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ModulAtion of gut Microbiota through nutritional interventions in Behçet's syndrome pAtients: the MAMBA Study

Settore Scientifico Disciplinare MED/09

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SUMMARY

Abstract

Background and aims of the study:

Behçet's syndrome (BS) is a systemic inflammatory disorder characterized by a wide range of clinical manifestations with no gold-standard therapy. Although the pathogenesis of BS is currently unknown, gut microbiota (GM) is recognized to deeply influence the course of the disease. Recent evidence suggests that the GM in BS has low biodiversity with a significant depletion in butyrate production, one of the most representative short-chain fatty acids (SCFAs), contributing to the inflammatory state.

The aim of the present project is to investigate whether a tailored dietary intervention could ameliorate the clinical manifestations and modulate the GM of BS patients.

Patients and methods

This is a randomized, open, cross-over study that involved BS patients, who were randomly assigned to one of three different isocaloric diets for 3 months: a lacto-ovo-vegetarian diet (VD), a Mediterranean diet (MD), or a Mediterranean diet supplemented with butyrate (MD-Bt). Anthropometric measurements, body composition, blood, and fecal samples were obtained from each participant at the beginning and the end of each intervention phase. The primary outcomes were the change from baseline of the BS gastrointestinal and systemic symptoms. Changes from baseline in antioxidant profile and in GM composition were considered as secondary outcomes.

Results

A cohort of 38 patients were analysed. Following the three nutritional interventions, a reduction in clinical BS activity was detected, though without statistical significance. Also, a remarkable improvement in gastrointestinal symptoms was observed in all three groups. Of major note, VD allowed to significantly reduce the daily glucocorticoids dosage, with a significant reduction in the proportion of patients receiving steroids. A significant improvement in the redox status was

detected after VD and MD+Bt interventions, both in terms of ROS production, plasma lipid peroxidation, and total antioxidant capacity, while no effect of MD was observed. On the other hand, these 3-month butyrate-enriched diets did not affect GM composition.

Conclusion

A long-term supportive treatment based on dietary and lifestyle issues, might be able to improve the patients' wellbeing, to allow steroid spare, and to reduce the oxidative stress sustaining BS-related thrombotic events. This could have a high impact on BS-related morbidity and mortality, considering the high cardiovascular risk in chronic inflammatory conditions such as BS.

1. Introduction

Behçet's syndrome (BS) is a chronic, multisystemic inflammatory disorder (1), characterized by recurrent oral and genital ulcers, skin lesions, as well as a sight-threatening intraocular inflammation called panuveitis (2). BS is thought to share both autoimmune and autoinflammatory disease features caused by an aberrant population of T helper 1 (T_H1) and T helper 17 (T_H17) cells in combination with hyper-activated neutrophils (3)(4), however the exact etiology of the disease is however not yet clear. In common with autoimmune diseases BS shares class I MHC association. However, in contrast to autoimmune disorders, BS has clinical features that seem to be mostly autoinflammatory.

BS natural course of disease is characterized by spontaneous remissions and exacerbations and it is usually more active during the first years from onset (5)(1).

Despite oral ulcers, genital aphthosis and uveitis indeed represent the classical clinical triad described for the first time in 1937 by the Turkish dermatologist Hulusi Behçet (6), blood vessels (both arterial and venous) of every caliber might be involved, together with articular joints, gastrointestinal system and central nervous system, outlining the complex clinical picture which characterizes BS (5)(1). This spectrum of heterogeneous manifestations not only distinguishes different subsets of patients, but it might also characterize different disease phases in the same subject (7).

Traditionally, the first description of the literature was attributed to Hulusi Behçet, who described the disease and recognized nosologically an independent disease entity (8)(6). However, the first report was made by Hippocrates of Kos (460-477 A.C.), who described in the "Epidemion" an endemic disease in Asia minor with same characteristics of BS (9). Since then the disease has been reported worldwide and in addition to the features described above, an inflammatory vasculitis is now recognized which may involve most tissues of the body, and from 2012 BS is

classified among vasculitis by the 2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitis, in the group of Variable Vessel Vasculitis (10).

1.1 Epidemiology

Despite BS is usually consider ubiquitous, this disease is better known as the "Silk Road Disease", a term coined for the first time by Ohno in 1982 (11) referring to a peculiar geographical distribution of BS patients, which might reflect a genetic disease predisposition spread by nomadic tribes or traders as they travelled between the Arabic world and the Orient along the ancient Silk Roads (12).

BS is estimated to globally affects 10,3 people every 100.000 inhabitants, with a a great geographical variability, with different prevalence from one world region to another (13). The highest prevalence of disease occurs across Asia in countries between 30° and 45° latitude north, where it runs a more severe course than elsewhere (14), with cases rarely reported from central and southern Africa, Greenland, and Australia (15)(16)(17).

This fascinating geographical correlation suggest a tight relationship between environment and genetic susceptibility in BS pathogenesis (figure 1), which might explain how the prevalence of BS in the Mediterranean area, the Middle East and Far East might be higher due to the continuous effect of external agents (including some pathogens or environmental factors) on a predisposing genetic substrate (18)(19).

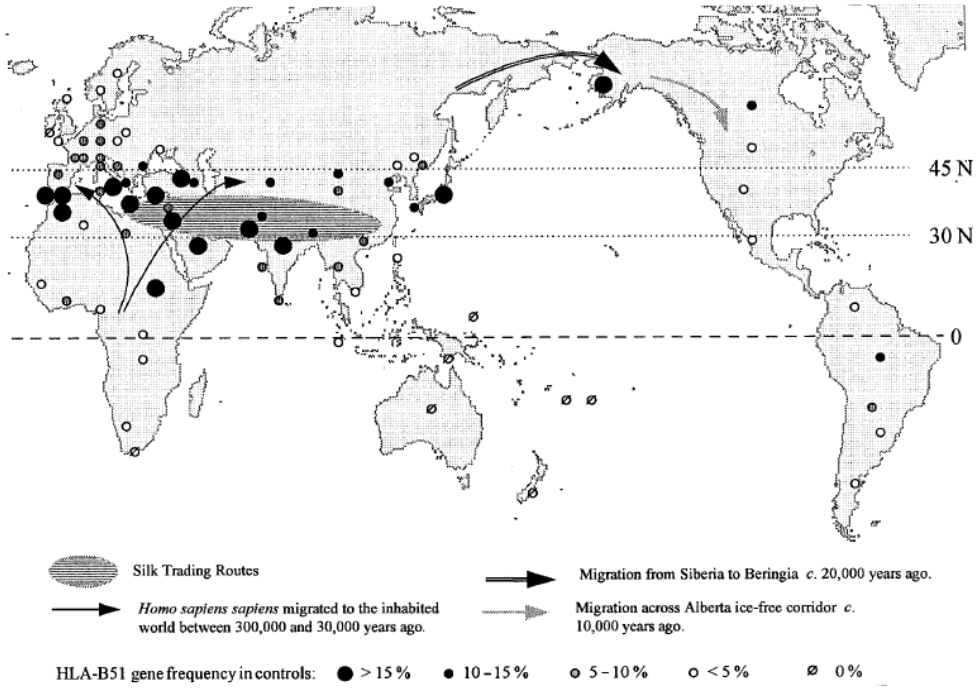

Figure 1*. World distribution of HLA-B51. The grey area represents the ancient Silk Road area. Modified from Verity et al. 1999 (12).*

Conversely, estimating these prevalence values is further complicated by changes in the migratory fluxes observed in the last decades. European countries with low BS prevalence, such as France and Germany, faced waves of immigration from North Africa, able to deeply modify the epidemiologic asset of BS in the native population.

Nowadays, in Europe BS prevalence highly differs from region to region, as reported in table 1: from the highest prevalence to the lowest, they were Turkey with 20 to 420 in 100,000 inhabitants, Italy 15.9 (20), Spain 7.5 , France 7.1, Germany 4.9 in Berlin municipality while in the whole Germany it is only 1.1, Sweden 4.9, Portugal 1.5, and UK 0.64 (21).

Country	Prevalence per 100,000
Turkey	20-420
Iran	80
Saudi Arabia	20
Iraq	17
Israel	15.2
Japan	13.5
Spain	7.5
France	7.1
Egypt	6.7
USA	5.2
Sweden	4.9
Italy	3.8-15.9
Germany	2.26
Portugal	1.53
UK	0.64

***Table** 1. Worldwide prevalence of Behçet's syndrome. Prevalence is expressed number of patients/ 100,000 inhabitants. Modified From Verity et al (12).*

Generally, BS is more frequent in males, between the second and fourth decade of life (with a peak in the third decade), while it rarely occurs in children and in subjects over 55 years old (21)(22). However, females are reported to be more frequently affected by BS in some Asian countries such as Japan and Korea (23).

Usually, BS diagnosis occurs during the adult life, however some patients might develop the first BS manifestations during their childhood, usually after 16 years of age (24). Despite the sporadic nature of BS, the existence of hereditary forms has been anecdotical reported, especially in Turkey and Japan (25).

1.2 Etiopathogenesis

Although the pathogenesis of BS is still unknown, it has been now suggested that genetic susceptibility, trigger factors, and immunological abnormalities could play a decisive role in BS development (26). Over the past year substantial advances have been done in the understanding of the genetic and immunology of BS. It has been supposed that triggering infectious factors

might participate in the outbreak of BS in genetically predisposed patients (figure 2). Moreover, two large genome-wide association study (GWAS) conducted in Turkey and Japan reported association between single nucleotide polymorphism (SNP) of interleukin (IL)-10 and IL-23R/IL-12RB2 genes in BS patients. Furthermore, the perturbations of T cell homeostasis in BS is recently becoming increasing important. In particular the promotion of T_H17 responses and the T Regulatory (T_{reg}) cells seems to correlate with BS activity and inflammatory cells within BS inflammatory lesions included mostly neutrophils, T_H1 and T_H17 cells, and cytotoxic $CD8^+$ and γδ T cells (27).

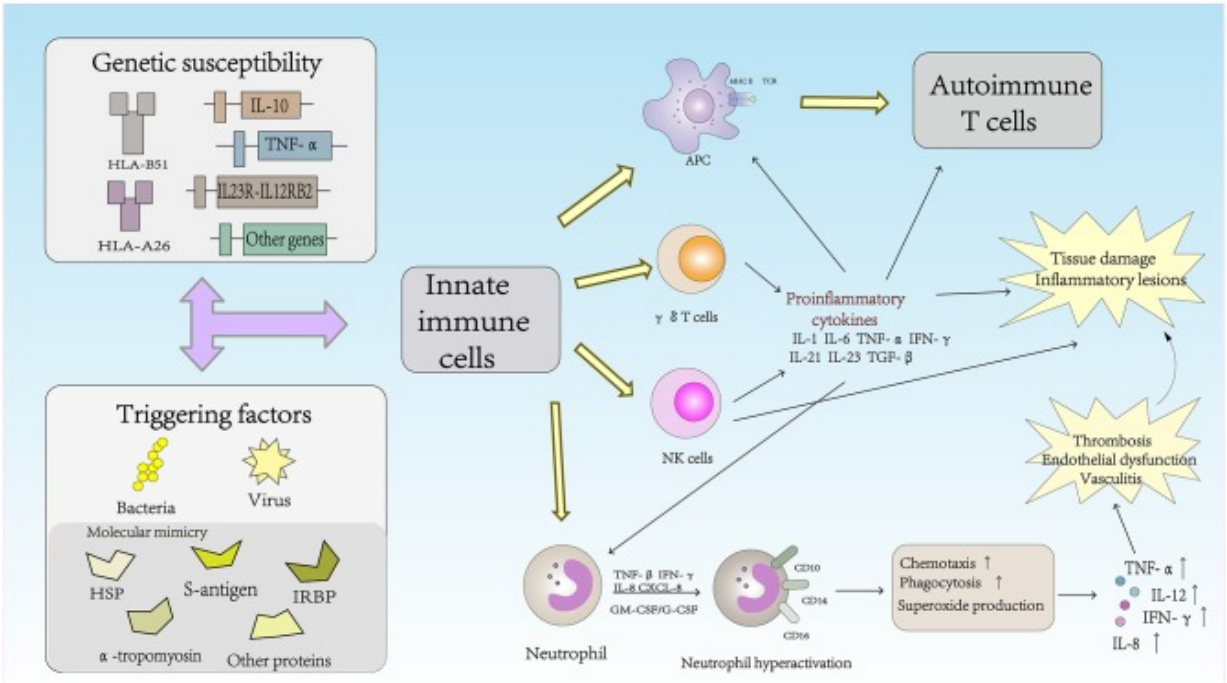

Figure 2. *Main BS pathogenic mechanisms hypothesis (26).*

1.2.1 Genetic Factors

Histocompatibility leucocyte antigen (HLA)-B*51 represents the strongest genetic risk factor for BS to date. It was initially reported in the Japanese (11) and reproduced among several other ethnic groups. A meta-analysis reported an overall odds ratio of 5.78 (95% CI=5.00–6.67) for HLA-B*51 carriers to develop BS, independently of the ethnicity (28).

However, in the last years, several studies performed on HLA-B*51-negative patients showed a significant association of BS with other loci, such as HLABw4-80I (29), an epitope present on B locus-derived proteins, characterized by the presence of an isoleucine at amino-acid position 80 in the a1 helix of the HLA-B*04. Additionally, the same authors found an association with HLA-A*26 independent from HLA-B*51, which was confirmed in other studies (30). Finally, Hughes et al. (31) demonstrated that HLA-A*03, B*15, B*27, B*49 and B*57 also contribute to BS risk independently, although this has not been replicated to the moment. Minor evidences exist on HLAB/MICA and at the region between HLA-F and HLA-A (32).

This association of HLA and BS led to classify this syndrome as a MHC-I-opathy. This concept was supported also by the evidence, by genome-wide association studies (GWAS), of mutations in ERAP1 (encoding for endoplasmic reticulum aminopeptidase 1, implicated in antigen presentation) in BS patients, similarly to ankylosing spondylitis and psoriatic arthritis. ERAP1 exerts functions as a trimming agent for endocytosed antigens before their loading on MHC class I molecules (33).

Given the truly complex genetic load and phenotypic clusters of BS, there have been relatively few epigenetic studies in this disease. In this line, GWAS in BS patients allowed to identify extra-HLA genetic risk factors. Remmers et al. (30) identified, by analyzing 311,459 SNPs in 1,215 BS patients and 1,278 controls, two novel susceptible loci for BS: IL23R-IL12RB2 and IL-10. Recently, association between IL-10 polymorphisms and BS was also demonstrated in Chinese patients. These data emphasize the possible role of IL-10 in BS pathogenesis and raise the question of possible participation of adaptive immunity, especially T_H17 and T_{reg} cells (34).

Copy number variation (CNV) of Complement component C4 genes was also investigated in BS, and what has been pointed out in this analysis is an increased frequency of more than 2 copies of the C4A gene in BS patients and this represents a risk factor independent from HLA-B*51. Moreover, it has also been demonstrated that BS patients with high C4A copy number had increased production of IL-6, an important mediator of the innate immunity acting as an acute

phase reactant (35).

Furthermore, a whole-exome sequencing (WES) analysis revealed heterozygous single-nucleotide variants (SNVs) in the genes encoding IL-1 receptor-associated kinase 4 (IRAK4), nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain-containing 14 (NRP14) and melanoma antigen-encoding gene E2 (MAGEE2), in BS patients (36).

More recently, non-synonymous variants (NSVs) in a gene involved in innate immune response (i.e., Toll-like receptor 4, TLR4) have been reported in BS; also, the familial Mediterranean fever (MEFV) gene Met694Val mutation seems to confer BS risk in the Turkish population. Thus, the disease-associated NSVs in TLR4 and MEFV genes support the hypothesis of the correlation between innate immune response and bacterial sensing mechanisms in the pathogenesis of BS (37).

All these evidence suggest the presence of a more complex BS genetic background, relying on both HLA and non-HLA correlated polymorphisms. These genetic variants definitely represent predisposing factors but are still not enough to determine BS manifestations by themselves.

1.2.2 Infectious Triggers

Genetic abnormalities are not sufficient to explain the outbreak of BS, especially when a meta-analysis presenting regarding BS risk due to HLA-B51 have been demonstrated the healthy control group had a high carriage rate (18%) of HLA-B51(28).

For quite some time, the hypothesis of an environmental trigger in genetically predisposed BS patients has been encouraged. Several infectious agents have been investigated, especially bacteria (Streptococcus, Mycoplasma, Helicobacter pylori) and viruses (Herpes simplex virus 1 and 2, Hepatitis virus, Parvovirus B19), as reported in table 2. Given the high frequency of oral ulcer (38), the increase rate of oral ulcers after dental interventions (19) and the decrease of some symptoms with penicillin or minocycline (39)(40), a putative role of oral flora has been supported in the pathogenesis of BS. The most investigated microorganisms were Streptococci especially

Streptococcus sanguis. The proportion of S sanguis in the oral flora of BS is increase compared to healthy controls (41). Interestingly, uncommon serotypes of S. sanguis with unique characteristics were isolated (18) and significant antibody responses to these streptococci were observed. KTH-1 streptococcal antigen could active γδ T cells in BS patients, but this response was also obtained with E. coli, Staphylococcus aureus and non-peptidic antigen share by various bacteria (42)(43). These results led to the hypothesis that the T lymphocytes of BS patients are hyperactive against bacterial antigen and not to a specific bacterial species. Various other bacterial species like Mycoplasma fermentans, H. pylori, Mycobacteria or Borrelia burgdorferi were occasionally reported as putative BS inducer but without strong level of evidence (27).

The viral hypothesis was supposed by Behçet in 1937, when he identified intra and extranuclear inclusion in aphthous and hypopyon samples suggesting a viral etiology to BS. Since then, many Herpesviridiae have been investigated (Herpes Simplex Virus (HSV) -1, Cytomegalovirus (CMV), Varicella Zoster virus (VZV), Epstein-Barr virus (EBV)). In HSV -1 DNA has been isolated in peripheral blood mononuclear cells (PBMC), and in oral and genital ulcers from BS patients (44). Higher proportion of serum antibodies against HSV1 was also reported in BS patients (45), however, in the absence of clinical efficacy of anti-viral therapy this hypothesis seems unlikely. The prevalence of Hepatitis virus A, B, C, E and G has been investigated in BS without any positive conclusions (46). The role of Parvovirus B19 was valuated with conflicting results (47). Conversely, no association has been detected between BS development and other bacterial or viral species such as Borrelia burgdorferi, Helicobacter pylori, CMV, EBV, Parvovirus B19, VZV and hepatitis viruses.

	Most frequent	Less frequent
Bacteria	*Streptococcus sanguis*	*Mycoplasma fermentans, Helicobacter pylori, Borrelia burgdorferi*
Virus	*Herpes simplex virus 1*	*Epstein Barr Virus, Cytomegalovirus, Varicelle Zona Virus, Parvovirus B19, Hepatitis virus* A, B, C, E and G.
Molecular Mimicry	Heat-shock protein 60 kDa	αβ-crystallin, heat-shock protein 70 kDa, retinal-S antigen, α-tropomyosin

Table 2: *Main infectious trigger investigated in BS pathogenesis. Modified from Pineton et al.(27)*

Finally, a direct role in BS development could be represented by some molecular mimicry based on sequence homology between microbial and human peptides (autoantigens). Several autoantigens have been described in BS patients, including heat-shock proteins (HSP) HSP 60 kDa and HSP 70 kDa, the *S antigen*, the interphotoreceptor retinoid binding protein (IRBP), α-tropomyosin and αβ-crystallin (27)(26). For example, HSP expression in active skin lesion of BS (erythema nodosum, mucocutaneous ulcers) is increased (48) and it seems that an immune reactivity against the S antigen and IRBP, retina-specific autoantigens, might be involved in the pathogenesis of BS ocular manifestations (26).

Despite some microbial infections being considered as triggering factors for BS, there's no evidence that this syndrome represents the result of a direct viral or bacterial infection.

However, infectious agents and probably microbiome alterations (could represent underlying disease causes leading to immune system imbalance in genetically predisposed subjects (49).

1.2.3 Immunopathogenesis

As already mentioned, immune system imbalance represents the main basis for BS development. Several pathogens can activate innate immunity, especially neutrophils and γδT cells, and acquired immunity following antigen processing and presentation to naïve T lymphocytes by antigen presenting cells (APCs). The first response begins in the mucosa of patients with BS due to the recognition by γδT cells of bacterial Hsp largely homologous to human HSP, with non-

MHC restricted mechanisms, and such homology maintains the immune response with a mechanism of molecular mimicry. γδT cells produce large amounts of TNF-α and IFN-γ, with subsequent activation of macrophages and secretion of CXCL8, IL-1 and TNF-α (1). A second way of activation of the immune response is also possible: antigen presentation by APC to naïve CD4T cells (in the context of MHC class II) leads to the production of IL-12 inducing a T_H1 response. Recently, some data also demonstrate a possible role of T_H17 cells and their cytokines in BS pathogenesis. Also, it is known that a milieu consisting of IL-6 or IL-1 with IL-23 is crucial for the differentiation of naïve T lymphocytes into T_H17 cells, and increased levels of IL-6 and IL-1 are documented in BS (50)(27). Furthermore, $CD4^+$ T cells producing IL-21 (crucial for the maintenance of differentiation of T_H17) are significantly increased in the peripheral blood of patients with BS (51).

1.2.3.1 Role of T Cells and Relationship with Gut Microbiota

Several studies have demonstrated an excessive T_H1 cell activation and infiltration in the peripheral blood, the gastrointestinal tract and the skin of BS patients. In this context, the most important cytokines implicated in the disease pathogenesis are IL-12 and IL-10. IL-12 causes $CD4^+$ T cell to differentiate into T_H1 cell promoting autoimmunity with different pathways (52), and IL-10 (anti-inflammatory cytokine) which is consistently decreases in BS with corresponding loss of the related gene expression (53)(54).

T_H17 cell, an important subset of cells implicated in effector mechanisms, secrete IL-17 in the presence of IL-23 and usually cause activation and recruitment of neutrophils and macrophages. The regulation of excessive activation of effector T cells are usually regulated by T_{reg} cells, through the Transforming Growth Factor-β (TGF-β) pathway. The loss of balance between T_H17 and T_{reg} is what it could be observed in BS patients (55).

As reported in figure 3, T_H17 cell immune response in BS patients might be dominant as the result of the growth of T_H17 to T_{reg} ratio, although both cell subsets could be overstimulated (56). This underlines once again the importance of T_H17 in the innate immune response.

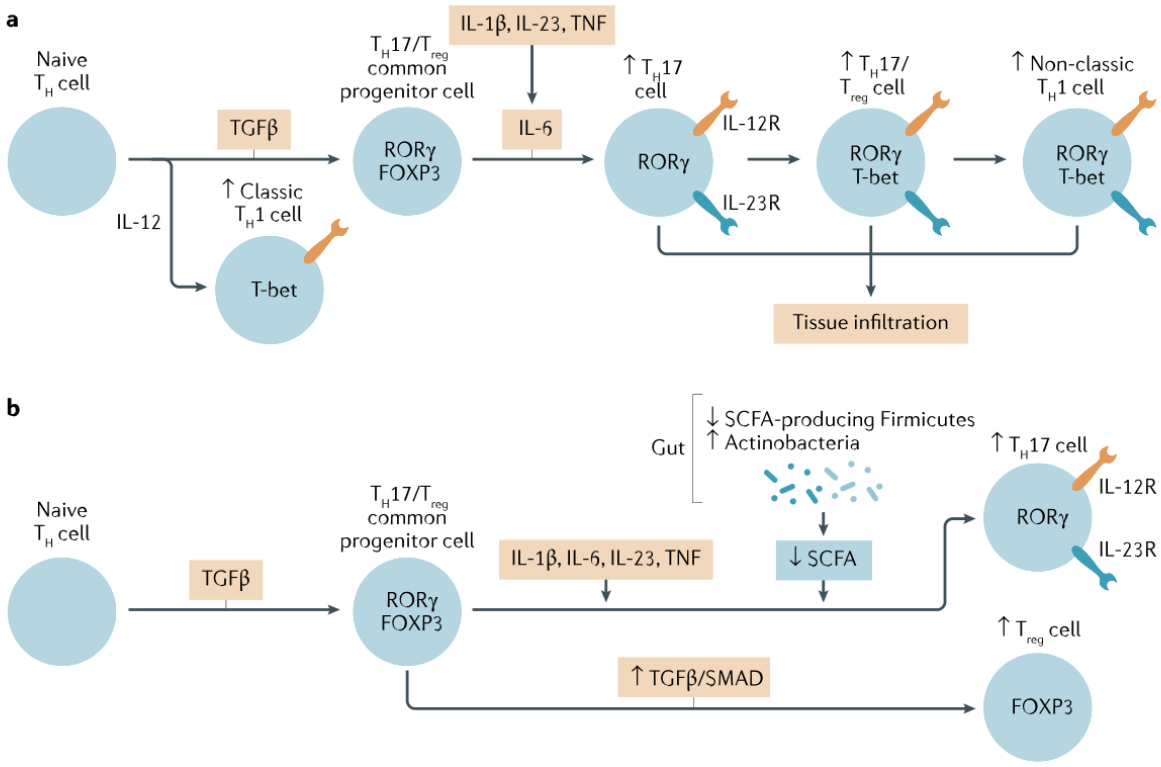

Figure 3. *Schematic representation of skewed helper T cell responses observe in patients with Behçet syndrome.*

T_H17 and T_{reg} have both been reported to be induced and activated by intestinal bacteria in BS (57). Next-generation sequencing techniques revealed a significant modification in the physiological intestinal bacterial flora of BS patients, possibly as an environmental factor (figure 3), not only in terms of bacterial species composition, but also in terms of gene function (58). Metagenomic studies have shown an abundance of bacterial species of the genus *Bifidobacterium* and *Lactobacillus* and a decrease in short-chain fatty acid-(SCFA) producing bacteria, such as *Megamonas hypermegale* and *Butyrivibrio* spp. These concepts were also confirmed by studies regarding stool-samples in which it has been demonstrated a decrease in SCFA-producing bacteria and a decrease in short-chain fatty acid concentration, such as butyric acid (59).

Propionic and butyric acid are SCFAs, produced by fermentation of dietary fiber by specific gut flora. Both are considered to be among the most important mediators of activity between peripheral blood T cell dysfunction and changes in the intestinal flora. In particular, SCFA promote T_{reg} cell differentiation via the epigenome forkhead box protein P3 (FOXP3), which is the master gene of T_{reg} cells (60). The decrease production of SCFAs compared with healthy control and the dysbiosis associated with this decrease is considered to cause the relative predominance of the master gene RORC, promoting further T_H17 cell differentiation. Such T_H17 differentiation is suggested to be further accelerated by inflammatory cytokines such as IL-6 and TNF in BS (figure 3).

1.2.3.2 Role of Neutrophils

Neutrophilscertainly represent the main cells involved in the pathogenesis of the different BS clinical manifestations. Their ability of chemiotaxis, phagocytosis and production of reactive oxygen species (ROS) play a key role in the BS pathogenesis. In particular, neutrophils seem to contribute to the pathogenesis of BS vascular events through protein (especially of fibrinogen) oxidative modification (61).

BS is indeed frequently complicated by atherothrombotic events (in particular venous thrombotic events, but also aneurisms) and, similarly to other types vasculitis, its histopathology is characterized by neutrophil and lymphocyte-rich vascular/perivascular infiltrates (51).

In BS patients, an hyperactivation of neutrophils has been demonstrated non only in the circulation, but also into the infiltrate of involved tissues and organ (such as nodular and papular skin lesions, synovial biopsies and intestinal ulcer biopsies) (33). Interestingly, neutrophils seem to have also a crucial role in the thrombotic mechanisms. In fact, the increase ROS produced by neutrophils are thought to modify the secondary structure of fibrinogen and the architecture of the fibrin clot making it less susceptible to plasmin-induce lysis (62)(63). Moreover, recent studies demonstrated a role of neutrophil extracellular traps (NETs) in pro-thrombotic mechanisms. The

levels of active NETs were found to be higher in patients with vascular BS than controls or in those with inactive disease (63). The assessment of NET components (such as cell-free DNA (cfDNA) and the neutrophil enzyme myeloperoxidase) in patients' serum or in purified neutrophils demonstrated higher levels of cfDNA and myeloperoxidase-DNA complexes in BS patients with vascular involvement as compared to those without vascular involvement. Moreover, purified neutrophils from BS patients exhibited spontaneous NETosis, differently from those of healthy subjects (63), suggesting the importance of neutrophil activation in the pathologic disease mechanisms.

1.3 Clinical Manifestations

As already mentioned above, clinical BS manifestation were firstly described as a triad characterized by oral aphthae, genital ulcers and uveitis (64).

Later, BS was recognized as a systemic inflammatory disease with variable clinical manifestations: characterized not only by muco-cutaneous and ocular disease, but also cardiovascular, musculoskeletal, gastrointestinal and neurologic involvement (1).

Currently, Behçet's heterogeneous spectrum of clinical manifestations makes the identification of a unique and nosologically distinct condition basically impossible: the disease is therefore considered as a "syndrome". Specific clinical manifestations also have different prevalence: in particular, mucocutaneous and ocular ones are the most frequent and are considered ad distinctive syndrome signs since BS first description (65).

1.3.1. Mucocutaneous Manifestations

Mucocutaneous manifestations can be considered as the distinctive sign of BS and are frequently present since disease onset. Their identification may allow for an early diagnosis and subsequent treatment (66). Nevertheless, none of the skin or mucosa lesion that patients presents is highly

specific for BS by itself, but the recurrent nature of a lesion, the occurrence of different types of lesion in the same patient and the presence of organ involvement lead to a diagnosis of BS. Recurrent oral ulcer are common in the general population and oral ulcer of BS are usually not distinguishable from those in the general population (67). Oral ulcers in BS are usually minor, and they are located on the mucosal lining of the cheeks, lips, the tongue and the gengiva (figure 4). They generally heal spontaneously without scarring during 1 to 3 weeks, despite the natural course of individual oral ulcers is not well described.

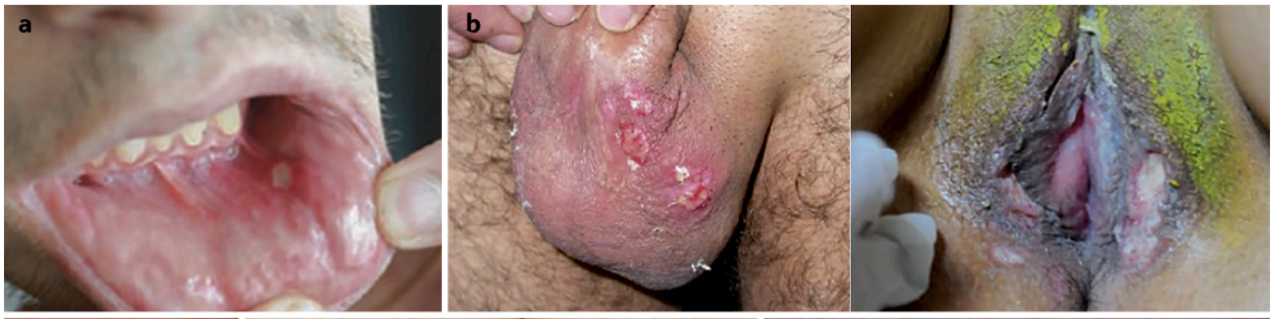

Figure 4. *Mucocutaneous manifestation of Behçet syndrome (BS);* ***a)*** *oral ulcers in BS patients can usually not be distinguished from those in the general population; they are predominantly < 1 cm in diameter and located on the mucosal lining on the cheeks, lips, tongue and gingiva;* ***b)*** *genital ulcers can occur on the scrotum in men and major labia in women with BS. Modified from Yazici et al. 2021 (33).*

In BS patients usually occur also genital ulcers, typically on the scrotum and penis in males and on the vulva or vaginal mucosa in females (figure 4). Genital ulcers are more specific for BS and the presence of recurrence oral and genital ulcers are adequate for a diagnosis of BS according to ICBD (68). Genital ulcers have a greater tendency to scar, resolve during 2 to 4 weeks, and may recur less frequently than oral ulcers. Differential diagnosis with HSV lesions, bowel disease, Reiter syndrome, Sweet syndrome, erythema multiforme and bullous disease need to be excluded. Combination of oral and genital aphthous ulcers is named bipolar or complex aphthosis, and other conditions, including gluten-sensitive enteropathy, ulcerative colitis, Crohn disease, drug eruptions, immunodeficiency syndromes, and hereditary periodic fever syndromes such as mevalonate kinase disease, should be considered in its differential diagnosis (33).

Erythema nodosum–like lesions are painful erythematous nodules usually seen bilaterally in the pretibial area in patients with BS and they may lead to a slowly healing hyperpigmentation (figure 5). Papulopustular skin findings as acne- or folliculitis-like lesions are also common. Histological examination shows a mixed type panniculitis with septal and lobular panniculitis and findings of neutrophilic vasculitis (69).

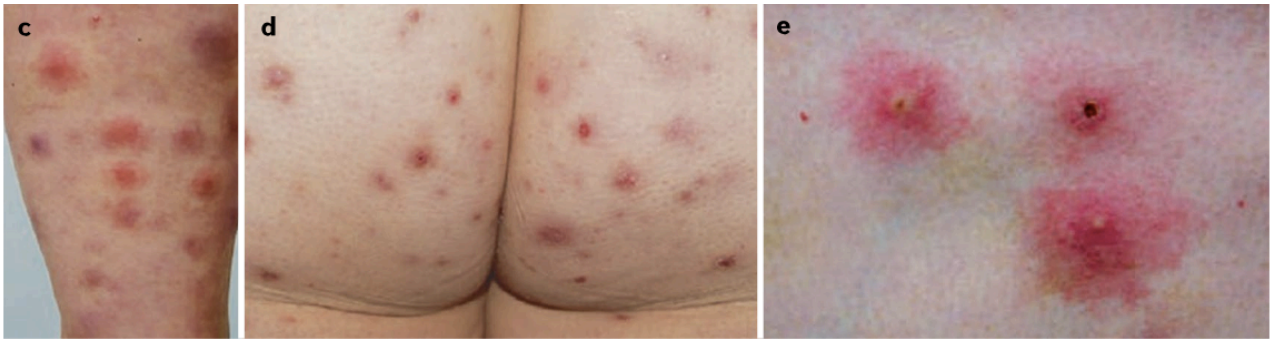

Figure 5.. *Mucocutaneous manifestation of Behçet syndrome (BS);* ***c)*** *painful erythema nodosum-like lesion occur on the lower of patients with BS, and can lead to slowly healing hyperpigmentation;* ***d)*** *and* ***e)*** *papulopustular lesions frequently occur in patients with BS but are often not specific for the disorder. Modified from Yazici et al. 2018 (1).*

Pathergy reaction is an important skin finding that represent hyperreactive inflammatory response feature of BS. Patients develop an erythematous papulopustular lesion after 20-gauge needle puncture to the forearm at 48 hours. Equally, oral or genital aphthae can be induced with needle prick trauma (70).

Finally, superficial thrombophlebitis, which is actually the most common lesion within the spectrum of vascular involvement in BS, may present as a cord-like painful, erythematosus lesion. Ultrasonography is helpful in the differentiation of these lesions and biopsy provides definitive diagnosis. The importance of this differentiation lies in the association between superficial thrombophlebitis and deep vein thrombosis (71).

1.3.2. Ocular Manifestations

Ocular manifestations are the most problematic manifestation of BS given of their frequency and morbidity. About half of patients with BS develop non-granulomatous posterior or panuveitis, usually affecting both eyes, and isolated anterior uveitis is rare (72)(24). Sudden onset of diffuse vitritis caused by posterior segment involvement is a common feature, and the appearance of inferior peripheral precipitates during resolution is very typical. In patients with serious posterior segment involvement, hypopyon may also develop in the anterior chamber.

Retinal vasculitis is one of the hallmarks of BS, affecting veins, capillaries and arteries. Area of superficial retinal infiltrates and necrosis with satellite haemorrhages are particularly alarming. Recurrent branch retinal vein occlusions, retinal hemorrhages, peripheral retinal occlusive periphlebitis, necrotizing retinopathy, and optic neuritis can also be found.

The ocular manifestations affect the functional prognosis as both eyes may be damaged in a short period of time. Young men are particularly at risk, although the severity in children must not be underestimated.

1.3.3 Articular Manifestations

Arthritis in BS is usually reported in about half of the patients, usually as recurrent non-deforming, mono- or oligoarthritis affecting the knee and ankle joints and less frequently the elbows and wrists. Usually, it resolves within days or a few weeks, but some patients may also develop chronic arthritis. Rarely, patients with BS can develop axial disease (73). Fibromyalgia and fatigue may be troubling in BS patients, somewhat more prominently in those with musculoskeletal involvement than in those without (74).

1.3.4. Cardiovascular Manifestations

BS has been classified as a variable vessel vasculitis affecting all types and sizes of blood vessels with a thrombotic tendency. Vasculitis or a form of vasculopathy seems to be operative in several manifestation such as retinal vasculitis in the eyes, vasculitis of the mesenteric arteries in the gastrointestinal system, perivascular inflammation and capillary or venous compromise in the brain and a neutrophilic or lymphocytic vasculitis observe in 50% of mucocutaneous biopsies (75).

Unlike other systemic vasculitis, BS is characterized by the contemporaneous involvement of both arteries and veins of all sizes and presents a unique tendency for aneurysm formation. Within BS, a specific cluster of patients suffering from recurrent inflammatory thromboses involving the venous and, more rarely, the arterial vasculature, called the 'vascular cluster' or 'angio-Behcet's' has been identified (33). Vascular events affect up to 40% of BS patients, with a higher prevalence in males , and they usually present as early disease manifestations. Superficial venous thrombosis and deep vein thrombosis (DVT) are the most frequent vascular manifestations, together affecting 15–40% of BS patients, and eventually leading to post-thrombotic syndrome in the most severe cases. Venous thrombotic events in BS mainly involve the superior or the inferior limbs, although thrombosis of atypical sites is an important and quite specific clinical feature of BS as well (76). Arterial involvement, less common than venous disease, is considered a peculiar vascular feature of BS, which being one of the only chronic inflammatory diseases causing aneurysms that can affect peripheral, visceral and pulmonary arteries. Venous thrombosis is often detected in patients with aneurysms or pseudoaneurysms. About 80% of the patients with pulmonary arterial involvement have been found to have concomitant venous thrombosis, mostly DVTs, suggesting that events on both the venous and the arterial side of the vascular tree are probably sustained by similar pathogenic mechanisms.

The vascular involvement in BS has a major impact on morbidity and long-term mortality, and has been identified as the leading cause of death in these patients (77).

Regard cardiac involvement, some patients may develop intracardiac thrombus. It has been described also the precence of pericarditis, myocarditis, and coronary artery aneurysms. (78).

In a retrospective study, recurrent vascular events were observed in 35.4% of the patients, whereas a prospective survey of the same centre found a recurrence rate for deep vein thrombosis of 45% at 24 months (79).

1.3.5. Neurological Manifestations

Neurological involvement occurs in 5% of BS patients, with a higher reported prevalence in males. Parenchymal neurological manifestations (neuro-Behçet) approximately represent 80-90% of cases, while the remaining cases are correlated to cerebral venous thrombosis (80).

Differently from other disease manifestations, cerebral involvement at parenchymal level typically appears after 5 years from onset. Parenchymal manifestations usually show involvement of the brain stem, diencephalic-telencephalic junction and basal ganglia, while the spinal cord is rarely involved, and both the cerebral cortex and cerebellum are generally excluded.

The most common manifestations are characterized by focal symptoms (usually in acute forms) or headache/ behavior disorders (usually linked to chronic disease forms). Magnetic resonance imaging (MRI) represents a key exam for differential diagnosis, and the presence of brain stem lesions represents a pretty specific BS sign.

Cerebrospinal Fluid (CSF) examination usually presents pleocytosis and abnormal protein levels, in absence of oligoclonal bands. Specific markers (both laboratory and clinical ones) able to distinguish between neuro-Behçet and Multiple Sclerosis (MS) lesions are still lacking (81). Along with vascular involvement, parenchymal neurologic manifestations are the main cause of mortality and morbidity, with death or loss of autonomy overall affecting up to 60% of patients.

Cerebral venous sinus thrombosis (CVST) occurs in up to 14% of BS patients. Sagittal and transverse sinuses are more frequently affected, despite all cerebral vessels being potentially

involved. Usually, cerebral vascular involvement is preceded by peripheral thrombotic events in 25% of the cases. On the other hand, CVST represent an onset manifestation in up to 15% of BS patients with vascular involvement. Patients with cerebral vascular involvement usually show no parenchymal disease manifestations (82).

1.3.6.Gastrointestinal Manifestations

Gastrointestinal involvement is one of the least frequent BS manifestations, affecting approximately 5% of patients. It equally affects both males and females and represents the most frequent disease manifestation in the Far East, reaching 50% of patients in some regions of Japan (65). Gastrointestinal manifestations usually appear 5-10 years following oral ulcers onset, even if they can also sometimes represent the presenting symptom of BS. Symptoms usually vary from mild to severe manifestations ascribable to ischemic events and /or perforations. Lesions mainly affect the terminal ileo (in 95% of cases) and less frequently the perineal and rectal region (65). Esophageal ulcers are more common in males and present clinically with substernal chest pain dysphagia (83). BS gastrointestinal involvement shares many similarities with Chron's disease and intestinal tuberculosis. In this scenario, differential diagnosis is mainly based on endoscopic examination, with round or oval ulcers larger than 1 cm and with discrete margin usually being suggestive of BS (84).

1.3.7 Co-existing BS Involvement and Major Disease Phenotypes

The above-mentioned organ involvements rarely occur as discrete BS manifestations, and commonly are clustered. This clinical perception has been supported in the past two decades by a number of cluster analyses and association studies identifying significant associations among specific disease manifestations (65).

The positive and negative associations identified among different clinical and demographic

features within BS are illustrated in figure 6. Of note, given the relatively low prevalence of gastrointestinal manifestations, no specific cluster analysis has focused on possible clusters of BS organ involvement frequently occurring in patients with gastrointestinal BS (7).

The first phenotype described in BS in the mucocutaneous and articular one. Skin-mucosa ulcerations are the most common, and usually the earliest, manifestations of BS, and recurrent oral and genital lesions are the hallmark of this syndrome. While one third of the BS population presents with only recurrent mucocutaneous symptoms, a not negligible proportion of patients presents both mucocutaneous and articular involvements. The association between acne and arthritis has been demonstrated in past decades, but it is suggested that also enthesitis was part of this clinical association (1).

Indeed, BS shares with seronegative spondyloarthritides (SpA) common pathogenetic mechanisms and genetic susceptibility, including the interleukin (IL)-23 and IL-17 pathways. Moreover, the involvement of major histocompatibility complex (MHC) class I alleles both in BS and in SpA [human leukocyte antigen (HLA)-B*51 and HLA-B*27, respectively] led to the unifying concept of "MHC-I-opathies" (65).

A second phenotype is the peripheral vascular and extra-parenchymal neurological phenotype. Superficial venous thrombosis (SVT) and deep vein thrombosis (DVT) are the most frequent vascular manifestations of BS, affecting altogether up to 40% of patients. DVT mainly involves the inferior, but also the superior limbs, while venous thrombosis of atypical locations (TAL) have been described. At the cerebral level, non-parenchymal vascular central nervous system (CNS) involvements include cerebral venous sinus thrombosis (CVST), arterial occlusion, and/or aneurysms. CVST represents 10–30% of all neurological BS manifestations. The concomitant presence of both cerebral arterial manifestations and CVST is extremely rare. In an analysis of 88 patients with CNS disease, a significant association was found between peripheral vascular disease and extra-parenchymal CNS involvement (i.e., dural sinus thrombi), while a poor association was found between parenchymal neurological and peripheral vascular involvements.

In a retrospective study involving 21 BS patients with CVST, the presence of extra cranial thrombosis was documented in 52% of patients. In a cohort study on 820 patients, CVST was reported in 64 cases. Among them, the presence of concomitant extra-neurological vascular lesions was significantly more frequent than in patients without CVST.

The concomitant presence of central and peripheral vascular involvements is probably sustained by common thrombogenic mechanisms. Namely, inflammation-induced thrombosis has been described in BS, with neutrophils playing a critical role in promoting oxidative stress, inflammation, and consequent endothelial dysfunctions. In this context, immunosuppression represents a key strategy for the therapeutic management of central and peripheral vascular involvements (85).

The third phenotype is characterized by the parenchymal CNS and ocular involvement. In a study conducted on 200 neuro-BS out-patients, 162 had parenchymal CNS involvement. In a first post-mortem study on a BS patient with parenchymal involvement, a cell infiltration was found around the central retinal artery within the optic nerve. Eye involvement is present in around half of BS patients, with a higher prevalence in males, and a lower prevalence among elderly. Ocular involvement is one of the most disabling complication in BS. In a retrospective observational study on 295 BS patients, a significant association between posterior uveitis and parenchymal CNS involvement was reported. Furthermore, male sex, eye disease, HLA-B51 positivity, and neurologic involvement are features identifying a specific cluster of BS patients. Although the pathogenetic mechanisms sustaining the concomitant occurrence of ocular and neurological BS involvements have never been described, the embryogenic process and the involvement of the neural tube and neural crest in the organogenesis of the eye might account for this association (65).

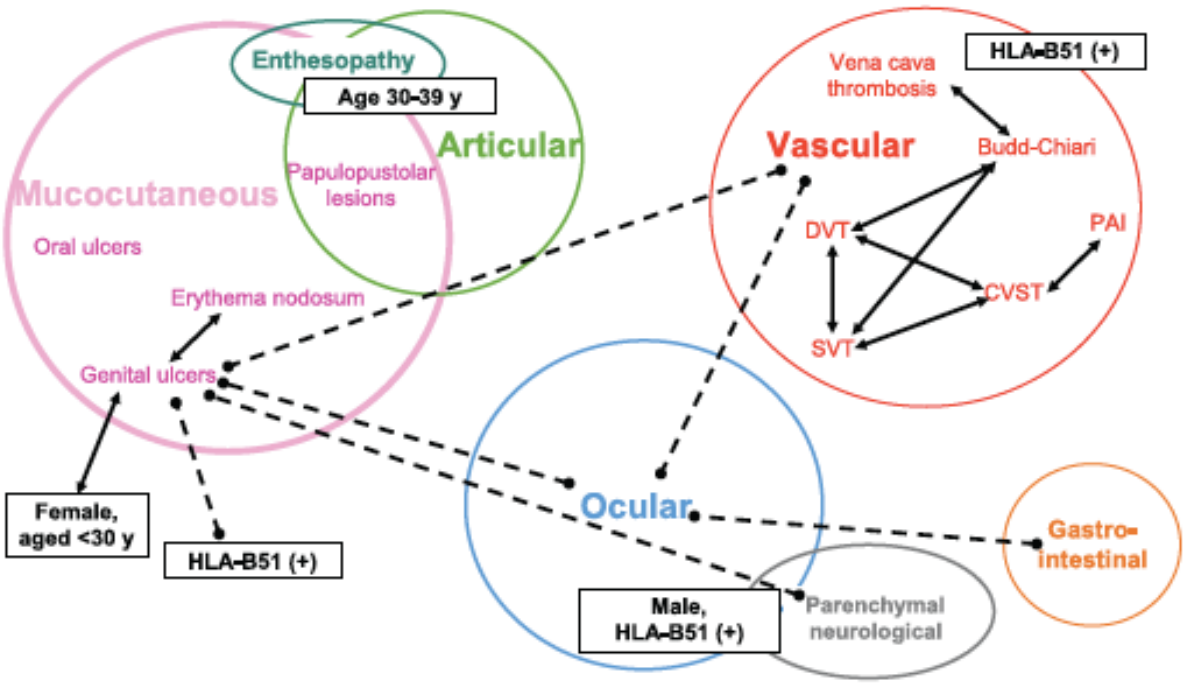

Figure 6. *Associations of different clinical involvements, and demographic and genetic patients' features (7).*

1.4 Diagnosis

During the last century, several classification or diagnostic criteria have been developed to help clinicians in everyday patient management and in conducting studies to better understand this complex condition. Moreover, no specific laboratory test, imaging or biopsy findings are available to definitively establish a certain diagnosis. Furthermore, as the clinical manifestations are different and sometimes insidious, differential diagnosis might be quite complex.

The International Study Group (ISG) criteria (1990) are the most widely used criteria (table 3), although there were two important limitation (86). First, approximatively 80% of BS patients evaluated for formulate the criteria were from Middle East, where gastrointestinal related BS symptoms were infrequent. Second, the ISG criteria do not consider symptom prevalence, which in fundamental given the setting in which these criteria are being used.

Mandatory criteria (required)	Minor criteria (at least 2 required)
• Recurrent oral apthous ulcers (at least 3occurrences in a 12-month period	• Recurrent genital ulcers • Eye lesions • Skin lesions (ie, erythema nodosum) • Positive pathergy test

***Table 3**: International Study Group (ISG) criteria (1990) for diagnosis of Behçet disease --which requires the presence of oral ulceration plus any two of genital ulceration, typical defined eye lesions, typical defined skin lesions, or a positive pathergy test (86).*

With the collaboration of 27 countries, a new set of criteria with higher sensitivity and specificity was formulated in 2014: The International Criteria for Behçet's disease (ICBD) (69). The aim of these new criteria is to take into consideration the complex multiplicity of potential BS clinical manifestations, also including CNS and vascular system involvement in order to allow for diagnosis formulation even in absence of oral aphthae (table 4). These characteristics allowed to increase ISG criteria sensitivity while maintaining good disease specificity. A score is assigned to each sign/symptom and BS diagnosis is only confirmed if the total ranking of all criteria is equal or higher than 4 (table 4).

Sign/symptom	Points
Ocular lesions	2
Genital aphthosis	2
Oral aphthosis	2
Skin lesions	1
Neurological manifestations	1
Vascular manifestations	1
Positive pathergy test*	1*

*Pathergy test is optional and the primary scoring system does not include pathergy testing. However, where pathergy testing is conducted one extra point may be assigned for a positive result.

***Table 4**. International Criteria for Behçet Disease – point score system: scoring ≥4 indicates Behcet's diagnosis (68).*

Once again, the main reported limitation of both ISG and ICBD diagnostic criteria is that they do

not take into account patients' environmental and genetic risk factors (such as geographical region of origin) (1).

1.5 Treatment

The basic principles in BS treatment are to suppress inflammation promptly and prevent damage and relapses. Since the disease has a heterogeneous nature, its treatment varies according to the type of involvement. Mucocutaneous and joint involvement in BS patients may reduce the quality of life but do not result in permanent damage. Conventional treatment is the first choice in these patients. On the other hand, immunosuppressive treatment is mandatory in patients with major organ involvement (84). Otherwise, it can cause morbidity or mortality.

As mentioned above, BS is a multisystemic vasculitis, characterized by different clinical involvements, including mucocutaneous, ocular, vascular, neurological, and gastrointestinal manifestations. Based on this concept, BS can be hardly considered as a single clinical entity and growing evidence supports that, within BS, different phenotypes, characterized by clusters of co-existing involvements, can be distinguished. However, tailoring the treatments on patient's specific phenotype, rather than on single disease manifestation, could represent a valid strategy for a personalized therapeutic approach to BS patients (84). The most important therapeutic approaches for different BS phenotypes are summarized in figure 7.

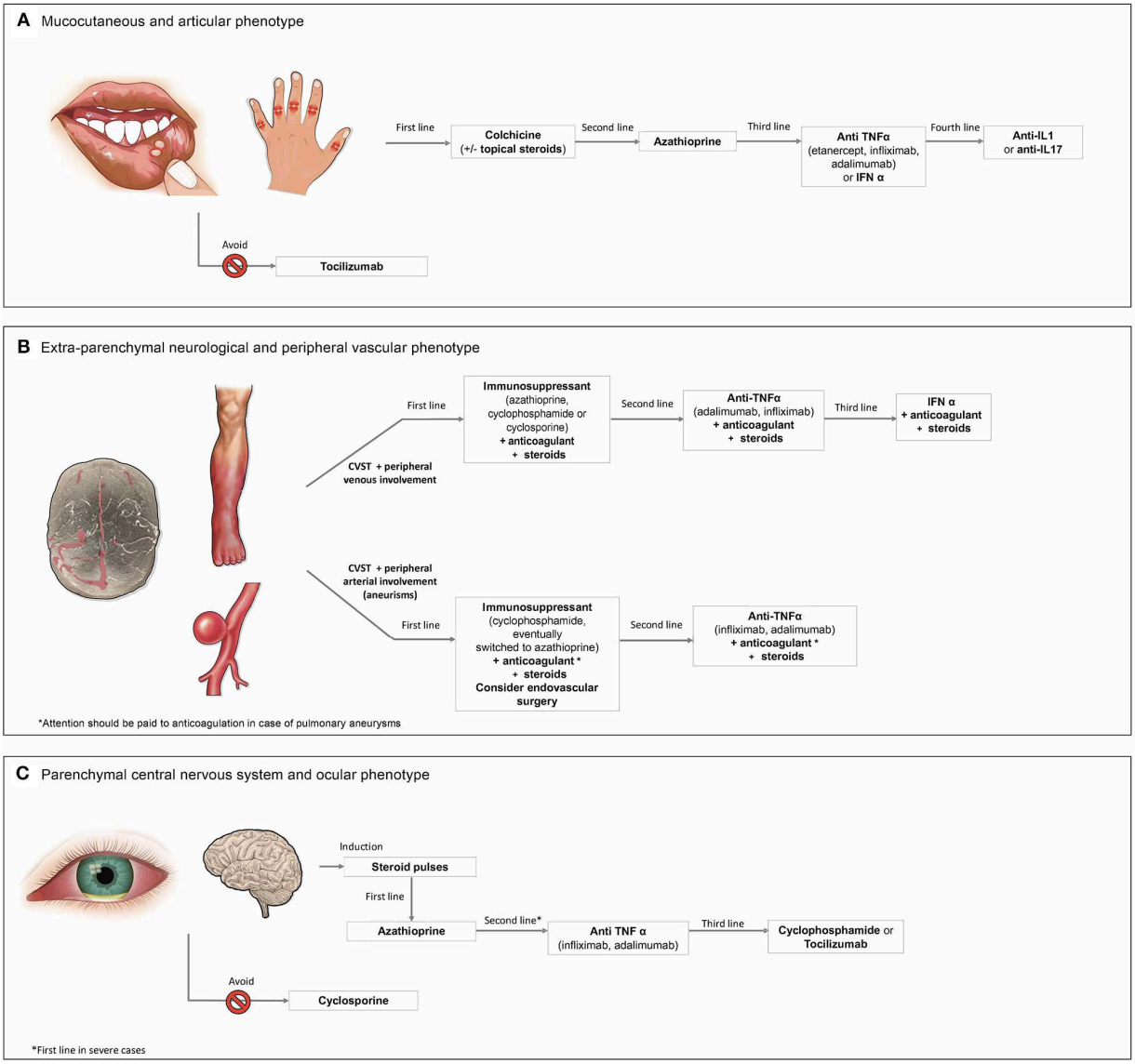

Figure 7. *Therapuetic approach to different clinical phenotypes of Behçet's syndrome* (84).

In patients newly diagnosed with BS and presenting "mucocutaneous and articular phenotype," first-line treatment should be based on colchicine. Two randomized controlled trials (RCTs) showed that colchicine led to a significant improvement of oral and genital ulcers, erythema nodosum, and articular symptoms (87)(88).

The 2018 EULAR recommendations support the use of colchicine as first-line systemic treatment, especially when the dominant lesions are erythema nodosum or genital ulcers (89).

In patients intolerant or resistant to colchicine, azathioprine (AZA) can represent an effective second-line treatment. Efficacy of AZA for oral and genital ulcers and for arthritis or mild ocular

involvement was documented (90).

In patients inadequately controlled with, or intolerant to, the aforementioned synthetic immunosuppressive regimen, the use of biologic strategies, with anti- TNF-α, or interferon (IFN) a should be considered. Among anti- TNF-α agents, only etanercept (ETN) has been studied in a trial on 40 BS patients with mucocutaneous disease and/or arthritis, showing a significant decrease of oral ulcers, nodular, and papulopustular lesions (91). However, data on the efficacy of ETN on arthritis were not conclusive, and the effects of this drug on genital ulcers were comparable with those in the placebo group. Conversely, the use of adalimumab (ADA) and infliximab (IFX) is supported by different observational studies and case series (92).

The efficacy of IFNα in the "mucocutaneous and articular phenotype" was reported in a retrospective observational study on 18 BS patients, treated for 12 weeks (93). Later on, in an RCT, IFNα was shown to control oral and genital ulcers, papulopustular lesions, erythema nodosum-like manifestations, and articular symptoms, while improving the severity and the frequency of ocular attacks (94). Of note, the safety profile of this drug deserves some attention, since adverse events including flulike syndrome, leukopenia, transient elevation of liver enzymes, as well as psychiatric disorders have been reported (94).

Some evidence (although not consistent) supports the use of IL-1 inhibitors anakinra (ANA) or canakinumab (CANA) (95)(96). In a recent case series of five BS patients with active and refractory mucocutaneous and articular manifestations, the anti-IL17 agent secukinumab was associated with a consistent improvement of both mucocutaneous and articular involvements (97).

Regarding other promising treatments, growing evidence supports the use of ustekinumab (98) and apremilast (99) for the control of mucocutaneous involvements.

Regarding patients with BS and presenting "extra-parenchymal neurological and peripheral vascular phenotype", high-dose glucocorticoids are the mainstay treatment for rapid induction of remission. There is no consensus on the use of additional anticoagulants or immunosuppressants, since recurrence is infrequent with this manifestation. Accordingly to the severity of this clinical

involvement, the first-line treatment should be based also on immunosuppressants such as AZA, cyclophosphamide (CYC) or cyclosporine (CSA) (100). In patients with refractory peripheral venous thrombosis, anti- TNF-α, namely, ADA, or IFX, should be used, alone or in combination with traditional disease-modifying anti-rheumatic drugs (DMARDs) (1)(100). Eventually, IFN a can be considered a therapeutic approach in selected cases, however, the role of this treatment for the control of CNS vascular involvements is still unclear. No RCT has determined the optimal therapeutic management of "parenchymal central nervous system and ocular phenotype". The induction treatment of acute severe neuro-BS is mainly based on high-dose corticosteroids, followed by the gradual tapering of the oral doses over 3–6 months. As first-line treatment AZA should be used, according to current EULAR recommendations (89). In case of severe ocular and parenchymal CNS involvements, the use of second-line options, namely, anti-TNF-α drugs, should be considered as first-line treatment. Further therapeutic options for this phenotype are CYC or TZC. According to a 10-year longitudinal study, CYC in association with AZA and prednisolone, was the best treatment for retinal vasculitis, before opting for biologic agents (101). The anti-IL6R TCZ is a promising treatment in the "parenchymal neurological and ocular phenotype." Results from its effectiveness for refractory neuro-BS (102), while a recent retrospective study on 11 patients with refractory uveitis reported rapid and sustained ocular improvement in all the patients (103). As for other nonbiologic alternatives, IFN a is highly effective for ocular control (89), and might have a potential role also for refractory neuro- BS. Notably, the use of CSA should be avoided in the "parenchymal neurological and ocular phenotype" due to an increased risk of CNS manifestations (104).

2. Gut Microbiota

The human gut microbiota (GM) – the enormous community of symbiont microorganisms inhabiting our gut – has been recognized as a key factor for human health and homeostasis (105). The role of GM in our physiology is so profound that human beings have been reconsidered as super-organisms, being the result of millennia of co-evolution with their microbial counterpart (106). The recent adoption of germ-free mouse models allowed to disclose several aspects of the human biology which rely on the mutualistic interaction with our "microbial organ," including their role on the energetic homeostasis, the estrogen equilibrium and the function of the immune system (60).

In particular, dynamic interactions between GM and a host's innate and adaptive immune systems have been shown to be essential for maintaining intestinal homeostasis and inhibiting inflammation. GM metabolizes proteins and complex carbohydrates, synthesizes vitamins, and produces an enormous number of metabolic products that can mediate the cross-talk between gut epithelium and immune cells. As a defense mechanism, gut epithelial cells produce a mucosal barrier to segregate microbiota from host immune cells and reduce intestinal permeability. Thus, an impaired interaction between gut bacteria and the mucosal immune system can lead to an increased abundance of potentially pathogenic gram-negative bacteria and their associated metabolites, disrupting the epithelial barrier and increasing susceptibility to infections.

Gut dysbiosis, i.e. the negative alteration in gut microbial composition, can therefore result in dysregulate immune responses, causing inflammation, oxidative stress, and insulin resistance. Over time, chronic dysbiosis and the leakage of microbiota and their metabolic products across the mucosal barrier may therefore contribute to an increased prevalence of cardiovascular diseases, autoimmune diseases, inflammatory bowel diseases, and a variety of cancers (107).

2.1 Gut Microbiota Metabolites: Focus on Short-Chain Fatty Acids

Under normal circumstances, GM produces metabolites to communicate with the immune system and to modulate immune responses (108). These metabolites play key roles in inflammatory signaling, interacting both directly and indirectly with host immune cells (109). Among the different GM metabolites, some bacteria, including *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, and *Anaerostipes butyraticus* (110), digest complex carbohydrates via fermentation, producing short-chain fatty acids (SCFAs) (111).

SCFAs, consisting mainly of butyrate, propionate, and acetate (109), are microbiota-derived metabolites enriched in the gut lumen that regulate host immune cells and provide a carbon source for colonocytes (112). By binding G-protein coupled receptors (GPCRs) and altering gene expression via reducing the activity of histone deacetylases (HDACs), SCFAs are essential for reducing local inflammation, protecting against pathogen infiltration, and maintaining intestinal barrier integrity.

SCFAs have multiple functions that are tissue or cell type dependent. For example, in addition to regulating cellular turnover and exerting barrier functions that maintain intestinal epithelium physiology, SCFAs also exhibit anti-inflammatory properties on host immune cells, regulating the expression of pro-inflammatory cytokines such as TNF, IL-12, IL-6 through activation of macrophages and DCs (113).

Among SCFAs, butyrate was shown to have a role as an anti-inflammatory agent, primarily via inhibition of nuclear factor κB (NF-κB) activation (114), thus being involved in a critical step to reduce inflammatory responses (115). Indeed, NF-κB activation is known to regulate cellular genes involved in early immune inflammatory responses, including IL-1β, TNF-α, IL-2, IL-6, IL-8, IL-12, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), T cell receptor-α (TCR-α), and MHC class II molecules (114). Butyrate inhibition NF-κB by inhibiting

HDACs, via induced Zn2+ binding in their active site (116), enhancing histone H3 acetylation at conserved FOXP3 promoter and enhancers sequences, inducing vigorous gene expression and functional maturation (107).

Moreover, butyrate's anti-inflammatory effects are also mediated by the upregulation of peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor highly expressed in colonic epithelial cells, and the inhibition of IFNγ signaling. Butyrate also represents the main energy source for colonocytes (117) and, stimulating the release of mucins, it also exerts a protective role strengthening the gut epithelial barrier.

2.2 Gut Microbiota and the Immune System

The maintenance of immunologic homeostasis in the mucosal layer is a demanding task requiring the discrimination between billions of beneficial commensals and pathogenic invaders. Gut microbiota communities change in composition in relation to different gastro-intestinal tract, and within the distinct lamina of intestinal mucus. The proximal small intestine is much less immunologically active than the ileum and colon, and the colonization of enteric microorganisms in the gut promotes an increase in permeability that allows macromolecules and antigens to pass from the intestine to the bloodstream, which may cause immune-mediated pathologic conditions (107). Gut permeability has been closely linked to both commensal microbiota and elements of the mucosal immune system, and is influenced by many factors including alterations of mucus layers, epithelial damage, and changes in the composition of gut bacteria (118), as gut microbial fermentation products and cellular components play pivotal roles in maintaining epithelial integrity.

Gut homeostasis is mediated by the preponderance of obligate anaerobic members of *Firmicutes* and *Bifidobacteriaceae*, whereas an increase in facultative anaerobic *Enterobacteriaceae* is a common marker of gut dysbiosis (119). Under gut homeostatic conditions, intestinal epithelial cells (IECs) regulate immune response and alter the local environment through the uptake of

SCFAs, using both passive and active mechanisms. SCFAs, particularly butyrate, promote an oxygen free environment by stimulating PPAR-γ, disrupting the pH balance and inhibiting the colonization of pathogens (figure 8). Specifically, the IECs synthesize peroxisome proliferator-activated receptor gamma (PPAR-γ), stimulated by butyrate. PPAR-γ helps maintain a local hypoxic environment by encouraging oxidative phosphorylation in colonocytes and oxidation of SCFAs by the mitochondria (119).

The obligate anaerobic SCFA-producing bacteria grow vigorously in such an environment, while the facultative anaerobic enteric pathogens' growth is suppressed (119). Concurrently, PPAR-γ activation decreases NOS2 levels in IECs, hindering the manufacture of both inducible NO synthase and nitrate, critical sources of energy for facultative anaerobic pathogens (119). Moreover, propionate provides resistance to the expansion of pathogenic bacteria in a PPAR-γ independent manner, proposing some similarity in SCFAs effects. Indeed, SCFAs mediate the intracellular acidification of pathogens, which is protective against pathogen infection.

Conversely, under pathologic conditions, the inhibition of the PPAR-γ -signaling pathway stimulates metabolic reprogramming, gut dysbiosis, and SCFAs exhaustion (120). This encourages colonocyte metabolism to adopt anaerobic glycolysis, called the Warburg effect, and limits the oxidative metabolism, increasing the concentration of oxygen, nitrate, and lactate in the gut lumen (119).

Additionally, virulence factors common to *Enterobacteriaceae*, such as Salmonella or Shigella, stimulates the migration of neutrophils through the epithelium, reducing the abundance of SCFAs. This acts as a negative feedback loop, encouraging pathogen growth, and demonstrating a causal interaction connecting microbiota-derived metabolism and gut epithelial health (107).

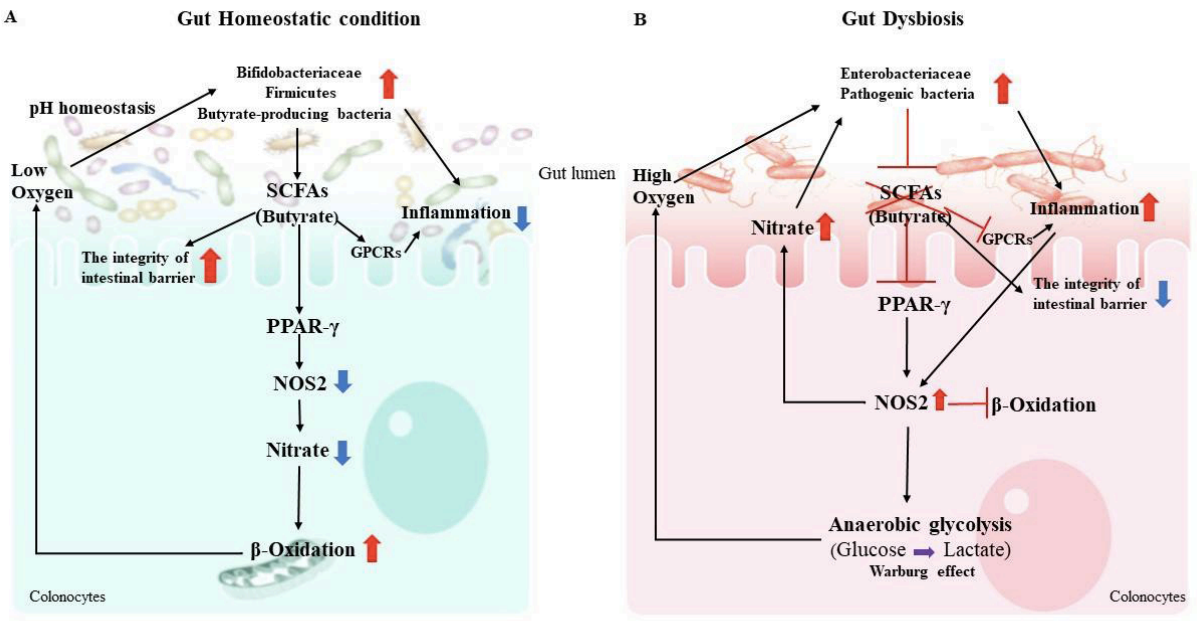

Figure 8. *The interaction between microbiota-derived metabolism in the gut epithelium. (A) In the gut homeostatic condition, gut microbiota, especially butyrate-producing bacteria, metabolizes fiber into fermentation products such as short chain fatty acids (SCFAs). (B) During gut dysbiosis, Enterobacteriaceae use virulence factors to stimulate neutrophil migration through the epithelium, reducing SCFA-producing bacteria, decreasing the abundance of short-chain fatty acids in the lumen (107).*

Host-body defense systems use multiple mechanisms to discourage colonization by pathogens. Recognition of gut microbiota initiates with two pattern recognition receptor systems (PRRs): nucleotide-binding oligomerization domain receptors (NODs) and toll-like receptors (TLRs) (121). PRRs are highly expressed in IECs, macrophages, and intestinal DCs. PRRs identify microbe or pathogen associated molecular patterns (MAMPs or PAMPs) on pathogens and commensals alike (107). After a microbe has been identified or has overrun the epithelium, an immunologic response targeted to the microbe is mounted (121). Upon PAMP recognition, PRRs activate a variety of intracellular signaling pathways, using chains of ligands, transcription factors, and kinases to signal the presence of infection in the host and trigger changes in gene expression that alter levels of a range of pro-inflammatory and anti-microbial cytokines, chemokines, and immunoreceptors (122).

The transfer of bacterial antigens to nearby lymphoid tissue induces the activation of T and B cells, while the activation of dendritic cells (DCs) leads to the production pro-inflammatory

cytokines (123). As a result, pro-inflammatory immune responses are activated through naive T cell differentiation.

It has been clearly shown that gut-associated lymphoid tissues (GALT) promote the production of IgA following microbiota colonization. IgA performes a fundamental task in mucosal homeostasis in the gut, functioning as the dominant antibody (124). GALT is a tissue consisting of Peyer's patches (PPs), plasma cells, and lymphocytes from the mesenteric lymph nodes and lamina propria. GALT maintaines the immune response via up-take of gut luminal antigens through M-cells, activating antigen-specific immune responses (124). Indeed, the depletion of IgA regulation results in dysregulation of gut microbiota, which in turn causes immune system dysfunction.

A breakdown in these regulatory mechanisms and in the cross-talk between the GM and the immune system might result in a set of chronic inflammatory conditions that are collectively known as Inflammatory Bowel Disease (IBD). The relationship between mucosal immune dysfunction and IBD is illustrated by the fact that both Crohn's Disease (CD) and Ulcerative Colitis (UC) are associated with genes that are critical in maintenance of the epithelial barrier and the regulation of innate and adaptive immune responses. The etiology of IBD is complex and is believe to be the consequence of genetic factors, the host immune system, and environmental factors such as the microbiota. Stressors such as defined infections have been also proposed to contribute to the induction of these disorders (108). The immune system is not only controlled by its symbiotic relationship with the microbiota but is also exquisitely sensitive to the nutritional status of the host. Evidence now exists for a multi-directional interaction between the diet, immune system and commensal microflora. The intestine serves as the primary site of nutrient absorption in the body. As such, the immune system and commensal microbiota are sensitive to changes in diet. Dietary control of immune cells is mediated both by metabolic requirements as well as direct sensing of food- derived metabolites.

2.3 Gut Microbiota and Behçet Syndrome

Gut microbiota ecology is dynamic, changing in response to age, diet, geographical location, medication use, and ingress and egress of microbiota. Most bacteria are introduced through environmental exposure, and some are transient and lack the capacity to permanently colonize the intestinal environment or are outcompeted by commensal microbes. The health of the microbial community at a site can be ascertained in terms of stability, diversity, resistance and resilience. In other words, it can be characterized in terms of the richness of the ecosystem, its vulnerability to compositional and functional change, and its capability of reestablishing itself to its original state. Thus, the ecological balance of the microbial community can be disturbed by loss of diversity, thriving of pathobionts, or withering of commensals (figure 9) (125).

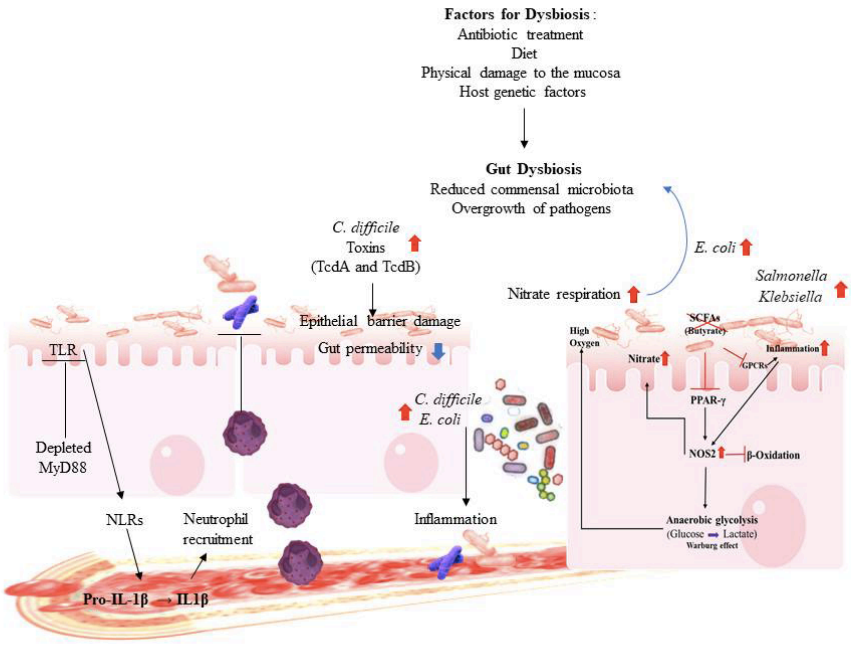

Figure 9. *Gut dysbiosis. Multiple factors including diet, antibiotic treatment, and host genetic can interrupt the community of commensal microbial, resulting in increased colonization by pathogens and the outgrowth of indigenous pathobionts (107).*

Abundant evidence suggests that the gut microbiota may be involved in the initiation and amplification of disease progression in patients with autoimmune diseases (126).

The association between gut microbiota imbalance and autoimmune diseases is probably due to several mechanisms that may impact the human immune system and function. For instance, modulating the host immune response and activation of APCs, including DCs, may evoke antigen presentation and cytokine production, subsequently affecting T cell differentiation and function. Furthermore, this might lead to a disruption in the homeostasis between T_H17 cells and T_{reg} cell (figure 10). There may also be similarities between reactions to foreign antigens and self-antigens, attributable to antigenic mimicry; consequently, pathogen-derived autoreactive T and B cells are activated, promoting autoimmunity. Moreover, the permeability of the intestinal mucosa has been shown to be altered in autoimmune diseases, as expression of tight junction proteins is modulated.

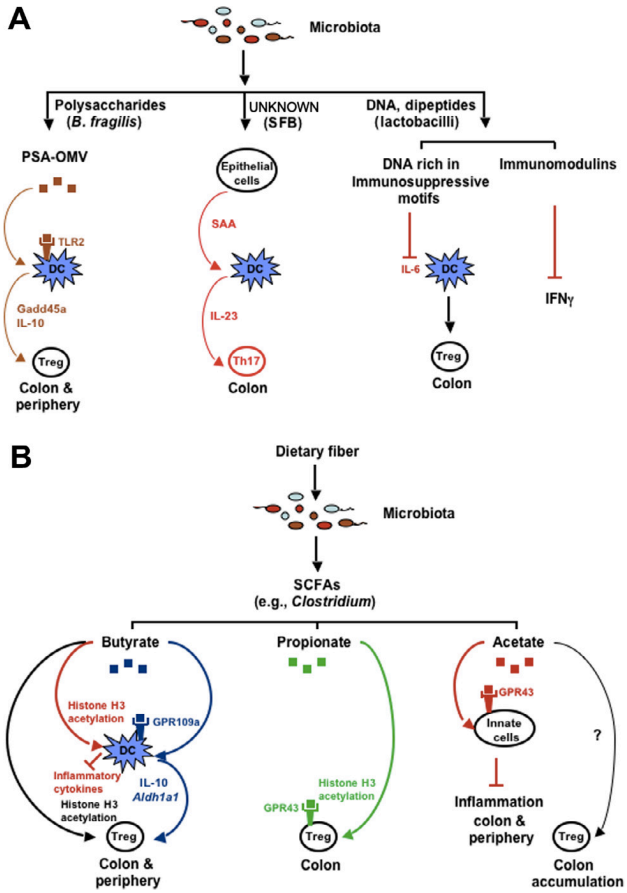

Figure10. *Gut commensal influence on the immune response. (A) The molecules expressed and produced by commensals affect the inflammatory response and T_{reg} differentiation. (B) Dietary fiber influences the gut microbiota composition, and in turn, the metabolites produced by commensals affect the inflammatory response and T_{reg} differentiation/accumulation, Aldha1: aldehyde dehydrogenase isoform 1A1; DC: dendritic cells; Gadd45a: growth arrest and DNA damage inducible alpha; GPR: G-protein-coupled receptor; NLR4: NLR family CARD domain*

containing 4; PSA-OMV: polysaccharide A-associated outer membrane vesicles; SAA: serum amyloid A; SCFAs: short-chain fatty acids; TLR: Toll-like receptor; T_{reg}: regulatory T cells (127).

Similarly to what observed for other autoimmune diseases, growing studies have shown oral and intestinal microbiota composition perturbations in patients with BS, with possible association with several extra-gastrointestinal manifestations (128)(129).

These findings were corroborated by a study on mouse model of autoimmune uveitis that received fecal microbiota transplantation with feces from BS patients. Notably, animal experiments revealed significantly exacerbated uveitis activity and increased the production of inflammatory cytokines including IL-17 and IFN-γ following fecal transplant, thereby confirming the association of the gut microbiome composition with BS pathogenesis (130).

In the Rheuma-BIOTA study (131), significant differences were noted in the composition of intestinal microbiota in patients with BS compared with healthy adults. *Actinomyces, Libanicoccus, Collinsella, Eggerthella, Enetrohabdus, Catenibacterium, and Enterobacter* were significantly higher in the BS group than in general population. Moreover, *Bacteroides, Cricetibacter, Alistipes, Lachnospira, Dielma, Akkermansia, Sutterella, Anaerofilum, Ruminococcease-UCG007, Acetanaerobacterium, and Copropaacter* were significantly lower in the BS group than in the control group. Furthermore, it was also found that the different clinical forms of BS present differences also in terms of GM composition.

Moreover, an Italian study on reported an overall reduced intestinal bacterial diversity and reduced butyrate-producing bacteria in patients with BS (60). By comparing GM structure and SCFAs content in BS patients and healthy controls, it has been described a peculiar distortion of the GM profile in BS, as well as the consequent impact in terms of pattern of SCFAs production. In particular, the BS-associated GM was characterized by a significant reduction of the total bacterial diversity compared to a healthy intestinal ecosystem. Furthermore, a significant depletion of *Roseburia* and *Subdoligranulum* (Clostridium cluster) in the GM of BS patients was

observed. Belonging to the Clostridium clusters XIVa and IV, respectively, these microorganisms are well-known butyrate producers of the human GM and are generally associated with a healthy GM structure (132). Strengthening these findings, Consolandi et al demonstrated a significant decrease of butyrate production in Behçet's patients (60).

More recently, a Dutch study reported that in patients with BS, the abundance of Barnesiellaceae and Lachnospira is decreased. Barnesiellaceae might exert protective anti-inflammatory effects by reducing the level of TNF-α, one of the key and targeted cytokines of BS, and the decrease in butyric acid production may be regulated by reducing the abundance of Lachnospira, thereby affecting T-cell differentiation and causing inflammation in BS. GM participate in the occurrence and development of BS mainly by Tregs and affecting the balance of Th_{17}/T_{reg} cells, but there are also some bacteria that play a role through other mechanisms (133).

Tecer, examining fecal microbial flora of 7 BS patients with uveitis, identified that, in these patients, the fecal microbiota consisted mainly of Firmicutes as a phylum, Clostridia as a class, Clostridiales as an order and Prevotella copri as a species. Veionellaceae, Succinivibrionaceae families and Succinivibrio, Mitsuokella genera were found to be dominant in the gut microbiota of BS patients who were fed by different diet and probably had different genetical factors (134).

Taken together, these data provide experimental evidence supporting a dysbiotic structure of the GM ecosystem in BS; characterized by a low biodiversity and by a depletion of key butyrate-producing members, the GM of Behçet's patients deviates from a mutualistic layout, resulting in an overall decrease of butyrate abundance in the gut.

As extensively described above, butyrate is a key microbial metabolite in the context of the GM–host mutualism, with a well-consolidated role as a modulator of the host immune function. In particular, butyrate exerts an important role for the maintenance of the host immune homeostasis (115), showing both systemic and local immuno-modulating properties, while also promoting the integrity of gut barrier.

While circulating butyrate prompts the generation of extrathymic T_{reg} (135), gut butyrate has been reported to inhibit local pro-inflammatory cytokine (115).

T-lymphocytes producing INF-γ and IL-17, together with neutrophils, are thought to represent the main effector cells in the pathogenesis of BS (26). Interestingly, butyrate is able to promote differentiation of T_{reg} via several mechanisms, thus influencing immune regulation and altering the mucosal immune response (119); the butyrate impairment in BS patients could favor a reduced T_{reg} mediated control, thus promoting a powerful immuno-pathological T cell responses.

To underline the close association between GM dysbiosis and the immune response, Kim and colleagues reported a significantly higher abundance of fecal Bacteroides uniformis in BS patients with active disease than in healthy controls and patients with stable disease. In particular BS patients with uveitis had a different GM composition (in terms of taxa abundance), compared to those without uveitis (136).

Interestingly, GM dysbiosis, similarly to the one detected in BS, has been previously observed in Inflammatory Bowel Disease (IBD), whose patients are generally characterized by a reduction of GM ecosystem diversity, the decrease of butyrate producers belonging to the Clostridium clusters IV and XIVa, and the corresponding decrease of butyrate production in the gut [29,51]. These deviations from a GM mutualistic structure have been hypothesized to contribute to IBD onset and progression through the establishment of a self-sustained pro-inflammatory loop in the gut (126).

Beside the central role is played by intestinal microbiota, several studies have demonstrated that also the salivary microbiota might influence the auto-immune mechanisms sustaining BS pathogenesis. Namely, a study by Ye and colleagues found an increased abundance of Haemophilus parainfluenzae in BS salivary microbiota, while the most depleted species included Alloprevotella rava and species in the genus Leptotrichia. However, the precise composition of the salivary micriobiome and its changes are difficult to be evaluated, given that several environmental factors might influence it (including smoking, periodontal treatments, …) (130).

3. Oxidative Stress and Behçet Syndrome

Several studies have demonstrated that increased ROS generation by both vascular and immune cells occurs in cardiovascular diseases and that oxidative stress contributes to vascular damage and atherogenesis (61). Chronic inflammation is a well-known risk factor for the development of thrombosis. However, the details of the complex crosstalk between inflammation and hemostasis are far to be elucidated (137).

As already mentioned above, among the systemic inflammatory diseases characterized by thrombotic tendency, BS is a peculiar systemic vasculitis, in which cardiovascular events affect up to 45% of BS patients involving both arterial and venous vessels of all sizes, but deep and superficial vein thrombosis of the lower extremities are the most common vascular manifestations of the disease (78).

An overall imbalance in blood redox status (assessed by ischemia-modified albumin, advanced oxidation protein products, and pro-oxidant/antioxidant balance) has been reported in BS. Moreover, systemic inflammation more than usual thrombophilic factors is thought to be the main trigger of thrombosis in this condition and seems to be mainly mediated by T lymphocytes, monocytes, neutrophils, and proinflammatory cytokines along with endothelial cell dysfunction (61). Based on these pathogenetic concepts and clinical experience, the EULAR recommendations for the management of BS suggest that thrombosis should be treated with immunosuppression rather than anticoagulation (89), as an inflammation-induced thrombosis.

Indeed, the array of processes related to inflammatory thrombosis comprises pathways that are not fully responsive to anti-thrombotic management (directed at the extrinsic pathway and generation of thrombin) (138). It is now widely accepted that a strict relationship among inflammation, endothelial dysfunction and oxidative stress exists (139). In particular, neutrophils enhance the risk, severity and adverse outcome of thrombosis acting as a modulator of several processes: causing the rupture of atherosclerotic plaque, inducing platelet activation, possible

tissue factor carriage, altering the antithrombotic function of the endothelium and inhibiting the response to fibrinolytic agents (140). In addition, a dose-dependent relationship between neutrophil activation, circulating nucleosomes and development of deep vein thrombosis has been reported.

Accordingly, a global blood redox alteration (revealed by ischemia-modified albumin, advanced oxidation protein products, and overall pro-oxidant/antioxidant balance) has been reported in BS patients. Specifically, lipid peroxidation markers in serum, erythrocytes and neutrophils and decreased levels of antioxidant enzymes (glutathione peroxidase, catalase) have been reported in BS patients and have been even indicated as prognostic tools in this disease.

Serum from BS patients also exhibits increased ROS levels, mainly represented by O^{2-} and H_2O_2 and clear signs of a massive release of neutrophil extracellular traps (NETosis) (141). Enhanced levels of plasma MPO activity have been found in BS patients, beside raised levels of plasma nitrate/nitrite, which are substrates for MPO and induce the reactive nitrogen dioxide (NO_2) oxidizing agent generation. MPO-dependent nitrate/nitrite depletion, leads to the reduction of these substrates for nitric oxide synthase reactions and consequent decrease in the production of nitric oxide (NO), a crucial modulator of smooth muscle contraction and vasodilation.

Fibrinogen, a plasma protein particularly susceptible to oxidation, plays a crucial role not only in autoimmunity and coagulation, but also in inflammatory processes. The ability of fibrinogen to contribute to the inflammatory response rely on its specific interaction with integrins, which are leukocyte cell surface adhesion receptors expressed on neutrophils, monocytes, macrophages, and several subsets of lymphocytes. Accordingly, some authors recently showed neutrophil hyperfunction, increased ROS production and endothelial cell dysfunction associated to an impaired fibrinolysis in BS patients (71) (Figure 11).

To clarify the possible relationship among these processes, a recent study by our group, performed in a large population of BS patients, was undertaken to elucidate the mechanisms of inflammation-induced thrombosis. In that study, fibrinogen oxidative modifications, fibrinogen protein

structure, fibrinogen function (assessed in terms of thrombin-dependent fibrin polymerization and fibrin susceptibility to plasmin-induced lysis) and possible blood ROS sources were explored. The findings clearly indicated that BS patients show a global redox status impairment along with an enhanced fibrinogen carbonylation. Moreover, clot structure revealed, in BS, a modified architecture mostly characterized by a tight fibrin network composed of filaments with slightly decreased average fiber size.

In another series of experiments aimed to clarify the mechanisms of fibrinogen oxidation, it was shown that purified fibrinogen was markedly carbonylated when incubated with neutrophils derived from BS patients, but not with monocytes or lymphocytes from the same patients. This went along with a significant increase in NADPH oxidase activity which was specifically evident only in the neutrophil fraction. Importantly, in BS patients, the extent of fibrinogen oxidation appeared significantly correlated with neutrophil-derived ROS, but not with lymphocyte- or monocyte-derived ROS. Fibrinogen oxidation was also strictly related to fibrinogen function which resulted significantly affected both in terms of polymerization and in terms of plasmin-induced lysis.

Notably, these oxidative alternations in fibrinogen structure were associated with a significant impairment also in fibrinogen function; indeed, fibrinogen polymerization and fibrin susceptibility to plasmin-induced lysis was markedly affected in BS patients when compared to control subjects. This is in line with the findings reporting that clots composed of thin fibers and reduced pores appear more thrombogenic. All these features strictly correlate with inflammation and oxidative stress (71).

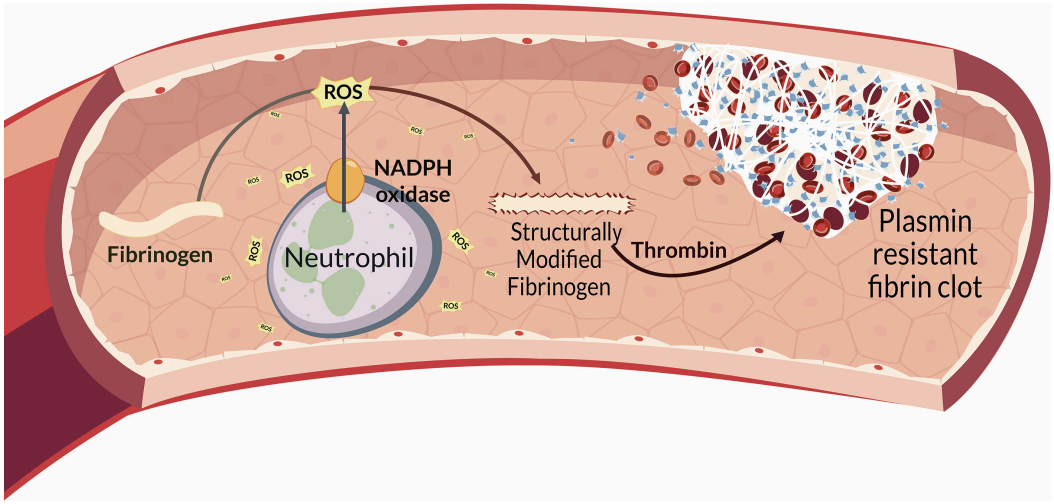

Figure 11. *Schematic representation of the NADPH oxidase (NOX2) enzyme complex. NOX2 activation depends on the assembly of four cytosolic proteins (p47phox, p67phox, p40phox, and Rac2) with two transmembrane proteins representing the catalytic core of NOX2 (i.e., p22phox and gp91phox), which form the flavocytochrome b558 complex. The assembly of cytosolic subunits with membrane-bound cyt b558 complex induces the transfer of electrons from cellular NADPH to molecular oxygen and the consequent formation of O−.2 . Once activated, about 10 nmol/min O−. 2 per million neutrophils are produced during the oxidative burst (71).*

4. Background and Aim of the Study

BS has been recently classified at the crossroad between autoimmune and autoinflammatory syndromes, although the pathogenesis remains still unclear. BS is characterized by a wide range of potential clinical manifestations with no gold-standard therapy (65). GM has been found to influence several metabolic and immunological condition, and perturbed GM profiles have been indicating a fascinating link between intestinal microbes and health status. Recent evidences indicated that a peculiar dysbiosis of the GM ecosystem is present in BS patients and might correspond to specific changes in profiles of SCFAs production (60). Distinctly, GM ecosystem in BS showed a low biodiversity, as already showed in several chronic disorders (60)(107). It has been proved that in BS there is a significant depletion of well-known butyrate producers, *Roseburia* and *Subdoligranulum*, and a consequent decrease of butyrate production. Butyrate, preferentially used by colonocytes as fuel, might induce T_{reg} cell differentiation via several mechanisms. Hence, butyrate impairment in BS could favor a reduced T_{reg} activity, inducing an imbalance between pro-inflammatory and anti-inflammatory cells (127).

In this context, growing evidence suggest that high-fiber dietary patterns are able to promote a more favorable GM profile, and could be key mediators of microbial diversity (61)(143). The high adherence to a lacto-ovo-vegetarian diet -including high intake of non-refined cereals, fruit, vegetables and legumes– seems to be associated with a beneficial GM profile, with enrichment in fiber-degrading bacteria and increase of fecal SCFA (144). In a similar way, other dietary patterns have been shown to modulate GM dysbiosis, by supporting the recovery of a balanced microbial community of health-promoting SCFA-producing members with the decrease of pro-inflammatory groups (145)(146). Current evidence indicates that the consumption of certain fibers, such as inulin and resistant starch, leads to specific GM rearrangements with the production of more butyrate than others (147). All these findings suggest that the adherence to a controlled dietary profile such as lacto-ovo-vegetarian diet, possibly enriched in substrates with potential for

butyrate production, may select butyrate-producing bacteria – especially *Roseburia* and *Faecalibacterium prausnitzii*- which could reverse the proinflammatory dysbiosis observed in BS.

A randomized cross-over dietary intervention-controlled trial was used to prove that.

The specific aims of the study are:

a) to conduct a dietary intervention randomized controlled trial in order to investigate whether a lacto-ovo-vegetarian diet enriched in substrates with potential for butyrate production or a Mediterranean diet supplemented with butyrate could be beneficial for GM and for the amelioration of the clinical manifestations and disease severity of patients with BS
b) to evaluate the effects of these interventions on inflammatory markers, endogenous butyrate production and oxidative stress
c) to validate and extend the preliminary results regarding GM ecosystem dysbiosis in BS patients.

5. Patients and Methods

5.1 Study Design

This is a randomized, open-label, cross-over clinical trial conducted at the Careggi University Hospital, Florence (Italy). A cross-over design was adopted to allow comparison of a lacto-ovo-vegetarian diet (VD), a Mediterranean diet supplemented with butyrate (MD-Bt), and a Mediterranean diet without any supplement (MD), as control, within the same individual. Participants act as their own controls in cross-over studies, so individual differences are controlled for, making the error variance smaller and subsequently reducing the sample size required to find a significant effect due to increased statistical power. The study design followed the Standard protocol recommendation for interventional trials (SPIRIT guidelines (148). The study was approved by the Local Ethic Committee (ref. n.: 12773/OSS; date of approval 04/12/2018).

5.2 Eligibility Criteria

Inclusion criteria comprised diagnosis of BS according to ICBD international criteria (68), age 18– 65 years, signed informed consent, also to participate in a study where one of the proposed diets was a vegetarian pattern.

Only patients on stable corticosteroids and colchicine treatment, defined as no increase in the daily corticosteroids and/or colchicine dosage in the previous 3 months, were considered eligible.

Exclusion criteria included pregnancy or lactation; concomitant presence of serious illness or unstable conditions (other immune-mediated or autoimmune diseases, including inflammatory bowel diseases); chronic viral infections; malignancies; recent myocardial infarction; chronic liver disease; current or recent (past 6 months) participation in a weight loss treatment program or use of weight loss medication; adoption of a vegetarian diet for the past 3 months; current or previous (past 6 months) symptomatic confirmed infection with SARS-CoV-2, assessed by nasal

swab and seroconversion; antibiotic, prebiotic, or probiotic use in the past 3 months. The latter was assessed during the screening period, by standardized pharmacological anamnesis.

5.3 Interventions

This was a clinical randomized study with a cross-over design, composed by three interventional periods separated by two wash-out periods. After a 2-week run-in period –to assess participants' eligibility and to collect demographic details, signed informed consent, and 3-day dietary records - the eligible participants were randomly assigned to follow a 3-month dietary profile with a VD, a MD or a MD-Bt.

The VD contained inulin and resistant-starch-rich foods, eggs, and dairy, in addition to plant-based food, but did not contain meat, poultry, or fish. The MD was based on all the food categories and provided two portions per week of fish and three portions per week of fresh and processed meat (one of which consisted of fresh or processed red meat). The MD-Bt was similar to the MD but supplemented with 1.8 g/day of oral butyrate. The three different dietary patterns were isocaloric and related to the participants' nutritional requirements, with about 50–55% of energy derived from carbohydrates, < 30% from fats, and 15–20% from proteins. Participants could prepare their meals or eat at restaurants. Alcoholic beverages were limited to two per day for men and one per day for women. Interventions have been delivered by a dietitian through face-to-face, individual counseling sessions at the Careggi University Hospital. Participants provided with a detailed, 1-week menu plan with portions expressed in grams or milliliters as appropriate, and tips and information on the food groups that can be included and those that cannot.

5.3.1. Strategies to Improve Adherence

Adherence to the interventions was promoted using behavior change strategies including self-monitoring, and regular phone calls for dietary counseling. In particular, participants received at

least one unannounced phone call during each intervention, in which participants were recalled on their last 24-h diet period. Furthermore, participants were provided with a detailed one-week menu plan for each dietary period with all foods expressed in weight and/or volume measures, and a hand-out containing details on their assigned diet, including food groups that can be included and ones that should be avoided. The vegetarian menu plan also included recipes for preparing meals.

5.4 Outcome Measures and Assessment

5.4.1 Primary Outcomes

Primary outcomes were assessed through validated questionnaires, to analyze the progression of BS. They included:

- Clinical response of BS, assessed by the BDCAF (149) (https://www.behcetdiseasesociety.org/behcetwsData/Uploads/files/BehcetsDiseaseActivityForm.pdf), corticosteroid use and blood inflammatory parameters. The BDCAF assessed the presence of oral and genital ulceration, skin, joint, and gastrointestinal involvement, presence of fatigue and headache, using a 5-point scale according to the duration of symptoms, with 0 meaning no symptoms and 4 meaning symptoms for 4 weeks. The presence of eye, large vessel, or CNS involvement was documented with "yes/no" answers. In addition, patients rated on a 7-point scale how active they felt. Similarly, clinicians completed a 7-point rating scale to assess their opinion of overall disease activity, with lower scores representing better outcomes. Inflammatory parameters included C-reactive protein (CRP), expressed mg/L (normal value: 0-5 mg/L), and erythrocyte sedimentation rate (ESR), expressed as mm/h (normal value:2-30 mm/h). Glucocorticoids daily dosage was expressed as prednisone or prednisone equivalents mg/day.

- Severity of gastrointestinal symptoms assessed by the Symptom Severity Scale (SSS) modified form. The SSS was a multidimensional rating scale assessing overall symptom severity on a visual analogue scale (VAS). An overall score was calculated from six items: pain severity, pain frequency, abdominal bloating, dissatisfactory bowel habit, abdominal heaviness, and life interference. The modified SSS ranged from 0 to 600, with higher scores meaning more severe symptoms.
- Improvement of gastrointestinal-related BS symptoms assessed by the Global Assessment of Improvement Scale (GAI) modified form. The GAI assessed improvement of symptoms of BS using a 7-point scale, with higher scores meaning an improvement in the symptoms. The severity of abdominal pain, severity of abdominal distention, satisfaction with bowel habits, severity of headache, severity of exhaustion, severity of nausea, attention disorder, muscle/joint pain, and quality of life were investigated in response to the following question: "Compared to the way you felt before you entered the study, have your symptoms over the past 7 days been: 1) "Substantially Worse", 2)" Moderately Worse, 3)" Slightly Worse", 4)" No Change", 5)" Slightly Improved", 6)" Moderately Improved" or 7) "Substantially Improved".

5.4.2 Secondary Outcomes

Secondary outcomes were measured in blood and stool samples.

Blood samples were analyzed to assess:

- Change from baseline in reactive oxygen species (ROS) assessed by flow cytometry. In particular, leukocyte subpopulations (lymphocyte, monocyte, and granulocyte) ROS were measured [61].
- Change from baseline in plasma total antioxidant capacity (TAC) assessed by fluorometry, using oxygen radical absorbance capacity [61].

- Change from baseline in the lipid peroxidation markers assessed by spectrophotometry. It will estimated using the thiobarbituric acid reactive substances (TBARS) assay kit [61].

Stool samples were analyzed to assess:

- Changes in the composition of the GM, assessed by 16S rRNA gene-based next-generation sequencing on the Illumina MiSeq platform. Total microbial DNA was extracted from feces using the repeated bead-beating plus column Method (150). The V3 and V4 hypervariable regions of the 16S rRNA gene were sequenced following the Illumina protocol for 16S Metagenomic Sequencing Library Preparation.
- Change from baseline in fecal SCFAs assessed by gas chromatography - mass spectrometry (GC-MS). The metabolomic analysis of fecal waters was performed after sample preparation involving solid phase microextraction (SPME), followed by GC-MS analysis to detect the volatile metabolites (60).

5.5 Recruitment, Allocation and Follow-up

Patients were recruited at the Behçet Center of the Careggi University Hospital, Florence, Italy, or using advertisements on local media, newspapers, social media and websites.

This was a clinical randomized study with a cross-over design, composed by three interventional periods separated by two wash-out periods. After a 2-week run-in period –to assess participants' eligibility and to collect demographic details, signed informed consent, and 3-day dietary records - the eligible participants were randomized 1:1:1 to the three intervention arms through a web-based online randomization procedure and were assigned to follow a 3-month dietary profile with a VD, a MD or a MD-Bt.

The study design is depicted in figure 12. There were six clinical evaluations of the study population: at baseline before starting the nutritional interventions (T0); 3 months after the onset of the first nutritional intervention (T1); at the end of the first wash-out period, lasting 1.5 months, when individuals were allowed to resume their normal eating habits; at the onset of the second

intervention (T2) at 7.5 months after the onset of the study; at the end of the second nutritional intervention (T3); at the beginning of the third nutritional intervention (T4), and at the end of the third nutritional intervention (T5).

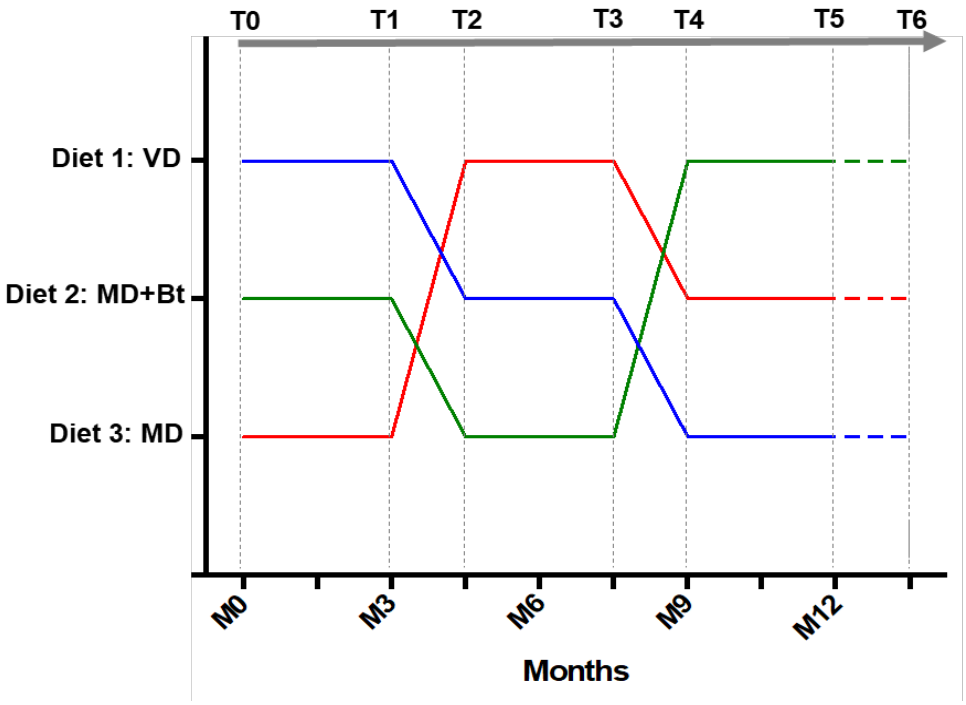

Figure 12: *Time-line of the MAMBA study. VD, vegetarian diet; MD, Mediterranean diet; MD-Bt, Mediterranean diet supplemented with butyrate.*

During the baseline visit, participants were instructed on the objectives and methods of the clinical trial and they were asked not to alter their physical exercise habits during the study. Anthropometric measurements, body composition, data on BS disease activity, clinical manifestations, and ongoing pharmacological treatments, and blood, urine, and stool samples were obtained from each participant at each follow-up timepoint (T0-T5).

5.6 Data Collection and Management

Follow-up assessments and data collection were performed at the Behçet Center and undertaken at the Unit of Clinical Nutrition of the Careggi University Hospital, Florence, Italy, by trial personnel. All participants were examined between 7.30 and 11.30 a.m. after a 12 h-fasting period.

Data were collected in an electronic database. Identifiable data or other documents were not recorded in the database and participants were identified by a unique trial ID only. Hard copies of data sheets linking the participant identification number to the person's contact details was kept securely in a locked filing cabinet in a locked office, accessible only to key research team members. Multiple strategies were employed to improve data quality during data collection, including accurate recruitment, a structured and time-limited protocol, the inclusion of a run-in period, the limitation of the burden and inconvenience of data collection to the participants, the development of a trusting and collaborative relationship between research units and participants, and double data-entry.

5.7 Anthropometric Measurements and Body Composition

Weight and height were measured using a stadiometer. Body mass index (BMI) was calculated as weight (kilograms)/ height (meters squared). Individuals were classified as overweight if their BMI was more than 25 kg/m2 but less than 30 kg/m2, and obese if their BMI was 30kg/m2 or more. Body composition was determined using a bioelectrical impedance analyzer (TANITA, model BC 420 MA) at the beginning and the end of each intervention phase.

5.8 Statistical Analysis

Continuous variables were expressed as mean plus/minus SD, or as median value and interquartile range (IQR), as appropriate according to data distribution. Normality of data distribution was

tested using the Shapiro-Wilk W test. Categorical variables were presented as numbers and percentages. All data were treated as paired samples from a crossover study. Continuous endpoints were analyzed within each dietary intervention group using the Student's t test for paired comparisons or the Wilcoxon signed rank test, as appropriate, to test whether the changes were statistically significant. Dichotomic endpoints were analyzed within each dietary intervention group using the McNemar test paired comparisons.

For the GM analysis, alpha-diversity was assesseed using different metrics, including the Faith's phylogenetic diversity, Chao1, observed species, and Shannon index. Beta-diversity was estimated by weighted and unweighted UniFrac distances (QIIME), which were used as input for Principal Coordinates Analysis (PCoA). Analyses were computed using the R packages Made4 and Vegan.

A p value <0.05 was considered statistically significant. Statistical analyses on clinical and laboratory parameters were performed using the software STATA v.14 (STATACorp, USA), and PRISM GraphPad v 9.12. Analysis on GM were performed using the software R, using packages vegan, stats, and made4and.

6. Results

6.1 Patient's characteristics

In this study, we enrolled 45 BS patients who fulfilled the ICBD criteria for BS (68). Of them, one patient discontinued the study during the first nutritional intervention because she discovered to be pregnant, and other two patients were lost to follow-up during the first nutritional intervention for poor compliance. Four patients recently started the trial, and the first nutritional intervention was still ongoing at the moment of data analysis (figure 13).

Therefore, a total of 38 patients who had completed at least one nutritional intervention were included in the analysis (figure 13). Of them, 15 patients had completed only the first intervention, 16 patients had completed two interventions, and only 7 patients had completed all the three interventions.

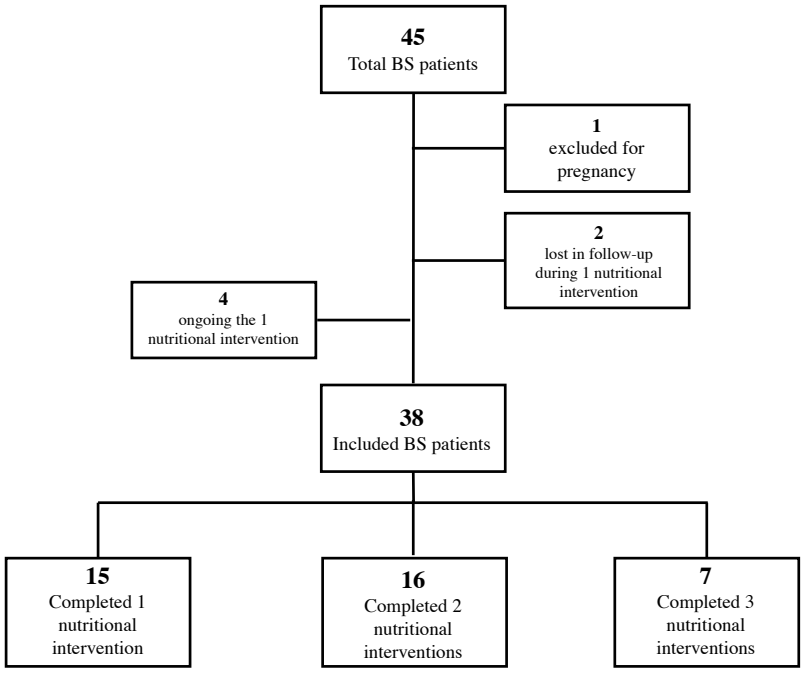

Figure 13. *Study flow-chart.*

The main demographic and clinical characteristics of the 38 BS patients are reported in table 5.

The median age was 44.5 (IQR 32-55) years and female gender was predominant (71%). Regarding clinical manifestations in the whole clinical history, 37 out of 38 patients (97%) had oral ulcers, 27 had cutaneous manifestations (71%), and 26 had ocular involvement (68%). Other frequent manifestations included articular and intestinal involvement (58% each), genital ulcer (45%), vascular events (42%) and neurological involvement (26%). Nine patients (24%) reported a positive pathergy test, and 17 patients (45%) presented HLA-B51 positivity.

At time of inclusion in the study, the median disease duration was of 40 (IQR 12.2-58.6) months. All patients presented active disease, the median BDCAF being 6 (IQR 4-8).

As for blood inflammatory parameters, the median level of ESR was 18 (IQR 15-28) mm/h, while the median level of CRP was 2,7 (IQR 1,5-4,6) mg/L.

Considering ongoing pharmacological treatments at study entry, 17 out of 38 patients (45%) were receiving glucocorticoids, at a median daily dosage of 5 mg/day (IQR 2.5-6.25 mg/day), 16 (42%) were receiving traditional DMARDs, 25 (66%) were treated with biologic DMARDs, and 16 (42%) were treated with colchicine.

Considering anthropometric characteristics, the median body weight at study entry was 72 (IQR 61-81) kg, with a median BMI of 26 (IQR 21-29) and a median fat mass of 30% (IQR 22-39).

Number of patients	**38**
Age, median (IQR)- *years*	44.5 (32-55)
Female, n (%)	27 (71)
Main Clinical Manifestations	
Oral ulcers, n (%) Papulo-pustular or nodular skin lesions, n (%) Ocular lesions, n (%) Joint involvement, n (%) Intestinal manifestations, n (%) Genital ulcers, n (%) Vascular manifestations, n (%) Neurological manifestations, n (%)	37 (97) 27 (71) 26 (68) 22 (58) 22 (58) 17 (45) 16 (42) 10 (26)
Positive pathergy test, n (%)	9 (24)
HLA-B51 positivity, n (%)	17 (45)
Disease duration, median (IQR) - *months*	40 (12.2-58.6)
Laboratory test	
ESR, median (IQR) - *mm/h* CRP, median (IQR) - *mg/L*	18 (15-28) 2,7 (1,5-4,6)
Ongoing treatments at study entry	
Glucocorticoids, n (%) DMARDs, n (%) Biologics, n (%) Colchicine, n (%)	17 (45) 16 (42) 25 (66) 16 (42)
Physical characteristics	
Weight, kg, median (IQR) Height, cm, median (IQR) BMI, median (IQR) % Fat mass, median (IQR)	72 (61-81) 171 (161-176) 26 (21-29) 30 (22-39)

Table 5. Main clinical features of 38 Bechet's patients enrolled, at study baseline. *All results, unless otherwise specified, are expressed as median (interquartile range, IQR).*

6.2 Primary Outcomes

Primary outcomes were assessed on a total of 21 patients who completed the VD intervention, 21 who completed the MD+Bt intervention, and 26 who completed the MD intervention.

The effects of the three nutritional interventions on BS disease control are reported in figure 14 and in figure 15.

BS disease activity, assessed by the BDCAF score, did not appear to be significantly influenced by the three nutritional interventions (figure 14A). Specifically, the mean BDCAF was of 5.8 (SD

2.4) before the VD intervention and 5.7 (SD 2.9) after VD (p=0.883). Similarly, in the MD+Bt group, the mean BDCAF was of 5.2 (SD 2.6) before the intervention and 4.9 (SD 3.1) after the intervention (p=0.557), while in the MD group the mean BDCAF was 5.1 (SD 2.7) before the intervention and 4.9 (SD 2.3) after the intervention (p=0.584).

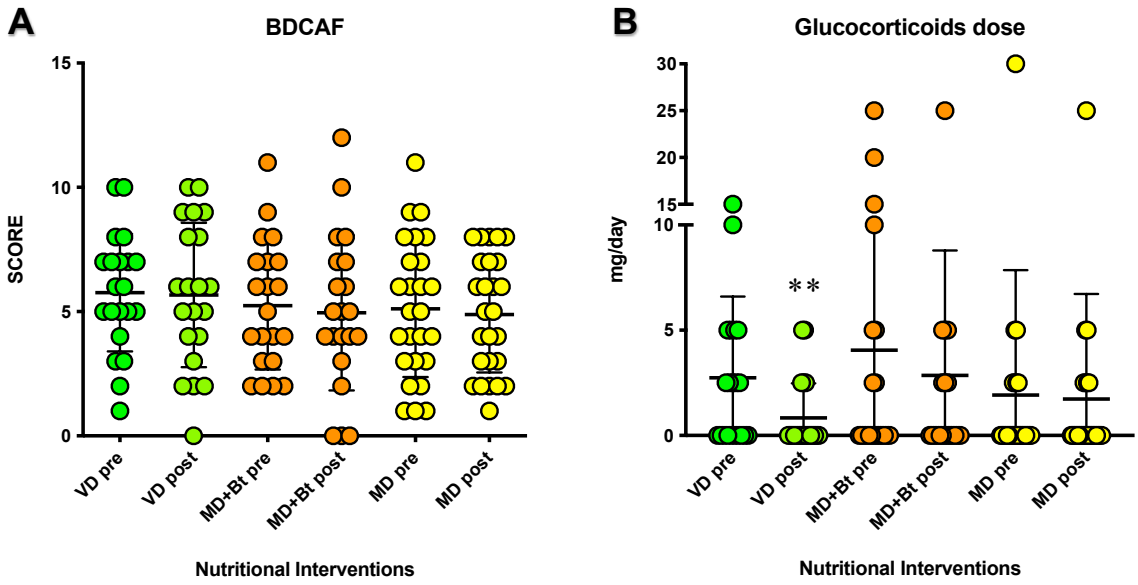

Figure 14. *Effect of three different nutritional interventions on clinical parameters in Behcet's syndrome patients (BS).* ***A)*** *represents BS current activity form (BDCAF) assessed before and after each nutritional intervention.* ***B)*** *represents daily dose of prednisolone equivalents (mg/d) assessed before and after each nutritional intervention. VD: vegetarian diet; MD+Bt: Mediterranean diet plus butyrate; MD: Mediterranean diet. P were calculated by Wilcoxon signed-rank test.*

Notably, the VD intervention was associated with a significant steroid sparing effect (figure 14B). Namely, the glucocorticoids dosage decreased from 2.5 (IQR 0-5) mg/day before the intervention to 0 (IQR 0-5) mg after the intervention (p=0.005) and the proportion of patients receiving glucocorticoids decreased from 11/21 (52.4%) to 5/21 (23.8%) (p=0.014).

Use glucocorticoids at baseline (i.e., before the beginning of the nutritional intervention) was less common in the MD+Bt and MD groups as compared to the VD group; in both interventions, no significant changes in the daily glucocorticoids dosage nor in the proportion of patients receiving glucocorticoids was detected following the interventions [for the MD+Bt group: the glucocorticoids dosage was from 0 (IQR 0-5) mg/day before the intervention to 0 (IQR 0-2.5) mg

after the intervention (p=0.130), and the proportion of patients on glucocorticoids therapy was 8/21 (38.1%) both before and after the intervention (p=1.000); for the MD: the glucocorticoids dosage was from 0 (IQR 0-2.5) mg/day before the intervention to 0 (IQR 0-2.5) mg after the intervention (p=0.317), and the proportion of patients on glucocorticoids therapy was 7/26 (26.9%) both before and after the intervention (p=1.000)].

On the other hand, none of the three nutritional interventions was associated with a DMARDs-sparing effect, and no patient was able to discontinue traditional and/or biological immunosuppressant treatments during the study.

Regarding blood inflammatory parameters (figure 15), CRP levels were only mildly increased at the beginning of the interventions (figure 15A), and they did not appear to be significantly influenced by the three nutritional interventions. Specifically, the median CRP level was of 3.2 (IQR 1.7-4.5) mg/L before the VD intervention and 2.8 (IQR 2.1-4.7) mg/L after VD (p=0.356). In the MD+Bt group, the median CRP level was of 2.9 (IQR 2.1-6.2) mg/L before the intervention and 2.7 (IQR 1.7-4.9) mg/L after the intervention (p=0.056), while in the MD group the median CRP level was 2.1 (IQR 1.7-2.6) mg/L before the intervention and 1.9 (IQR 1.1.-2.4) mg/L after the intervention (p=0.161).

Similarly, no statistically significant reduction in ESR levels was detected (figure 15B) following VD intervention, the median ESR level varying from 18 (IQR 15-24) mm/h before the intervention to 18 (IQR 15-20) after the intervention (p=0.052). Moreover, ESR levels did not appear to be significantly influenced by MD+Bt or MD interventions [for MD+But group: 18 (IQR 16-30) mm/h before treatment vs 20 (IQR 15-24) mm/h after the intervention, p=0.094; for MD group: 13 (IQR 12-18) before treatment vs 12 (IQR 10-19) after the intervention, p=0.353].

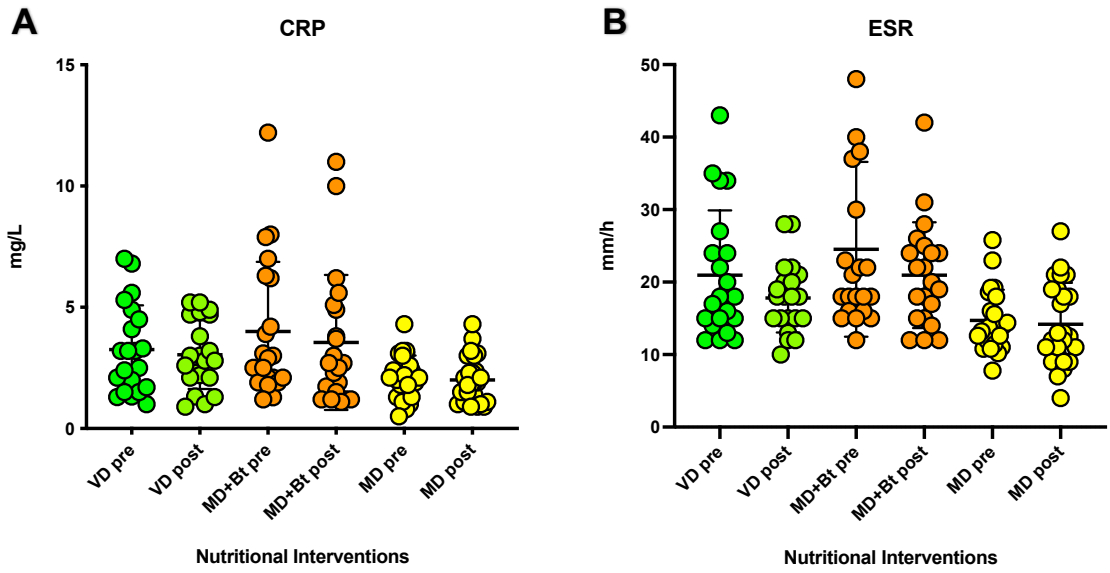

Figure 15. *Effect of three different nutritional interventions on clinical parameters in Behcet's syndrome patients (BS).* ***A)*** *represents C-reactive protein (CRP) levels (mg/dL) assessed before and after each nutritional intervention.* ***B)*** *represents erythrocyte sedimentation rate (ESR, expressed as mm/h) assessed before and after each nutritional intervention. VD: vegetarian diet; MD+Bt: Mediterranean diet plus butyrate; MD: Mediterranean diet. P were calculated by Wilcoxon signed-rank test.*

The effect of the three nutritional interventions on the severity of gastrointestinal symptoms, assessed by the Symptom Severity Scale (SSS) modified form, is reported in figure 16A.

A statistically significant reduction in the SSS score was observed following all the three interventions; namely, the mean SSS score decreased from 239.5 (SD 103.9) to 166.7 (SD 93.6) in the VD group (p=0.004), from 192.9 (SD 103.9) to 140.9 (SD 79.1) in the MD+Bt group (p=0.004) and from 195.8 (SD 96.2) to 163.1 (SD 81.9) in the MD+Bt group (p=0.023).

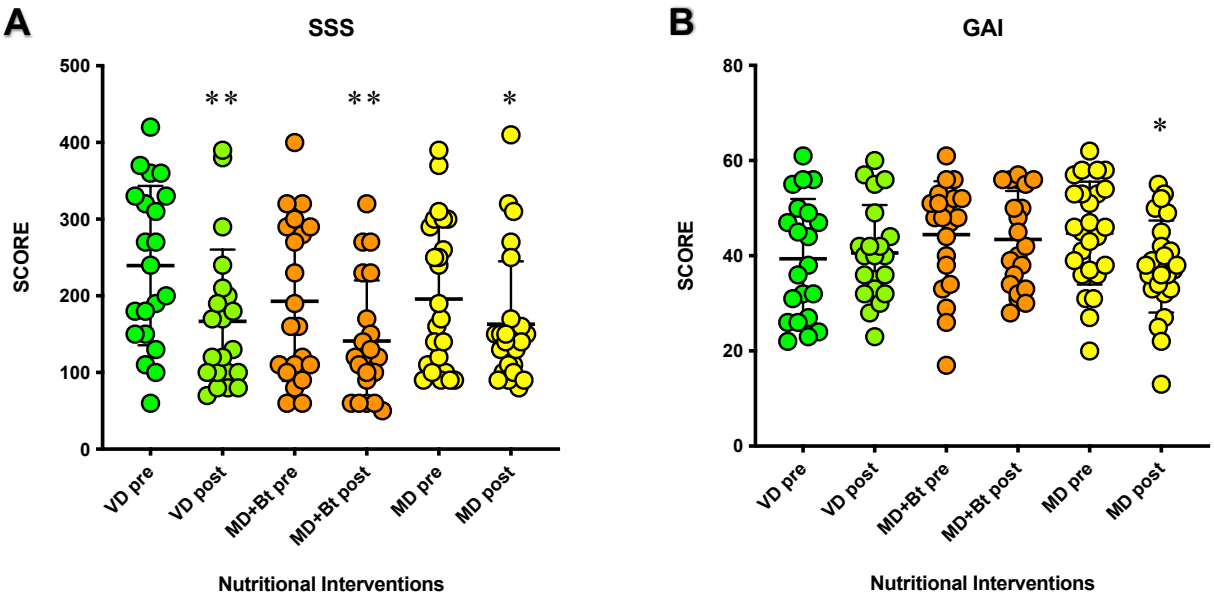

Figure 16. *Effect of three different nutritional interventions on gastrointestinal symptoms in Behcet's syndrome patients (BS).* ***A)*** *represents the variation of gastrointestinal symptoms evaluated by Symptom Severity Scale (SSS) assessed before and after each nutritional intervention.* ***B)*** *represents Global Assessment of Improvement Scale (GAI), following the three nutritional intervention. VD: vegetarian diet; MD+Bt: Mediterranean diet plus butyrate; MD: Mediterranean diet. P were calculated by Wilcoxon signed-rank test.*

The variation in gastrointestinal-related BS symptoms, assessed by the Global Assessment of Improvement Scale (GAI), following the three nutritional interventions are reported in figure 16B. No significant improvement in the GAI score was detected following VD or MD+Bt interventions [for the VD group: mean GAI of 39.3 (SD 12.5) before the intervention and 40.6 (SD 10.1) after the intervention, p=0.723; for the MD+Bt group: mean GAI of 44.4 (SD 11.2) before the intervention and 43.4 (SD 10.2) after the intervention, p=0.730]. Conversely, the mean GAI score significantly decreased following the MD intervention, from 44.8 (SD 10.8) to 37.7 (SD 9.7) (p=0.013).

6.3 Secondary Outcomes

At time of data analysis, results of laboratory assessment of redox status were available only in a subgroup of patients; thus, secondary outcomes on redox status were assessed on 16 out of 21

patients who completed the VD intervention, 16 out of 21 who completed the MD+Bt intervention, and 16 out of 26 who completed the MD intervention.

Figure 17 reports the levels of intracellular lymphocyte, monocyte and granulocyte ROS in the serum of BS patients before and after each nutritional intervention.

Regarding VD group, a statistically significant reduction in lymphocyte ROS was observed following the 3 months of intervention (the mean level being 1619.9 (SD 328.5) RFU before the intervention and 1016.3 (SD 230.2) RFU after treatment, $p<0.001$), figure 17A. Similarly, also monocyte and granulocyte ROS levels significantly decreased following this diet [for monocyte ROS: 3194.6 (SD 668.6) RFU before treatment vs 2327.5 (SD 446.1) RFU after the intervention, $p<0.001$; for granulocyte ROS: 4207.9 (SD 786.3) RFU before treatment vs 2833 (SD 779.4) RFU after the intervention, $p<0.001$], figure 17 B-C.

As for MD+Bt group, a statistically significant reduction in lymphocyte ROS (figure 17A) was observed following the 3 months of butyrate supplementation (the mean level being 1595.1 (SD 423.9) RFU before the intervention and 1019.4 (SD 200.0) RFU after treatment, $p<0.001$).

Similarly, also monocyte and granulocyte ROS levels significantly decreased following this diet [for monocyte ROS: 3263.1 (SD 791.1) RFU before treatment vs 2504.2 (SD 731.2) RFU after the intervention, $p<0.001$; for granulocyte ROS: 4751.6 (SD 798.0) RFU before treatment vs 2765 (SD 721.0) RFU after the intervention, $p<0.001$], figure 17 B-C.

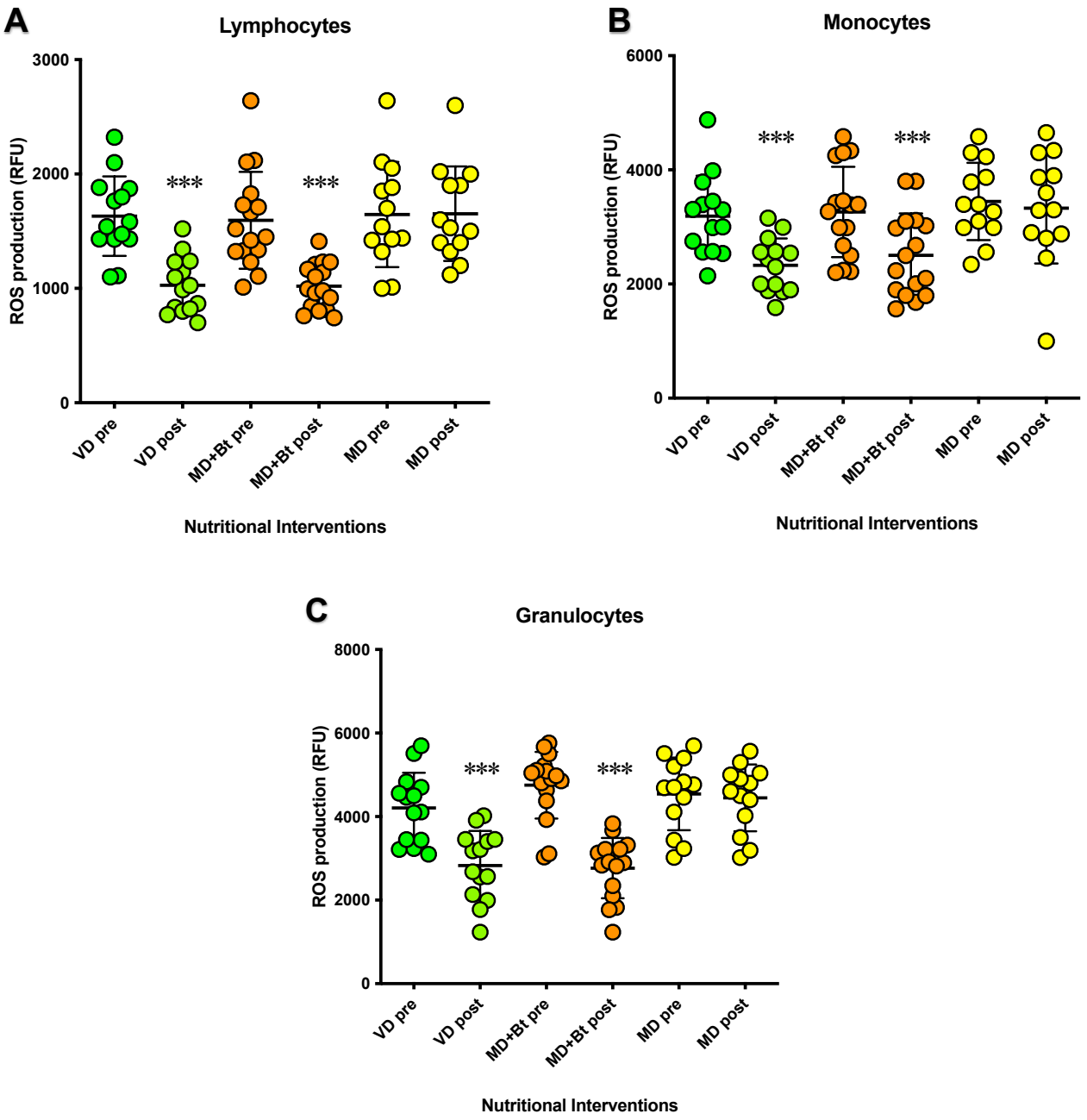

Figure 17. *Effect of three different nutritional intervention on oxidative stress markers in Behcet's syndrome patients (BS).* ***A)*** *Intracellular lymphocyte reactive oxygen species (ROS) production assessed before and after each nutritional intervention.* ***B)*** *Intracellular monocyte reactive oxygen species (ROS) production assessed before and after each nutritional intervention.* ***C)*** *Intracellular granulocytes reactive oxygen species (ROS) production production assessed before and after each nutritional intervention. VD: vegetarian diet; MD+Bt: Mediterranean diet plus butyrate; MD: Mediterranean diet. P were calculated by Wilcoxon signed-rank test.*

Conversely, lymphocyte, monocyte and granulocyte ROS production (respectively figure 17 A, B and C) did not appear to be significantly influenced by MD intervention [for lymphocyte ROS: 1649.8 (SD 418.3) RFU before treatment vs 1644.5 (SD 372.1) RFU after the intervention, p=0.824; for monocyte ROS: 3460.6 (SD 610.2) RFU before treatment vs 3336.6 (SD 867.5)

RFU after the intervention, p=0.511; for granulocyte ROS: 4539.3 (SD 783.1) RFU before treatment vs 4445.7 (SD 722.9) RFU after the intervention, p=0.059].

Figure 18 reports the total antioxidant capacity (TAC) levels assessed before and after each nutritional intervention.

Regarding the VD group, a statistically significant increase in the plasma TAC was observed following the 3 months of intervention (the mean level being 14.0 (SD 1.9) ORAC before the intervention and 16.1 (SD 2.0) ORAC after treatment, p<0.001). Similarly, in the MD+Bt group, plasma TAC significantly improved following the intervention (the mean level being 14.6 (SD 1.9) ORAC before the intervention and 15.7 (SD 1.9) ORAC after treatment, p<0.001). Conversely, in the MD group, a significant impairment in the TAC was observed following the intervention, the mean TAC level decreasing from 15.2 (SD 1.9) to 14.9 (SD 1.8) (p=0.015).

The variations in lipid peroxidation markers before and after each nutritional intervention are reported in figure 18.

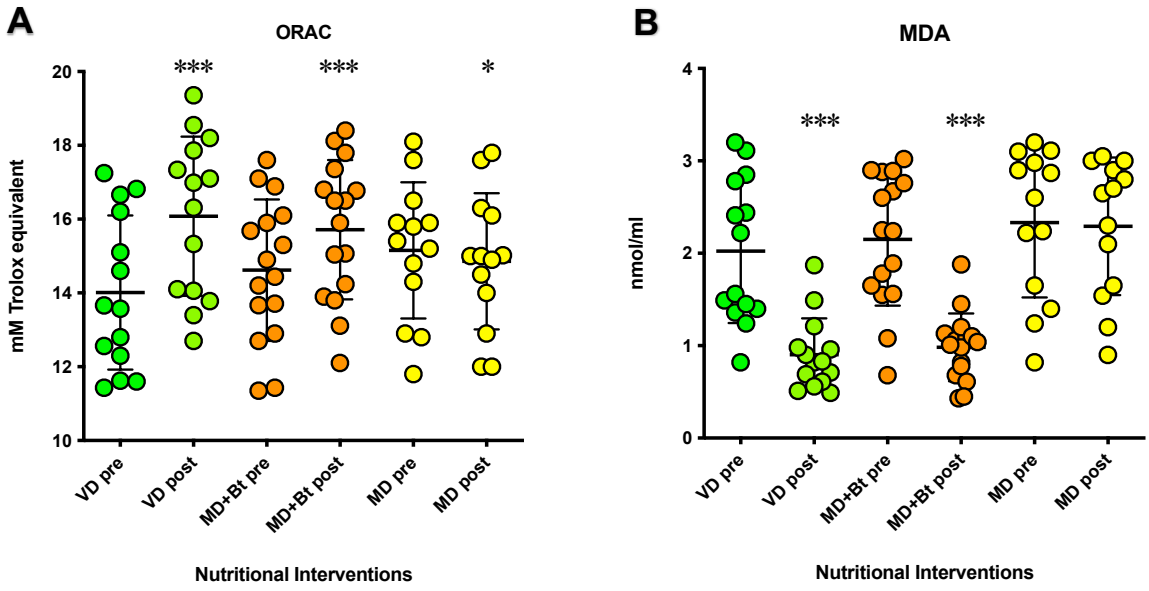

Figure 18. *Effect of three different nutritional intervention on oxidative stress markers in Behcet's syndrome patients (BS). Plasma total antioxidant capacity (**A**) and plasma lipid peroxidation in terms of MDA (malondialdehyde) content (**B**), in BS patients, assessed before and after each specific nutritional intervention. VD: vegetarian diet; MD+Bt: Mediterranean diet plus butyrate; MD: Mediterranean diet. P were calculated by Wilcoxon signed-rank test*

A significant reduction in plasma lipid peroxidation was observed following both VD and MD+Bt interventions, the mean levels decreasing from 2.0 (SD 0.7) MDA to 0.9 (SD 0.4) MDA in the VD group ($p<0.001$) and from 2.2 (SD 0.7) MDA to 1.0 (SD 0.4) MDA in the MD+Bt group ($p<0.001$), respectively. Conversely, levels of plasma lipid peroxidation did not appear to be significantly influenced by the MD intervention, the mean levels being 2.3 (SD 0.7) MDA before the intervention and 2.3 (SD 0.6) MDA following the intervention ($p=0.195$).

Focusing of analysis conducted on fecal samples, at time of data analysis GM composition was evaluated only in 21 patients, 7 for each group of nutritional intervention. Fecal samples were assessed before and after the specific nutritional intervention. No significant changes in GM composition were observed (figure 19), although more members of the *Clostridium XIVa, Roboutsia* and *Eggerthella genera* were detected after butyrate supplementation or vegetarian diet.

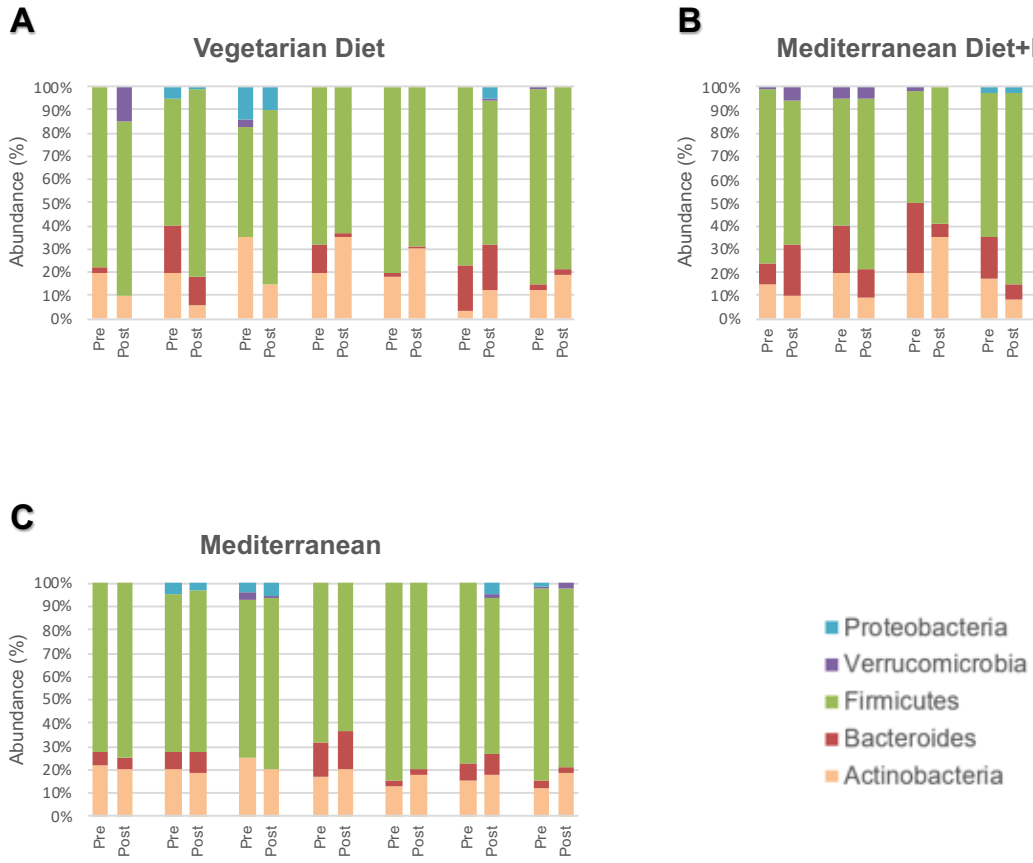

Figure 19. *Effect of three different nutritional intervention on gut microbiota composition in Behcet's syndrome patients (BS).* Fecal gut microbiota composition at phylum level before (pre) and after (post) lacto-ovo-vegetarian diet (**A**), Mediterranean diet and oral butyrate supplementation (**B**) and Mediterranean diet (**C**).

6.4 Anthropometric Assessment

All patients received an iso-caloric diet, with a dietary intake ranging between 1400 and 2000 kcal/day, according to the patient's requirement. During follow-up, no patient declared to have changed its daily routine, in terms of physical activity or smoking habit.

During follow-up, no statistically significant difference was found in terms of body weight or BMI, the mean variation in the BMI being 0.5 (SD 0.02) in the VD group, 0.2 (SD 0.1) in the MD+But group, and 0.1 (SD 0.1) in the MD group. Accordingly, also the body composition, assessed in terms of fat mass, muscle mass and body water, did not change throughout the different nutritional interventions (*data not shown*).

6.5 Safety and Adherence

During follow-up, the mean adherence reported by the patients was of 95% (SD 3%) for VD, 94% (SD 2%) for MD+Bt, and 99% (SD 3%) for MD.

Despite weekly phone calls for dietary counseling and adherence promotion, 3 out of 38 patients who completed at least one nutritional intervention, resulted to be poorly adherent to the proposed interventions.

Specifically, two patients were lost to follow-up during the VD diet, due to difficulties in adhering to a vegetarian regimen. One patient was lost to follow-up during the MD+Bt diet, due to poor tolerance to butyrate supplementation with an increase in the number of episodes of diarrhea.

No major adverse events were reported during the trial. None of the patients reported significant disease relapses requiring an increase in the daily dosage of glucocorticoids exceeding 20% or the introduction or increase in immunosuppressants. Moreover, no significant variation in the gluco-lipidic profile was detected following the three nutritional interventions.

7. Discussion

BS is a systemic inflammatory disorder characterized by a wide range of potential clinical manifestations affecting different organs and tissues (33), with higher risk of mortality due to vascular-thrombotic and neurological affections. The etiology remains unclear, and although various mechanisms have been proposed, it is not yet clear whether the microbiome has a role in this process (151).

A recent study demonstrated that GM in BS patients is characterized by low biodiversity and specific changes in the profiles of SCFA production (60). In particular, a significant depletion of butyrate producers - such as *Roseburia* and *Subdoligranulum* - and a consequent decrease in butyrate production was detected. Butyrate is the preferred fuel for colonocytes and one of the most representative SCFAs. It induces T_{reg} cell differentiation through several mechanisms, so butyrate impairment in individuals with BS is thought to reduce T_{reg} cell-mediated control, promoting immunopathological T cell responses (127) sustaining thrombo-inflammation.

Increased evidence suggests that dietary patterns characterized by increased amounts of plant-based foods, such as a MD or VD, enriched in substrates with potential for butyrate production, have positive effects on health status, possibly modulating GM and the production of its metabolites (144). Indeed, dietary patterns rich in non-refined cereals, fruits, vegetables, and legumes have been found to promote a healthier GM profile due to the large amount of dietary fiber. These fermentable substrates are sources of metabolic fuel for the fermentation of GM, which, in turn, results in end products - mainly SCFAs, among which butyrate - with a multifactorial role in host health (152).

In our knowledge, no studies have systematically evaluated the effects of tailored nutritional intervention on BS. Thus, the aim of this randomized crossover 3-arms controlled trial was to understand the effect of a VD (enriched in fermentable substrates for butyrate production),

MD-Bt (with exogenous butyrate supplementation), or MD on the manifestations of BS and, possibly, the role of the intestinal microbiota as a mediator of dietary effects in individuals with BS.

Our results demonstrate that, in general, a close dietary control and a regular follow-up display benefic effects on clinical disease manifestations, although without reaching statistical significance. Indeed, all three nutritional interventions were associated with a reduction in the BDCAF score, though without statistical significance, while inflammatory markers did not appear to be significantly influenced by the three dietary regimens.

Accordingly, also the evaluation of gastrointestinal symptoms specifically related to BS showed a similar trend, with a reduction in the GAI score following all three nutritional interventions, and achieving statistical significance only in the MD group, which might be due to a higher sample size in this interventional group.

Moreover, a significant improvement in gastrointestinal symptoms (assessed by SSS) was detected for all interventional groups, with a greater reduction in the SSS score after VD or MD+Bt diets.

Notably, despite no statistically significant reduction in the BDCAF score or the inflammatory markers, the VD intervention allowed to significantly reduce the daily glucocorticoids dosage as well as the percentage of patients receiving steroids. In the other two interventional groups, glucocorticoids therapy remained stable over follow-up. No difference in terms of concomitant immunosuppressive therapy (traditional or biologic) was detected in all groups.

While the effects of tailored butyrate-enriched diets on clinical parameters were poor, this interventional trial clearly showed that both VD and MD+Bt can exert significant effects on the reduction of the redox status, which is impaired by a neutrophil-dependent mechanism (via ROS) in BS. Indeed, ROS levels as well as plasma lipid peroxidation significantly decreased following butyrate supplementation (either via endogenous or exogenous supplementation), paralleled by a

significant improvement in plasma total antioxidant capacity, while MD had no effect on these parameters.

On the other hand, a 3-month butyrate-enriched diet did not affect GM composition and SCFAs production, suggesting that longer nutritional interventions are needed for scratching microbial resilience. The mutual relationship between SCFA and microbial composition is a long-term process. Thus, we can reasonably assume that a 3-months dietary intervention determines an enhance in SCFAs biosynthesis by the pre-existing microbial community, while a longer period is needed to observe significant variation in the quali-quantitative composition of the microbial community. Pending these results, our preliminary data suggest the benefits of tailored dietary interventions for cardiovascular prevention.

It is worth considering that the results from this study might suffer some limitations, mostly related to the small sample of BS patients included, and to the fact that not all enrolled patients completed all the three nutritional interventions. This might have decreased the power of the study to detect significant variations in the considered endpoints, while also precluding the possibility of conducting subgroup analyses.

However, Behçet's syndrome is a rare disease; moreover, we applied stringent inclusion criteria, as all patients had no traditional cardiovascular risk factors, were on stable treatment with corticosteroids and colchicine in the last 3 months before enrolment, and had no history of infections or of antibiotics and/or pre-/probiotics use in the previous 3 months. In addition, only a small proportion of BS patients meeting the inclusion criteria and eligible for the study was willing to participate and to maintain the dietary regimens for the required period. Thus, although the small sample size represents a considerable limit of this study, the results are strengthened by the fact of having included a cohort of highly homogeneous, highly adherent to the dietary interventions patients.

Secondly, the findings from this study are limited by the short duration of each nutritional intervention (i.e., 3 months), which might have prevented a deep change of microbiome

composition. However, when designing the trial, we believed that longer nutritional interventions might have negatively affected patients' adherence. In this study, adherence was routinely assessed by unannounced phone calls, which allowed to closely monitor compliance as well as to motivate patients' adherence to the different interventions.

Finally, another limitation is related to the open-label nature of this trial. Indeed, as participants and clinicians blinding was not feasible, reporting and detection bias cannot be excluded, particularly for subjective outcomes (as questionnaires on symptoms).

To overcome the limitations from this study, future multicenter nutrition interventional trial on a wider cohort of BS patients, with longer nutritional interventions, are advocated to better elucidate the benefits of long term tailored diets in Behçet syndrome. A possible post-hoc analysis might explore also if a specific disease phenotype is associated with a better response to the different nutritional interventions.

8. Conclusions

BS is a rare disease, and the available treatments are not specifically tailored therapies. Beyond the real progress made in the treatment of this disease in the past decade, the management of BS remains a challenge for physicians. Increasing evidence demonstrates that it is a disorder with an extremely wide spectrum of clinical features that can respond to certain specific treatments which, however, can be ineffective on other manifestations or even worsen some features. A treatment based on dietary and lifestyle issues, able to restore immune system homeostasis, could have a high impact on cost sustainability in the treatment of such a chronic and disabling inflammatory condition.

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