transcription factors in the immune system through several mechanisms such as blocking the DNA-binding activity of transcription factors,

recruiting transcriptional co-repressors and promoting protein sumoylation (Shuai, 2006). SHP-1 negatively regulates cytokine signalling pathways by binding via their SH2 domains to phosphotyrosine residues of cytokine receptors and dephosphorylating signalling components such as the IL4 receptor, JAK2 (Wormald and Hilton, 2004).

In this project, we encountered some limitations which may cause difficulties in interpretation of the results and needs to be considered for future experiments:

1) RAS was used as a disease control in this project since their oral lesions are quite similar to BD. Unfortunately; we were not able to recruit a large number of volunteers to have reasonable sample size for robust statistical analysis. The reason for this is that, individuals who referred to dental hospital for treatment of RAS are reluctant to donate samples for research. We were therefore restricted to a small number of generous colleagues, with RAS, who were willing to donate samples. Our BD patients were always very willing to donate samples (with the understandable exception of punch biopsies) and were always very interested in the research being carried out into their disease. Ideally, since most of the investigations in this project were systemic and on blood samples, samples from systemic auto immune or auto inflammatory diseases might make a more suitable disease control group.

2) In this project the findings on mRNA expression by qPCR and protein relative measurements by western blot, had weak correlations. Generally protein concentration is nearly close to phenotypic level, expresses the physiological status of a cell. However, transcription regulation, due to the regulation control at different levels between transcription and translation, is insufficient by itself to completely describes protein abundance (MacKay *et al.*, 2004). Several studies have been performed to compare mRNA expression and their corresponding protein levels and they found

imperfect and variable correlation between mRNA and protein levels (Shebl *et al.*, 2010). Therefore care must be taken in interpreting data about a protein when mRNA expression results are used.

3) Since SOCS1 and SOCS3 levels are regulated by post-transcriptional mechanisms, the qPCR experiments on buccal mucosal cells for mRNA level should have been confirmed by other protein detection methods such as immunohistochemical staining of oral ulcer biopsies. Unfortunately, BD is a rare disease in the UK, and recruiting enough patients has been always a problem and biopsy material was scarce. There are several studies being carried out in the department and some information on the expression of SOCS by immunohistochemical staining was recently carried out by Dr Sonia Vartoukian. There is some evidence of increased staining of SOCS proteins on the borders of the epithelium close to ulcer lesions and also in the basal lamina propria (data not shown). These findings will be part of a joint publication which is to be submitted for publication.

4) One of our main problems in this project was the stratification of our patient cohort; first, recruiting BD patient without any medication or categorising them into groups with the same type and dose of medication and secondly classifying them in groups with similar clinical symptoms proved very difficult and resulted in very small subgroups . This would have made statistical analysis extremely difficult.

5) There were some problems in sample preparation which could bias our results and gave some outliers and/or large standard deviations. As discuss earlier, BD is a rare disease in the UK and it takes time to gather enough cohort. For this reason some of our samples taken over a period of two years and there is some concern that storage of

samples may lead to some degradation and affect our results depending on type of the sample and the experimental method.

Altogether, SOCS expression studies in this project showed considerable changes of SOCS1-3 in BD. However these alterations were different in different cell compartments. In order to have a clearer picture of the SOCS function in BD, more experiments required looking at each individual cytokines and its pathway and comparing them with appropriate controls. However, the results do suggest the importance of investigating both the local environment of the oral mucosa and the systemic compartment together.

It is not clear if the activity in the local oral mucosa influences systemic activity or *vice versa*. However, from a clinical perspective Professor Fortune has observed that those patient who are able to control their mouth ulcers seem to have fewer sytemic manifestaions (personal communication and unpublished observsations).

6.2 Cytokines and Behçet disease

Since one of the key immunological features of BD is alterations in cytokine levels, the investigation of cytokine profile in the serum or saliva of these patients helps to elucidate the aetiopathogenesis of BD and also might provide diagnostic or activity markers for the disease. Therefore, the serum cytokine profile in a UK cohort of 58 BD patients was investigated. Since many samples gave undetectable values and some had very large range of values, making comparisons were very difficult. Generally our results showed elevated levels of IL-8, TNF- α and IL-1 β in BD patients compared to

healthy controls but all failed to reach significance. A similar study on serum cytokine profile of UK cohort has been done by Curnow *et al.*, 2008. There are similarities and differences between the results of these studies. They found higher level of IL-8 and TNF- α in BD compared to HCs and lower level in relapsed than quiet patients which were in agreement with our findings. However they reported higher level of IL-6 as well which were undetectable in our experiments. However, we were able to detect high numbers of IL-6 secreting cells in BD samples using the ELISPOT method. On the other hand, while IL-10 and IL-4 were undetectable in the Curnow study, they were positive in 24 and 26 out of 79 of our samples respectively. We could not conclude any specific cytokine profile suggesting either a Th1 or Th2 profile, which was in agreement with the study of Curnow *et al* 2008, and also similar to previous findings of our group when investigating chemokine and cytokine expression in ulcers of BD patients (Dalghous *et al.*, 2006). Other studies on serum cytokine level in BD have been controversial. Several studies have confirmed higher level of proinflammatory cytokines such as IL-1 α , IL-1R, and IL-1 β in the serum of patients with BD (Zhou *et al.*, 2012, Düzgün *et al.*, 2005). In addition, genetic studies have established significant association of IL-1 polymorphism with increased susceptibility for BD (Karasneh *et al.*, 2003).Düzgün *et al* have reported significant higher serum level of TNF- α and its soluble receptor (TNFR1) in patient with active BD in comparison with controls (Düzgün *et al.*, 2005). The association of TNF- α polymorphisms with Behçet's disease in various ethnic groups supported the involvement of TNF- α (Touma *et al.*, 2010). Evereklioglu *et al* reported significant higher level of IL-8 and TNF- α in active BD patients than those in inactive phases of the disease(Evereklioglu *et al.*, 2002). This finding is completely different from Curnow *et al.*, 2008 and our study. In our largest study, using 58 serum samples there appeared to be a small significant difference between BD relapsed and quiet ($P<0.0406$). However, there were several samples where

no IL-8 was detected and this may have skewed the result. If only those sample where IL-8 was detectable were compared, there was no difference. We further investigated a small cohort of matched serum and saliva samples and again there was no significant difference between BD quiet and relapsed. However, when PBMCs were cultured with or without Human HSP70 there was a significant differences in the levels of IL-8 produced. The IL-8 level were significantly higher in unstimulated BD-RE cultures than the HCs while following stimulation, the stimulation index was significantly higher in HCs than BD suggesting that further stimulation of BD-RE could not induce further secretion of this pro-inflammatory cytokine.

As stated earlier, Th1 cells are responsible for directing cell mediated immunity by releasing cytokines such as IFN- γ and IL-12. Most of the studies are in favour of Th1 polarization of the immune response in Behcet's disease (Frassanito *et al.*, 1999, Zhou *et al.*, 2012). In our study, IFN- γ and IL-12p70 was found in only 7 and 9 out of 79 samples. The Curnow study supported our results and found very similar results.

IL-4, IL-5, IL-13 and IL-10 are produced by Th2 cells to promote humoral immunity. Remmers (2010) reported the association of IL-10 variant with decreased levels of both mRNA and protein expression in BD (Remmers *et al.*, 2010). Generally, there is no data regarding a possible role of IL-4, IL-5 in BD. Our results did not detect any IL-4 and IL-5 and mean level of IL-10 slightly decreased. In Curnow study IL-4 and IL-10 were undetectable. Although autoantibodies to several self-proteins are known to be induced in BD and these Th2 cytokines might support their production (Pineton de Chambrun *et al.*, 2012). However, we were able to demonstrate a decreased ability of PBMC's fro BD pateints to produce IL-10 in culture following stimulation with Human HSP70,

suggesting that the capacity for the production of suppressive cytokines is somehow depressed in BD compare with healthy controls.

The cytokine analysis was repeated but this time with 37 matched serum and saliva samples. Interestingly, the range of cytokines in serum and saliva were very different and some seemed to be more abundant in the saliva than serum. Saliva IL-6 was significantly higher in BD patients and RAS compared to HCs. Saliva IL-6 levels were also significantly higher in relapsed BD versus quiet BD and was higher in the saliva of patients with ulcers than without it. Saliva TNF- α level was higher in BD relapsed and patient with oral ulcer compared with HCs. The data on salivary cytokine profile in Behçet disease is limited. In a study on salivary cytokines in patients with recurrent aphthous ulceration, significant differences in salivary TNF- α between healthy controls and patients with acute RAU especially during the remission period were found. (Boras *et al.*, 2006). They did not report any differences in salivary IL-6 between all three groups. Our data did not show any differences between RAS and healthy controls in both TNF- α and IL-6. In the analysis of salivary cytokines the contribution of any serum transudate was not taken into account and in future experiments it would be important to measure the levels of serum albumin, for example, as a potential measure of the amount of transudation. Similarly the oral hygiene of patients needs to be monitored as levels of gingivitis and periodontal disease will contribute to serum transudation.

It has been suggested that HSP70 might mediate innate and adaptive immune responses and contribute in proinflammatory cytokine activation and tissue destruction in BD (Birtas-Atesoglu *et al.*, 2008). HSP70 has ability to bind with high affinity to the plasma membrane, activating nuclear factor (NF)- κ B and upregulation the expression of pro-

inflammatory cytokines such as TNF- α , IL-1 β and IL-6 in human monocytes (Asea *et al.*, 2000). Significant up regulation of both free human HSP70 and anti-HSP70 antibodies in patients with BD have been observed (Birtas-Atesoglu *et al.*, 2008). Our group has also shown that HSP70 was elevated in the saliva of BD patients and this correlated with serum IgG specific for the antigen (Bergmeier *et al.*, 2009). As pointed out earlier, subcutaneous injection of HSP peptide 336-51 with adjuvant and oral and nasal administration without adjuvant are able to induce experimental autoimmune uveitis in rats (Phipps *et al.*, 2003). Furthermore, heat shock proteins have been considered as candidate self-antigens in Behçet's disease. In this study, cytokine secretion profile was investigated in PBMCs from BD patients, following 24 hours stimulation with HSP70. It was demonstrated that anti-HSP70 antibodies can develop and IL-8 and TNF α production was induced by HSP70 in human peripheral blood monocytes, possibly through activation via TLR-4 (Yokota *et al.*, 2006a, Yokota *et al.*, 2006b).

Surprisingly, IFN- γ , IL-17, IL-4, IL-5 and TNF- β all fell outside of the detection range. Both IL-8 and IL-10 were significantly upregulated following stimulation in HCs and BD cell cultures. Comparing study groups in unstimulated cells showed significant upregulation of IL-8 in BD-RE and overall combined BD compared with HCs. In contrast, following stimulation, BD or RE displayed a significant decrease in IL-8 in comparison to HCs which is contrary to expectations. For IL-10, there was no significant difference in the spontaneous secretion of IL-10 in unstimulated cultures. Measurement of the stimulation index again showed significant decrease in BD compared to HCs suggesting that BD cells were less able to respond to stimulation by Human HSP70.

The results obtained from the cytokine profile experiments in BD (chapter 4) are in agreement with some of the previous studies but not all of them. There are several possible explanations for these differences and some contradictory results:

1) There are complications in patient selection as Behçet disease is multi-systemic and recruiting patients who display only one organ involvement or similar multi organ involvements is difficult. Similarly, there are differences in categorizing patients in different studies. Some studies consider “active” as having activity in even one organ and some considering active or relapsed patient as having at least activity in three organs as described by the International Study Group.

2) Our cohort was largely white British in origin, therefore the role of environmental or genetic factors in the aetiopathogenesis of BD will be different from studies carried out in other countries with different genetic and environmental backgrounds. The RAS subjects used in this study were self identified as healthy and their ulcers when present were all of the minor type. The subjects were not on any medication for their condition.

3) Using different methodologies is another important factor which affects comparing results from different experiments. ELISA techniques are widely accepted as the gold standard to quantify cytokine expression. ELISAs are well-matched for single parameter analysis, but it is time-consuming and expensive when performing multi-parameter analyses. A multiplex bead array assay (MBAA) was used in this project. This system is able to distinguish simultaneously up to 20 different protein targets in just single small volume sample. Moreover, MBAA assays are easy to perform and reproducible. Although the majority of studies have presented good correlations between MBAA and ELISAs for the cytokines tested, the degree of correlation differs extensively due to

(possibly) the methods of comparison and the type of antibodies used (Elshal and McCoy, 2006). All in all, it is recommended that any study which involves consecutive follow up of patients, or samples from different time points, are better carried out using only a single technique, one platform, and one commercial company for all samples (Khan *et al.*, 2004).

4) Some of the differences in type of cytokines responses resulted from studies on cellular production of cytokines and following stimulations such as LPS or PHA. PHA is a polyclonal stimulator and while LPS is also able to activate many cell types it does so through specific binding to such molecules as CD14 and TLR4. Therefore the activation pathways and resulting cytokines may be very different.

5) The effect of type of treatment in BD patient on cytokine responses is complicated. Azathioprine works by inhibiting purine synthesis, which is required for proliferation, and iNOS production, that leads to inhibition of cell migration (Curnow *et al.*, 2008). It has been suggested that Colchicine inhibits neutrophil activity and migration, but it does not affect CXCL8 levels (Miller EJ, 1993). As a result these drugs may inhibit the migration of leucocytes into inflammatory locations, but not essentially the chemokines and cytokines implicated in this process (Curnow *et al.*, 2008).

6) There are some important considerations in sample collection which cause variation in the results such as detection range, serum versus plasma, several rounds of freeze-thawing and sample storage time. It has been shown that cytokines including IL-13, IL-15, IL-17 and CXCL8 began to be degraded within one year of storage, while levels of IL-1 α , IL-1 β , IL-5, IL-6, and IL-10 were degraded by over 50% in 2-3 years (de Jager *et al.*, 2009). In a small pilot study we have observed greater proteolytic activity in the

saliva of BD patients which may contribute to loss of cytokines (data not shown). This needs to be further investigated.

It is generally accepted that cytokines play critical roles in the initiation and perpetuation of Behçet disease. From this study, no classic Th1, Th2 or Th17 response was concluded. Higher levels of proinflammatory cytokines TNF- α and IL- β were observed. Serum IL-8 was in the past considered as a disease activity marker, since it has been shown to be significantly higher in quiet BD patient than relapsed (Durmazlar *et al.*, 2009). However, the data in this study does not support those findings. Salivary IL-6 can also consider as an activity marker in BD patient with oral ulcer. IL-6 has been associated with the incution of a Th17 profile but Th17 cytokines wer not readily detectable in this stusdy. Further investigation, especially functional assays is needed to understand the pathological and clinical effect of cytokines in BD.

6.3 STAT Phosphorylation profile

Cytoplasmic and nuclear transcription factors play an important role in regulation of T cell differentiation and cytokine pathways. The, STAT transcription factor family especially STAT1, STAT2, STAT3, STAT4, STAT5a/b and STAT6 are involved in modulating inflammatory responses. Apart from their important role in JAK-STAT signalling pathway, STATs gene family are critical for Th cells differentiation. It is now clear that this pathway might directly relate to human disease including Behçet's disease. Polymorphisms of the genes encoding IL-23, JAK2, TYK2, and STAT3 have all been linked to autoimmune diseases such as psoriatic arthritis, Grave's disease, Crohn's disease and multiple sclerosis (o'shea and plunge 2012). GWAS studies have shown associations of IL23R/IL-12RB2, IL-12 and STAT4 with Behçet disease

(Remmers *et al.*, 2010). Therefore, transcriptional regulation of Th1/Th17 polarization in BD was investigated in this project.

In order to activate and bind to DNA, most of transcription factors including STATs must be phosphorylated in advance. Thus, the phosphorylation profiles of kinases and their protein substrates were analysed in BD by the Proteome Profiler Human Phospho-Kinase Array. Using this method, we were able to look at a total of 38 kinase phosphorylation sites simultaneously. Our results showed marked difference of STATs family in BD patient compared to healthy controls. STAT1, STAT2, STAT3 and STAT4 were significantly higher in BD compared to HCs. STAT6 were greater in BD than HCs but failed to reach significance. All the STAT5 proteins: STAT 5a, STAT 5b and STAT 5a/b displayed a significant rise in BD patients compared to HCs.

There are very a very limited number of publications regarding STATs activity in BD and the existing studies have been performed using different methodologies, therefore comparison of our results with previous findings were quite difficult. This study has attempted to investigate the transcription factors in PBMCs and in CD4⁺ T cells using two different methodologies and our findings are interesting but not conclusive.

The implication of the Th1 /Th17 polarization and increased level of Th1 or Th17 cytokines in Behcet's disease was subjected to several studies (Frassanito *et al.*, 1999, Zhou *et al.*, 2012). Recent studies have revealed simultaneous increased level of both Th1- and Th17- cytokines in these patients (Shimizu *et al.*, 2012).

Differentiation into Th1 cells is dependent on STAT1 and STAT4 acting via T-bet to produce the signature cytokine of Th1, namely IFN γ . This process is inhibited by SOCS1 and probably activated by SOCS3. For a Th17 profile, ROR γ t is dependent on

STAT3 activation and is inhibited by SOCS 3 but stimulated by SOCS1. STAT 5 acts to upregulated the induction of Tregs through Foxp3 and is probably inhibited by SOCS1 but the role of SOCS 3 is yet to be unraveled (Yoshimura *et al* 21012). The experiments carried out in this study were designed to investigate this profile in BD compared with HC and RAS subjects.

The findings of the current study are consistent with previous studies confirming Th1- and Th17 polarization of BD. Increased phosphorylation of STAT 1 and 4 would be consistent with activation of a Th1 differentiation pathway and increased phosphorylation of STAT 3 is in accordance with Th17 differentiation pathway.

From all the STATs, only STAT6 did not show any remarkable differences in BD. This result may be explained by the fact that STAT6 is involved in the development of Th2 Cells differentiation and mediating responses to IL-4 and most of the previous studies did not suggested Th2 polarization in BD. Moreover, there is no data available regarding the possible role of classical Th2 cytokines; IL-4, IL-5 and IL-13 in BD. 10 Our data showed significant upregulation of STAT5 proteins which are associated with Tregs differentiation, in BD. Increased number of CD4+CD25+ Treg cells in the peripheral circulation of active BD patients have been reported (Hamzaoui *et al.*, 2006). They suggested that high level of CD4+CD25+ T cells could be important in the regulatory process of the inflammation in active BD. The investigation of Tregs was beyond the scope of the project, but the results suggest the role of STAT 5 in the differentiation of these cells and would be a very interesting further study.

It has been suggested that STAT 2 is associated with the defence against virus as STAT2 deficient mice display an increased susceptibility to viral infection (Park *et al.*, 2000). Our results showed higher level of STAT2 in BD than HCs, but this finding

needs to be confirmed using larger sample size and further methodology as no data has been reported so far.

We identified seven new kinase phosphorylation sites including AMPK α 1, Yes, Fyn, Fgr, FAK, RSK1/2/3 and CRBE which revealed significant increase in BD compared to HCs. To our knowledge these proteins have not been investigated in BD.

Protein kinase (AMPK)- α 1 has a key regulatory role in NF- κ B-dependent gene expressions in inflammatory signalling mediated by and TNF- α stimulation (Jung *et al.*, 2012) . The important role of proinflammatory cytokines including TNF- α in BD has been discussed earlier and TLR4 is the primary recognition molecule for LPS and HSP which both are considered as triggering factors in BD by driving TNF α production..

Yes, Fyn and Fgr are the members of Src family kinase which are non-receptor tyrosine kinases. Many studies have shown the various roles of Src family in macrophage-mediated innate immunity, such as phagocytosis, the production of inflammatory cytokines/mediators, and the induction of cellular migration (Byeon *et al.*, 2012). In this respect investigation of these signalling molecules could be important in finding the pathogenesis of many inflammatory diseases. The infiltration of inflammatory cells into the oral mucosa might be dependent on the upregulation of these molecules.

Ribosomal s6 kinase (rsk) is a family of protein kinases involved in signal transduction and regulates diverse cellular processes, such as cell growth, cell motility, cell survival and cell proliferation (Anjum and Blenis, 2008). Again, migration will be dependent on changes that allow cells to move through tissue and may be key to the infiltration of cells producing pro-inflammatory cytokines at sites of pathology.

CREB (cAMP response element-binding protein) is another transcription factor and mostly investigated in brain. In neurons, a wide range of extracellular stimuli are able to activate CREB family members. CREB-dependent gene expression has been implicated in different functions ranging from development to implication in disease (Bonnie and David, 2002). Since many aspects of the pathology of BD involve neurological involvement, this might be a signature of this end of the BD spectrum of pathologies.

These results were very exciting as they have revealed new targets of investigation and may also reveal drug targets for better management of the disease.

We analysed phosphorylation status of STAT1, STAT2, STAT3, STAT5 and the transcription factors T-bet and ROR γ t in CD4⁺ T Cells following activation with IFN- α . STAT1, STAT5a and STAT5b showed significant upregulation following stimulation with IFN α but STAT 3 did not show any change following stimulation.

Our finding did not reveal any significant differences in pSTAT 3 and pSTAT 5 between study groups. We found lower mean stimulation index of pSTAT5 in BD-RE in comparison to HCs but it failed to reach significance. T-bet showed higher level in BD-RE compared to BD-Q and ROR γ t slightly increased in BD compared to HCs but both just failed to reach significance.

JAK-STAT signalling is one of the best characterized IFN-signalling pathways (Stark, 2007). IFN- α can activate several members of the STAT transcription factor family. STAT1, STAT2, STAT3 and STAT5 have been found to be phosphorylated by IFNs-I (Stark, 2007)

In a study on 73 BD patients in Tunisia , significant higher levels of mRNA relative expression for IL-17 and ROR γ t in active BD patients when compared to healthy controls and to remission BD patients has been reported (Hamzaoui *et al.*, 2011). In a study by Li (2003), upregulation of T-bet mRNA was detected in BD patients compared to controls but no significant difference was found between BD patients with active uveitis and normal controls regarding the expression of either T-bet mRNA or its protein after stimulation with PHA (Li *et al.*, 2003).

The results of these two methods did not support each other. Proteome profiler is a semi quantitative assay with multi analyse profiling but less sensitive while Phosflow is quantitative, single cell analysis method with high sensitivity. In Proteome profiler we looked at PBMCs but in Phosflow analysis CD4+Tcells were considered. Moreover in Proteome profiler, only three samples were examined in each study group while in Phosflow; around eight samples exist in each group. To gain comprehensive results, more preliminary optimization experiments and larger sample size are needed.

7: Conclusion and Future work

The present study was designed to determine the SOCS expression in Behçet's disease. Altogether, SOCS 1-3 expression showed considerable changes in Behçet's disease. Up-regulation of SOCS expression might considerably affect cellular reaction to antiinflammatory cytokines in BD which leads to disease progression.

.However these alterations were quite different in different cell compartments and localization. This is an important observation as using peripheral blood to investigate disease in local tissues may not give correct information. In order to have clearer picture of the SOCS function in BD, more experiments are required looking at each individual

cytokine pathway and its regulation by SOCS and comparing them with appropriate controls.

From cytokine studies in this project, no classic Th1, Th2 or Th17 response was concluded. Higher levels of proinflammatory cytokines TNF- α and IL- β were observed.. Salivary IL-6 can also be considered as an activity marker in BD patient with oral ulceration. Further investigations, especially functional assays are needed to understand the pathological and clinical effect of cytokines in BD.

This project showed significant upregulation of STAT1, STAT2, STAT3, STAT4 and STAT5 in BD compared to HCs which is consistent with involvement of a Th1, Th17 and Treg cells differentiation pathways.

We identified seven new kinase phosphorylation sites including AMPKa1, Yes, Fyn, Fgr, FAK, RASK123 and CRBE in BD, all involve in signalling pathways of inflammatory cytokines and mediators. To our knowledge these proteins have not been investigated in BD.

Addressing cytokine regulation will greatly boost our understanding of the immune pathogenesis of Behçet's disease and may considerably contribute to the study on strategy of the new treatment to prevent exacerbation of symptoms in these patients.

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Appendixes

Appendix 1	Ethical Approval of “Immunoregulation at the mucosal barrier”
Appendix 2	Patient Invitation Information
Appendix 3	Healthy Volunteer Information
Appendix 4	Written Consent Form
Appendix 5	Clinical Disease Activity Forms
Appendix 6	Patient Perception form
Appendix 7	Average expression stability value (M) of reference genes

Appendix 1: Ethical Approval of “Immunoregulation at the mucosal barrier”



Central Office for Research Ethics Committees (COREC)

NOTICE OF SUBSTANTIAL AMENDMENT

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) at <http://eudract.emea.eu.int/document.html#guidance>.

To be completed in typescript by the Chief Investigator in language comprehensible to a lay person and submitted to the Research Ethics Committee that gave a favourable opinion of the research (“the main REC”). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available at <http://www.corec.org.uk/applicants/apply/amendments.htm>.

Details of Chief Investigator:	
Name:	Professor Farida Fortune
Address:	Clinical & Diagnostic Oral Sciences Blizard Building 4 Newark Street Whitechapel, London E1 2AT
Telephone:	020 7882 7158/4
E-mail:	f.fortune@qmul.ac.uk
Fax:	

Full title of study:	Immunoregulation at the mucosal barrier
Name of main REC:	East London & the City
REC reference number:	P/03/122
Date study commenced:	November 2003
Protocol reference (if applicable), current version and date:	
Amendment number and date:	Amendment 2, 14 th November 2006

Notice of amendment (non-CTIMP), version 3.1, November 2005

Type of amendment (indicate all that apply in bold)

(a) Amendment to information previously given on the REC application form

Yes No

If yes, please refer to relevant sections of the REC application in the "summary of changes" below.

(b) Amendment to the protocol

Yes **No**

If yes, please submit either the revised protocol with a new version number and date, highlighting changes in bold, or a document listing the changes and giving both the previous and revised text.

(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study

Yes **No**

If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold.

Is this a modified version of an amendment previously notified to the REC and given an unfavourable opinion?

Yes **No**

Summary of changes

Briefly summarise the main changes proposed in this amendment using language comprehensible to a lay person. Explain the purpose of the changes and their significance for the study. In the case of a modified amendment, highlight the modifications that have been made.

If the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study, supporting scientific information should be given (or enclosed separately). Indicate whether or not additional scientific critique has been obtained.

New staff to be added for indemnity:

This refers to section 7.7 of the original application : Insurance & Indemnity
Dr Lesley Bergmeier, Senior Lecturer in Applied Mucosal Immunology.
Dr Eleni Hagi-Pavli, Lecturer in Oral Sciences.
Mrs Noha Seoudi, SPR in Oral Microbiology.

Staff to be removed from indemnity:

Dr Paula Farthing, Prof. David Williams, Dr Supriya Kapas.

Any other relevant information

Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.

No additional ethical issues

List of enclosed documents No additional documents – changes outlined in Summary of Changes above.

Document	Version	Date

Declaration

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Chief Investigator: F. Fortune

Print name: F. FORTUNE

Date of submission: 14th November 2006

East London and The City Research Ethics Committee 1

Aneurin Bevan House, 81 Commercial Road, London E1 1RD

Telephone Number: 020 7 655 6718

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Email Address: Sandra.Burke@nelondon.nhs.uk

Professor Farida Fortune
Clinical & Diagnostic Oral Sciences
Blizard Building
4 Newark Street
Whitechapel
London E1 2AT

29 January 2007

Dear Professor Fortune

Study title: Immunoregulation at the Mucosal Barrier
REC reference: P/03/122

Amendment number: 2
Amendment date: 14 November 2006

The above amendment was reviewed at the meeting of the Sub-Committee of the REC held on 24 January 2007.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Notice of Substantial Amendment (non-CTIMPs)		14 November 2006
Covering Letter		

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are Dr A. T. Tucker and Prof A Johnston.

An advisory committee to London Strategic Health Authority

All investigators and research collaborators in the NHS should notify the R&D Department for the relevant NHS care organisation of this amendment and check whether it affects research governance approval of the research.

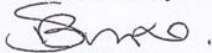
Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

P/03/122:

Please quote this number on all correspondence

Yours sincerely



Sandra Burke
Senior Committee Co-ordinator
East London & The City Research Ethics Committees

Copy to: R&D Department

Appendix 2: Patient Invitation Information

INVITATION TO PARTICIPATE (Patients)

Immunoregulation at the mucosal barrier (Version 1: February 2004)

Bart's and The London Queen Mary's School of Medicine and Dentistry, University of London

We invite you to take part in a research study which we think may be important. The information which follows tells you about it. It is important that you understand what is in this leaflet. It says what will happen if you take part and what the risks might be. Try to make sure you know what will happen to you if you decide to take part. Whether or not you do take part is entirely your choice and any decision will not affect the treatment you receive. Please ask any questions you want to about the research and we will try our best to answer them.

Why have we approached you?

You have been invited to participate in this study because you have an inflammatory condition which may affect the mouth. We wish to collect some tissue from your mouth (biopsy), saliva, urine and blood samples.

This is because we think that inflammation in the mouth causing ulceration may have a more widespread effect in determining whether you may develop other features of your particular illness. Mouth ulceration may predict severity or activation of your disease. You are unlikely to benefit directly from this research yourself, but we hope that the results of these studies will benefit similar patients in the future.

What would I do in the study if I took part?

If you agree to participate in this study you will initially be asked to complete a confidential health questionnaire; you will also undergo a physical examination, including blood pressure, pulse rate and heart rhythm. We will also require a routine

blood sample from your arm (about an egg-cup full, 50 ml), a samples of spit (1/2 egg cup, 25 ml) and a urine sample (10 ml). A drug of abuse screen will also be performed

If you are female, we will ask your permission to perform a pregnancy test; we would prefer that you do not to take part in this study if you are pregnant. None of the tests we intend to do will harm an unborn baby, but the mucosa (tissue) heals less well in pregnancy. We would prefer to avoid biopsies in pregnancy unless absolutely necessary. Please inform us if you think you may be pregnant.

There are several components to the study:

On entry to the study we will take a blood sample and saliva. The blood sample will be taken as part of your routine screen. We will take 50 ml (1 egg cup full) of blood, 25 ml (1/2 egg-cup) of spit and 10ml of urine. Blood tests may cause a very small discomfort when the needle is put into the vein, but this is momentary. The saliva will be collected by asking you to spit into a small container over a period of not more than 5 mins. You will collect the urine sample in private.

Another two blood tests requiring the same amount of blood will be taken over the period of study.

A small mouth biopsy (6 mm, about the size of the tip of your small finger).will be taken from the inside of your cheek or lip in an area where there is an ulcer or the tissue is very inflamed. You will be given a pain killing injection which will numb the area to be biopsied. This is similar to having an injection at your dentist. This painkilling injection does sting, but only momentarily, and it is very effective and works quickly. You will not be able to feel any discomfort for the rest of the procedure. A stitch will be used to hold the mucosa together afterwards; this will need to be removed by us 10 days after the procedure. Each mouth sample will be frozen and stored prior to examination

using molecular or cellular technology. We would like to see what is happening in the inflamed and ulcerated areas.

You are very unlikely to develop any problems following the above tests. Very occasionally, however, a mucosal biopsy or a blood test can be associated with bruising, bleeding or infection.

The above tests will involve you attending either the Royal London Hospital or St. Thomas' Hospital depending on where you are registered as a patient.

If you have any questions concerning this study please feel free to ask us .We can provide you with further information if you wish. Contact names and telephone numbers are given below.

Tissue samples taken from you may be stored for a period of no more than 5 years, and will not be used for any other purposes other than this study.

Similarly, any blood samples taken from you will only be used for this study.

You don't have to join the study. You are free to decide not to be in this trial or to drop out at any time. If you decide not to be in the study, or drop out, this will not put at risk your ordinary medical care.

What happens if you are worried or if there is an emergency? You will always be able to contact an investigator to discuss your concerns and/or to get help:

Name: Prof.F.Fortune

Address: Centre for Clinical and diagnostic Oral Science, Clinical Sciences Building, 2 Newark Street, London E1 2AT

Telephone number: 020-7882 7154/8

What happens if something goes wrong?

We believe that this study is basically safe and do not expect you to suffer any harm or injury because of your participation in it. However, Barts and The London NHS Trust has agreed that if your health does suffer as a result of your being in the study then you will be compensated. In such a situation, you will not have to prove that the harm or injury which affects you is anyone's fault. If you are not happy with any proposed compensation, you may have to pursue your claim through legal action.

Appendix 3: Healthy Volunteer Information

INVITATION TO PARTICIPATE (Healthy Volunteers)

IMMUNOREGULATION AT THE MUCOSAL BARRIER

Bart's and The London Queen Mary's School of Medicine and Dentistry, University of London

Version 1: February 2004

We invite you to take part in a research study which we think may be important. The information which follows tells you about it. It is important that you understand what is in this leaflet. It says what will happen if you take part and what the risks might be. Try to make sure you know what will happen to you if you decide to take part. Whether or not you do take part is entirely your choice. Please ask any questions you want to about the research and we will try our best to answer them.

You have been invited to participate in this study because you are a healthy individual.

We wish to collect some tissue from your mouth (biopsy), saliva, urine and blood samples.

This is because we think that inflammation in the mouth may have a more widespread effect in determining whether patients develop other features of their particular illness.

Mouth inflammation may predict severity or activation of systemic disease or disease at distant sites such as the eye.

Understanding the nature of this may allow development of preventative treatments or strategies to reduce systemic effects of oral inflammation in patients with oral mucosal disease. You are unlikely to directly benefit from this research yourself, but we hope that the results will benefit patients in the future.

We require healthy volunteers in order to find out whether the effects of inflammation on the mucosa of patients are different to the mucosa of healthy volunteers.

What would I do in the study if I took part?

If you agree to participate in this study you will initially be asked to complete a confidential health questionnaire; you will also undergo a physical examination, including blood pressure, pulse rate and heart rhythm. We will also require a routine blood sample from your arm (about an egg-cup full), a sample of spit (1/2 egg-cup) and a urine sample.

If you are female, we will ask your permission to perform a pregnancy test; as we would prefer you not to take part in this study if you are pregnant. None of the tests we intend to do will harm an unborn baby, but the skin heals less well in pregnancy and we would prefer to avoid surgery in pregnancy unless absolutely necessary. Please inform us if you think you may be pregnant.

There are several components to this study:

On entry to the study we will take a blood sample and saliva. The blood sample will be taken as part of your routine screen. We will take 50 ml (egg cup full) of blood, 25ml (1/2 egg cup of spit) and 10 ml of urine. Blood tests may cause a very small discomfort when the needle is put into the vein, but this is momentary. The 25 ml of saliva will be taken by asking you to spit into a small container over a period of not more than 5 mins. The urine sample will be collected in private. Another two blood tests requiring the same amount of blood will be taken over the period of study.

A small mouth biopsy will be taken from the inside of your cheek or lip. This will be 6mm (the size of the tip of your small finger). You will be given a pain killing injection which will numb the area to be biopsied. This is similar to having an injection at your dentist. This painkilling injection does sting, but only momentarily, and it is very effective and works quickly. You will not be able to feel any discomfort for the rest of the procedure. A stitch will be used to hold the mucosa together afterwards; this will need to be removed by us 10 days after the procedure. Each mouth sample will be

frozen and stored prior to examination using molecular or cellular technology. You are very unlikely to develop any problems following the above tests.

Very occasionally, however, a mouth biopsy or a blood test can be associated with bruising, bleeding or infection

If you are female we will ask your permission to perform a pregnancy test, as we would prefer you not to take part in this study if you are pregnant. None of these tests will harm an unborn baby, but the skin heals less well in pregnancy and we would prefer to avoid surgery in pregnancy unless absolutely necessary. Please inform us if you think you might be pregnant.

The above tests will involve you attending either the Royal London Hospital or St. Thomas' Hospital not both (this will depend on the site that you normally attend clinic). Tissue samples taken from you may be stored for a period of no more than 5 years, and will not be used for any other purposes other than this study.

Similarly, any blood samples taken from you will only be used for this study.

If you have any questions concerning this study please feel free to ask us .We can provide you with further information if you wish. Contact names and telephone numbers are given below.

You don't have to join the study. You are free to decide not to be in this trial or to drop out at any time. If you decide not to be in the study, or drop out, this will not put at risk your ordinary medical care.

What happens if you are worried or if there is an emergency?

You will always be able to contact an investigator to discuss your concerns and/or to get help:

Name: Prof.F.Fortune

Address: Centre for Clinical and Diagnostic Oral Science, Clinical Sciences Building, 2 Newark Street, London E1 2AT

Telephone number: 020-7882 7169

What happens if something goes wrong?

We believe that this study is basically safe and do not expect you to suffer any harm or injury because of your participation in it. However, we carry insurance to make sure that if your health does suffer as a result of your being in the study then you will be compensated. In such a situation, you will not have to prove that the harm or injury which affects you is anyone's fault. If you are not happy with any proposed compensation, you may have to pursue your claim through legal action.

Appendix 4: Written Consent Form

WRITTEN CONSENT FORM:

Title of research proposal:

REC Number:

Name of Patient/Volunteer (Block Capitals):

Address:

(Delete if unnecessary to the research project)

- The study organisers have invited me to take part in this research.
- I understand what is in the leaflet about the research. I have a
copy of the leaflet to keep.
- I have had the chance to talk and ask questions about the study.
- I know what my part will be in the study and I know how long it will take.
- I have been told about any special drugs, operations, tests or other checks
I might be given.
- I know how the study may affect me. I have been told if there are possible risks.
- I understand that I should not **actively** take part in more than 1 research study at a time.
- I know that the local North East London Health Authority
Research Ethics Committee has seen and agreed to this study.
- I understand that personal information is strictly confidential: I know the
only people who may see information about my part in the study are the research
team or an official representative of the organisation which funded the research.
- I understand that my personal information may be stored on a computer. If this
is done then it will not affect the confidentiality of this information. All such storage
of information must comply with the 1998 Data Protection Act.
- I know that the researchers will/might tell my general practitioner (GP)
about my part in the study.
- I freely consent to be a subject in the study. No-one has put pressure on me.
- I know that I can stop taking part in the study at any time.
- I know if I do not take part I will still be able to have my normal treatment.
- As a medical/ nursing student or qualified staff, I understand that agreement or

- Refusal to take part will make no difference to the results of my course or my career.
- I know that if there are any problems, I can contact:



Dr/Mr/Ms.....

Tel. No. Bleep No./Ext.

Patient's/Volunteer's: Signature

Witness's signature

Title of research proposal:

REC Number:

Name of Patient/Volunteer (Block Capitals):

Appendix 5: Clinical Disease activity Forms

Behçet's Disease Clinical Activity Information Form


Date of sample		
Name		
Hospital Number		
Diagnosis:		
Behçet's Phenotype	Active: (tick if applies)	Quiet: (tick if applies)
Mouth		
Genital		
Eyes		
Joints		
Skin: Folliculitis Erythema nodosum		
Vascular		
CNS		
GI tract		
Fatigue		
BD Activity	Relapse:	Quiet:
Date of last respiratory tract infection (cold/flu etc):		
Past Medical history:		
Current drugs and Dosage:		
Current smoker?	Y/N Cigs/day for years
Past smoker?	Y/N	Date of quitting:
Ulcer Severity Score:		
BDCAF score:		

Appendix 6: Patient Perception form



BEHÇET'S DISEASE CURRENT ACTIVITY FORM

Date of assessment: Name: Tel:
Centre: Sex: M/F DOB:
Clinician: Address:


Self rating scale of wellbeing over last 28 days
(Patient to tick face chosen) 
Self rating scale of wellbeing today
(Patient to tick face chosen) 

CLINICAL FEATURES	ACTIVITY	(Other clinical details)
Fatigue	0 1 2 3 4	
Headache	0 1 2 3 4	
Oral ulceration	0 1 2 3 4	
Genital ulceration	0 1 2 3 4	
Skin lesions	erythema nodosum or superficial thrombophlebitis pustules	0 1 2 3 4
		0 1 2 3 4
		0 1 2 3 4
Joints	arthralgia arthritis	0 1 2 3 4
		0 1 2 3 4
Gastrointestinal	nausea or vomiting or abdominal pain diarrhoea with (per rectum) altered/frank blood	0 1 2 3 4
		0 1 2 3 4

Eye Is there eye activity? Yes / No
Behçet's Oculopathy Index 0 1 2 3 (completed by Ophthalmologist)

CNS Is there new nervous system activity? Yes / No (ask question overleaf)
(If "yes", answer questions below)
Q 1. Yes / No Q 2. Yes / No Q 3. Yes / No Q 4. Yes / No Q 5. Yes / No

Major vessel Is there new major vessel activity? Yes / No (ask question overleaf)
(If "yes", answer questions below)
Q 1. Yes / No Q 2. Yes / No Q 3. Yes / No Q 4. Yes / No

Clinician's impression of activity over last 28 days: 

Intention to initiate or change treatment? Yes / No
Current medication on arrival: Change in medication:
.....
.....
.....
.....

(a)

Scoring system for Activity form

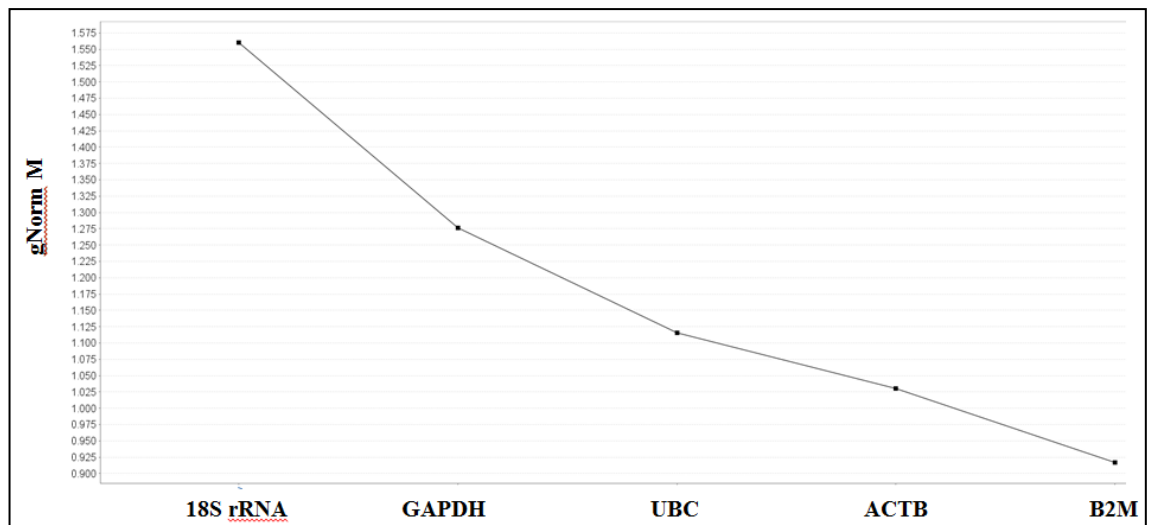
All scoring depends on the symptoms present over the preceding 4 weeks prior to assessment. Only clinical features that the clinician feels are due to Behçet's Disease should be scored.

- To complete the self rating scale of overall wellbeing for the last 4 weeks, please ask the patient the following question:
"Here are some faces expressing various feelings, thinking about your Behçet's disease only, which of these faces describes how you have been feeling over the last 4 weeks?"
To complete the self rating scale of wellbeing today, please ask the patient the following question:
"Here are some faces expressing various feelings, thinking about your Behçet's disease only, which of these faces describes how you feel today?"
- Scoring for fatigue, headache, oral and genital ulceration, skin lesions, joint symptoms, and gastrointestinal symptoms is based on duration of symptoms (*round up to nearest week*). Please ask the following question and fill in the blank with the organ system to be assessed:
"Over the last 4 weeks, for how many weeks in total have you had" ?
0 no symptoms
1 symptoms for 1 week (1 - 7 days in total)
2 symptoms for 2 weeks (8 - 14 days in total)
3 symptoms for 3 weeks (15 - 21 days in total)
4 symptoms for 4 weeks (22 - 28 days in total)
- Eye involvement
Eye activity may be present if the following symptoms are present: (1) red eye, (2) blurred vision, (3) painful eye.
Please ask the following question (*Tick if symptom present*):
"Over the last 4 weeks have you had a red eye, a painful eye, or blurred or reduced vision?"
If any of these symptoms are present, or if you feel there may be eye activity refer patient to ophthalmologist who will determine the eye score (Behçet's Oculopathy Index)
- Nervous system
Please ask the following question (*Tick if symptom present*):
"Over the last 4 weeks have you had any blackouts, difficulty with speech or hearing, double vision, weakness or loss of feeling in the face, arm or leg, memory loss or loss of balance?"
If the answer to all of these is "no" then answers to Q 1-5 are deemed negative; otherwise determine the following:
Q 1. Are there new symptoms or signs consistent with meningeal involvement?
Q 2. Are there new symptoms or signs consistent with isolated cranial nerve involvement?
Q 3. Are there new symptoms or signs consistent with brainstem or cerebellar involvement?
Q 4. Are there new symptoms or signs consistent with cerebral hemisphere involvement?
Q 5. Are there new symptoms or signs consistent with spinal cord involvement?
- Major vessel involvement (exclude neurological involvement)
Please ask the following question (*Tick if symptom present*):
"Over the last 4 weeks have you had chest pain, breathlessness, coughed up blood, or had any pain, swelling or discoloration of either the face, arm or leg?"
If the answer to all of these is "no" then answers to Q 1-4 are deemed negative; otherwise determine the following:
Q 1 Are there new symptoms or signs consistent with peripheral deep venous thrombosis?
Q 2 Are there new symptoms or signs consistent with central deep venous thrombosis?
Q 3 Are there new symptoms or signs consistent with peripheral arterial thrombosis/aneurysm?
Q 4 Are there new symptoms or signs consistent with pulmonary arterial thrombosis/aneurysm?

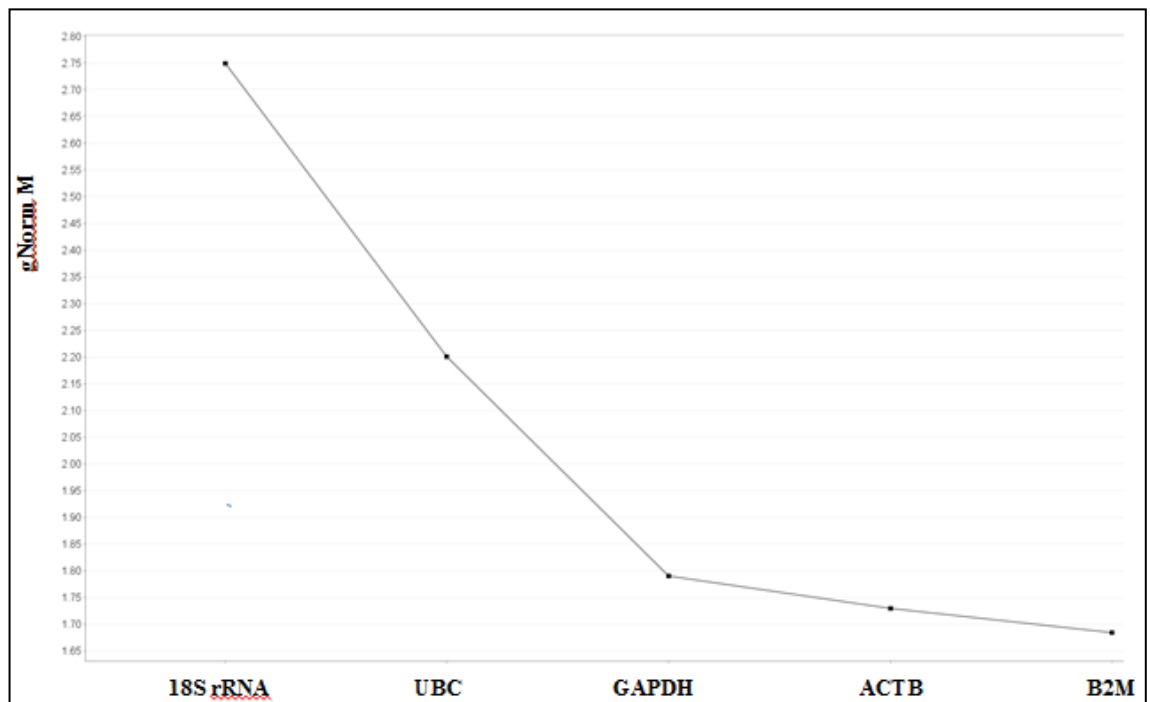
(b)

Appendix 7: Average expression stability value (M) of reference genes

Average expression stability value (M) of reference genes in Neutrophil samples



Average expression stability value (M) of reference genes in PBMC samples



Appendix 8: Graphs of the remaining Phospho-kinase activities with

no significant differences

