Why are Behçet's disease patients always exhausted?

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ABSTRACT

Objective. *Patients with Behçet's disease (BD) constantly complain of fatigue and many have problems with poor sleep. This ultimately has a major impact on all aspects of normal living. To attempt to understand this, Artificial Intelligence (AI) was used to identify potential biomarkers. These were alpha-melanocyte stimulating hormone (α-MSH), vasoactive intestinal peptide (VIP) and some inflammatory cytokines. We assessed the association of fatigue, quality of sleep and disease activity with circulating concentration of α-MSH, VIP and inflammatory cytokines.*

Methods. *There were 127 participants, 97 BD patients, and 30 healthy controls (HC). All completed the Multi-Dimensional Assessment of Fatigue questionnaire (MAF) and the Pittsburgh Sleep Quality Index (PSQI) on the day of their clinical assessment. Enzyme-linked immunosorbent assays (ELISA) were used to evaluate the serum concentrations of α-MSH, VIP and cytokines (IL-1β, IL-6, IL-10, and TNF-α).*

Results. *64% of BD patients experienced high fatigue scores, and 63% had poor quality of sleep. When BD and HC were compared the MAF and PSQI scores as well as the serum concentrations of α-MSH, VIP, and IL-6 were significantly higher in BD (p values were: 0.001, 0.001, 0.001, 0.004 and 0.036, respectively). Both α-MSH and IL-6 had significant impact on MAF and PSQI. Interestingly, VIP had a significant influence on PSQI and disease activity, but not on MAF.*

Conclusion. *A better understanding of these complex clinical and biochemical interactions between α-MSH, VIP and IL-6 might lead to the development of novel approaches to manage fatigue and sleep disorders as well as disease activity in BD patients.*

Introduction

Fatigue and disturbed sleep patterns are frequent symptoms in BD. They have a major impact on patients' psychology, social well-being, disease activity and quality of life (QoL) (1, 2). Medically, fatigue is often unexplained and BD patients often have difficulty in describing fatigue symptoms which may or may not be related to disease activity and/or drugs. It causes physical and psychological problems, which may be exacerbated by secondary contributory factors including depressive mood, sleep disturbances and an unhealthy lifestyle (3). Studies using fatigue questionnaires have shown that BD patients tend to have high fatigue scores throughout life (4-6). Fatigue is reported to be a common feature of a wide variety of conditions including chronic inflammation, infectious, neurological and psychiatric diseases and cancer (7). Furthermore, the quality of sleep is another major problem in BD patients that might have association with fatigue and the complexity of symptoms and signs of the BD activity (6). Anxiety and depression might also cause negative effects on the quality of sleep in BD (8). There is no specific biological molecule/biomarker to assess fatigue and sleep disturbance in patients. At present sleep questionnaires are the most common method to measure the quality and pattern of sleep in adults. In this study, to understand why most of the BD patients are always tired, we used mathematical modelling to identify any known or novel proteins or peptides which may contribute to poor quality of sleep, the increase of fatigue and to active systemic problems in BD. One of the key immune-pathological features of BD is the high level of pro-inflammatory Th1 cytokines (IL-2, IL-12, IL-18, IL-27, and IFNγ), and Th2 cytokines (IL-2, IL-10, IL13 and TNFβ/LTα) which play important roles

in the onset and perpetuation of the disease (9, 10). Cytokines such as IL-1, IL-6 and TNF-α are known to exert both paracrine and endocrine effects. Therefore, they mediate not only local but systemic responses to physiological and/or pathological stimuli. Moreover, the serum levels of cytokines elevated in patients with fatigue and poor sleep (11).

The α-MSH level in patients with chronic fatigue syndrome (CFS) was higher than in healthy groups and is considered to be caused partly by chronic stressful events (12). The authors suggested that α-MSH could be a potential biological marker for the diagnosis of CFS, at least during the first 5 years after its onset. In addition, α-MSH level may be higher in fatigue related conditions such as insomnia, sleep apnoea and inflammatory diseases caused by viral infections (12, 13). The effects of α-MSH may be due to an immunomodulatory/immunosuppressive activity (14-16), and may act as a suppressor of IFN-γ, TNF-α, IL-1, IL-6, IL-8 and induces the immunosuppressive cytokine IL-10 (17).

Vasoactive intestinal peptide (VIP), belongs to the secretin/glucagon superfamily of molecules and functions as a hormone, neurotransmitter, neurotrophic agent and immune modulator (18). It is important in limiting ongoing inflammatory and immune responses, preventing platelet aggregation and promoting resolution of inflammation, by downregulating a wide spectrum of inflammatory cytokines, chemokines and mediators of oxidative stress by influencing the Th1 to Th2 shift and suppressing pro-inflammatory activity through the Protein Kinase-A (PKA)/ Cyclic Adenosine 3',5'-monophosphate (cAMP) pathway (19-21). Following severe infection and/or significant physical exercise chronic fatigue may develop due to the loss of immunological tolerance to vasoactive neuropeptides (18).

The mathematical modelling was done and indicated that α-MSH, VIP and inflammatory cytokines may be important molecules influencing fatigue, sleep and disease activity. Therefore, in this study, we investigated whether α-MSH, VIP and inflammatory cytokines were similar in BD patients compared with healthy participants. We also scrutinised the association of these biological molecules with oral and genital ulceration activity as well as the Behçet's disease current activity form (BDCAF). The BD patients' prescribed medication was also assessed and correlated with the study outcomes.

Materials and methods

This prospective study included a cohort of 100 BD patients diagnosed according to the International Study Group Criteria (ISG 1990) and International Criteria of Behçet's Disease (ICBD 2014) criteria. The convenience sampling method was used to recruit study participants. 3 patients were excluded from the study due to incomplete clinical information (44 males: 53 females; mean age: 38.9±1.20 years). 30 healthy control participants (HC), (14 males: 16 females; mean age: 34±2.17 years) were invited and recruited to the study, and informed consents were obtained from all the participants.

Ethical approval for this study was granted by Queen Mary University of London Ethics of Research Committee (P/03/122) at Barts Health NHS Trust.

BD and HC groups were divided into eight subgroups based on fatigue score and the Pittsburgh Sleep Quality Index (PSQI): (61; 62%) BD had high fatigue scores and (33; 34%) BD low fatigue scores, (5; 17%) HC had high fatigue scores and (25; 83%) HC low fatigue scores while (38; 63%) BD patients reported poor PSQI, (22; 37%) BD had good PSQI, while (8; 27%) HC had poor PSQI and (22; 73%) HC had good PSQI.

Mathematical modelling for candidate molecules

We have applied an AI tool (THERA-Metrics Discovery AG, Stans, Switzerland) to collect and analyse information on the disease and the potentially related biomarkers. It is based on Computational Linguistics and Mathematical Graph Theory, a methodology defined to build a visual graph representation of knowledge. This software is a fully unbiased, data-driven system using peer-reviewed literature only, with no pre-defined knowledge structure, and automatically linking concepts gathered on drugs, pathways and biological activities with the pathophysiological signs and symptoms of diseases, thus creating novel working hypotheses based on biological and biochemical reasoning (22).

Clinical investigations

1. Multi-Dimensional Assessment of Fatigue (MAF)

MAF is a validated, patient self-reported scale to assess fatigue score in the preceding week, consisting of 16 items covering four dimensions of fatigue: 1) severity, 2) distress, 3) interference in activities of daily living (*e.g.*, household chores, cooking, bathing, dressing, working, socialising, sexual activity, leisure/recreation, shopping, walking, and other exercise), 4) frequency and change during the previous week. The items are used to calculate scores for each of the four dimensions and 15 of the 16 questions are used to calculate the global fatigue index (GFI) (23). The GFI ranges from 1 (no fatigue) to 50 (severe fatigue). In our cohort, the patients' scores were ranged from 1 (no fatigue), 2-20 (low fatigue), and 21 and over were (high fatigue) group, this classification was based on our cohort cut-off score of 20 with sensitivity of 88% and specificity of 76%. The subgroups are part of statistical analysis to interpret and explain the data, and to match the statistical analysis with PSQI subgroups in this study.

2. The Pittsburgh Sleep Quality Index (PSQI)

PSQI is a self-rated questionnaire which assesses the quality of sleep and disturbances over the last 4 weeks. A scale of 19 items produce seven "component" scores; 1) subjective sleep quality, 2) sleep latency, 3) sleep duration, 4) habitual sleep efficiency, 5) sleep disturbances, 6) use of sleeping medication, and 7) daytime dysfunction. A total PSQI score corresponding to the total of individual scores from the above seven components results in a range between (0-21), a score equal

to 5 or less indicated a good quality of sleep for each patient (1). The PSQI has a sensitivity of 89.6% and specificity of 86.5% for identifying cases with sleep disorder, using a cut-off score of 5 (24).

3. Outcomes measurements of BD activity

The clinical assessment of the BD cohort was carried out by using multiple outcome forms for monitoring patients in the 4 weeks prior to the clinic appointment; genital ulcer severity score (GUSS) and oral ulcer severity score (OUSS) forms (25) are validated tools to assess orogenital ulcers in BD patients that include the six ulcer characteristics (number, size, duration, ulcer-free period, pain and site). BD current activity form (BDCAF) is a well-established tool for the assessment BD activity in the clinic, which scores the history of clinical features on scale of 0-12: headache, mouth ulcer, genital ulcer, ocular symptoms, skin lesions, erythema nodosum, joint pain, joint swelling, blood vessel involvement, gastrointestinal, and central nervous system complications, which present over the four weeks prior to the day of assessment (26). This clinical information was recorded in the patients' medical files by clinicians or a senior nurse on the day the patients were present at the clinic. Information related to the treatment for each patient (non-steroidal anti-inflammatory drugs [NSAIDs], colchicine, azathioprine, mycophenolate mofetil (MMF), biologic agents, and/or corticosteroids) was also recorded.

Laboratory investigations

Enzyme-linked immunosorbent assay (ELISA) to measure serum concentration of α-MSH, VIP and specific four cytokines.

Blood was collected into 8mL vacutainers containing gel (BD Vacutainer® System, Devon, UK), and gently mixed by inversion, on the day of patients' clinical assessment. After centrifuged at 1000xg for 15min, the separated serum samples were aliquoted in single-use volumes to avoid multiple freeze-thaw cycles and stored at -80ºC until use. Serum concentrations of α-MSH, VIP (BD: HC= 83:30), IL-1β, IL-6, IL-10, and TNF-α (BD: HC= 52:30) were measured by ELISA, according to the manufacturer's instructions (α-MSH; VIP; Cloud-Clone Corp, USA. Cytokines: RandD Systems, bio-techne, Abingdon, UK). The standard curves were generated for both molecules as manufacturer's instructions. The minimum detectable dose (MDD) of human α-MSH and VIP were typically less than 48.8 pg/ml and 2.95 pg/ml respectively.

The levels of IL-1β, IL-6, IL-10, and TNF-α were quantified by using "Quantikine" Kits. The minimum detectable dose (MDD) of human IL-1β, IL-6, IL-10 and TNF-α were 1 pg/ml, 0.7 pg/ml, 3.9 pg/ml, and 1.6 pg/ml respectively. The subject samples were assayed in duplicate, values averaged and expressed as concentrations (pg/ml) relative to a 6-point standard curve as recommended by the manufacturer.

Stop solution (2N sulfuric acid) was added to each well. The optical density of the samples was analysed using 96-well microplate reader (BMG LABTECH, OPTIMA software version 2,00R3) set at the appropriate wavelengths for subtracting readings at 540 nm/570 nm from the readings at 450 nm. This subtraction step was done to correct the optical imperfections in the plate. The results averaged and expressed as concentrations (pg/ml). The results from the laboratory tests included alpha-MSH, VIP, IL1β, IL6, IL10 and TNF-α serum concentration which correlated statistically with the patients' clinical information, included BDCAF, disease activity and the fatigue, quality of sleep, oral ulcer severity OUSS, and genital ulcer severity scores GUSS.

Statistical analysis

Descriptive statistics of data were computed as mean ± SD, median, minimum and maximum value and qualitative variables presented as number (%). The parametric comparisons between groups were analysed by Independent sample t- test and ANOVA. Mann-Whitney test and Kruskal-Wallis were used for non-parametric data. Spearman's rank test and Pearson's coefficient were used to analyse non-parametric and parametric evaluations, respectively. The cut off serum concentration for biological molecules were determined using receiver operating characteristic (ROC) curve analysis. Accuracy was measured by the area under the ROC curve (AUC). An area of 1 represents a perfect test; an area of 0.5 represents a worthless test. *p*-value <0.05 was considered statistically significant. Regression analysis was also carried out and the Tables are presented in supplementary information.

Results

Ninety-seven BD patients (44 males: 53 females; mean age: 38.9±1.20 years) and 30 healthy participants (14 males: 16 females; mean age: 34±2.17 years) were enrolled in this study.

The participants were divided into eight subgroups; Group 1= 64% BD high fatigue score, Group 2= 36% BD low fatigue score, Group 3= 17% HC high fatigue score and Group 4= 83% HC low fatigue score, and Group 5= 63% BD poor PSQI, Group 6= 37% BD good PSQI, Group 7= 27% HC poor PSQI and Group 8= 73% HC good PSQI.

Descriptive results and disease presentation

Sixty-four BD patients had active disease on the day of clinical assessment, (29; 30%) had oral ulceration, (26; 27%) arthropathy, (20; 21%) skin lesions, (17; 18%) headaches and other central nerve system (CNS) symptoms, (4; 4%) genital ulceration, and (2; 2%) gastrointestinal tract (GIT) and (2; 2%) had vascular problems each. During this study none of the patients had any ocular symptoms (no ocular activity and/or the progress of ocular symptoms were controlled). In addition, none of the patients in this study had a positive pathergy test.

Colchicine and azathioprine were the most frequently prescribed medication in this cohort. (14; 15%) patients had colchicine alone, (7; 7%) patients had azathioprine alone, (5; 5%) had prednisolone, (6; 6%) had MMF and (8; 8%) had adalimumab (humira). (4; 4%) BD patients were taking anticoagulants and

Table I. The BD symptoms and immunosuppressive treatment at the time the serum samples were collected.

Systemic involvement	Patients (No; %)
Oral ulceration	29/97; 30%
Arthropathy	26/97; 27%
Skin lesions	20/97; 21%
Headaches and other central nerve system (CNS) symptoms	17/97; 18%
Genital ulceration	4/97; 4%
Gastrointestinal tract (GIT)	2/97; 2%
Vascular problems	2/97; 2%
Ocular lesions	Non
Positive pathergy test	Non
Grouped treatment protocols	
Colchicine and azathioprine	7/97; 7%
Colchicine and antidepressants	2/97; 2%
azathioprine and prednisolone	3/97; 4%
azathioprine and methotrexate	1/97; 1%
Mycophenolate mofetil and prednisolone	8/97; 8%
Mycophenolate mofetil and warfarin	2/97; 2%
Adalimumab and azathioprine	3/97; 4%
Infliximab and azathioprine	2/97; 2%
Adalimumab and Mycophenolate mofetil	2/97; 2%
Infliximab and prednisolone	2/97; 2%
Infliximab, azathioprine and colchicine	1/97; 1%
Infliximab, azathioprine and prednisolone	1/97; 1%
No medication	10/97; 10%

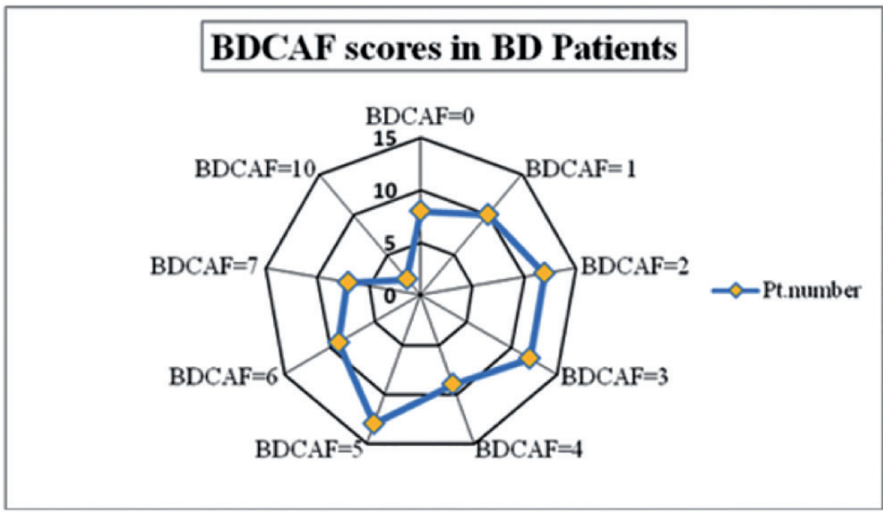

Fig. 1. Distribution of BDCAF scores in BD patients.
8 patients scored 0 (where no BD symptoms), 10 patients had a score of 1 (only one BD symptoms out of 12), 12 patients scored 2 (two BD symptoms out of 12) and scored 3 (three BD symptoms) each, 9 patients had a score of 4 (four BD symptoms out of 12) and a score of 6 (six BD symptoms out of 12) each, 13 patients scored 5 (five BD symptoms out of 12), 7 patients scored 7 (seven BD symptoms out of 12), and 2 patients had a score of 10 (ten BD symptoms out of 12).

(6; 6%) were taking aspirin. The rest of BD patients were on combination therapy outlined in (Table I).

The average of the BDCAF score of BD patients in the last 4 weeks of clinical appointment was (4.1±1.6; mean±SD), 8 patients scored 0 (no BD symptoms), 10 patients had a score of 1 (only one symptom of BD), 12 patients had score of 2 and 3 each, 9 patients scored 4 and 6 each, 13 patients had a score of 5, 7 patients had a score of 7, and 2 patients scored 10 (Fig. 1).

There were no significant age and gender differences between the patients and HC subjects (Table II). The average for the fatigue score in BD patients was (28.38±1.56; mean±SD) and the PSQI average for BD patients was (9.4±0.67; mean±SD) and both were statistically significant compared to HC groups (p=0.001), respectively. The α-MSH concentration (ng/ml) was significantly higher in BD patients compared to HC (p=0.001). In addition, there were significant differences of VIP concentration (pg/ml) between BD and HC (p=0.004). Table III displays the cytokines concentration in detectable serum samples of BD and HC participants. There was a statistically significant difference in the level of IL-6 cytokines between BD and HC groups (p=0.036). However, IL-1β, IL-10 and TNF-α were not significant between BD and HC, p values >0.05 (Table II).

Fatigue and PSQI with demographic data and clinical outcomes

When BD and HC fatigue groups (4 groups) were analysed based on gender. In the BD group, 46 out of 53 females had high fatigue scores compared with 15 out of 33 males. In the HC group, there were 3 out of 16 females with high fatigue compared with 2 out of 14 males.

Statistical analysis using the Chi-square test showed a significant difference between patients' gender and fatigue (p=0.001) in BD, whereas there was no significant difference between the healthy group (p=0.337).

Both males and females in the BD and HC cohorts segregated into distinct groups reporting low or high fatigue. There was no significant difference in the MAF scores for BD and HC based on gender. However, female BD patients reported significantly higher levels of fatigue compared with HC females in the high fatigue group (Fig. 2).

In BD, the BDCAF scores were significantly higher between both fatigue groups (high: low) and PSQI groups (worse: good) (p=0.001 each), and with clinician rating of disease activity (p value; 0.001 and 0.005, respectively). The BD fatigue groups (high: low) and PSQI groups (worse: good) had higher significant differences with OUSS and GUSS in patients (p values were: 0.001, 0.009, 0.006, and 0.018) respectively (Table IV).

Regression analysis was performed to

Table II. Clinical characteristics and study factors of BD patients and healthy controls.

Clinical factors	BD patients (minimum value-maximum value; median; mean ± SD)	HC participants (minimum value-maximum value; median; mean ± SD)	*p* value
Gender (M: F)	44:53	14:16	0.77
Age	20-68; 38.5; 38.9±1.20	21-60; 32; 36.1±2.17	0.163
BDCAF	0-10;3; 3.5±0.25	Not present	-
OUSS	0-46;15; 14.8±1.29	Not present	-
GUSS	0-31; 1; 4.35±1.1	Not present	-
Systemic activity			
Active	64	Not present	-
Quiet	33		
Outcomes measures			
Fatigue score	7-48; 32.35; 28.38±1.56	1-30.4; 10.8; 12.36±1.77	**0.001***
PSQI score	1-20; 11; 9.4±0.67	0-12; 4; 4.74±0.72	**0.001***
α-MSH concentration (ng/ml)	1.4-4.2; 3.06; 3.02±0.078	1.5-2.2; 1.92; 1.93±0.03	**0.001***
VIP (pg/ml)	36.6-323; 141.7; 148±77	66-124; 106.7; 104.1±16	**0.004***
Cytokines (pg/ml)			
IL-1β	0.21-.619; 0.29;0.36±0.1	0.044-3.9; 0.54; 1.13±0.5	0.339
IL-6	0.37-2.77; 1.59; 1.58±0.3	0.18-0.90; 0.34; 0.44±0.1	**0.036***
IL-10	3.12-6.25; 3.56; 4.79±0.7	0.85-24.5; 7.31; 10.9±7.1	0.470
TNF-α	0.61-26.1; 7.75; 9.57±4.1	0.09-16.8; 1.54; 2.41±0.9	0.152

PSQI: Pittsburgh sleep quality index; BDCAF: Behçet's disease current activity form; OUSS: oral ulcer severity score; GUSS: genital ulcer severity score; α-MSH: alpha-melanocyte stimulating hormone; VIP: vasoactive intestinal peptide; Not present: these are healthy controls without any manifestations. *p values <0.05.

Table III. Serum samples of BD and HC participants where cytokine concentration was detectable.

Cytokines (MDD) (pg/ml)	No. of positive samples (BD) [No. active BD: No. inactive BD]	No. of positive samples (HC)
IL-1β (1 pg/ml)	21/52 [13:6]	7/30
IL-6 (0.7 pg/ml)	47/52 [29:11]	4/30
IL-10 (3.9 pg/ml)	41/52 [29:8]	3/30
TNF-α (1.6 pg/ml)	23/52 [8:12]	17/30

MDD: minimum detectable dose.
Lowest detectable levels of cytokines on the standard curves are shown in pg/ml.

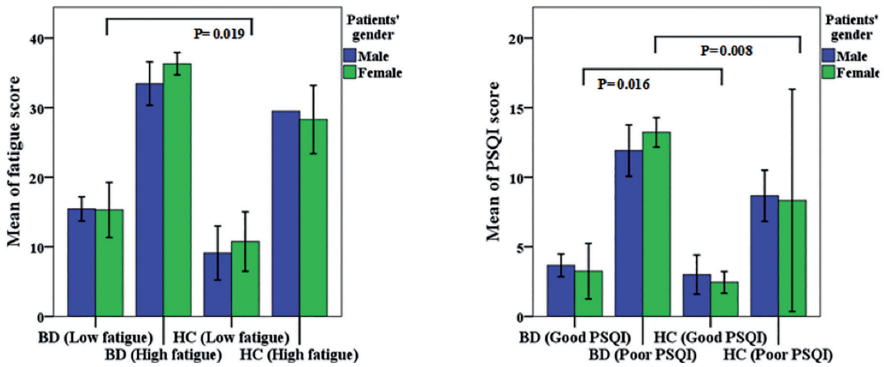

Fig. 2. Fatigue and quality of sleep in BD patients based on gender.
Low fatigue groups (males and females) of BD had a significant difference compared with HC groups of low fatigue (p=0.019). Moreover, the PSQI was greater in BD with good and poor PSQI comparing with HC of good and poor PSQI (p=0.016 and p=0.008) respectively.

test the influence of both BD symptoms (activity) and individual BD medication on fatigue and quality of sleep. Arthropathy was the only symptom that had a significant impact which increased the fatigue score and amplified PSQI score. There were no correlations or impact of the immunomodulatory medication on both the fatigue score and PSQI score.

Fatigue and disease activity in BD patients

Regression analysis was used to investigate the effect of the fatigue score (the predictor variable) on the BDCAF score (dependent variable). The outcome of this regression was: R= 0.554; R^2= 0.307; p=0.001, (Model 1; Table S1). The coefficient of the standardised beta value was 0.554. This indicates that fatigue in BD patients is significantly associated with an increase in the BDCAF score in this cohort, by a percentage of 55% of the variance. Additionally, the fatigue score (the predictor variable) was using to evaluate its impact on the clinician rating of disease activity (dependent variable) was: R= 0.50; R^2= 0.25; p=0.001 and beta value was 0.500, (Model 2; Table S1). This indicates that the fatigue score can increase the clinical disease activity by a 50% of the variance.

Quality of sleep and disease activity in BD patients

Model 3; Table S1 demonstrates that the regression analysis of PSQI (predictor variable) and BDCAF score (dependent variable). The result of this regression analysis was: R= 0.43; R^2= 0.19; p=0.001, and beta value was 0.432. This denotes that the PSQI is associated with an increase BDCAF score in BD by a percentage of 43% of variance. In addition, BD systemic activity (dependent variable) and PSQI (predictor variable): R= 0.37; R^2= 0.14; p=0.01, indicates that the PSQI is responsible for increasing BD systemic activity by a 37% of the variance, (Model 4; Table S1).

Association of α-MSH and VIP serum concentration with fatigue, quality of sleep and clinical outcomes

The mean of α-MSH concentration was significantly greater in BD patients who had high fatigue score (3.26±0.45 ng/ml) than those with low fatigue score (2.68±0.89 ng/ml) (p=0.01). In contrast, HC groups of high and low

Table IV. Clinical characteristics of BD patients and the differences between fatigue and PSQI groups.

Clinical outcomes	Pt. Fatigue groups (mean±SE low fatigue: mean±SE high fatigue (*p* values)	Pt. PSQI groups (mean±SE good PSQI: mean±SE worse PSQI (*p* values)
Fatigue score	(15.5±0.7: 35.5±0.76) *p*=**0.001***	(18.2±1.7:34.2±1.6) *p*=**0.001***
PSQI score	(5.57±0.83: 11.7±0.72) *p*=**0.001***	(3.6±0.33:12.7±0.5) *p*=**0.001***
BDCAF	(1.66±0.27: 4.41±0.28) *p*=**0.001***	(2.1±0.42:4.24±0.4) *p*=**0.001***
Systemic activity	(0.28±0.08: 0.71±0.06) *p*=**0.001***	(0.20±0.1:0.6±0.1) *p*=**0.005***
OUSS	(9.8±1.52:17.6±1.64) *p*=**0.001***	(11±1.9:19.1±2.3) *p*=**0.009***
GUSS	(1.03±1.03:5.9±1.4) *p*=**0.006***	(1.45±1.45:7.34±2) *p*=**0.018***

PSQI: Pittsburgh sleep quality index; BDCAF: Behçet's disease current activity form; OUSS: oral ulcer severity score; GUSS: genital ulcer severity score.
*p values<0.05.

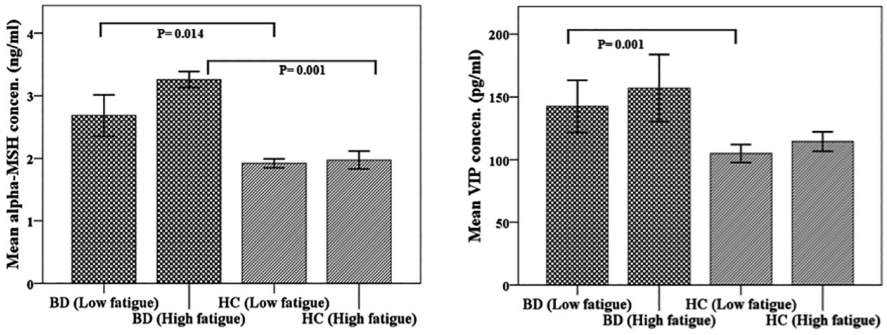

Fig. 3A. The differences of alpha-MSH and VIP serum concentration in fatigue groups of BD and HC participants.
α-MSH concentrations were significantly greater in BD high fatigue than low fatigue score, also the significant was noticed between BD and HC of high and low fatigue. In contrast, HC groups of high and low fatigue scores showed no significant results in α-MSH concentration (p=0.37). There were significant differences between α-MSH of BD low fatigue and HC low fatigue (p=0.014), and between BD high fatigue and HC high fatigue groups (p=0.001). Significant differences of VIP serum concentrations were observed between BD and HC with low fatigue scores (p=0.001).

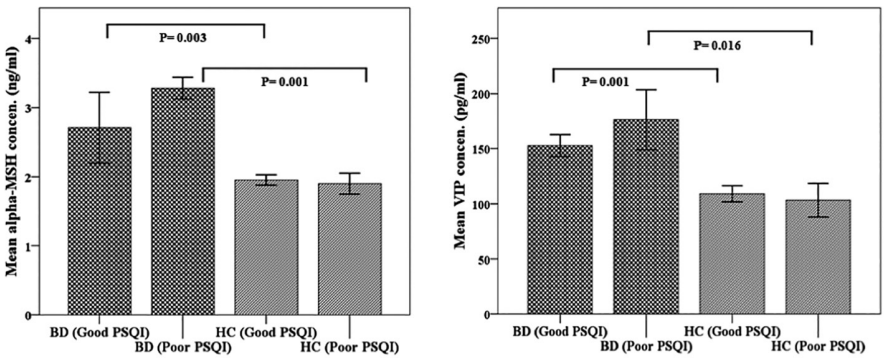

Fig. 3B. The differences of alpha-MSH and VIP serum concentration in PSQI groups of BD and HC participants.
The significant differences based on α-MSH were found between BD of good and poor PSQI; also, between BD good and poor PSQI group and HC of good and poor PSQI group (p=0.003, and p=0.001) respectively. The VIP serum concentrations were higher in BD with good and poor PSQI groups compared with HC of good and poor PSQI groups; p<0.05.

fatigue scores showed no significant differences in α-MSH concentration (p=0.37). Additionally, there were higher significant differences between α-MSH in BD low fatigue and HC low fatigue (p=0.014), and between BD high fatigue and HC high fatigue groups (p=0.001). There was higher significant difference of VIP serum concentrations in BD and HC with low fatigue scores (p=0.001) (Fig. 3A).

In terms of quality of sleep, the significant difference in α-MSH concentration was found between BD good and poor PSQI compared to HC (p=0.003, and p=0.001 respectively). The comparison of the VIP serum concentration in BD and HC between those with poor PSQI, and good PSQI groups had significant differences (p=0.016, and p=0.001 respectively) as shown in Figure 3B.

Pearson's correlation coefficient was applied to investigate the correlation between the study clinical outcomes variables with α-MSH and VIP concentrations. There was a positive correlation between α-MSH with PSQI, also between α-MSH with the fatigue score (R= 0.348, p=0.012 and R= 0.439, p=0.001, respectively). However, VIP concentration had a positive correlation with the PSQI score (R= 0.349, p=0.016). In addition, OUSS, GUSS, and BDCAF scores had positive correlations with the fatigue score and PSQI; p values were significant <0.05.

Fatigue score had a strong positive association with PSQI, therefore, multiple regressions were performed to investigate PSQI questionnaire variables and fatigue scores. This showed that the day-time dysfunction and sleep quality questions were the main variables which had significant impact to increase the fatigue score of BD patients (R= 0.749; p=0.001) and beta values were; 0.35 and 0.33, respectively (Table S2).

Based on the disease activity and the differences in α-MSH and VIP serum concentration, the data shows that VIP was significantly associated with disease activity (p=0.019). The Mann-Whitney test was performed to compare BD symptoms with VIP concentrations, and the only significant

association noted was between BD patients who had arthropathy and CNS symptoms (mean serum VIP= 66 pg/ml and 156 pg/ml respectively; p=0.043). ROC curve analysis suggests that the optimum α-MSH concentration cut-off point for BD patients with high fatigue was 2.89 ng/ml, had a sensitivity, specificity and accuracy of 79% 70% and 0.774, respectively (95% confidence interval 0.639-0.902, p=0.001) (Fig. S1).

The single regression analysis illustrations that the association between fatigue score (dependent variable) and α-MSH (predictor variable) was significant: R= 0.44; R^2= 0.193; p=0.001. The beta value suggests that (α-MSH) is responsible for about 44% of the variance to increase the fatigue score (Model 5; Table S3). The VIP concentration (predictor variable) does not have an impact on the fatigue score (dependent variable); p=0.152 (Model 6; Table S3).

The α-MSH concentration (predictor variable) had a positive association with PSQI (dependent variable): R= 0.35; R^2= 0.121; p=0.012, (Model 7; Table S3), and beta value suggests that the BD patients who had high serum concentration of α-MSH to can increase score of PSQI (poor quality of sleep) by 35% of the variance. Also, the VIP concentration (predictor variable) and PSQI (dependent variable) was significant: R= 0.343; R^2= 0.112; p=0.018. The beta value suggests that the VIP concentration was responsible for more than 34% of variance to increase score of PSQI (Model 8; Table S3).

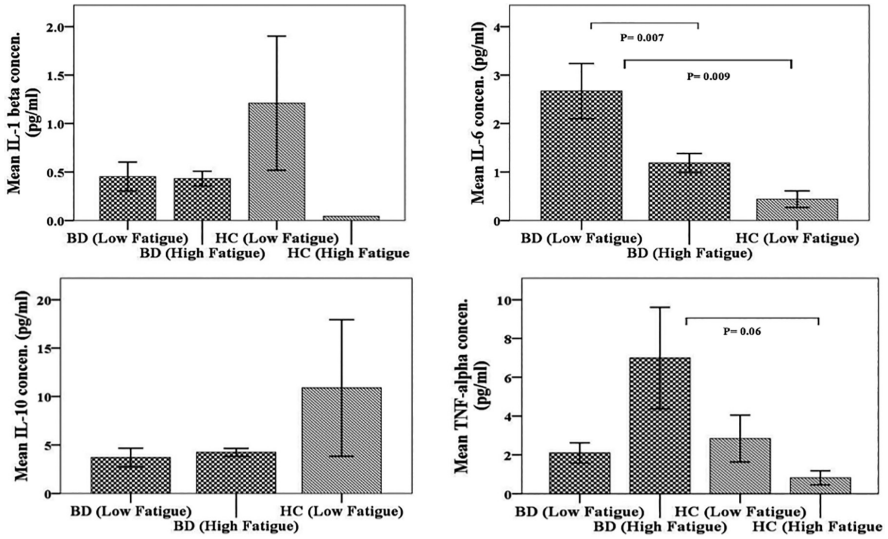

Fig. 4A. The differences of cytokines concentration in fatigue groups of BD and HC.
BD low fatigue group had higher concentration of IL-6 than BD high fatigue group and HC fatigue groups (p=0.007 and p=0.009) each. TNF-α just failed to reach significance between BD high fatigue group and HC high fatigue group (p=0.06). IL-10 and IL-1β were not significant between BD and HC of fatigue groups.

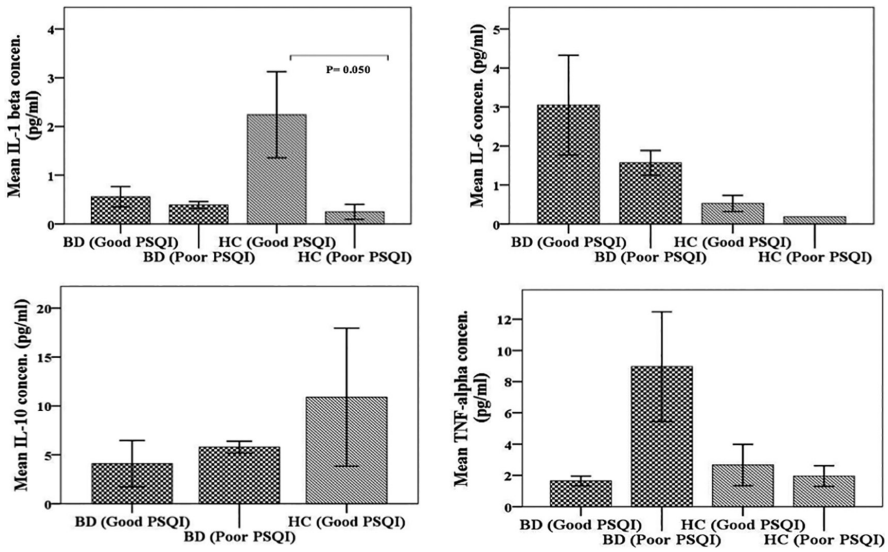

Fig. 4B. The differences of cytokines concentration in PSQI groups of BD and HC.
IL-1 β in HC of good and poor PSQI groups had a p=0.050. The mean value for IL-6 was greater in BD good PSQI than HC good PSQI group (p=0.08); however, the range for BD (Good PSQI) was wide so that the analysis just failed to reach significance. IL-10 was not significant between the most of the PSQI of BD and HC groups. p value of TNF-α concentration was not significant between BD groups of good and worse PSQI.

Association of IL-6 and TNF-α with fatigue, quality of sleep and clinical outcomes

The serum cytokines concentration did not illustrate any statistically significant differences in HC of fatigue groups and PSQI groups. However, in BD patients the serum cytokine concentration of IL-6 was significantly greater in low fatigue group than the patients of high fatigue group (3.02±2.19; p=0.007) (Table S4). However, the other cytokines IL-1β, IL-10 and TNF-α did not show any statistical significance between the low and high fatigue groups. Furthermore, IL-1β, IL-6 and IL-10 concentrations were very similar between PSQI groups of BD patients.

IL-1β concentration was significant only in HC good PSQI group comparing with HC poor PQSI group (p=0.05). Moreover, BD of low fatigue had greater levels of IL-6 than HC low fatigue group (p=0.009). IL-10 concentration was not significant between any of BD fatigue groups and PSQI groups nor between HC groups. P value of TNF-α concentration was 0.06 between BD and HC with high fatigue groups (Fig. 4A and 4B).

Spearman's correlation coefficient revealed that α-MSH had a moderate negative correlation with IL-6 (R= -0.368, p=0.030), whereas, α-MSH had a positive correlation with TNF-α

(R= 0.495, p=0.023). In addition, VIP did not show any statistical significance (Table S5).

Those cytokines; IL-1β, IL-6, IL-10, and TNF-α did not show any statistical significant with BD systemic activity (p=0.690, p=0.656, p=0.734 and p=0.137, respectively). However, TNF-α showed a positive significant correlation with BDCAF score (R= 0.515, p=0.012).

ROC curve analysis suggests that 1.3 pg/ml was the IL-6 cut off point of BD low fatigue group in this study, with a sensitivity, specificity and accuracy of 79%, 77% and 0.777, respectively (95% confidence interval 0.635-0.920; p=0.003) (Fig. S2).

Discussion

Presently, there are no available biomarkers to assist in the diagnosis or in treating fatigue or to improve the quality of sleep. In this study, most of our patients presented with fatigue and/or poor quality of sleep. It is therefore not surprising that quality of sleep pattern and daytime dysfunction items of the PSQI questionnaire had a significant impact on the fatigue score in patients with BD. The first question was addressed in this article is whether gender and age of patients had any association with fatigue and quality of sleep. Age appeared not to be a significant factor in either the BD or HC groups of fatigue or quality of sleep. However, gender was significant related to both fatigue and quality of sleep. Females with BD and the HC group had higher fatigue scores when compared to males. The explanation for the distinction between female and male scores may be related to the hormonal changes in females (during menstrual cycle and menopause) increasing the risk of fatigue (27).

As mentioned above, fatigue was a common problem in this cohort, and was seen predominantly in patients who suffered from arthropathy. Arthropathy is one of the most common manifestations in BD which contributes to reduced QoL (28). In addition, most the patients in this study also suffered from poor quality of sleep which may have contributed to further exacerbating their arthropathy induced pain. The BD systemic activity, OUSS, GUSS and BDCAF were found to be more severe when the fatigue and the poor quality of sleep scores increased. In addition, there is no specific medication that is routinely used to treat or relieve the fatigue experienced by patients that improves the quality of sleep. This study demonstrated that there was no association between any specific BD medication and the patients' experience of either fatigue or quality of sleep scores.

We also attempted to identify whether there was any association between the patients' systemic activity, fatigue and quality of sleep with specific biological molecules. To this end we used mathematical modelling to identify likely molecules. These were α-MSH and VIP. In addition, we investigated cytokines which are known to be associated with fatigue and sleep.

Alpha-MSH is a neuroimmuno-modulating peptide which has been found to be raised in the CSF (12). In previous studies, α-MSH serum concentrations were elevated during infective episodes or inflammatory disorders compared with healthy controls (12, 29, 30). α-MSH had a positive correlation with episodes of stress (31). Interestingly in BD psychosocial stress is appears to be one of the possible triggers in the aetiopathogenesis of Behçet's and it is also associated with episodes of relapse (2), despite the majority of patients in this study were white British, who tend to produce less melanin due to variations in their melanocyte-stimulating hormone receptors (32). In this study, the results demonstrate that the α-MSH serum concentrations were higher in the BD fatigue groups and in PSQI group compared with healthy participants. The high circulating concentration of α-MSH may be due to its anti-inflammatory effect in active disease, and the use of immune-modulatory medication (33). An additional factor may have been the timing of the collection of samples which was taken during daylight hours (11am-4pm) in both cohorts. Several other studies have already established that α-MSH production is increased during daylight and during exposure to UV light (34, 35).

When we further divided our BD cohort into those with high or low fatigue scores the α-MSH concentration for patients with high fatigue scores was (≥2.89 ng/ml), compared with BD patients with low fatigue scores. These results suggest that the level of α-MSH might be used to differentiate BD fatigue groups (low and high fatigue).

Biologically, VIP plays a role in the regulation of the immune cells to inhibit the inflammatory response. This function adds to its importance as a contender molecule to affect systemic activity in BD. The method of collection was standardised with patients at rest in the clinic as a recent study has detected that the salivary VIP concentration remarkably increased after a stress response induced by brief intense exercise lasting minutes (36). Previous studies have suggested that the normal VIP level in blood measured by radio immunoassay (RIA) or recycling immunoaffinity chromatography (RIC) was less than 100 pg/ml (37-39). However, both Luminex and ELISA methods used to measure levels in human serum gave similar results with the VIP concentration; 95±5 pg/ml and 100.4±45.7 pg/ml, respectively. In addition, VIP concentration in healthy fasting participants were reported as: 0-170 pg/ml or 0-190 pg/ml (40). In this study, the mean serum of VIP concentration of healthy controls was 104.1±16 pg/ml. However, the distribution of VIP serum concentration in BD patients was very variable, ranging (36 to >323 pg/ml) especially amongst inactive BD patients who had higher VIP concentration compared to active BD patients. The lowest levels of VIP tended to be more frequent in BD patients with arthropathy (mean= 66 pg/ml), whereas BD patients with CNS symptoms had much higher levels (mean= 156 pg/ml). This observation is in line with the findings of other groups (41). The heterogeneity in VIP concentration was also observed in patients who had immunomodulatory medication such as infliximab alone or combined with other medications but, interestingly, not prednisolone. Fur-

thermore, our data observed a positive association of VIP with the quality of sleep in BD cohort.

The increased levels of pro-inflammatory cytokines activate the hypothalamus and subsequently lead to "sickness behaviour". This can disturb eating behaviour, mood states, with the feelings of discomfort and low energy (42). In the present study, the association between BD patients' systemic activity, fatigue and quality of sleep with the serum concentration of some cytokines were investigated. It has found that IL-6 was the main cytokine component which was significantly different in BD patients compared with HC. This finding supports other studies which concluded that the dominant cytokine in auto-inflammatory diseases such as BD is IL-1β, and the secondary cytokine is IL-6 (43). We also found that IL-6 was higher in BD patients with low fatigue compared with the high fatigue group which might due to the effect of immunomodulatory medication.

Moreover, previous studies reported that increased levels of IL-1β and TNF-α may be associated with fever, fatigue, and sleep disturbances (44). Our data indicated that the IL-10 and IL-1β serum concentrations were not significantly different between BD fatigue and PSQI groups. TNF-α failed to demonstrate any significant difference between BD patients with increased of fatigue and poor quality of sleep groups (Table S4). However, this may have been because most of the BD patients were using immunomodulatory medication which decreases the level of TNF-α and IL-1β. This may have contributed to masking the differences between the fatigue and quality of sleep groups in BD cohort.

The main limitation of this study is that it is based on an observational cross-sectional design, with data collected from patients at a single point of time during the day, which did not allow us to investigate any diurnal changes. This may have played a role in masking the differences between the high and low fatigue groups. However, the current data has given us baseline information to carry out prospective follow-up studies with a larger sample size. In addition, controlled clinical trials will be needed to provide more information about the role of these molecules in BD.

In conclusion, in BD fatigue and poor quality of sleep both remain very challenging issues for both patients and attending physicians. In the present study, arthropathy in BD patients had a significant impact by increasing the fatigue score and causing poor quality of sleep. The complex interactions of α-MSH and VIP with IL-6 might lead to the development of novel approaches to treat symptoms of fatigue and sleeplessness in BD patients. The data also suggests that VIP might be used as a marker for CNS disease activity in BD patients. The findings indicate that further in vitro laboratory experiments in needed to understand the effect of α-MSH, VIP, and IL-6 on immune cells function in BD patients. It is important to test α-MSH, VIP, and IL-6 in the context of any new medication proposed for the treatment of fatigue. Currently available treatments targets are IL-1β, and TNF-α and their receptors. These are not completely successful in treating patients. In contrast, IL-6 is a more interesting target for BD patients. We suggest that continued use of AI mathematical modelling methods can provide essential information in the analysis of complex physiological processes, which would support novel drug discovery.

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