ORAL HEALTH STATUS OF BEHÇET'S SYNDROME PATIENTS IN THE UK

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ABSTRACT

Objectives

This thesis aimed to study the inter-relationship between the oral health status, aspects of the adaptive and innate immune response, and the oral microbiome of a well-defined cohort of Behçet's syndrome (BS) patients.

Methods

Patients thought to have BS were referred to one of the two centres participating in this study (St Thomas' Hospital and Royal London Hospital). Diagnosis was based on the International Study Group (ISG) criteria. Information about the frequency of the different symptoms of the disease along with the treatment protocol was collected on a data sheet and stored electronically in a database, in the period between January 2006 and August 2012. The data were then analysed at the end of the study period.

The quality of life (QoL) and oral health status of BS patients in the UK was compared to those from Turkey. Furthermore, the oral health status of BS patients in the UK was compared to that of healthy control (HC) volunteers and recurrent aphthous stomatitis (RAS) patients. Saliva and oral swabs were cultured and purified to homogeneity and bacteria were identified by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF). Salivary viral load and the serum immunoglobulin (Ig) G of the different herpes viruses were also examined.

In order to determine whether there were differences in the innate response efficacy in BS patients, the expression of key molecular determinants of pathogen recognition was also

analysed. Total ribonucleic acid (RNA) was purified from non-ulcerated buccal mucosal brush biopsies and analysed by real time polymerase chain reaction (qPCR) for the presence of toll-like receptor (TLR) 2 messenger RNA (mRNA) and TLR4 mRNA, and their splice variants. The functions of TLR2 and TLR4 were also investigated.

Results

In the investigated cohort of UK BS patients, all patients had a history of recurrent oral ulceration, 85.6% had dermatological lesions, 79.1% had rheumatological manifestations, 73.9% had genital ulceration, 68.6% had ocular involvement, 15% had neurological manifestations, and 10.5% had vascular involvement. The most frequent treatment was colchicine (54.2%), followed by topical corticosteroid therapy (53.6%) then azathioprine (43.8%).

The QoL of BS patients from the UK and Turkey was affected to a similar extent. The Turkish BS patients had generally poorer oral health status in comparison to the UK BS patients. Nevertheless, the UK BS patients had also generally poorer oral health status in comparison to HC volunteers. The oral health status of the UK BS patients was comparable to those suffering from RAS.

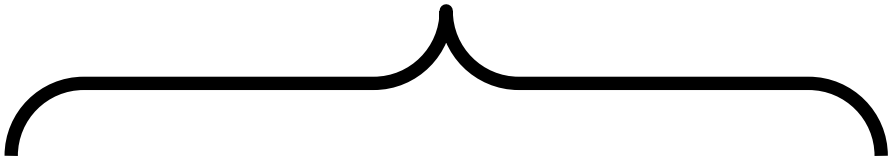
The oral mucosal and salivary microbial profile was variable between individuals in the same group and between individuals in different groups. The orally active BS patients' oral mucosa showed the highest microbial community complexity and diversity compared to all the other investigated groups. Moreover, the BS patients had statistically higher salivary Epstein-Barr virus (EBV) shedding compared to HC volunteers, but not to RAS patients.

Relapsed BS patients' oral mucosa expressed higher levels of TLR2 and TLR4 mRNA. Investigation of the known splice variants of both receptors revealed that TLR2 mRNA variants b, d and e, and TLR4 variants 3 and 4 are significantly elevated in relapsed BS patients. A significant defect in the response to cognate agonists of TLR1/2 heterodimer and TLR4 was also observed in the whole BS patient cohort. Furthermore, BS patients expressed a lower cytomegalovirus (CMV) IgG level in comparison to all the investigated groups.

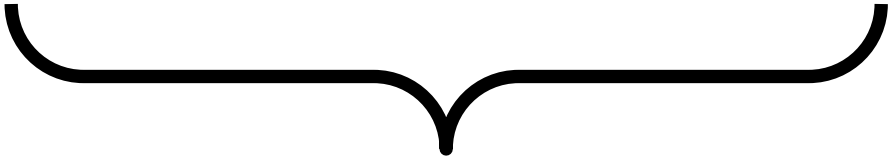
Conclusion

In conclusion, BS patients show higher levels of expression of some of the unusual splice variants of TLR2 and TLR4 mRNA, which might contribute to the observed functional defect in TLR1/2 heterodimer and TLR4. This defect in the key molecular determinants of pathogen recognition can lead to a failure in the adaptive immune responses' modulation, resulting in the observed decrease in the expression of CMV IgG in BS patients and indeed the increase in susceptibility to oral infections. Furthermore, it is envisaged that the reported discrepancy in the oral microbiome of BS patients can be targeted in the future by probiotics to restore the balance of the oral microbial community, leading to better oral health which in turn will enable a better control of the BS immune response.

DECLARATION



I hereby certify that the work embodied in this thesis is the result of my own investigation, except where otherwise stated.



LIST OF PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

MUNCU, G., NIAZI, S., STEWART, J., HAGI-PAVLI, E., GOKANI, B., **SEOUDI, N.,** ERGUN, T., YAVUZ, S., STANFORD, M., FORTUNE, F., DIRESKENELI, H. (2009) Oral Health And Related Quality Of Life Status In Patients From UK And Turkey: A Comparative Study In Behçet's Disease. *J Oral Pathol Me, 38(5), 406–9.*

SEOUDI, N., BERGMEIER, L. A., HAGI-PAVLI, E., BIBBY, D., CURTIS, M. A., FORTUNE, F. The Role of TLR2 and TLR4 in Behçet's Disease Pathogenesis (Manuscript accepted for publication in Innate Immunity)

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PRESENTATIONS

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ABBREVIATIONS

Abbreviation	Term
AE	elution buffer
AL	attachment level
AP	alkaline phosphatase
APC	antigen presenting cell
BAL	broncho-alveolar lavage
BC	before Christ
BDCAF	Behçet's disease current activity form
BP	base pair
BS	Behçet's syndrome
Caco	adenocarcinoma of the colon
CC	conjugate control
CD	cluster of differentiation
c-DNA	complementary deoxyribonucleic acid
CEJ	cemento-enamel junction
Cl	chloride
CLIA	chemiluminescent immunoassay
CLRs	C-type lectin receptors
CM	common mycobacterial
CMV	cytomegalovirus
CNS	central nervous system
CO_2	carbon dioxide
CpG	cytosine-phosphate-guanine
CRP	C-reactive protein
CTB	cholera toxin B
Cy.	cyclohexyl fluorophore
DAIBD	disease activity index for intestinal Behçet's disease
DAMPs	damage-associated molecular patterns
DCs	dendritic cells
DGGE	denaturing gradient gel electrophoresis
DMFT	decayed, missing, filled teeth
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
ds	double strand
E.coli	*Escherichia coli*
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELFA	enzyme-linked fluorescent assay

ELISA	enzyme-linked immunosorbent Assay
ESR	erythrocyte sedimentation rate
EU	ELISA Unit
EULAR	European League Against Rheumatism
F	female
F-H	ficoll-hypaque
FITC	fluorescein isothiocyanate
FMF	familial Mediterranean fever
FRET	fluorescence resonance energy transfer
FSL	synthetic lipoprotein of the N-terminal part of 44-kDa lipoprotein of *Mycoplasma salivarium*
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	genus control
GF	growth factors
GI	gingival index
H_2O	water
HC	healthy control
HCl	hydrochloric acid
Hel	human erythroleukemia
HHV	human herpes virus
HIV	human immunodeficiency virus
HKLM	heat-killed *Listeria monocytogenes*
HLA	human leucocyte antigen
HMIM	human microbial identification microarray
HRP	horseradish peroxidase
HSP	heat shock protein
HSV	herpes simplex virus
HUM70t (HSP70-Hom)	HSP70 gene
IBD	inflammatory bowel disease
IBDDAM	Iranian Behçet's disease dynamic activity measure
ICBD	international criteria for Behçet's disease
IFA	indirect fluorescent assay
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
IRAKs	interleukin-1 receptor-associated kinases
IRF	interferon response factor
ISG	International Study Group
kDa	kilodalton
LJ	Lowenstein-Jensen
LOX	lectin-like oxidized low-density lipoprotein

LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAGIC	mouth and genital ulcers with inflamed cartilage
Mal/TIRAP	MyD88 adaptor-like/TIR domain-containing adaptor molecule
MALDI-TOF	matrix-assisted laser desorption/ionisation-time of flight
MANOVA	multivariate analysis of variance
mg	milligram
$MgCl_2$	magnesium chloride
MGIT	mycobacterial growth indicator tubes
MHC	major histocompatibility complex
MHC I	MHC class I
MHC II	MHC class II
MIC	major histocompatibility complex class I Chain-related
min	minutes
ml	millilitre
Mr	marker
mRNA	messenger RNA
MyD88	myeloid differentiation primary response protein 88
n	number
NaCl	sodium chloride
NALC	N-acetyl-L-cysteine
NaOH	sodium hydroxide
NFκB	nuclear factor kappa B
NHS	national health service
NKG2D	natural killer-cell G2D
NLRs	nucleotide-binding oligomerization domain (NOD)-like receptors
O_2	oxygen
OHIP-14	oral health impact profile-14
OHQoL–UK	United Kingdom oral health-related quality of life
OHRQoL	oral health-related quality of life
Pam3CSK4	synthetic tripalmitoylated lipopeptide mimics acylated amino terminus of bacterial lipoproteins
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pH	power of hydrogen
PHA	phytohaemagglutinin
PHV	phocine herpes virus
PI	plaque index

PNM	primer-nucleotide mix
PPD	periodontal probing depth
propan-2-ol	isopropanol
PRRs	pattern recognition receptors
PVDF	polyvinylidenedifluoride
QoL	quality of life
qPCR	real time polymerase chain reaction
RAS	recurrent aphthous stomatitis
rh	recombinant human
RLRs	retinoic acid-inducible gene (RIG)-l-like receptors
RLU	relative light units
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RSV	respiratory syncytial virus
RT	reverse transcriptase
SARM	sterile alpha and HEAT/armadillo motif
SBI	sulcus bleeding index
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	seconds
Sem	standard error of mean
SLE	systemic lupus erythematosis
SNP	single-nucleotide polymorphism
SPR	solid phase receptacle
ss	single strand
STAT	signal transducer and activator of transcription
STR	stringent wash solution
SYBR	synergy brands
T	tween
TAMRA	tetramethylrhodamine
TBS	tris-buffered saline
TCR^+	T cell receptor positive
TFA	tri-fluoro-acetic-acid
Th	T helper
TIR	toll/interleukin-1 receptor
TLRs	toll-like receptors
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAM	tumour necrosis factor receptor-associated factor

TRIF	TIR domain-containing adaptor protein inducing interferon-ß
Tris	tris(hydroxymethyl)aminomethane
UC	universal control
v	volume
VAS	visual analogue scale
VCA	viral capsid antigens
VIDAS	vitek immuno-diagnostic assay system
VZV	varicella zoster virus
wt	weight
α	alpha
β	beta
γ	gamma
δ	delta
∞	until removing the samples from the cycler
–ve	negative
+ve	positive
°C	degree Celsius
5´UTR	5´ untranslated region

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DEDICATION

This thesis is lovingly dedicated to the memory of my father, grandfather and grandmother who will never be forgotten. They are the light that guides me through life.

This thesis is also dedicated to my mother and older sister who are my constant source of inspiration. They have given me the drive and discipline to tackle any task with enthusiasm.

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CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Behçet's syndrome (BS) is one of the vasculitides with devastating multi-systemic symptoms. It is an immune-related disease that was first described more than fifty years ago by the dermatologist Helusi Behçet. The disease affects young adults and severely compromises their quality of life (QoL). It was thought that an environmental factor triggers the heightened immune response in these patients, leading to the multi-systemic presentation of the disease.

There is consensus that the complex interplay between the commensal microbial community and the abnormal immune response is a fundamental root cause for triggering BS. Therefore, the expression of molecular determinants of pathogen recognition is a key to understanding the capacity of these patients to respond to microbial insult. In this thesis, the author aimed to facilitate the understanding of the disease by investigating the inter-relationship between BS patients' oral microbiome and the relevant aspects of their innate and adaptive immune response.

The thesis embraces seven chapters: Introduction, Materials and Methods, Behçet's Syndrome Patient Cohort in the UK, Oral Health Status and Quality of Life of Behçet's Syndrome Patients, Oral Mucosal Expression of Toll-Like Receptors 2 and 4, The Oral Microbiome of Behçet's Syndrome Patients, and Discussion.

Chapter 1 (Introduction): this chapter begins with an overview of the whole thesis, summarising the main findings and conclusions of this study. A review of literature is included in this chapter, which focuses on BS and reviews the available English written

literature in relation to the following: BS historical background, definition and diagnosis, diagnostic tests and activity markers, epidemiology, clinical picture, deferential diagnosis, aetiology, pathogenesis, and treatment. The aims and hypothesis that drive the clinical and laboratory elements of this study are detailed at the end of this chapter.

Chapter 2 (Materials and Methods): this chapter focuses on the methodology employed to study the clinical and laboratory elements of this thesis.

In summary, the oral health status and QoL of BS patients was assessed. Bacteria in saliva and oral swabs were cultured and purified to homogeneity and bacteria were identified by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry. Salivary viral load and the serum immunoglobulin (Ig) G antibody to the different herpes viruses [herpes simplex virus (HSV) 1, HSV2, cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella zoster virus (VZV), human herpes virus (HHV) 8] were also examined.

In order to determine whether there were differences in the innate immune response efficacy in BS patients, the expression of key molecular determinants of pathogen recognition were also analysed. Total ribonucleic acid (RNA) was purified from non-ulcerated buccal mucosal brush biopsies and checked by real time polymerase chain reaction (qPCR) for the presence of toll-like receptor (TLR) 2 messenger ribonucleic acid (mRNA), TLR4 mRNA and their splice variants. Total protein was purified from the same samples and checked for the presence of TLR2 and TLR4 protein using slot-blot and Western-blot. The functions of TLR2 and TLR4 were also investigated.

Chapter 3 (Behçet's Syndrome Patient Cohort in the UK): this chapter analyses the BS cohort. The prevalence of the different signs of the disease was assessed in a cohort of 153 BS patients from two main tertiary referral centres in the UK for BS patients' management. Recurrent oral ulceration were observed in 100% of the cases, followed by dermatological lesions (85.6%), rheumatological manifestations (79.1%), genital ulceration (73.9%) and ocular involvement (68.6%). Only 15% of the BS patients had neurological manifestations and 10.5% had vascular involvement.

Patients were prescribed multiple medications in 73.9% of the cases. A total of 3.3% of all BS patients were treated with topical corticosteroid as the sole treatment while 53.6% were treated with topical corticosteroid as part of their multiple therapies. Colchicine was the most frequent treatment, used in 54.2% of the BS patient cohort, followed by azathioprine (43.8%).

Chapter 4 (Oral Health Status and Quality of Life of Behçet's Syndrome Patients): this chapter presents data extracted from analysing the BS patient cohort, their QoL and oral health status. The QoL of BS patients from the UK and Turkey was affected to a similar extent. The Turkish BS patients had generally poorer oral health status compared to the UK BS patients. Nevertheless, the UK BS patients had also generally poorer oral health status compared to healthy control (HC) volunteers. The oral health status of the UK BS patients was comparable to those with recurrent aphthous stomatitis (RAS).

Chapter 5 (Oral Mucosal Expression of Toll-Like Receptors 2 and 4): this chapter presents the data extracted from analysing the expressions and functions of TLR2 and

TLR4 in BS patients. Relapsed BS patients' oral mucosa expressed a higher level of TLR2 and TLR4 mRNA. Investigation of the known splice variants of both receptors revealed that TLR2 mRNA variant b, d, and e, and TLR4 variants 3 and 4 are significantly elevated in relapsed BS patients. A significant defect in the response to cognate agonists of TLR1/2 heterodimer and TLR4 was also observed in the whole BS patient cohort.

Chapter 6 (The Oral Microbiome of Behçet's Syndrome Patients): this chapter presents data extracted from investigating the oral microbiome of BS patients. The oral mucosal and salivary microbial profile was variable between individuals in the same group and between individuals in different groups. The orally active BS patients' oral mucosa showed the highest microbial community complexity and diversity compared to all the other investigated groups. Moreover, BS patients had statistically higher salivary EBV shedding compared to HC volunteers, but not to RAS patients. Furthermore, BS patients expressed lower CMV IgG level compared to all the investigated groups.

Chapter 7 (Discussion): this chapter critically appraises the different aspects of the results obtained during this study and highlights the conclusion and scope for future work. The main points concluded from this study are as follows: (1) BS patients exhibit some innate immune dysfunction and/or dysregulation that can explain the observed abnormalities in some areas of the adaptive immune responses to oral infection; (2) It is envisaged that the reported discrepancy in the oral microbiome of BS patients can be targeted in the future by probiotics to restore the balance of the oral microbial community, leading to better oral health which in turn will enable a better control of the BS immune response.

1.2 REVIEW OF LITERATURE OF BEHÇET'S SYNDROME

1.2.1 HISTORICAL BACKGROUND

The constellate of the three clinical signs (orogenital ulceration and uveitis) which create the major components of what is now known as Behçet's syndrome (BS) was first described by Hippocrates in the fifth century before Christ (BC) in his 3rd *Epidemion* book. Subsequently, Benediktos Adamantiades presented the first case of relapsing iritis with hypopyon to the Medical Society of Athens in 1930. He identified three major signs of the disease: oral ulceration, relapsing iritis and hypopyon. In 1946, he acknowledged thrombophlebitis as the fourth major sign of the same disease. Adamantiades was the first to classify the disease into ocular, mucocutaneous and systemic subclasses and contributed to the first classification and diagnostic criteria (Zouboulis, 2002).

Dr Hulusi Behçet, a Turkish dermatologist born in Istanbul in 1889, graduated in 1910 from the Gulham Military Medical School and subsequently specialized in dermatology and syphilology. He was the first to formally describe the various manifestations of the disease, in the *Journal of Skin and Venereal Diseases* in 1936, by reporting a series of three cases (Verity et al., 1999a) (Figure 1.1). He initially described a triad of oral aphthae, genital ulcers, and hypopyon uveitis, and called it Behçet's triple complex syndrome (Gray, 1950). Behçet's triple complex syndrome was classically described at that time as a triad of the following signs: (1) recurrent oral ulceration ranging from small ulcers to large necrotic ulcers with inflamed sharp edges that heal with scar formation; (2) genital ulceration; (3) various eye lesions ranging from conjunctivitis and iritis to thrombosis of the central retinal vein (Gray, 1950). Behçet's disease was internationally documented at the International Congress of Dermatology in 1947 (Verity et al., 1999a).

Figure 1.1: Dr Hulusi Behçet, the Turkish dermatologist who formally described the various manifestations of BS [source: www.tiphastaligi.com].

1.2.2 DEFINITION AND DIAGNOSIS

Currently BS is classified with the vasculitides and defined as a chronic, relapsing, multi-systemic, idiopathic, inflammatory vasculitis that possibly will involve most tissues of the body, such as the mucocutaneous, ocular, musculoskeletal, vascular and central nervous system (CNS) (Escudier et al., 2006, Evereklioglu, 2005). The highest morbidity and mortality rates are observed with cardiovascular, gastrointestinal and neurological involvement (Talarico et al., 2012, Verity et al., 1999c).

The diagnosis of BS is based solely on a clinical expert opinion. It is well appreciated that the standardization of the diagnosis of BS is mandatory for accurate international epidemiological studies, treatment evaluation, and development of guidelines. Therefore,

sixteen different diagnostic and classification criteria have been proposed to date for the purpose of standardizing the diagnosis and classification of BS. Two out of these sixteen criteria are the product of an international collaboration (Davatchi, 2012, Lancet, 1990). The first diagnostic and classification criteria were developed in 1946 by Curth (Curth, 1946).

In the UK, researchers follow the International Study Group (ISG) diagnostic and classification criteria (sensitivity: 92% and specificity: 97%) to standardize the diagnosis of BS in research projects and ensure that only well-defined cases are included in the research (Lancet, 1990). The ISG criteria are the product of collaborative work between six different countries (France, Iran, Japan, Turkey, UK, and USA) and they aim to bring international consensus on BS diagnosis and classification. This diagnostic tool includes the main criterion and additional criteria. The diagnosis of BS is established when the main criterion and at least two of the additional criteria are satisfied (ISG, 1992, Lancet, 1990) (Table 1.1).

Table 1.1: The ISG diagnostic and classification criteria for BS (1990).

The main criterion

Recurrent episodes of ulceration on the oral mucosa (at least three times in a 12-month period)

The additional criteria

1- Ocular involvement
2- Recurrent episodes of genital ulceration
3- Skin involvement
4- Positive pathergy reaction

Some other criteria were developed subsequent to the ISG criteria to overcome their limitations, such as the inability to diagnose cases that have fewer than three episodes of recurrent oral ulceration per year, patients who do not have oral ulceration, and patients who do not develop oral ulceration until later in the disease. Furthermore, some countries with a high prevalence of BS did not take part in the international collaborative work leading to the development of the ISG criteria (Chang and Kim, 2003).

The most recent criteria are the international criteria for Behçet's disease (ICBD) which were proposed in 2006 and validated in 2010 (Davatchi et al., 2010a). The ICBD are the outcome of the collaboration of many countries including Austria, Azerbaijan, China, Egypt, Japan, Jordan, Libya, Morocco, Pakistan, Portugal, Russia, Saudi Arabia, Singapore, Spain, Taiwan, Thailand, Tunisia, Turkey, and USA. These criteria were created by analysing an international cohort of 2,556 BS patients and 1,163 controls. Initially, there were two formats for the ICBD: the traditional format and the classification tree. However, only the traditional format is currently recognized. According to the traditional ICBD, a patient is classified as having BS when scoring three or more points. Oral aphthous, skin manifestations, vascular manifestations, and a pathergy positive test each score one point in the ICBD system, while genital aphthous and ocular manifestations score two points each (Table 1.2).

Table 1.2: The ICBD diagnostic criteria for BS (2006).

Criteria scoring one point:

1- Oral aphthous
2- Skin manifestations
3- Vascular manifestations
4- Pathergy positive test

Criteria scoring two points:

1- Genital aphthous
2- Ocular manifestations

Davatchi et al. (2010) recently evaluated the sensitivity, specificity, and accuracy of several different diagnostic and classification criteria. The accuracy of the different criteria was as follows: 75.8% for Mason and Barnes criteria, 78.8% for O'Duffy criteria, 80.4% for Hewitt criteria, 84.3% for Hubault criteria, 85.3% for ISG criteria, 86% for Dilsen criteria, 86.2% for Dilsen revised criteria, 88.8% for Japan criteria, 89.6% for Japan revised criteria, 92.7% for Iran traditional criteria, 93.1% for Zhang criteria, 93.4% for Curth criteria, and 97.1% for the classification tree. Furthermore, 98.2% sensitivity and 95.6% specificity were reported for the ICBD in the Iranian BS patient cohort (Davatchi et al., 2010a, Davatchi, 2012). An international multi-centre study to examine the different criteria in different BS patient cohorts is mandatory to confirm these findings.

In the author's opinion, the ICBD criteria could be subject to criticism based on their potential for over diagnosing BS. For example, isolated idiopathic orogenital ulceration satisfy the ICBD criteria leading to a potential false positive diagnosis and therefore the specificity of the criteria is expected to be lower than the reported 95.6%.

1.2.3 DIAGNOSTIC TESTS

There is a lack of a universally recognized diagnostic laboratory test for BS. The pathergy test (reactivity of the skin to a needle prick or injection) forms a major component in the two sets of international diagnostic and classification criteria (ISG and ICBD). Classically, the pathergy test is performed by puncturing the skin with a 25- or 21-gauge needle, perpendicular to or diagonally to the skin, with or without the injection of normal sterile saline. Some centres use a set of three tests: 25-gauge needle with intradermal injection of one drop of normal sterile saline, 25-gauge needle without injection, and 21-gauge needle without injection (Davatchi et al., 2010b). The reaction is then monitored after 24 and 48 hours. However, the fact that there is a lack of a standardized measuring method in the pathergy reaction makes its value as a sole diagnostic test for BS uncertain.

Two different possible reactions are seen in the positive pathergy test: the classical papulopustular reaction, and the circular haemorrhagic reaction. The classical papulopustular reaction has sensitivity of 60%, specificity of 87%, and accuracy of 73% in an Iranian BS patient cohort, while the circular haemorrhagic reaction has sensitivity of 12%, specificity of 98%, and accuracy of 55% in the same cohort (Davatchi et al., 2010b).

Ozdemir et al. (2007) performed a study of a Turkish BS patient cohort to compare the results of the pathergy reaction in various body areas. This study concluded that no positive pathergy reaction was found in HC volunteers, but the pathergy test was positive in 18% of patients with RAS, and 85.7% of BS patients. In the above-mentioned patient cohort, the forearm was the most frequent site showing positive pathergy reaction, while the abdomen

was the least frequent site. At the same time, this study showed that multiple needle pricks increased the positive rate in BS (Ozdemir et al., 2007).

The pathergy test was also investigated in another Turkish BS patient cohort (n: 31) compared to familial Mediterranean fever (FMF) patients and HC volunteers. The positivity rate was 41.9% in BS patients, 0% in FMF patients, and 0% in HC volunteers (Aydin et al., 2009). In another study the positivity rate of the pathergy test was 8% in inflammatory bowel disease (IBD) patients (cohort of 93 Crohn's disease patients, and 130 ulcerative colitis patients) (Hatemi et al., 2008b). This highlights a good specificity of the test; however, a standardized test protocol along with a well-designed multi-centre study to examine the reproducibility and validity of the test in different BS patient cohorts is mandatory.

Davatchi et al. (2011) investigated the sensitivity and specificity of the pathergy test over time by analysing the test results recorded for BS patients enrolled in the registry of Tehran University of Medical Sciences (BS: n=6607 and HC: n=4292) during the last 35 years. They concluded that the test specificity has increased over time (from 86.6% to 98.4%) while the sensitivity has decreased over time (from 64.2% to 35.8%). The fact that the frequency of the positive pathergy reaction has declined over recent decades is undermining its value as a diagnostic test for BS (Davatchi et al., 2011, Davatchi et al., 2003). Interestingly, the rate of positive pathergy tests in the BS cohort from western countries such as the UK and Germany seems to be much lower than in the "Silk Road" countries such as Turkey and Iran. Currently, the rate of the pathergy test is 56% for patients from

Turkey, 54% for Iran, 53% for Morocco, 44% for Japan, 40% for Korea, 34% for Germany, and 32% for the UK (Davatchi et al., 2010b).

The histopathology of the papulopustular skin lesion in BS patients was investigated as a diagnostic marker. However, in a recent study, vasculitis (leukoclastic vasculitis or lymphocytic vasculitis) was recorded in only 23.8% of the investigated cases (n: 42). Superficial, deep perivascular inflammation and/or interstitial dermatitis was encountered in 64.3% of cases (Kalkan et al., 2009).

Other diagnostic markers were investigated, such as the thrombomoduline level for endothelial dysfunction in BS patients. However, the study was performed on a small number of patients and has not been substantiated by larger studies (Menashi et al., 2008).

1.2.4 ACTIVITY MARKERS

The availability of a reproducible and validated activity marker is of prime importance in monitoring and evaluating the different treatment strategies. This in turn will facilitate the development of evidence-based information that is useful for decision makers and guideline developers. This fact encouraged research aimed at establishing an activity scoring system and/or laboratory activity markers. Iranian and European scoring systems were suggested and refined, as a result and the International Scientific Committee approved the Behçet's disease current activity form (BDCAF), which is based on the history of the disease's clinical features. The BDCAF scores oral ulcerations, genital ulcerations, skin involvement, joint involvement, gastrointestinal involvement, fatigue, and headache in relation to the duration of symptoms. Large vessel and CNS involvement along with Behçet's oculopathy

index is also recorded. Patients' and clinicians' opinions on the overall disease activity are rated on a 7-point scale (Bhakta et al., 1999).

The BDCAF was validated and proven to have a good inter-observer reliability in assessing the general disease activity (Bhakta et al., 1999). A Turkish version of the BDCAF was also evaluated for reproducibility and validity (Hamuryudan et al., 1999). Subsequently, the score was subjected to re-evaluation in a multi-centre study (Turkey, Korea, and the UK) which led to a further refinement to allow comparison between countries and pooling of data (Lawton et al., 2004). However, the Iranian Behçet's disease dynamic activity measure (IBDDAM) that is based on an interval scale, evaluation of symptom history in the 12 months prior to the date of assessment, and an average activity score per month was preferred in some countries such as Turkey (Shahram et al., 2009). Recently, a disease activity index for intestinal Behçet's disease (DAIBD) was suggested and validated by a Korean research group; nevertheless; an international consensus needs to be established (Cheon et al., 2011).

C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and serum neopterin are significantly higher in active BS patients compared to inactive BS patients and HC volunteers; however, these activity markers are known to be elevated in other inflammatory as well as infectious diseases and hence they are not specific for BS (Coskun et al., 2005). Furthermore, the serum total level of homocysteine (a known risk factor for atherothrombogenesis) was also found to be elevated in active BS patients compared to inactive BS patients and HC volunteers (Sarican et al., 2007). Moreover, circulating endothelial cells and soluble tumour necrosis factor receptor (TNFR) 2 were suggested as

disease activity markers (Kutlay et al., 2008, Turan et al., 2008). However, to date there is a lack of international consensus on the use of a laboratory validated activity marker with an accurate positive/negative cut-off. Currently, BS activity determination is based solely on expert clinical judgement.

1.2.5 EPIDEMIOLOGY

A large proportion of BS patients live along the "Silk Road", or their ancestors used to live in those countries through which the "Silk Road" passed; these include Turkey, Iraq, Iran, Korea, and Japan amongst others (Figure 1.2). However, the current thinking is that the global prevalence of BS is higher than was documented previously (Yazici et al., 2008).

The "Silk Road" is a historical trade route that connected East, South and West Asia with the Mediterranean countries, the European world, and North and East Africa. The land road was supplemented by a sea route extending from the Red Sea to East Africa, India, China, and Southeast Asia. The classical concept of a single "Silk Road" is misleading as there were several routes that were frequently selected to transport a wide variety of commodities (Verity et al., 1999a). Not only had silk been traded from China along these routes since 206 BC, but also other produce, technologies, religions, and philosophies were exchanged. Moreover, some diseases such as bubonic plague were disseminated in the same way (Figure 1.2).

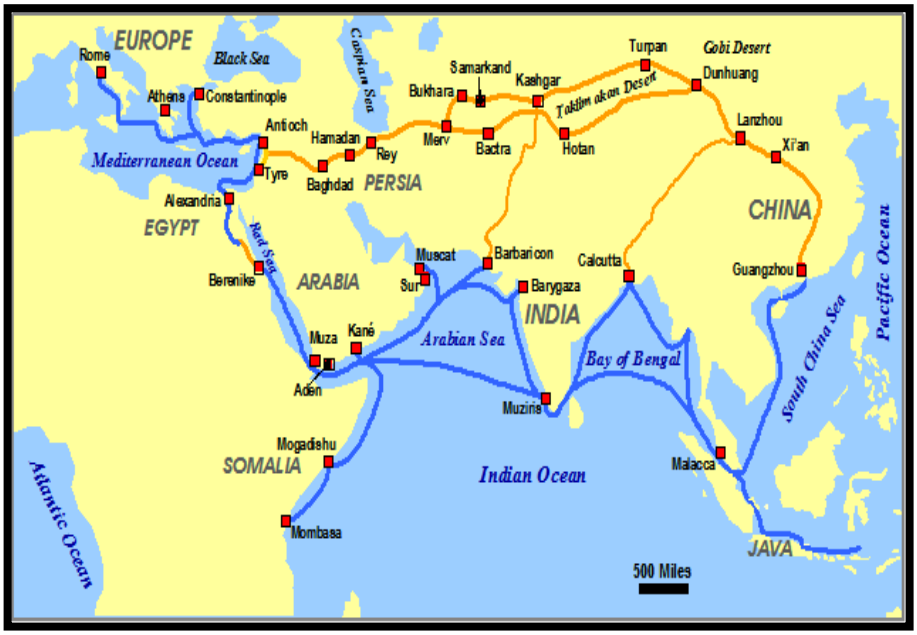


Figure 1.2: The geographic distribution of the "Silk Road" which is indicated by the yellow line [source: www.maximizingprogress.org/2008/06/silk-road].

The frequency of BS in adults is very variable throughout the world. There is a consensus that the global incidence and prevalence of BS are more frequent than was reported earlier in the literature (Yazici et al., 2008). A prevalence of 380/100,000 was reported in Turkey, 100/100,000 in Japan, 80/100,000 in Iran, 50-185/100,000 in the Druze populations (a unique religious community found primarily in Syria, Lebanon, Jordan, Israel, and the Palestinian territories), 7.1/100,000 in France, 5.2/100,000 in USA, 3.8/100,000 in Italy, 2.4/100,000 in the northern region of Israel, 2.26/100,000 in Germany, 0.64/100,000 in England and 0.24/100,000 in Northwest Spain (Calamia et al., 2009, Davatchi et al., 2010b, Davatchi et al., 2010c, Dejaco et al., 2009, Gonzalez-Gay et al., 2000, Jaber et al., 2002, Klein et al., 2010, Krause et al., 2007, Mahr et al., 2008, Salvarani et al., 2007, Zouboulis et al., 1997).

BS generally affects young adults between the second and fourth decade of life. However, the onset can occur at any age (Kone-Paut et al., 2011, Mundy and Miller, 1978). Mean age at onset for BS patients in countries which have a national survey for BS (Iran, Japan, China, Korea, and Germany) is 26.2, 35.7, 33.8, 29, and 26 years respectively (Davatchi et al., 2010b, Davatchi et al., 2010c, Zouboulis et al., 1997). The mean age at diagnosis was 33 in Italy, 31 in USA, 32.8 in Egypt, 29 in Korea, 25.6 in Turkey, 33.1 in India, 29.3 in Saudi Arabia, 29.4 in Iraq, 30.1 in Jordon, 26 in Lebanon, 30.7 in Israel, 20 in Greece, 25.7 in Portugal, 33 in Sweden, 28.03 in Brazil, 20.8 in Ireland, and 24.7 in Yorkshire, UK (Calamia et al., 2009, Chamberlain, 1977, Davatchi et al., 2010b, El Menyawi et al., 2008, Sachetto et al., 2011, Salvarani et al., 2007). Juvenile disease was recorded in 6.9% of BS patients in the German BS patient cohort (Zouboulis et al., 1997). A younger age at diagnosis was considered to be associated with a more severe clinical course, especially in relation to intestinal BS (Jung et al., 2011). However, late-onset BS (BS diagnosed after the age of 40) was deemed to have a good prognosis, especially in relation to the ocular involvement (Sungur et al., 2010).

The disease was classically described as being more prevalent in men than women in the ancient "Silk Road" countries, whereas the trend is reversed in Western Europe and the USA (Evereklioglu, 2005, Zouboulis et al., 1997). Currently, there seems to be almost no gender predilection in most BS patient cohorts. The male to female ratio of BS patients in countries which have a national survey for BS (Iran, Japan, China, Korea and Germany) is 1.19, 0.98, 1.34, 0.63, and 1.40 to 1 respectively (Davatchi et al., 2010b, Zouboulis et al., 1997). The male to female ratio was 1:1.19 in Brazil, 1:1 in Italy, 3.67:1 in Russia, 3.4:1 in Saudi Arabia, 3:1 in Iraq, 1.03:1 in Turkey, 1.8:21 in India, 0.64:1 in Israel, 2:1 in

Morocco, 1.4:1 in Greece, 1:1 in Portugal, 0.5:1 in Spain, and 0.67:1 in Sweden (Calamia et al., 2009, Davatchi et al., 2010b, Davatchi et al., 2010c, El Menyawi et al., 2008, Sachetto et al., 2011, Salvarani et al., 2007). Interestingly, late-onset BS was observed consistently to affect both genders equally (Sungur et al., 2010).

1.2.6 CLINICAL PICTURE

The major clinical manifestations of BS are reported in the literature as follows: ulcers in the oral cavity and on the genitals, acne-like papulopustular skin lesions, erythema nodosum, positive pathergy test, and ocular symptoms. Other symptoms that may occur include arthritis, venous thrombosis, gastrointestinal complications, and inflammation in the CNS, kidneys, lungs, and cardiovascular system (El Menyawi et al., 2008, Evereklioglu, 2004, Zouboulis et al., 2003).

BS is characterized by a chronic course with unpredictable exacerbations and remissions. Male gender, the human leucocyte antigen (HLA) B51, and a younger age at onset are all associated with more severe disease. The main complications of the disease are neurological, pulmonary, large vessel involvement, and bowel perforation (Zouboulis et al., 2003).

BS does not affect the life expectancy of the affected patients. However, mortality due to severe CNS involvements, pulmonary involvements, catastrophic bleeding from large vessels, bowel perforation, or as a complication of immunosuppressive therapy has been reported. Furthermore, the risk of visual loss was shown as 23% in an international multi-

centre study, despite the availability of modern treatment modalities (Evereklioglu, 2004, Evereklioglu, 2005, Kitaichi et al., 2007).

1.2.6.1 ORAL ULCERATION

Oral ulceration was typically found in almost all patients with BS. Interestingly, the initial presentation of the disease is by oral ulceration in most BS cases. In Iran, 82.1% of BS patients presented with oral ulceration as the initial disease manifestation (Davatchi et al., 2010c). Likewise, in Italian BS patients the primary finding was oral ulceration in 88% of the investigated cases (Salvarani et al., 2007).

Oral ulceration are usually round or oval, surrounded with a well-defined inflamed red margin, and with a yellowish white necrotic base. Minor (<10 mm in diameter), herpetiform (multiple 1–2 mm in diameter ulcerations that coalesce, forming a big ulcer with a ragged margin), and major (10–30 mm in diameter) aphthous ulcer-like lesions may present in BS patients (Figures 1.3–1.5). In addition, a mixed pattern was observed and documented in the literature. Major aphthous-like lesions are more common in BS patients than in RAS patients (Krause et al., 1999). Small ulcerations usually heal without scarring; however, large ulcerations are more prone to heal with scar formation (64%) (Davatchi et al., 2010b).

Oral ulceration of BS are typically associated with increased tissue oedema and are surrounded with a large erythematous border, compared to RAS oral ulcerations. BS also involves the soft palate and the oropharynx more often (Kokturk, 2012, Main and Chamberlain, 1992).

The global rate of oral ulcerations in BS patients is 94.5% (Kitaichi et al., 2007). However, a relatively higher percentage was reported in population-based studies (97% in Iran, 98% in Japan, 98% in China, 99% in Korea, 99% in Germany, 100% in USA, 100% in Italy, and 100% in Egypt) (Calamia et al., 2009, Davatchi et al., 2010b, Davatchi et al., 2010c, El Menyawi et al., 2008, Salvarani et al., 2007, Zouboulis et al., 1997).

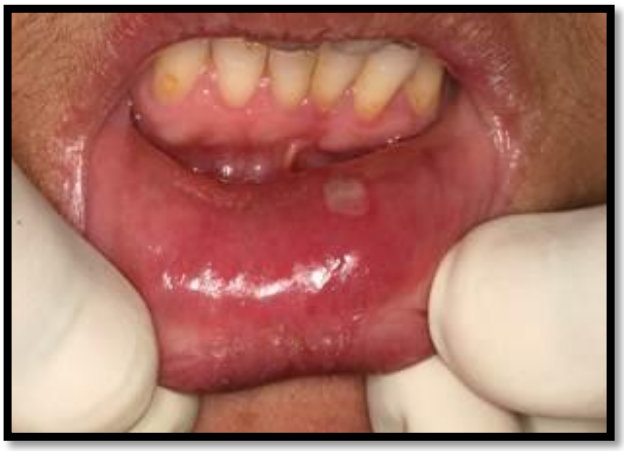


Figure 1.3: Oral ulceration (minor aphthous ulcer-like lesion) in BS patient [source: Royal London Hospital with patient consent].

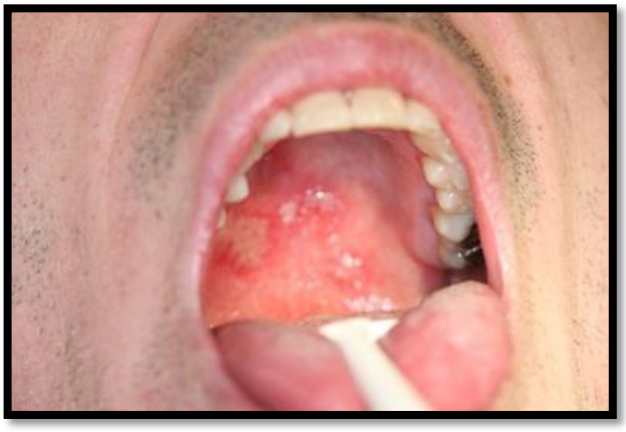


Figure 1.4: Oral ulceration (herpetiform aphthous ulcer-like lesion) in BS patient [source: Royal London Hospital with patient consent].

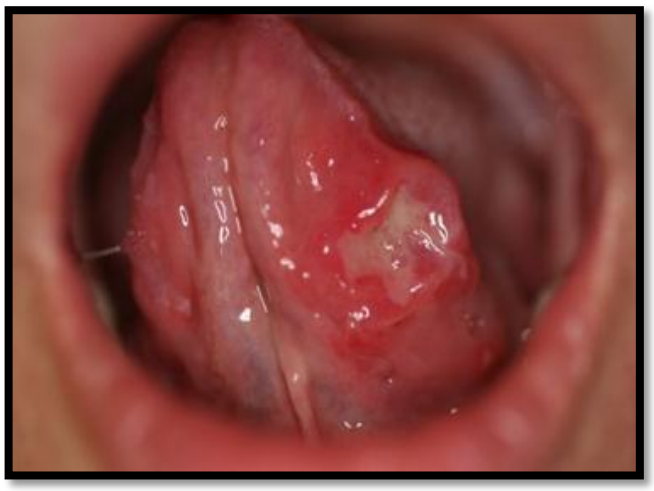


Figure 1.5: Oral ulceration (major aphthous ulcer-like lesion) in BS patient [source: Royal London Hospital with patient consent].

The histopathological picture of the BS oral ulceration is nonspecific, with lymphocyte, macrophage, and neutrophils infiltration at the base of the ulcer. Leukocytoclastic and lymphocytic vasculitis were also observed (Chun et al., 1990, Kokturk, 2012).

Oral ulcerations can be caused by a number of different oral conditions, therefore the clinician should be aware of the relevant differential diagnosis. Traumatic ulceration, RAS, hematinic deficiency, infections (herpes simplex, syphilis, tuberculosis, human immunodeficiency virus (HIV), herpangina, and hand–foot–mouth disease), squamous cell carcinoma, necrotizing sialometaplasia, cyclic neutropenia, graft versus host disease, radiotherapy-induced mucositis, osteoradionecrosis, lichen planus, lichenoid reaction, systemic lupus erythematosis, pemphigus, pemphigoid, mouth and genital ulcers with inflamed cartilage (MAGIC) syndrome, Reiter's syndrome, Sweet's syndrome, and IBD (Table 1.3).

Table 1.3: Differential diagnosis of the oral ulceration of BS.

CONDITIONS PRESENTING WITH ORAL ULCERATIONS	
Traumatic ulceration	Osteoradionecrosis
RAS	Lichen planus
Hematinic deficiency	Lichenoid reaction
Herpes simplex	SLE
Syphilis	Pemphigus
Tuberculosis	Pemphigoid
HIV	MAGIC
Herpangina	Reiter's syndrome
Hand–foot–mouth disease	Sweet's syndrome
Radiotherapy-induced mucositis	Inflammatory bowel disease
HIV: Human immunodeficiency virus; IBD: inflammatory bowel disease; MAGIC: mouth and genital ulcers with inflamed cartilage syndrome; RAS: recurrent aphthous stomatitis; SLE: systemic lupus erythematosis	

1.2.6.2 GENITAL ULCERATION

Genital ulceration are considered to be the second most common manifestation of BS and sometimes they are the initial finding of the disease. In Iran, 10% of BS patients presented with genital ulceration as the initial disease manifestation (Davatchi et al., 2010c). Likewise, 16% of the Italian BS patients reported genital ulceration as the initial finding of their disease (Salvarani et al., 2007).

The clinical picture of genital ulceration resembles the oral ulceration. The main differences are that genital ulceration recur less frequently, take longer to heal, heal with scar formation, and are usually bigger in size. In females suffering from BS, genital ulceration might be seen on the vulva, vagina, and cervix. Interestingly, genital ulceration are observed more prominently during the premenstrual or menstrual phases of the ovarian cycle. Furthermore, genital ulceration recur less frequently during pregnancy but activity increases during the post-partum period. In male BS patients, genital ulceration are

observed mainly on the scrotum and less often on the shaft of the penis or the meatus (Davatchi et al., 2010b, Kokturk, 2012) (Figure 1.6).

The frequency of genital ulceration in BS patients is as follows: 61.4% in a multi-centre study, 65% in Iran, 73% in Japan, 76% in China, 83% in Korea, 75% in Germany, 77% in Italy, 62% in USA, and 96.8% in Egypt (Calamia et al., 2009, Davatchi et al., 2010b, Davatchi et al., 2010c, El Menyawi et al., 2008, Kitaichi et al., 2007, Salvarani et al., 2007, Zouboulis et al., 1997).

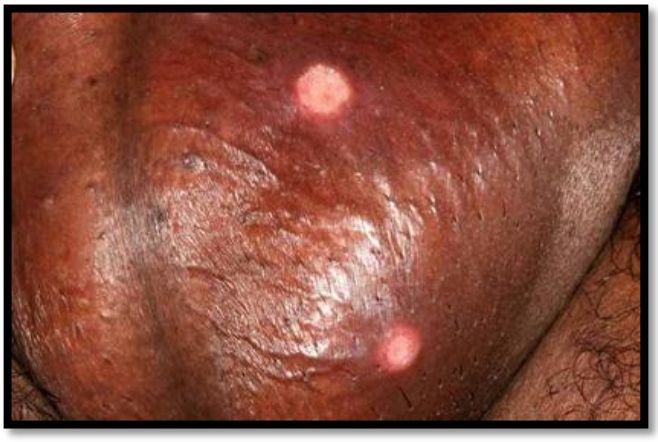


Figure 1.6: Genital ulceration in a male BS patient [source: Royal London Hospital with patient consent].

1.2.6.3 OTHER FORMS OF ULCERATION

BS patients may also suffer from anal ulceration, with very similar characteristics to the genital ulceration. Moreover, conjunctival ulceration were reported in the literature (Davatchi et al., 2010b).

1.2.6.4 CUTANEOUS MANIFESTATIONS

Cutaneous manifestations of BS may present in the form of papulopustular, erythema nodosum-like lesion, superficial thrombophlebitis, pathergy reaction, or skin ulcer. The frequency of cutaneous manifestations is as follows: 69.5% in a multi-centre study, 66% in Iran, 87% in Japan, 69% in China, 84% in Korea, 81.5 in Germany, 100% in Italy, 100% in USA, and 55.5% in Egypt (Calamia et al., 2009, Davatchi et al., 2010b, Davatchi et al., 2010c, El Menyawi et al., 2008, Kitaichi et al., 2007, Salvarani et al., 2007, Zouboulis et al., 1997). It is the second most common initial manifestation in the Italian BS patient cohort (33.3%) (Salvarani et al., 2007).

Papulopustular lesions are the most common cutaneous lesion in BS patients and typically present with elevated erythema and a dome-shaped pustule in the centre. They are believed to be a type of vasculitis with a sterile pustule. Papulopustular lesions are observed mainly in the lower limbs and pubis. However, they can affect anywhere in the body including palms, soles, face, trunk, and the back. Their frequency is as follows: 57% in Iran, 31% in China, and 62% in Germany (Davatchi et al., 2010b) (Figures 1.7 and 1.8).

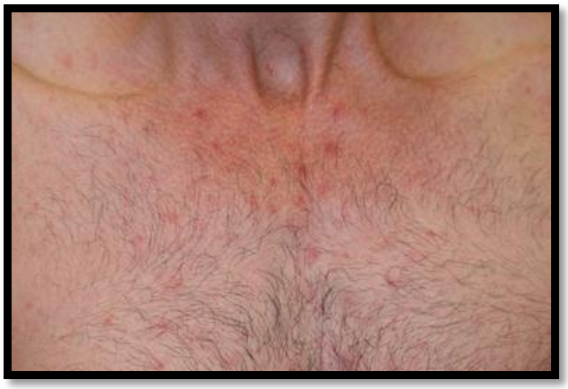


Figure 1.7: Papulopustular lesions in BS patient [source: Royal London Hospital with patient consent].

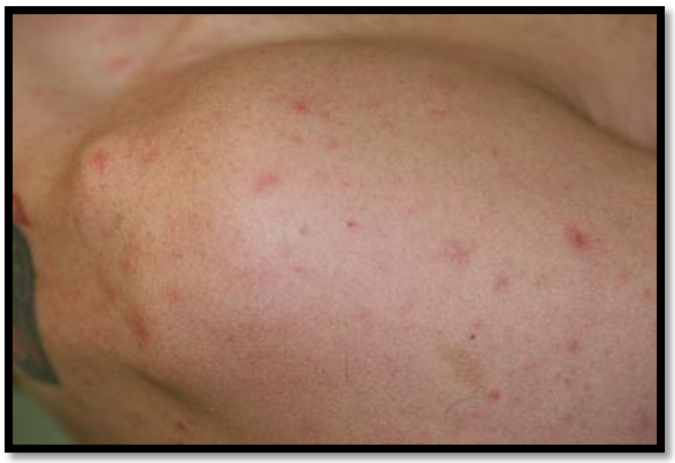


Figure 1.8: Papulopustular lesions in BS patient [source: Royal London Hospital with patient consent].

Erythema nodosum-like lesions are the second most common cutaneous manifestation of BS. They are painful, subcutaneous nodules of different sizes and are characterized by severe oedema and erythema around them (Figures 1.9 and 1.10). They frequently affect the lower limbs. However, they may affect anywhere in the body including buttocks, face, and neck. These lesions take a few weeks to heal and subsequently leave hyperpigmented areas. The erythema nodosum-like lesions frequency in BS is as follows: 22% in Iran, 38% in China, and 42% in Germany (Davatchi et al., 2010b, Davatchi et al., 2010c, Kokturk, 2012). The histological appearance of an erythema nodosum-like lesion is variable including leukocytoclastic vasculitis, neutrophil vascular reaction, lymphocytic vasculitis, lymphohistocytic septal/lobular panniculitis, granulomatous panniculitis, or acute necrotizing panniculitis (Kim and LeBoit, 2000, Lee et al., 1997).

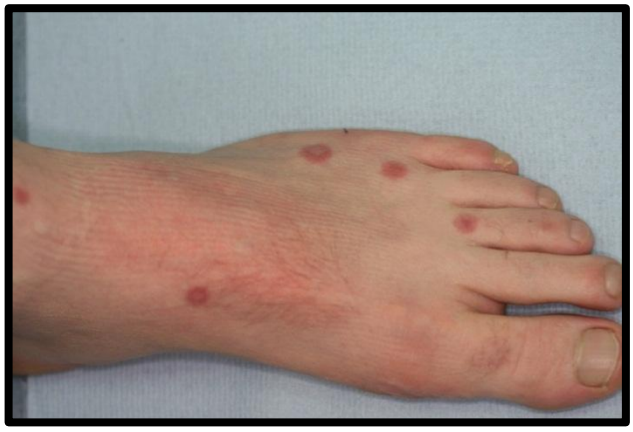


Figure 1.9: Erythema nodosum-like lesion in BS patient [source: Royal London Hospital with patient consent].

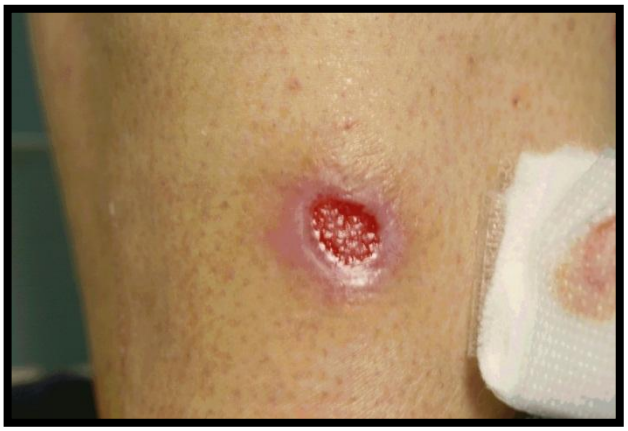


Figure 1.10: Ulcerated erythema nodosum-like lesion in BS patient [source: Royal London Hospital with patient consent].

Superficial thrombophlebitis is also one of the characteristic cutaneous lesions of BS. It presents as a recurrent painful subcutaneous nodule with erythema of the overlying skin. It is observed mainly in the lower extremities and classically heals in a few days (Kokturk, 2012, Lee et al., 1997).

1.2.6.5 OCULAR MANIFESTATIONS

Uveitis (involving the anterior segment, posterior segment, or both) is the main ocular manifestation in BS patients. The intraocular inflammation is usually bilateral and occurs as the initial manifestation of BS in only 8.7% of the cases. Uveitis frequency in BS is as follows: 55% in Iran, 69% in Japan, 63.5% in China, 45.1% Korea, 53% in Germany, 55.6% in Italy, 47.6% in Egypt, and 62% in USA (Calamia et al., 2009, Davatchi et al., 2010b, Davatchi et al., 2010c, El Menyawi et al., 2008, Kitaichi et al., 2007, Salvarani et al., 2007, Zouboulis et al., 1997).

Anterior uveitis is an inflammation of the anterior chamber of the eye leading to pain, photophobia, and visual disturbance. Hypopyon is the most classical form of anterior uveitis in BS patients, presenting as visibly whitish thick exudates with sediment in the lower part of the anterior chamber of the eye (Figure 1.11). Recurrent attacks of anterior uveitis can lead to cataract or glaucoma (Davatchi et al., 2010b, Soloway and Weissgold, 1996).

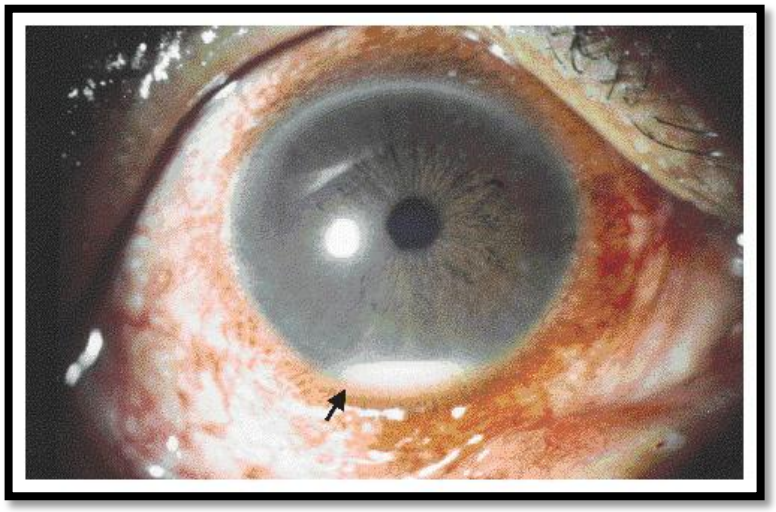


Figure 1.11: Hypopyon in BS patient [source: (Soloway and Weissgold, 1996)].

Recurrent posterior uveitis and retinal vasculitis are the most important causes of visual loss in BS patients. Men tend to have posterior uveitis more frequently than women. Consequently, poor visual acuity as a complication of BS ocular manifestations is seen in men more often than women (24.8% versus 18.9%). Unfortunately, despite of the availability of the modern treatment modalities, a quarter of BS patients who suffer from ocular manifestations go blind at one point of their disease course (Kitaichi et al., 2007).

1.2.6.6 JOINT MANIFESTATIONS

Arthralgia, arthritis and synovitis are the main articular manifestations in BS patients. Joint manifestations frequency in BS is as follows: 33% in Iran, 57% in Japan, 30% in China, 38% in Korea, 59% in Germany, 50% in Italy, 36.5% in Egypt, and 46% in USA (Calamia et al., 2009, Davatchi et al., 2010b, Davatchi et al., 2010c, El Menyawi et al., 2008, Kitaichi et al., 2007, Salvarani et al., 2007, Zouboulis et al., 1997).

BS patients' joint manifestations are typically non-erosive, non-deforming, and recurrent, and each attack takes a few weeks to resolve. Rarely, chronic polyarticular arthritis, osteonecrosis, and ankylosing spondylitis are seen in BS patients (Kokturk, 2012).

1.2.6.7 GASTROINTESTINAL MANIFESTATIONS

Ulcerations affecting the gastrointestinal tract at any site from the oesophagus to the anus can occur in BS patients. However, the terminal ileum and the caecum are the sites most affected, by deeply penetrating ulcerations that carry a high tendency to perforation. BS gastrointestinal manifestations present with acute abdominal pain, diarrhoea, constipation, or proctorrhagia. The frequency of gastrointestinal manifestations in BS is as follows: 7%

in Iran, 15.5% in Japan, 9% in China, 7.3% in Korea, 12% in Germany, and 19% in Egypt (Davatchi et al., 2010b, Davatchi et al., 2010c, El Menyawi et al., 2008, Kokturk, 2012).

1.2.6.8 VASCULAR MANIFESTATIONS

BS is classified as systemic vasculitis affecting all vessels. It can easily be differentiated from other vasculitis by following one of the international criteria for BS diagnosis and classification. BS arterial involvement is very rare, but very similar to that of Takayasu's arteritis. However, neutophils and lymphocytes infiltration of the vasa vasorum is significantly higher in BS compared to Takayasu's arteritis (Kokturk, 2012, Talarico et al., 2012) (Table 1.4).

Table 1.4: BS is a vasculitis that can affect all types of vessels (large, medium, and small).

LARGE VESSEL VASCULITIS	**MEDIUM VESSEL VASCULITIS**	**SMALL VESSEL VASCULITIS**
Behçet's Syndrome	Behçet's Syndrome	Behçet's Syndrome
Polymyalgia rheumatica	Kawasaki disease	Wegener's granulomatosis
Takayasu's arteritis	Cutaneous vasculitis	Cutaneous vasculitis
Giant cell arteritis	Polyarteritis nodosa	Polyarteritis nodosa
		Henoch-Schönlein purpura
		Churg-Stauss syndrome

The clinical vascular manifestation frequency is as follows: 8.9% in Iran, 8.9% in Japan, 7.7% in China, 1.8% in Korea, and 13% in Germany. However, a colour duplex sonography study showed that vascular involvement is about 79% in the 100 BS patients studied (63% arterial involvement, 55% venous involvement, and 39% both arterial and venous) (Davatchi et al., 2010b, Morelli et al., 1997).

Venous involvement including superficial thrombophlebitis, deep venous thrombosis and large venous thrombosis was documented in BS patients. Occlusion of the suprahepatic vein (Budd-Chiari syndrome) carries a high risk of mortality. Likewise, thromboses of the superior and inferior vena cava and the dural sinuses have poor prognosis. Arterial involvements including thrombosis, aneurysms, and pulse weakness were observed in BS patients (Kokturk, 2012)

1.2.6.9 PULMONARY MANIFESTATIONS

Pulmonary manifestations of BS are rare but carry a high risk of morbidity and mortality. Vasculitis of the pulmonary arteries, veins, and septal capillaries is the main cause of the pulmonary manifestation in BS patients, leading to aneurysm, thrombotic occlusion, haemorrhage, pleural effusion, pulmonary infarct, or pulmonary fibrosis. The main pulmonary symptom in BS patients is haemoptysis. The frequency of pulmonary manifestations in BS patients is as follows: 0.3% in Iran, 2.2% in China, 11% in Italy, 33% in USA and 3.6% in Germany (Calamia et al., 2009, Davatchi et al., 2010b, Davatchi et al., 2010c, Kokturk, 2012, Salvarani et al., 2007).

1.2.6.10 CARDIAC MANIFESTATIONS

Cardiac manifestations of BS are very rare. Myocarditis, valvular lesions, pericarditis, ventricular aneurysms, intracardiac thrombosis, and coronary vasculitis were reported in the literature. The frequency of the cardiac manifestations is as follows: 0.5% in Iran, 4% in China, and 3.2% in Germany (Davatchi et al., 2010b, Davatchi et al., 2010c, Kokturk, 2012).

1.2.6.11 NEUROLOGICAL MANIFESTATIONS

Neurological manifestations of BS are rare but carry considerable risk of morbidity and mortality. Aseptic meningoencephalitis is the classical manifestation observed in Neuro-Behçet. However, behavioural changes, seizures, headache, benign intracranial hypertension, diencephalic dysfunction, aphasia, pseudobulbar palsy, brainstem syndromes, cranial nerve palsies, hemiplegia, cerebellar syndromes, myelopathy, and mononeuritis were reported in the literature as BS neurological manifestations. The frequency of neurological manifestations of BS is as follows: 9% in Iran, 11% in Japan, 6.4% in China, 4.6% in Korea, and 11% in Germany (Davatchi et al., 2010b, Davatchi et al., 2010c, Kokturk, 2012).

1.2.7 TREATMENT

The aim of the current treatment recommendations in BS is to prevent irreversible damage that mostly occurs early in the course of the disease, especially in the high-risk groups such as young age of onset, male gender, and HLA-B51. Treatment should also aim to prevent exacerbations of mucocutaneous and joint involvement that usually does not cause damage but affects the QoL (Calamia and Kaklamanis, 2008, Evereklioglu, 2004, Fresko and Yazici, 2008, Hatemi et al., 2008a, Kappen et al., 2008).

1.2.7.1 EULAR RECOMMENDATIONS

Several treatment recommendations were recently developed by a multi-disciplinary committee of the European League Against Rheumatism (EULAR), based on the best available evidence (Hatemi et al., 2008a):

1. Azathioprine and systemic corticosteroids are the treatment of choice for BS patients suffering from inflammatory eye disease affecting the posterior segment.

2. If the eye disease is severe, and that is defined as 0.2 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 ciclosporin or infliximab is prescribed in combination with azathioprine and corticosteroids; alternatively interferon alpha (IFNα) with or without corticosteroids is another appropriate treatment strategy.

3. There is no evidence-based guideline for the management of major vessel disease in BS. In case of acute deep vein thrombosis in BS, immunosuppressive agents such as corticosteroids, azathioprine, cyclophosphamide or ciclosporin are recommended. However, for the management of pulmonary and peripheral arterial aneurysms, cyclophosphamide and corticosteroids are recommended.

4. Similarly, there is no evidence of the benefit of anticoagulants, anti-platelet or anti-fibrinolytic agents in the management of deep vein thrombosis or the arterial lesions of BS.

5. There is no evidence-based recommendation for the management of gastrointestinal involvement of BS. Sulfasalazine, corticosteroids, azathioprine, TNFα antagonists and thalidomide should be tried as a first line therapy before proceeding to surgical treatment, except in emergencies.

6. Colchicine is the drug of choice for BS patients with arthritis.

7. There is no evidence-based guideline for the management of CNS involvement in BS. For parenchymal involvement, corticosteroids, IFN, azathioprine, cyclophosphamide, methotrexate, and TNFα antagonists can be tried. For dural sinus thrombosis, corticosteroids are recommended.

8. Ciclosporin should not be used in BS patients with CNS involvement unless necessary for intraocular inflammation.

9. The decision to treat skin and mucosa involvement will depend on the severity of the lesions. Topical therapy such as local corticosteroids should be the first line of treatment for isolated oral and genital ulcers, and acne-like lesions. Colchicine should be preferred when the dominant lesion is erythema nodosum-like lesions. To note, leg ulcers in BS might have different causes. Therefore, treatment should be designed in relation to the cause. Azathioprine, INF, and TNFα antagonists may be considered in resistant cases.

1.2.7.2 THE UK'S BS CENTRES OF EXCELLENCE RECOMMENDATIONS

A new treatment guidelines were suggested in 2012 by the three BS centres of excellence in the UK (unpublished). The drug pathway is as follows:

1.2.7.2.1 MUCOCUTANEOUS DISEASE

1. The initial therapy (oral cochicine, topical steroid, topical non-steroidal therapy, or mixed topical steroid and antimicrobial therapy) is expected to be effective in 70% of the cases.
2. If the initial therapy was ineffective, the step up therapy (azathioprine, mycophenolate, tacrolimus, or steroid) is expected to be effective in 66% of the cases.
3. If the step up therapy was ineffective, a 6 months course of TNF inhibitor therapy (infliximab 5mg/kg for 4 doses then swap to either adalimumab 40mg once a week or etanercept 50mg once a week) is expected to be effective. TNF inhibitor therapy should be re-initiated during flare up periods.

1.2.7.2.2 BS EYE DISEASE

1. The initial therapy (topical, intraocular, intavenous, or oral steroid) followed by maintenance therapy (oral steroid, azathioprine, or ciclosporin) is expected to be effective in 80% of the cases.
2. If the initial therapy was ineffective, a 24 months course of TNF inhibitor therapy (infliximab 5mg/kg for 4 doses then swap to either adalimumab 40mg once a week or etanercept 50mg once a week) is expected to be effective. TNF inhibitor therapy should be re-initiated during flare up periods.
3. If the TNF inhibitor therapy was ineffective, a 6 months course of INF-α should be considered.

1.2.7.2.3 MAJOR ORGAN DISEASE

Major organ disease is defined as BS with CNS involvements, peripheral neurological disease, or major vessel thrombosis.

1. The initial therapy (intavenous steroid and azathioprine) is expected to be effective in 50% of the cases.
2. If the initial therapy was ineffective, a 6 months course of TNF inhibitor therapy (infliximab 5mg/kg for 4 doses then swap to either adalimumab 40mg once a week or etanercept 50mg once a week) is expected to be effective. TNF inhibitor therapy should be reinitiated during flare up periods.
3. If the TNF inhibitor therapy was ineffective, cyclophosphamide or INF-α should be considered.

1.2.8 AETIOLOGY AND PATHOGENESIS

The aetiology of BS remains obscure. Bacterial and viral aetiology were suggested in the literature from over fifty years ago (Denman et al., 1980, Gray, 1950). However, epidemiologic and molecular studies proved that both genetic and environmental factors contribute to the disease susceptibility (Evereklioglu, 2005).

BS is one of the chronic, relapsing, multi-systemic, idiopathic, inflammatory problems. Therefore, it is crucial to understand precisely how the inflammation is initiated and to trace the activation events that occur within the cells of the innate immune system. It is also necessary to identify individual molecules of environmental origin that act as inducers of the inflammatory process, and the host receptor molecules that detect them (Huyton et al., 2007).

1.2.8.1 HYPOTHESIS

Lehner (1999) postulated that microbial infection induces a stress response that leads to upregulation of the heat shock protein (HSP) as well as the major histocompatibility complex class I chain-related (MIC) gene A product, which in turn will stimulate gamma delta (γδ) and alpha beta (αβ) T cell receptor positive (TCR^+) cells, neutrophils and B cells. These activated T cells, B cells and neutrophils will induce cytokines to modulate the immune response. The end result of these complex cellular and cytokine immune interactions in HLA-B51 or genetically related subjects is to induce pathological changes consistent with BS (Lehner, 1999) (Figure 1.12).

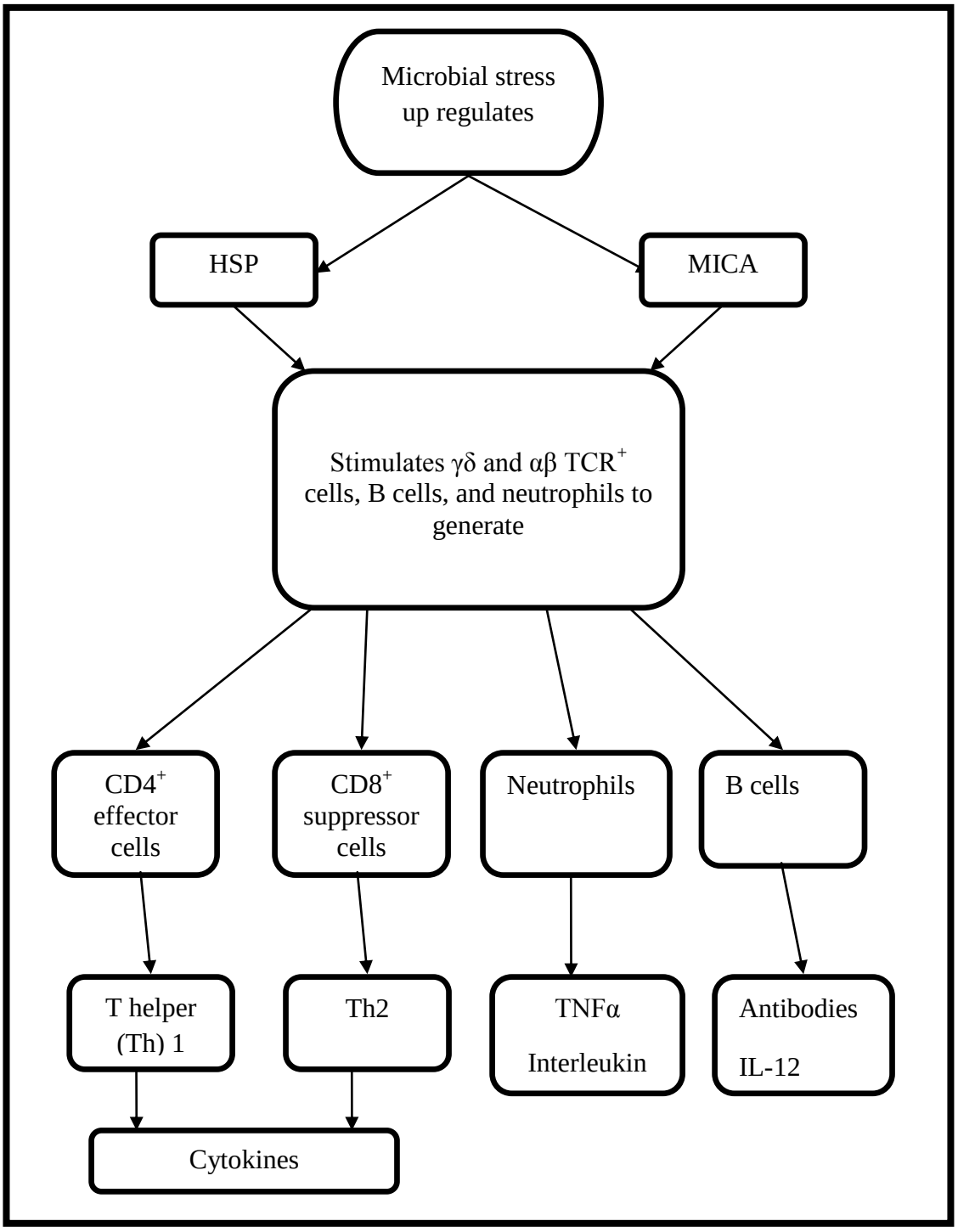


Figure 1.12: Lehner's Hypothesis for BS aetiology and pathogenesis [source: redrawn from (Lehner, 1999)].

1.2.8.2 GENETICS

The major histocompatibility complex (MHC) genes are the most polymorphic genes present in the genome, explained by the fact that more than 250 alleles have been identified for some HLA loci by serologic assays. Moreover, the molecular sequencing has shown that a single serologically defined HLA allele may consist of slightly different multiple variants (Abbas and Lichtman, 2005) (Figure 1.13). There are 21 different alleles from B5101 to B5121 serving to encode MHC class I, which participates during the presentation of endogenous antigens to $CD8^+$ cytotoxic-suppressor T lymphocytes (Abbas and Lichtman, 2005). Additionally, the HLA-B51 gene plays a role in neutrophil function (Fortune, 2003, Lehner, 1999, Yamashita, 1997).

Interestingly, the MHC class I HLA genotype B5 and its subclass (split antigen) B51 allele on chromosome 6p21 (HLA-B5101) is strongly associated with the susceptibility to BS (Bettencourt et al., 2008, Krause and Weinberger, 2008, Mizuki et al., 2002, Takemoto et al., 2008). Furthermore, HLA-Cw16 was also associated with BS susceptibility, while HLA-B27 was associated with the severity of the disease in a Portuguese BS cohort (Bettencourt et al., 2008). Indeed, the HLA-B51 genotype and BS have a close geographic distribution. However, around a third of BS patients do not possess the gene. HLA-B51 was considered to be a strong risk factor for developing severe ocular BS in particular (Verity et al., 1999c). However, other genes such as Factor V Leiden were also implicated as an additional risk factor for the development of ocular disease in BS patients (Verity et al., 1999b).

To date, it is still not clear whether HLA-B51 is pathogenic to BS or whether it is in linkage disequilibrium to the causative gene (Ahmad et al., 2003, Mizuki et al., 1995) (Figure 1.13). Therefore, recent studies have focused on studying MICA, TNFα and HSP genes as these genes are located on chromosome 6 in close proximity to HLA-B51 (Ahmad et al., 2003, Akman et al., 2008b, Arayssi et al., 2008, Durrani and Papaliodis, 2008, Hughes et al., 2005, Karasneh et al., 2005, Mizuki et al., 1995).

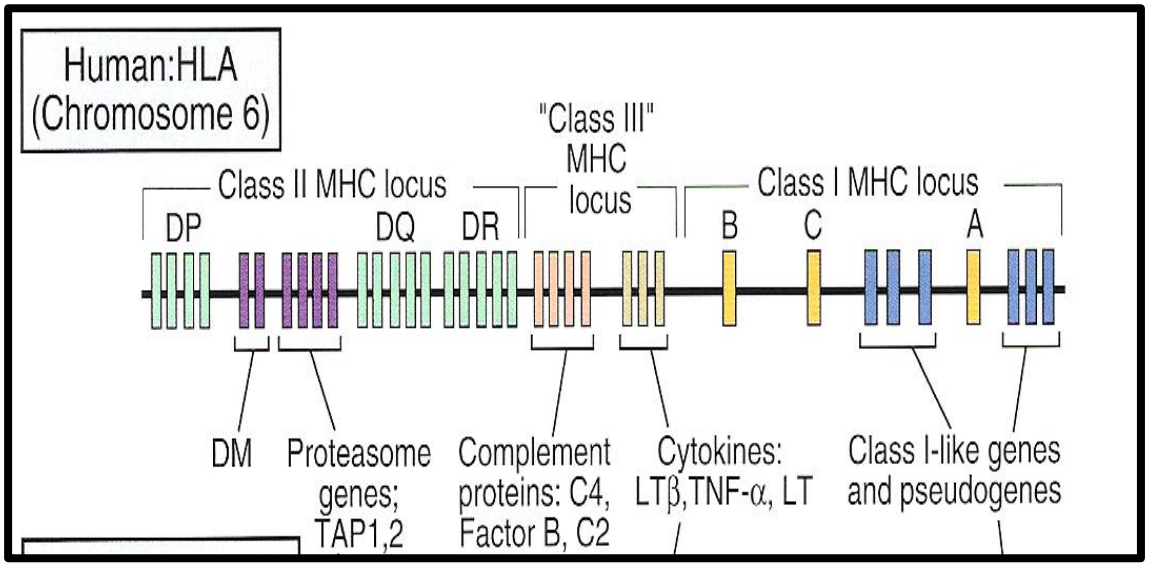


Figure 1.13: The proximity of Class I and Class II MHC Locus in chromosome 6p21 [source: (Abbas and Lichtman, 2005)].

The MIC genes occupy the region between the HLA-B and TNF genes. Seven MIC loci exist (A–G), with only MICA and MICB encoding proteins which show homology with the classical HLA molecule. MIC encoded proteins do not combine with ß2 microglobulin and are not expressed on normal circulating lymphocytes but they are expressed on the surface of freshly isolated gastric epithelium and endothelial cells, and fibroblasts. MICA and MICB act as ligands for activating the natural killer-cell G2D (NKG2D) receptor which is

also found on γδ T cells and αß $CD8^+$ T cells (Abbas and Lichtman, 2005). Interestingly, a triplet repeat microsatellite polymorphism in the transmembrane region of the MICA gene has been found in Caucasian BS patients and other patients with Turkish, Korean, Italian, and Iranian origins (Hughes et al., 2005, Mizuki et al., 2002, Park et al., 2002, Picco et al., 2002).

Recent genetic investigations suggested that TNF genes are an important risk factor in BS pathogenesis. Two extended HLA haplotypes which contain the TNF promoter polymorphism-1031C gene were identified and correlated to BS. The TNF promoter polymorphism-1031C allele was found to be associated with BS even in individuals who did not carry either HLA-B51 or HLA-B5701 (Ahmad et al., 2003, Arayssi et al., 2008).

A comparison between patients with BS and HC volunteers revealed no significant difference in the frequency of polymorphism in one of the HSP 70 genes HUM70t (HSP70-Hom) (Mizuki et al., 1995). Furthermore, the single-nucleotide polymorphism (SNP) analysis of TLR2 and TLR4 genes was not significantly associated with BS in Caucasian, Turkish, and Tunisian cohorts (Bacanli et al., 2006b, Ben Dhifallah et al., 2009, Durrani et al., 2011, Tomiyama et al., 2009). However, one SNP in the TLR4 gene but not TLR2 was associated with BS and six other SNPs had an effect on its clinical features in the Japanese BS patient cohort (Meguro et al., 2008, Tomiyama et al., 2008). Likewise, TLR4 haplotype TAGCGGTAA was considered a susceptibility gene for HLA-B51 positive BS patients and for the complication of arthritis in the Korean cohort (Horie et al., 2009).

Polymorphisms in other genes were also suggested as being associated with the risk of developing BS, such as the increased frequency of E469 in the intercellular adhesion molecule-1 gene, increased frequency of IL-1α-889C, and the decreased frequency of 620W in the gene encoding protein tyrosine phosphatase type 22 (Akman et al., 2008a, Baranathan et al., 2007, Verity et al., 2000).

Recently, an association at the IL10 and IL23R-IL12RB2 locus was identified. Furthermore, BS was found to be associated with the IL10 gene variant (rs1518111A allele), which is known to cause a diminished mRNA expression and a low protein production (Mizuki et al., 2010, Remmers et al., 2010, Xavier et al., 2012). IL10 is a known suppressor for inflammatory cytokines; therefore, desregulation of its production will lead to a status of heightened inflammatory response. Interestingly, the same variant was previously associated with severe juvenile rheumatoid arthritis (Crawley et al., 1999)

1.2.8.3 INFECTIOUS AETIOLOGY

The aetiology of BS has not been completely identified. However, an infectious aetiology was postulated more than fifty years ago (Denman et al., 1980, Eglin et al., 1982, Gray, 1950). A viral cause for BS was first postulated by Hulusi Behçet, and later Denman et al. (1980) and then Eglin et al. (1982) showed by in situ DNA-DNA hybridization that part of the HSV1 genome is transcribed in mononuclear cells of some patients with BS (Denman et al., 1980, Eglin et al., 1982, Gray, 1950).

The current evidence in the literature suggests that the herpes virus family is the most probable virus associated with BS. Herpes virus DNA and serum antibodies against the

virus have been found in a higher proportion of patients with BS than in HC volunteers. However, none of these viral infectious agents were proven to cause BS and no study was able to establish whether the organisms were the cause or consequence of the lesions (Direskeneli, 2001).

The level of IgG antibody against HSV1 was found to be significantly increased in patients who had BS (Lee et al., 2005). However, no statistical significance was observed between PCR results of HSV1 DNA in the saliva of BS patients and of HC volunteers (Lee et al., 1996). At the same time, administration of high doses of acyclovir in association with plasma exchanges failed to produce positive treatment results, and treatment with acyclovir alone failed to alleviate the frequency and severity of oral and genital ulceration or other BS features in a randomized, double-blind, placebo-controlled, crossover trial (Davies et al., 1988).

In addition to HSV1, the CMV has been studied in relation to BS. The mean titre of IgG and IgA antibodies to CMV was significantly lower in BS patients than in HC volunteers. However, the number of patients having IgM antibody against CMV was similar in both BS patients and HC volunteers (Lee et al., 2005). In a recent study, CMV DNA was detected in biopsy specimens from the oral mucosa of BS patients (Sun et al., 1996).

The involvement of streptococcus infection has also been suggested (Lehner et al., 1991, Mumcu et al., 2007b). The serum antibody titre and delayed hypersensitivity of patients with BS against *Streptococcal* antigens were reported to be significantly higher than those of HC volunteers (Kaneko et al., 1997, Kaneko et al., 2008). An increase in the

concentration of oral streptococci in BS patients has been demonstrated, more especially the uncommon serotypes of *Streptococcus sanguis* (KTH-1, strain BD113-20) (Lehner et al., 1991, Yoshikawa et al., 1998). Interestingly, some streptococcal antigens that are associated with HSP can be recognized by human γδ T cell lines. Indeed, the previous research in this field proved that the microbial HSP65 shows homology with the human mitochondrial HSP60 and shares antigenicity with this oral mucosal antigen (Lehner et al., 1991, Lehner, 1997).

In a recent study, *Staphylococcus aureus*, *Moraxella* spp., and *Streptococcus* spp. were found to colonize the conjunctiva of BS patients more significantly than those of the HC volunteers (Gunduz et al., 2008). *Staphylococcus aureus* and *Prevotella* spp. were also found to colonize the pustular lesions of BS patients more frequently than HC volunteers (Hatemi et al., 2004). Furthermore, treatment of eight BS patients suffering from active mucocutaneous symptoms with azithromycin for four weeks was reported to be effective in decreasing folliculitic lesions and speeding up the healing of oral ulcers (Mumcu et al., 2005).

Interestingly, the incidence of bacteraemia and viraemia was also investigated in relation to BS patients. No bacterial DNA was isolated from the collected blood samples from BS patients. However, EBV, CMV and HHV8 were detected more frequently in the BS patient cohorts (Irschick et al., 2011).

It is still not entirely clear whether the change in the microbial community triggers the heightened immune response in BS patients, or whether the increase in the detected

imbalance in the microbial diversity and load results from a raised host susceptibility to infection due to a defect in the immune response (Fortune, 2003). However, the speculation that an infectious antigen might contribute to the aetiology of BS is supported by the following facts:

1. The HLA-B51 gene, which has an association with BS, encodes MHC class I that participates during the presentation of endogenous antigens to $CD8^+$ cytotoxic-suppressor T lymphocytes (Bettencourt et al., 2008, Krause et al., 2008, Takemoto et al., 2008).
2. Recently, BS was found to be associated with the IL10 gene variant (rs1518111A allele), which is known to cause a diminished mRNA expression and a low protein production, and potentially leads to heightened inflammatory response to microbial insult (Mizuki et al., 2010, Remmers et al., 2010, Xavier et al., 2012).
3. The major part of the immunopathogenesis in BS is believed to be a T-cell-mediated immune response, especially by γδ T cells; they are a fascinating population of T cells that participate in early responses against microorganisms by identifying a wide variety of antigens and they can respond to non-peptide antigens in a non-MHC restricted fashion (Fortune et al., 1990).
4. There is an increase in the concentration of oral streptococci and increase in the IgG antibody against HSV1 in BS, which remains an area of interest for further research as no causative correlation has been established yet (Hamza et al., 1990, Yoshikawa et al., 1998).

1.2.8.4 HEAT SHOCK PROTEIN

HSPs form the most abundant group of proteins inside cells and are expressed in a variety of intracellular locations, such as cytosol of prokaryotes and in the cytosol, nuclei, endoplasmic reticulum, mitochondria and chloroplasts of eukaryotes. Their expression can be induced as a result of heat shock or other forms of stress, including exposure to toxins, oxidative stress and glucose deprivation (Srivastava, 2002).

There are ten families of HSPs. Each family consists of anywhere from one to five closely related proteins, whereas there is no obvious homology among the individual HSP families. The proposed function of HSPs is folding and unfolding of proteins, degradation of proteins, assembly of multi-subunit complexes, thermo-tolerance, and buffering of expression of mutations. Furthermore, HSP molecules are peptide binding proteins and are associated with antigenic epitopes (Srivastava, 2002) (Figure 1.14).

HSPs are highly conserved immunogenic proteins synthesized in large amounts when cells are exposed to stressful stimuli. They are often immunodominant antigens produced in bacteria and mammalian cells by a variety of stresses. In infections, HSP release could support induction of immunity. Extensive sequence homology between microbial and human HSPs has led to the concept that HSPs might be involved in the aetiology and pathogenesis of autoimmune disorders. It has long been suspected that these proteins act as danger signals and act as endogenous immune stimulators that are released during infections by intrinsic cells of the body, and that alert the immune system to the need to induce immune responses (Lang et al., 2005).

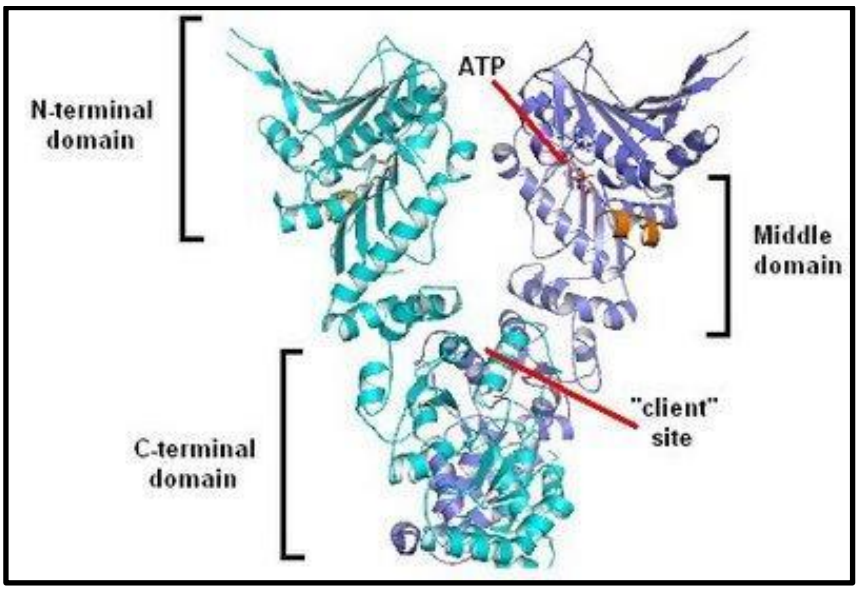


Figure 1.14: Different members of HSPs family contain a highly conserved N-terminal ATPase domain, a conserved C-terminal hydrophobic peptide binding domain and more variable alpha-helical domains [source: (Dollins et al., 2007)].

When a pathogen infects the cells, it replicates in the infected cells and can destroy them, releasing abundant HSPs that carry information about the internal environment. The released HSPs in turn can bind to specific receptors on the antigen presenting cell (APC) such as CD91, TLRs, CD36, CD40, LOX1 (Srivastava, 2002). Endocytosis of HSP-peptide complexes occurs after the HSPs interact with APCs through one of the above-mentioned receptors. Endocytosis will be followed by the processing of the HSP-peptide complex and their presentation by MHC class I (MHC I) molecules to $CD8^+$ T cells or by MHC class II (MHC II) molecules to $CD4^+$ T cells. HSPs can also activate the antigen nonspecific pathway, which lead to innate immune response activation (Srivastava, 2002).

The MHC I or the MHC II pathway will be activated if HSPs bind to CD91 or LOX1 receptors on the APC. However, when the HSPs interact with the APC through TLRs,

CD40 or CD36, the antigen nonspecific pathway will be activated, which leads to translocation of nuclear factor kappa B (NFκβ) into the nuclei of macrophages and dendritic cells (DCs) (Srivastava, 2002).

HSPs can act as an adjuvant to exogenous antigen and induce the activation of the APC through the classical MHC I antigen processing pathway, a phenomenon known as cross-presentation that can be used in vaccine development (Susumu et al., 2008).

Nevertheless, the release of HSPs is not specific to infections only but also occurs in response to physical stimuli, such as heat or irradiation, and in non-infectious inflammatory diseases. It is known now that HSPs released from injured tissue may aggravate autoimmune responses (Srivastava, 2002).

Many research groups have indicated that HSPs have been implicated in the aetiology of BS. Indeed, a significant increase in serum IgA antibodies to the mycobacterial HSP65 was found in BS. Furthermore, the HSP65 of *Mycrobacterial tuberculosis* shows significant homology with human HSP60 and cross-reacts with that produced by strains of *Streptococcus sanguis*. These observations are consistent with the presence of an increased population of γδ T cells, which could recognize and respond to HSPs and which were found in the peripheral blood of patients with BS. Furthermore, γδ T cells increased during periods of active disease (Direskeneli et al., 1996, Fortune, 2003, Pervin et al., 1993). Moreover, HSP60 is a known ligand for TLR2 and TLR4 and possibly acts as an endogenous danger signal to the immune system, with rapid inflammatory cytokine release and the enhancement of adaptive Th1-type responses (Takeda et al., 2003).

Two types of immune responses to HSPs have been proposed. One type has foreign epitopes not shared by self-HSP, inducing a conventional response to infection, and the other has conserved epitopes recognized by a tightly regulated autoreactive T cell population. Both the T cell and B cell responses in BS fall into the second type, as immune responses were detected against both foreign and homologous human determinants (Direskeneli et al., 1996, Pervin et al., 1993).

Recently, T cell epitope mapping has identified four peptides (p111-125, p154-172, p219-233 and p311-325) derived from the sequence of the HSP65. Interestingly, p336-351 is significantly associated with BS in Britain, Japan and Turkey. These peptides can specifically stimulate TCR $\gamma\delta^+$ lymphocytes from BS patients and they show significant homology with the corresponding peptides derived from the human HSP60 (Lehner, 1997).

A rat model of uveitis was investigated and the development of uveitis was successfully prevented by oral tolerization with the 336-351 peptide linked to recombinant cholera toxin B subunit (CTB) (Lehner et al., 2003). This strategy was adopted in a phase I/II clinical trial by oral administration of p336-351 three times a week, followed by gradual withdrawal of all immunosuppressive drugs used to control the disease in eight patients with BS. Oral administration of p336-351-CTB had no adverse effect and withdrawal of the immunosuppressive drugs showed no relapse of uveitis in five out of the eight patients. After tolerization was discontinued, three patients remained free of relapsing uveitis for 10–18 months after cessation of all treatment (Stanford et al., 2004).

The human HSP70 protein and anti-HSP70 antibodies were also investigated and both were found to be elevated in the serum of BS patients. HSP70 mediates innate and adaptive immune responses and may participate in pro-inflammatory cytokine activation and tissue destruction in BS. It also can take a role in the cross-presentation process previously explained (Birtas-Atesoglu et al., 2008).

Based on the above background information, one can conclude that a heightened responsiveness to microbial antigens in genetically susceptible hosts might be the best hypothesis to explain BS aetiology. It is possible that microbial HSPs stimulate the production of self-HSPs. This, in turn, may serve as an endogenous danger signal with rapid augmentation of the inflammatory reaction (Lehner, 1999).

1.2.8.5 TOLL-LIKE RECEPTORS

TLRs are a receptor-based system for the recognition and discrimination of microbial pathogens by sensing their Pathogen-Associated Molecular Patterns (PAMP). TLR1, the first TLR, was identified in 1994, and was so named because of its close homology with the known Drosophila Toll receptor system (Gay and Gangloff, 2007). TLR4 was identified in 1997, and TLRs 2, 3, and 5 were cloned shortly thereafter. TLRs are mainly expressed on the cells associated with innate immunity such as granulocytes, monocytes, macrophages and dendritic cells. However, some TLR expression was demonstrated on epithelial and endothelial cells (Takeda et al., 2003).

TLRs act as surface proteins that sense the presence of microbial infection by binding to the characteristic patterns within molecules on the outside of bacteria, fungi and viruses, and

even to forms of DNA and RNA. This receptor system is thought to provide the specificity for the innate immunity, allowing it to recognize and differentiate between gram negative (–ve) bacteria, gram positive (+ve) bacteria or fungal infection (Takeda et al., 2003).

The TLRs are type I integral membrane glycoprotein, consisting of a characteristic extracellular leucine-rich repeat (LRR) domain and intracellular toll/interleukin-1 receptor (TIR) domain. These conserved domains have helped to identify and classify 13 distinct mammalian TLRs, 10 of which are found in humans (Huyton et al., 2007) (Figure 1.15).

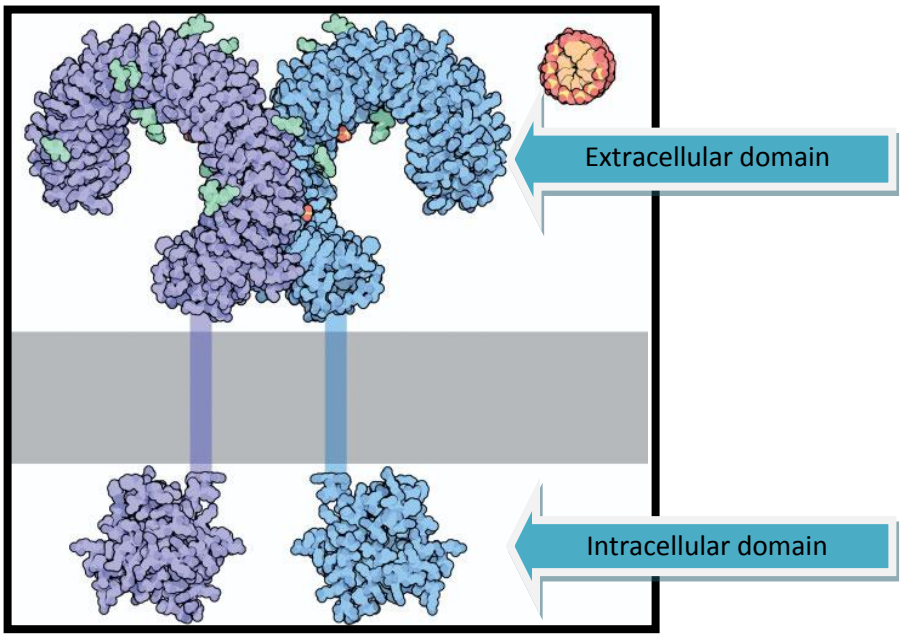


Figure 1.15: Structure of TLR [source: (Goodsell, 2006)].

TLR2 and TLR4 jointly act as two of the major innate immune receptors recognizing most of the oral microbial commensals and pathogens. It is known that some HSP families, which might play a pivotal role in BS pathogenesis, are ligand to TLR2 and TLR4 (Binder

et al., 2004). Therefore, Table 1.5 focuses on the description of the TLR2 and TLR4 distribution, their activators, ligand and adaptors.

Table 1.5: TLR2's and TLR4's distribution, activators, ligand, and adaptors [source: (Takeda et al., 2003)].

TOLL-LIKE RECEPTOR	**CELL TYPES**	**ACTIVATORS**	**LIGAND**	**ADAPTORS**
TLR2	Macrophage Dendritic cell Granulocyte	Gram +ve bacteria Fungi Parasite Self Viruses	B-glucans HSP70 Necrotic cells Peptidoglycan Porins Posphalipomannan Teichoic acid Zymosan	Mal MyD88
TLR4	Macrophage Dendritic cell T cell Granulocyte B cell	Gram –ve bacteria Fungi Self Viruses	*Chamydia pneumonia* CMV Glycoproteins Human HSP60/70 HSP60 LPS Mannan O-linked mannosyl residue Teichoic acid RSV	Mal/TIRAP and MyD88 TRAM and TRIF

HSP: heat shock protein; LPS: lipopolysaccharide; Mal: MyD88 adaptor-like; Mal/TIRAP: MyD88 adaptor-like/TIR domain-containing adaptor molecule; MyD88: myeloid differentiation primary response protein 88; RSV: respiratory syncytial virus; TRAM: tumour necrosis factor receptor-associated factor; TRIF: TIR domain-containing adaptor protein inducing interferon-β

Activation of TLRs triggers a cascade of protein kinases that result in a well-characterized innate host recognition pathway leading to the nuclear translocation of NF-κB and transcription of cytokine-encoding genes (Takeda et al., 2003). The NF-κB pathway is of great significance because it is involved in the regulation of about 40% of human genes and

is particularly involved in the production of pro-inflammatory cytokines, chemokines, and Growth Factors (GF) as well as inducible enzymes, including the inducible Nitric Oxide Synthase (iNOS) (Takeda et al., 2003) (Figure 1.16).

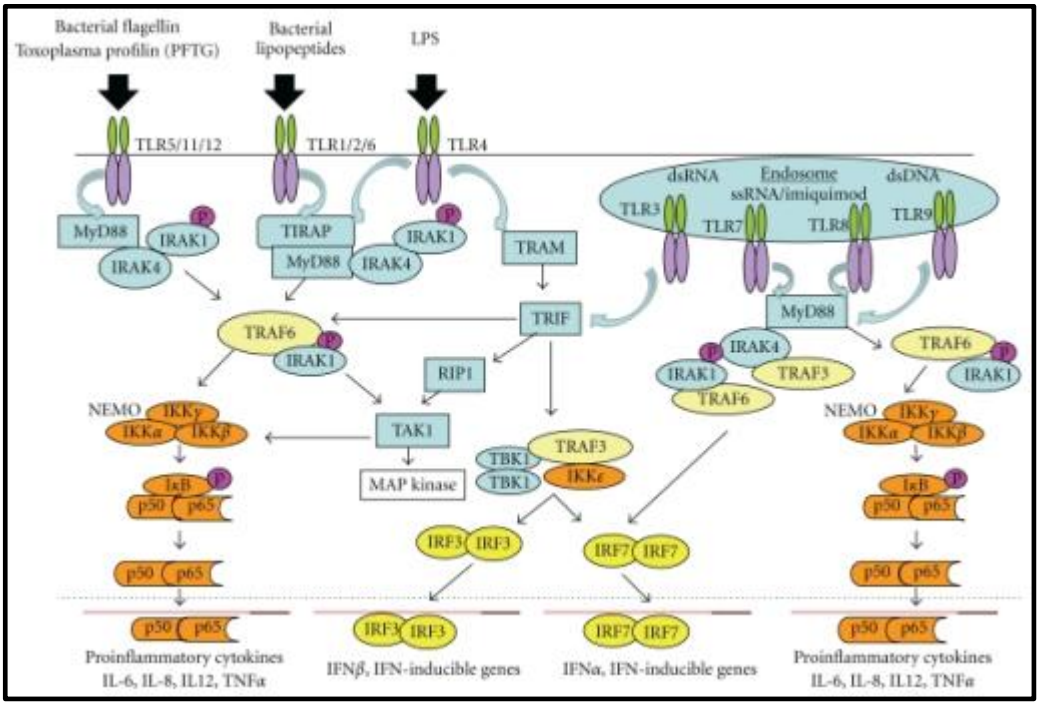


Figure 1.16: The cascade of protein kinases triggered by activation of TLRs [source: (Zhu and Mohan, 2010)].

The first step in TLR signalling is stimulus-induced dimerization or oligomerization. The next step is the recruitment of adaptors to the TIR domains of the dimerized receptor. There are a total of five adaptors which are: myeloid differentiation primary response protein 88 (MyD88); MyD88 adaptor-like/TIR domain-containing adaptor molecule (Mal/TIRAP); tumour necrosis factor receptor-associated factor (TRAM); TIR domain-containing adaptor protein inducing interferon-ß (TRIF); and sterile alpha and HEAT/Armadillo motif protein (SARM). MyD88 is used by TLRs 5, 7, 8 and 9. However, TLR2 requires Mal and MyD88,

whereas TLR4 can use either Mal/TIRAP and MyD88 or TRAM and TRIF to signal to NF-κB or antiviral responses mediated through interferon response factor (IRF) 3 respectively. At the same time, TLR3 uses TRIF exclusively for IRF3-directed signals (Gay and Gangloff, 2007).

Next in the signalling hierarchy are the interleukin-1 receptor-associated kinases (IRAKs). There are four IRAKs; IRAK2 and IRAKM are not active phosphotransferases and function as negative regulators, whereas IRAK4 and IRAK1 autophosphorylate and are recruited by association with MyD88 into the post receptor signalling complex (Gay and Gangloff, 2007). The precise events thereafter which lead to downstream signalling are not completely understood: they lead either to releases of NF-κB which then relocalize to the nucleus or to the activation of the second branch of the TLRs' pathway leading to antiviral responses such as the production of interferons, chemokines and anti-inflammatory cytokines. Interestingly, TLR4 and TLR3 can activate the antiviral response pathway. In doing so, TLR4 requires the adaptor molecules TRAM and TRIF, whereas TLR3 requires TRIF alone (Gay and Gangloff, 2007).

More interestingly, the activation of different TLRs can result in different reactions within the cell. As an example, activation of TLR2 induces the transcription of genes encoding TNF-α and IL-1ß less effectively than those binding to TLR4, and results in no transcription of genes encoding IFN-γ or IL-12. Moreover, TLR2 signalling can also activate pro-apoptotic pathways resulting in inflammatory cell death (Takeda et al., 2003).

An elevated expression of TLR4 (mRNA and protein) but not TLR2 in peripheral blood mononuclear cells (PBMCs) of BS patients was initially observed, irrespective of the disease activity (Kirino et al., 2008). Later on, TLR2 and TLR4 expression in the PBMCs of active BS patients were highly elevated compared to HC volunteers. Interestingly, in the same study the serum vitamin D levels were found to be lower in active BS patients. Therefore, vitamin D levels were inversely correlated with the expressions of TLR2, TLR4, and with the clinical indicators (Do et al., 2008). In a functional study, vitamin D was found to suppress the protein and mRNA expression of both receptors (Do et al., 2008). Furthermore, decreased TLR2 expression was noted in PBMCs of BS patients after stimulation with HSP60 and lipopolysaccharide (LPS) (Yavuz et al., 2008). The total expression of TLR2 and TLR4 was also found to be elevated in the intestinal and buccal mucosa of BS patients and broncho-alveolar lavage (BAL) (Durrani et al., 2011, Hamzaoui et al., 2012, Nara et al., 2008).

Because of the essential role of TLRs in innate immune recognition, these receptors are currently being explored as possible therapeutic targets. The use of antibodies or small molecules that block recognition or signalling by the TLRs may play a role in damping the exaggerated inflammatory responses in diseases such as sepsis and autoinflammatory or autoimmune diseases (Goodsell, 2006).

1.3 AIMS AND HYPOTHESIS

1.3.1 CLINICAL AIMS

1. To analyse the frequency of the different signs and symptoms of the BS patient cohort in the UK and compare it to the international published data. To analyse the treatment protocol used to treat the BS patient cohort in the UK.
2. To correlate the QoL to the oral health status and disease activity in the UK's BS patient cohort and compare it to a Turkish BS patient cohort.
3. To compare the oral health status of the UK's BS patient cohort with RAS patients and HC volunteers.

1.3.2 LABORATORY AIMS

1. To investigate aspects of the oral mucosal innate immune response in BS patients by focusing on the difference in expression of TLR2 and TLR4, mRNA and protein in the buccal mucosa of BS patients, RAS patients, and HC volunteers. Moreover, to determine the difference in expression of TLR2 and TLR4, mRNA and protein, in the oral mucosa of BS patients during inactive and relapsed periods.
2. To investigate the level of expression of the different splice variants of TLR2 and TLR4 in the oral mucosa of BS patients, RAS patients, and HC volunteers. Understanding the difference between inactive and relapsed periods of BS activity was of great interest.
3. To investigate the function of TLR2 and TLR4 in vitro upon treatment with different agonists.

4. To determine the difference in buccal mucosal and salivary microbiome between BS patients, RAS patients, and HC volunteers. Moreover, to investigate any difference in the ulcerated and non-ulcerated mucosal microbiome in BS patients.

1.3.3 HYPOTHESIS

It was hypothesized that the complex interplay between the oral microbiome (environmental factors) and aspects of the innate immune responses such as TLRs in genetically susceptible patients leads to enhanced inflammatory responses and consequently the amplified tissue damage observed in BS patients (Figure 1.17).

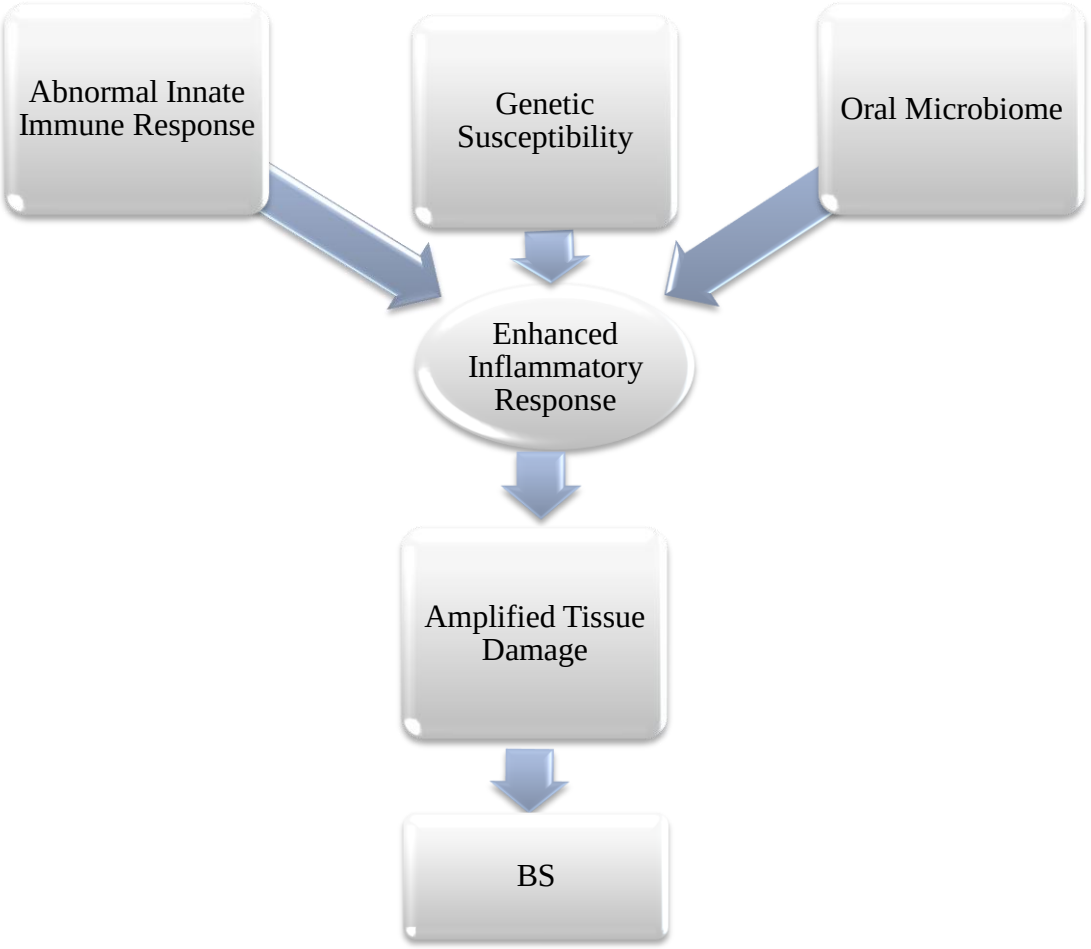


Figure 1.17: BS is caused by the interaction of a number of factors leading to amplified tissue damage.

This study attempted to examine the following null hypothesis:

1. There are no differences in the QoL, oral health status and disease activity in the UK's BS patient cohort compared to a Turkish BS patient cohort.
2. There are no differences in the oral health status of the UK's BS patients, RAS patients, and HC volunteers.
3. There are no differences in the expression of TLR2 and TLR4, mRNA, and protein in the buccal mucosa of BS patients, RAS patients, and HC volunteers.
4. There are no differences in the expression of TLR2 and TLR4, mRNA, and protein in the buccal mucosa of BS patients during inactive and relapsed periods.
5. There are no differences in the level of expression of the different mRNA splice variants of TLR2 and TLR4 in the buccal mucosa of BS patients, RAS patients, and HC volunteers.
6. There are no differences in the level of expression of the different mRNA splice variants of TLR2 and TLR4 in the buccal mucosa of inactive and relapsed BS patients.
7. There are no differences in the function of TLR2 and TLR4 expressed on BS patients' PBMC compared to HC volunteers.
8. There are no differences in the buccal mucosal and salivary microbiome between BS patients, RAS patients, and HC volunteers.
9. There are no differences in the ulcerated and non-ulcerated mucosal microbiome in the BS patient cohort.

CHAPTER 2

MATERIALS AND METHODS

2.1 SECTION 1: CLINICAL METHODS

This clinical study investigated aspects of the oral health status of Behçet's syndrome (BS) patients and compared it to recurrent aphthous stomatitis (RAS) patients and healthy control (HC) volunteers. BS patients were accessible through specialized clinics in the Royal London Hospital and St Thomas' Hospital. These clinics specialize in diagnosing, managing, and investigating patients who are classified according to the International Study Group (ISG) criteria for BS diagnosis (*The Lancet*, 1990). A small cohort of Turkish BS patients was also compared to the UK patients in the quality of life (QoL) and oral health analysis part of the study. The RAS patients and HC volunteers were accessible through the Oral Medicine Clinic at the Royal London Hospital.

2.1.1 ETHICAL APPROVAL

The project is part of the immunoregulation at the mucosal barrier programme, with ethical approval granted by the East London and The City Research Ethics Committee, Reference P/03/122. Ethical approval has been granted for St Thomas' Hospital, and the Turkish centre independently.

2.1.2 SUBJECTS

There are three groups in this clinical study, namely BS patients, RAS patients and HC volunteers.

2.1.2.1 GROUP NUMBER 1: BS PATIENTS

BS patients were recruited mainly from the two UK centres mentioned above. The Turkish cohort was included in the QoL and oral health status analysis only.

2.1.2.1.1 UK COHORT

To avoid selection bias, all patients attending the research clinic who were over 18 years old were invited to take part in the study. If the patient wished to consider participation in the study, it was explained to them and they were provided with full details. An informed consent was subsequently obtained (Appendix 1).

2.1.2.1.2 TURKISH COHORT

Similarly to the UK cohort, all patients attending the research clinic who were over 18 years old were invited to take part in the study. Patient recruitment and investigation in Turkey were conducted by the Turkish collaborators.

2.1.2.1.3 BS PATIENTS' INCLUSION CRITERIA

All BS patients were diagnosed and classified according the ISG criteria for BS patient diagnosis (Lancet, 1990).

2.1.2.1.4 BS PATIENTS' EXCLUSION CRITERIA

1. Not fulfilling the ISG criteria for BS patient diagnosis
2. Pregnancy
3. Age under 18 years.

2.1.2.1.5 BS PATIENTS' STRATIFICATIONS

BS patients were clinically assessed in a consultant-led examination and then classified according to the overall activity of the disease into the following groups:

1. Relapsed status that was defined as severe disease activity in more than two of the BS symptoms despite treatment.
2. Minor activity status that was defined as some disease activity in less than three of the BS symptoms.
3. Inactive status that was defined as the absence of any symptoms at the time of sampling.

BS patients were further stratified according to the oral activity into orally active and orally inactive groups.

1. Orally active BS is a state of oral activity where patients presented to the clinic with oral ulcerations.
2. Orally inactive BS is a state of oral inactivity where patients presented to the clinic with no oral ulcerations.

The different disease activities, other than the oral ulceration, were also examined along with the treatment protocol.

2.1.2.2 GROUP NUMBER 2: HC VOLUNTEERS

This group consists of HC volunteers, recruited using advertisements displayed on appropriate notice boards in the Dental Institute of The Royal London Hospital and the Centre of Clinical and Diagnostic Oral Sciences of Barts and The London School of Medicine and Dentistry. Every effort has been made to match the age and gender of the control group to that of the patient group.

2.1.2.2.1HC VOLUNTEERS' INCLUSION CRITERIA

1. Healthy individuals who do not suffer from chronic diseases or do not receive regular medications.
2. Healthy individuals who matched the BS patient group in age and gender.

2.1.2.2.2 HC VOLUNTEERS' EXCLUSION CRITERIA

1. Chronic disease
2. Regular medications
3. Pregnancy
4. Age under 18 years
5. History of recurrent oral ulceration.

2.1.2.3 GROUP NUMBER 3: RAS PATIENTS

This group consists of RAS patients who experience recurrent oral ulcerations but do not fulfil the ISG criteria of BS patient diagnosis.

2.1.2.3.1 RAS PATIENTS' INCLUSION CRITERIA

The RAS patient group includes patients who have a history of recurrent oral ulcerations of indefinable cause.

2.1.2.3.2RAS PATIENTS' EXCLUSION CRITERIA

1. Patients with a history of recurrent oral ulcerations accompanied by any other systemic manifestation such as skin lesions, eye problems or genital ulcerations.

2. Patients with a history of recurrent oral ulcerations with identifiable cause such as trauma, mucocutaneous disease, or vitamin deficiency.
3. Age under 18 years.
4. Pregnancy.

2.1.3 QUESTIONNAIRES

2.1.3.1 CLINICIAN'S OBSERVATION FORM FOR DISEASE ACTIVITY

The clinician's observation form for disease activity was used to record the activity of the disease in each session after a consultant-led clinical assessment (Appendix 2). Oral, genital, dermatological, ophthalmological, vascular, and neurological activities were recorded. The clinician's opinion on the overall activity of the disease was also recorded and each case was classified as relapsed or inactive.

2.1.3.2 QoL AND ORAL HEALTH STATUS QUESTIONNAIRE

QoL was assessed using the oral health impact profile-14 (OHIP-14) while the oral health status was assessed by dental examination using dental and periodontal indices. This questionnaire aimed to highlight the correlation between QoL, oral health, and disease activity.

2.1.3.2.1 OHIP-14

A 5-point Likert-type scale was used in scoring each item of the OHIP-14. Responses were coded 0=never, 1=hardly ever, 2=occasionally, 3=fairly often, 4=very often. Patients' responses were added up to produce an OHIP-14 total score. Total OHIP-14 scores ranged

from 0 (no impact) to 56 (all of the oral health problems were experienced very often). High scores indicated a poor oral health-related QoL (Appendix 3).

2.1.3.2.2 DENTAL AND PERIODONTAL INDICES

Oral health was evaluated by dental and periodontal indices including the decayed, missing, filled teeth (DMFT) index, plaque index (PI), gingival index (GI), sulcus bleeding index (SBI), periodontal probing depth (PPD) and attachment level (AL) (Appendix 4).

2.1.3.2.2.1 DMFT INDEX

The DMFT index is a general indicator of the dental health status of a population: the lower the index, the better the dental health. The DMFT score for any individual can range from 0 to 32, in whole numbers. The index gives equal weight to missing, untreated, decayed, and well-restored teeth (Miyamoto et al., 2006).

2.1.3.2.2.2 PI

The PI is a general indicator of oral hygiene. The score ranges from 0 to 3. Score 0 means no plaque, score 1 means a film of plaque adhering to the free gingival margin and adjacent area of the tooth, score 2 means moderate accumulation of soft deposits within the gingival pocket of the tooth and gingival margin which can be seen with the naked eye, and score 3 means an abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.

The PI was calculated for each examined tooth by adding the plaque scores for the 4 different surfaces (buccal, lingual/palatal, mesial, and distal) and dividing the score by 4.

The index for each patient was then calculated by adding the indices for all teeth and dividing by the number of teeth (Loe, 1967).

2.1.3.2.2.3 GI

The GI was developed more than 40 years ago to describe the clinical severity and location of gingival inflammation (Table 2.1) (Loe, 1967). The GI score ranges from 0 to 3 with score 0 representing clinically normal gingiva with no bleeding on probing, and score 3 corresponding to severe gingival inflammation evidenced by marked redness, hypertrophy, oedema, ulceration, and spontaneous bleeding. Score 1 indicates mild gingival inflammation while score 2 points towards the presence of moderate gingival inflammation.

Table 2.1: Loe (1967) GI scoring system.

APPEARANCE	**BLEEDING**	**INFLAMMATION**	**SCORE**
Normal	No bleeding	None	0
Slight change in colour, and mild oedema with slight change in texture	No bleeding	Mild	1
Redness, hypertrophy, oedema, and glazing	Bleeding on probing/pressure	Moderate	2
Marked redness, hypertrophy, oedema, ulceration	Spontaneous bleeding	Severe	3
[source: (Loe, 1967)]			

2.1.3.2.2.4 SBI

The SBI was used to supplement the data obtained from the GI in assessing the presence of gingivitis (Table 2.2) (Muhlemann and Son, 1971). The SBI score ranges from 0 to 5 with score 5 indicating severe gingival inflammation while score 0 represents clinically normal gingiva.

Table 2.2: Muhlemann and Son (1971) SBI scoring system.

APPEARANCE	SULCUS PROBING	SCORE
Healthy	No bleeding	0
Apparently healthy with no change in colour and no swelling	Bleeding on probing	1
Change in colour due to inflammation; no swelling or macroscopic oedema	Bleeding on probing	2
Change in colour due to inflammation; slight oedematous swelling	Bleeding on probing	3
Obvious swelling	Bleeding on probing	4
Spontaneous bleeding; changes in colour; marked swelling with or without ulceration	Bleeding on probing	5
[source: (Muhlemann and Son, 1971)]		

2.1.3.2.2.5 PPD

The PPD was measured between the gingival margin and the base of the pocket in six points (mesiobuccal, mid buccal, distobuccal, distolingual, mid lingual, mesiolingual) of each examined tooth (Figure 2.1). An average of the six readings was calculated for each tooth.

2.1.3.2.2.6 AL

The AL was measured between the cemento-enamel junction (CEJ) and the base of the pocket in six points (mesiobuccal, mid buccal, distobuccal, distolingual, mid lingual, mesiolingual) of each examined tooth (Figure 2.1). An average of the six readings was calculated for each tooth.

2.1.3.2.2.7 RECESSION

Recession was calculated by subtracting the PPD from the AL.

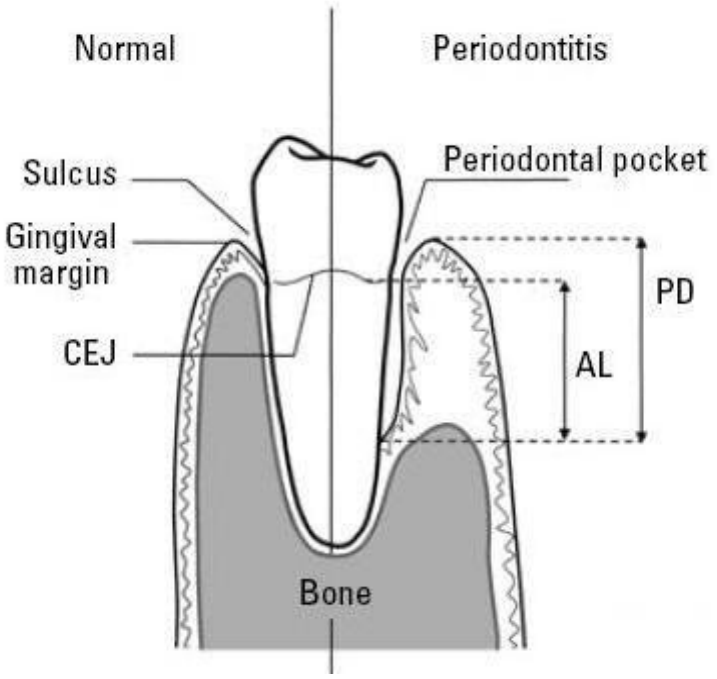


Figure 2.1: The difference between PPD and AL. PPD is the measurement between the gingival margin and the base of the pocket while AL is the measurement between the CEJ and the base of the pocket [source: www.medscape.com].

2.1.4 TYPE OF SAMPLES COLLECTED

2.1.4.1 SALIVA

Un-stimulated, whole saliva samples (1ml) were collected from BS patients, RAS patients, and HC volunteers. Samples were collected in a sterile universal container and processed using the following protocol: the saliva samples were centrifuged at 3300 rpm at 4 °C for 15 minutes. The supernatant was divided into 200 µl aliquots in eppendorfs and stored at -80 °C.

Salivary sediments were either re-suspended in 1 ml of Amies liquid transport medium and stored at 4 °C to be cultured on different bacteriological culture media within 48 hours of

collection or they were lysed by adding 600 µl of lysis buffer (Purescript, Qiagen, UK). For the purpose of deoxyribonucleic acid (DNA) purification, the salivary sediment samples were mixed with the lysis buffer by pipetting up and down, then they were incubated at 65 °C for 15 minutes and stored at -80 °C until further analysis.

2.1.4.2 BRUSH BIOPSY

The base of the ulcer and the non-ulcerated buccal mucosa from BS patients, RAS patients, and HC volunteers were brush biopsied with a nylon bristle buccal cytology brush (Flowgen, UK) using 10 strokes with 2 different brushes. Brushes were dipped up and down 10 times in a 1.5 ml eppendorf containing 600 µl of cell lysis solution (Purescript-Gentra, Qiagen, UK) or 600 µl of normal saline. The samples in the lysis buffer were incubated at 65 °C for 15 minutes then stored at -80 °C until DNA, ribonucleic acid (RNA), and protein isolation was carried out. The samples in normal saline were used to grow mycobacterium according the protocol described later on.

2.1.4.3 ORAL MUCOSAL SWABS

Amies liquid transport medium swabs (Copan, UK) were collected from ulcerated and non-ulcerated oral mucosa of the same cohorts of patients and controls. Swabs were stored at 4 °C and processed within 48 hours of collection by culturing on different culture media, following the protocol detailed below.

2.1.4.4 SERUM SAMPLES

Blood samples were collected from the same cohorts of BS patients, RAS patients, and HC volunteers in tubes coated with pro-coagulant to shorten the coagulation time. The tubes

also contained separation gel that solidifies and forms a barrier to separate serum from red blood cells. Serum was obtained from clotted blood by centrifugation at 3300 rpm at 4 °C for 15 minutes. Samples were divided into 200 µl aliquots and stored at -80 °C. The serum samples were then tested for the presence of herpes viruses immunoglobulin (Ig) G according to the protocol detailed in chapter 2, section 2.2.3.4.

2.1.4.5 WHOLE BLOOD SAMPLES

Whole blood samples were collected in tubes coated with ethylenediaminetetraacetic acid (EDTA) to avoid coagulation of the blood during transport time from the clinic to the laboratory. Upon reaching the laboratory, the peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over ficoll-hypaque (F-H) (1700 rpm for 35 minutes). The cells were then tested according to the toll-like receptor (TLR) function study protocol detailed in chapter 2, section 2.2.5.

2.2 SECTION 2: LABORATORY METHODS

2.2.1 SAMPLE PREPARATION

2.2.1.1 DNA, RNA, AND PROTEIN EXTRACTION FROM BRUSH BIOPSIES AND SALIVARY SEDIMENTS

DNA, RNA, and protein were isolated using the Purescript kit (Gentra, Qiagen, UK) following the manufacturer's protocol as follows.

2.2.1.1.1 PROTEIN AND DNA PRECIPITATION

A 200 µl protein-DNA precipitation solution was added to the cell lysate prepared as mentioned above from saliva sediments and brush biopsies. The content of the tube was mixed by inverting it gently about 10 times and it was then placed on ice for 5 minutes. The samples were subsequently centrifuged at 15,000 rpm for 3 minutes to allow the formation of tight pellets of protein and DNA.

2.2.1.1.2 RNA EXTRACTION

RNA in the supernatant fluid was poured into a sterile 1.5 ml eppendorf containing 600 µl of 100% isopropanol (propan-2-ol) and 1 µl of glycogen solution (Gentra, Qiagen, UK). The sample was mixed by inverting the tube almost 50 times and centrifuged at 15,000 rpm for 3 minutes; the RNA and glycogen mixture was visible at the end of this step as a small translucent pellet. The supernatant was poured off and the tube drained on clean absorbent paper. 100 µl of 70% ethanol (v/v) was then added to the tube, which was inverted several times to wash the pellet, followed by centrifuging the samples at 15,000 rpm for 1 minute. The ethanol was poured off and the samples allowed to air dry at an angle for 15 minutes. A 20 µl RNA hydration solution was added and the tube was placed on ice for at least 30

minutes. The sample was centrifuged for 5 seconds to spin down the entire sample and then stored at -80 °C until the complementary Deoxyribonucleic Acid (c-DNA) preparation.

2.2.1.1.3 HUMAN DNA AND PROTEIN EXTRACTION

A 600 µl cell lysis solution was added to the cell pellet and gently pipetted up and down until the cells were suspended then 3 µl of RNAse A (4mg/ml) was added and the sample mixed by inverting the tube 25 times. It was then incubated at 37 °C for 15 minutes to degrade the RNA. The samples were allowed to cool by placing them on ice for 1 minute then 200 µl of protein precipitation reagent were added and the samples vortexed vigorously at high speed for 20 seconds to mix the protein precipitation reagent uniformly with the cell lysate.

The samples were then incubated on ice for 30 to 60 minutes and were centrifuged at 13,000 rpm for 3 minutes to allow the protein to form a tight pellet. Supernatant containing DNA was carefully removed into fresh tubes. The protein pellets were then re-suspended in 200 µl of sterile protease inhibitor cocktail solution (Roche Applied Science, UK). The supernatant samples were centrifuged at 13,000 rpm for 1 minute in 600 µl of 100% isopropanol (propan-2-ol). The supernatant was then discarded and 600 µl of 70% ethanol were added to the DNA pellets to wash the samples. The samples were centrifuged as before. The supernatants were carefully discarded and samples left to air dry. Each DNA sample was dissolved in 20 µl of DNA hydration solution and was rehydrated by incubating it for 1 hour at 65 °C or alternatively overnight at room temperature. All samples were stored at -80 °C until further analysis.

2.2.1.1.4 BACTERIAL DNA EXTRACTION

For the purpose of bacterial DNA purification, the following steps followed the steps detailed in section 2.2.1.1.2, as follows:

A 600 µl cell lysis solution was added to the cell pellet and gently pipetted up and down until the cells were suspended. A 1.5 µl lytic enzyme solution was then added and the tube inverted about 25 times to mix its contents. The samples were incubated at 37 °C for 1 hour to digest the cell walls and were occasionally inverted during the incubation. Then 3 µl of RNAse A (4mg/ml-wt/v) were added and the sample mixed by inverting the tube 25 times and incubating it at 37 °C for 1 hour to degrade the RNA. The samples were allowed to cool by placing them on ice for 1 minute then 200 µl of protein precipitation reagent were added and the samples vortexed vigorously at high speed for 20 seconds to mix the protein precipitation reagent uniformly with the cell lysate.

The samples were then incubated on ice for 30 to 60 minutes and were centrifuged at 13,000 rpm for 3 minutes to allow the protein to form a tight pellet. Supernatant containing DNA was carefully removed into fresh tubes. The protein pellets were then discarded (Roche Applied Science, UK). The supernatant samples were centrifuged at 13,000 rpm for 1 minute in 600 µl of 100% isopropanol (propan-2-ol). The supernatant was then discarded and 600 µl of 70% ethanol were added to the DNA pellets to wash the DNA. The samples were centrifuged as before. The supernatants were carefully discarded and samples left to air dry. Each DNA sample was dissolved in 20 µl of DNA hydration solution and was rehydrated by incubating it for 1 hour at 65 °C or alternatively overnight at room temperature. All samples were stored at -80 °C until further analysis.

2.2.1.2 NUCLEIC ACID PURIFICATION FROM SALIVA SAMPLES

Nucleic acid in 300 µl of all the saliva samples was simultaneously purified along with 6 water samples as a negative control. All samples were processed in the BioRobotMDx workstation using the QIAamp DNA extraction kit, following the manufacturer's protocol (Qiagen, UK) (Figure 2.2). This method utilized the selective binding properties of a silica-based membrane to purify DNA samples. Purified DNA samples were then eluted in elusion buffer (AE) (10 mM Tris Cl, 0.5 mM EDTA, pH 9) and were free from protein, nucleases, and other contaminants or inhibitors. All samples were subsequently spiked with phocine herpes virus (PHV) DNA to act as an internal control during the real time polymerase chain reaction (qPCR) test. Samples were then stored at -80 °C until further analysis.

Figure 2.2: BioRobotMDa workstation employed for automated nucleic acid purification from salivary supernatant samples (Qiagen, UK) [source: Virology Unit at Barts and The London NHS Trust].

2.2.1.3 QUANTIFICATION OF DNA, RNA, AND PROTEIN

DNA, RNA, and protein quantification were determined by using the NanoDrop spectrophotometer, according to the manufacturer's protocol (Labtech, UK) (Figure 2.3). Using fibre optic technology and surface tension, the NanoDrop spectrophotometer holds 1 µl of sample between two optical surfaces to define the path length in a vertical orientation.

The upper and lower optical surfaces of the microspectrophotometer sample retention system were cleaned at the beginning of each reading session by pipetting 1 µl of clean deionised water onto the lower optical surface. The lever arm was then closed and tapped a few times to bathe the upper optical surface, followed by reopening the lever arm to allow meticulous cleaning with a clean tissue.

The NanoDrop software was opened on the required module, followed by initiation of the spectrophotometer by placing 1 µl of deionised water onto the lower optic surface, lowering the lever arm, and selecting "initialize" in the NanoDrop software. A blank measurement was performed by loading 1 µl of the buffer used to re-suspend the DNA, RNA, or protein samples before the first sample measurement and between every 5 measurements. Both optical surfaces were cleaned before measuring the first sample and in between each sample.

Figure 2.3: NanoDrop spectrophotometer [source: Labtech, UK].

2.2.1.4 c-DNA PREPARATION

RNA was transcribed to c-DNA by using the high-capacity c-DNA reverse transcription kit (Applied Biosystems, UK) according to the kit protocol. To synthesize single-stranded c-DNA from the previously purified RNA, a 10 µl reverse transcription master mix was prepared for each RNA sample, as shown in table 2.3, then 10 µl of RNA sample was added. The reverse transcription was performed in the thermal cycler using the thermal cycler conditions shown in table 2.4. c-DNA samples were then stored at -80 °C until further analysis.

Table 2.3: Master mix for c-DNA preparation.

COMPONENT	**VOLUME REACTION PER SAMPLE**
10x RT Buffer	2.0 µl
25x dNTP Mix (100 mM)	0.8 µl
10x RT Random Primers	2.0 µl
MultiScribeReverse^{TM}Transcriptase	1.0 µl
RNase Inhibitors	1.0 µl
Nuclease-free H_2O	3.2 µl
Total	10.0 µl
dNTP: deoxyribonucleotide triphosphate; H_2O: water; RT: reverse transcriptase	

Table 2.4: The thermal cycler condition for c-DNA preparation.

	STEP 1	**STEP 2**	**STEP 3**	**STEP 4**
Temperature (°C)	25 °C	37 °C	85 °C	4 °C
Time	10 min	120 min	5 sec	∞
°C: degree Celsius; min: minutes; sec: seconds; ∞: until removing the samples from the cycler				

2.2.2 ORAL MUCOSAL EXPRESSION OF TLR2 AND TLR4

2.2.2.1 qPCR FOR TLR2 AND TLR4 mRNA DETECTION

2.2.2.1.1 PRIMERS FOR THE qPCR

TaqMan probe was used for the detection of TLR2, TLR4 and 18S ribosomal RNA (18S rRNA) in general (TLR2 Hs00152932_m1, TLR4 Hs00370853_m1 and 18S rRNA Hs99999901_s1). The TaqMan probe for TLR2 mRNA was a general TLR2 probe for the common 5´ untranslated region (5´UTR) region of the five transcripts on the 2–3 exon boundaries, while for TLR4 mRNA the general TLR4 probe for the common 5´UTR region of the three transcripts on the 1–2 exon boundary was employed (Applied Biosystems, UK). 18S rRNA was used as a housekeeping gene for the purpose of calculating the relative expression of TLR2 and TLR4 mRNA.

These probes are a sequence detection system applying TaqMan chemistry, also known as fluorogenic 5´ nuclease chemistry. The TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles. By using these probes, a specific hybridization between probe and target generates a fluorescent signal which is considered one of the advantages of this technology. TaqMan probes can also be labelled with different distinguishable reporter dyes that allow amplification of two distinct sequences in one reaction tube. This property leads to post-

PCR processing elimination and reduces assay labour and material costs. However, the disadvantage of TaqMan chemistry is that the synthesis of different probes is required for different sequences.

2.2.2.1.2 THE THERMAL CYCLE PROTOCOL

The cycle used in this experiment was composed of three stages, namely denaturation, annealing and extension/elongation. Stage one is 1 cycle at 50 °C for 2 minutes, stage two is 1 cycle at 95 °C for 10 minutes, and stage three is composed of 50 cycles, with each cycle formed of two steps, 95 °C for 0.15 minutes followed by a cooling step at 60 °C for 1 minute (Figure 2.4). Samples were tested in triplicate to confirm the reliability of the technique at the Barts and the London School of Medicine and Dentistry's Genome Centre.

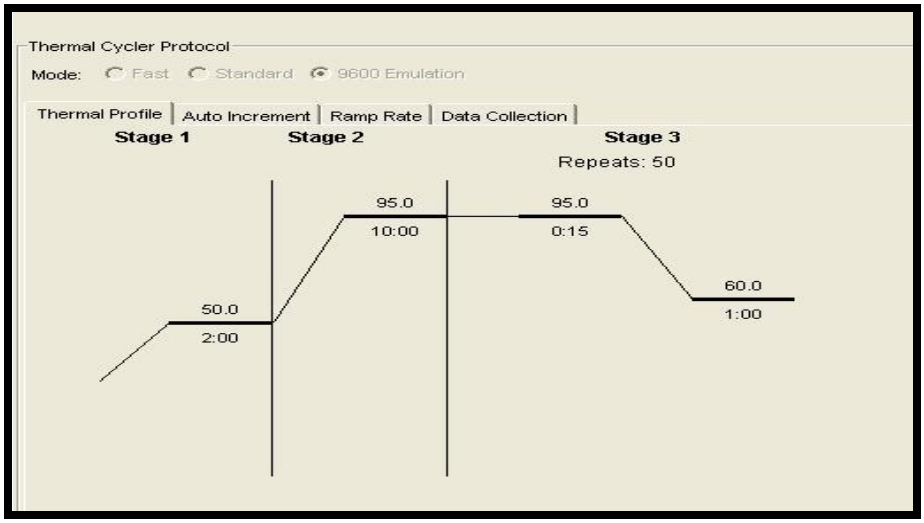


Figure 2.4: The thermal cycle protocol used in the TaqMan qPCR experiment for the detection of TLR2, TLR4, and 18s rRNA.

2.2.2.2 qPCR FOR THE DETECTION OF TLR2 AND TLR4 mRNA SPLICE VARIANTS

2.2.2.2.1 PRIMERS FOR THE qPCR

Specific primers designed and validated in-house were used in this experiment to detect the relative expression of TLR2 and TLR4 mRNA splice variants in relation to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All primers were designed to pass by exon boundaries and examined for its ability to amplify a single product (Table 2.5). Validation steps proved that each primer pair detected a single product and that negative control is consistently negative (Figure 2.5).

SYBR Green master mix was used, which contains a fluorescent DNA binding dye that helps in quantifying the amount of c-DNA in the examined sample by measuring the increased fluorescence through the PCR cycle (Roche Applied Science, UK). Each reaction consisted of 7 µl of x1 SYBR Green master mix, 1 µl of the primer mix that consisted of the forward and the reverse primers, and 2 µl of the sample c-DNA. Each qPCR cycle included at least one negative control and five positive controls in serial dilutions, creating the standard curve used for the quantification (Figures 2.6 and 2.7). Samples were tested in duplicate to test the reliability of the technique.

2.2.2.2.2 THE THERMAL CYCLE PROTOCOL

The thermal cycle was composed of three stages, namely denaturation, amplification and melting analysis. Stage one consisted of 1 cycle at 95 °C for 5 minutes. Stage two consisted of 45 cycles with each cycle formed of three steps: melting at 95 °C for 10 seconds, annealing at 60 °C for 10 seconds, and extension at 72 °C for 10 seconds, followed by the

three stages of the melting analysis (95 °C for 5 seconds, 60 °C for 60 seconds, 60 °C to 99 °C gradual heating, and finally 40 °C for 20 seconds).

Table 2.5: q PCR primers for GAPDH, TLR2 and TLR4 splice variants.

TOLL-LIKE RECEPTOR MRNA	**PRIMER**	**PRODUCT SIZE**	**START**	**STOP**
TLR2 variant a (AF424051.1) F	GAGTTCTCCCAGTTTCTCTTTTAAC	110	98	122
TLR2 variant a (AF424051.1) R	CCCACAGGTACCTTCACTTGG	110	207	187
TLR2 variant b (AF424049) F	CCCTTTTGCTTACTTCCTAGTCCCG	107	1	25
TLR2 variant b AF424049) R	GGGAGAACTCCGAGCAGTCACCT	107	107	85
TLR2 variant c (AF424053.1) F	CCCTTTTGCTTACTTCCTAGTCCC	206	1	24
TLR2 variant c (AF424053.1) R	GTGCTTCAACCTTCACTTGGTCAC	206	206	183
TLR2 variant d (AF424052.1) F	CCCTTTTGCTTACTTCCTAGTCCC	119	1	24
TLR2 variant d (AF424052.1) R	CCTTTGGATCCTGGGAGAACTCC	119	119	97
TLR2 variant e (AF424050) F	CCCTTTTGCTTACTTCCTAGTCCCG	114	1	25
TLR2 variant e AF424050) R	TCAACCTGGGAGAACTCCGAGCAG	114	114	91
TLR4 variant 1 (NM_138554.3) F	GCCCTGCGTGGAGGTGGTTC	292	372	391
TLR4 variant 1 (NM_138554.3) R	TCCAGAAAAGGCTCCCAGGGCT	292	663	642
TLR4 variant 3 (NR_024168.1) F	CCTGCGTGGAGACTTGGCCC	203	374	393
TLR4 variant 3 (NR_024168.1) R	TGAGAAGGGGAGGTTGTCGGGG	203	576	555
TLR4 variant 4 (NR_024169.1) F	AGCCCTGCGTGGAGGTGTGA	126	371	390
TLR4 variant 4 (NR_024169.1) R	TCCAGAAAAGGCTCCCAGGGCT	126	496	475
GAPDH (NM_002046.3) F	TGACGCTGGGGCTGGCATTG	143	987	1006
GAPDH (NM_002046.3) R	GGCTGGTGGTCCAGGGGTCT	143	1129	1110
F: forward; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; R: reverse; TLR: toll-like receptor				

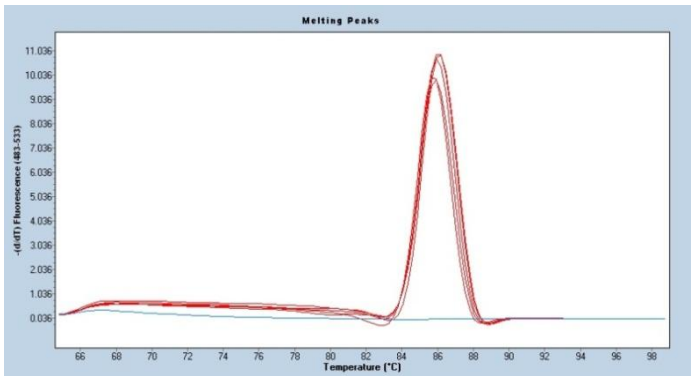


Figure 2.5: Melting temperatures of five positive controls and one negative control, showing single product amplification from the positive control samples and no product amplified from the negative control sample.

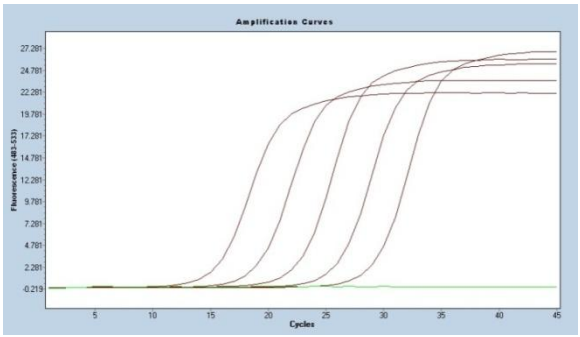


Figure 2.6: Serial dilutions of five positive control samples and one negative control sample.

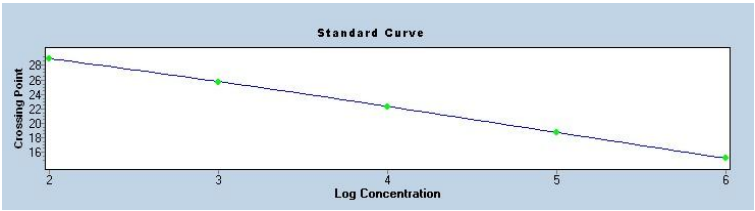


Figure 2.7: Standard curve created from five positive control samples and one negative control sample.

2.2.2.3 SLOT-BLOT FOR TLR2 AND TLR4 PROTEIN DETECTION

2.2.2.3.1 ANTIBODIES

Two different primary antibodies were used for TLR4: TLR4 (H-80), a rabbit polyclonal antibody raised against amino acids 242-321 of TLR4 of human origin (Santa Cruz Biotechnology, USA) and polyclonal goat anti-human TLR4 which was produced in goats immunized with purified, recombinant human TLR4 (rh TLR4) extracellular domain (R&D Systems, UK). Simultaneously, two different antibodies were used for TLR2: TLR2 (H-175), a rabbit polyclonal antibody raised against amino acids 180-354 mapping near the N-terminus of TLR2 of human origin (Santa Cruz Biotechnology, USA) and TLR2.1, a mouse monoclonal antibody raised against TLR2 cells of human origin which can be used in Western-blot, flow cytometry and immunofluorescence. The TLR2.1 antibody was generated using either full-length fusion proteins or transfected cell lines; therefore, the exact amino acid sequences of the epitopes it recognizes are not mapped or reported (eBioscience, USA). ß-Actin antibody (Sigma-Aldrich, USA), a rabbit antibody raised to ß-Actin of human origin, was used as a control antibody.

The optimization stage proved that the following antibodies are more specific in detecting the tested proteins:

1. Monoclonal mouse anti-human TLR2.1 antibody (eBioscience, USA)
2. Polyclonal goat anti-human TLR4 antibody (R&D Systems, UK)
3. Rabbit anti-human ß-Actin antibody (Sigma-Aldrich, USA)

Three different species-specific secondary antibodies were used: donkey anti-goat IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology, USA), anti-rabbit-HRP (Pierce, USA), and anti-mouse-HRP (Pierce, USA).

2.2.2.3.2 POSITIVE CONTROLS

Three types of positive control cells were used: Hel 92.1.7 cell lysate for TLR4 (Insight Biotechnology Limited, UK), Caco-2 cell lysate for TLR2 (Insight Biotechnology Limited, UK), and peripheral blood mononuclear cells (PBMC) used as a control for the two receptors.

2.2.2.3.3 SLOT-BLOT ANALYSIS

Protein samples were quantified using the NanoDrop spectrophotometer as mentioned above (Labtech, UK). 12.5% (w/v) purified protein samples were prepared in a sodium dodecyl sulphate (SDS) buffer composed of 0.32 M Tris-HCl pH 6.8, 3.5% SDS, 8.7% glycerol, 1.8% 2-mercaptoethanol and 0.1% bromophenol blue. The Bio-Dot SF (Bio-Rad, USA) apparatus was assembled according to the manufacturer's instructions and a nitrocellulose membrane was placed in the apparatus after pre-wetting it in Tris-buffered saline (TBS; 20 mM Tris, pH 7.5 and 500 mM NaCl). The membrane was rehydrated with 100 µl of TBS per well to ensure uniform binding of the antigen. The wells were filled with 200 µl of the 12.5% (w/v) purified protein in SDS buffer (25 µg/200 µl). The samples were allowed to filter through the membrane under vacuum. The wells were then washed with 200 µl of TBS using a gentle vacuum. The membrane was removed from the apparatus and blocked by incubating it in a blocking buffer containing dried milk powder (5% wt/v), 0.32 M Tris-HCl pH 8.0 (1% v/v), 5 M NaCl (3% v/v) and Tween 20 (0.05% v/v) for at least 1 hour at room temperature with agitation.

TLR2 and TLR4 were detected by incubating the membrane with the primary antibody (TLR2 at 1:500 and TLR4 at 1:1000: eBioscience, USA and R&D systems, USA

respectively) overnight at 4 °C. The membrane was washed with TBS-Tween (TBST) buffer containing 0.32 M TRIS-HCL pH 8.0 (1% v/v), 5 M sodium chloride (3% v/v), and Tween 20 (0.05% v/v) for 60 minutes. A bound antibody was detected using an HRP conjugated species-specific antibody at 1:1000 for 1 hour at room temperature with agitation.

The membrane was developed by using chemiluminescent detection reagents such as the enhanced chemiluminescent plus (ECL plus, BioRad, UK) reaction, which is based on the enzymatic generation of an acridinium ester. This technique produces more light emission of longer duration than the ECL reaction, which was based on the oxidation of the cyclic Diacylhydrazide. The signal generated by the ECL reaction was then quantified by using densitometry analysis and expressed as a relative expression in relation to the negative control.

2.2.2.4 WESTERN-BLOT FOR TLR2 AND TLR4 PROTEIN

2.2.2.4.1 ANTIBODIES

The same primary and secondary antibodies used in the slot-blot analysis were also used in the Western-blot analysis.

2.2.2.4.2 POSITIVE CONTROLS

The same positive controls used in the slot-blot analysis were also used in the Western-blot analysis.

2.2.2.4.3 WESTERN-BLOT ANALYSIS

All proteins samples were quantified by using the NanoDrop spectrophotometer (Labtech, UK). The samples were prepared for loading in the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by adding buffer composed of 0.32 moles (M) Tris-HCl pH 6.8 (28% v/v), SDS (3.5% w/v), glycerol (8.7% v/v), 2-mercaptoethanol (1.8% v/v) and bromophenol blue (0.1% v/v). 1 µg/µl protein samples were prepared (25 µg protein in a 25 µl reducing buffer) in order to load equal volumes of protein to each well. All protein samples were allowed to denature at 100 °C for 5 minutes. Proteins were separated by SDS-PAGE in a 4–20% linear gradient ready-casted gel (Invitrogen, UK) by using electrophoresis at 150 volts (constant voltage) for 1–2 hours. Separated protein samples were transferred to a polyvinylidenedifluoride (PVDF) membrane (Immobilon membrane, Sigma-Aldrich, USA) at 30 volts (constant voltage) for 1 hour at 4 °C in the cold room or on ice (Corbett and Dunn, 1993, Dunn and Bradd, 1993). The membrane was blocked by incubating it in a blocking buffer containing dried milk powder (5% wt/v), 0.32 M Tris-HCl pH 8.0 (1% v/v), 5 M NaCl (3% v/v) and Tween 20 (0.05% v/v) for at least 1 hour at room temperature with agitation.

Detection of TLR2 and TLR4 was achieved by incubating the membrane with the primary antibody overnight at 4 °C. The TLR2 and TLR4 antibody was prepared by adding it to the blocking buffer in the desired concentration (TLR2 at 1:500 v/v, and TLR4 at 1:1000 v/v). The membrane was then washed by using a wash buffer containing 0.32 M Tris-HCl pH 8.0 (1% v/v), 5 M NaCl (3% v/v), and Tween 20 (0.05% v/v) for 60 minutes. After washing, the membrane was incubated with the species-specific secondary antibody

engaged to a HRP at a concentration of 1:1000 v/v for 1 hour at room temperature with agitation. The membrane was developed by using ECL plus (ECL plus-BioRad, UK).

2.2.2.4.4 OPTIMIZATION

The Western-blot optimization took a substantial amount of time and effort. The optimization experiment was as follows: Hel 92.1.7 cell lysate was used as positive control for TLR4 (Insight Biotechnology Limited, UK), Caco-2 cell lysate was used as positive control for TLR2 (Insight Biotechnology Limited, UK), and PBMC were used as positive control for both proteins. The first lane was used for the molecular weight ladder (Kaleidoscope marker, Bio-Rad, USA), then Hel 92.1.7 cell lysate in 20, 50, 100 and 150 µg/25 µl were added, each in a different lane, followed by PBMC in 20, 25 and 50 µg/25 µl, followed by another molecular weight ladder, then Caco-2 cell lysate in 20, 50, 100 and 150 µg/25 µl were added, each in a different lane, followed by PBMC lysate in 20, 25 and 50 µg/25 µl, then finally a last molecular weight ladder (Tables 2.6 and 2.7).

Table 2.6: Optimization experiment for TLR4 using positive control samples.

LANE	LYSATE	PRIMARY ANTIBODY	SECONDARY ANTIBODY
1	Molecular weight marker	Polyclonal goat anti-human TLR4 antibody (1:1000)	Donkey anti-goat antibody-HRP (1:1000)
2	Hel 92.1.7 – 20 µg		
3	Hel 92.1.7 – 50 µg		
4	Hel 92.1.7 – 100 µg		
5	Hel 92.1.7 – 150 µg		
6	Monocytes-20 µg		
7	Monocytes-25 µg		
8	Monocytes-50 µg		
9	Molecular weight marker		

Table 2.7: Optimization experiment for TLR2 using positive control samples.

LANE	LYSATE	PRIMARY ANTIBODY	SECONDARY ANTIBODY
9	Molecular weight marker	Monoclonal mouse anti-human TLR2.1 antibody (1:500)	Anti-mouse antibody-HRP (1:1000)
10	Caco-20 µg		
11	Caco-50 µg		
12	Caco-100 µg		
13	Caco-150 µg		
14	Monocytes-20 µg		
15	Monocytes-25 µg		
16	Monocytes-50 µg		
17	Molecular weight marker		

The membrane was cut into two and the first half was incubated with TLR4 antibody, while the second half was incubated with TLR2 antibody. Multiple bands were observed for TLR2 and TLR4 in each experiment, which was repeated twice with the first set of antibodies then a third time with the second set of antibodies from a different company (Figure 2.8).

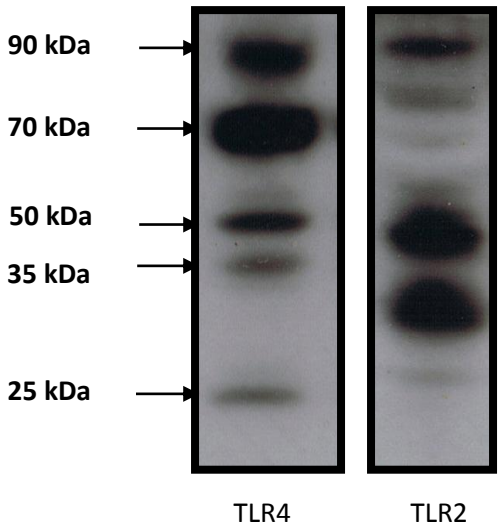


Figure 2.8: Western-blot analysis of TLR2 and TLR4 protein showing multiple bands, which may represent multiple splice variants.

2.2.2.5 TLR FUNCTIONAL STUDY

PBMC were isolated by density gradient centrifugation over F-H (1700 rpm for 35 minutes). The isolated cells were incubated at 37 °C overnight. Each sample was plated in twelve different wells of a 96 well plate in a density of $2x10^5$/well. The incubated cells were treated by different agonists such as 0.1 µg of Pam3CSK4, 10^8 HKLM, 0.2 µg *E.coli* K12 LPS and 1 µg FSL-1 (InvivoGen, USA) to examine the function of TLR1/2 heterodimer, TLR2, TLR4, and TLR2/6 heterodimer respectively. Two wells of each sample were treated with phytohaemagglutinin (PHA) as a positive control. Another two wells were performed as a negative control and were not treated by any agonist. The cells plus agonists were incubated at 37 °C overnight. The level of tumour necrosis factor alpha (TNF-α) in the culture supernatant was quantified by using enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (R&D Systems, UK). The stimulation index was calculated for each treatment by dividing the level of TNF-α produced after treatment by the spontaneous TNF-α secretion.

2.2.3 THE ORAL MICROBIOME

2.2.3.1 MALD-TOF ANALYSIS

The oral and salivary microbiome was analysed by matrix-assisted laser desorption/ionisation-time of flight (MALDI–TOF) analysis after culturing the samples on different bacteriological culture media in different environments to recover the maximum number of cultivable bacteria.

2.2.3.1.1 CULTURE TECHNIQUES

Amies transport medium swabs (Copan, UK), collected from ulcerated and non-ulcerated oral mucosa, and re-suspended salivary sediments samples were diluted in sterile saline at 10^2 and 10^4 and cultured on 7 different culture media: blood agar, chocolate agar, colistin nalidixic acid agar, MacConkey agar, gonococcus agar, Sabouraud agar and fastidious anaerobic agar (Figures 2.9–2.11).

Blood agar, chocolate agar, and gonococcus agar plates were incubated at 37 °C for 48 hours in a CO_2 enriched environment. Colistin nalidixic acid agar, MacConkey agar, and Sabouraud agar plates were incubated at 37 °C for 48 hours in an O_2 enriched environment. The fastidious anaerobic agar plates were incubated at 37 °C for 7 days in an anaerobic environment.

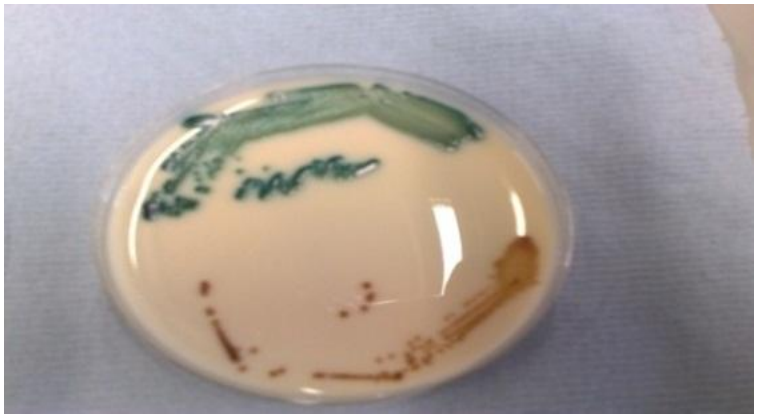


Figure 2.9: Two different *Candida* species grow on *Candida* chromogenic agar.

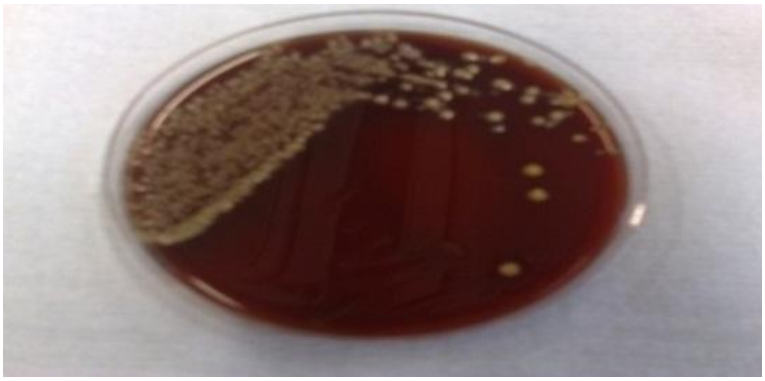


Figure 2.10: Mixed oral flora grows on chocolate agar plate.

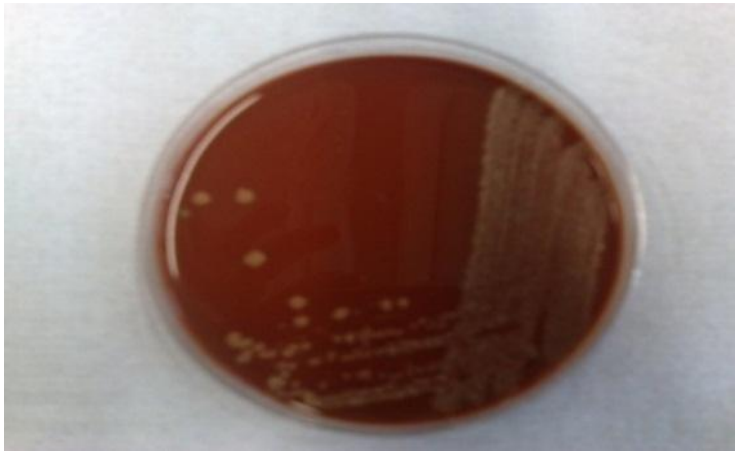


Figure 2.11: Mixed oral flora grows on blood agar plate.

2.2.3.1.2 BACTERIAL QUANTIFICATION

Bacteria which have grown on blood agar plates were quantified in colony-forming units by using the standard plate count. The 10^2 dilution was obtained by transferring 100 µl of the initial sample into 9.9 ml of sterile saline. The 10^2 dilution was then manually vortexed to distribute the bacteria evenly by grasping the tube between the palms of both hands and rotating quickly. The 10^4 was created by the transfer of 100 µl of the 10^2 dilution sample into 9.9 ml of sterile saline and mixing the sample as mentioned above. 100 µl of each

dilution was aseptically transferred to the surface of the blood agar plate and distributed evenly using a sterile bacteriological spreader.

The blood agar plates were incubated at 37 °C for 48 hours in a CO_2 enriched environment. At the end of the incubation period, all plates containing 30 to 300 colonies were selected and the colonies were counted. Plates with fewer than 30 colonies or more than 300 colonies were considered unquantifiable.

Further dilutions were prepared in cases when the 10^4 dilution grew more bacterial colonies than the maximum quantifiable colony number. The original sample was used in cases when the 10^2 dilution grew fewer bacterial colonies than the minimum quantifiable colony number.

2.2.3.1.3 BACTERIAL PURIFICATION

Isolated colonies were re-cultured in the same culture environments to homogeneously purify them before peptide isolation.

2.2.3.1.4 BACTERIA PEPTIDE PURIFICATION

Peptides were purified by the acetonitrile precipitation method according to the manufacturer's instructions (Bruker Daltonics, Germany). Five colonies from each purified bacteria were placed into an eppendorf tube and mixed with 300 µl of sterile deionised water by briefly vortexing the tube. 900 µl of absolute ethanol were added to each tube and mixed gently by inverting the tube several times.

A tight pellet was created by centrifuging at maximum speed for 2 minutes. The supernatant was removed and all the residual ethanol was allowed to evaporate by leaving the eppendorf tube open for 10–15 minutes. The pellet was thoroughly re-suspended in 25 µl of formic acid. 25 µl of acetonitrile was then added to each sample and mixed carefully by inverting the tube several times. The tubes were centrifuged at maximum speed for 2 minutes. The supernatant containing the purified bacterial peptides was transferred to a sterile tube and saved at -80 °C until further analysis.

2.2.3.1.5 MALDI-TOF MATRIX PREPARATION

250 µl of the basic organic solvent (2.5% (v/v) tri-fluoro-acetic-acid (TFA), 50% (v/v) acetonitrile and deionised water) was added to 10 mg/ml alpha cyanohydroxycinnamic acid (Bruker Daltonics, Germany) and vortexed thoroughly until the crystals were completely dissolved.

2.2.3.1.6 MALDI-TOF TARGET PLATE PREPARATION

The polished steel 96 target plate was thoroughly cleaned by overlaying its surface with 70% aqueous ethanol for 5 minutes at room temperature (Figure 2.12). The target plate was then rinsed with tap water and wiped with a paper towel moistened with 70% aqueous ethanol. Subsequently, sterile deionised water was used to wipe the plate, followed by overlaying it with 100 µl of 80% TFA and quickly wiping it scrupulously. Lastly, the target plate was rinsed with sterile deionised water, wiped with a paper towel and left to air dry for 15 minutes.

2.2.3.1.7 MALDI-TOF ANALYSIS

A MALDI–TOF analysis using the MALDI Biotyper was conducted on a 96 target plate by applying 1 µl of each peptide sample to a different target, followed by 1 µl of the crystallised molecules in the MALDI matrix (Bruker Daltonics, Germany) to protect the peptides and help in the ionisation process by the laser beam (Figure 2.12).

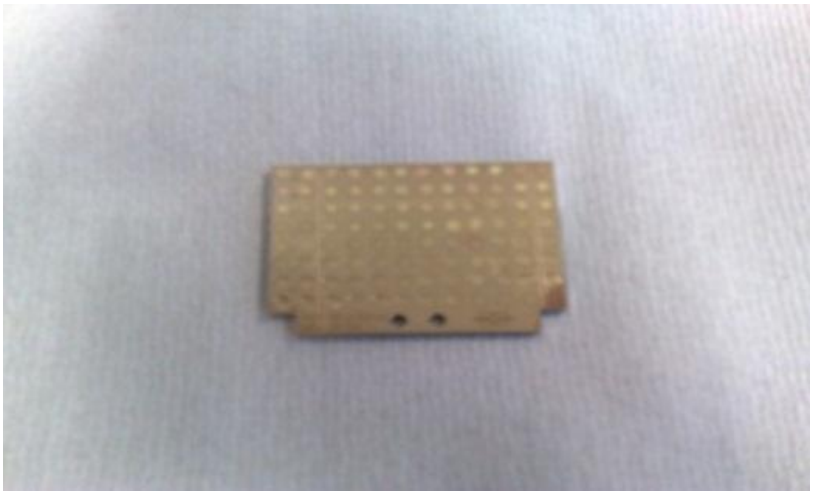


Figure 2.12: Target plate of the MALDI-TOF machine (Bruker Daltonics, Germany) [source: Medical Microbiology Unit at Barts and the London NHS Trust].

MALDI–TOF applies nitrogen-pulsed laser ionisation to the peptide samples to ionise and separate them based on their mass/charge ratio (Figure 2.13). The resulting spectra formulate the protein finger print (2,000–20,000 Dalton range) which is then compared to a database of known spectra to identify and type the different bacteria being analysed. A match factor for each sample was automatically calculated by the MALDI Biotyper based on the number and amplitude of the peaks that match.

Figure 2.13: MALDI-TOF (Bruker Daltonics, Germany) [source: Medical Microbiology Unit at Barts and The London NHS Trust].

2.2.3.1.8 MALDI-TOF DATA INTERPRETATION

A score of 1.7 or above was considered a reliable identification of the investigated microorganism (Tables 2.8 and 2.9). Scores below 1.7 were a driver for repeating the peptide isolation or checking the purity of the isolated organism. In cases when no peaks were found, 1:10 and 1:100 dilutions were prepared from the peptide sample to exclude the possibility that the sample was much too concentrated to be read by the MALDI–TOF analysis. Due to the reported inability of MALDI-TOF in identifying *S. mitis* from *S. pneumoniae*, optochin susceptibility and bile solubility tests were used to check the identification accuracy of any microorganism reported as *S. pneumoniae* by MALDI-TOF (Kok et al., 2011).

Table 2.8: MALDI-TOF data interpretation.

Analyte Name	Analyte ID	Organism (best match)	Score Value	Organism (second best match)	Score Value
A1 (++)		Neisseria flavescens	2.097	Neisseria flavescens	1.99
A2 (+)		Lactobacillus rhamnosus	1.89	Lactobacillus rhamnosus	1.857
A3 (+)		Kingella denitrificans	1.857	Kingella denitrificans	1.732

Table 2.9. The MALDI-TOF typing data for one microorganism investigated.

Rank (Quality)	Matched Pattern	Score Value	NCBI Identifier
1 (++)	Neisseria flavescens DSM 17633T DSM	2.097	484
2 (+)	Neisseria flavescens C1 2 PGM	1.99	484
3 (+)	Neisseria perflava DSM 18009T DSM	1.956	33053
4 (+)	Neisseria subflava DSM 17610T DSM	1.878	28449

2.2.3.2 MYCOBACTERIAL ANALYSIS

Brush biopsies (Flowgen Bioscience, UK) from ulcerated and non-ulcerated oral mucosa and unstimulated saliva samples were investigated in the mycobacterial analysis.

2.2.3.2.1 SAMPLE DECONTAMINATION

Saliva samples were decontaminated using the NaOH-NALC [2% sodium hydroxide (NaOH), 1.45% trisodium citrate and 0.5% N-acetyl-L-cysteine (NALC)] decontamination method as follows: 6 ml of the prepared NaOH-NALC were added to each salivary sample in a 50 ml Falcon tube and mixed by briefly vortexing and inverting the tube several times. The sample was allowed to be incubated with the NaOH-NALC for 30 minutes with

periodic gentle vortexing. Subsequently, each sample was diluted (1:40 dilution) with phosphate buffer (pH 6.8) followed by centrifugation at 3000 xg for 30 minutes. The supernatant was discarded and 1.5 ml of sterile phosphate buffer was added to re-suspend the pellet.

Brush biopsy samples were treated with the 4% sulphuric acid decontamination method as follows: 2 ml of the acid was mixed with the brush biopsy sample by vortexing and then incubated for 30 minutes with periodic gentle mixing. Subsequently, each sample was diluted (1:40 dilution) with phosphate buffer and processed as mentioned above for the salivary samples.

2.2.3.2.2 CULTURING TECHNIQUES

0.25 ml of each decontaminated sample was cultured on Lowenstein-Jensen (LJ) slopes (Figure 2.18) and 0.5 ml was inoculated into mycobacterial growth indicator tubes (MGIT) which contained 4 ml of Middlebrook 7H9 broth base with a fluorescent indicator (Figures 2.14 and 2.15) (Becton Dickinson Diagnostic Instrument Systems, USA). MGIT bottles were incubated on the MGIT instrument which reads each tube hourly and triggers an alarm when growth is detected (Figure 2.16) (Becton Dickinson Diagnostic Instrument Systems, USA). All samples were allowed to be incubated for 6 weeks.

Figure 2.14: LJ culture media [source: The National Mycobacterium Reference Laboratory].

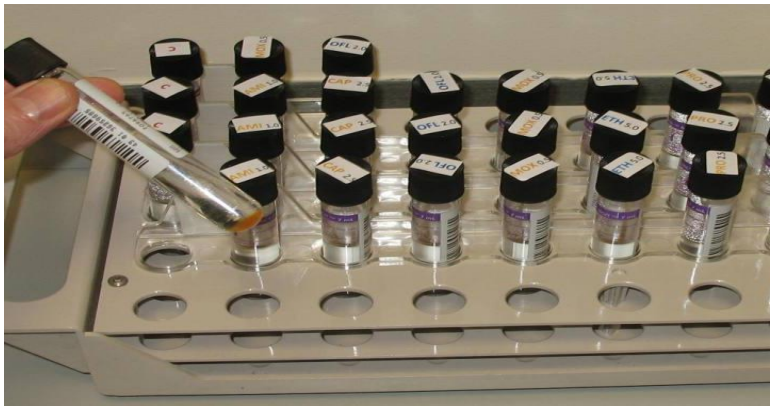


Figure 2.15: MGIT bottles (Becton Dickinson Diagnostic Instrument Systems, USA) [source: The National Mycobacterium Reference Laboratory].

Figure 2.16: MGIT instrument (Becton Dickinson Diagnostic Instrument Systems, USA) [source: http://www.bd.com/ds].

2.2.3.2.3 MYCOBACTERIAL IDENTIFICATION

The isolated mycobacterium was identified by the PCR and DNA-DNA hybridization technique using the GenoType Mycobacterium Common Mycobacterial (CM) kit, following the manufacturer's instructions (Hain Lifescience, Germany).

The above-mentioned kit allows the identification of the following *Mycobacterial* species: *M. avium, M. chelonae, M. abscessus, M. fortuitum, M. gordonae, M. intercellulare, M. scrofulaceum, M. interjectum, M. kansasii, M. malmoense, M. peregrinum, M. marinum, M. ulcerans, M. tuberculosis complex* and *M. xenopi.*

The identification procedure was divided into three stages: DNA isolation, multiplex amplification with biotinylated primes, and reverse hybridization.

2.2.3.2.3.1 DNA ISOLATION

DNA was isolated from bacteria grown on LJ slopes or MGIT tubes. Bacterial colonies that had grown on LJ slopes were diluted directly with 300 µl of sterile deionised water. In the case of *Mycobacteria* grown on MGIT tubes, pellets were created by centrifuging 1 ml of the medium for 15 minutes at 10000 xg prior to the dilution step. Diluted samples were incubated for 20 minutes at 95 °C then for 15 minutes in an ultrasonic bath. Subsequently, the samples were centrifuged for 5 minutes at full speed and 5 µl of the supernatant were used in the PCR reaction.

2.2.3.2.3.2 DNA AMPLIFICATION

To amplify the isolated DNA, 45 µl of master mix were prepared for each DNA sample, as shown in table 2.10, then 5 µl of DNA sample were added. The amplification was performed in the thermal cycler using the thermal cycler conditions shown in table 2.11.

Table 2.10: Master mix for mycobacterial DNA amplification.

COMPONENT	**VOLUME REACTION PER SAMPLE**
PNM	35 µl
10 x polymerase incubation buffer	5 µl
$MgCl_2$	1 µl (1.5 mM final concentration)
Thermostable DNA polymerase	1 µl
Nuclease-free H_2O	3 µl
$MgCl_2$: magnesium chloride; PNM: primer-nucleotide mix	

Table 2.11: Thermal cycler conditions for mycobacterial DNA amplification.

	STEP 1 1 CYCLE	**STEP 2 10 CYCLES**		**STEP 3 20 CYCLES**			**STEP 4 1 CYCLE**
Temperature	95 °C	95 °C	58 °C	95 °C	53 °C	70 °C	70 °C
Time	5 min	30 sec	2 min	25 sec	40 sec	40 sec	8 min
°C: degree Celsius; min: minutes; sec: seconds							

2.2.3.2.3.3 HYBRIDIZATION

The hybridization stage included the following steps: chemical denaturation of the amplified product, hybridization of the single-stranded, biotin-labelled amplicons to membrane-bound probes, stringent washing, streptavidin conjugated alkaline phosphatise (AP) conjugation and AP mediated staining reaction.

2.2.3.2.3.3.1 CHEMICAL DENATURATION STEP

The chemical denaturation was created by mixing 20 µl of the amplified sample with 20 µl of the denaturation solution then incubating the mixture at room temperature for 5 minutes. 1 ml of warm hybridization buffer (45 °C) was added to each denatured sample and shaken gently until a homogeneous colour was created.

2.2.3.2.3.3.2 HYBRIDIZATION OF THE SINGLE-STRANDED, BIOTIN-LABELLED AMPLICONS TO MEMBRANE-BOUND PROBES STEP

A GenoType Mycobacterium CM DNA strip was added to each sample and allowed to be completely covered with the solution. The strips were incubated for 30 minutes at 45 °C in a shaking water bath.

2.2.3.2.3.3.3 STRINGENT WASHING STEP

At the end of the incubation period, all the hybridization solution was aspirated and 1 ml of the stringent wash solution (STR) was incubated with the DNA strip for 15 minutes at 45 °C. The DNA strip was then removed and rinsed with 1 ml of rinse solution for 1 minute at room temperature.

2.2.3.2.3.3.4 AP CONJUGATION STEP

1 ml of the diluted conjugate containing AP was added to each DNA strip and incubated for 30 minutes at room temperature on a shaking platform. The DNA strip was then washed twice for 1 minute each time with 1 ml of deionised water on a shaking platform at room temperature.

2.2.3.2.3.3.5 AP MEDIATED STAINING STEP

1 ml of the diluted substrate containing dimethyl sulfoxide was added to each DNA strip and incubated at room temperature in the dark for 20 minutes. The reaction was then stopped by rinsing the DNA strip twice with deionised water.

Each DNA strip contains 16 DNA probes, conjugate control (CC) and marker (Mr) (Figure 2.17). The first strip is the CC followed by the universal control (UC), which indicates the presence of bacteria, then the genus control (GC) that indicates the presence of mycobacteria in the tested sample. The other 14 bands are specific DNA probes used to identify the isolated mycobacterium. The hybridized strips were interpreted according to the manufacturer's instructions, as illustrated in Figure 2.18.

Figure 2.17: DNA strip with 16 DNA probes, CC and Mr.

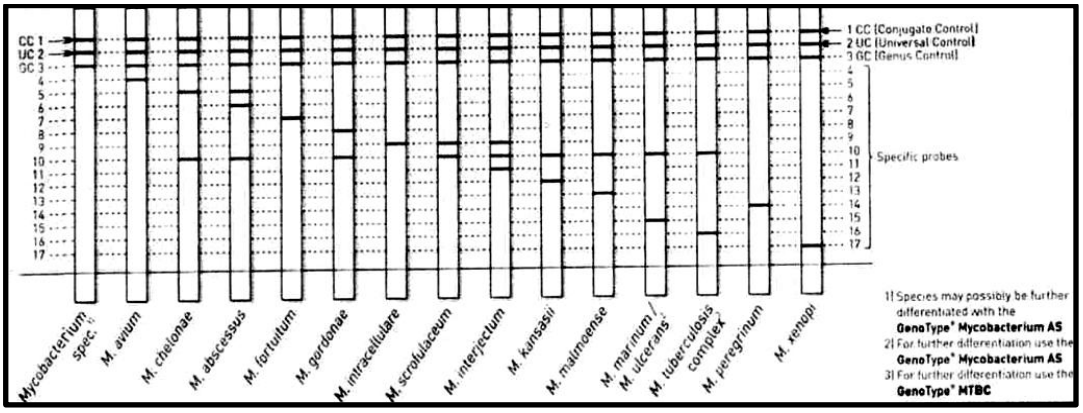


Figure 2.18. GenoType Mycobacterium CM kit (source: Hain Lifescience, Germany).

2.2.3.3 qPCR OF THE HERPES VIRUSES

2.2.3.3.1 HSV1, HSV2 AND VZV qPCR

Multiplex real time amplification of herpes simplex virus (HSV) 1, HSV2 and varicella zoster virus (VZV) DNA using glycoprotein B-specific primers was performed. The 143 bp, 140 bp and 97 bp products were detected in qPCR by the use of TaqMan probes that are labelled at the 5´ end with FAM fluorophore (FAM), JOE fluorophore (JOE) and cyclohexyl fluorophore (Cy.) respectively and at the 3´ end with the quencher BHQ fluorophore (BHQ) 3 (Table 2.12). The condition of the qPCR was as follows: 2.5 µl of 1x PCR buffer contained 1.5 mmol/L $MgCl_2$ (Qiagen, UK), 5 µl of $MgCl_2$ (25 mmol/L), 7.5 µl of dNTPs (6.25 mmol/L of each nucleotides), 1 µl of the forward and reverse primers (15 pmol/µl), 1 µl of TaqMan probe (5 pmol/L) and 0.25 µl of HotStarTaq polymerase (0.25 IU/µl) made up to a final volume of 20 µl with sterile water (Applied Biosystems, UK). 5 µl of extracted DNA were added to each reaction. The PCR cycling condition was 2 minutes at 50 °C, 10 minutes at 95 °C and 60 cycles of 15 seconds at 95 °C and 15 seconds at 60 °C. All samples were analysed in duplicate and the average viral load was calculated by

using the sequence detection system software available on the ABI 7700 platform (Applied Biosystems, UK).

2.2.3.3.2 CMV qPCR

Real time amplification of cytomegalovirus (CMV) DNA using glycoprotein B-specific primers was performed. The 150 bp product was detected in qPCR by the use of a TaqMan probe which is labelled at the 5´ end with 6-carboxyfluorescein-FAM and at the 3´ end with the quencher tetramethylrhodamine (TAMRA) (Table 2.12). The conditions of the PCR were as follows: 2.5 µl of $MgCl_2$ (25 mmol/L), 7.5 µl of dNTPs (96.25 mmol/L each nucleotide), 1 µL of each primer and 0.25 µL of HotStarTaq polymerase (0.25 IU/µl) (Applied Biosystems, UK) made up to a final volume of 20 µl with sterile water. 5 µl of sample DNA, positive control or negative control were added to each reaction. Each qPCR run included positive control, negative control and dilution series of cloned CMV DNA in triplicate of $1\text{-}10^4$ genomes. PCR cycling conditions were 2 minutes at 50 °C, 10 minutes at 95 °C, and 60 cycles of 15 seconds at 95 °C and 15 seconds at 60 °C (Mattes et al., 2005).

2.2.3.3.3 EBV qPCR

Real time amplification of Epstein-Barr virus (EBV) DNA using glycoprotein B-specific primers was performed. The 96 bp product was detected in qPCR by the use of a TaqMan probe which is labelled at the 5´ end with JOE and at the 3´ end with BHQ1 (Table 2.12). The conditions of the PCR were as described above in the CMV qPCR experiment.

2.2.3.3.4 HHV8 qPCR

Primers and probes for the detection of human herpes virus (HHV) 8 nucleic acid were chosen according to the previously published data (Stamey et al., 2001) (Table 2.12). They were synthesized by the standard phosphoramide chemistry techniques. Probes were labelled at the 5' end with the reporter molecule 6-carboxyfluorescein-FAM and at the 3' end with the quencher 6-carboxyletramethylehodamine. TaqMan probes were synthesized with a 3' phosphate group to block extension by Taq polymerase (Applied Biosystems, UK). The qPCR conditions were as described above in the CMV qPCR experiment.

2.2.3.3.5 PHV qPCR

Real time amplification of PHV DNA using glycoprotein B-specific primers was performed as an internal control. The 101 bp product was detected in qPCR by the use of a TaqMan probe which is labelled at the 5´ end with 6-carboxyfluorescein-FAM and at the 3´ end with the quencher BHQ1 (Table 2.12). The condition of the PCR and the PCR cycling condition were as described above in the CMV qPCR experiment.

Table 2.12: qPCR primers for HSV1, HSV2, CMV, EBV, HHV8 and PHV.

TARGET	PRIMERS AND PROBES		PRODUCT SIZE/BP
HSV1	F	TATTGGTGCGATGGCGACAC	143
	R	CTTTCCGCATGTGGGCTCTC	
	P	FAM-CCCCGCCCCATACCCTACCCGC-BHQ1	
HSV2	F	AGCATCCCGATCACTGTGTACTA	140
	R	GCGATGGTCAGGTTGTACGT	
	P	JOE-CAGTGCTGGAACGTGCCTGCCGC-BHQ1	
VZV	F	GCCCGTCTATTCCATTCAGCAA	97
	R	CCCGCAAACTTGTAGAACTGTTG	
	P	Cy-CACACGACGCCTCCGCCGCAG-BHQ3	
CMV	F	GAGGACAACGAAATCCTGTTGGGCA	150
	R	TCGACGGTGGAGATACTGCTGAGG	
	P	FAM-CAATCATGCGTTTGAAGAGGTAGTCCACG-BHQ1	
EBV	F	GGCCAGAGGTAAGTGGACTTTAAT	96
	R	GGGGACCCTGAGACGGG	
	P	JOE-CCCAACACTCCACCACACCCAGGC-BHQ1	
HHV8	F	TCGGTGGCGATGCTTTAGAC	97
	R	TGAAGCAGACGATGCTTTGC	
	P	TCGTAACCCCCGTCTACTTTCCCCG	
PHV	F	CGTTCCAACAACACAACCTACTG	101
	R	CTCTCATATCATCATTCAACTCAGTGT	
	P	JOE-ACCACCAATTACTCCTAGTCCACCACCG-BHQ1	

BP: base pair; CMV: cytomegalovirus; EBV: Epstein-Barr virus; F: forward; HHV8: human herpes virus 8; HSV: herpes simplex virus; P: probe; PHV: phocine herpes virus; R: reverse; VZV: varicella zoster virus.

2.2.3.4 IMMUNOASSAYS FOR THE DETECTION OF IgG TO HERPES VIRUSES

2.2.3.4.1 HSV1 ELISA

A 100 µl of the 1/101 diluted serum samples were added to the readymade recombinant-gG1-antigen coated wells (Biokit, Spain). The antibodies in the samples were allowed to combine with the antigens attached to the well during a one hour incubation period at 37 °C. The wells were then washed to remove all residual test specimens and then enzyme-

labelled antibodies to human IgG (conjugate) were added. The wells were incubated for 30 minutes at 37 °C to allow the conjugate to bind immunologically to the anti-HSV1 IgG antibodies that had combined with the antigen in the well during the first incubation period. After another wash to eliminate the unbound material, an enzyme substrate solution containing a chromogen was added. If the sample contained anti-HSV1 IgG, the solution developed a blue colour and the intensity of the colour was proportional to the number of antibodies in the specimens.

The samples were tested in duplicate and seven control samples were tested simultaneously to validate the assay and extract quantifiable data. The mean absorbance of the low positive control was calculated and considered to be the cut-off value, followed by dividing the sample absorbance by the cut-off value to give a ratio absorbance/cut-off, which is proportional to the concentration of the HSV1 IgG antibodies in each tested specimen.

2.2.3.4.2 HSV2 ELISA

HSV2 IgG was detected and quantified using a similar method to the HSV1 IgG ELISA. However, the wells of the HSV2 IgG ELISA kit were coated with recombinant-gG2-antigen instead of recombinant-gG1-antigen (Biokit, Spain).

2.2.3.4.3 VZV ELISA

VZV IgG was detected and quantified using a similar method to the HSV1 IgG ELISA. However, the wells of the VZV IgG ELISA kit were coated with VZV antigen from partially purified extract of human fibroblast infected with VZV, strain ELLEN (ATCC) (Diamedix, USA). To determine the ELISA unit (EU)/ml, the following formula was used

following the manufacturer's instructions (absorbance of calibrator x absorbance of sample = EU/ml of sample).

2.2.3.4.4 CMV ELFA

CMV IgG was quantified by an automated quantitative two-step enzyme immunoassay sandwich method with a final fluorescent detection, enzyme-linked fluorescent assay (ELFA) (bioMérieux, France).

All the assay steps were performed automatically by the vitek immuno-diagnostic assay system (VIDAS) instrument, according to the manufacturer's instructions (Figure 2.19). The solid phase receptacle (SPR) coated internally by the CMV antigen (strain AD169) acted also as a pipetting device for the assay. Fluorescence was measured twice for each sample tested; the first reading was a background reading of the serum and the second one was taken after the incubation period with the antigen. The instrument, using calibration curves that are stored by the machine, automatically calculated the results and expressed them in AU/ml. The samples were tested in duplicate, along with calibrator and control samples to validate the assay.

Figure 2.19: VIDAS instrument (bioMérieux, France) [source: Virology Unit at Barts and The London NHS Trust].

2.2.3.4.5 EBV CHEMILUMINESCENT IMMUNOASSAY

EBV viral capside antigens (VCA) IgG was quantified in the investigated serum samples by the LIAISON analyzer, following the manufacturer's instructions. The samples were tested in duplicate, along with calibrator and control samples, to validate the assay (DiaSorin, Italy).

The fully automated LIAISON immunoassay was used with its magnetic microparticle technology, chemiluminescence with flashlight kinetics, and an isoluminol derivative as labels. The p18 synthetic peptide is the major component used for coating magnetic particles (solid phase) and a mouse monocolonal antibody is linked to an isoluminol derivative (isoluminol-antibody conjugate). The cycle has two incubation periods: VCA antibodies in the sample were allowed to bind to the solid phase during the first incubation period, while the antibody conjugate reacted with VCA IgG already bound to the solid phase during the second incubation period. The unbound material was removed in a wash

cycle after each incubation period. Subsequently, the starter reagents were added and a flash chemiluminescence reaction was induced. The light signal was measured by a photomultiplier as relative light units (RLU) indicating VCA IgG concentration in tested samples.

30 µL of each sample were used for this analysis. All reagents required for the assay (magnetic particles, luminescence-labelled tracer, two calibrators, diluent and assay buffer) were provided ready to use and assembled in one integrated reagent cartridge identifiable by a bar-coded label providing information such as lot number, expiry date and recalibration data (Figure 2.20). The analyzer automatically calculated the antibody concentrations and expressed it as U/ml.

Figure 2.20: LIAISON analyzer (DiaSorin, Italy) [source: Virology Unit at Barts and The London NHS Trust].

2.2.3.4.6 HHV8 INDIRECT FLUORESCENT ASSAY

An indirect fluorescent antibody staining method was employed to semi-quantify the HHV8 IgG antibodies in the serum samples (Advanced Biotechnologies, Columbia). The 1:40 diluted serum samples were incubated with HHV8 infected cells on a slide, allowing the antibody in the serum sample, if present, to bind with the antigen present in the infected cells, forming immunological complex. Incubation was done in a moist chamber for 30 minutes at 37 °C. After the wash step (5–10 minutes in a wash buffer), the anti-human IgG conjugated to the dye fluorescein isothiocyanate (FITC) was added to each well on the slide and allowed to bind to the antibody-antigen complexes for 30 minutes at 37 °C.

The slides were then washed (5–10 minutes in wash buffer), mounted and blindly evaluated under a fluorescent microscope by two different investigators. Each slide contained ten tests; the positive and negative controls and wash buffer were tested simultaneously with the seven patients' samples to validate the assay. The positive control was given a semi-quantitative score equal to 4 and the negative control was given a score of 0 (Figures 2.21 and 2.22). Each patient's samples were scored relative to the positive and negative controls. The results were checked for consistency and re-evaluated if there were any discrepant results between the two investigators.

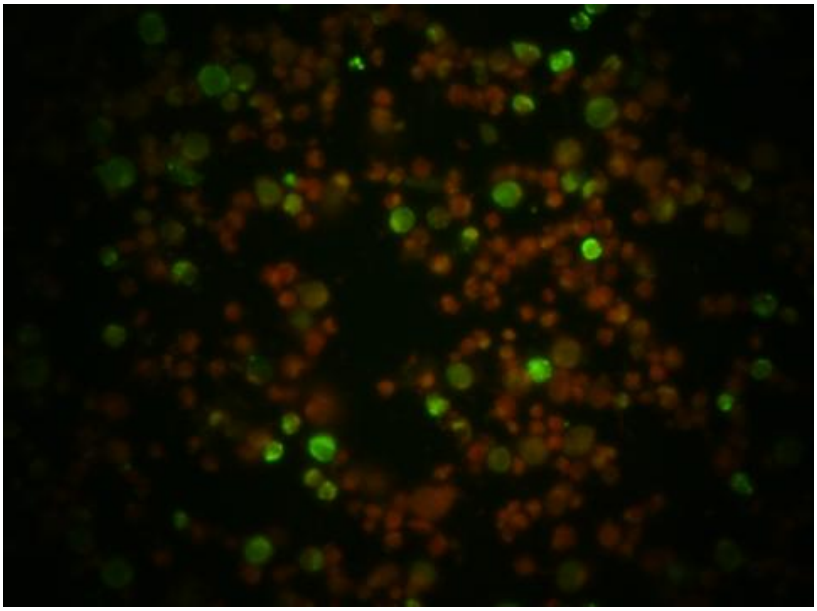


Figure 2.21: HHV8 indirect fluorescent assay (positive control sample).

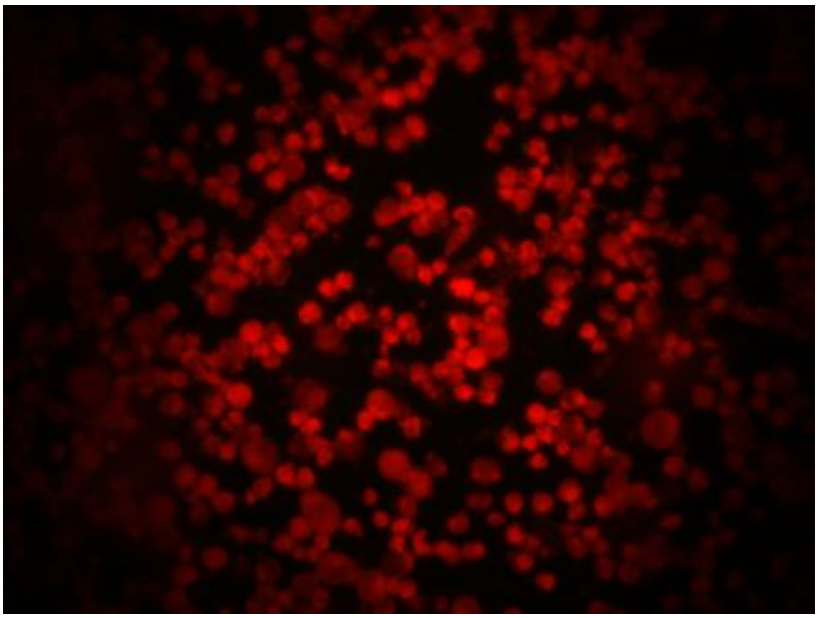


Figure 2.22: HHV8 indirect fluorescent assay (negative control sample).

2.2.4 STATISTICAL ANALYSIS

The statistical power calculation was investigated using StateMate 2 software to determine the minimum number of samples required in each part of the study. This practice ensured avoiding type I statistical error (rejecting the potentially true null hypothesis), and type II statistical error (failure to reject the potentially false null hypothesis) (GraphPad, USA).

Mean, median, range, minimum, maximum, standard deviation (SD), standard error of mean (Sem), and percentage (%) were used in the descriptive statistics. The obtained data were analysed by Mann-Whitney-U when comparing quantitative data of two groups, Kruskal-Wallis when comparing quantitative data of more than two groups, and non-parametric multivariate analysis of variance (MANOVA) with Bonferroni post tests when comparing multiple variants in relation to different groups. Differences in the rate were analysed using Chi-square tests and the correlation was analysed by using Spearman test. The GraphPad Prism® statistical package was used (GraphPad, USA).

CHAPTER 3

BEHÇET'S SYNDROME PATIENT COHORT IN THE UK

3.1 ABSTRACT

3.1.1 BACKGROUND

Classically, Behçet's syndrome (BS) was considered more prevalent in the Far East and the Mediterranean region along what historically was known as the "Silk Road" (between the 30th and 45th degree latitudes in Asian and European countries). BS's highest prevalence is still along the "Silk Road", especially in Turkey (380/100,000). However, recently, a higher than expected prevalence was reported in France (7.1/100,000), proving that this syndrome is not as rare outside the "Silk Road" boundaries as was previously thought.

3.1.2 OBJECTIVES

To analyse the clinical picture and treatment protocol of BS in a cohort of patients from two of the largest tertiary referral centres in the UK for BS patient management and care. The clinical picture of the UK BS patients was then compared to the published large series from other countries.

3.1.3 METHODS

Patients thought to have BS were referred to one of the two centres participating in this study (St Thomas' Hospital and Royal London Hospital). Patients were then examined and classified according to the International Study Group (ISG) criteria by a multi-disciplinary team including consultants from Oral Medicine, Ophthalmology, Rheumatology, and Immunology. Information about the frequency of the different symptoms of the disease along with the treatment protocol was collected on a data sheet and stored electronically in a database in the period between January 2006 and August 2012. The information was then analysed at the end of the study period.

3.1.4 RESULTS

The male/female (M/F) ratio was 0.5:1.0. All BS patients had a history of recurrent oral ulceration, 85.6% had dermatological lesions, 79.1% had rheumatological manifestation, 73.9% had genital ulcerations, 68.6% had ocular involvement, 15% had neurological manifestations, and 10.5% had vascular involvement. The most frequent treatment was colchicine (54.2%) followed by topical corticosteroid therapy (53.6%) then azathioprine (43.8%).

3.1.5 CONCLUSION

BS in the UK is more prevalent in females. The most frequent symptoms are mucocutaneous, rheumatological, and ocular manifestations. In this study, the rate of ocular and rheumatological manifestations is higher than expected, which might highlight a referral bias due to the nature of the study centres (Oral Medicine, Rheumatology, Ophthalmology, and Immunology multi-disciplinary clinic).

3.1.6 DECLARATION

The ethical approval application was performed by Professor Fortune. The evaluation and diagnosis of patients were undertaken in a consultant-led examination.

The author has performed the data analysis in this chapter, and contributed to the data collection and management. The research and clinical team of the Centre for Clinical and Diagnostic Oral Sciences also contributed to data collection and management.

3.2 INTRODUCTION

A syndrome can be defined as a collection of signs and symptoms that frequently emerge together despite the lack of a known cause. Behçet's syndrome (BS) is a collection of mucocutaneous, ocular, rheumatological, vascular, and neural symptoms with mysterious cause; therefore, we prefer to describe this condition as a syndrome rather than a disease. The underlying pathological mechanism of BS is vasculitis that affects all types and sizes of vessels (Talarico et al., 2012).

Currently, there are five nationwide surveys of BS (Iran: 5,059 patients; Japan: 3,316 patients; China: 1,996 patients; Korea: 1,527 patients; Germany: 590 patients) (Davatchi et al., 2010b). In Europe, the highest prevalence of BS was reported recently in France (7.1/100,000 adults) highlighting increasing prevalence outside the "Silk Route" boundaries (Mahr et al., 2008). The male/female (M/F) ratio is almost equal in the five nationwide surveys; however, the classically reported trend was pointing towards an increase predelection for men than women in countries along the "Silk Route" (Davatchi et al., 2010b, El Menyawi et al., 2008). On the other hand, a reverse pattern was reported in countries outside the ancient "Silk Route" boundaries (Chamberlain, 1977, Jankowski et al., 1992). Mucous membrane manifestations are the most prevalent manifestations of the syndrome, followed by dermatological, ocular, rheumatological, neurological, gastrointestinal, vascular, pulmonary, and cardiac manifestations (Davatchi et al., 2010b, Davatchi et al., 2010c, El Menyawi et al., 2008, Salvarani et al., 2007, Zouboulis et al., 1997).

To date there is no curative treatment for BS. However, good control over the disease flare up can be established by using immunosuppressive medications aiming at preventing damage caused by recurrent episodes of inflammation during the flare up attacks. In 2008 the European League Against Rheumatism (EULAR) developed nine different recommendations for BS management and care based on the best available evidence and expert opinion (Hatemi et al., 2008a). The EULAR recommendations are discussed in chapter 1, section 1.2.7.

New and promising treatment modalities including biological agents such as interferon alpha 2 a (INF-α2a) and tumour necrosis factor alpha (TNF-α) inhibitors were suggested in the literature (Pipitone et al., 2006). INF-α2a has immunomodulatory properties leading to a decrease in the circulating gama delta (γδ) T cells, enhanced human leukocyte antigen (HLA) 1 expression on peripheral blood mononuclear cells (PBMC), and inhibited T cell adhesion to endothelial cells (Treusch et al., 2004). The TNF-α inhibitors are either monocolonal antibodies such as infliximab and adalimumab, or fusion protein receptors such as etanercept. Infliximab and etanercept have been reported to have an effective therapeutic outcome in the treatment of BS (Melikoglu et al., 2005, Ohno et al., 2004).

Oral tolerization with the 336-351 sequence of heat shock protein (HSP) 60 linked to recombinant cholera B-toxin was studied in a phase I/II clinical trial with promising results in a small cohort of BS patients. Following the oral tolerization, 3 out of 5 patients remained free of relapsing uveitis for 10–18 months subsequent to stopping all treatment (Stanford et al., 2004). However, this study needs to be supplemented by a phase III trial before adopting this technique in the clinical treatment of patients suffering from BS.

This part of the thesis is a hospital-based study analysing the clinical picture of BS patients in the UK cohort, aiming at a better understanding of this syndrome in a country outside the "Silk Road" boundaries. The clinical picture and treatment protocol of BS patients who were enrolled in two tertiary referral centres in the UK were analysed in the period between January 2006 and August 2012, and compared to the published data from other countries.

3.3 METHODOLOGY

3.3.1 ETHICAL APPROVAL

Ethical approval was granted by the East London and The City Research Ethics Committee (Reference P/03/122) to study BS aetiology as a part of the immunoregulation at the mucosal barrier programme. Subsequently, ethical approval has been granted for St Thomas' Hospital independently.

3.3.2 CLINICAL CENTRES

The BS patients were recruited from two main centres in London, St Thomas' Hospital and Royal London Hospital. These centres subsequently became a Centre of Excellence for the management and care of BS patients. Both centres are multi-disciplinary in nature, embracing the following specialities: Oral Medicine, Ophthalmology, Rheumatology, and Immunology. The centres work in close association with the International Society of Behçet's Disease (ISBD).

3.3.3 PATIENT DIAGNOSIS

For the purpose of research standardization, all BS patients enrolled in this study were diagnosed according to the International Study Group (ISG) classification and diagnostic criteria for BS (Lancet, 1990). The BS patients' inclusion criteria, exclusion criteria, and stratification are detailed in chapter 2, section 2.1.2.2. All patients aged 18 years old and over were invited to take part in the study. A written invitation was handed to them and the study was explained fully to each patient who wished to participate. A consent form was filled in and signed (Appendix 1). Disease signs and activity were recorded in each clinical appointment, along with the overall disease activity and the current treatment protocol, on the clinician's observation form for disease activity (Appendix 2).

3.3.4 CLINICAL DATABASE

A clinical database was developed in order to record demographic data, date of clinical assessment, disease symptoms, and disease activity in relation to each clinical sign. The overall disease status was recorded to differentiate between inactive and relapsed cases. Furthermore, the treatment plan along with any changes in the current treatment protocol was recorded for each clinical visit.

3.3.5 STATISTICAL ANALYSIS

Data were analysed using Prism software (GraphPad, USA). Mean and percentages were used as descriptive statistics.

3.4 RESULTS

3.4.1 MALE/FEMALE RATIO

A total of 153 BS patients, diagnosed according to the ISG criteria for BS classification and diagnosis, were registered on the clinical database in the period between January 2006 and August 2012 (Lancet, 1990). About 32% of those patients were male and 68% were female with male/female (M/F) ratio of 0.5:1 (Figure 3.1).

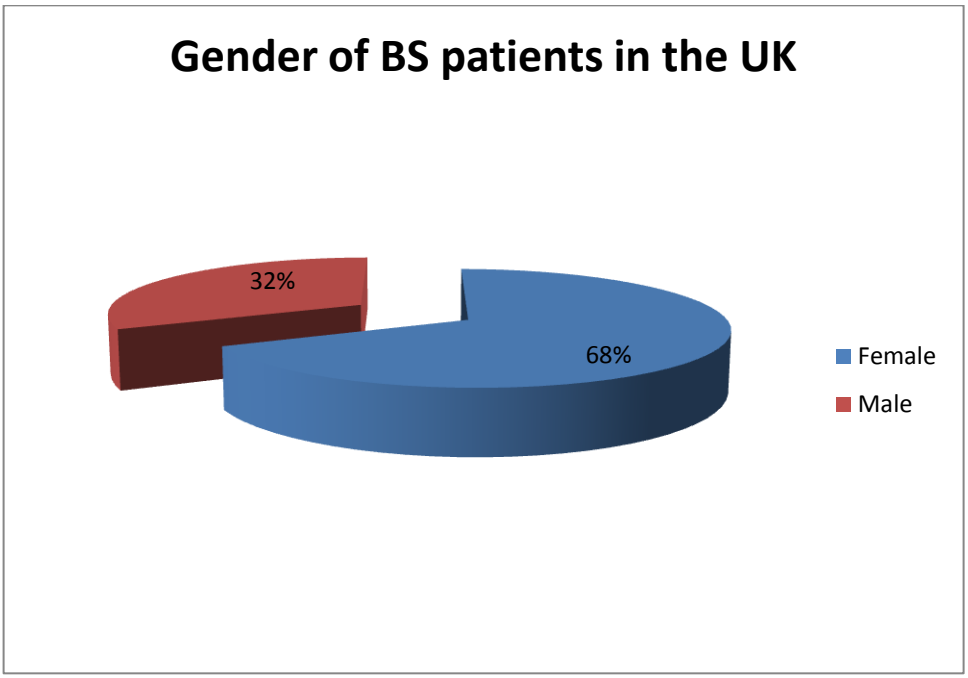


Figure 3.1: Gender of the UK's BS patients (M/F: 49/104).

3.4.2 ETHNIC BACKGROUND

About two-thirds of the BS patients identified themselves as European. They reported no known ancestors from the "Silk Route" area (93/1153: 60.8%). Only 7.8% classified themselves as Turkish (11/120: 9.1%). The percentage of African, Asian, mixed Asian and European, and other ethnic origins were as follows: 3.3%, 3.3%, 1.3% and 1.3% respectively. About a quarter of the BS patients did not disclose their ethnic background

(Tables 3.1 and Figure 3.2). The mean age at analysis was 42.85 years of age (mean age: 42.84 ± 11.65; range: 18–77 years old).

Table 3.1: The ethnic background of the BS patient cohort in the UK.

ETHNIC ORIGIN	NUMBER OF CASES	PERCENTAGE
African	5	3.3%
Asian	5	3.3%
European	93	60.8%
Asian/European	2	1.3%
Turkish	12	7.8%
Others	2	1.3%
Not specified	34	22.2%

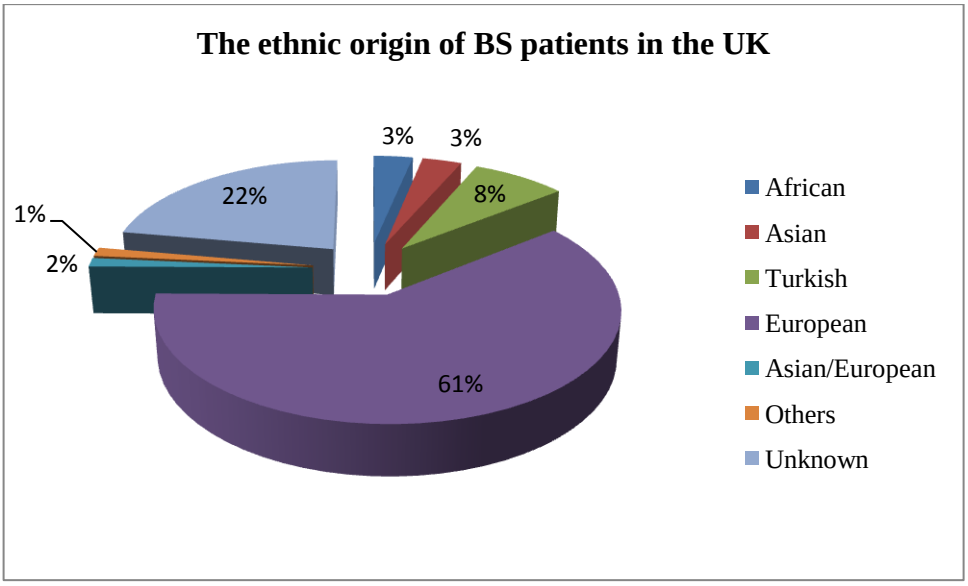


Figure 3.2: The ethnic origin of BS patients in the UK.

3.4.3 DISEASE SYMPTOMS

BS patients were diagnosed according to the ISG criteria for BS patient classification and diagnosis. Therefore, a total of 100% of the BS patients had a history of recurrent oral ulceration of a major, minor or herpetiform nature, while 85.6% had dermatological lesions

such as folliculitis (85.6%) or erythema nodosum-like lesions (82.4%), 79.1% had rheumatological manifestations, 73.9% had genital ulceration, and 68.6% had ocular involvement. Only 15% of the BS patient cohort had neurological manifestations, and 10.5% had vascular involvement (Table 3.2).

Table 3.2: Clinical manifestations of the UK's cohort of BS patients (n=153).

ORGAN INVOLVEMENT	**n**	**%**
Oral ulcer	153/153	100%
Genital ulcer	113/153	73.9%
Folliculitis	131/153	85.6%
Erythema nodosum-like lesion	126/153	82.4%
Cutaneous involvement	131/153	85.6%
Ocular involvement	105/153	68.6%
Rheumatologic involvement	121/153	79.1%
Vascular involvement	16/153	10.5%
Neurologic involvement	23/153	15%
n: number; UK: United Kingdom; %: percentage		

3.4.4 TREATMENT PROTOCOL

Treatment protocol was variable based on the subset of BS signs and symptoms according to the EULAR recommendation for the management of BS patients (Hatemi et al., 2008a). Patients were prescribed multiple medications in 73.9% of the cases (113/153) while only 15.7% of the cases were treated by single medication (24/153) and a further 10.5% did not need to be actively treated at the time of their presentation to the BS patients' specialist clinic (16/153). A total of 3.3% of the UK's BS patient cohort were treated with topical corticosteroid as the sole treatment (5/153) while 53.6% were treated with topical corticosteroid as part of their multiple therapies (82/153). Colchicine was the most frequent treatment, used in 54.2% of the BS patient cohort (83/153), followed by topical corticosteroid therapy (82/153: 53.6%) then azathioprine (67/153: 43.8%) (Table 3.3).

Systemic corticosteroid was mostly used as emergency treatment during relapsed episodes only (58/153: 37.9%), while mycophenolate, ciclosporin, anti-TNF, and methotrexate were used in a small number of patients as follows: 15.7%, 5.9%, 2%, and 1.3% respectively (Table 3.3).

Table 3.3: The different treatment modalities used to manage the UK's cohort of BS patients (n=153).

TREATMENT PROTOCOL	**n**	**%**
Systemic corticosteroid	58/153	37.9%
Colchicine	83/153	54.2%
Azathioprine	67/153	43.8%
Mycophenolate	24/153	15.7%
Ciclosporin	9/153	5.9%
Anti-TNF	3/153	2%
Methotrixate	2/153	1.3%
Topical corticosteroid treatment	82/153	53.6%
Triple therapy mouthwash (topical antifungal, antibacterial, and corticosteroid treatment)	23/153	15%
Topical treatment only	5/153	3.3%
No treatments	16/153	10.5%
n: number; TNF: Tumor Necrosis Factor; UK: United Kingdom; %: percentage		

3.5 DISCUSSION AND CONCLUSION

A cohort of 153 BS patients was successfully recruited. All patients in the cohort were diagnosed according to the ISG criteria to ensure research standardization by strictly including well-defined cases only (ISG, 1992, Lancet, 1990). The disease was classically described as having a greater predilection for men than women in the ancient "Silk Road" countries, whereas the trend is reversed in Western Europe and the USA (Evereklioglu, 2005, Zouboulis et al., 1997). Our study confirms this pattern as the M/F ratio was 0.5:1.0

in the UK BS patient cohort. BS patients in Korea, Israel, Spain, and Sweden have a similar M/F ratio to that of the UK cohort (Table 3.4).

Table 3.4: M/F ratio of the UK BS patient cohort compared to the published data from other countries [source: (Davatchi et al., 2010b)].

COUNTRY	M/F RATIO	REFERENCE
Brazil	1.00:1.19	(Sachetto et al., 2011)
China	1.34:1.00	(Wang et al., 2010)
Germany	1.40:1.00	(Zouboulis et al., 1997)
Greece	1.40:1.00	(Vaiopoulos et al., 2010)
India	1.80:2:00	Reviewed in (Davatchi et al., 2010b)
Iran	1.19:1.00	(Davatchi et al., 2010c)
Iraq	3.00:1.00	(Al-Rawi and Neda, 2003)
Israel	0.64:1.00	(Krause et al., 2007)
Italy	1.00:1.00	(Salvarani et al., 2007)
Japan	0.98:1.00	(Kaneko et al., 2003)
Korea	0.63:1.00	(Yi et al., 2009)
Morocco	2.00:1.00	Reviewed in (Davatchi et al., 2010b)
Portugal	1.00:1.00	(de Souza-Ramalho et al., 1991)
Russia	3.67:1.00	Reviewed in (Davatchi et al., 2010b)
Saudi Arabia	3.40:1.00	Reviewed in (Davatchi et al., 2010b)
Spain	0.50:1.00	(Gonzalez-Gay et al., 2000)
Sweden	0.67:1.00	(Ek and Hedfors, 1993)
Turkey	1.03:1.00	(Tuzun et al., 1996)
UK	0.50:1.00	Seoudi PhD thesis
M/F: male/female; UK: United Kingdom		

The most frequent symptoms in the UK BS patient cohort were mucocutaneous, rheumatological, and ocular manifestations. The high UK rate of oral ulceration (100%) was expected as all BS patients were diagnosed according to the ISG criteria for BS diagnosis and classification. The UK rate of genital ulceration (73.9%) was greater than that reported in Iran (65%) and Germany (64%), similar to that of the Japan (73%) but less than that of China (76%) and Korea (83%).

The UK rate of cutaneous involvements (85.6%) was similar to that of Korea (84%), slightly less than that reported in Japan (87%), but greater than that reported in Iran (66%), China (69%), and Germany (81%) (Table 3.5). Interestingly, the UK rate of ocular involvements (68.6%) was similar to that reported in Japan (69%) but greater than that reported in Iran (55%), China (35%), Korea (51%), and Germany (53%). Likewise, the UK rheumatological manifestations (79.1%) were remarkably higher than those reported in Iran (33%), Japan (57%), China (30%), Korea (38%), and Germany (53%).

This study is a hospital-based not population-based study, and as such invites a referral bias due to the specialized multi-disciplinary nature of the study centres (Oral Medicine, Rheumatology, Ophthalmology, and Immunology clinic). It is logical and indeed expected that it is the very complicated cases that are referred to these centres rather than the simple ones (Table 3.5).

Table 3.5: The clinical picture of BS patients in the UK cohort compared to the published data from other countries [source: (Davatchi et al., 2010b)].

	CHINA	**GERMANY**	**IRAN**	**JAPAN**	**KOREA**	**UK**
n	1,996	590	5,059	3,316	1,527	153
Oral ulcer	98%	98%	97%	98%	99%	100%
Genital ulcer	76%	64%	65%	73%	83%	73.90%
Folliculitis	31%	62%	57%	N/R	N/R	85.60%
Erythema nodosum-like lesion	38%	42%	22%	N/R	N/R	82.40%
Cutaneous involvement	69%	81%	66%	87%	84%	85.60%
Ocular involvement	35%	53%	55%	69%	51%	68.60%
Rheumatologic involvement	30%	53%	33%	57%	38%	79.10%
Vascular involvement	7.70%	13%	8.90%	8.90%	1.80%	10.50%
Neurologic involvement	6.50%	11%	9%	11%	4.60%	15%
n: number; N/R: not reported; UK: United Kingdom; %: percentage						

In agreement with the EULAR recommendation, the systemic corticosteroid was mostly used as emergency treatment during relapsed episodes only (37.9%). The frequent use of colchicine (54.2%) reflected the high rate of rheumatological (79.1%) and dermatological manifestations (85.6%) observed in this cohort of BS patients. The use of azathioprine was also elevated (43.8%), reflecting the high rate of ocular manifestations (68.6%) recorded in BS patients in the UK cohort.

In this BS patient cohort only two patients from the 153 cases had lost their useful eyesight as a complication of their disease. This might reflect the success of the treatment protocol used. However, it might also reflect the nature of the cohort (68% female and 60.8% Caucasian ethnic origin). It was documented previously that male BS patients have a worse visual prognosis compared to female patients. In addition, patients with poor vision were reported more frequently in countries such as Japan, India, and Iran compared to the UK, Tunisia, Germany, Greece, Turkey, and Italy (Kitaichi et al., 2007).

In conclusion, this part of the thesis analysed the different signs and symptoms of BS patients in a well-defined cohort. In the UK cohort of BS patients, we observed female predilection. The most frequent symptoms were mucocutaneous, rheumatological, and ocular manifestations. However, the high rate of ocular and rheumatological involvements might highlight a referral bias due to the nature of the study centres.

CHAPTER 4

ORAL HEALTH STATUS AND QUALITY OF LIFE OF BEHÇET'S SYNDROME PATIENTS

4.1 ABSTRACT

4.1.1 BACKGROUND

Identifying patients' perception of the disease burden helps to direct the clinical and research resources in the right direction. Behçet's syndrome (BS) patients usually suffer from recurrent oral ulcerations as the initial sign of the disease; therefore, studying the impact of their oral health status on their quality of life (QoL) is crucial.

4.1.2 OBJECTIVES

This part of the thesis is aimed at identifying the impact of the oral health of Behçet's syndrome (BS) patients from two different countries (UK and Turkey) on their QoL.

4.1.3 METHODS

The QoL of BS patients in the UK was compared to that of BS patients in Turkey. A total of 31 BS patients from the UK (M/F: 13/18, mean age: 41.8±11.5) and 31 BS patients from Turkey (M/F: 13/18, mean age: 41.5±10.3) were recruited and studied. Both patient cohorts were diagnosed according to the International Study Group (ISG) criteria for BS classification and diagnosis and closely matched in age and gender (*The Lancet*, 1990). The oral health status of the UK cohort of BS patients was compared to that of the Turkish cohort. Furthermore, the oral health status of 55 BS patients in the UK (M/F: 23/32, mean age: 42.6±12.3) was compared to that of 15 healthy control (HC) volunteers (M/F: 9/6, mean age: 39.7±13.8) and 6 recurrent aphthous stomatitis (RAS) patients (M/F: 3/3, mean age: 46.5±7).

4.1.4 RESULTS

The QoL of BS patients from the UK and Turkey was affected to a similar extent. Interestingly, the number of oral ulcers per month was higher in the UK patients; however, this might reflect a referral bias as UK patients were seen at two of the main tertiary referral centres in the UK for BS management and care. Turkish patients had generally poorer oral health status, which can be explained by the lower utilization rate of the dental services. The oral health status of BS patients form the UK was poorer than HC volunteers but similar to those patients suffering from RAS.

4.1.5 CONCLUSION

BS negatively affects the QoL and the oral health status. In a syndrome with unknown aetiology such as BS that starts with recurrent oral ulceration, studying the oral cavity will be a vital stage in identifying the aetiology of the disease and therefore increasing the potential for discovering more efficient treatment modalities.

4.1.6 DECLARATION

The ethical approval application was performed by Professor Fortune. The evaluation and diagnosis of patients were undertaken in a consultant-led examination.

The author performed the dental assessment and data analysis of the UK patient and volunteer cohorts. She also contributed to data collection, and management of the UK patient and volunteer cohorts. The research and clinical team of the Centre for Clinical and Diagnostic Oral Sciences contributed to data collection and management of the UK BS patients.

The dental assessment, data collection, and data analysis of the Turkish BS patient cohort was conducted by Dr Mumcu's team, following the same protocol used in the UK centres. Dr Mumcu's team also contributed to the comparative analysis between the Turkish and the UK BS patient cohorts.

4.2 INTRODUCTION

Establishing patients' perception of their disease burden and its effect on their quality of life (QoL) is crucial in informing clinical decision-making and improving management strategies. It also identifies areas of need, aiming to redirect the clinical and research resources in patient-focused directions (McGrath et al., 2003, Mumcu et al., 2006, Mumcu et al., 2007a). The health-related QoL can be identified by using either a generic questionnaire or a disease-specific questionnaire (Bernabe et al., 2010, Gilworth et al., 2004, Touma et al., 2011). The impact of a particular symptom on the QoL can also be examined using a symptom related QoL questionnaire (Mumcu et al., 2006, Mumcu et al., 2007a, Onal et al., 2010).

Behçet's syndrome (BS) is one of the chronic diseases characterized by vasculitis affecting different vessels in almost all body organs, causing multi-systemic symptoms such as mucocutaneous, gastrointestinal, musculoskeletal, neurological, ophthalmic, and vascular involvements (Bernabe et al., 2010, Escudier et al., 2006, Kitaichi et al., 2007). Previous studies examined the QoL of BS patients by using generic and disease-specific instruments and highlighted the considerable negative impact of the syndrome (Bernabe et al., 2010, Gilworth et al., 2004).

Active oral ulcerations are amongst the most important and very early symptoms in BS (Davatchi et al., 2010c, Mumcu et al., 2006). Therefore, the oral health-related QoL of BS patients was also examined by two different instruments, namely the oral health impact profile 14 (OHIP-14), and the United Kingdom oral health-related quality of life (OHQoL-UK) (McGrath et al., 2003, Mumcu et al., 2006, Mumcu et al., 2007a) (Tables 4.1 and 4.2). However, this part of the thesis is the first study to examine the oral health-related QoL of BS patients from the UK and compare it to patients from Turkey.

Poor oral health was previously observed in BS patients with a possible cause or effect relationship (Karacayli et al., 2009). Nevertheless, this part of the thesis is the first study to compare the oral health of BS patients to healthy control (HC) volunteers and recurrent aphthous stomatitis (RAS) patients.

Table 4.1: Questions in the OHIP-14.

BECAUSE OF PROBLEMS WITH YOUR TEETH, DENTURE OR MOUTH HAVE YOU
had trouble pronouncing words?
felt your sense of taste has worsened?
had painful aching in the mouth?
found it uncomfortable to eat any foods?
been self-conscious?
felt tense?
had an unsatisfactory diet?
had to interrupt meals?
found it difficult to relax?
been a bit embarrassed?
been irritable with other people?
had difficulty doing your usual jobs?
felt life in general was less satisfying?
been totally unable to function?

Table 4.2: Questions in the OHQoL-UK.

WHAT EFFECT, IF ANY, DO YOUR TEETH, GUMS, MOUTH OR DENTURE HAVE ON YOUR
comfort?
breath odour?
eating?
appearance?
general health?
speech?
smiling/laughing?
ability to relax/sleep?
confidence?
mood?
carefree manner?
personality?
social life?
romantic relationships?
work/usual activities?
finances?

4.3 METHODOLOGY

4.3.1 PATIENT COHORTS

There are three groups in this part of the thesis, namely the BS patient cohorts (UK and Turkey), the RAS patients and the HC volunteers. Patients and volunteers were recruited and classified according to the protocol detailed in chapter 2, section 2.1.2.

4.3.2 DATA ACQUISITION

Data was recorded in two different data collection forms: QoL questionnaire and oral health status questionnaire.

4.3.2.1 QUALITY OF LIFE QUESTIONNAIRE

The OHIP-14 is one of the internationally validated and widely used oral health-related quality of life (OHRQol) indicators. It reflects the patient's own views about their health

and oral status by using a 14-item questionnaire to measure self-reported functional limitation, discomfort and disability related to the investigated oral condition.

A 5-point Likert-type scale is used to score 14 different items, such as the effect of the disease on the physical function of the mouth and in particular on speaking, taste sensation, food intake, and movement. The OHIP-14 also investigates the effect of the disease on the psychosocial symptoms and psychological wellbeing of the patients. Its scores range from 0 to 56, with score 0 indicating no impact of the disease on oral health while score 56 indicates severe impact on all aspects of oral health (Appendix 3).

4.3.2.2 ORAL HEALTH STATUS QUESTIONNAIRE

Oral assessment was carried out by a trained dentist and evaluated by dental and periodontal indices including the decayed, missing, filled teeth (DMFT) index, plaque index (PI), gingival index (GI), sulcus bleeding index (SBI), periodontal probing depth (PPD) and attachment level (AL) (Appendix 4).

4.3.3 STATISTICAL ANALYSIS

A statistical power calculation using the StatMate 2 program (GraphPad, USA) and based on the previous published literature investigating the QoL by using OHIP-14 indicated that a sample size of 30 in each group has a 95% power to detect a difference between means of 13.46 with a significant level (alpha) of 0.05 (two tailed) (Mumcu et al., 2007a). For the purpose of the oral health assessment, a statistical power calculation based on the previous published literature investigating BS patients' oral status by using similar dental indices indicated that a sample size of 6 in each group has a 95% power to detect a difference

between means of 4.14 with a significant level (alpha) of 0.05 (two tailed) (Karacayli et al., 2009). The author aimed to investigate the maximum sample size achievable during the time of the PhD course and using the available resources.

Data were analysed using Prism software (GraphPad, USA). Mean, standard deviation (SD) and standard error of mean (Sem) were used in the descriptive statistics. Non-parametric tests such as the Mann-Whitney-U test, Kruskal-Wallis test, and Spearman correlation test were used in this study due to the non-normal distributed data. P value of less than 0.05 was considered significant.

4.4 RESULTS

4.4.1 QUALITY OF LIFE

No significant difference was found in OHIP-14 scores between patients from the UK (22.7±14.4) and Turkey (20.4±14.3) (Mann-Whitney, p=0.709). In addition, OHIP-14 subscale scores looking at physical, psychosocial, and psychological symptoms were similar in patients from the UK (8.7±5.2, 8.3±5.5 and 5.7±4.3 respectively) and Turkey (7.01±5.7, 6.6±5.5 and 7.1±4.9 respectively) (Mann-Whitney, p=0.219, p=0.311 and p=0.248 respectively) (Tables 4.3 and 4.4).

Table 4.3: OHIP-14 scores: a comparison between the BS patient cohort from the UK and the cohort from Turkey.

	UK PATIENTS (n=31)		**TURKISH PATIENTS (n=31)**	
	Mean	**SD**	**Mean**	**SD**
OHIP-14 Score	22.7	14.4	20.2	14.3
n: number; OHIP-14: oral health impact profile-14; SD: standard deviation; UK: United Kingdom.				

Table 4.4: Oral ulcer-related subscales in OHIP-14: a comparison between the BS patient cohort from the UK and the cohort from Turkey.

	UK PATIENTS (n=31)		**TURKISH PATIENTS (n=31)**	
	Mean	**SD**	**Mean**	**SD**
Subscale 1 (Physical symptoms)	8.7	5.2	7.01	5.7
Subscale 2 (Psychosocial symptoms)	8.3	5.5	6.6	5.5
Subscale 3 (Psychological symptoms)	5.7	4.3	7.1	4.9
n: number; OHIP-14: oral health impact profile-14; SD: standard deviation; UK: United Kingdom.				

The overall disease severity score was similar in both groups (Turkey: 4.9±1.6 versus UK: 5.1±1.8) (Mann-Whitney, p=0.983). The healing times of oral ulcers and oral ulcer-related pain were also similar in patients from the UK (8.6±5.2 days and 50.5±36.2 respectively) and Turkey (7.2±2.1 days and 42.7±40.7 respectively) (Mann-Whitney, p>0.05). However, the number of oral ulcers per month was significantly higher in the UK patients (3.3±2.8) compared to the Turkish patients (1.5±2.5) (Mann-Whitney, p=0.014) (Table 4.5). The OHIP-14 score correlated with the healing time of oral ulcers in the UK patients (r=0.4, p=0.04) and the number of oral ulcers in patients from Turkey (r=0.4, p=0.012).

Table 4.5: Disease severity score and oral ulcer-related factors in the BS patient cohort from the UK and the corhort from Turkey.

	UK PATIENTS (n=31)		**TURKISH PATIENTS (n=31)**	
	Mean	**SD**	**Mean**	**SD**
Number of oral ulcers	3.3	2.8	1.5	2.5
Healing time of oral ulcers in days	8.6	5.2	7.2	2.1
Oral ulcer-related pain in 100 mm VAS	50.5	36.2	42.7	40.7
Disease severity score	5.1	1.8	4.9	1.6
SD: standard deviation; VAS: visual analogue scale.				

4.4.2 ORAL HEALTH STATUS

4.4.2.1 COMPARATIVE STUDY OF THE UK AND TURKISH BS COHORTS

The number of filled teeth (1.1±1.6) and the frequency of tooth brushing (1.01±0.7) were significantly lower in BS Turkish patients when compared to those from the UK (5.6±4.2 and 1.9±0.6 respectively) (Mann-Whitney, $p<0.0001$). However, the scores of the SBI and PPD were higher in Turkish patients (1.7±1.2 and 2.7±0.6 respectively) than in the patients from the UK (1.1±0.7 and 2.1±0.4) (Mann-Whitney, $p=0.004$ and $p<0.0001$ respectively) (Tables 4.6).

Table 4.6: Oral health and related factors in the UK and Turkish cohorts of BS patients.

	UK PATIENTS (n=31)		**TURKISH PATIENTS (n=31)**	
Oral health and related factors	**Mean**	**SD**	**Mean**	**SD**
Carious teeth	0.6	0.9	1.1	1.8
Extracted teeth	3.4	3.3	4.5	4.4
Filled teeth	5.6	4.2	1.1	1.6
Total number of teeth	24.6	5.6	23.4	4.4
PI	0.9	0.8	1.3	1.2
GI	1.1	0.8	1.6	1.2
SBI	1.1	0.7	1.7	1.2
PPD	2.1	0.4	2.7	0.6
AL	3.3	1.01	3.9	1.5
Frequency of tooth brushing	1.9	0.6	1.01	0.7
Last dental visit (months)	5.1	7.2	28.6	23.7
AL: attachment level; BS: Behçet's syndrome; GI: gıngıval index; PPD: periodontal probing depth; PI: plaque index; SD: standard deviation; SBI: sulcus bleeding index.				

The period of time since the last dental visit was significantly less for the UK's BS patients than for those from Turkey (5.1±7.2 months versus 28.6±23.7 months) (Mann-Whitney, $p<0.0001$) (Tables 4.7 and 4.8). No significant correlation was observed between OHIP-14 and the scores of dental and periodontal health and the time since the last dental visit in either of the patient groups.

4.4.2.2 ORAL HEALTH STATUS IN THE UK'S BS PATIENTS COMPARED TO THE CONTROLS

The oral health status was studied in a cohort of 55 BS patients (F/M: 32/23, mean age: 42.6±12.3) and compared to that of 15 HC volunteers (F/M: 6/9, mean age: 39.7±13.8) and 6 RAS patients (F/M: 3/3, mean age: 46.5±7). The oral health status was assessed using the DMFT, PI, GI, SBI, PPD, and AL.

Using non-parametric statistics, Mann-Whitney to compare two groups and Kruskal-Wallis to compare three groups, the BS patient cohort showed a statistically higher DMFT score compared to the HC volunteers but there was no statistically significant difference between BS patients and RAS patients (Mann-Whitney, p=0.0162 and p=0.9585 respectively) (Figures 4.1 and Table 4.7).

There was a statistically significant increase in the GI score observed for the BS patient cohort compared to the HC volunteers but there was no statistically significant difference between BS patients and RAS patients (Mann-Whitney, p=0.0056 and p=0.7870 respectively). The same pattern was observed in relation to SBI, PPD and AL (SBI: Mann-Whitney; p=0.0002 and p=0.1488 respectively), (PPD: Mann-Whitney, p=0.039 and p=0.3891 respectively), (AL: Mann-Whitney, p=0.015 and p=0.3351 respectively). However, there was no statistically significant difference in PI between BS patients, HC volunteers and RAS patients (Kruskal-Wallis, p=0.2756). Oral hygiene was good in the three groups, with PI ranged between 0 and 2.4 for all three investigated groups (Figure 4.1 and Table 4.7).

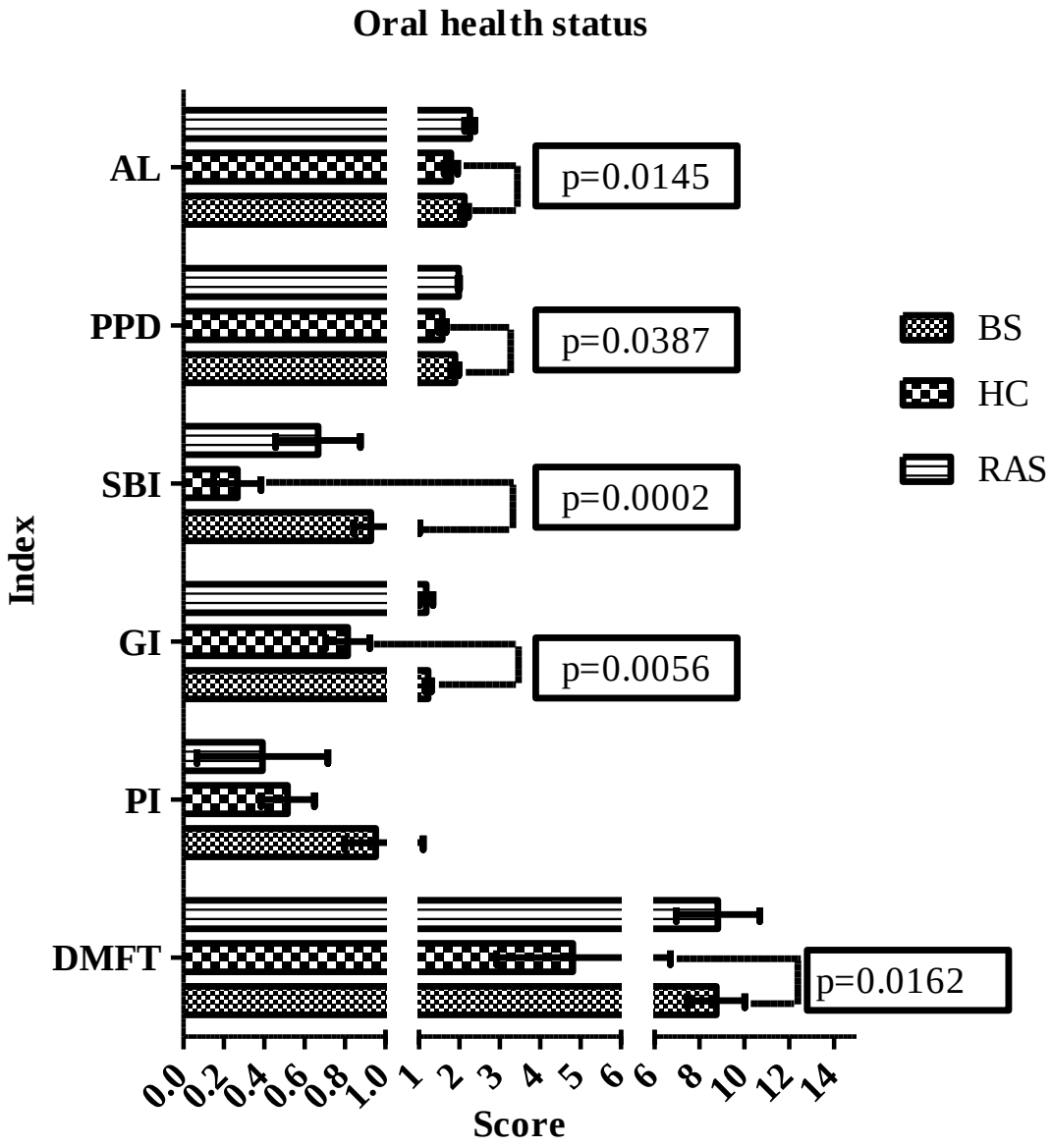


Figure 4.1: Oral health status of BS patients compared to RAS patients and HC volunteers. Attachment level (AL), decayed, missing, filled teeth (DMFT) index, gingival index (GI), plaque index (PI), periodontal probing depth (PPD), and sulcus bleeding index (SBI) were investigated in all groups.

Table 4.7: Oral health status in the UK cohort of BS patients compared to RAS patients and HC volunteers.

	BS (n=55)		**HC (n=15)**		**RAS (n=6)**	
Indices	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
DMFT	9.18	7.31	4.81	7.56	8.83	4.54
PI	0.93	0.78	0.52	0.52	0.56	0.84
GI	1.17	0.59	0.81	0.42	1.18	0.40
SBI	1.01	0.55	0.27	0.46	0.67	0.52
PPD	1.99	0.43	1.58	0.36	1.98	0.05
AL	2.79	0.75	1.79	0.66	2.26	0.30
AL: attachment level; BS: Behçet's syndrome; DMFT: decayed; missing, filled teeth; GI: gingival index; n: number; PI: plaque index; PPD: periodontal probing depth; RAS: recurrent aphthous stomatitis; SBI: sulcus bleeding index; SD: standard deviation.						

4.5 DISCUSSION AND CONCLUSION

The presence of discomfort, pain, poor oral function, and dissatisfaction with oral health are the main areas for impaired oral QoL (McGrath et al., 2003). The OHIP-14 was used in this part of the thesis as a tool to compare 31 cases of BS in the UK to an equal number of cases in Turkey. This questionnaire was previously validated and proven to be a reliable tool in oral disease as a patient-centred outcome measure (McGrath et al., 2003, Mumcu et al., 2006, Mumcu et al., 2007a).

BS negatively affected the QoL of patients from the UK to a similar extent to those from Turkey. In a recent study, BS was proven to affect the QoL similarly to other chronic conditions such as multiple sclerosis and arthritis. The symptoms of the disease exhibited a negative impact on the QoL, with the strongest impact related to joint problems followed by neurological symptoms, pathergy reaction, and stomach and bowel problems (Bernabe et al., 2010).

It is documented in the literature that the dental and periodontal treatment were associated with a short-term flare up of oral ulceration; however, it was thought that good oral hygiene along with frequent dental maintenance can deliver a long-term benefit by decreasing the frequency and number of oral ulcerations or even decreasing the prevalence of the disease (Direskeneli and Mumcu, 2010, Karacayli et al., 2009). The role of the oral pathogen and/or commensals in the patho-aetiology of BS was questioned and investigated in the literature. However, to date there is no proven causative correlation (Fortune, 2003, Lehner, 1997).

In this part of the thesis, we observed an increase in the number of oral ulcers in BS patients from the UK. However, this might reflect a referral bias as the UK patients were seen at two of the main tertiary referral centres in the UK for BS patient management and care, where the most complicated cases are expected to be referred. The SBI and PPD scores in the Turkish BS cohort were higher than those in the BS patient cohort from the UK. This fact proved that BS patients in Turkey had generally poorer oral health, which can be explained by the lower utilization rate of dental services.

We also observed statistically higher DMFT, GI, SBI, PPD, and AL scores in the UK BS patient cohort compared to HC volunteers. However, there were no statistical differences between BS and RAS patients in all the measured indices. This can be due to the difficulty of maintaining good oral hygiene during the recurrent attacks of oral ulceration or possibly a status of increased susceptibility to oral pathogens in both disease groups (BS and RAS). Nevertheless, the PI was low in all the examined groups from the UK, highlighting favourable oral hygiene awareness and/or the availability of dental care. The fact that the

oral health status of BS and RAS patients was poorer than in HC volunteers despite the equal PI might implicate a possible impairment in the immune responses to oral pathogens and/or commensals in both BS and RAS patients, leading to more susceptibility to dental and periodontal disease.

The results of the QoL study are published in *The Journal of Oral Pathology and Medicine* (Mumcu et al., 2009c) (Appendix 5).

CHAPTER 5

ORAL MUCOSAL EXPRESSION OF TOLL-LIKE RECEPTORS 2 AND 4

5.1 ABSTRACT

5.1.1 BACKGROUND

Toll-like receptors (TLRs) are pattern recognition receptors (PRR) playing a pivotal role in sensing exogenous pathogen and endogenous danger signals. Furthermore, their role in the pathogenesis of inflammatory and immune-related diseases is gradually being unravelled. Behçet's syndrome (BS) is one of the immune-related diseases with enhanced inflammatory reaction as the characteristic pathological feature, but its aetiology remains unknown. The activation or desregulation of the innate immune response was hypothesized in BS in response to a yet unknown environmental factor such as one of the oral pathogens or commensals.

5.1.2 OBJECTIVES

This part of the thesis aimed at investigating the expression and function of TLR2 and TLR4 in BS, given that jointly these two receptors are capable of identifying most of the oral microbial commensals and pathogens.

5.1.3 METHODS

A total of 87 subjects were included in this study: 55 BS patients, 24 healthy control (HC) volunteers and 8 recurrent aphthous stomatitis (RAS) patients. Non-lesional oral mucosal brush biopsies were collected. Total ribonucleic acid (RNA) was purified and analysed by real time polymerase chain reaction (qPCR) for the presence of messenger ribonucleic acid (mRNA) of TLR2 and TLR4 along with their splice variants. Peripheral blood mononuclear cells (PBMC) from BS patients and healthy individuals were treated in vitro with different TLR2 and TLR4 agonists to assess the function of these pattern recognition receptors.

5.1.4 RESULTS

TLR2b, TLR2d, TLR2e, TLR4.3, and TLR4.4 are significantly elevated in relapsed BS patients. A significant defect in the response to cognate agonists of TLR1/2 heterodimer and TLR4 was also observed in the whole cohort of BS patients.

5.1.5 CONCLUSION

The expression of unusual splice variants of TLR2 and TLR4 might explain the observed defect in the function of these receptors in BS patients.

5.1.6 DECLARATION

The ethical approval application was performed by Professor Fortune. The evaluation and diagnosis of patients were undertaken in a consultant-led examination.

The author performed all data analysis in this chapter. She also contributed to sample collections, sample processing, experimental assays, data collection, and data management. The research and clinical team of the Centre for Clinical and Diagnostic Oral Sciences contributed to sample collection, data collection and data management.

The Genome Centre performed the qPCR analysis for TLR2 and TLR4. The Barts and The London NHS virology unit guided the author in the qPCR analysis for TLR2 and TLR4 splice variants. The research team of the Centre for Clinical and Diagnostic Oral Sciences contributed to the design and optimization of the protein analysis and the functional study.

5.2 INTRODUCTION

The most serious consequences of Behçet's syndrome are blindness, catastrophic gastrointestinal bleeding or large vessel disease, especially when presenting in untreated young adults (Yazici and Esen, 2008). The lack of a universally recognized diagnostic laboratory test results in the diagnosis of BS being based solely on clinical criteria. Oral bacterial and viral aetiology have been suggested (Hamza et al., 1990, Lehner et al., 1991, Sun et al., 1996, Tojo et al., 2003a, Tojo et al., 2003b) and the evidence points towards both genetic and environmental factors as contributors to the disease process (Lehner, 1999).

Pattern recognition receptors (PRRs) are responsible for sensing the presence of microorganisms through the recognition of some structures conserved among microbial species called the pathogen-associated molecular patterns (PAMPs). They also able to recognize endogenous molecules released from damaged cells known as damage-associated molecular patterns (DAMPs). There are four different classes of PRR that can broadly be classified into two main groups as follows: (1) transmembrane proteins such as toll-like receptors (TLRs) and C-type lectin receptors (CLRs); (2) cytoplasmic proteins such as the retinoic acid-inducible gene (RIG)-l-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Takeuchi and Akira, 2010).

TLRs form one of the transmembrane proteins groups of the PRR. As such, they act as first line of defence against microbes and danger signals. Lipopolysaccharides (LPS) produced by gram negative bacteria, peptidoglycans of gram positive bacteria, human and microbial heat shock proteins (HSPs), and viruses are all potential ligands for TLRs (Ariza et al., 2009, Compton et al., 2003, Gay and Gangloff, 2007) (Table 5.1).

Table 5.1: The localisation and ligand for the different TLRs [source: (Takeuchi and Akira, 2010)].

TLR	**LOCALISATION**	**LIGAND**	**ORIGIN OF THE LIGAND**
TLR1	Plasma membrane	Triacyle lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoteichoic acid Peptidoglycan porins Phosphalipomannan β-glucans Zymosan DAMPs (HSP)	Bacteria, viruses, parasites, fungus, self
TLR3	Endolyosome	dsRNA	Virus
TLR4	Plasma membrane	LPS Mannan O-linked mannosyl residue DAMPs (HSP Fibrinogen, fibrinectin Hyaluronan)	Bacteria, viruses, fungus, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyle lipoprotein Zymosan	Bacteria, fungus, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Viruses, bacteria, self
TLR9	Endolysosome	CpG-DNA	Viruses, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
CpG-DNA: cytosine-phosphate-guanine-deoxyribonucleic acid; DAMP: Damage-associated molecular patterns; ds: double strand; HSP: heat shock protein; LPS: lipopolysaccharides; RNA: ribonucleic acid; ss: single strand; TLR: toll-like receptors			

Interestingly, TLR2 and TLR4 jointly are capable of identifying most of the oral microbial commensals and pathogens. These receptors are mainly expressed on the cells associated with innate immunity such as granulocytes, monocytes, macrophages and dendritic cells.

However, expression of some TLRs has been demonstrated on epithelial and endothelial cells (Takeda et al., 2003).

Activation of TLRs triggers a cascade of protein kinases that result in a well-characterized innate host recognition pathway leading to the nuclear translocation of nuclear factor kappa B (NF-κB) and transcription of cytokine-encoding genes (Binder et al., 2004, Takeda et al., 2003). More interestingly, the activation of different TLRs can result in different reactions within the cell and different ligands may induce a relatively more aggressive response when bound to the same receptor (Coats et al., 2005).

TLR2 messenger ribonucleic acid (mRNA) has five different splice variants, one of which lacks exon II, while TLR4 mRNA is known to have three mRNA splice variants, one of which might have inhibitory activity (Haehnel et al., 2002, Jaresova et al., 2007). All TLR2 mRNA splice variants contain the complete sequence of exon III with no changes in the putative open reading frame; however, the differences in the 5´ untranslated sequences influence the stability of the mRNA, which can affect the extent of TLR2 protein translation. TLR2 mRNA variant e is highly expressed in freshly isolated monocytes and the splicing rapidly changes upon adherence to the longer splices (Haehnel et al., 2002). Furthermore, the presence of an early stop codon in TLR4 mRNA variant 3 and 4 makes them subject to a non-sense decay mechanism that should prevent the production of truncated non-functional protein (Brogna and Wen, 2009, Jaresova et al., 2007).

Single-nucleotide polymorphism (SNP) analyses of TLR2 and TLR4 genes were not significantly associated with BS in the Caucasian, Turkish, and Tunisian cohorts (Bacanli

et al., 2006a, Ben Dhifallah et al., 2009, Durrani et al., 2011, Tomiyama et al., 2009). However, one SNP in the TLR4 gene was associated with BS and six other SNPs had an effect on its clinical features in the Japanese BS cohort (Meguro et al., 2008). Likewise, the TLR4 haplotype TAGCGGTAA was considered as a susceptibility gene for HLA-B51 positive BS patients and for the complication of arthritis in the Korean cohort (Horie et al., 2009).

An elevated expression of TLR4 (mRNA and protein) but not TLR2 in the peripheral blood mononuclear cells (PBMCs) of BS patients was initially observed, irrespective of the disease activity (Kirino et al., 2008). Later on, an elevated expression of TLR2 and TLR4 mRNA in the PBMCs of active BS patients was highlighted (Do et al., 2008). Interestingly, vitamin D was found to suppress the protein and mRNA expression of both receptors (Do et al., 2008). Furthermore, decreased TLR2 expression was noted in the PBMCs of BS patients after stimulation with HSP60 and LPS (Yavuz et al., 2008). The total expression of TLR2 and TLR4 was also found to be elevated in the intestinal and buccal mucosa of BS patients and broncho-alveolar lavage (BAL) (Durrani et al., 2011, Hamzaoui et al., 2012, Nara et al., 2008). Furthermore, NOD2 expression showed correlation with TLR2 and TLR4 in BAL samples from BS patients (Hamzaoui et al., 2012). However, to date no study has investigated the differential expression and function of TLR2 and TLR4 splice variants in the oral mucosa of BS.

5.3 METHODOLOGY

5.3.1 PATIENT SELECTION AND ETHICAL APPROVAL

All patients were diagnosed according to the International Study Group (ISG) criteria for BS and informed consent was obtained. The patient cohort was recruited from the outpatient clinic at the Royal London Hospital and St. Thomas' Hospital with the appropriate local ethical approval. BS patients were stratified according to the overall disease activity, following a consultant-led assessment, into the following groups: (1) relapsed status (severe disease activity in more than two of the BS symptoms despite treatment), (2) minor activity status (some disease activity in less than three of the BS symptoms), (3) inactive status (the absence of any symptoms at the time of sampling), (4) non-relapsed (minor activity BS group + inactive BS group). Moreover, the different disease activity and the treatment protocol were also examined.

A total of 87 subjects were included in this part of the thesis: 55 BS patients (13 relapsed BS, 24 minor activity BS, 18 inactive BS) (F/M: 33/22, mean age: 43.3±13), 24 HC (F/M: 14/10, mean age: 39.7±13.8) and 8 recurrent aphthous stomatitis (RAS) (F/M: 5/3, mean age: 46.5±7).

5.3.2 SAMPLES

A total of 87 brush biopsies from non-lesional oral mucosa from 13 relapsed BS patients, 24 minor activity BS, 18 inactive BS, 24 HC volunteers, and 8 RAS patients were collected using a nylon bristle cytology brush (Flowgen, UK) (Fedele, 2009). All samples were made anonymous according to ethical guidelines. Total RNA and protein were purified simultaneously from the same samples according to the Purescript kit protocol (Purescript-

Gentra, Qiagen, UK). For the purpose of the functional study, a total of 46 blood samples from 10 relapsed BS patients, 21 inactive BS patients, and 15 healthy individuals were also collected.

5.3.3 EXPRESSION OF TLR2 AND TLR4 mRNA BY qPCR

Total RNA was transcribed to complementary deoxyribonucleic acid (c-DNA) using the high-capacity c-DNA reverse transcription kit (Applied Biosystems, UK) according to the manufacturer's instructions. All samples were tested in a real time polymerase chain reaction (qPCR) experiment using TaqMan probes for TLR2 mRNA (general TLR2 probe for the common 5´ untranslated region (5´UTR) region of the five transcripts on the 2-3 exon boundary), TLR4 mRNA (general TLR4 probe for the common 5´UTR region of the three transcripts on the 1-2 exon boundary) (Applied Biosystems, UK). 18 S rRNA acted as a housekeeping gene for the relative expression analysis (Figures 5.1 and 5.2)

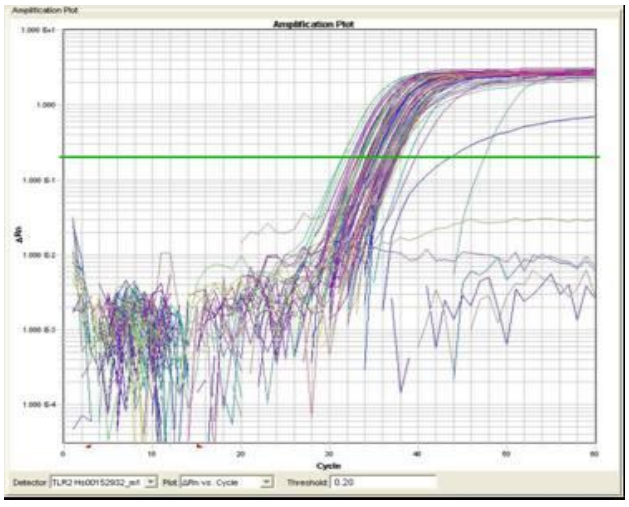


Figure 5.1: qPCR of the TLR2 mRNA in general.

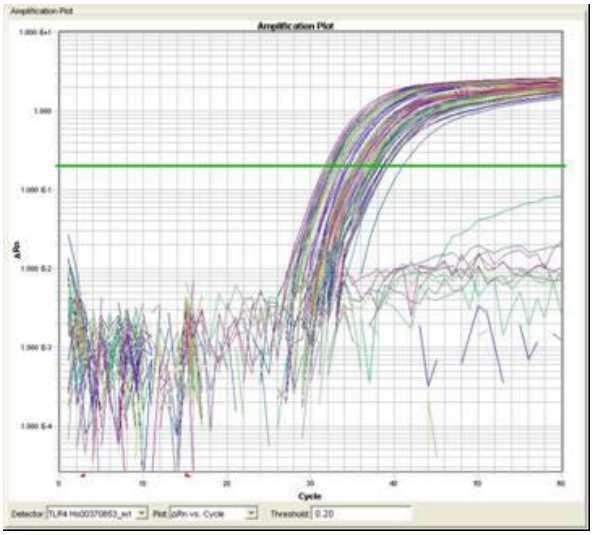


Figure 5.2: qPCR of the TLR4 mRNA in general.

5.3.4 EXPRESSION OF SPLICE VARIANTS OF TLR2 AND TLR4 mRNA BY qPCR

A total of 35 positive c-DNA samples from the same cohort investigated in the previous experiment (11 relapsed BS patients, 12 non-relapsed BS patients, 12 HC volunteers) were tested for the presence of the different splice variants of TLR2 mRNA and TLR4 mRNA by using specific primers designed and validated in-house (chapter 2, section 2.2.2.2, Table 2.5). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acted as a housekeeping gene for the relative expression analysis. SYBR Green master mix was used in this analysis (Roche Applied Science, UK). and the thermal cycle parameters were as follows: one cycle at 95 °C for 5 minutes followed by 45 cycles (melting at 95 °C for 10 seconds, annealing at 60 °C for 10 seconds and extension at 72 °C for 10 seconds) and one melting analysis cycle (95 °C for 5 seconds, 60 °C for 60 seconds, 60 °C to 99 °C gradual heating and finally 40 °C for 20 seconds).

5.3.5 SLOT-BLOT ANALYSIS

A total of 42 protein samples from 14 relapsed BS patients, 14 non-relapsed BS patients, and 14 HC volunteers were quantified by using the NanoDrop spectrophotometer (Labtech, UK). 12.5% purified protein samples were prepared in sodium dodecyl sulphate (SDS) buffer and analysed according to the protocol detailed in chapter 2, section 2.2.2.3.

5.3.6 WESTERN-BLOT ANALYSIS

A total of 30 protein samples from the cohort investigated by Slot-blot analysis **(**8 relapsed BS patients, 12 non-relapsed BS patients, and 10 HC volunteers) were prepared for loading in the sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analysed according to the protocol detailed in chapter 2, section 2.2.2.4.

5.3.7 TLR FUNCTIONAL STUDY

A total of 46 blood samples from 10 relapsed BS patients, 21 non-relapsed BS patients and 15 healthy individuals were analysed. PBMC were isolated by density gradient centrifugation over ficoll-hypaque (F-H) (1700 rpm for 35 minutes) and processed according to the protocol detailed in chapter 2, section 2.2.2.5.

5.3.8 STATISTICAL ANALYSIS

The statistical power was calculated using the StatMate 2 program (GraphPad, USA). Based on the average of the standard deviation (SD) of the investigated 4 groups in the qPCR pilot study (SD average=0.002207), a sample size of 8 in each group has a 95% power to detect a difference between means of 0.0043 with a significant level (alpha) of

0.05 (two tailed). The author aimed to investigate the maximum sample size achievable during the time of the PhD course and using the available resources.

The GraphPad Prism® statistical package was used in the data analysis (GraphPad Software Inc., USA). Mean, median, minimum, maximum, range, SD, standard error of mean (Sem), and percentages (%) were used to describe the data. The data were analysed by Mann-Whitney-U when comparing quantitative data of two groups, and Kruskal-Wallis when comparing quantitative data of more than two groups. Differences in the rate were analysed using Chi-square tests. Correlation was analysed by using Spearman test.

5.4 RESULTS

Clinical characteristics and medications used in the treatment protocol are summarized in Table 5.2. Of the 55 BS patients, 13 were relapsed, 24 had minor activity, and 18 were inactive. Oral activity was observed in 50.9%, followed by rheumatologic involvement (38.2%), skin involvement (25.5%), genital ulceration (18.2%), ocular lesions (10.9%), and CNS involvement (3.6%). Colchicine was used in the treatment of 43.6% of BS, followed by azathioprine (36.4%), and systemic steroids (34.5%). Topical oral steroid treatment was used in 43.6% of BS.

Table 5.2: Clinical characteristics of BS patients.

VARIABLES	**VALUE**
Clinical characterisations	**Case number (%)**
Relapsed (activity in more than two of BS symptoms)	13 (23.6)
Minor activity (activity in less than three of BS symptoms)	24 (42.6)
Non-relapsed (relapsed group + minor activity group)	42 (76.4)
Inactive (absence of any activity of BS symptoms)	18 (32.7)
Oral ulcers	28 (50.9)
Genital ulcers	10 (18.2)
Skin lesions	14 (25.5)
Ocular lesions	6 (10.9)
Rheumatologic involvement	21 (38.2)
Vasculitis	0 (0)
CNS involvements	2 (3.6)
Medications used	**Case number (%)**
Systemic corticosteroid	19 (34.5)
Topical corticosteroid	24 (43.6)
Colchicine	24 (43.6)
Azathioprine	20 (36.4)

5.4.1 TLR2 AND TLR4 GENE TRANSCRIPTION IN THE ORAL MUCOSA

The relative expression of TLR2 and TLR4 was measured by qPCR. 18S ribosomal ribonucleic acid (rRNA) was used as a housekeeping gene for the relative expression analysis. Data from BS patients' samples were compared to data from RAS patients, and HC volunteers. The BS patient cohort was further subdivided into relapsed, minor disease activity, and inactive status for further analysis. A sub-analysis was performed to investigate the difference in gene transcription in relation to the different system (oral, ocular, cutaneous, rheumatological, vascular, etc.) activity of BS patients.

5.4.1.1 THE RELATIVE EXPRESSION OF TLR2 AND TLR4 mRNA IN GENERAL

There was a significant increase in the expression of TLR2 mRNA in relapsed BS patients compared to HC volunteers ($p=0.0129$). Similarly, relapsed compared with inactive BS

patients (p=0.0260), relapsed compared with minor activity BS patients (p=0.0396), and relapsed compared with non-relapsed BS patients (p=0.0155). Likewise, there was increase in the expression of TLR2 in relapsed BS patients compared to RAS patients (p=0.0214). However, when HC volunteers were compared with the full cohort of BS patients (relapsed and non-relapsed) there was no significant difference (p=0.3107). RAS patients expressed higher levels of TLR2 in comparison to HC volunteers; however, the difference was not statistically significant (Figure 5.3).

The expression of TLR4 (Figure 5.3) showed a similar pattern with a significant increase in expression of relapsed BS patients versus HC volunteers (p=0.0019), relapsed compared to inactive BS patients (p=0.0041), relapsed compared with minor activity BS patients (p=0.0256), relapsed compared with non-relapsed BS patients (p=0.0043), and relapsed BS patients compared to RAS patients (p=0.0024). There was no significant difference in the expression of the HC volunteers and the whole of the BS patients cohort for TLR4 (p=0.3107). RAS patients also expressed higher levels of TLR4 in comparison to HC volunteers; however, this was not statistically significant. The level of expression of TLR4 appeared higher than TLR2 but again was not significant (Figure 5.3).

Examining the ocular activity showed that ocular active BS patients expressed statistically higher levels of TLR2 and TLR4 in their buccal mucosa brush biopsies than ocular inactive BS patients (p=0.0389 and p=0.0497 respectively). However the number of the ocular active patients was very small (n=4). Furthermore, orally active BS patients expressed higher levels of TLR2 and TLR4 in their buccal mucosa brush biopsies in comparison to orally inactive BS patients but the differences were not statistically significant. The other

systemic activities (skin, genital, rheumatologic...etc) and treatment protocol did not significantly affect the expression of TLR2 and TLR4 mRNA (Data not shown).

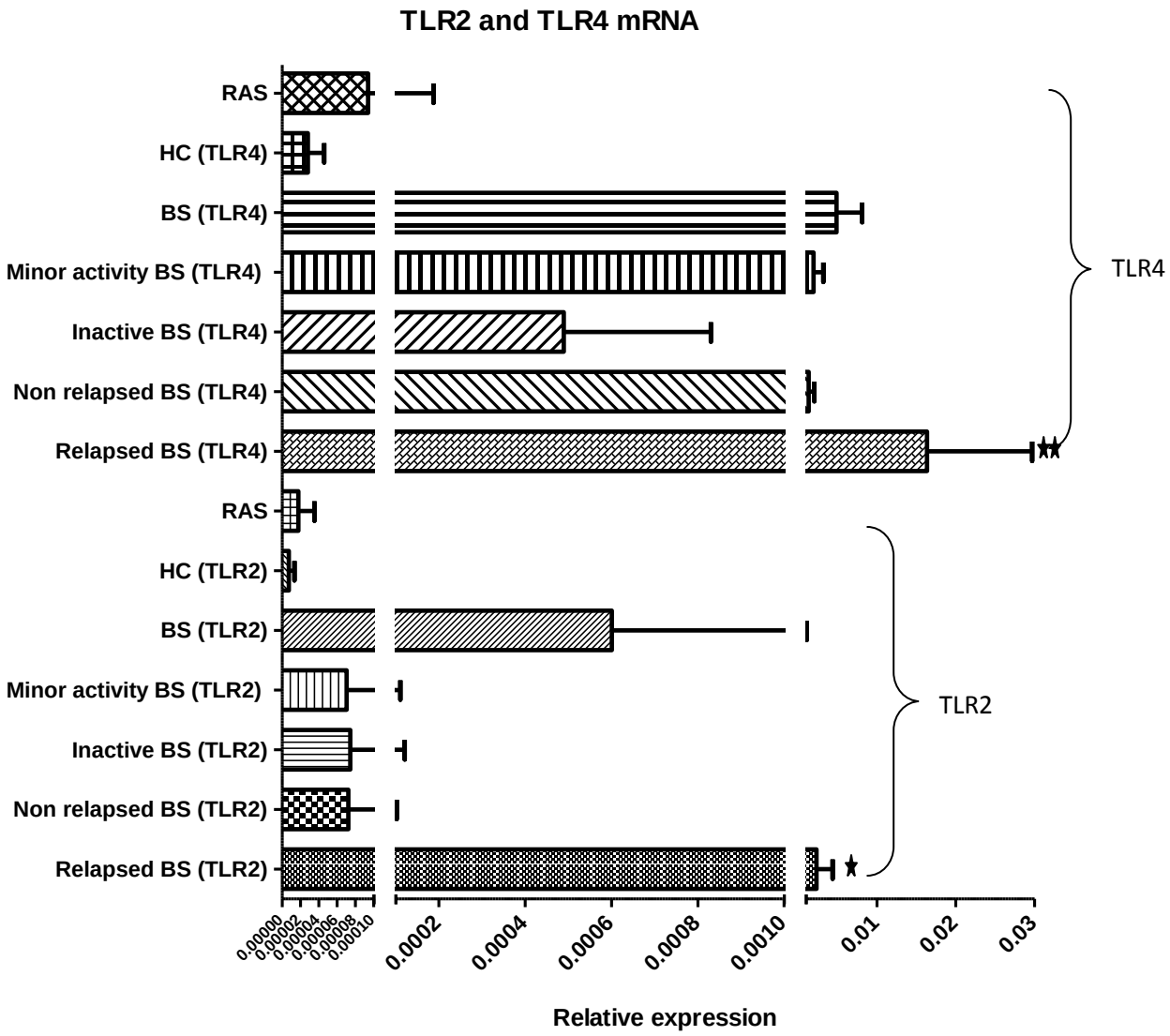
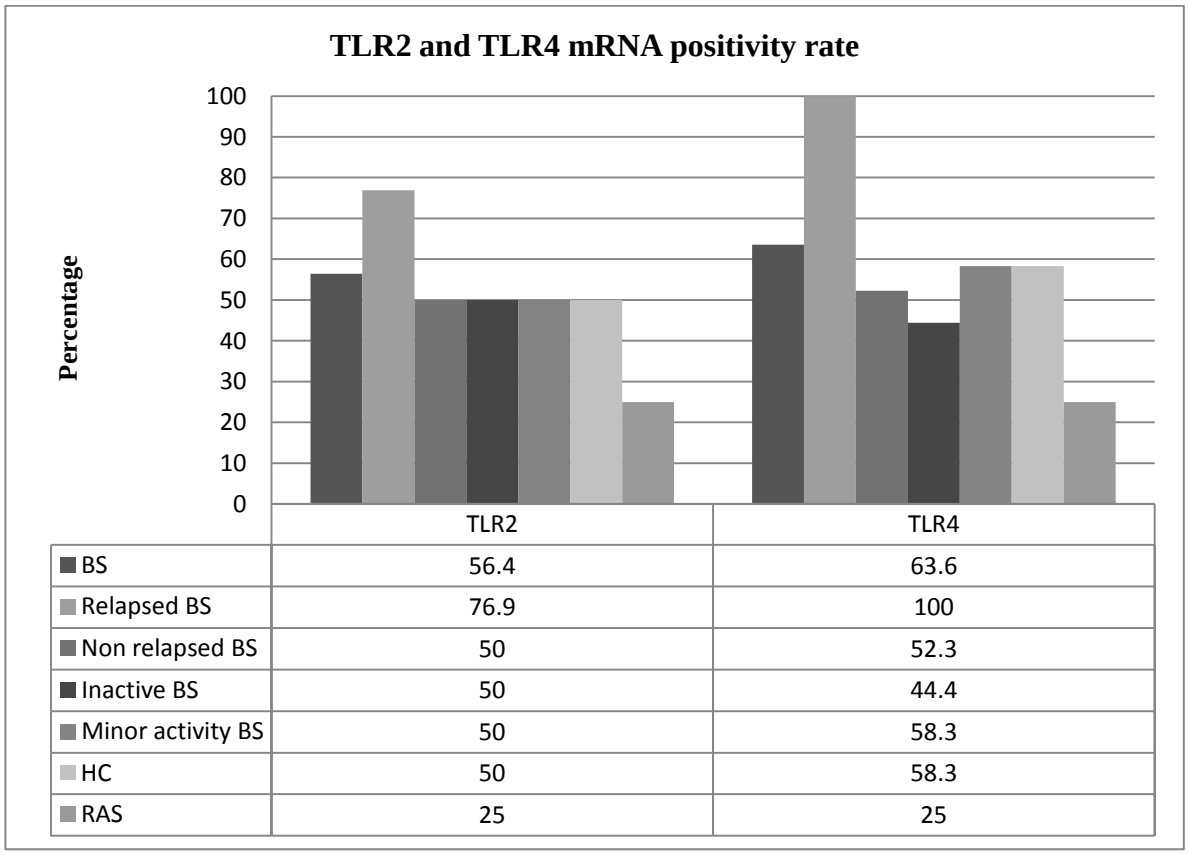


Figure 5.3: The relative expression of TLR2 and TLR4 mRNA (BS: n=55, relapsed BS: n=13, non-relapsed BS: n=42, inactive BS: n=18, minor activity BS: n=24, HC: n=24, and RAS: n=8).

The rate of positivity was high for relapsed BS patients compared with all the other groups [TLR2: 76.9% (10/13) and TLR4: 100% (13/13)]. There were no statistical significant differences in positivity rate between HC volunteers, non-relapsed BS patients, minor activity BS patients, inactive BS patients and RAS patients (Figure 5.4).



	TLR2	TLR4
BS	56.4	63.6
Relapsed BS	76.9	100
Non relapsed BS	50	52.3
Inactive BS	50	44.4
Minor activity BS	50	58.3
HC	50	58.3
RAS	25	25

Figure 5.4: TLR2 and TLR4 mRNA positivity rate in the non-lesional oral mucosa (BS: n=55, relapsed BS: n=13, non-relapsed BS: n=42, inactive BS: n=18, minor activity BS: n=24, HC=24, RAS=8).

5.4.1.2 THE RELATIVE EXPRESSION OF TLR2 AND TLR4 mRNA SPLICE VARIANTS

TLR2a and TLR2c expression was broadly similar in all groups. The expression of TLR2b, TLR2d, and TLR2e were higher in relapsed BS patients compared to HC volunteers ($p=0.0321$, $p=0.0272$ and $p=0.0134$ respectively). However, there were no statistical significant difference in the expression levels of TLR2b, TLR2d, and TLR2e between relapsed and non-relapsed BS patients ($p=0.1985$, $p=0.1213$ and $p=0.4887$ respectively) (Figure 5.5).

The relative expression of TLR4 variant 1, 3 and 4 mRNA was measured in the same cohorts. All investigated groups had broadly similar levels of TLR4.1 expression. Relapsed BS patients expressed higher levels of TLR4.3 compared to non-relapsed BS patients, and HC volunteers ($p=0.0127$, and $p=0.0002$ respectively). Likewise, relapsed BS patients expressed higher levels of TLR4.4 compared to non-relapsed BS patients, and HC volunteers ($p=0.0373$, and $p=0.0127$ respectively) (Figure 5.5).

The rate of positivity was high for TLR2 variant a, b, d, and e. However, TLR2 variant c was low in all the investigated groups (Figure 5.6). Expression of TLR4 splice variants 3 and 4 was infrequent in HC volunteers (1/12: 8.3%) and non-relapsed BS patients (3/12: 25% and 4/12: 33.3% respectively) compared to relapsed BS patients (9/10: 90%) (Figure 5.7).

Descriptive statistical analysis including mean, median, SD, Sem, and percentages of TLR2 and TLR4 mRNA and their splice variants is presented in table 5.3 and 5.4.

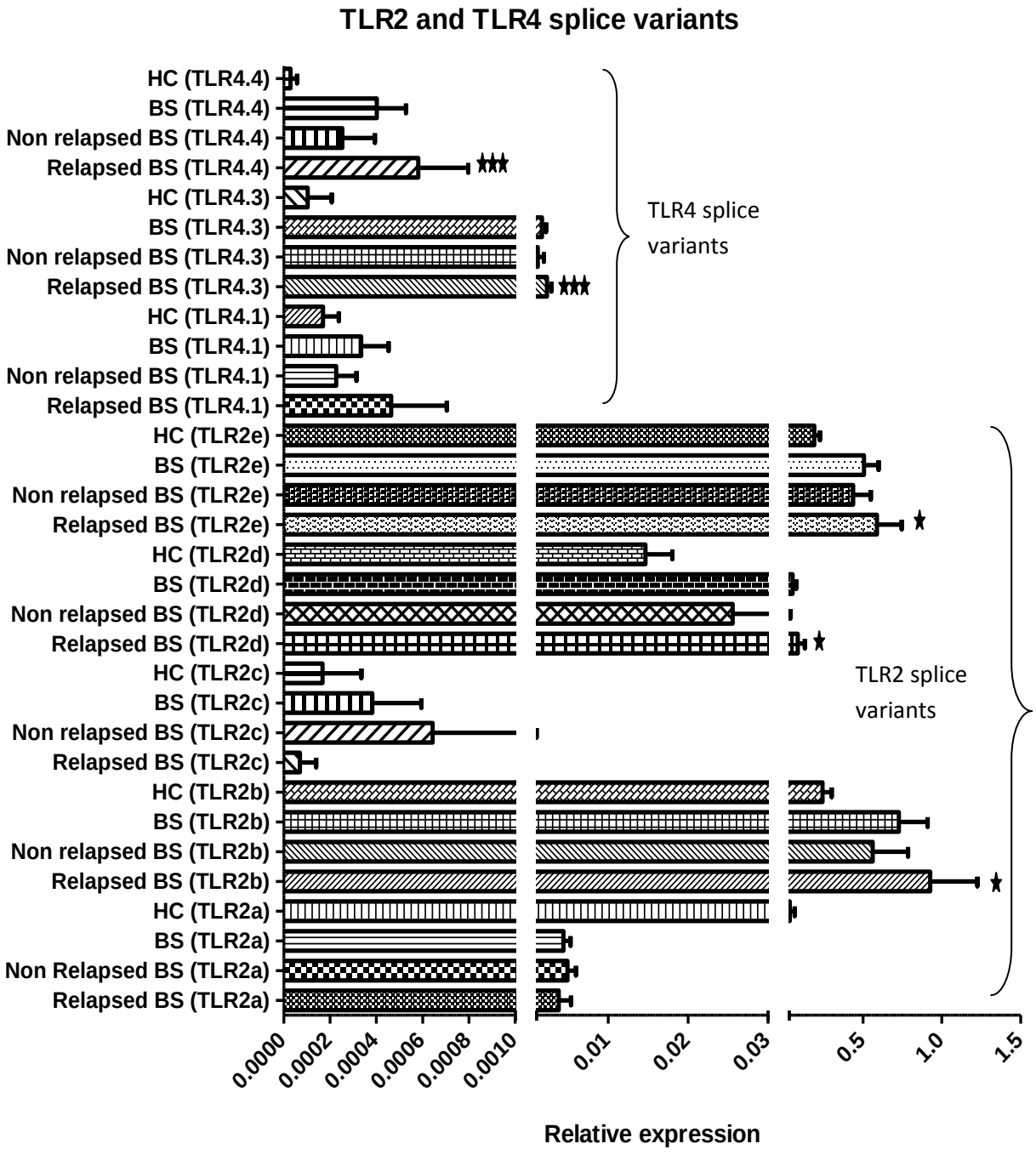


Figure 5.5: The relative expression of TLR2 splice variants a-e and TLR4 splice variants 1, 3 and 4 (BS: n=22, relapsed BS: n=10, non-relapsed BS: n=12, and HC: n=12).

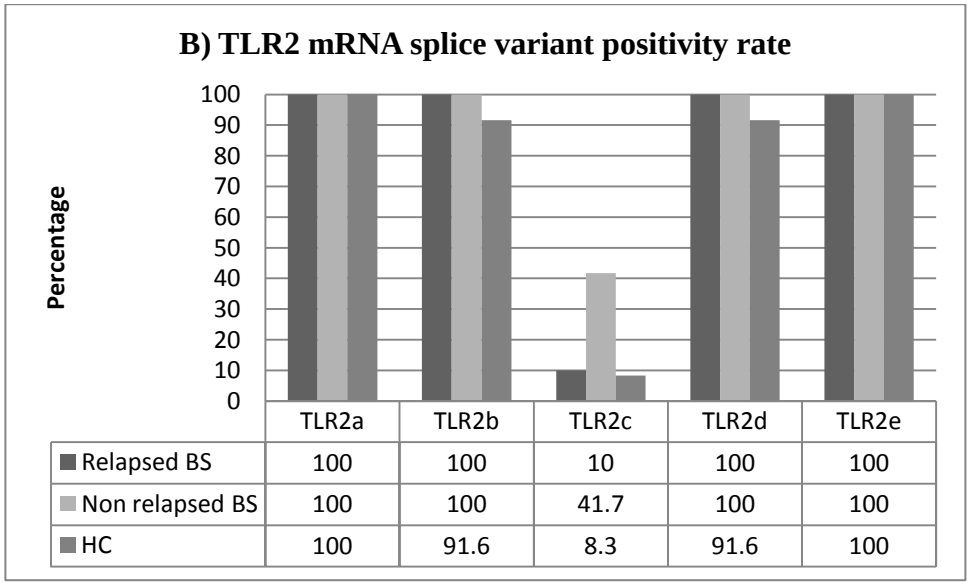


Figure 5.6: TLR2 mRNA splice variants positivity rate (BS: n=22, relapsed BS: n=10, non-relapsed BS: n=12, HC: n=12).

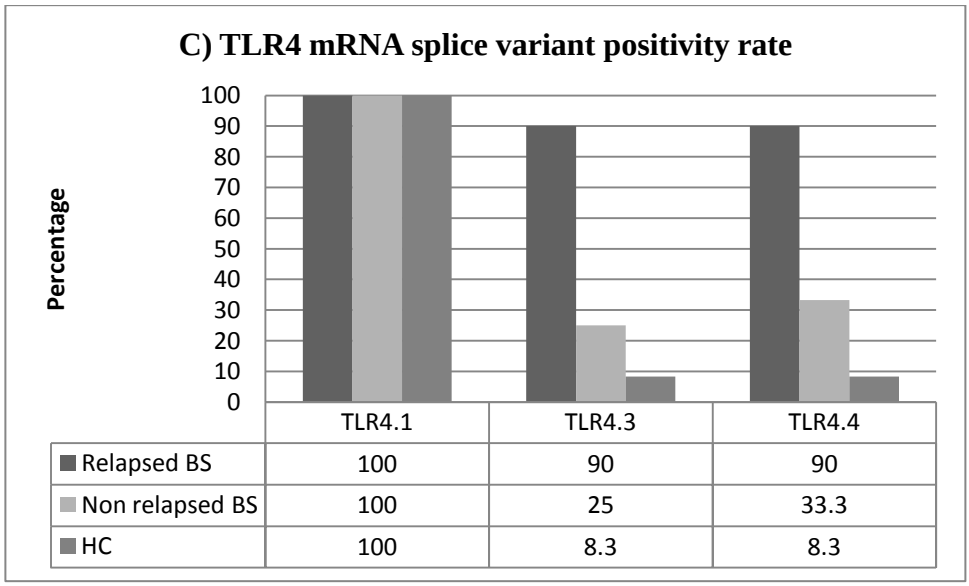


Figure 5.7: TLR4 mRNA splice variants positivity rate (BS: n=22, relapsed BS: n=10, non-relapsed BS: n=12, HC: n=12).

Table 5.3: mRNA quantification of TLR2 and their splice variants.

	GROUP	MEDIAN	MINIMUM	MAXIMUM	MEAN	SD	Sem	%
TLR2 mRNA	Relapsed BS (n=13)	3.380e-006	0	0.02693	0.002307	0.007409	0.0020055	76.9% (10/13)
	Non-relapsed BS (n=42)	3.047e-008	0	0.0009257	7.211e-005	0.0001943	2.997e-005	50% (21/42)
	RAS (n=8)	0	0	0.00014	1.769e-005	4.942e-005	1.747e-005	25% (2/8)
	HC (n=24)	1.130e-007	0	0.0001470	7.405e-006	2.986e-005	6.095e-006	50% (12/24)
TLR2a mRNA	Relapsed BS (n=10)	0.0020	0.000201	0.0158	0.003802	0.004854	0.001535	100% (10/10)
	Non-relapsed BS (n=12)	0.003853	0.000608	0.01225	0.004880	0.003549	0.001053	100% (12/12)
	HC (n=12)	0.00177	0.00017	0.0158	0.004390	0.004168	0.0008887	100% (12/12)
TLR2b mRNA	Relapsed BS (n=10)	0.6357	0.02212	3.020	0.9266	0.9442	0.2986	100% (10/10)
	Non-relapsed BS (n=12)	0.3210	0.007765	2.868	0.5608	0.7785	0.2247	100% (12/12)
	HC (n=12)	0.2307	0	0.6077	0.2412	0.2061	0.05949	91.6% (11/12)
TLR2c mRNA	Relapsed BS (n=10)	0	0	0.0007	7.000e-005	0.0002214	7.000e-005	10% (1/10)
	Non-relapsed BS (n=12)	0	0	0.003875	0.0006435	0.001292	0.0003729	41.7% (5/12)
	HC (n=12)	0	0	0.003875	0.0003828	0.0009901	0.0002111	8.3% (1/12)
TLR2d mRNA	Relapsed BS (n=10)	0.03328	0.00314	0.4745	0.08407	0.1414	0.04473	100% (10/10)
	Non-relapsed BS (n=12)	0.01695	0.000788	0.1448	0.02560	0.03852	0.01112	100% (12/12)
	HC (n=12)	0.01164	0	0.0400	0.01466	0.01166	0.003366	91.6% (11/12)
TLR2e mRNA	Relapsed BS (n=10)	0.4945	0.1052	1.772	0.5873	0.4992	0.1579	100% (10/10)
	Non-relapsed BS (n=12)	0.3628	0.0186	1.473	0.4360	0.3925	0.1133	100% (12/12)
	HC (n=12)	0.1750	0.03675	0.4166	0.1892	0.1233	0.03558	100% (12/12)

BS: Behçet's syndrome; HC: healthy control; RAS: recurrent aphthous stomatitis; SD: standard deviation; Sem: Standard error of mean.

Table 5.4: mRNA quantification of TLR4 and their splice variants.

	GROUP	MEDIAN	MINIMUM	MAXIMUM	MEAN	SD	Sem	%
TLR4 mRNA	Relapsed BS (n=13)	0.0004994	9.100e-008	0.1743	0.01634	0.04820	0.01337	71.4% (13/13)
	Non-relapsed BS (n=42)	1.084e-007	0	0.02726	0.001322	0.004672	0.0003411	52.3% (22/42)
	RAS (n=8)	0	0	0.000749	9.365e-005	0.0002648	9.362e-005	25% (2/8)
	HC (n=24)	2.335e-007	0	0.0003347	2.801e-005	8.721e-005	1.780e-005	58.3% (14/24)
TLR4.1 mRNA	Relapsed BS (n=10)	0.0002295	1.700e-006	0.00254	0.0004641	0.0007582	0.0002398	100% (10/10)
	Non-relapsed BS (n=12)	7.730e-005	3.170e-006	0.00102	0.0002262	0.0003056	8.823e-005	100% (12/12)
	HC (n=12)	8.060e-005	1.700e-006	0.000751	0.0001697	0.0002351	6.786e-005	100% (12/12)
TLR4.3 mRNA	Relapsed BS (n=10)	0.00207	0	0.004765	0.002324	0.001709	0.0005405	90% (9/10)
	Non-relapsed BS (n=12)	0	0	0.00917	0.001150	0.002753	0.0007948	25% (3/12)
	HC (n=12)	0	0	0.001245	0.0001038	0.0003594	0.0001038	8.3% (1/12)
TLR4.4 mRNA	Relapsed BS (n=10)	0.0003835	0	0.002175	0.0005810	0.0006826	0.0002158	90% (9/10)
	Non-relapsed BS (n=12)	0	0	0.001454	0.0002531	0.0004855	0.0001402	33.3% (4/12)
	HC (n=12)	0	0	0.0003475	2.896e-005	0.0001003	2.896e-005	8.3% (1/12)

BS: Behçet's syndrome; HC: healthy control; RAS: recurrent aphthous stomatitis; SD: standard deviation; Sem: Standard error of mean.

5.4.1.3 TLR2 AND TLR4 mRNA CORRELATION STUDY

There was positive correlation between TLR2b and TLR2e in all the investigated groups (relapsed BS patients: p=0.0001 and r=0.9758, non-relapsed BS patients: p=0.0139 and r=0.6853, HC volunteers: p=0.0004 and r=0.8531). Additionally, TLR2b positively correlated with TLR2d and TLR2e (p=0.0220 and r=0.6503 & p=0.0016 and r=0.8042 respectively) in non-relapsed BS patients (Figure 5.8).

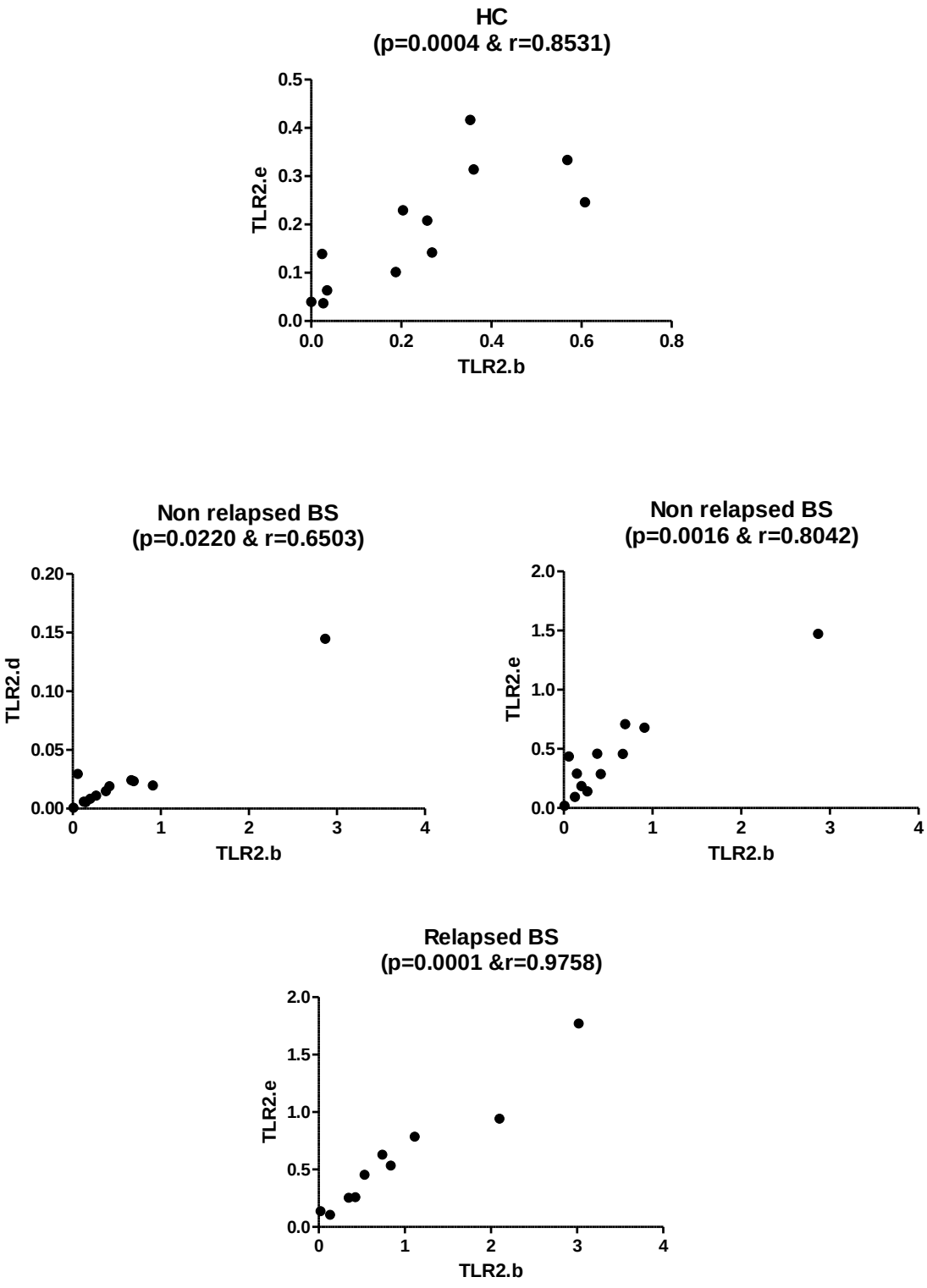


Figure 5.8: TLR2 correlation study (BS: n=22, relapsed BS: n=10, non-relapsed BS: n=12, HC: n=12).

There was positive correlation between the total TLR4 and TLR4.1 in all the investigated groups (relapsed BS patients: p=0.0330 and r=0.6727, non-relapsed BS patients: p=0.0008 and r=0.8322, HC volunteers: p=0.0004 and r=0.8531). Additionally, TLR4.4 positively correlated with total TLR4 (p=0.0289 and r=0.6848) and TLR4.1 (p=0.0330 and r=0.6727) (Figure 5.9).

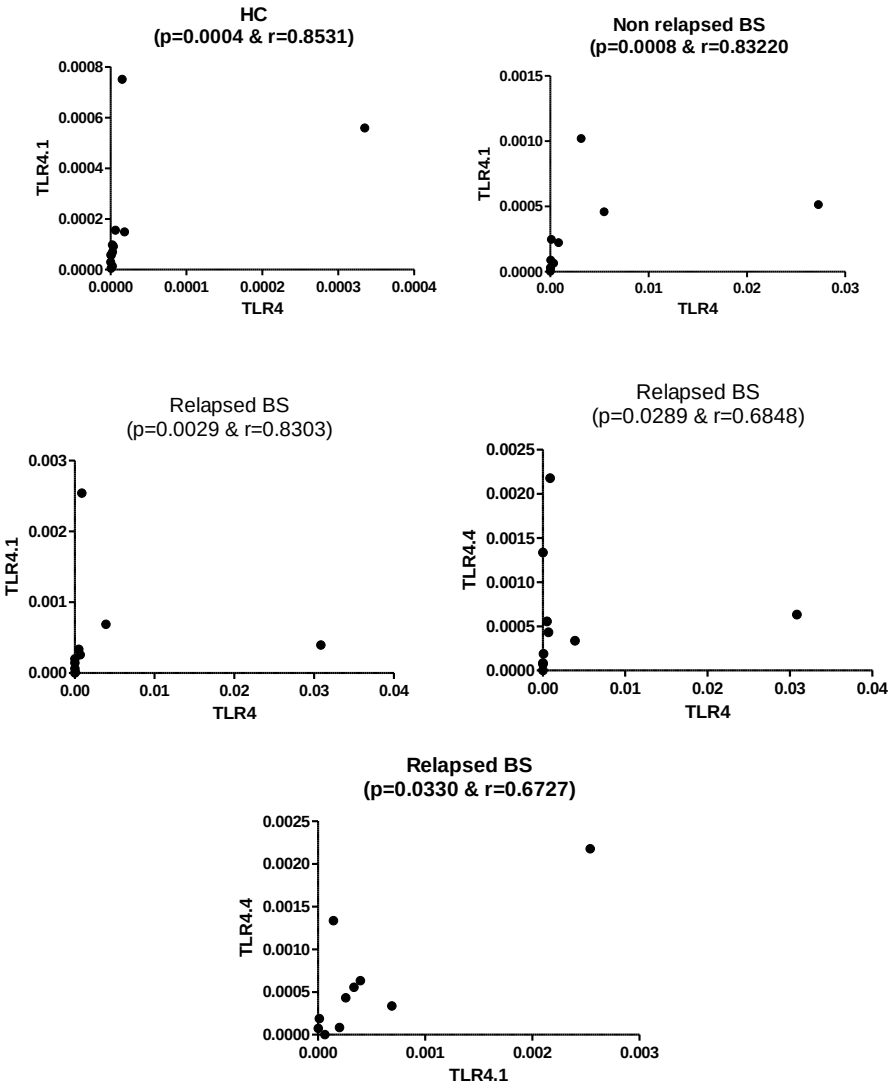


Figure 5.9: TLR4 correlation study (BS: n=22, relapsed BS: n=10, non-relapsed BS: n=12, HC: n=12).

5.4.2 TLR2 AND TLR4 GENE TRANSLATION IN THE ORAL MUCOSA

Total protein was purified from oral mucosal brush biopsies of 42 subjects (14 relapsed BS patients, 14 non-relapsed BS patients, and 14 HC volunteers) for the purpose of TLR2 and TLR4 detection by using immunoassays.

5.4.2.1 TLR2 AND TLR4 TOTAL PROTEIN

Slot-blot analysis of lysates of oral mucosal brush biopsies was carried out for TLR2 and TLR4 in 42 samples from 14 relapsed BS patients, 14 non-relapsed BS patients, and 14 HC volunteers. There was no statistically significant difference in the expression of the TLR2 protein between relapsed BS patients, non-relapsed BS patients and HC volunteers (Kruskal-Wallis, p=0.2937). There was also no statistically significant difference in the expression of the same protein between the whole BS patient cohort, including relapsed and non-relapsed presentation, and HC volunteers (Mann-Whitney, p=0.3158) (Table 5.5, and Figures 5.10-5.11).

Table 5.5: Slot-blot for TLR2 protein in oral mucosal brush biopsies.

	NON-RELAPSED BS PATIENTS	**RELAPSED BS PATIENTS**	**BS PATIENT COHORT**	**HC VOLUNTEERS**
n	14	14	28	14
Median	1.030	1.020	1.025	1.150
Min	1.000	1.000	1.000	1.000
Max	4.200	1.200	4.200	4.400
Mean	1.647	1.054	1.350	1.442
SD	0.9876	0.07239	0.7506	0.9081
Sem	0.2639	0.01935	0.1419	0.2427
BS: Behcet's syndrome; HC: healthy control; SD: standard deviation; Sem: standard error of mean; Max: maximum; Min: minimum; n: number.				

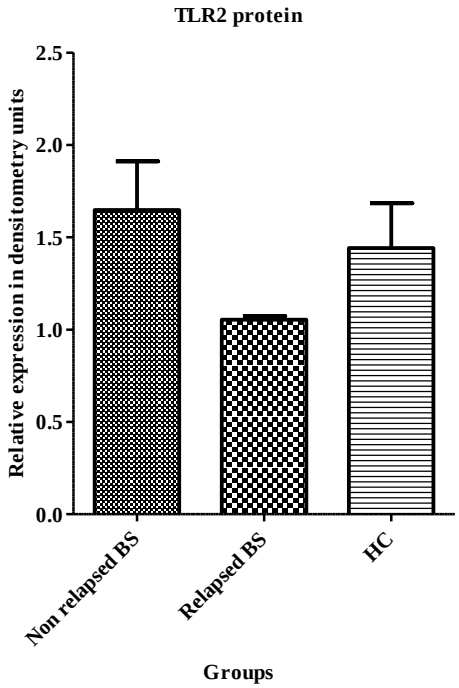


Figure 5.10: TLR2 protein expression in lysate from oral mucosal brush biopsies using Slot-blot analysis. A comparative study between 14 non-relapsed BS patients, 14 relapsed BS patients, and 14 HC volunteers.

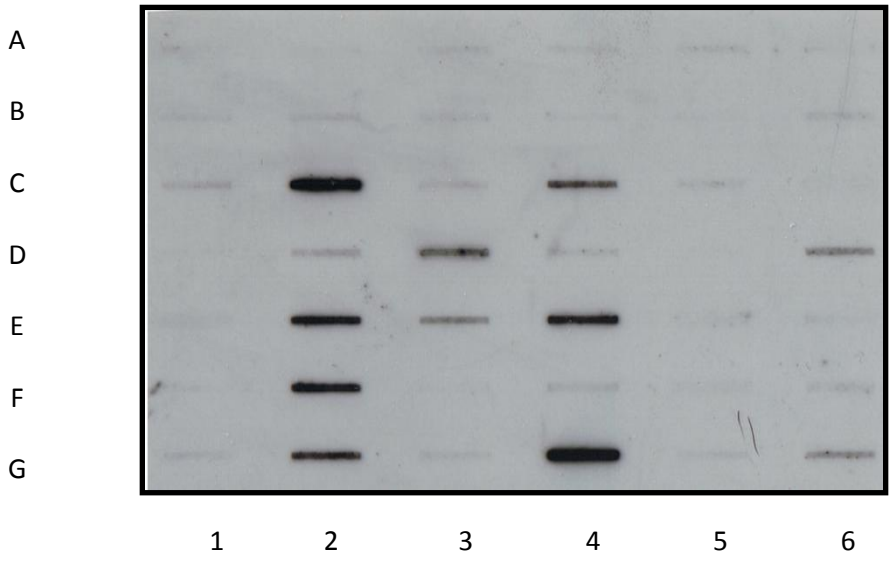


Figure 5.11: TLR2 Slot-blot for 42 protein samples from oral mucosal brush biopsies of 14 non-relapsed BS patients, followed by 14 HC volunteers and 14 relapsed BS patients.

The pattern observed for TLR2 protein was also observed for TLR4 protein. There was no statistically significant difference between relapsed BS patients, non-relapsed BS patients, and HC volunteers (Kruskal-Wallis, p=0.4673). There was also no statistically significant difference in the expression of the same protein between the whole BS patient cohort and HC volunteers (Mann-Whitney, p=0.4329) (Table 5.6 and Figures 5.12–5.13).

Table 5.6: Slot-blot for TLR4 protein in oral mucosal brush biopsies.

	NON-RELAPSED BS PATIENTS	**RELAPSED BS PATIENTS**	**BS PATIENT COHORT**	**HC VOLUNTEERS**
n	14	14	28	14
Median	1.000	1.040	1.005	1.000
Min	1.000	1.000	1.000	1.000
Max	1.400	1.800	1.800	1.900
Mean	1.077	1.156	1.116	1.153
SD	0.1322	0.2310	0.1890	0.3069
Sem	0.03534	0.06174	0.08202	0.08202
BS: Behcet's syndrome; HC: healthy control; SD: standard deviation; Sem: standard error of mean; Max: maximum; Min: minimum; n: number.				

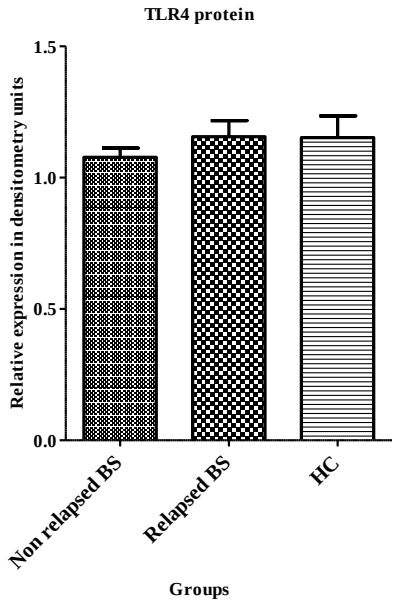


Figure 5.12: TLR4 protein expression in lysate from oral mucosal brush biopsies using Slot-blot analysis.

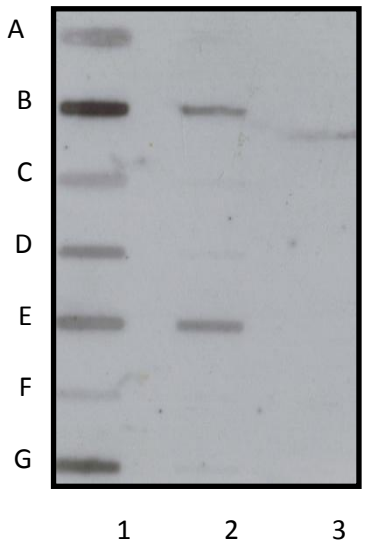


Figure 5.13: TLR4 Slot-blot for 7 protein samples from oral mucosal brush biopsies.

A1, B1, C1, D1, E1, F1 and G1: 7 different samples from BS patients.

A2, B2, C2, D2, E2, F2 and G2: 1:5 dilutions of the same samples.

A3, B3, C3, D3, E3, F3 and G3: negative control samples.

5.4.2.2 TLR2 AND TLR4 PROTEIN ISOFORMS

Western-blot analysis of protein lysates from oral mucosal brush biopsies was carried out for TLR4 in 20 BS patients (8 relapsed BS patients and 12 non-relapsed BS patients) and 10 HC volunteers. TLR4 showed heterogeneous bands with 90% of HC volunteers showing a band at ~90kDa. The 70kDa band was also strong in 90% of samples from HC volunteers, while the 50kDa band appeared in 50% of relapsed BS patients' samples. Additionally, 75% of the relapsed BS patients' samples had a band at about 35kDa and 12.5% had a band on 25kDa. There was a significantly higher expression of 70kDa band in the HC volunteers compared to BS patients (relapsed and non-relapsed) (Chi-square, p=0.0107), while band 35kDa was significantly highly expressed in relapsed BS patients (Chi-square, p=0.0097) (Table 5.7 and Figure 5.14).

The TLR2 protein expression also appeared to be somewhat heterogeneous despite a constant amount of protein loading in all cases of 1 µg/ml. Three main bands were observed for TLR2 at about 90 kDa, 50 kDa and 30 kDa (Figure 5.14).

Table 5.7: TLR4 Western-blot.

TLR4 WESTERN-BLOT BAND SIZE	**RELAPSED BS (n=8)**	**NON-RELAPSED BS (n=12)**	**HC VOLUNTEERS (n=10)**
≈90 kDa	87.5% (7/8)	83% (10/12)	90% (9/10)
≈70 kDa	75% (6/8)	50% (6/12)	90% (9/10)
≈50 kDa	50% (4/8)	33% (4/12)	40% (4/10)
≈35 kDa	75% (6/8)	50% (6/12)	40% (4/10)
≈25 kDa	12.5% (1/8)	8.3% (1/12)	30% (3/10)
BS: Behcet's syndrome; n: number; HC: healthy control.			

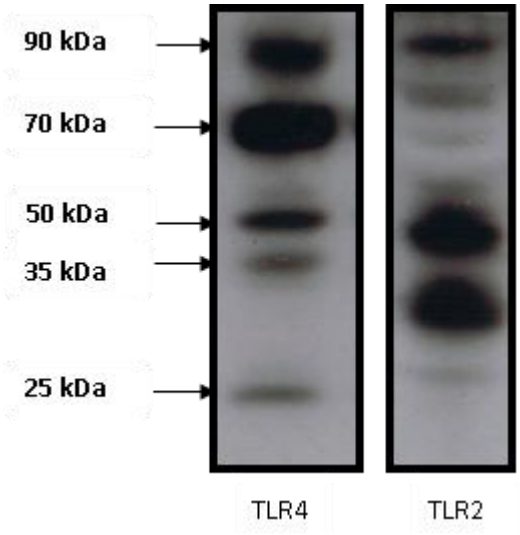


Figure 5.14: Western-blot for TLR4 and TLR2 protein. TLR4 Western-blot demonstrated 5 different bands at 90, 70, 50, 35 and 25 kDa, while TLR2 Western-blot showed 3 main bands at 90, 50 and 30 kDa.

5.4.3 TLR2 AND TLR4 FUNCTION IS DAMPENED IN BS PATIENTS

A total of 46 blood samples from 10 relapsed BS patients, 21 non-relapsed BS patients, and 15 healthy individuals were analysed. PBMC were treated with the different cognate agonist for TLR1/2 heterodimer, TLR2, TLR4, and TLR2/6 heterodimer. The level of spontaneous secretion of TNF-α was quantified along with its post treatment levels (R&D Systems, UK). The spontaneous tumour necrosis factor alpha (TNF-α) secretion was elevated in relapsed BS patients PBMC supernatant; however, this difference was not statistically significant (Figure 5.15). All three groups responded equally to phytohaemagglutinin (PHA) treatment. After the treatment with the different TLRs' cognate agonists, PBMC from the whole BS patient cohort (relapsed and non-relapsed) failed to respond to TLR4 agonist efficiently compared to healthy individuals (Mann-Whitney, p= 0.0093). Also, there was observed decrease in the response to TLR1/2 agonist by BS PBMC (Mann-Whitney, P=0.0500) (Figure 5.16).

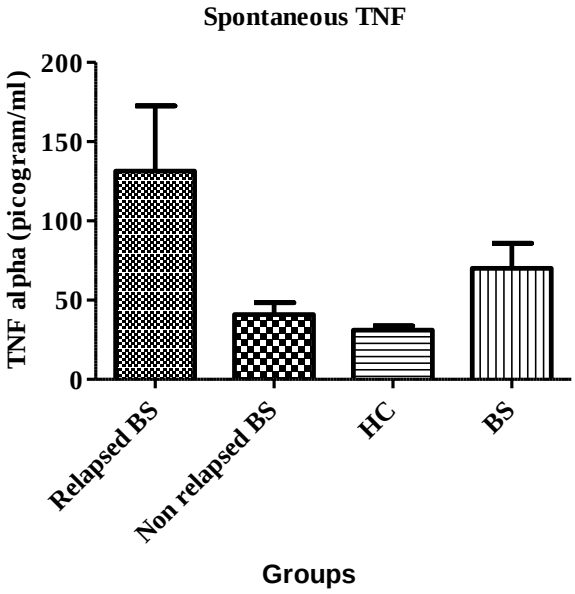


Figure 5.15: The spontaneous TNF-α secretion in relapsed BS patients PBMC supernatant, non-relapsed BS patients, and HC volunteers.

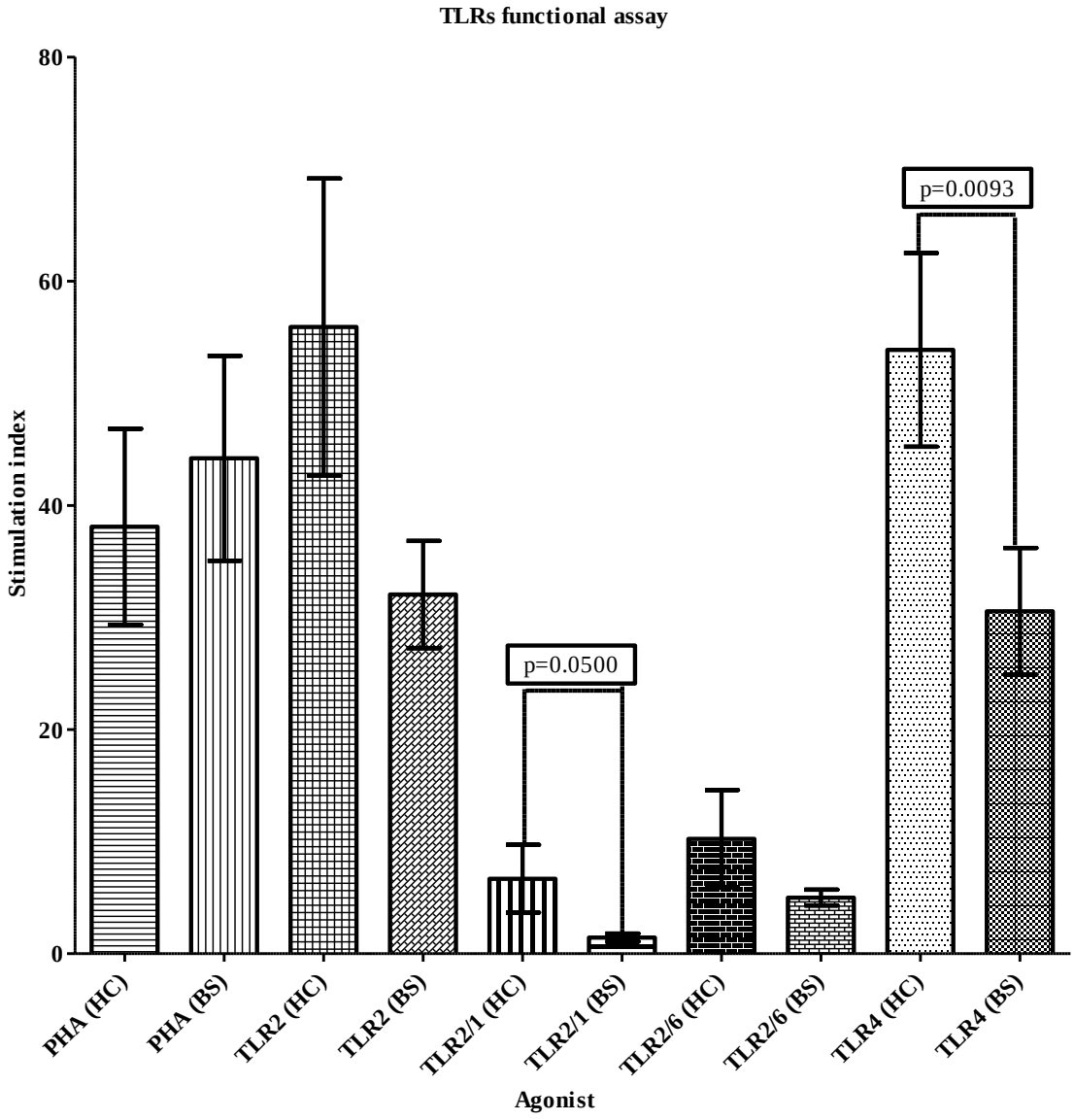


Figure 5.16: TNF-α secretion by BS patients and HC volunteers PBMC upon stimulation with PHA and TLRs agonists.

5.5 DISCUSSION AND CONCLUSION

Enhanced inflammatory reaction is a recognized characteristic pathological feature of BS with well-recorded high serum pro-inflammatory cytokine levels such as TNFα and interleukin 6 (IL6) (Evereklioglu et al., 2002). These cytokines can be triggered by the TLRs' cascade that leads to the translocation of NF-κB and transcription of cytokine-encoding genes after binding to a challenging antigen, either endogenous or exogenous (Gay and Gangloff, 2007). Furthermore, oral bacterial and viral aetiology was suggested in the literature for BS (Hamza et al., 1990, Lehner et al., 1991, Sun et al., 1996, Tojo et al., 2003b). Interestingly, TLR2 and TLR4 jointly are capable of sensing most of the oral microbial commensals and pathogens. Therefore, the aim of this part of the thesis was to study the differential expression of TLR2, TLR4, and their splice variants in relation to BS. The author investigated the oral mucosa in particular because it is deemed to be an important site of the disease activity for BS, based on the fact that most BS patients experience recurrent episodes of oral ulceration and they are considered to be the initial sign of the disease in more than 80% of patients (Davatchi et al., 2010b).

The results of this part of the thesis indicate that TLR2 and TLR4 mRNA expression increases in the oral mucosa of BS patients during the relapsed phase, highlighting the importance of these markers in monitoring the disease activity. This is consistent with another study that showed elevated expression of TLR2 and TLR4 in the PBMCs of active BS patients (Do et al., 2008).

Given the higher levels of TLR2 and TLR4 in relapsed disease, one of their ligands may play an important role in the pathogenesis of BS. However, it is also possible that multiple antigens contribute to the activity of the disease, with some of them acting as shared ligand for both TLR2 and TLR4.

This part of the thesis is the first study to investigate the relative expression of the different mRNA splice variants of TLR2 and TLR4, and it points towards possible innate immune abnormalities in BS patients. Interestingly, TLR2 mRNA variant b, which is known to be able to transcribe to the full-length protein, was high in relapsed BS patients' oral mucosa. Furthermore, TLR2 mRNA variant d, one of the unusual splice variant possibly affecting the stability of the mRNA, was also predominantly elevated in relapsed BS patients' oral mucosa. The elevated TLR2 mRNA variant e in relapsed BS patients' oral mucosa was of great importance as this fact highlights the high monocyte infiltration in their non-lesional oral mucosa.

The upregulation of the unusual mRNA splice variants of TLR4 may lead to incomplete protein translation. TLR4 mRNA splice variant 3 and 4 were significantly elevated in the whole BS patient cohort. TLR4 variant 3 has extra exon (compared to splice variant 1), which results in a frameshift and an early stop codon; the translated protein from this splice variant is expected to be significantly truncated and a candidate for nonsense decay. Interestingly, TLR4 mRNA splice variant 4 lacks an exon (compared to variant 1), resulting in a frameshift and an early stop codon; the translated protein of this splice variant is also expected to be significantly truncated and a candidate for nonsense decay.

Interestingly, the correlation study pointed toward a positive correlation between TLR2b (known to translate to full length protein) and TLR2e (known to be expressed by fresh monocytes) in all investigated groups. It is documented in the literature that TLR activation can induce chemokine production and leukocyte recruitment (Parker et al., 2004). However contradictory observations were also documented pointing towards inhibition of leukocyte chemotaxis following TLR activations by a high concentrations of their agonists (Yi et al., 2012).

The dampened response of BS PBMC TLR4 upon stimulation with its agonist is one of the novelties of this part of the thesis and could explain the higher level of TLR4 variant 3 and 4 in relapsed BS patients. In addition, the decrease in the BS PBMC response upon treatment with TLR1/2 agonist might highlight a defect in the heterodimer formation between these two receptors. The results are consistent with the available literature indicating the decrease in TLR2 expression in PBMCs upon treatment with LPS and HSP (Yavuz et al., 2008).

Examination of the slot-blot data suggests that protein expression of TLR2 and TLR4 is not raised during relapsed BS compared to the non-relapsed phase. This appears to highlight a defect in the translation of the mRNA that was expected in view of the elevated level of expression of the unusual mRNA splice variants of TLR2 and TLR4. However, it is not unprecedented for protein and mRNA levels of the same gene/protein to show a poor correlation in the same tissue (Dixon et al., 2004).

In the Western-blot analysis for TLR2 and TLR4, the author consistently observed multiple bands despite the use of protease inhibitor cocktail tablets (Roche Applied Science, UK) to remove the potential for protein degradation. Koch et al. (2007) also demonstrated by Western-blot the presence of 5 protein bands for TLR4 around the sizes 130, 110, 72, 50, 26 kDa. His interpretation was that the band at 130 kDa represents a glycosylated TLR4 isoform, expressed at the cell surface, and the band at 110 kDa represents a partially glycosylated form, which is unable to translocate to the surface. However, the bands at lower molecular weights may represent translation products of the TLR4 mRNA splice variants that contain an early stop codon (Koch et al., 2007, Ohnishi et al., 2003). These mRNA splice variants should be candidates for the non-sense decay mechanism, preventing them from being translated into truncated

non-functional protein. Therefore, we believe that the abundance of band 35kDa of TLR4 in relapsed BS patients may indicate either a defect in the non-sense decay mechanism or enhanced proteolysis in this cohort.

We conclude that there is a shift towards expressing some of the unusual mRNA splice variants of TLR2 and TLR4 by BS patients' oral mucosa, which explains the observed defect in TLR4 and TLR1/2 heterodimer function and indicates abnormalities in the innate immune response to oral microbes in this syndrome. However, the exact protein product of the TLR2 and TLR4 mRNA splice variants is not completely understood, which in turn invites further research to investigate the function of these splices in health and disease

CHAPTER 6

THE ORAL MICROBIOME OF BEHÇET'S SYNDROME PATIENTS

6.1 ABSTRACT

6.1.1 BACKGROUND

Behçet's syndrome (BS) is one of the devastating vasculitides with unknown aetiology. Currently, there is a consensus that the complex interplay between the commensal microbial community and the abnormal immune response is a fundamental root cause triggering the complex clinical presentation of BS.

6.1.2 OBJECTIVES

This study investigates the oral microbiome in BS patients and compares it to recurrent aphthous stomatitis (RAS) patients and healthy control (HC) volunteers.

6.1.3 METHODS

Eighty-one saliva samples and oral swabs from BS patients and controls were cultured and bacteria were identified by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF). Molecular identification of cultured Mycobacterium was established. Salivary viral load and the serum immunoglobulin G (IgG) of the different herpes viruses were quantified from a further 89 saliva samples and matched serum samples.

6.1.4 RESULTS

Sixty different oral bacteria, five fungi and four mycobacteria were identified. Simultaneously, the presence of six different herpes viruses was inspected. The oral mucosal and salivary microbial profile was variable between individuals in the same group and between individuals in different groups. The orally active BS patients' oral mucosa showed the highest microbial community complexity and diversity compared to all the other investigated groups. Salivary shedding of Epstein-Barr virus (EBV) was statistically significantly higher in BS patients compared to HC volunteers, independent

of their oral disease activity (Mann-Whitney, p=0.0057). Furthermore, the cytomegalovirus (CMV) IgG expression was significantly deficient in BS patients' serum samples compared to RAS patients and HC volunteers (Kruskal-Wallis, p=0.0450).

6.1.5 CONCLUSION

The lower level of expression of CMV IgG could be caused by a defect in the cross-talk between innate and adaptive immune responses, given the previously mentioned defect in the toll-like receptor (TLR) 1/2 heterodimer function that act as an initial receptor to sense CMV. This in turn will lead to a status of increased susceptibility to CMV infection.

Despite the uncertainty about whether the reported differences in the microbiome of BS patients are of a causative or reactive nature, these differences might prove to be of great importance as restoring the balance of the microbial community is expected to promote rapid recovery from oral ulcerations and potential better control of the immunological response.

6.1.6 DECLARATION

The ethical approval application was performed by Professor Fortune. The evaluation and diagnosis of patients were undertaken in a consultant-led examination.

The author has performed all data analysis in this chapter. She also contributed to sample collections, sample processing, experimental assays, data collection, and data management. The research and clinical team of the Centre for Clinical and Diagnostic Oral Sciences contributed to sample collection, data collection and data management.

The Barts and the London NHS Medical Microbiology unit guided the author in the MALDI-TOF analysis. The National Mycobacterium Reference Laboratory kindly funded the mycobacterial analysis and guided the author throughout its stages. The Barts and The London NHS virology unit guided the author in the real time Polymerase Chain Reaction (qPCR) and immunoassays for the herpes viruses.

6.2 INTRODUCTION

Investigating the aetiology and pathogenesis of chronic diseases is challenging but it can yield crucial information to help create new treatment modalities. The role of the oral bacteria in the pathogenesis of many life threatening systemic diseases such as brain abscess, endocarditis and glomerulonephritis is well documented in the literature. Recently, the importance of oral and gut commensals in the pathogenesis of immune-related diseases has been highlighted in autoimmune encephalomyelitis, rheumatic arthritis, pancreatic diseases, and inflammatory bowel diseases (Docktor et al., 2012, Farrell et al., 2012, Liao et al., 2009, Mueller et al., 2009, Ochoa-Reparaz et al., 2009, Wolf and Curtis, 2008). Furthermore, oral tolerization and probiotic therapy were suggested as treatment modalities with their potential benefit in treating immune-related diseases and some oral ulcerative diseases (Stanford et al., 2004, Sun et al., 2009, Tasli et al., 2006).

Behçet's syndrome (BS) is a multi-systemic immune-related disease characterized by recurrent oral ulcers as the initial sign of the disease in more than 80% of the cases (Davatchi et al., 2010b). Because of this observation, it is thought that the oral environment may play a very important role in its aetiology and pathogenesis. To date, the consensus is that the disease is triggered by a profound inflammatory response to an

undefined environmental factor in a genetically susceptible host, but as yet the aetiology remains poorly understood (Lehner, 1999).

A bacterial aetiology was suggested in the literature a long time ago. The uncommon serovars of *Streptococcus sanguis* KTH1 and KTH3 were thought to be a potential aetiological factor in BS based on their frequent isolation from this group of patients. Furthermore, BS patients showed marked skin reactivation to these antigens and there were lymphoproliferative responses observed against KTH1 and KTH3 whole cell antigen. However, in subsequent studies there were no differences in the uncommon serovars KTH1 and KTH3 biochemical properties compared to *Streptococcus oralis* isolated from healthy control (HC) volunteers, therefore, it was reclassified as *Streptococcus oralis* (Lehner et al., 1991, Narikawa et al., 1995).

The cross reactivity of bacterial heat shock proteins (HSP) with their mammalian counterparts was also studied in relation to BS patients (Lehner et al., 1991). The serum antibody titre and delayed-type hypersensitivity against *Streptococcal* antigens were reported as significantly higher in BS patients than in those of the controls. Therefore, it was suggested that hypersensitivity to *Streptococcal* species may act as a trigger for the inflammatory response (Kaneko et al., 1997, Kaneko et al., 2008). Indeed, an oral tolerization with the peptide (p36-351) of HSP60 was investigated in a phase I/II clinical trial, with promising results (Stanford et al., 2004).

In a recent study, *Staphylococcus aureus*, *Moraxella*, and *Streptococcus* were found to colonize the conjunctiva of BS patients significantly more than in a HC group (Gunduz et al., 2008). Likewise, *Staphylococcus aureus* and *Prevotella* spp. were more commonly isolated from the skin lesions of BS patients (Hatemi et al., 2004).

Furthermore, treatment of eight BS patients suffering from active mucocutaneous symptoms with azithromycin for four weeks was reported to be effective in decreasing folliculitic lesions and speeding up the healing of oral ulcers (Mumcu et al., 2005). In another study, minocycline successfully reduced the frequency of clinical symptoms in BS patients and indeed reduced the in vitro production of the pro-inflammatory cytokines by their peripheral blood mononuclear cells (PBMCs) when stimulated with *Streptococcal* antigen (Kaneko et al., 1997). Moreover, probiotic treatment in the form of *Lactobacillus brevis* CD2 lozenges seemed to be beneficial in controlling the oral ulceration of BS patients in a pilot study (Tasli et al., 2006)

A viral aetiology was also postulated more than fifty years ago by Hulusi Behçet. Later, Denman et al. (1980) and then Eglin et al. (1982) showed by in situ DNA-DNA hybridization that at least part of the herpes simplex virus (HSV) 1 genome is transcribed in mononuclear cells of some patients with BS (Denman et al., 1980, Eglin et al., 1982, Gray, 1950). However, administration of high doses of acyclovir in association with plasma exchanges failed to produce positive treatment results and treatment with acyclovir alone failed to alleviate the frequency and severity of oro-genital ulceration or other BS features in a randomized, double-blind, placebo-controlled, crossover trial (Davies et al., 1988).

In other studies, the level of immunoglobulin (Ig) G antibody against HSV1 was found to be significantly increased in patients who had BS but there was no HSV1 DNA found in peripheral blood leukocytes and oral smears from the same cohort (Nomura et al., 1998). Also, there was no statistically significant increase in HSV1 DNA observed in the saliva of BS patients compared to HC volunteers (Lee et al., 1996).

In addition to HSV1, the human cytomegalovirus (CMV) has been studied in relation to BS. CMV DNA was detected in biopsy specimens from the oral mucosa of BS patients (Sun et al., 1996). In another study, the mean titre of IgG and IgA antibodies to CMV was significantly lower in BS patients than in HC volunteers. However, the number of subjects having IgM antibody against CMV was similar in BS patients and HC volunteers (Lee et al., 2005). Nevertheless, these studies have not been substantiated.

Epstein-Barr virus (EBV) was also suggested as a potential cause of BS. EBV DNA was isolated in the pre-ulcerative stage of oral ulceration of patients with BS and recurrent aphthous stomatitis (RAS) in a very small cohort (Sun et al., 1998).

To date, the oral microbial profile of BS patients has not been fully identified. This part of the thesis is attempting to fill that gap in the literature by investigating the oral microbiome of the BS patient cohort in the UK and comparing it to HC volunteers and RAS patients. We also investigated the oral microbial profile of BS during orally active and inactive phases of the disease, and in ulcer sites compared to non-ulcer sites in the same patients.

6.3 MATERIALS AND METHODS

6.3.1 PATIENT COHORTS AND SAMPLE COLLECTIONS

The International Study Group (ISG) criteria were employed to diagnose and stratify all BS patients. Informed consent was obtained with appropriate local ethical approval. BS patients were stratified according to the oral activity of the disease into orally active (presented with oral ulceration at the time of sample collection) and orally inactive disease status. A total of 89 subjects were invited to this part of the study [54 BS (M/F: 19/35 mean age: 44±11), 28 HC (M/F: 13/15, mean age: 34±10.5) and 7 RAS (M/F:

3/4, mean age: 49.7±8)]. Out of those, 14 BS patients and 3 RAS patients had oral ulcerations at the time of sampling. None of these investigated subjects showed any signs of oral infection, nor were they treated with any topical or systemic antimicrobials at the time of sampling or during the week before sampling.

Eighty-one samples (45 oral swabs (Copan, UK) from ulcerated and non-ulcerated oral mucosa and 36 unstimulated saliva samples) from 10 orally active BS patients, 10 orally inactive BS patients, 10 HC volunteers, and 6 RAS patients (50% orally active) from the above-mentioned cohorts were cultured and bacteria were identified by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF). Molecular identification of cultured Mycobacterium was established from another 81 samples from the same patient and volunteer cohorts (45 brush biopsies (Flowgen Bioscience, UK) from ulcerated and non-ulcerated oral mucosa and 36 unstimulated saliva samples).

Herpes viruses' nucleic acid was quantified using real time polymerase chain reaction (qPCR) with specific primers in 89 saliva samples, while the specific IgG response to the different herpes viruses was measured by quantitative immunoassays in matching serum samples from the same cohorts of patients and volunteers.

6.3.2 ORAL MICROBIAL ANALYSIS

Oral mucosal swabs and salivary samples were diluted in sterile saline at 10^{-2} and 10^{-4} and cultured on 7 different culture media: blood agar, chocolate agar, colistin nalidixic acid agar, MacConkey agar, gonococcus agar, Sabouraud agar and fastidious anaerobic agar. Blood agar, chocolate agar and gonococcus agar plates were incubated at 37 °C for 48 hours in a CO_2 enriched environment. Colistin nalidixic acid agar, MacConkey agar and Sabouraud agar plates were incubated at 37 °C for 48 hours in an O_2 enriched

environment. The fastidious anaerobic agar plates were incubated at 37 °C for 7 days in an anaerobic environment. Bacteria grown on blood agar plates were quantified in colony-forming units. Isolated colonies were re-cultured in the same culture environments to purify them before peptide isolation.

Peptides were purified by the acetonitrile precipitation method according to the manufacturer's instructions (Bruker Daltonics, Germany). MALDI-TOF analysis using the MALDI Biotyper was conducted in a 96 target plate by applying 1 µl of each peptide sample to a different target followed by 1 µl of the crystallised molecules in the MALDI matrix, to protect the peptides and help in the ionisation process by the laser beam (Bruker Daltonics, Germany).

MALDI-TOF applied nitrogen-pulsed laser ionisation to the peptide samples to ionise and separate them based on their mass/charge ratio. The resulting spectra formulated the protein finger print (2,000–20,000 Dalton range) which was then compared to a database of known spectra to identify and type the different bacteria being analysed. A match factor for each sample was automatically calculated by the MALDI Biotyper based on the number and amplitude of the peaks that matched. A score of 1.7 or above was considered a reliable identification of the investigated microorganism.

6.3.3 MYCOBACTERIAL ANALYSIS

For the purpose of mycobacterial analysis, all investigated brush biopsy samples were decontaminated using the 3% oxalic acid decontamination method while all saliva samples were treated with the 2% sodium hydroxide and N-acetyl-L-cysteine decontamination method.

All samples were cultured on Lowenstein-Jensen (LJ) slopes and mycobacterial growth indicator tubes (MGIT) following the manufacturer's instructions (Becton Dickinson Diagnostic Instrument Systems, USA). MGIT bottles were incubated on the MGIT instrument which reads each tube hourly and triggers an alarm when growth is detected (Becton Dickinson Diagnostic Instrument Systems, USA). All samples were allowed to be incubated for 6 weeks.

An isolated mycobacterium was identified by the PCR and DNA-DNA hybridization technique using the GenoType Mycobacterium CM kit following the manufacturer's instructions (Hain Lifescience, Germany).

6.3.4 q PCR FOR HERPES VIRUSES

6.3.4.1 NUCLEIC ACID PURIFICATION FROM THE SALIVA SAMPLES

To investigate HSV1, HSV2, EBV, CMV, varicella zoster virus (VZV), and human herpes virus (HHV) 8, nucleic acid in 300 µl of all the saliva samples was simultaneously purified, along with 6 water samples as negative control. All samples were processed by the BioRobot MDx workstation using a QIAamp DNA extraction kit following the manufacturer's protocol (Qiagen, UK). This method utilized the selective binding properties of a silica-based membrane. Purified DNA samples were then eluted in elusion buffer (AE) and were free from protein, nucleases and other contaminants or inhibitors. All samples were subsequently spiked with phocine herpes virus (PHV) DNA to act as internal control during qPCR test.

6.3.4.2 HSV1, HSV2 AND VZV qPCR

Multiplex real time amplification of HSV1, HSV2, and VZV DNA using glycoprotein B-specific primers was performed as described in chapter 2, section 2.2.3.4.

6.3.4.3 CMV qPCR

Real time amplification of CMV DNA using glycoprotein B-specific primers was performed according to the previously published method (Mattes et al., 2005).

6.3.4.4 EBV qPCR

Real time amplification of EBV DNA using glycoprotein B-specific primers was performed as described in chapter 2, section 2.2.3.4.

6.3.4.5 HHV8 qPCR

Primers, probes, PCR conditions, and cycles were adopted from the previously published method (Stamey et al., 2001).

6.3.4.6 PHV qPCR

Real time amplification of PHV DNA using glycoprotein B-specific primers was performed as internal control. The conditions of the PCR were described in chapter 2, section 2.2.3.4.

6.3.5 IMMUNOASSAYS

6.3.5.1 HSV1 ELISA

To detect the presence of HSV1 IgG antibodies, a 100 µl of the 1/101 diluted serum samples were added to the recombinant-Gg1-antigen coated wells and then processed according to the enzyme-linked immunosorbent assay (ELISA) manufacturer's instructions (Biokit, Spain). The samples were tested in duplicates along with seven control samples to validate the assay. According to the manufacturer's instructions, the mean absorbance of the low positive control was calculated and considered to be the cut-off value. The samples' absorbance was then divided by the cut-off value to give a

ratio absorbance/cut-off which is proportional to the concentration of the HSV1 IgG antibodies in each investigated specimen (Biokit, Spain).

6.3.5.2 HSV2 ELISA

The HSV2 IgG ELISA method is similar to HSV1 IgG ELISA apart from the fact that the wells were coated with recombinant-gG2-antigen instead of recombinant-gG1-antigen (Biokit, Spain).

6.3.5.3 VZV ELISA

The VZV IgG ELISA method is similar to HSV1 and HSV2 IgG ELISA but the wells were coated with VZV antigen from partially purified extract of human fibroblast infected with VZV, strain ELLEN (ATCC) (Diamedix, USA). To determine the ELISA unit (EU)/ml, the following formula was used following the manufacturer's instructions (absorbance of calibrator x absorbance of sample = EU/ml of sample).

6.3.5.4 CMV ELFA

An automated quantitative two-step enzyme immunoassay sandwich method with a final fluorescent detection, enzyme-linked fluorescent assay (ELFA), was employed to detect CMV IgG (bioMérieux, France). All of the assay steps were performed automatically by vitek immuno-diagnostic assay system (VIDAS) instrument according to the manufacturer's instructions (bioMérieux, France). The instrument, using calibration curves that are stored by the machine, automatically calculated the results and expressed them in AU/ml.

6.3.5.5 EBV CLIA

The quantification the IgG antibodies to EBV viral capside antigen (VCA) in the serum samples was performed on the LIAISON Analyzer using chemiluminescent immunoassay (CLIA) following the manufacturer's instructions (DiaSorin, Italy). The samples were tested in duplicates, along with the calibrator and control samples to validate the assay. The analyser automatically calculated the antibody concentrations and expressed them as U/ml.

6.3.5.6 HHV8 IFA

A semi-quantification of the IgG antibodies to HHV8 in the serum samples was investigated by utilizing indirect fluorescent assay (IFA) following the manufacturer's instructions (Advanced Biotechnologies, Columbia). The investigated samples were scored in relation to the positive and negative controls. Results were blindly recorded by two different investigators and then checked for consistency and re-evaluated if there were any discrepant results between the two investigators.

6.3.6 STATISTICAL ANALYSIS

The statistical power was calculated using the StatMate 2 program (GraphPad, USA). For the purpose of the viral analysis, the statistical power calculation was based on the previously published information about the prevalence of HSV1 in London in people of the age of 30 or above (54%) (Smith and Robinson, 2002). A sample size of 12 in each group has 95% power to detect an increase of 0.5 with a significant level (alpha) of 0.05 (two tailed). However, one can use an unequal number in the samples of each of the investigated groups without losing any statistical power if the total number of investigated subjects is increased proportionally. For example, the statistical power from investigating a sample size of 12 in three different groups (total number

investigated 36) will be equal to investigating a sample size of 54 in one group, 28 in another, and 7 in the third group (total number investigated 89).

Due to the extent and cost of the analysis performed for each sample for the bacteriological and mycobacterial identification, the author investigated a sample size in concordance with other published papers investigating the human oral microbiome (Farrell et al., 2012, Dang et al., 2012).

The GraphPad Prism® statistical package was used in the data analysis (GraphPad Software Inc., USA). Mean, median, minimum, maximum, range, standard deviation (SD), standard error of mean (Sem) and percentages (%) were used to describe the data. The data were analysed by Mann-Whitney-U when comparing quantitative data of two groups, and Kruskal-Wallis when comparing quantitative data of more than two groups. Differences in the rate were analysed using Chi-square tests. Non-parametric multivariate analysis of variance (MANOVA) with Bonferroni post tests when comparing multiple variants in relation to different groups were also used.

6.4 RESULTS

6.4.1 ORAL MUCOSAL AND SALIVARY MICROBIAL LOAD

There was no statistically significant difference in the microbial load colonizing the oral mucosa of orally active BS patients, orally inactive BS patients, RAS patients and HC volunteers (Kruskal-Wallis, p=0.2283). However, there was a slight increase in the bacterial load colonizing the ulcerative mucosa of orally active BS patients compared to the non-ulcerative mucosa of the same patients, but this difference was not statistically significant (Mann-Whitney, p=0.4595) (Figures 6.1 and 6.2).

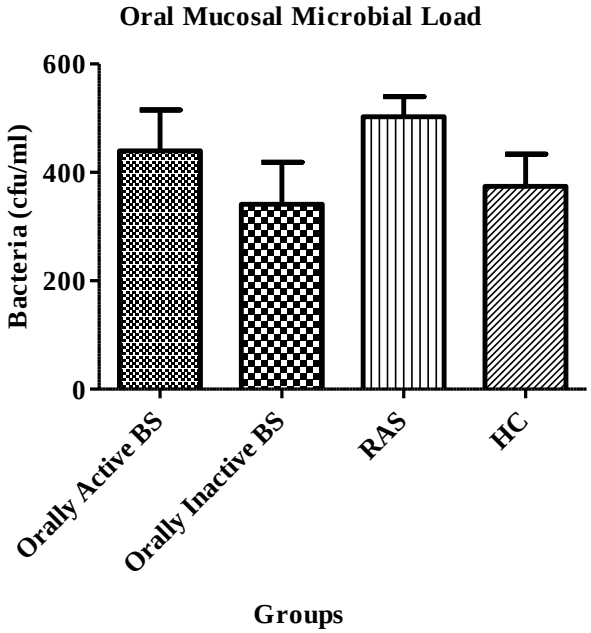


Figure 6.1: The oral mucosal microbial load of orally active BS patients, orally inactive BS patients, RAS patients, and HC volunteers.

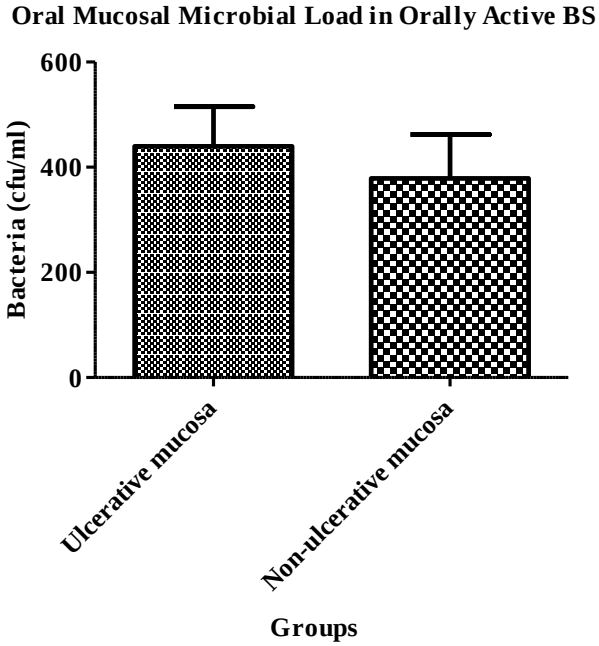


Figure 6.2: The oral mucosal microbial load of ulcerated oral mucosa and non-ulcerated oral mucosa of orally active BS patients.

There was no statistically significant difference in the salivary microbial load of orally active BS patients, orally inactive BS patients, and RAS patients (Kruskal-Wallis, p=0.1216). There was an observed decrease in the salivary microbial load of RAS patients compared to HC volunteers. However, this difference was not statistically significant. Nevertheless, the salivary microbial load of the orally inactive BS patients was significantly decreased compared to HC volunteers (Mann-Whitney, p=0.0185) (Figure 6.3).

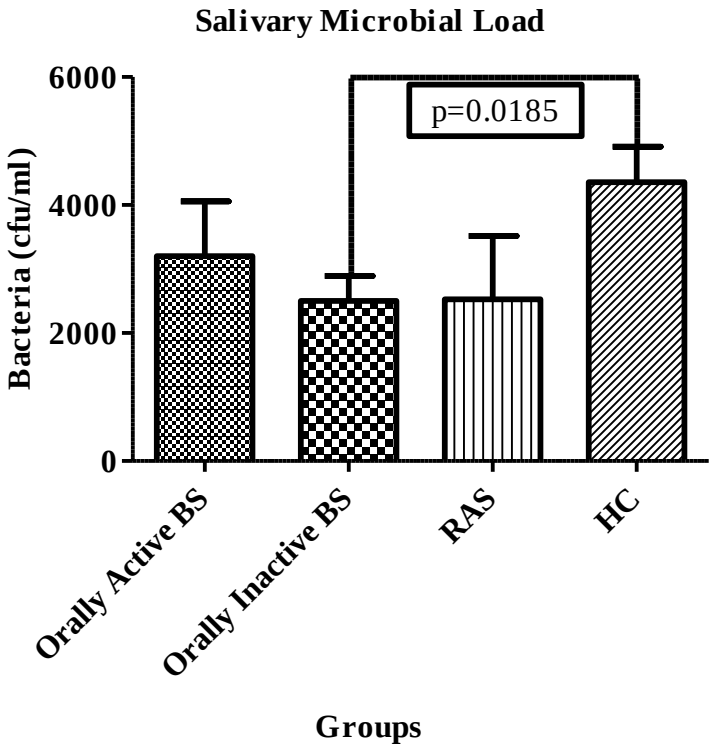


Figure 6.3: The salivary microbial load of orally active BS patients, orally inactive BS patients, RAS patients, and HC volunteers.

6.4.2 ORAL AND SALIVARY MICROBIAL COMPLEXITY

There was no statistically significant difference in the oral mucosal complexity in the four investigated groups (Kruskal-Wallis, p=0.1378). However, there was a noticeable increase in the complexity of the bacteria isolated from the ulcerative mucosa of orally

active BS patients compared to all the other groups investigated. Moreover, there was a noticeable increase in the complexity of the bacteria isolated from the ulcerative mucosa of orally active BS patients compared to the non-ulcerative mucosa of the same patients, but this difference was not statistically significant (Mann-Whitney, p=0.5532) (Figures 6.4 and 6.5).

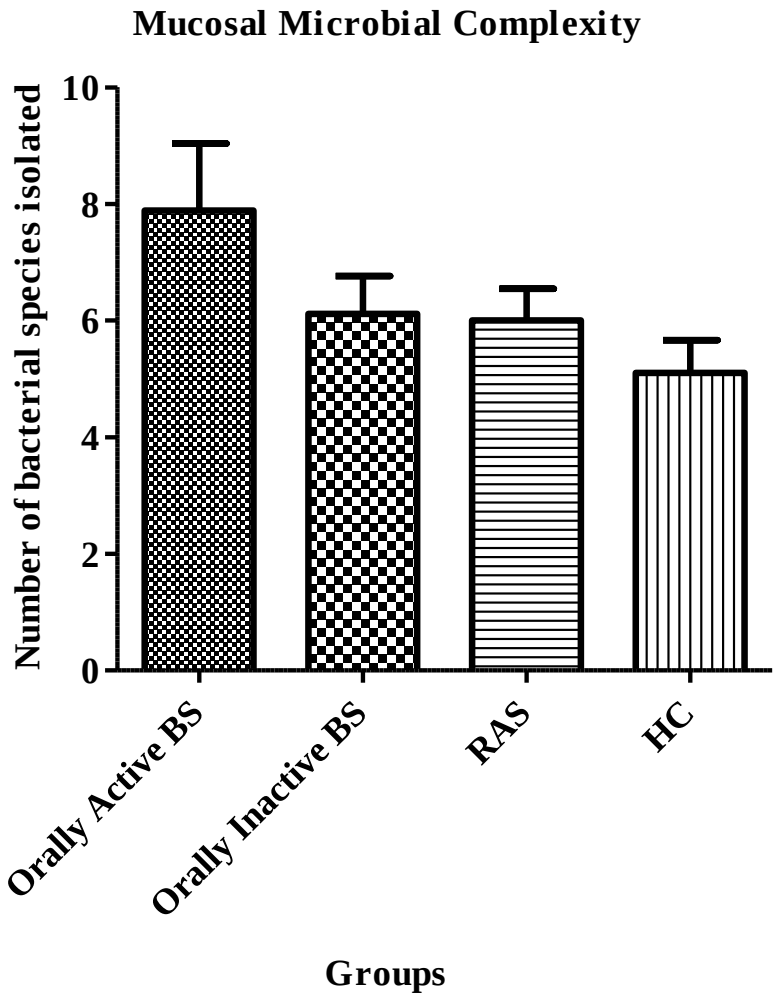


Figure 6.4: The oral mucosal microbial complexity in orally active BS patients, orally inactive BS patients, RAS patients, and HC volunteers.

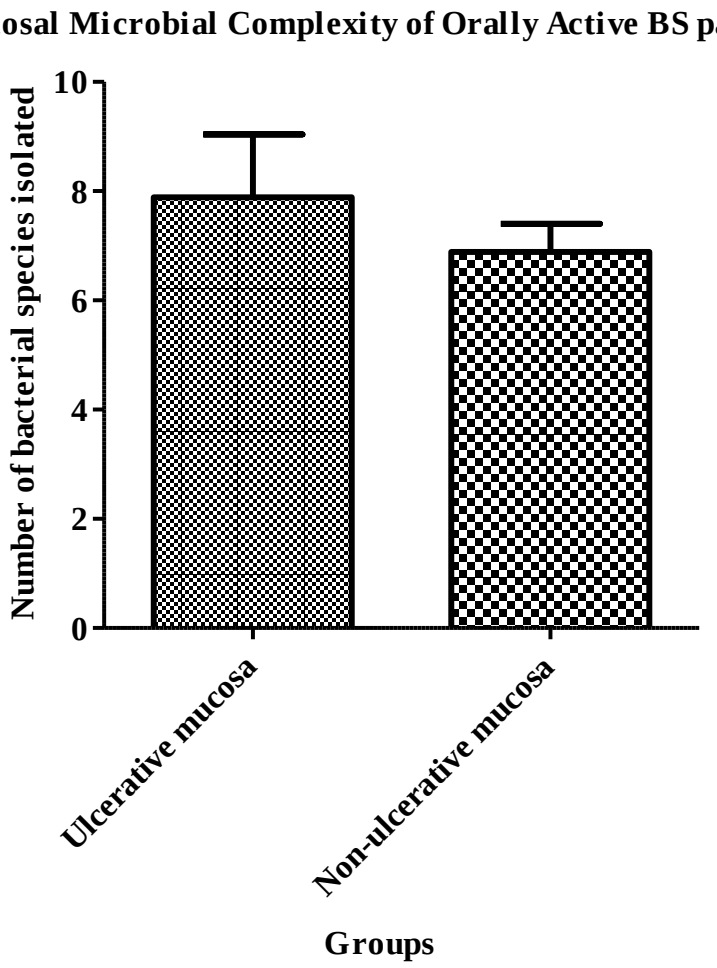


Figure 6.5: The oral mucosal microbial complexity of ulcerated and non-ulcerated sites of orally active BS patients.

Looking at the salivary microbial complexity in orally active BS patients, orally inactive BS patients, RAS patients, and HC volunteers, there was no statistically significant difference in the four investigated groups (Kruskal-Wallis, p=0.1555) (Figure 6.6).

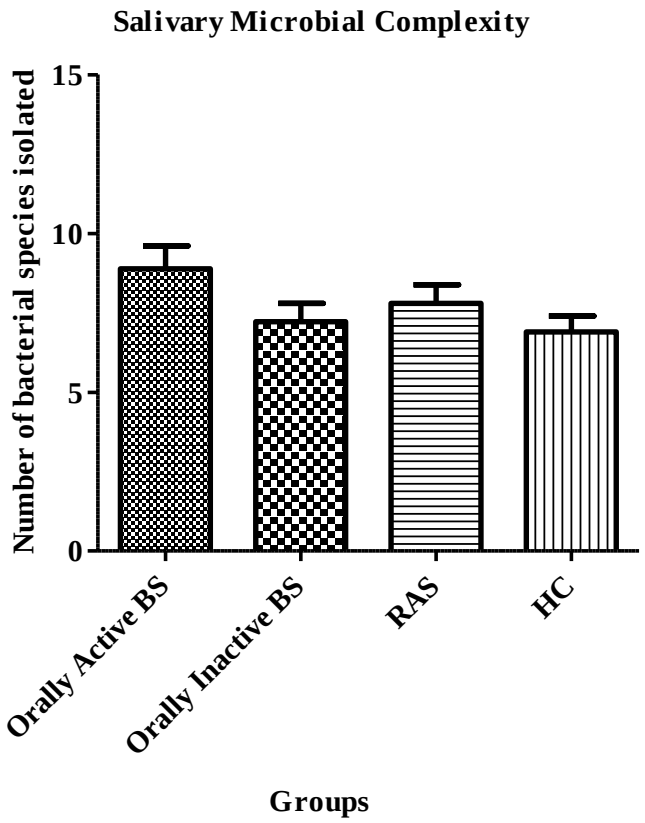


Figure 6.6: The salivary microbial complexity of orally active BS patients, orally inactive BS patients, RAS patients, and HC volunteers.

6.4.3 ORAL MUCOSAL MICROBIAL ANALYSIS

The taxonomic proportion of species that were detected in the oral mucosal samples of subjects in each patient group were analysed from 905 successfully isolated, purified and identified microorganisms obtained by MALDI-TOF analysis from 81 different samples. Each sample was bacteriologically cultured on 7 different culture media and allowed to be incubated on 3 different growth environments to maximize the possibility of identifying all the culturable bacteria in each sample. From these 80 samples, 60 different oral bacteria and 5 fungi were identified to the species level. There was great variability between individuals in each group and also between individuals in different groups. Interestingly, variability was also noticed in different sites (ulcerated and non-ulcerated mucosa) in the same individual.

The microbial profile of the non-ulcerated mucosa in orally active BS patients was the most variable with 31 different bacterial species in 14 different bacterial genera identified as follows: *Actinomyces odontolyticus, Candida albicans, Capnocytophaga sputigena, Corynebacterium durum, Gemella haemolysans, Kingella denitrificans, Kocuria palustris, Lactobacillus fermentum, Lactobacillus* sp., *Neisseria cinerea, Neisseria flavescens, Neisseria mucosa, Neisseria perflava, Prevotella melaninogenica, Rothia aeria, Rothia dentocariosa, Rothia mucilaginosa, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus australis, Streptococcus constellatus, Streptococcus gordonii, Streptococcus mutans, Streptococcus oralis, Streptococcus mitis, Streptococcus pseudopneumoniae, Streptococcus salivarius, Streptococcus sanguinis, Streptococcus vestibularis, Veillonella atypica,* and *Veillonella dispar*. *Rothia dentocariosa* was statistically significantly more likely to colonize the non-ulcerated mucosa of orally active BS patients compared to the ulcerated mucosa of orally active BS patients, ulcerated mucosa of RAS patients, and the oral mucosa of HC volunteers (MANOVA, $p<0.05$) (Figure 6.7).

In total, 14 different genera were represented in the oral microbiome of the non-ulcerated sites in orally active BS patients. In contrast, only 9 genera were detected in the HC volunteers' oral mucosal microbiome. The oral microbiome of the non-ulcerated sites in orally active BS patients included all the genera detected in HC volunteers as well as *Actionmyces, Candida, Haemophilus, Lactobacillus,* and *Prevotella*. *Neisseria* and *Veillonella* were isolated more frequently from HC volunteers (Chi-square, $p=0.0159$: 88.9% vs 75% and $p<0.0001$: 22.2 vs 12.5% respectively) while *Rothia* showed higher representation in the non-ulcerated mucosa of orally active BS patients (Chi-square, $p<0.0001$: 87.5% vs 44.4%) (Figure 6.8).

The microbial profile of the ulcerated mucosa in orally active BS patients was slightly less diverse than that of the non-ulcerated mucosa, with 23 different bacterial species in 12 different bacterial genera identified as follows: *Actinomyces naeslundii, Capnocytophaga ochracea, Capnocytophaga sputigena, Corynebacterium durum, Gemella haemolysans, Haemophilus parainfluenzae, Kingella denitrificans, Neisseria cinerea, Neisseria flavescens, Neisseria macacae, Propionibacterium acnes, Rothia aeria, Rothia dentocariosa, Rothia mucilaginosa, Staphylococcus aureus, Streptococcus anginosus, Streptococcus cristatus, Streptococcus mutans, Streptococcus oralis, Streptococcus mitis, Streptococcus pseudopneumoniae, Streptococcus salivarius*, and *Streptococcus sanguinis*. *Rothia dentocariosa* was statistically significantly less likely to colonize the ulcerated mucosa of orally active BS patients compared to the non-ulcerated mucosa of orally active BS patients (MANOVA, $p<0.05$). Interestingly, colonization of the ulcerative mucosa of BS patients with *Streptococcus salivarius* was statistically significantly higher than of the ulcerative mucosa of RAS patients (MANOVA, $p<0.05$) (Figure 6.7).

In total, 12 different genera were represented in the oral microbiome of the ulcerated sites in orally active BS patients. In contrast, only 9 genera were detected in the HC volunteers' oral mucosal microbiome. The oral microbiome of the ulcerated sites in orally active BS patients included all the genera detected in HC volunteers as well as *Actinomyces, Kocuria, and Propionibacterium*. *Neisseria* and *Veillonella* were isolated more frequently from HC volunteers (Chi-square, $p<0.000$: 188.9% vs 63% and $p<0.0001$: 22.2 vs 13% respectively) while *Rothia* showed higher representation in the ulcerated mucosa of orally active BS patients (Chi-square, $p=0.0069$: 75% vs 44.4%) (Figure 6.8). However, the proportion of *Rothia denticariosa* in relation to the other

species of *Rothia* was less than that seen in the non-ulcerated sites of orally active BS patients (Figure 6.9). There was a noticeable shift towards *Rothia mucilaginosa* (50%) as it was isolated more frequently than *Rothia denticariosa* (37.5%) from the ulcer sites of orally active BS patients.

The microbial profile of the oral mucosa in orally inactive BS patients was similar in complexity to that of those showing oral activity with 25 different bacterial species in 9 different bacterial genera identified as follows: *Actinomyces naeslundii, Candida africana, Candida albicans, Candida dubliniensis, Gemella haemolysans, Kingella denitrificans, Neisseria flavescens, Neisseria macacae, Neisseria mucosa, Neisseria perflava, Prevotella melaninogenica, Rothia aeria, Rothia dentocariosa, Rothia mucilaginosa, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus anginosus, Streptococcus australis, Streptococcus constellatus, Streptococcus gordonii, Streptococcus mutans, Streptococcus oralis, Streptococcus mitis, Streptococcus salivarius,* and *Streptococcus vestibularis*.

In total, 9 different genera were represented in the oral mucosal microbiome of the orally inactive BS patients; they included all the genera detected in HC volunteers, (excluding *Capnocytophaga, Corynebacterium*, and *Veillonella)* as well as *Actionmyces, Candida,* and *Prevotella. Neisseria* and *Veillonella* were isolated more frequently from HC volunteers (Chi-square, $p=0.0009$: 88.9% vs 44.4% and $p<0.0001$: 22.2 vs 0% respectively) while *Rothia* showed higher representation in the oral mucosa of orally inactive BS patients (Chi-square, $p<0.0001$: 77.8% vs 44.4%) (Figure 6.8).

The microbial profile of the oral mucosa in HC volunteers was less diverse in comparison to the oral microbial profile of BS patients of both orally active and orally

inactive status, with 16 different bacterial species in 9 different bacterial genera identified as follows: *Capnocytophaga haemolytica, Corynebacterium durum, Gemella haemolysans, Kingella denitrificans, Neisseria flavescens, Neisseria mucosa, Rothia aeria, Rothia dentocariosa, Rothia mucilaginosa, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus anginosus, Streptococcus australis, Streptococcus gordonii, Streptococcus mitis, Streptococcus salivarius,* and *Veillonella parvula* (Figure 6.7).

The microbial profile of the oral mucosa of RAS patients was less diverse in comparison to the oral microbial profile of BS patients of both orally active and orally inactive status, with 17 different bacterial species in 9 different bacterial genera identified as follows: *Actinomyces naeslundii, Capnocytophaga ochracea, Haemophilus influenzae, Haemophilus parainfluenzae, Lactobacillus gasseri, Lactobacillus paracasei, Neisseria flavescens, Rothia dentocariosa, Rothia mucilaginosa Staphylococcus hominis, Staphylococcus epidermidis, Streptococcus cristatus, Streptococcus gordonii, Streptococcus mutans, Streptococcus oralis, Streptococcus mitis,* and *Streptococcus salivarius*. Similarly to the pattern observed in BS patients' oral mucosa, *Rothia dentocariosa* was statistically significantly more likely to colonize the non-ulcerated mucosa of RAS patients compared to the ulcerative mucosa of RAS patients (MANOVA, $p<0.05$). Likewise, *Streptococcus mitis* was statistically significantly more likely to colonize the non-ulcerated mucosa of RAS patients compared to ulcerative mucosa of RAS patients (MANOVA, $p<0.05$) (Figure 6.7).

In total, 9 different genera were represented in the microbiome of oral mucosa of RAS patients and they matched only 5 genera from those that were detected in HC volunteers (*Capnocytophaga, Neisseria, Rothia, Staphylococcus,* and *Streptococcus*).

Corynebacterium, *Gamella*, *Kingella* and *Veillonella* were missing from the RAS non-ulcerated oral mucosal microbiome. However, *Actionmyces*, *Haemophilus*, *Lactobacillus*, and *Prevotella* appeared to replace them. *Neisseria* and *Veillonella* were isolated more frequently from HC volunteers (Chi-square, $p<0.0001$: 88.9% vs 25% and $p<0.0001$: 22.2 vs 0% respectively) while *Rothia* showed slightly higher representation in the oral mucosa of RAS patients (Chi-square, $p=0.0564$: 50% vs 44.4%) (Figure 6.8).

There was an observed increase in the colonization of orally active BS patients' ulcerated oral mucosa sites with *Streptococcus sanguinis* compared to HC volunteers' oral mucosa (Chi-square, $p<0.0001$: 25% vs 0%). A much more diverse range of *Streptococcus* species was isolated from BS patients' oral mucosa, with 10 different species isolated from the non-ulcerated oral mucosa of orally active BS patients, 8 different species isolated from the ulcerated oral mucosa of orally active BS patients, and 10 different species isolated from the non-ulcerated oral mucosa of orally inactive BS patients. On the other hand, only 5 different *Streptococcus* species were isolated from the oral mucosa of HC volunteers, and 6 from the oral mucosa of RAS patients.

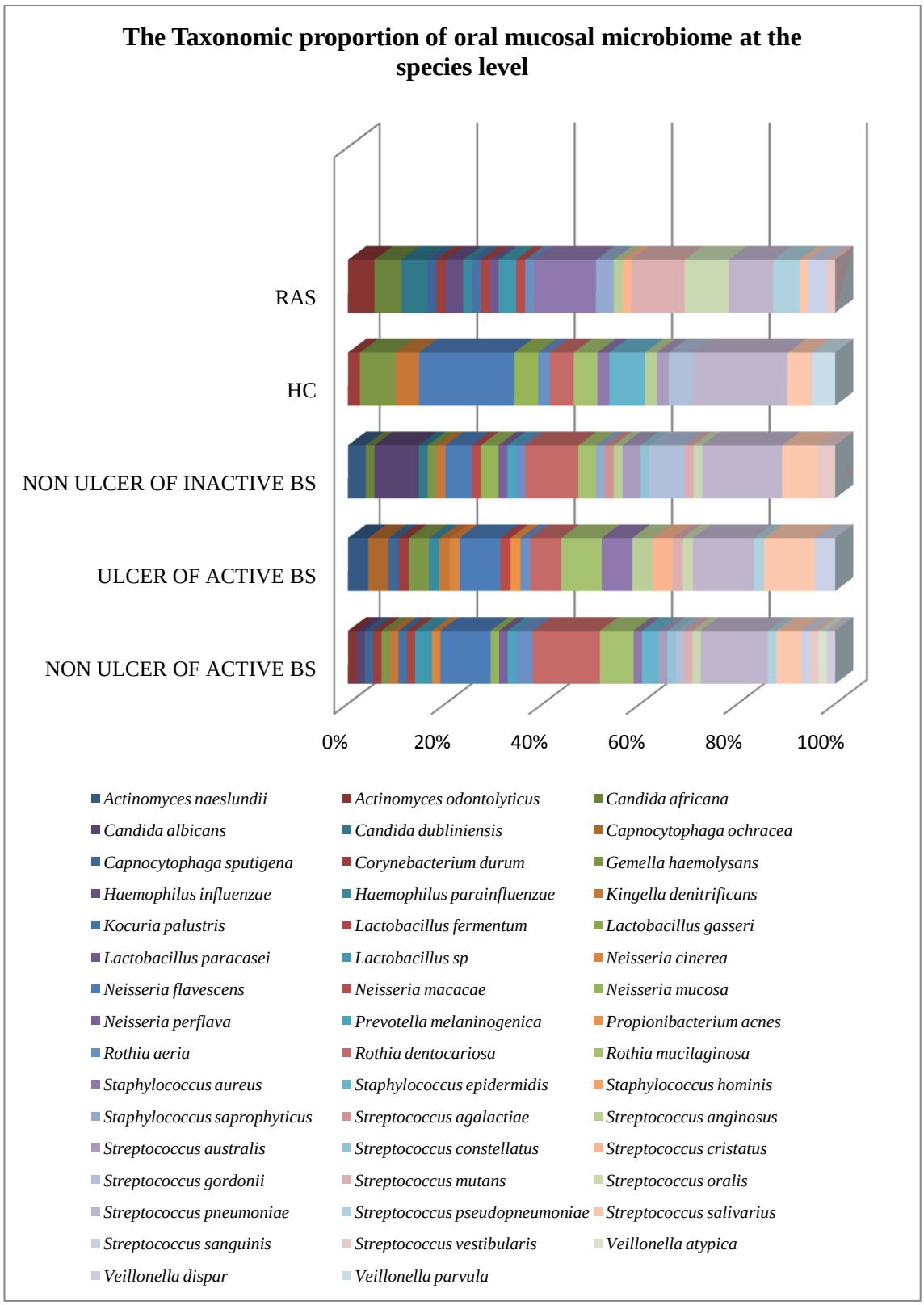


Figure 6.7: The taxonomic proportion of the oral mucosal microbiome at the species level in orally active BS patients, orally inactive BS patients, HC volunteers, and RAS patients. A much more diverse oral microbial profile was observed in BS patients' oral mucosa.

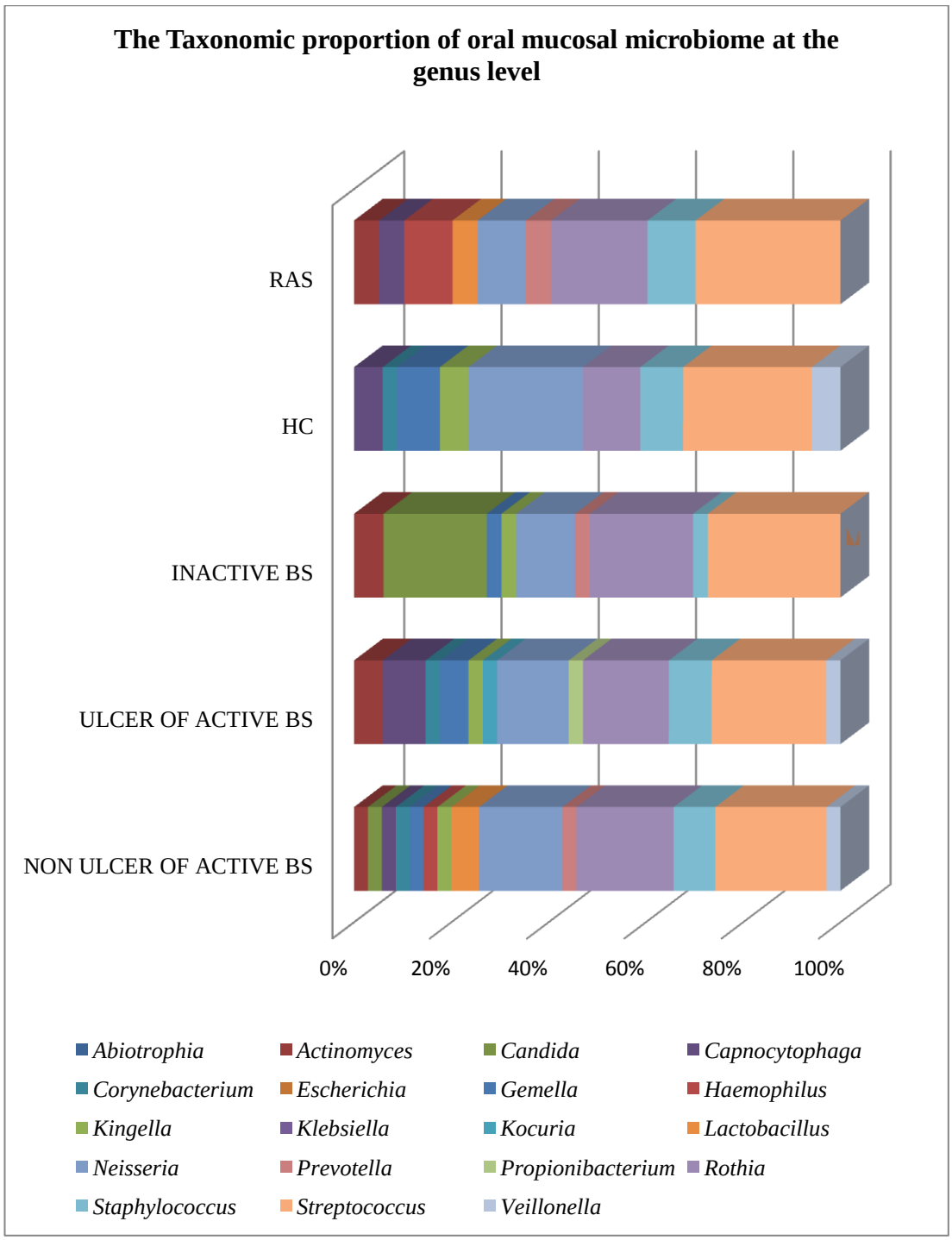
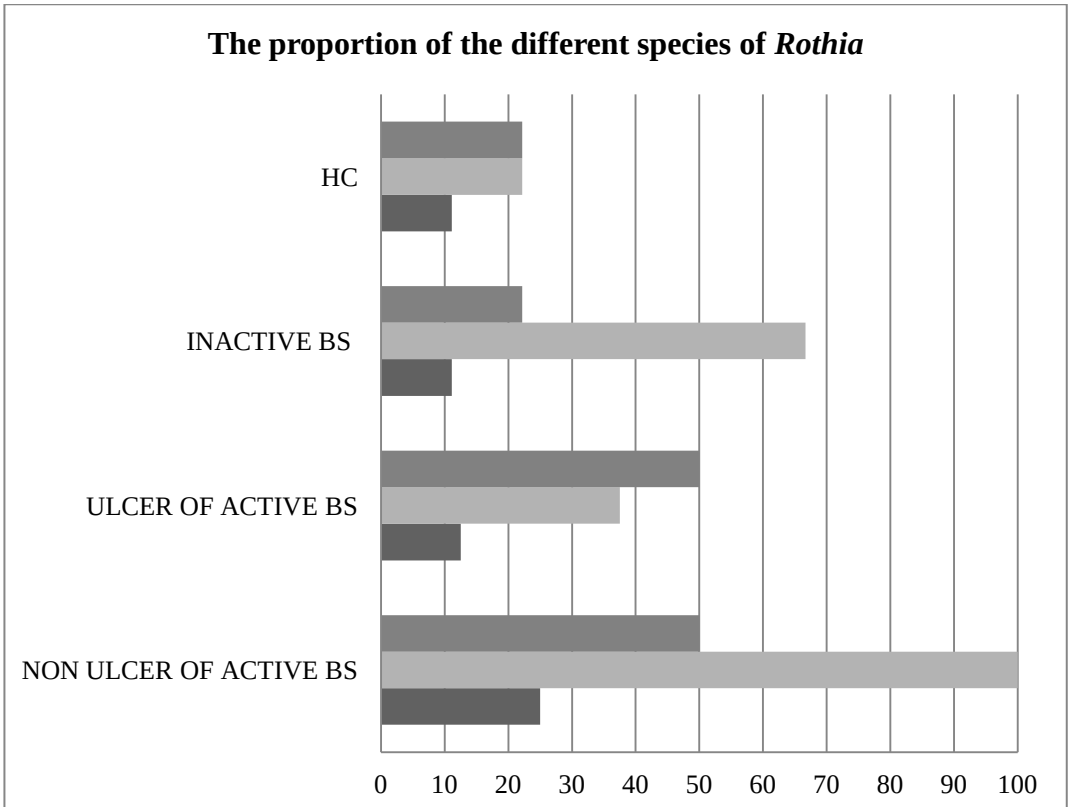


Figure 6.8: The taxonomic proportion of the oral mucosal microbiome at the genus level in orally active BS patients, orally inactive BS patients, HC volunteers, and RAS patients. A much more diverse oral microbial profile was observed in BS patients' oral mucosa.



	NON ULCER OF ACTIVE BS	ULCER OF ACTIVE BS	INACTIVE BS	HC
Rothia mucilaginosa	50	50	22.2	22.2
Rothia dentocariosa	100	37.5	66.7	22.2
Rothia aeria	25	12.5	11.1	11.1

Figure 6.9: *Rothia* in ulcer sites of orally active BS patients. The proportion of *Rothia denticariosa* in relation to the other species of *Rothia* was less than that seen in the non-ulcerated sites. Data presented in percentages.

6.4.4 SALIVARY MICROBIAL ANALYSIS

The taxonomic proportion of species that were detected in the salivary samples of subjects in each patient group were analysed. Similarly to the pattern observed in the oral mucosal microbial analysis, there was great variability between individuals in each group and also between individuals in different groups.

The salivary microbial profile of orally active BS patients was complex, with 29 different bacterial species in 14 different bacterial genera identified as follows: *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Candida albicans*, *Candida metapsilosis*, *Candida parapsilosis*, *Capnocytophaga ochracea*, *Capnocytophaga sputigena*, *Escherichia coli*, *Gemella haemolysans*, *Haemophilus parainfluenzae*, *Kingella denitrificans*, *Kocuria rhizophila*, *Neisseria flavescens*, *Neisseria perflava*, *Prevotella buccae*, *Prevotella oralis*, *Rothia aeria*, *Rothia dentocariosa*, *Rothia mucilaginosa*, *Staphylococcus aureus*, *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Streptococcus anginosus*, *Streptococcus australis*, *Streptococcus cristatus*, *Streptococcus mitis*, *Streptococcus salivarius*, *Streptococcus sanguinis*, and *Veillonella parvula* (Figure 6.10).

In total, 14 different genera were represented in the salivary microbiome of the orally active BS patients. In contrast, only 9 genera were detected in the saliva of HC volunteers. The salivary microbiome of the orally active BS patients included all the genera detected in HC volunteers (except *Haemophilus*) as well as *Actinomyces*, *Candida*, *Echerichia*, *Gamella*, *Kocuria*, and *Prevotella. Neisseria* were isolated more frequently from HC volunteers (Chi-square, $p<0.0001$: 100% vs 66.7%) while *Staphylococcus* showed higher representation in the orally active BS patients' saliva (Chi-square, $p=0.0002$: 55.6% vs 33%) (Figure 6.11).

The salivary microbial profile of orally inactive BS patients was slightly less complex compared to the orally active BS patients, with 24 different bacterial species in 11 different bacterial genera identified as follows: *Abiotrophia defectiva*, *Candida africana*, *Candida albicans*, *Candida dubliniensis*, *Capnocytophaga ochracea*, *Kingella denitrificans*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Neisseria flavescens*,

Neisseria mucosa, *Neisseria perflava*, *Prevotella buccae*, *Rothia aeria*, *Rothia dentocariosa*, *Rothia mucilaginosa*, *Staphylococcus aureus*, *Streptococcus anginosus*, *Streptococcus australis*, *Streptococcus cristatus*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus salivarius*, *Streptococcus sanguinis*, and *Veillonella atypica* (Figure 6.10).

Topical and/or systemic steroid treatment rate was 68% in BS patients who had *Candida albicans* isolated from their saliva at the time of the sampling (Chi-square, p=0.0038). Therefore, unsurprisingly, *Candida albicans* was statistically significantly more isolated from the saliva of orally inactive BS patients compared to HC volunteers and RAS patients (MANOVA, p>0.05).

In total, 11 different genera were represented in the salivary microbiome of the orally inactive BS patients. In contrast, only 9 genera were detected in the saliva of HC volunteers. The salivary microbiome of the orally inactive BS patients included all the genera detected in HC volunteers (except *Haemophilus*) as well as *Abiotrophia*, *Candida*, and *Prevotella*. *Neisseria* were isolated more frequently from HC volunteers (Chi-square, p<0.0001: 100% vs 63.6%) (Figure 6.11).

The salivary microbial profile of HC volunteers included 25 different bacterial species in 9 different bacterial genera identified as follows: *Capnocytophaga haemolytica*, *Capnocytophaga ochracea*, *Haemophilus parainfluenzae*, *Kingella denitrificans*, *Lactobacillus paracasei*, *Neisseria elongata*, *Neisseria flavescens*, *Neisseria mucosa*, *Neisseria perflava*, *Rothia aeria*, *Rothia dentocariosa*, *Rothia mucilaginosa*, *Staphylococcus aureus*, *Staphylococcus capitis*, *Staphylococcus epidermidis*, *Streptococcus anginosus*, *Streptococcus australis*, *Streptococcus dysgalactiae*,

Streptococcus gordonii, *Streptococcus oralis*, *Streptococcus peroris*, *Streptococcus mitis*, *Streptococcus salivarius*, *Veillonella atypica*, and *Veillonella parvula* (Figure 6.10).

The salivary microbial profile of RAS volunteers was less complex than the salivary microbial profile of BS patients and HC volunteers, with 18 different bacterial species in 10 different bacterial genera identified as follows: *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Haemophilus parainfluenzae*, *Klebsiella oxytoca*, *Lactobacillus paracasei*, *Neisseria flavescens*, *Neisseria perflava*, *Neisseria subflava*, *Prevotella nigrescens*, *Rothia dentocariosa*, *Staphylococcus pasteuri*, *Staphylococcus warneri*, *Streptococcus anginosus*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus salivarius*, *Streptococcus sanguinis*, and *Veillonella atypica* (Figure 6.11).

There was an observed increase in the salivary *Streptococcus sanguinis* in orally active BS patients (44.4%) compared to orally inactive BS patients (9.1%) (Chi-square, $p<0.0001$), RAS patients (0%) (Chi-square, $p<0.0001$), and HC volunteers (20%) (Chi-square, $p<0.0001$). Contradicting the pattern observed in the oral mucosal microbial analysis, the salivary *Streptococcus* species were similar in diversity in orally active BS patients (6 different species), orally inactive BS patients (7 different species), HC volunteers (8 different species), and RAS patients (5 different species).

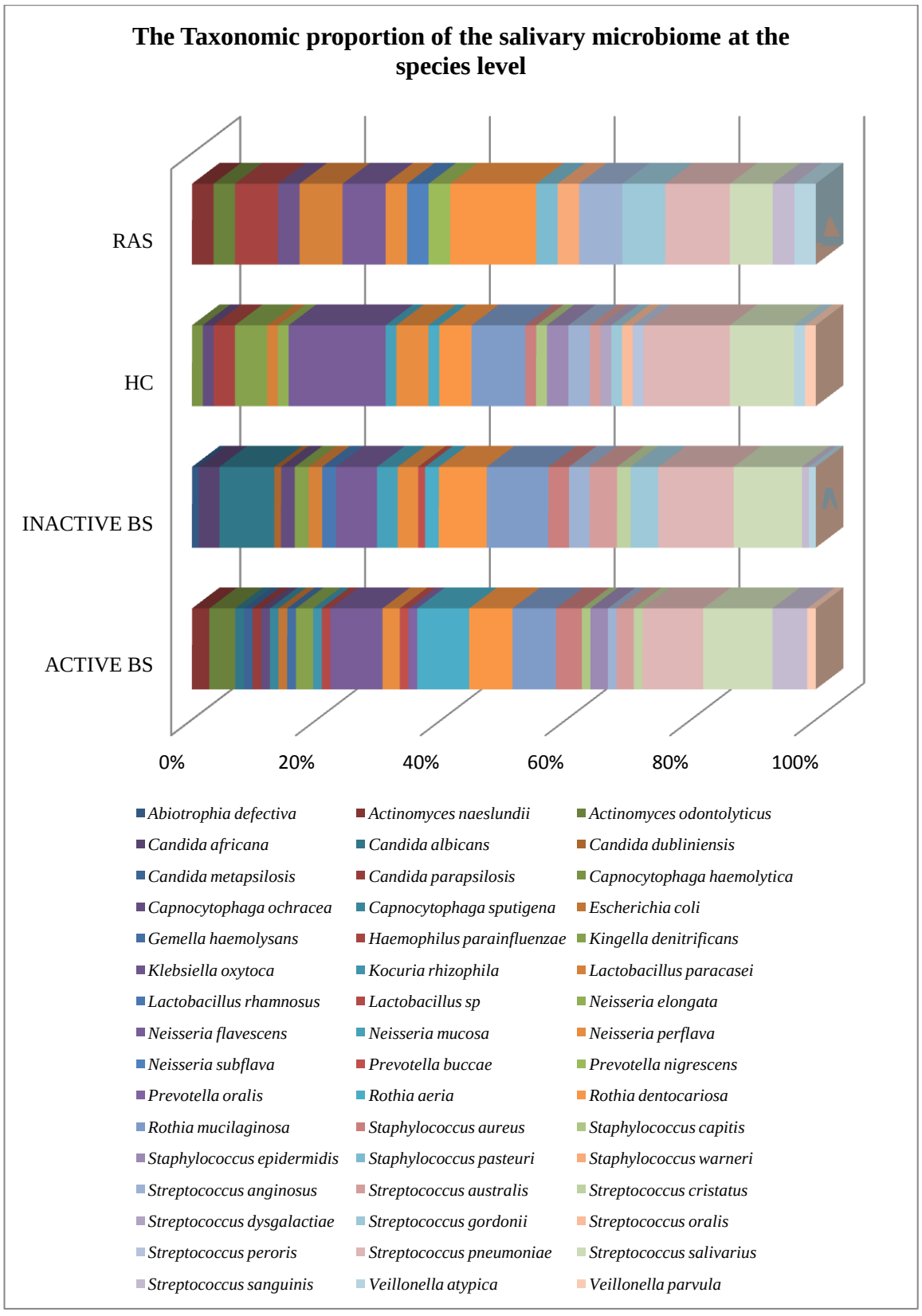


Figure 6.10: The taxonomic proportion of the salivary microbiome at the species level in orally active BS patients, orally inactive BS patients, HC volunteers, and RAS patients.

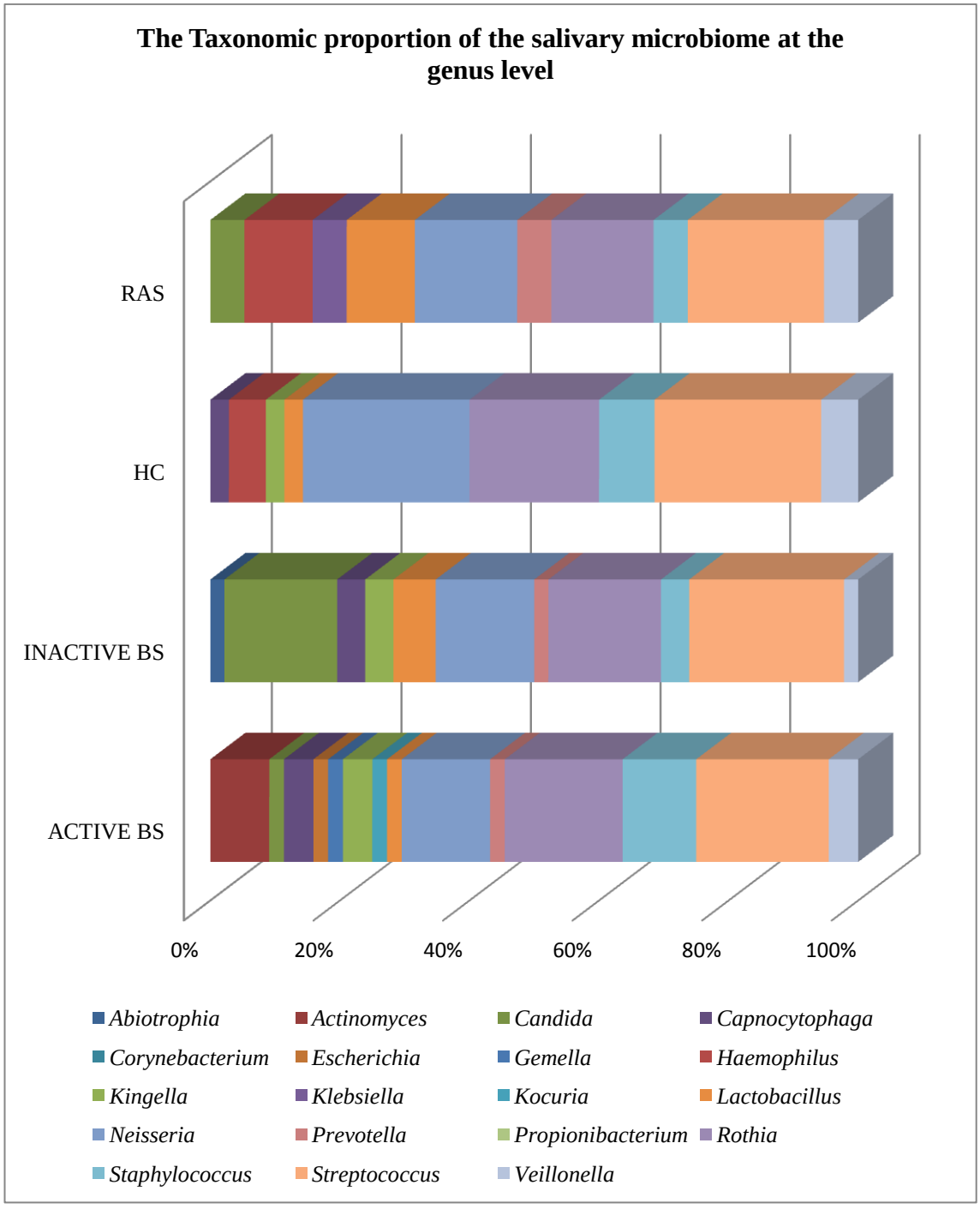


Figure 6.11: The taxonomic proportion of the salivary microbiome at the genus level in orally active BS patients, orally inactive BS patients, HC volunteers, and RAS patients.

6.4.5 MYCOBACTERIAL ANALYSIS

Two different species of non-tuberculous mycobacteria (*Mycobacterium gordona* and *Mycobacterium chelonae*) were isolated from saliva samples of four orally active BS

patients. *Mycobacterium avium intracellulare* was isolated from one orally inactive BS patient's saliva. There was no mycobacterium isolated from any of the brush biopsies of orally active and orally inactive BS patients. *Mycobacterium gordonii* and *Mycobacterium Kansasii* were isolated from two HC volunteers' saliva samples. *Mycobacterium gordonii* was isolated from one orally inactive RAS patient's saliva sample.

6.4.6 HERPES VIRUS ANALYSIS

6.4.6.1 HSV1

There was no statistically significant difference in the HSV1 IgG level of expression between BS patients, RAS patients, and HC volunteers (Kruskal-Wallis, p=0.6697). Mean and standard deviation were as follows: BS patients (0.87 ratio absorbance/cut-off ± 0.92), RAS patients (0.75 ratio absorbance/cut-off ± 0.89), and HC volunteers (0.96 ratio absorbance/cut-off ± 0.98). There was also no statistically significant difference in the prevalence of HSV1 infection between the three investigated groups, with positivity rate ranges between 46% and 57% (Chi-square, p=0.2892) (Table 6.1). Only one BS patient's saliva was positive for HSV1 by qPCR.

6.4.6.2 HSV2

There was no statistically significant difference in HSV2 IgG level of expression between BS patients, RAS patients, and HC volunteers (Kruskal-Wallis, p=0.9297). Mean and standard deviation were as follows: BS patients (0.16 ratio absorbance/cut-off ± 0.36), RAS patients (0.21 ratio absorbance/cut-off ± 0.45), and HC volunteers (0.12 ratio absorbance/cut-off ± 0.22). There was also no statistically significant difference in the prevalence of HSV2 infection between BS patients and HC volunteers (Chi-square,

p=0.2507) and BS patients compared to RAS patients (Chi-square, p=0.2507) (Table 6.1). The salivary HSV2 viral load was negative for all three groups.

6.4.6.3 VZV

There was no statistically significant difference in the VZV IgG level of expression between BS patients, RAS patients, and HC volunteers (Kruskal-Wallis, p=0.6054). Mean and standard deviation were as follows: BS patients (98.7 EU/ml ± 35.1), RAS patients (91.9 EU/ml ± 50.6), and HC volunteers (106.8 EU/ml ± 38.5). There was also no statistically significant difference in the prevalence of VZV infection between the three investigated groups, with positivity rate ranges between 86% and 100% (Chi-square, p=0.6978) (Table 6.1). The salivary VZV viral load was negative for all three groups.

6.4.6.4 CMV

There was a statistically significant decrease in the CMV IgG level of expression in BS patients' samples compared to those of RAS patients and HC volunteers (Kruskal-Wallis, p=0.0450). Mean and standard deviation were as follows: BS patients (30.67 AU/ml ± 35.2), RAS patients (55.6 AU/ml ± 46.8) and HC volunteers (48.32 AU/ml ± 37.9) (Figure 6.12). Nevertheless, there was no statistically significant difference in the prevalence of CMV infection between the three investigated groups, with a positivity rate as follows: BS patients (50%), RAS patients (71%) HC volunteers (64%) (Chi-square, p=0.2206) (Table 6.1). The salivary CMV viral load was negative for all three investigated groups.

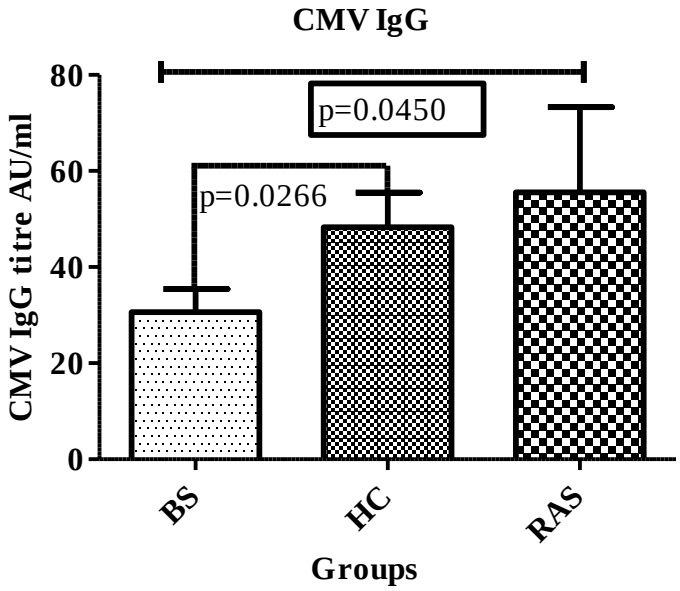


Figure 6.12: CMV IgG level of expression.

6.4.6.5 EBV

There was no statistically significant difference in the EBV IgG level of expression between BS patients, RAS patients, and HC volunteers (Kruskal-Wallis, p=0.9742). Mean and standard deviation were as follows: BS patients (412.3 U/ml ± 285.0), RAS patients (399.4 U/ml ± 345.8), and HC volunteers (403.2 U/ml ± 255.5). There was also no statistically significant difference in the prevalence of EBV infection between the three investigated groups, with positivity rate ranges between 86% and 96% (Chi-square, p=0.1181) (Table 6.1).

Interestingly, there was a statistically significant increase in salivary shedding of EBV in the saliva of the BS patients compared to HC volunteers (Mann-Whitney, p=0.0057). However, there was no statistically significant difference between BS patients and RAS patients (Chi-square, p=0.4407). Mean and standard deviation of the absolute shedding of the virus were as follows: BS patients (2.3 log ± 2.3), RAS patients (1.4 log ± 2.1), and HC volunteers (0.89 log ± 1.7) (Figure 6.13). There was also a statistically

significant increase in the positivity rate in the BS patients (55%) compared to HC volunteers (25%) (Chi=square, p<0.0001), but not RAS patients (43%) (Chi=square, p=0.0926)

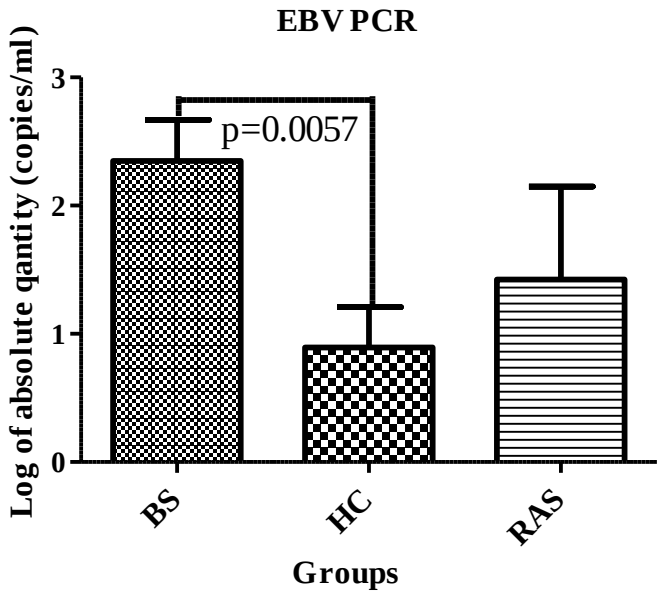


Figure 6.13: The salivary shedding of EBV.

There was no statistically significant difference in the EBV salivary shedding of orally active BS patients (n=14) compared to orally inactive BS patients (n=40) (Mann-Whitney, p=0.135). Mean and standard deviation of the absolute shedding of the virus were as follows: orally active BS patients (1.8 log ± 2.4) and orally inactive BS patients (2.8 log ± 2.4). There was also no statistically significant difference in the positivity rate in orally active BS patients (43%) compared to orally inactive BS patients (65%) (Chi-square, p=0.1348) (Figure 6.14).

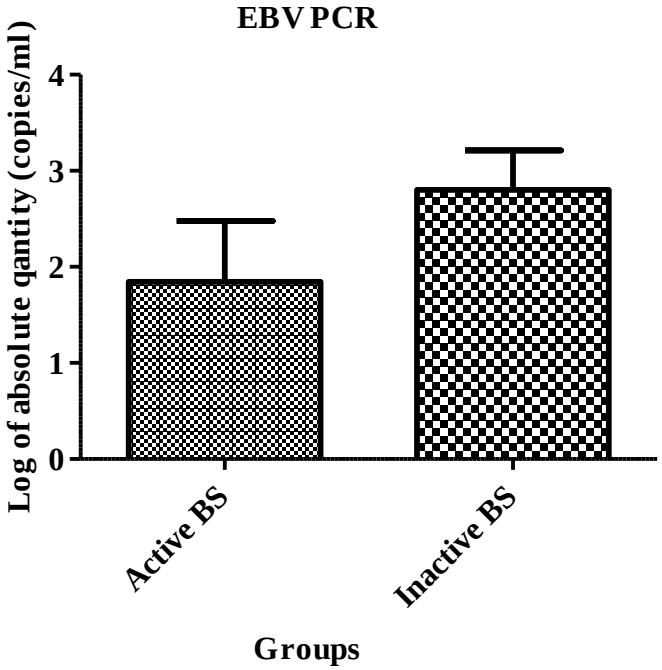


Figure 6.14: The salivary shedding of EBV in orally active BS patients compared to orally inactive BS patients.

6.4.6.6 HHV8

There was no statistically significant difference in the HHV8 IgG level of expression between BS patients, RAS patients, and HC volunteers (Kruskal-Wallis, p=0.3150) (Figure 6.15). The HHV8 immunoassay used in this study is a semi-quantitative assay, as mentioned before in section 6.3.5.6. Six BS patients' samples and one HC volunteer's sample gave nonspecific binding and therefore were excluded from the analysis (Figure 6.16). The prevalence of the HHV8 is reported in table 6.1. Salivary HHV8 qPCR was negative for all three investigated groups.

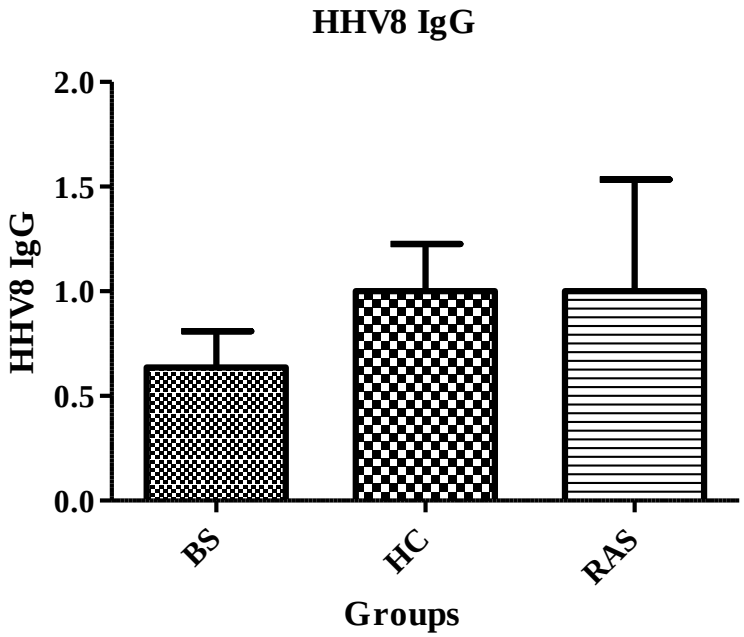


Figure 6.15: HHV8 IgG level of expression.

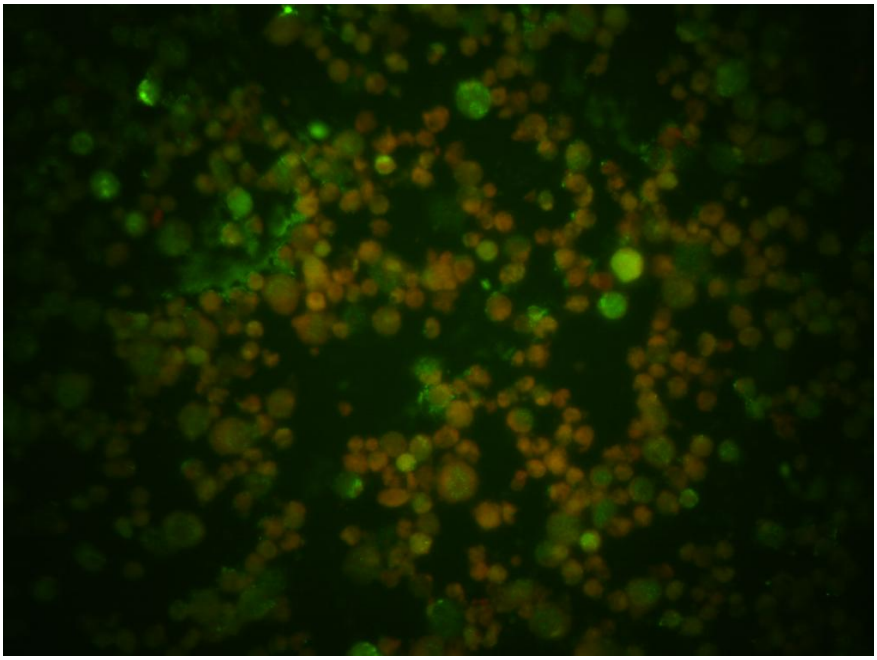


Figure 6.16: Nonspecific binding of HHV8 infected cells to molecules in the serum samples of a BS patient (IFA).

Table 6.1: Prevalence of herpes virus infections in BS patients, RAS patients, and HC volunteers.

	BS (n=54)	**RAS (n=7)**	**HC (n=28)**
HSV1	(29/54) 53.7%	(14/28) 50%	(3/7) 42.9%
HSV2	(5/54) 9%	(1/7) 14%	(1/28) 4%
CMV	(27/54) 50%	(5/7) 71%	(19/28) 64%
EBV	(48/54) 89%	(6/7) 85.7%	(27/28) 96.4%
VZV	(54/54) 100%	(6/7) 85.7%	(28/28) 100%
HHV8	(14/44) 32%	(3/7) 42%	(13/27) 48%
CMV: cytomegalovirus; EBV: Epstein-Barr virus; HHV8: human herpes virus 8; HSV1: herpes simplex virus 1; HSV2: herpes simplex virus 2; VZV: varicella voster virus.			

6.4.6.7 VALIDATION OF THE IMMUNOASSAY

All samples were analysed in duplicate. Negative control, positive control and calibrator samples were tested each time along with the investigated samples. In order to validate the results, the value of these control samples was checked against the validation range suggested by the manufacturer's instructions. For the HHV8 IFA, the slide was blindly evaluated by two different investigators followed by re-evaluation of the discrepant samples.

6.4.6.8 VALIDATION OF THE qPCR

All extracted DNA from clinical samples and negative control samples was spiked with PHV as an internal control, according to the method described in chapter 2, section 2.2.3.4. Samples were then tested by qPCR for the presence of PHV. All samples were positive for PHV, which excluded the presence of PCR inhibitors in the reaction.

6.5 DISCUSSION AND CONCLUSION

It is estimated that the human oral cavity may be inhabited by about 700 different aerobic and anaerobic bacterial species, which produce a huge number of different pathogen-associated molecular patterns (PAMPs) such as peptides and polysaccharides that can interact with each other and the host immunity to maintain a stable symbiotic microenvironment during health (Bik et al., 2006, Paster et al., 2001). If this balance is disturbed, the symbiotic relationship will shift to allow the induction of a pathogenic process leading to various disease symptoms.

It is well appreciated that the human microbiome includes a "core" microbiome which is common between most individuals and a "variable" microbiome that evolves in response to lifestyle, and phenotypic and genotypic differences (Bik et al., 2006, Turnbaugh et al., 2007). The bacterial genera and species that dominate the mouth vary considerably between healthy individuals. However, in general the healthy human mouth is inhabited by nine main bacterial phyla (Firmicutes, Proteobacteria, Bacteroidetes, Actionbacteria, Fusobacteria, TM7, Spirochaetes, and Synergistes) (Bik et al., 2010). On the genera level, *Streptococcus* is known to be the most abundant genus, followed by *Haemophilus*, *Neisseria*, *Prevotella*, *Veillonella*, and *Rothia* (Figure 6.17).

Interestingly, recently there are data indicating that the oral microbiome changes considerably in certain chronic diseases such as pancreatic disease and inflammatory bowel disease, offering the potential for using the salivary microbiome as useful non-invasive biomarkers (Docktor et al., 2012, Farrell et al., 2012). In addition, there is some evidence of the impact of the human immunodeficiency virus (HIV) infection and

its treatment with antiretroviral medication on the community structure of the oral microbiome (Dang et al., 2012).

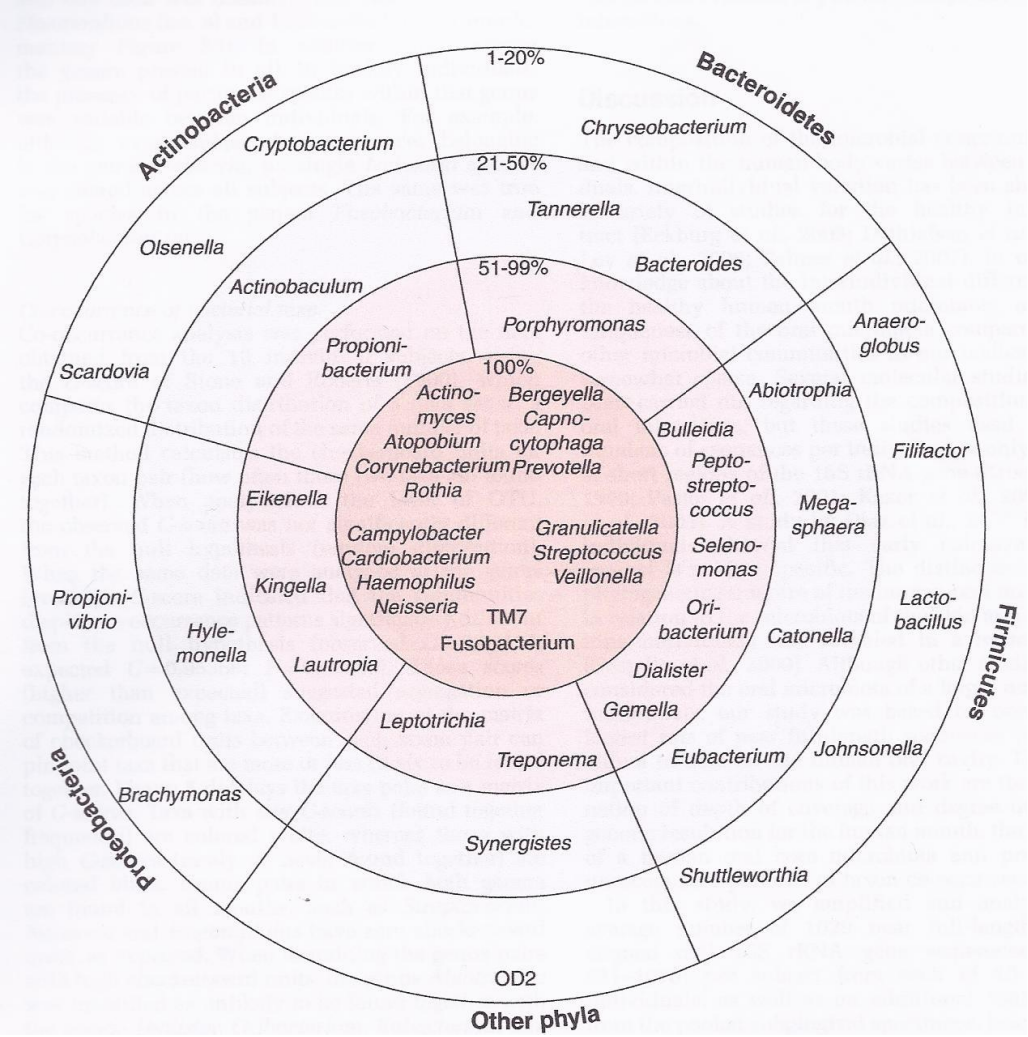


Figure 6.17: Oral community members. The inner circle highlights the bacterial genera found in 100% of the investigated subjects in the 2010 study by *Bik et al*. The genera in the second circle were present in 51%–99% of subjects, followed by the third circle (21%–50%) and the fourth circle (1%–20%) [source: (Bik et al., 2010)].

This part of the thesis is the first study to investigate the oral and salivary microbiome of BS patients in great depth. There is some evidence in the literature pointing towards a possible increase in the *Streptococcus* load and the existence of an unfavourable shift in the cutaneous and conjunctival flora (Gunduz et al., 2008, Hatemi et al., 2004, Isogai et

al., 1990, Lehner et al., 1991). The author observed a decrease in the oral mucosal microbial load in orally active BS patients, although there was a tendency for increased complexity and diversity within the microbial community isolated from these patients. The phyla Actinobacteria including the genera *Rothia*, *Actinomyces*, and *Propionibacterium* was represented more frequently in BS patients and RAS patients compared to HC volunteers. On the other hand, *Neisseria* and *Veillonella* were more represented in HC volunteers. Furthermore, the ulcer sites of orally active BS patients and RAS patients seemed to be less able to support the growth and multiplication of *Rothia dentocariosa*.

Interestingly, the ulcer sites of BS patients seemed to be colonized with *Streptococcus* more frequently than the ulcer sites of RAS patients. Also the complexity of the *Streptococcus* community was enhanced in the oral mucosa of BS patients in general. In concordance with the published study investigating the bacterial diversity in aphthous ulcers, *Prevotella* was isolated more frequently in RAS patients' oral mucosa (Marchini et al., 2007). Indeed, the genera *Prevotella* was also more frequently isolated from BS patients. There was also an increase in the salivary *Candida albicans* in BS patients. However, it is evidenced in the literature that the topical and systemic application of corticosteroid can decrease the salivary total IgA; therefore it is not unprecedented to observe an increase in *Candida albicans* colonization in a patient cohort that is frequently treated with topical and systemic preparations of corticosteroid (68%) (Fukushima et al., 2005).

There was no statistically significant difference in the prevalence of HSV1, HSV2, VZV, and HHV8 in BS patients compared to HC volunteers and RAS patients. Moreover, the salivary viral load of these viruses was negative in the three investigated

groups, apart from only one BS patient who was positive for HSV1. These results contradict some of the earlier studies especially regarding HSV1 prevalence, but the validated methodology used in this study along with the bigger cohort substantiate the previous published results (Denman et al., 1980, Eglin et al., 1982, Lee et al., 1996, Nomura et al., 1998). In addition, our results are supported by the fact that BS was not successfully treated by administration of high doses of acyclovir in association with plasma exchanges or treatment with acyclovir alone (Davies et al., 1988).

There was a statistically significant decrease in the level of expression of CMV IgG in the serum samples of BS patients. This result is in concordance with the previously published study (Lee et al., 2005). Interestingly, the prevalence of CMV in our BS patients was only 50%, while a much higher prevalence of the CMV infection was reported from countries along the "Silk Route", reaching 97% in those aged between 17 and 40 (Uyar et al., 2008). The initial sensing of CMV is suggested to be through toll-like receptor (TLR) 1/2 heterodimer (Sato et al., 2006). Given the known influence of TLR on the adaptive immune response modulation, it is very possible that the observed decrease in the CMV IgG is a consequence of the previously mentioned potential dysfunction of the TLR1/2 heterodimer in BS patients (Palm and Medzhitov, 2009). Therefore, a status of increased susceptibility to CMV infection is expected in those patients.

The prevalence of EBV in BS patients was comparable to that of HC volunteers and RAS patients. However, the salivary viral load of BS patients was significantly higher compared to HC volunteers, but similar to RAS patients. Interestingly, the initial sensing of EBV is suggested to be through TLR2 independently from TLR1 or TLR6 (Trinchieri and Sher, 2007). It is also documented that the released EBV-encoded

dUTPase from infected cells into the extracellular environment acts as a ligand for TLR2, resulting in the classical signalling cascade of TLRs activating nuclear factor kappa B (NFκβ) and inducing the production of pro-inflammatory cytokines such as interleukin 6 (IL6) and tumour necrosis factor alpha (TNFα) (Ariza et al., 2009). IL6 in turn supports the latent infection and persistence of EBV through its potent activation effect on signal transducer and activator of transcription (STAT) 3 gene signalling that regulates the Epstein-Barr nuclear antigen (EBNA) 1 transcription, which plays a crucial role in the maintenance of the EBV episome in infected cells (Tsao et al., 2012). Interestingly, enhanced inflammatory reaction is a recognized pathological feature of BS with well-recorded high serum pro-inflammatory cytokine levels such as TNFα and IL6 (Evereklioglu et al., 2002).

In conclusion, the lower level of expression of CMV IgG is of great interest as it correlates with the previously investigated defect in the TLR1/2 heterodimer function that acts as the initial receptor sensing CMV. Furthermore, despite the uncertainty about whether the reported differences in the microbiome in health and disease are of a causative or reactive nature, restoring the balance of these differences might prove to be of great importance in treating the oral ulceration and controlling the immunological response in BS patients and RAS patients.

CHAPTER 7

DISCUSSION

Behçet's syndrome (BS) is a multi-systemic immune-related disease with major systemic involvement which may result in blindness, stroke, and major vascular accident (Davatchi et al., 2010b). It is a debilitating chronic disease that severely affects patients' quality of life (QoL) (Fortune, 2003, Mumcu et al., 2009b, Bernabe et al., 2010). However, it was not clear from the available literature whether the cross-cultural differences could modify the impact of this disease on patients' quality of life (QoL). Moreover, the effect of the oral health status on the patients' perception of their disease burden was of particular interest.

Although, BS is markedly prevalent in areas surrounding the old "Silk Road" trading routes in the Middle East and Central Asia (Davatchi et al., 2010b), recently it was documented in the literature that its prevalence in other areas around the world is greater than previously thought (Yazici et al., 2008). Furthermore, the prevalence of BS in the UK was estimated to be 0.65/100,000 (Chamberlain, 1977, Dejaco et al., 2009). Indeed, the author documented that over a 6-year period there were 153 well-defined BS patients enrolled at two of the UK's centres for the management and care of BS patients. Therefore, increasing the awareness of the disease among health care professionals and the public is mandatory.

BS patients most frequently suffer from recurrent oral ulceration, which is usually the initial sign of the disease. Because of this observation, it is thought that the oral environment may play a very important role in the aetiology and pathogenesis of the disease (Davatchi et al., 2010b). To date the consensus is that BS is triggered by a profound inflammatory response to an undefined environmental factor in a genetically susceptible host (Lehner, 1999). Therefore, in well-defined cohorts, investigating the inter-relationship between the oral microbiome and the relevant aspects of the innate

and adaptive immune response was considered a key to understanding the capacity of these patients to respond to microbial insult.

This thesis focused on four main areas: (1) studying the different symptoms of BS patients along with the treatment protocol used in two of the main centres for BS patients' management and care in the UK; (2) investigating the oral health status and QoL of BS patients from two different ethnic origins; (3) examining the capacity of the BS patients' oral mucosa for expressing the different splice variants of toll-like receptor2 (TLR2) and toll-like receptor4 (TLR4); and (4) identifying the oral microbiome of BS patients.

7.1 DISCUSSION

7.1.1 BEHÇET'S SYNDROME PATIENT COHORT IN THE UK

The most frequent symptoms in the UK BS patient cohort were mucocutaneous, rheumatological, and ocular manifestations. Oral ulceration was reported in 100% of investigated cases for the reason that all BS patients were diagnosed according to the International Study Group (ISG) criteria (Lancet, 1990). The UK rate of genital ulceration (73.9%) was greater than that reported in Iran (65%) and Germany (64%), similar to that of Japan (73%), but less than that of China (76%) and Korea (83%). Interestingly, the UK rate of ocular involvements (68.6%) was similar to that reported in Japan (69%), but greater than that reported in Iran (55%), China (35%), Korea (51%), and Germany (53%). Likewise, the UK rheumatological manifestations (79.1%) were remarkably higher than those reported in Iran (33%), Japan (57%), China (30%), Korea (38%), and Germany (53%) (Sachetto et al., 2011, Salvarani et al., 2007, Calamia et al., 2009, Davatchi et al., 2010c, Davatchi et al., 2010b, El Menyawi et al., 2008).

The systemic corticosteroid was mostly used as emergency treatment during relapsed episodes only (37.9%). The frequent use of colchicine (54.2%) reflected the high rate of rheumatological (79.1%) and dermatological manifestations (85.6%) observed in this cohort of BS patients. The use of azathioprine was also elevated (43.8%), reflecting the high rate of ocular manifestations (68.6%) recorded in BS patients in the UK. Only two patients from the 153 cases had lost their useful eyesight as a complication of their disease. This might reflect the success of the treatment protocol used. However, it might also reflect the nature of the cohort (68% female and 60.8% of Caucasian ethnic origin). Indeed, it was documented previously that male BS patients have a worse visual prognosis compared to female patients. In addition, poor vision was reported more frequently in relation to BS patients in countries such as Japan, India and Iran compared to those from the UK, Tunisia, Germany, Greece, Turkey, and Italy (Kitaichi et al., 2007).

7.1.2 ORAL HEALTH STATUS AND QUALITY OF LIFE ANALYSIS OF BEHÇET'S SYNDROME PATIENTS

The oral health impact profile 14 (OHIP-14) that was used in this part of the thesis was previously validated and proven to be a reliable tool in oral disease as a patient-centred outcome measure (McGrath et al., 2003, Mumcu et al., 2006, Mumcu et al., 2007a). By using this tool, it was proven that the oral health status of BS negatively affects the QoL of patients from the UK to a similar extent to those from Turkey. It was also documented in the previously published literature that BS affects the QoL similarly to other chronic conditions such as multiple sclerosis and arthritis (Bernabe et al., 2010).

An increase in the number of oral ulcers in BS patients from the UK was observed compared to patients from Turkey. However, this might reflect a referral bias as the UK

patients were recruited from two of the main tertiary referral centres in the UK for BS patient management and care, where the most complicated cases are expected to be referred. The sulcus bleeding index (SBI) and periodontal probing depth (PPD) in the Turkish BS cohort were higher compared to the BS patient cohort from the UK. This fact can be explained by the reported lower utilization rate of dental services by the Turkish BS patients.

A statistically higher score in all the oral health indices except the plaque index (PI) was observed in the UK BS patient cohort compared to healthy control (HC) volunteers. On the other hand, there were no statistical differences between BS and recurrent aphthous stomatitis (RAS) patients in all the measured indices. This can be due to the difficulty in maintaining good oral hygiene during the recurrent attacks of oral ulceration or possibly a status of increased susceptibility to oral pathogens in both disease groups (BS and RAS). However, the fact that the oral health status of BS and RAS patients was poorer than HC volunteers, despite the equal PI, might implicate a possible impairment in the immune responses to oral pathogens and/or commensals in both disease groups, leading to more susceptibility to dental and periodontal disease.

7.1.3 ORAL MUCOSAL EXPRESSION OF TOLL-LIKE RECEPTORS 2 AND 4

TLR2 and TLR4 messenger ribonucleic acid (mRNA) expression increases significantly in the oral mucosa of BS patients during the relapsed phase. This result is consistent with another study that showed elevated expression of TLR2 and TLR4 in the peripheral blood mononuclear cells (PBMCs) of active BS patients (Do et al., 2008).

An investigation into the different mRNA splice variants of TLR2 and TLR4 concluded with the following points. (1) TLR2 mRNA variant b, which is known to be able to

translated to the full-length protein, was particularly high in relapsed BS patients' oral mucosa. (2) TLR2 mRNA variant d, one of the unusual splice variant possibly affecting the stability of the mRNA, was predominantly elevated in relapsed BS patients' oral mucosa. (3) TLR2 mRNA variant e was also elevated in relapsed BS patients' oral mucosa, potentially highlighting the high monocyte infiltration in the non-lesional oral mucosa of these patients. (4) TLR4 mRNA splice variant 3 was significantly elevated in relapsed BS patients' oral mucosa; this splice variant has extra exon (compared to splice variant 1), which results in a frameshift and an early stop codon, and the translated protein from this splice variant is expected to be significantly truncated and a candidate for nonsense decay. (6) TLR4 mRNA splice variant 4 was also elevated in relapsed BS patients' oral mucosa. TLR4 mRNA splice variant 4 lacks an exon (compared to variant 1), resulting in a frameshift and an early stop codon; the translated protein of this splice variant is also expected to be significantly truncated and a candidate for nonsense decay.

The total protein expression of TLR2 and TLR4 was not raised during relapsed BS compared to the non-relapsed phase, confirming the expected defect in the translation due to the observed elevated level of expression of the unusual mRNA splice variants of TLR2 and TLR4. The protein characterization also established the presence of multiple isoforms for TLR2 and TLR4. At the same time, the abundance of band 35kDa of TLR4 in relapsed BS patients may indicate either a defect in the non-sense decay mechanism or enhanced proteolysis in this cohort.

The decrease in the BS PBMCs' response upon treatment with TLR1/2 agonist might highlight a defect in the heterodimer formation between these two receptors. These results are consistent with a published study illustrating the decrease in TLR2 expression in PBMCs upon treatment with lipopolysaccharide (LPS) and heat shock

protein (HSP) (Yavuz et al., 2008). In addition, the dampened response of BS PBMC TLR4 upon stimulation with its agonist could be explained by the higher level of TLR4 mRNA variant 3 and 4 in relapsed BS patients.

7.1.4 THE ORAL MICROBIOME OF BEHÇET'S SYNDROME PATIENTS

There was an observed decrease in the oral mucosal microbial load in orally active BS patients although there was a tendency for increased complexity and diversity within the microbial community isolated from these patients, with an observed shift towards Actinobacteria phyla including *Rothia, Actinomyces*, and *Propionibacterium*, and Bacteroidetes phyla, in particular *Prevotella.* Moreover, some of the very common commensal bacteria such as *Neisseria*, and *Veillonella* were more represented in HC volunteers than in BS patients. This shift might explain the previously observed less favourable oral health status in BS patients compared to HC volunteers despite the fact that their PI was very similar. It is also worth mentioning that a very similar oral health status was observed in RAS patients; however, RAS patients' oral microbiome was less diverse despite showing a very similar microbiological shift compared to BS patients. Furthermore, the ulcer site of orally active BS patients and RAS patients seemed to be less able to support the growth and multiplication of *Rothia dentocariosa*.

In concordance with the previously published data, the ulcer sites of BS patients seemed to be colonized with *Streptococcus* more frequently than the ulcer sites of RAS patients. Also, the complexity of the *Streptococcus* community was enhanced in the oral mucosa of BS patients in general (Isogai et al., 1990, Lehner et al., 1991).

TLR1/2 heterodimer plays an important role in the initial sensing of cytomegalovirus (CMV) and the consequent modulation of the adaptive immune response efficiently to

defend the host against this infection. Therefore, the observed defect in this heterodimer function in relation to BS patients can explain the recorded decrease in the CMV IgG in the same patient cohort (Palm and Medzhitov, 2009, Sato et al., 2006).

The persistent shedding of salivary Epstein-Barr virus (EBV) in BS patients during both phases of oral activity (orally active and orally inactive periods) can aid in understanding the disease process. Knowing the process that preserves EBV and promotes latency in the host cells, one can appreciate the complex nature of the interaction between the microbial insult and the human immune defence. If this interaction is faulty or exaggerated, a pathological process is expected to be initiated. The initial sensing of EBV is through TLR2 independently from TLR1 or TLR6; this in turn will lead to the classical signalling cascade of TLRs, inducing the production of interleukin 6 (IL6) as one of the pro-inflammatory cytokines that support latent infection and persistence of EBV. Interestingly, enhanced inflammatory reaction is a recognized pathological feature of BS with a well-recorded high IL6 (Ariza et al., 2009, Evereklioglu et al., 2002, Trinchieri and Sher, 2007, Tsao et al., 2012).

7.2 SIGNIFICANCE

7.2.1 BEHÇET'S SYNDROME PATIENT COHORT IN THE UK

This part of the thesis highlighted that BS is actually not as rare in western countries as was previously thought as there were 153 well-defined cases of BS patients enrolled at only two centres for the management and care of BS patients in the UK. This fact in turn will be useful in resource allocation and decision-making. Increasing the awareness of BS clinical diagnosis and management among health care professionals is crucial to allow early diagnosis and prevent its possible detrimental complications such as

blindness (Chamberlain, 1977, Jankowski et al., 1992, Mahr et al., 2008, Yazici et al., 2008).

7.2.2 ORAL HEALTH STATUS AND QUALITY OF LIFE ANALYSIS OF BEHÇET'S SYNDROME PATIENTS

Establishing patients' perception of their oral disease burden and its effect on their QoL is crucial in informing clinical decision-making and improving management strategies. It also identifies areas of need, aiming to redirect the clinical and research resources in a patient-focused direction (McGrath et al., 2003, Mumcu et al., 2006, Mumcu et al., 2007a). This part of the thesis highlighted the fact that the oral health status of BS patients negatively affects their QoL, independently from any cross-cultural and ethnic differences. This fact emphasizes the importance of increasing the resources directed towards improving the oral health status for those patients and also towards researching the aetiology of the disease, aimed at discovering new and more effective treatment strategies.

7.2.3 ORAL MUCOSAL EXPRESSION OF TOLL-LIKE RECEPTORS 2 AND 4

The significant increase in TLR2 and TLR4 mRNA expression in the oral mucosa of BS patients during the relapsed phase can be very valuable in two main areas: (1) the potential future utilization of these markers to monitor the disease activity; and (2) the possibility of informing future research directions in unravelling the aetiology of the disease, as it is possible that one of the TLR2 and TLR4 ligands is playing a key role in the aetiopathogenesis of BS.

Additionally, the observed shift towards expressing some of the unusual mRNA splice variants of TLR2 and TLR4 by BS patients' oral mucosa might explain the defect in

TLR4 and TLR1/2 heterodimer function and indicates the potential presence of some abnormalities in the innate immune response to oral microbes in this syndrome.

7.2.4 THE ORAL MICROBIOME OF BEHÇET'S SYNDROME PATIENTS

Traditional microbiological techniques are available to examine the oral microbiome including culturing and biochemical identification; however, these techniques are laborious and pricey and do not easily lend themselves to systematic analysis of large patient cohorts. Studies have tended to be restricted to certain microbial genera, thereby limiting the investigation of the oral microbiome and ignoring the other genera that act as dependent and independent variants (Mumcu et al., 2009a, Narikawa et al., 1995). This part of the thesis is the first attempt to date to examine the whole microbiome in relation to BS patients and to fill this gap in the literature.

The two main points concluded from this part of the study might prove to be of great significance in understanding the disease process and modifying the future clinical management of BS patients. (1) Despite the uncertainty about whether the reported differences in the microbiome in health and disease are of a causative or reactive nature, restoring the balance of these differences might prove to be of great importance in treating the oral ulceration and controlling the immunological response in BS patients and indeed in RAS patients. (2) The observed abnormalities in salivary shedding of EBV and the adaptive immune response to CMV correlate well with the documented defect in TLR2 function and in unravelling some areas of the BS disease process.

7.3 LIMITATIONS

7.3.1 BEHÇET'S SYNDROME PATIENT COHORT IN THE UK

The ISG criteria were used to classify and diagnose all cases enrolled in this study. While the ISG criteria are a very useful tool to ensure that only well-defined cases are enrolled in the research study, the tool has its limitations. It is unable to diagnose cases where patients have fewer than three episodes of recurrent oral ulceration per year, do not have oral ulceration, or develop oral ulceration later in their disease (Chang and Kim, 2003).

This study is a hospital-based not population-based study, and as such invites a referral bias due to the specialized multi-disciplinary nature of the study centres (Oral Medicine, Rheumatology, Ophthalmology, and Immunology clinic). It is logical and indeed expected that it is the very complicated cases that are referred to these centres rather than the simple ones.

7.3.2 ORAL HEALTH STATUS AND QUALITY OF LIFE ANALYSIS OF BEHÇET'S SYNDROME PATIENTS

Again, the UK part of this study is hospital-based at specialized multi-disciplinary centres (Oral Medicine, Rheumatology, Ophthalmology, and Immunology clinic), which invited referral bias. The Turkish part of the study was also hospital-based, but in a Rheumatology clinic. Therefore, a difference in the severity of the cases was expected. Despite these differences, the reported impact of the oral health status on the QoL was similar in both cohorts.

7.3.3 ORAL MUCOSAL EXPRESSION OF TOLL-LIKE RECEPTORS 2 AND 4

The sample sizes in certain assays were small; however, a statistical power calculation based on the pilot study proved that a sample size of 8 in each group has a 95% power to detect a difference between means of 0.0043 with a significant level (alpha) of 0.05 (two tailed) (StatMate 2: GraphPad, USA).

The exact protein product of the TLR2 and TLR4 mRNA splice variants is not completely understood, which in turn invites further research to investigate the function of these splices in health and disease.

7.3.4 THE ORAL MICROBIOME OF BEHÇET'S SYNDROME PATIENTS

Real time polymerase chain reaction (qPCR) using specific primers, matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry, denaturing gradient gel electrophoresis (DGGE), cloning and sequencing, next-generation sequencing, and the human oral microbial identification microarray (HOMIM) are different techniques that can be used to facilitate systematic mining of the oral microbiome (Bik et al., 2010, Colombo et al., 2009, Eigner et al., 2009, Paster et al., 2001, Socransky et al., 2004, Wade, 2011). Considering the advantages and disadvantages of each method, MALDI-TOF was chosen in this part of the thesis for its quick and accurate identification of most culturable microorganisms and for its ability to give accurate typing information. MALDI-TOF identifies the viable culturable bacteria, fungi and mycobacteria, but with two limitations: (1) unculturable oral bacteria are not recognized by this technique; and (2) *Streptococcus mitis* is usually misidentified as *Streptococcus pneumonia* (Risch et al., 2010). However, the author tried to overcome the second limitation by performing optochin susceptibility and bile

solubility tests to check the identification accuracy of any microorganism reported as *S. pneumoniae* by MALDI-TOF (Kok et al., 2011).

Due to the extent and cost of the analysis performed for each sample for the purpose of bacteriological and mycobacterial identification, the sample size in the MALDI-TOF analysis and the mycobacterial analysis was relatively small, although from well-defined cohorts. Therefore, it is crucial to interpret these results carefully as a preliminary indication of the impact of BS and RAS on the community structure of the oral microbiome. In fact, variation in diet, oral hygiene and sampling time are factors that can significantly affect the oral microbial community structure in a single patient and/or healthy individual (Dewhirst et al., 2010).

7.4 SCOPE FOR THE FUTURE

7.4.1 BEHÇET'S SYNDROME PATIENT COHORT IN THE UK

A population-based study to determine the prevalence of BS in the UK will be of great importance. In addition, a more detailed data collection sheet to capture more information about the different signs and symptoms of the disease, such as the nature of ocular involvements (anterior segment or posterior segment), will be valuable.

7.4.2 ORAL HEALTH STATUS AND QUALITY OF LIFE ANALYSIS OF BEHÇET'S SYNDROME PATIENTS

Similar cross-cultural comparative studies investigating BS patients' QoL using a generic questionnaire or disease-specific questionnaire will be of interest to highlight the impact of other symptoms of the disease on the QoL of patients from different ethnic and cultural backgrounds (Bernabe et al., 2010, Touma et al., 2011, Gilworth et al., 2004). A population-based study will be more useful in controlling the confounding

factors and referral bias created in hospital-based studies. However, these types of studies are costly and control of the quality of diagnosis of the enrolled patients is difficult.

7.4.3 ORAL MUCOSAL EXPRESSION OF TOLL-LIKE RECEPTORS 2 AND 4

Investigating the presence of the TLR2 and TLR4 mRNA splice variants in other immune-related diseases as well as in different cell types is of great importance. Furthermore, studying the properties of the translated protein from each mRNA splice variant will help in understanding their biological functions. It is also important to establish if these isoforms affect the protein expression on the cell surface, and indeed their capability of forming certain heterodimers and interacting with other receptors and adaptor molecules.

Outside the PhD project, the author is currently trying to understand the interaction between TLR2 and TLR1 or TLR6 on the surface of the PBMCs isolated from BS patients during the different disease activity, by using flow cytometry-based fluorescence resonance energy transfer (FRET) analysis.

7.4.4 THE ORAL MICROBIOME OF BEHÇET'S SYNDROME PATIENTS

The differences in the oral microbial community structure of ulcerated and non-ulcerated mucosa of BS patients are of great interest, as restoring the balance of the microbial community can promote rapid recovery from oral ulceration. Supplementing these data with a molecular analysis of the unculturable bacteria by using HOMIM analysis and/or next-generation sequencing will ensure the complete identification of the oral microbiome of BS, including unculturable microorganisms, during the different oral activities of the disease.

Indeed the author has collected all the required samples for the molecular analysis as follows: saliva samples and swabs from ulcerated and non-ulcerated BS oral mucosa from BS patients during orally active and orally inactive phases of the disease. Samples have also been collected from RAS patients and HC volunteers, with informed consent under the ethical approval for this study of the disease. Microbial deoxyribonucleic acid (DNA) was isolated using a DNA isolation kit (Gentra, Qiagen, UK). All samples were checked for the presence of DNA by NanoDrop spectrophotometer (Labtec, UK) and for the presence of bacterial DNA by using general bacterial primers for 16S ribosomal DNA gene. The samples have been stored at -80° ready for the HOMIM and/or next-generation sequencing analysis. The author also has applied for funding to support this analysis, hoping to embark on it during her postdoctoral training.

7.5 CONCLUSION

BS is a devastating chronic disease affecting patients' QoL. The oral health status of these patients has a negative effect on their QoL. Investigating the oral environment is a very important research area that can unravel the mysterious disease aetiology and pathogenesis of BS, allowing the future discovery of new, more useful treatment modalities. Indeed, in this thesis the author has highlighted that the higher level of expression of some of the unusual splice variants of TLR2 and TLR4 mRNA in the oral mucosa might explain the observed functional defect in TLR1/2 heterodimer and TLR4, which in turn can be expected to cause a failure in the adaptive immune response modulation resulting in the observed decrease in the expression of CMV IgG and the increased susceptibility to oral infections (Figure 7.1). Furthermore, it is envisaged that the reported discrepancy in the oral microbiome of BS patients can be targeted in the future by probiotics to restore the balance of the oral microbial community, leading to

better oral health which in turn will potentially enable a better control of the BS immune response.

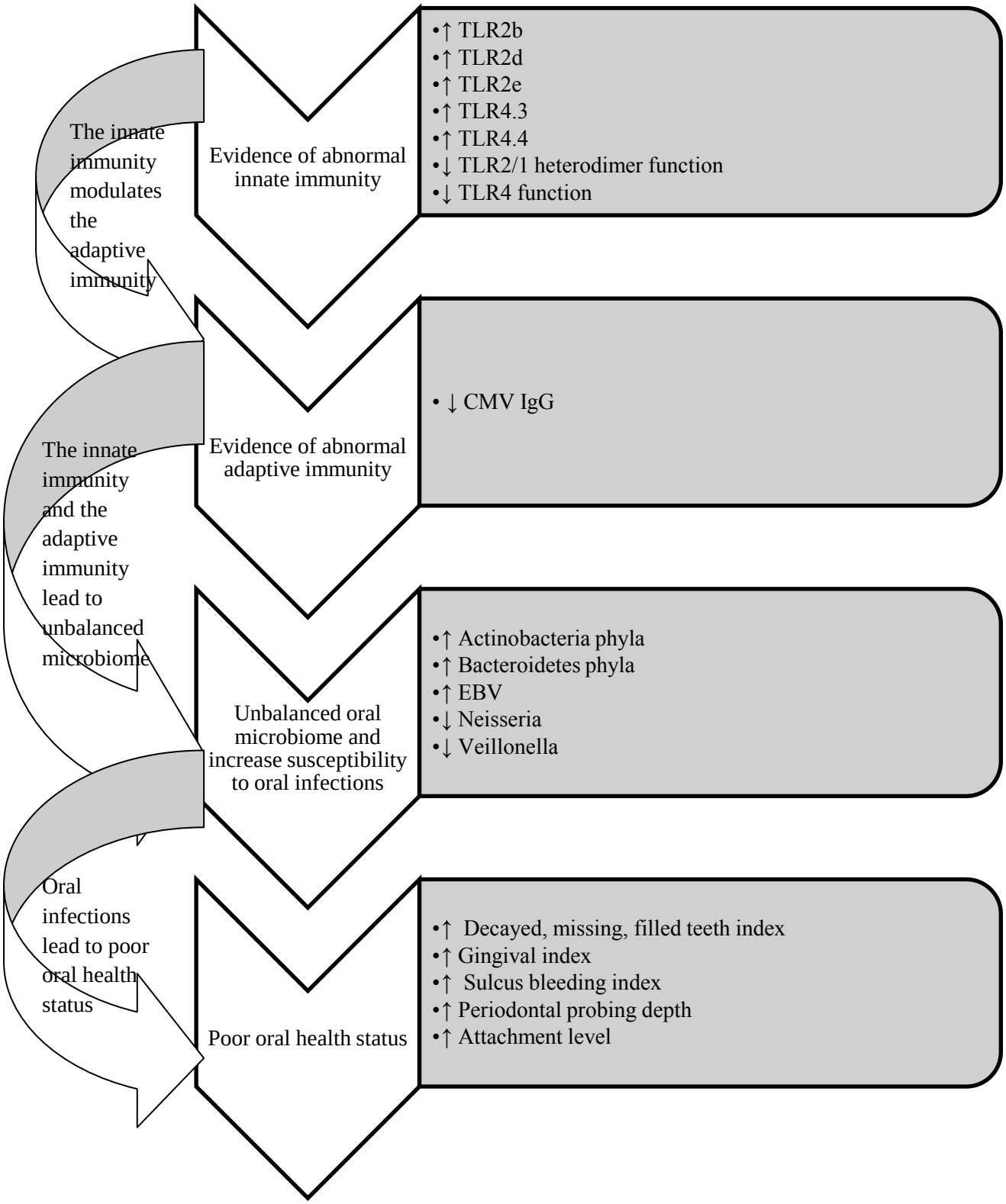


Figure 7.1: Schematic presentation of the main findings of this thesis in relation to BS patients.

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APPENDICES

APPENDIX (1) CONSENT FORM

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 drugs 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.

INVITATION TO PARTICIPATE (Healthy Volunteers)

IMMUNOREGULATIONAT 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.

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 .....

APPENDIX (2) CLINICIAN'S DISEASE ACTIVITY FORM

Date of Sample

Name

Hospital Number

Demographics

Age

Behcet's Phenotype:	Active	Quiet
Mouth		
Genital		
Eyes		
Joints		
Skin: Folliculitis		
Erythema nodosum		
Vascular		
CNS		

BD Activity	Relapse	Quiet

Current Drugs

Other Remarks:

APPENDIX (3) QUALITY OF LIFE QUESTIONNAIRE

INSTRUCTIONS:

This set of questions asks for your views about your health and oral health. This information will helps keep track of how you feel and how well you are able to do your usual activities. Answer every question by marking the answer as indicated. If you are unsure about how the answer a question please give the best answer you can.

OHIP					
Because of problems with your teeth, denture or mouth have you	**Never**	**Hardly ever**	**Occasionally**	**Fairly often**	**All the times**
1. Had trouble pronouncing words	0	1	2	3	4
2. Felt sense of taste has worsened	0	1	2	3	4
3. Had painful aching in the mouth	0	1	2	3	4
4. Found it uncomfortable to eat any food	0	1	2	3	4
5. Have been self-conscious	0	1	2	3	4
6. Felt tense	0	1	2	3	4
7. Had an unsatisfactory diet	0	1	2	3	4
8. Had to interrupt meals	0	1	2	3	4
9. Found it difficult to relax	0	1	2	3	4
10. Have been a bit embarrassed	0	1	2	3	4
11. Have been irritable with other people	0	1	2	3	4
12. Had difficulty doing unusual jobs	0	1	2	3	4
13. Felt life in general was less satisfactory	0	1	2	3	4
14. Have been totally unable to function	0	1	2	3	4

APPENDIX (4) ORAL HEALTH STATUS QUESTIONNAIRE

Patient Name:

Age:

Gender:

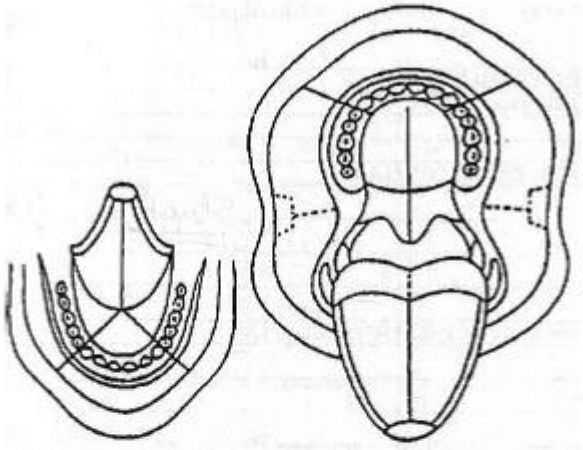
Date of the examination:

Date of onset of the disease:

Name of Dentist:

Types of Samples collected during this session:

Number, site and size of oral ulceration:



M: Missing, F: Filling, Cr Crown, B: Bridge, P: Partial denture, T: Total denture

17	16	15	14	13	12	11	21	22	23	24	25	26	27
47	46	45	44	43	42	41	31	32	33	34	35	36	37

Plaque index

17	16	15	14	13	12	11	21	22	23	24	25	26	27
47	46	45	44	43	42	41	31	32	33	34	35	36	37

Gingival index

17	16	15	14	13	12	11	21	22	23	24	25	26	27
47	46	45	44	43	42	41	31	32	33	34	35	36	37

Sulcus Bleeding Depth

17	16	15	14	13	12	11	21	22	23	24	25	26	27
47	46	45	44	43	42	41	31	32	33	34	35	36	37

Periodontal Pocket depth

17	16	15	14	13	12	11	21	22	23	24	25	26	27
47	46	45	44	43	42	41	31	32	33	34	35	36	37

Attachment loss

17	16	15	14	13	12	11	21	22	23	24	25	26	27
47	46	45	44	43	42	41	31	32	33	34	35	36	37

APPENDIX (5) PUBLISHED PAPER

MUNCU, G., NIAZI, S., STEWART, J., HAGI-PAVLI, E., GOKANI, B., **SEOUDI, N.,** ERGUN, T., YAVUZ, S., STANFORD, M., FORTUNE, F., DIRESKENELI, H. (2009) Oral Health And Related Quality Of Life Status In Patients From UK And Turkey: A Comparative Study In Behçet's Disease. *J Oral Pathol Me, 38(5), 406–9.*

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Oral health and related quality of life status in patients from UK and Turkey: a comparative study in Behcet's disease

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BACKGROUND: The aim of this study was to evaluate and compare oral health-related quality of life (oral QoL) in patients from UK and Turkey with Behcet's disease (BD).

METHODS: Thirty-one BD patients from UK (F/M: 18/13, mean age: 41.8 ± 11.5 years) and Turkey (F/M: 18/13, mean age: 41.5 ± 10.3) who were matched according to age and gender were included in the study. All patients had active oral ulcers. Oral QoL was assessed by Oral Health Impact Profile-14 (OHIP-14). Oral health was evaluated by dental and periodontal indices.

RESULTS: No significant difference was found in OHIP-14 scores between patients from UK (22.7 ± 14.4) and Turkey (20.4 ± 14.3) ($P = 0.709$). The OHIP-14 score correlated with the healing time of oral ulcers in UK ($r = 0.4$, $P = 0.04$) and the number of oral ulcers in Turkey ($r = 0.4$, $P = 0.012$). The number of oral ulcers per month was significantly higher in UK (3.3 ± 2.8) compared with that in Turkey (1.5 ± 2.5) ($P = 0.014$). However, the number of filled teeth and frequency of tooth brushing were significantly lower in patients from Turkey compared with those in UK ($P = 0.000$). Similarly, the duration since the last dental visit (5.1 ± 7.2 months) was significantly lower in UK compared with that in Turkey (28.6 ± 23.7 months) ($P = 0.000$).

CONCLUSIONS: Oral QoL was similar in patients from UK and Turkey with active oral ulcers. However, the number of oral ulcers was observed to be higher in UK. As expected, a lower utilization rate of dental services might have led to a poorer oral health in patients from Turkey.

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Keywords: Behcet's disease; OHIP-14; oral health; quality of life; Turkey; UK

Introduction

Behçet's disease (BD), a multi-systemic inflammatory disorder, is presented by muco-cutaneous, ocular, arthritic, vascular, central nervous and gastrointestinal system involvement. It is more prevalent along the ancient Silk Route from the Mediterranean to East Asian countries, compared with Europe and USA. Ethnic diversity can influence the progression, severity and clinical manifestations of BD (1–5).

Oral ulcers are usually the first sign and the main Classification Criteria (97–100%) in BD, causing an unpredictable course with remission and exacerbations. They are either observed by physicians in clinical examinations or their presence is reported by patients (at least three times a year for classification) (6). The effects of oral ulcers on oral health status (7) and oral health-related quality of life (oral QoL) in Turkish BD population have been reported in our previous studies (8, 9). Yet, comparative studies have not previously been conducted in different populations, even though ethnic diversity is thought to be an important factor of the disease course in BD. Therefore, the aim of this study was to evaluate and compare the status of oral QoL in patients from UK and Turkey with BD.

Materials and methods

Thirty-one BD patients from UK (all Caucasian) (F/M: 18/13, mean age: 41.8 ± 11.5 years) and Turkey (F/M: 18/13, mean age: 41.5 ± 10.3) who were classified according to ISG criteria (10) and matched according to age and gender were included in the study.

Patients from UK with BD were recruited from a specialist tertiary clinic run jointly by the Department of Oral Medicine, Royal London Hospital, London and

the Medical Eye Unit, St Thomas' Hospital, located in Greater London. They were usually referred by their General Practitioner and were examined between November 2006 and April 2007. During the same time span, BD patients from Turkey was examined in the Behçet's disease outpatient clinic of Marmara Medical School Hospital located in Uskudar region, one of 39 districts in Istanbul, a province of Turkey. Patients with the ethnic diversities of Turkey and UK and representing the general BD profile in these countries were selected.

All patients had active oral ulcers in the previous 3 months. Clinical manifestations of BD patients are presented in Table 1. Oral ulcer-related pain status was evaluated by 100-mm visual analogue scale.

Oral QoL was assessed by the Oral Health Impact Profile-14 (OHIP-14) originally developed in Australia in English. A 5-point Likert-type scale was used in scoring each item of the OHIP-14. Responses were coded 0 = 'never', 1 = 'hardly ever', 2 = 'occasionally', 3 = 'fairly often', 4 = 'very often'. Item responses were summed to produce an OHIP-14 total score. Total OHIP-14 scores ranged from 0 (no impact) to 56 (all of the oral health problems were experienced very often). High scores indicated a poor oral QoL (11). Turkish version of OHIP-14 was found to be valid and reliable in our previous study (8). In addition, OHIP-14 subscale scores that were structured in active patients by factor analysis were also calculated (9). Reference period for both patient groups was the previous month in OHIP-14. Oral health was evaluated by dental and periodontal indices including plaque index, gingival index, sulcus bleeding index (SBI), periodontal probing depth (PPD), clinical attachment level, the number of extracted teeth, carious teeth and filled teeth (12).

The research project was approved by the Human Research Ethics Committee of the University of Marmara in Istanbul, Turkey and Queen Mary in London, UK.

Statistical analysis

Data were analysed by using spss 11.5 software (SPSS Inc., Chicago, IL, USA). Mean and standard deviations were used in the tables. Non-parametric tests such as Mann–Whitney *U*-test and the Spearman correlation test were used in the analysis because of non-normal distributed data and number of patients in both groups. Chi-square test was also carried out in the evaluation of clinical spectrum between patient groups. A *P*-value of <0.05 is accepted to be significant. In addition, internal reliability of OHIP-14 and subscales in both patient groups was evaluated by Cronbach-alpha values.

Table 1 Scores of OHIP-14 and oral ulcer-related subscales in patients from UK and Turkey

	UK patients (n = 31)		*Turkish patients (n = 31)*		
	Mean	*SD*	*Mean*	*SD*	*P-value*[a]
OHIP-14 score	22.7	14.4	20.4	14.3	0.709
Oral ulcer-related subscales in OHIP-14					
Subscale 1 (physical symptoms)	8.7	5.2	7.01	5.7	0.219
Subscale 2 (psycho-social symptoms)	8.3	5.5	6.6	5.5	0.311
Subscale 3 (psychological symptoms)	5.7	4.3	7.1	4.9	0.248

OHIP-14, Oral Health Impact Profile-14.
[a]Mann–Whitney *U*-test was used in the analysis.

Results

Oral QoL of life

No significant difference was found in OHIP-14 scores between patients from UK (22.7 ± 14.4) and Turkey (20.4 ± 14.3) ($P = 0.709$). In addition, OHIP-14 subscales scores regarding physical, psycho-social and psychological symptoms were similar in patients from both UK (8.7 ± 5.2, 8.3 ± 5.5 and 5.7 ± 4.3, respectively) and Turkey (7.01 ± 5.7, 6.6 ± 5.5 and 7.1 ± 4.9, respectively) ($P = 0.219$, $P = 0.311$ and $P = 0.248$, respectively) (Table 1).

Cutaneous involvement was more common in patients from Turkey (100% vs. 77.5%) ($P = 0.005$) (Table 2), whereas the number of oral ulcers per month was significantly higher in UK (3.3 ± 2.8) in comparison with that in Turkey (1.5 ± 2.5) ($P = 0.014$) (Table 3). The disease severity score was similar between both groups (Turkey: 4.9 ± 1.6 vs. UK: 5.1 ± 1.8) ($P = 0.983$). The healing times of oral ulcers and oral ulcer-related pain status were also similar in patients from UK (8.6 ± 5.2 days and 50.5 ± 36.2, respectively) and Turkey (7.2 ± 2.1 days and 42.7 ± 40.7, respectively) ($P > 0.05$) (Table 3). The OHIP-14 score correlated with the healing time of oral ulcers in UK patients ($r = 0.4$, $P = 0.04$) and the number of oral ulcers in patients from Turkey ($r = 0.4$ $P = 0.012$). Treatment protocols significantly differed between UK (colchicine: 25%, immunosuppressives: 71.4%, no medication: 3.6%) and Turkey (64.5% and 35.5%, respectively) ($P = 0.008$).

The number of filled teeth (1.1 ± 1.6) and the frequency of tooth brushing (1.01 ± 0.7) were significantly lower in Turkish patients when compared with

Table 2 Clinical manifestations of patients from UK and Turkey with Behçet's disease

	UK patients (n = 31)		*Turkish patients (n = 31)*		
Organ involvement	*n*	*%*	*n*	*%*	*P-value*[a]
Oral ulcer	31	100	31	100	Not analysed
Genital ulcer	29	93.5	31	100	0.151
Cutaneous involvement	24	77.5	31	100	**0.005***
Ocular involvement	8	25.8	11	35.5	0.409
Rheumatologic involvement	13	41.9	19	61.3	0.127
Vascular involvement	5	16.1	7	22.6	0.520
Gastrointestinal involvement	2	6.5	1	3.2	0.438
Neurological involvement	0	0	2	6.5	0.151
Pathergy reaction	17	54.8	19	61.3	0.607

[a]Chi-square test was used in the analysis.
*Statistically significant.

Table 3 Disease severity score and oral ulcer-related factors in patients from UK and Turkey

	UK patients (n = 31)		*Turkish patients (n = 31)*		
	Mean	*SD*	*Mean*	*SD*	*P-value*[a]
Number of oral ulcer	3.3	2.8	1.5	2.5	**0.014***
Healing time of oral ulcers	8.6	5.2	7.2	2.1	0.369
Oral ulcer-related pain evaluated by VAS	50.5	36.2	42.7	40.7	0.433
Disease severity score	5.1	1.8	4.9	1.6	0.983

VAS, visual analogue scale.
[a]Mann–Whitney *U*-test was used in the analysis.
*Statistically significant.

Table 4 Oral health and related factors in patients from UK and Turkey

	UK patients (n = 31)		*Turkish patients (n = 31)*		*P-value*[a]
Carious teeth	0.6	0.9	1.1	1.8	0.204
Extracted teeth	3.4	3.3	4.5	4.4	0.388
Filled teeth	5.6	4.2	1.1	1.6	**0.000***
Total number of teeth	24.6	5.6	23.4	4.4	0.170
Plaque index	0.9	0.8	1.3	1.2	0.133
Gingival index	1.1	0.8	1.6	1.2	0.09
Sulcus bleeding index	1.1	0.7	1.7	1.2	**0.04***
Periodontal pocket depth	2.1	0.4	2.7	0.6	**0.000***
Clinical attachment level	3.3	1.01	3.9	1.5	0.103
Frequency of tooth brushing	1.9	0.6	1.01	0.7	**0.000***
Last dental visit (months)	5.1	7.2	28.6	23.7	**0.000***

[a]Mann–Whitney *U*-test was used in the analysis.
*Statistically significant.

those in UK (5.6 ± 4.2 and 1.9 ± 0.6, respectively) ($P = 0.000$). The scores of SBI and PPD were also higher in Turkish patients (1.7 ± 1.2 and 2.7 ± 0.6, respectively) than in those of UK (1.1 ± 0.7 and 2.1 ± 0.4) ($P = 0.04$ and $P = 0.000$, respectively). The duration from the last dental visit (5.1 ± 7.2 months) was significantly lower in UK than that in Turkey (28.6 ± 23.7 months) ($P = 0.000$) (Table 4). No significant correlation was observed between OHIP-14 and scores of dental and periodontal health and duration of last dental visit in both patient groups ($P > 0.05$).

In OHIP-14, Cronbach-alpha values were found to be 0.9523 in UK patients and 0.9271 in Turkish patients. Cronbach-alpha values were also calculated for physical symptoms, psycho-social symptoms and psychological symptoms of OHIP-14 in both UK and Turkey (0.8735, 0.8847 and 0.8448 in UK patients vs. 0.8286, 0.8969 and 0.8230 in Turkish patients).

Discussion

Oral QoL reflects people's experiences influencing their well-being or satisfaction of life (13). Oral health, problem-based dental visits because of dental and oral mucosal problems and oral hygiene practice are important variables influencing oral QoL (14). The OHIP-14 is a reliable questionnaire in oral disease as a patient-centred outcome measure. The presence of discomfort and pain, poor oral functions and dissatisfaction with oral health are the main areas for impaired oral QoL (15–17).

In this study, scores of OHIP-14, subscales of OHIP-14, pain status and healing time of oral ulcers were found to be similar in active patients from UK who were mainly under immunosuppressive treatment and from Turkey using mainly colchicine. The number of oral ulcers in UK patients and the healing times of oral ulcers in Turkish patients were related with poor oral QoL status. The presence of oral ulcers was reported to be a risk factor for poor oral QoL status in patients both from Turkey (8, 9) and UK (18).

Poor oral health, lower utilization of dental services and infrequent tooth brushing were reported in Turkish patients when compared with those in the UK. As expected, a lack of oral hygiene practice and low rate of utilization of dental services might have led to a poorer oral health status in Turkish patients. Oral health belief of populations, personal oral hygiene practices and dental care delivery systems are important component of patient's oral health (19). Therefore, these differences between patients from UK and Turkey can be predicted.

The number of oral ulcers was found to be higher in patients from UK compared with those in Turkey. Moreover, oral ulcers were more active under immunosuppressive treatment in UK. This was unexpected as BD is known to have a more severe disease course in patients originating from the Mediterranean countries. However, the disease severity score reflecting general disease status was similar in both patient groups. This suggests that there may a referral bias between the two institutions associated with the disease manifestations of BD patients as the tertiary referral centre in UK is an oral clinic whereas the Turkish centre is based in a rheumatology department. A second explanation may be the geographical variables and ethnical diversity, which are previously implicated for the different disease spectrums in patients with BD, especially reported between Caucasian and Middle-Eastern patients (20). Patients from UK had both good oral health and a severe oral ulcer activity, whereas poor oral health and less severe oral ulcer activity were seen in patients from Turkey in the present study. Oral QoL conditions were similar in both patient groups. As oral QoL status is affected by both poor oral health, including dental and periodontal problems, and oral mucosal pathologies such as oral ulcers (15, 16), similar oral QoL conditions could result as an outcome of different oral conditions in patients from UK and Turkey.

Poor oral health was found to be related to an increased severity of disease in Turkish BD patients, in our previous study (7). However, a similar trend was not present in UK. Oral environment, associated with infection-related factors including *Streptococcus* spp., herpes simplex virus and oral hygiene status, is possibly only one of diverse aetiopathogenetic factors in BD, as genetic background and immune mechanisms are also crucial (1–5).

Limited number of studies evaluated disease patterns between UK and Turkey in the literature (21, 22). When clinical spectrum and HLA associations were compared between patients from UK and Turkey, different HLA associations (21) and an absence of pathergy phenomenon (22) among the patients from UK were observed. Another study comparing ethnical differences in Germany observed a more severe disease spectrum in BD patients with Turkish origin (23).

Consequently, patients from both UK and Turkey with active oral ulcers had the same impairment in their oral QoL status in our study. The number of oral ulcers was observed to be higher in UK. However, as expected, lack of tooth brushing and a lower utilization rate of dental services might have led to a poorer oral health in patients from Turkey.

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