Tracing Behçet's disease origins along the Silk Road: an anthropological evolutionary genetics perspective

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ABSTRACT

Objective. *Behçet's disease is a multifactorial vasculitis that shows its highest prevalence in geographical areas historically involved in the Silk Road, suggesting that it might have originated somewhere along these ancient trade routes. This study aims to provide a first clue towards genetic evidence for this hypothesis by testing it via an anthropological evolutionary genetics approach.*

Methods. *Behçet's disease variation at ancestry informative mitochondrial DNA control region and haplogroup diagnostic sites was characterised in 185 disease subjects of Italian descent and set into the Eurasian mitochondrial landscape by comparison with nearly 9,000 sequences representative of diversity observable in Italy and along the main Silk Road routes.*

Results. *Dissection of the actual genetic ancestry of disease individuals by means of population structure, spatial autocorrelation and haplogroup analyses revealed their closer relationships with some Middle Eastern and Central Asian groups settled along the Silk Road than with healthy Italians.*

Conclusion. *These findings support the hypothesis that the Behçet's disease genetic risk has migrated to western Eurasia in parallel with ancestry components typical of Silk Road-related groups. This provided new insights that are useful to improve the understanding of disease origins and diffusion, as well as to inform future association studies aimed at properly accounting for the actual genetic ancestry of the examined Behçet's disease samples in order to minimise the detection of spurious associations and to improve the identification of genetic variants with actual clinical relevance.*

Introduction

Behçet's disease (BD) is a multifactorial vasculitis whose pathogenic mechanisms are far from being exhaustively elucidated (1-4). Its susceptibility was proved to be strongly associated with the HLA-B*51 antigen (5, 6) and, to a lesser extent, with other HLA class I loci (7) in several ethnic groups. Significant associations with non-HLA genes were also reported, even though not all were replicated in populations with different ancestries (8-10). Accordingly, common polymorphisms pointed out by genotyping-based association studies cannot fully explain BD heritability and are also unable to account for remarkable geographical variation of its prevalence. Rare and potentially population-specific variants, together with still underestimated environmental risk factors, could thus account for at least a fraction of BD missing heritability and worldwide differential occurrence (11-13). However, some of the association signals not replicated in all populations might also represent spurious associations due to different ancestry proportions of cases and controls not revealed by traditional genetic association studies.

Although BD is widespread throughout the world, its prevalence shows considerable geographical variation, with peaks being observed in Turkey, Israel, Iran and Iraq. Moreover, substantial prevalence was reported also for some southern European populations, such as Italians (14, 15) and Greeks, and in many other Mediterranean regions (*e.g.* Egypt, Lebanon, Syria, Jordan, Saudi Arabia), whereas it is quite low in Central and Northern Europe, as well as in the United States (1, 16). To the best of our knowledge, no reliable epidemiologic data are available for central Asia and the Indian subcontinent. This

peculiar distribution spanning from the Far East to the Mediterranean basin ensured to BD the epithet of "Silk Road disease", since it affects more significantly populations historically involved in these ancient trade routes.

Accordingly, it has been hypothesised that BD was initially diffused to the North-Western European coasts, including the British Isles, by Phoenician traders and, subsequently, in a more substantial manner along the East-West caravan routes traced by Marco Polo (17). Epidemiological data (1, 16) indeed suggest that BD hypothetical risk variants might have emerged somewhere along these trade routes and would have migrated in parallel with ancestry components typical of Silk Road-related groups during population movements historically occurred between Asia and the Mediterranean basin. That being so, signatures of Silk Road-related ancestry should characterise BD individuals irrespective of their declared descent. Unfortunately, this potentially determines their appreciable genetic discordance with respect to healthy controls simply matched for latest geographical origins, thus seriously challenging the identification of reliable risk variants by means of genetic association studies.

Until now, investigation of HLA-B*51 frequency patterns has been only used to genetically confirm the hypothesis of a Silk Road-related origin of BD, providing quite conflicting results. In fact, distribution of populations showing high HLA-B*51 occurrence turned out to be much wider than that of Silk Road-related ones, with some Amerindian groups being characterised by outstanding frequency for this locus in conjunction with complete absence of the disease, and South-Eastern Asian and Indian populations involved in maritime Silk Road trades showing neither high HLA-B*51 nor high BD prevalence (17). Nevertheless, pathogen-driven adaptation is known to have strongly influenced diversity at HLA genes, shaping their population variation patterns in function of surrounding pathogen landscapes (18).

Therefore, to more accurately test the hypothesis of Silk Road-related BD origins by avoiding potential confounding effects due to the action of natural selection and/or recombination arising from the analysis of autosomal loci involved in the disease susceptibility, the present study focused on the characterisation of mitochondrial DNA (mtDNA) variation in a large sample of unrelated individuals affected by BD and belonging to a restricted geographical area (*i.e.* the Italian peninsula). In fact, investigation of mtDNA diversity is particularly suited for tracing records of past evolutionary and demographic processes occurred during the history of human populations (19-22). Moreover, being not associated with increased BD susceptibility, mtDNA appeared to be an effective tool to search for potential genetic differences between healthy and disease Italian subjects exclusively due to different ancestry proportions.

In accordance with the hypothesis mentioned above, we can assume that signatures of Silk Road-related ancestry should be transmitted together with the BD genetic risk, thus resulting appreciable also in disease individuals of Italian descent. Accordingly, setting observed BD profiles within the Italian and Eurasian mitochondrial landscapes could reveal their potential genetic relationships with populations historically involved in the Silk Road. This is expected to provide a first clue towards a genetic evidence useful to improve the understanding of BD origins and patterns of diffusion and to inform future association studies aimed at properly accounting for the actual genetic ancestry of examined BD samples, in order to minimise the detection of spurious associations.

Materials and methods

Ethics statement

The present study involved 185 unrelated BD patients who have attended one of the nine Italian referral centres for BD over a period of seven years (2000–2008). All patients fulfilled the criteria established by the International Study Group for BD (ISGB) (23) and were of Italian origins for several generations. A written informed consent was collected from each of them and the study was designed according to the ethical principles for medical research involving human subjects stated by the World Medical Association Declaration of Helsinki. The Ethics Committee of the Azienda Ospedaliera Arcispedale Santa Maria Nuova also approved the described research protocol.

Reference population samples

In order to set the observed BD mtDNA variation into the context of the Italian and Eurasian mitochondrial landscapes, a reference dataset was generated by collecting sequence data for the Hyper Variable Segments I and/or II (HVS-I and/or HVS-II) of the mtDNA control region from literature. Accordingly, a panel of 8,495 mtDNA sequences from individuals belonging to 84 populations from Italy, Balkans, Caucasus, Middle East, Central, Southern and Eastern Asia, as well as North Africa, which can be sufficiently representative of genetic variation observable within the Italian peninsula and along or around the main ancient Silk Road routes, was obtained (Supplementary Table I).

Sequencing and genotyping experiments

DNA was extracted from peripheral white blood cells using a Genomic DNA purification kit (Gentra Systems, Inc., Minneapolis, MN, USA). Variation at the mtDNA HVS-I and HVS-II regions was investigated by sequencing a total of 749 base pairs (bp), encompassing nucleotide positions from 15,975 to 155.

mtDNA haplogroups were inferred on the basis of the diagnostic sites observed in the D-loop region by following recommendations reported in Bandelt *et al*. (24), as well as by taking advantage from the mtDNA tree Build 12 (http://www.phylotree.org/) (25) and from literature data. They were also confirmed by a hierarchical analysis on the mtDNA coding region by testing a total of 22 single nucleotide polymorphisms (SNPs) by means of two different multiplex-PCR (26-28). An exhaustive description of both sequencing and genotyping protocols and of procedures applied to detect polymorphic sites is provided in *Supplementary Materials and Method*s.

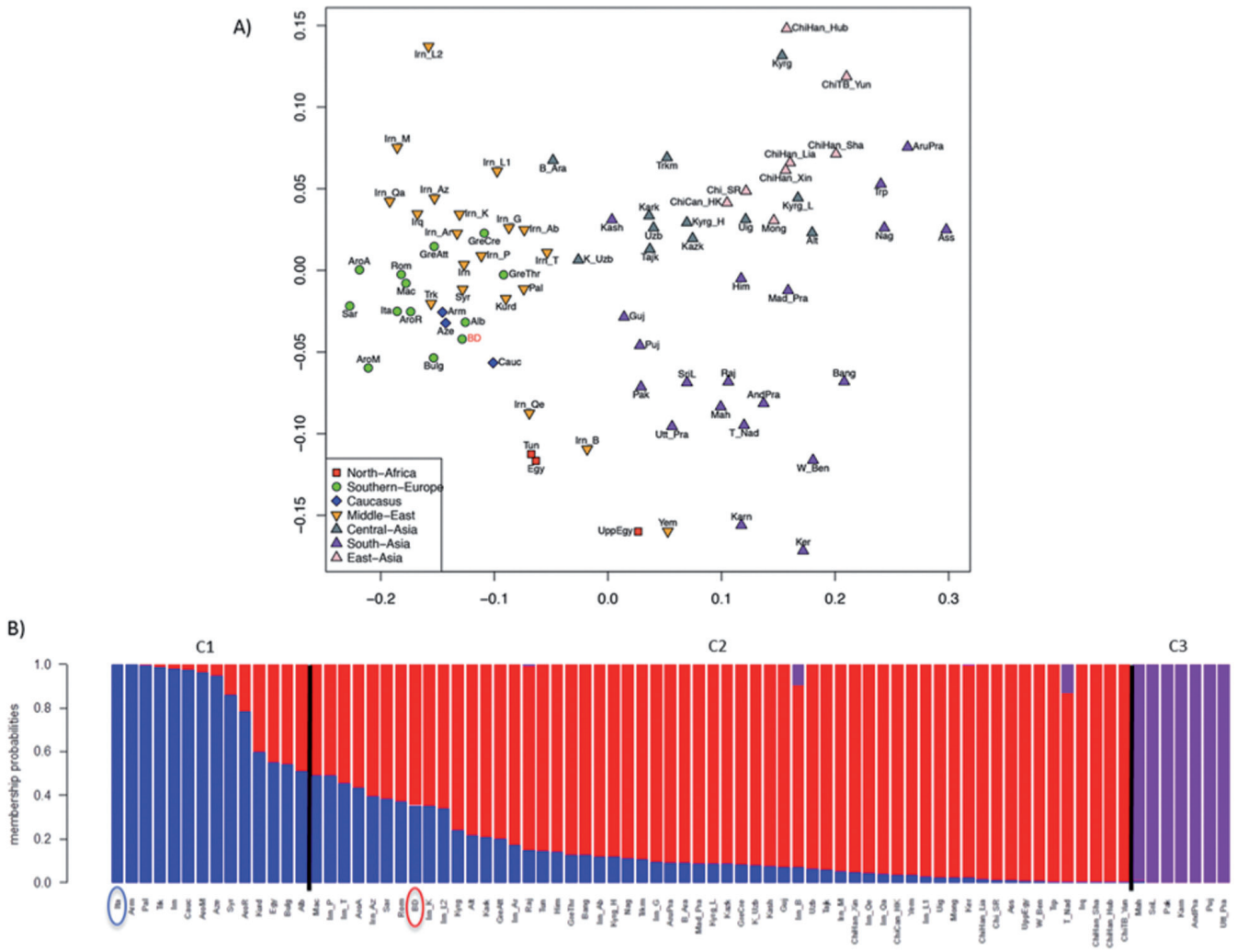

Fig. 1. A. MDS representation of genetic distances computed between BD and 79 reference populations. Groups from Southern Europe, Caucasus, Middle East, Northern Africa, Southern, Central and Eastern Asia are shown with different colours and symbols. **B.** Cluster membership probabilities of BD and reference populations computed by DAPC.

Statistical analyses

Estimates of basic descriptive statistics for the BD sample and for reference populations, such as nucleotide (π) and haplotype diversity (H), mean number of pairwise differences (MPD) and number of polymorphic sites (S), as well as Tajima's *D* and pairwise F_{st} genetic distances among groups, were computed on HVS-I data (encompassing nucleotide positions from 16,024 to 16,383) using the Arlequin package (29) v. 3.5.1.2. An F_{st} genetic distance matrix was obtained and used for a graphical representation by means of a non-metric multidimensional scaling (MDS), by filtering for outlier populations exceeding two standard deviations along at least one of the considered dimensions.

Genetic relationships among non-outlier groups were then explored via a non-hierarchical clustering algorithm based on Gaussian mixture models implemented in the R *mclust* package (30) and applied to principal components (PCs) computed by principal components analysis (PCA) that accounted for about 80% of variation.

To test robustness of the identified population groups, evaluation of posterior cluster membership probabilities for each population to belong to a given cluster was achieved by means of discriminant analysis of principal components (DAPC) (31) using the R *adegenet* package.

A series of spatial principal component analysis (sPCA) (32) was carried out on both the reference and the whole dataset (*i.e.* including the BD sample) using the R *adegenet* library to further explore whether Silk Road involvement or geographical features structured the observed mtDNA variability and according to the identification of potential local (*i.e.* negative) or global (*i.e.* positive) spatial autocorrelations.

Fisher's exact test was used to compare haplogroup frequencies between BD and healthy Italian samples.

A more detailed description of the applied statistical methods is provided in *Supplementary Materials and Methods*.

Results

Summary statistics of HVS-I variability for the 183 successfully sequenced BD patients and for reference popula-

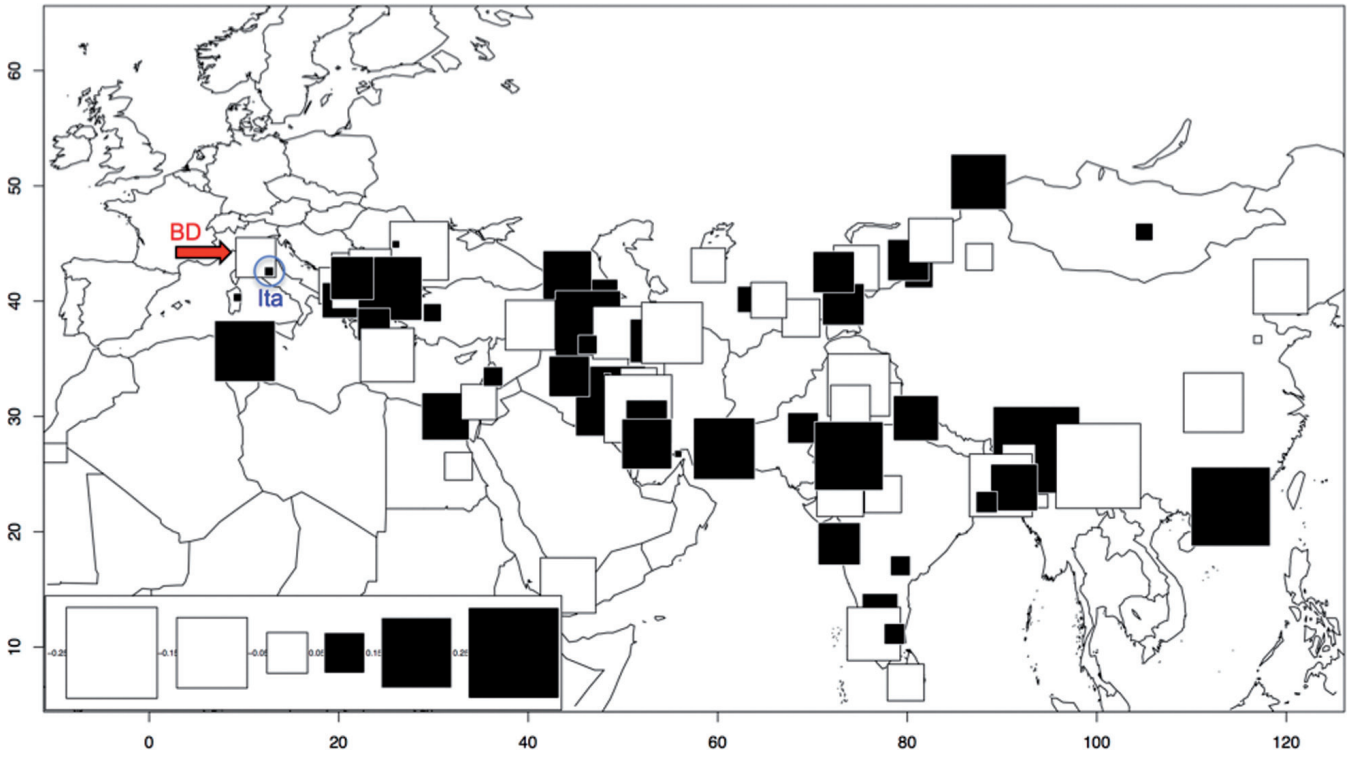

Fig. 2. Dissection of the strongest local sPCA eigenvalue computed on a reduced dataset of 77 populations along a gradient of spatial distribution and graphically represented on a geographical map. Large white squares represent the most negative values, while large black squares represent the most positive ones, with low absolute values for intermediate results being represented by small squares.

tions are reported in Supplementary Table I. BD nucleotide and haplotype diversities and MPD lay within the range of values observed for healthy Italian samples. A similar pattern was observed also for the BD Tajima's *D* in accordance to what found for healthy Italians.

Low but significant F_{st} genetic distances between disease subjects and almost all examined groups, including healthy Italians (p=0.016) were observed (Supplementary Table II A-B). Non-significant distances were found only between BD and populations from Albania (p=0.559), Macedonia (p=0.176), Attic peninsula (p=0.085), Crete (p=0.579), as well as with Aromuns from Romania (p=0.058), Kurds from East Turkey (KET, p=0.151) and Khoremian Uzbeks (KU, p=0.146). This reflected what observed also for healthy Italians, with the exception of BD close genetic affinity to KET and KU, which indeed exhibited significant F_{st} values when compared to the Italian population (p=0.023 and p=0.022).

The MDS performed on 79 non-outlier populations revealed a partial overlap between Southern European and Middle Eastern population groups, mainly due to positions occupied by Greek samples, albeit these clusters appeared to be appreciably distinguishable. BD subjects turned out to be closer to populations from the Caucasus, as well as to Albanians, Syrians and KET, than to healthy Italians (Fig. 1A).

Clustering analysis on computed PCs identified three main population clusters: C1 was constituted by samples from the Caucasus, all Southern European groups with the exception of Greeks from Thrace and Crete, Kazakhs and 40% of Middle Eastern populations; C2 was made up of the two Greek samples mentioned above, all Central Asian samples but Kazakhs, East Asian and North African groups, as well as 55% of Middle Eastern populations and 50% of the South Asian samples; C3 included the remaining 50% of examined South Asian groups and Baloch people from South-Eastern Iran. DAPC applied to the observed population groups pointed out that BD and healthy Italian samples were appreciably distant within the genetic landscape of C1 samples, with the former being closer to some Middle Eastern groups (*e.g.* Iranian Turkmens, Kurds, Azeri, Pars) than to the latter (Supplementary Fig. 1). In fact, according to posterior cluster membership probabilities healthy Italians were confirmed to unequivocally belong to C1 (99.99% probability), while BD showed greater probability to cluster with C2 populations (65%) than with C1 ones (35%) (Fig. 1B).

No statistical significance was obtained by means of sPCA for neither a global nor a local structure (Global_test = 0.048, p=0.395; Local_test = 0.053, p=0.459) when the dataset of reference populations was analysed. Nevertheless, a tendency of examined sequences to be more similar among neighbouring populations than among randomly distributed groups could be hypothesised according to computed eigenvalues. sPC1 indeed represented the strongest global eigenvalue as in the case of positive spatial autocorrelation and pointed to a rough east-west cline enabling to appreciably differentiate the group of Southern European,

Caucasian and Middle-Eastern samples from the bulk of Asian ones, with Central Asian and North-Eastern Indian populations occupying an intermediate position and showing low absolute sPC1 values (Supplementary Fig. 2). When sPCA was repeated including BD, a significantly negative spatial autocorrelation was found (Local_test = 0.150, p=0.002). Interpolation of the strongest local score highlighted the presence of several population pairs, included BD and healthy Italians, for which geographical proximity was not coupled by equivalent genetic affinity (Supplementary Fig. 3). Observed negative spatial autocorrelation remained significant also when the most divergent neighbouring groups (*i.e.* Turkmens and KU) were removed (Local_test = 0.132, p=0.006), revealing increased BD genetic affinity with some geographically distant and Silk Road-related groups (*e.g.* people from Kyrgyzstan, Tajikistan, Uzbekistan and Palestine) than with healthy Italians (Fig. 2).

Finally, 18 main mitochondrial lineages (macro-Hgs) were identified in the BD sample (Supplementary Table III), with typical West Eurasian ones accounting for the great majority of BD mtDNAs (94.6%) and typical East Eurasian ones being nearly absent. West Eurasian H and U macro-Hgs were the predominant lineages (41.6% and 20%), followed by the K (7.6%) and T lineages (6.5%). Within the macro-Hg H, 16.8% of the total examined BD mtDNAs were not assigned to a specific sub-lineage, whereas 24.9% belonged to 15 different H sub-clades. Among them, H1 (7.5%), H2 (2.2%), H5 (4.3%), H6 (2.2%) and H20 (1.62%) were the most represented. Frequencies of H1 and H20 were significantly different between BD and healthy Italians, with H1 being under-represented (p=0.044) and H20 being over-represented (p=0.049) in the former. As for macro-Hg U, its overall occurrence was found to be significantly increased in Italian disease individuals with respect to healthy ones (20% *vs.* 12.1%, p=0.006). Typical J and T European lineages were also found in the BD sample, but with unusual lower frequencies in comparison to the overall Italian sample (4.9% *vs.* 9.1%, p=0.074 and 6.5% *vs.* 11.9%, p=0.035).

Discussion

The present study was aimed at providing a first clue for genetic evidence supporting the hypothesis of BD origin historically occurred in populations settled along the caravan routes traced by Marco Polo and of the subsequent diffusion of BD risk variants to Western Eurasia along the so-called Silk Road. For this purpose, an anthropological evolutionary genetics approach was applied in the belief that disentangling the role of evolutionary, demographic and cultural factors in shaping patterns of human populations genetic structure represents an invaluable resource also for biomedical and clinical research.

Assessment of mtDNA variation in a large sample of BD subjects of Italian descent was used, coupled with its contextualisation in the Italian and Eurasian mitochondrial landscapes, to investigate the presence of appreciable Silk Road-related ancestry components in individuals affected by the disease. BD mtDNA profiles were compared with nearly 9,000 sequences representative of diversity observable within the Italian peninsula and in ethnic groups spanning from the Chinese Xinjiang region to Turkey and Southern Europe, passing through Kyrgyzstan, Tajikistan, Uzbekistan, Northern Iran, Armenia and Kurdistan, selected as reliable proxies for the populations historically involved in the Silk Road (33).

Sequence analysis highlighted the absence of unusual BD genetic diversity in comparison to healthy Italian and Eurasian populations, as well as of comparable excess of singletons and low frequency variants in their mtDNA pools according to significantly negative Tajima's D estimates (Supplementary Table I). Together with the bulk of the observed BD mtDNA haplogroups, this points to ancient processes of demographic growth, plausibly related to human expansion from an Italian refugium after the Late Glacial Maximum (34, 35), which left significant traces in the mtDNA of both healthy and diseases samples suggesting that they actually share an ancestral genetic background. This provide further support also to the evidence that mtDNA variation is not associated with increased BD susceptibility, as highlighted by recent GWAS (9). Accordingly, specific genetic differences between Italian healthy and disease subjects are expected to be due exclusively to different ancestry proportions established in historical times. In fact, since we have investigated a neutral maternally inherited genetic locus, also the presence of divergent patterns of variation at disease and healthy subjects as a consequence of the action of natural selection and/or recombination can be ruled out.

Interestingly, significant genetic distances were found between BD and healthy Italians, with the former showing considerable genetic affinity to KET and KU populations and a peculiar position in the MDS representation that suggested their relationships also to people from Syria and the Caucasus (Fig. 1A). Population groups identified by clustering analysis roughly reflected such samples distribution and, although the BD sample clustered within the bulk of Southern European and Caucasian populations (C1), it appeared to considerably diverge from healthy Italians, occupying an intermediate position between C1 and C2 populations in close proximity with Iranian groups (Supplementary Fig. 1). In fact, DAPC posterior cluster membership probabilities revealed significantly different probabilities for Italian healthy (99.99%) and BD (35%) subjects to belong to the cluster that includes populations from the Caucasus and non-Silk Road-related groups, as well as a small percentage of Middle Eastern samples. On the contrary, BD individuals showed a probability of 65% to belong to the cluster including most of Middle Eastern and Central Asian samples, again showing a pattern comparable to those of some Iranian groups (Fig. 1B).

To explore how far mtDNA affinity among the studied populations correlates with their geographic proximity or is influenced by their involvement in the Silk Road, a series of sPCA was performed. This led to the achievement of different results for the dataset of reference populations and for that including

also disease individuals. An appreciable tendency of reference sequences to be more similar among neighbouring populations than among randomly distributed groups was indeed found. Dissection of the strongest global eigenvalue along a gradient of spatial distribution thus revealed a small corridor of gradual transition from Asian populations to Southern European, Caucasian and Middle Eastern ones, depicting a rough east-west cline of variation (Supplementary Fig. 2). On the contrary, when also BD was included in the analysis a significantly negative spatial autocorrelation was found, pointing to increased genetic dissimilarity among neighbouring populations with respect to what observed for the reference dataset. Dissection of the strongest local eigenvalue indeed highlighted the presence of several population pairs, including BD and healthy Italians, for which geographical proximity was not coupled by equivalent genetic affinity. In particular, BD showed a pattern similar to those of some Silk Road-related groups (*e.g.* people from Kyrgyzstan, Tajikistan, Uzbekistan and Palestine) that is completely at the opposite with respect to that characterising healthy Italians (Fig. 2).

Reliable correlations between patterns of BD variation and disease origins and diffusion along the main Silk Road routes was also provided by the analysis of haplogroup profiles. As expected, West Eurasian H and U macro-haplogroups predominantly characterised BD mtDNAs, confirming that disease individuals shared common ancient ancestors with healthy Italians. However, some peculiar H sub-lineages (*i.e.* H6 and H20) scarcely represented in the Italian population (35, 36) were observed at appreciable frequencies in BD, with the former being generally frequent in western Caucasus, Syria, Arabian Peninsula (37, 38) and in Chinese populations settled along the Silk Road (39, 40) and the latter being widespread in populations from Turkey, Syria, Iran and Armenia (38, 41). Increased frequencies of these lineages in BD could be interpreted as signatures of genetic contributions to the Italian mtDNA pool plausibly consistent with historical people movements from Silk Road-related regions. On the contrary, typical Western European H1 (42, 43), as well as Western Eurasian T (44, 45) and J (46, 47) lineages, showed unusual lower frequencies in the BD sample with respect to the overall Italian one, again highlighting characteristics of the disease mtDNA background that are quite peculiar for subjects of non-admixed Italian descent.

Conclusion

The observed increased genetic relationships of Italian BD patients with some Middle Eastern and Central Asian groups settled along the Silk Road with respect to healthy Italians support the hypothesis that relatively recent ancestors of disease subjects received genetic contributions consequent to Silk Road-related population movements occurred between Asia and the Mediterranean area. In addition to appreciably modify the Italian ancestral mtDNA background of these individuals, such migratory events reasonably transmitted to them also BD autosomal risk variants, thus contributing to spread the disease genetic risk to the Italian peninsula. These findings should be used to inform future association studies aimed at properly accounting for the actual genetic ancestry of examined patients (48). This will enable to minimise the detection of spurious associations due to different ancestry proportions of disease and control subjects, promising to improve the identification of variants with actual clinical relevance and to accelerate the translation of obtained knowledge into concrete medical applications. Moreover, identification of plausible Middle Eastern and Central Asian candidate BD source populations could be exploited to design targeted studies focused on the investigation of autosomal variation surrounding the known disease susceptibility loci to further advance the understanding of the full spectrum of BD risk variants and of the disease aetiology.

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