A genome-wide association study identifies an association between variants in EFCAB4B gene and periodontal disease in an Italian isolated population

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Background and Objective: Periodontitis in one of the most prevalent dental diseases. Despite numerous studies have investigated its aetiopathogenetic factors, few works have focused on its genetic predisposition and most of them took into account only candidate genes. Therefore, we conducted a Genome Wide Association Study in an Italian isolated population aimed at uncovering genetic variants that predispose to this disorder.

Methods: Diagnosis of chronic periodontitis was made following the criteria of the American Academy of Periodontology. Patients with chronic periodontitis were grouped into different categories: slight, severe, localized and generalized. A control group composed by people without signs of periodontitis or gingivitis was defined. DNA was genotyped using 370k Illumina chips. Linear mixed model regression was used to test the association between each single nucleotide polymorphism (SNP) (independent variable) and the periodontitis status (dependent variable), controlling for confounders sex, age and smoking. The genomic kinship matrix was also used as random effect.

Results: Four SNPs on the gene EFCAB4B resulted significantly associated to localized periodontitis ($P < 5 \times 10^{-8}$), with the best hit on the rs242016 SNP ($P = 1.5 \times 10^{-8}$).

Conclusion: We have identified a novel significant association between the EFCAB4B gene and localized periodontitis. These results open a new perspective in the understanding of genetic factors contributing to this common disorder.

KEYWORDS
gene expression, genetic(s), inflammation and innate immunity, periodontitis

1 | INTRODUCTION

Periodontal disease consists in a progressively destructive change leading to loss of bone and periodontal ligament due to chronic inflammation of the supporting tissues of the teeth. Clinically there are plaque and calculus and is characterized by pocket formation and/or recession of the gum. In the advanced stages, the tooth loses its support until it is lost.[1]

This pathologic condition affects almost half of the adult population after 30 years of age[2] and many epidemiological studies have evaluated how its prevalence is higher in male compared to female and increases with age.[3] The percentage of individuals with healthy periodontium (absence of inflammation and probing depths <4 mm) decreases with increasing age and does not represent more than 10% of the adults.[4] In the Italian population, prevalence of chronic periodontitis has been estimated as

about 60%, while between 10% and 14% show either severe or advanced forms,[5,6] in agreement with the prevalence in the global population.[7]

It is well known that periodontitis is a complex inflammation-based disease with a multifactorial aetiology. The main cause of this inflammation is found in the pathogenic bacterial species that may influence the onset and progression of the disease in combination with risk factors as smoking, diabetes and stress, or genetic predisposition.[8] Although bacterial accumulation is the condition sine qua non for the insurgence of the inflammation,[9] it was demonstrated that there is no correlation between the amount of plaque and the severity of periodontitis.[10] This suggests that the host response and its genetic contribution is a key factor in developing the periodontal disease. A population study in more than 10 000 Swedish twin pairs revealed that the genetic contribution to the risk of periodontal disease is 39% in women and 33% in men,[11] and studies in adult twins have confirmed that the host susceptibility is influenced by genetic factors.[11-13]

A gene can be present in the genome in different forms defined as alleles. Allelic modifications may produce normal or altered proteins resulting in any, minor or big change in their functions, or sometimes in suppression of function. A genetic polymorphism occurs when an allele is present in at least the 1% of population, and it is considered the usual variant of a gene in a population. The most common types of genetic polymorphisms are the single nucleotide polymorphisms (SNPs). The change in protein function that a polymorphism produces is normally small, but its effect can be enhanced by environmental exposures, ie, diet, smoking or infective factors, increasing the risk of developing a specific disease.

Most recently, a genome-wide association approach was used to identify new genetic risk factors for periodontal disease. Different suggestive loci were identified,[14-17] but only few studies have shown genome-wide significant results associated to periodontal disease or to related phenotypes.[18-20] In Schaefer et al, association in a German population between aggressive periodontitis and rs1537415 in the glycosyltransferase gene GLT6D1 was reported.[19] In another study, genome-wide significant signals were detected analysing periodontal complex traits derived from information on levels of periodontal pathogens and tissue inflammatory response [interleukin (IL)-1β].[18]

Recently, Munz et al identified new loci (SIGLEC5 and DEFA1A3) involved in innate and adaptive immunity response as the susceptibility factor for aggressive and chronic periodontitis.[20]

However, to date these results on periodontitis were controversial and more studies are needed to clarify the genetic contribution on the different forms of periodontitis.

Therefore, in this study we perform a Genome Wide Association Study (GWAS) in a genetic isolate population in northern Italy, aimed to identify new genes associated to different categories (slight, severe, localized, generalized) of chronic periodontal disease.

2 | MATERIAL AND METHODS

2.1 | Participants

Data of 826 individuals aged 18-89 years were collected between March 2008 and November 2008. Thanks to the 'Friuli Venezia Giulia Genetic Park' project, aimed at analysing a series of villages located in the north-east of Italy (San Martino del Carso, n = 232; Erto e Casso, n = 378; Clauzetto, n = 377; Illegio, n = 340; Sauris, n = 412; Val di Resia, n = 1021), showing evidence of isolation due to geographical, historical, linguistic and/or cultural factors. A detailed description of these villages is reported in Esko et al[21] and in Xue et al.[22] Briefly, a strong signal for genetic isolation and a high level of inner structure resulting in increased genetic similarity was reported. Moreover, these villages showed a divergence time from the closest general population of about 150 generations ago (~1000 years).

Participants gave written informed consent and the ethics committee of IRCCS Burlo Garofolo/ethics committee of the University of Trieste approved the study (Prot. CE/V-78, 06/08/2007).

2.2 | Periodontal status assessment

Medical family and individual history was first recorded. All the adults were subjected to an accurate oral examination in which periodontal examinations included measurements of periodontal pocket depth, plaque index, bleeding on probing index, clinical attachment loss and gingival recession on all teeth using a periodontal probe PCP12 (Hu-Friedy, Chicago, IL, USA). Four sites on each tooth were assessed: mesial; buccal; distal; and lingual. Moreover, the overall number of teeth present in the mouth was recorded. An additional X-ray examination (panoramic radiography) was collected. Wisdom teeth were excluded.

Diagnosis was made following the criteria of the American Academy of Periodontology[23] and subjects were then categorized as summarized in Table 1.

2.3 | Sample collection and phenotype definition

A common group of controls composed by people without signs of periodontitis or gingivitis was defined. Then, patients with diagnosis of chronic periodontitis were classified into different categories: 'slight' (people that fell into group 3 or 4); 'severe' (group 5 or 6); 'localized' (group 3 or 5); and 'generalized' (groups 4 and 6). Finally, the last group was named 'all', which included all people affected with chronic periodontitis (groups 3-6). A total number of 224 subjects were excluded, including patients with clinical signs of gingivitis (n = 93), aggressive periodontitis (n = 10), edentulous (n = 93) and patients with incomplete data (n = 28).

2.4 | Genotyping and imputation

For each sample, DNA was extracted from peripheral blood and genotyping was carried out using Illumina 370k high density SNP array (Illumina, Inc., San Diego, CA, USA). Genotype imputation was

conducted after standard QC using SHAPEIT2 for the phasing step[24] and IMPUTE2 for the imputation[25] using the 1000 Genomes phase I v3 reference set.[26]

2.5 | Statistical analysis

For statistical analysis, we treated the logistic trait (0 = controls, 1 = cases) as though it was quantitative, which corresponds to the Armitage Trend Test. We thus used a linear mixed model regression where the periodontitis status was the dependent variant while the SNP dosages were the independent variable. Sex, age and smoking status were included in the model as covariates. The genomic kinship matrix was used as random effect to take into account the non-independence of the samples as they are coming from an isolated population. Genomic kinship was estimated using the ibs function from the GenABEL R package. Linear regression was conducted using MixABEL and the GRAMMAR+ method.[27] We considered significant all SNPs that exhibited $P < 5 \times 10^{-8}$. Odds ratios were assessed using standard logistic regression, as no method can estimate odds ratios when inbreeding is present, while standard errors for confidence intervals were derived using the corrected test statistic and the beta from the logistic regression.

3 | RESULTS

The main features of participants are shown in Table 2. The mean age of the study sample was 51.39 ± 16.22 (range 18-89 years); 44% (n = 363) of the participants were males and 56% (n = 463) were females; 20% of subjects were current smokers.

In our sample, 160 subjects without signs of periodontitis or gingivitis formed the control group, while 442 were affected by chronic

TABLE 1 Criteria of the American Academy of Periodontology followed to classify the periodontal health status of subjects involved in the study

Code	Condition	Criteria
0	Healthy	BoP <25%
1	Gingivitis	BoP >25%
2	Aggressive PD	Tooth mobility and pockets of incisors and molars, age <35 y
3	Chronic localized PD slight or moderate	<30% with support loss <1/3 of the root
4	Chronic generalized PD slight or moderate	>30% with support loss <1/3 of the root
5	Chronic localized PD severe	<30% with support loss >1/3 of the root
6	Chronic generalized PD severe	>30% with support loss >1/3 of the root
7	Edentulous	
8	Not evaluable	No rx, incomplete data

BoP, bleeding on probing; PD, pocket depth.

TABLE 2 Subject characteristics and periodontal disease status

	All (n = 826)	Males (n = 363)	Females (n = 463)
Age (y) (mean ± SD)	51.3 ± 16.2	51.8 ± 15.7	51.0 ± 16.6
Smoking status (n)			
Yes	164	73	91
No	662	290	372
Periodontal status (n)			
Controls (code 0)	160	58	102
All (code 3 + 4 + 5 + 6)	442	214	228
Slight (code 3 + 4)	273	131	142
Severe (5 + 6)	169	83	86
Localized (3 + 5)	207	98	109
Generalized (code 4 + 6)	235	116	119
Others (code 1 + 2+7 + 8)	224	91	133

'Others' refers to subjects with gingivitis, edentulous, not evaluable or aggressive periodontitis. All these subjects were excluded from the statistical analysis.

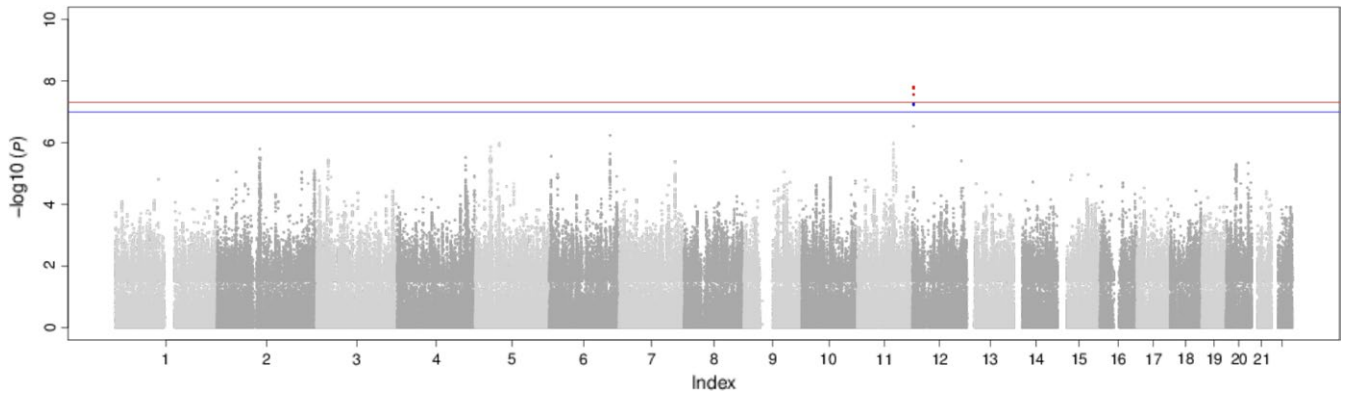

FIGURE 1 Manhattan plot of the genome-wide association study results. Each dot represents a single nucleotide polymorphism, with *x*-axis showing chromosomal positions and *y*-axis showing log10 (*P*-value)

TABLE 3 Results for the SNPs in EFCAB4B gene significantly associated to localized periodontitis

SNP	Chr	Position	Other allele	Coded allele	MAF	n	*P*	OR	CI
rs242016	12	3788260	G	A	0.21	367	1.54×10^{-8}	3.7	2.32-6.29
rs242014	12	3789135	C	T	0.20	367	1.63×10^{-8}	3.7	2.32-6.29
rs10491972	12	3789949	A	G	0.20	367	1.70×10^{-8}	3.7	2.32-6.29
rs242002	12	3807915	G	T	0.21	367	2.73×10^{-8}	3.6	2.28-6.13

Chr, chromosome number; CI, 95% confidence interval values; MAF, minor allele frequency; n, total number of samples used in the analysis; OR, odds ratio; *P*, *P*-value; SNPs, single nucleotide polymorphisms.
First column reports the name of the SNP; the 2nd the chromosome number; the 3rd the position of the polymorphism expressed in base pairs; the 4th and the 5th the alternative and the coded allele respectively. Sixth column reports the frequency of the coded allele.

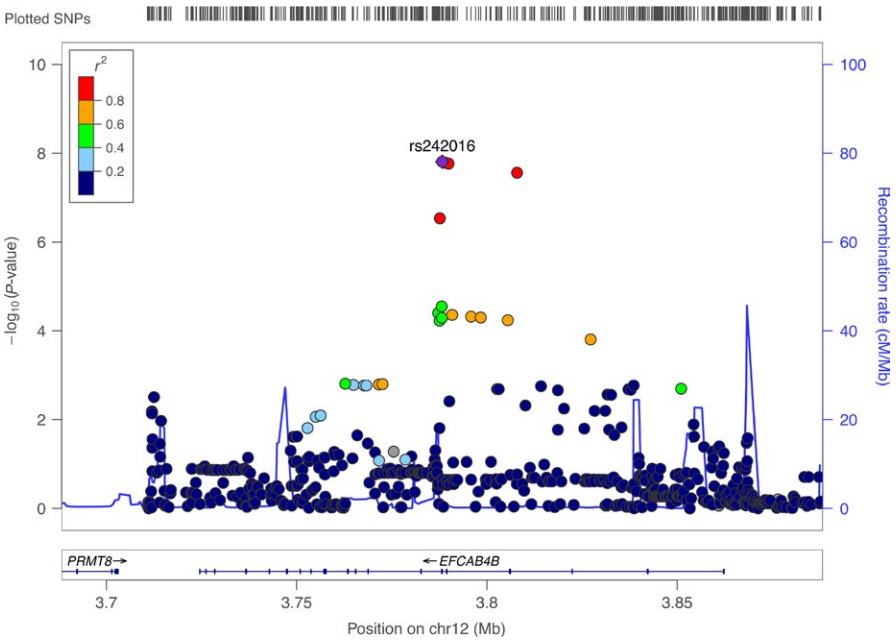

FIGURE 2 Regional association plot for the top hit of genome-wide association study on periodontal disease. Plot made using the tool Locus Zoom. SNPs are plotted with their *P* values (as −log10 values) as a function of genomic position. Estimated recombination rate are plotted to reflect the local linkage disequilibrium structure around the associated SNPs. SNPs, single nucleotide polymorphisms

periodontitis. Within the group of patients with chronic periodontitis were then differentiated: 273 individuals with slight periodontitis, 169 with severe periodontitis; 207 with localized periodontitis; and 235 with generalized periodontitis.

Mean age differ significantly ($P < 0.05$) between control group (36.87 ± 14.04) and the group of subjects with chronic periodontitis (54.63 ± 12.41), as well as the groups of subjects with slight (51.59 ± 12.12), severe (59.54 ± 11.28), localized (51.83 ± 12.56) and generalized (57.10 ± 11.76) periodontitis.

Case-control genome-wide association analysis on all these groups of periodontitis identified four SNPs significantly associated only to localized periodontitis. Figure 1 shows the Manhattan plot of the results on localized periodontitis across the genome. Results and detailed information for the significant SNPs are reported in Table 3.

The best hit was on rs242016 ($P = 1.5 \times 10^{-8}$, OR = 3.7). In addition, rs242014 ($P = 1.6 \times 10^{-8}$, OR = 3.7), rs10491972 ($P = 1.7 \times 10^{-8}$, OR = 3.7) and rs242002 ($P = 2.7 \times 10^{-8}$, OR = 3.6) reached statistical significance. All the identified SNPs fall inside the EF-hand calcium binding domain 4B (EFCAB4B) gene, localized on chromosome 12.

Figure 2 shows the regional association plot for the top hit of GWAS results.

No significant associations were found for the other subclassifications of periodontitis (all, severe, slight, generalized).

The possible functional role of the identified SNPs was explored using Haploreg v4,[28] showing that rs242002 is located inside enhancer regions active in numerous tissues, suggesting an involvement in affecting gene expression (http://archive.broadinstitute.org/mammals/haploreg/detail_v4.1.php?query=&id=rs242002).

4 | DISCUSSION

This study investigated the association among different subtypes of chronic periodontal disease and SNPs thanks to a GWAS. The advantage of running a study in geographically isolated towns is that these populations are consequentially also genetically isolated; thus, they have a restricted environmental, phenotypic and genetic heterogeneity, and this helps to identify the impact of genetic variants on multifactorial diseases, such as periodontal disease. In the present study, association among SNPs and chronic periodontitis (distinguished in slight, severe, localized and generalized periodontitis) was performed, founding for the first time an association between SNPs into the EFCAB4B gene and localized chronic periodontitis.

The function of SNPs we identified is still unknown. rs242016 is a synonymous SNP, while rs242014, rs10491972 and rs242002 are intron variants. Ad showed from Haploreg v4, a possible involvement of rs242002 in gene expression could be hypothesized.

Little is known also about EFCAB4B. This gene, localized on chromosome 12, codes for a protein involved in Ca^{2+} regulation during inflammation.[29] EFCAB4B is a Ca^{2+} binding protein highly expressed in T cells. It is a component of a ternary complex of proteins, which are essential elements in store-operated Ca^{2+} entry through Ca^{2+} release-activated Ca^{2+} channels in immune cells.[30] The Ca^{2+} influx across these channels is important for the activation, proliferation and cytokine production of these cells.[31]

Moreover, EFCAB4B is also largely expressed in minor salivary glands, as reported in the GTEX portal[32] (Figure S1). Considering the saliva itself produces proteins with immune activator/modulator properties (reviewed in Fabian et al),[33] further studies will be needed to investigate its link with this protein.

The link between the expression of the EFCAB4B protein in these tissues and periodontitis should be investigated in further studies to find out if its presence can be useful to monitor the onset and progression of the disease.

Other GWAS studies found association between SNPs in EFCAB4B gene and other pathological inflammatory conditions such as in non-alcoholic fatty liver disease.[34]

Moreover, it has already been investigated if certain polymorphisms of proteins involved in the inflammatory response may increase or decrease a person's risk for periodontitis. Many are the candidate gene studies that investigated alteration of genes encoding for inflammatory mediators that can be periodontitis-associated, such as IL-4, IL-6, tumour necrosis factor, etc.;[35-38] that is, it was found that the polymorphism of the IL-1 gene cluster is associated with the severity of periodontitis in non-smokers, and moreover it distinguished patients affected by severe periodontitis from those with mild disease.[39] Periodontal disease is also itself a risk factor for other inflammatory diseases. Chronic inflammation of the periodontal tissues continuously releases into the circulation blood inflammatory mediators involved in the progression of diseases such as coronary heart disease,[40] diabetes,[41] pre-term birth and low birth weight infants.[42]

The results of our study suggested a role for the EFCAB4B gene in localized periodontitis through the possible involvement of the inflammatory mechanism. It can be hypothesized that this gene can be crucial during the early stages of periodontal disease, when it is localized, influencing the beginning of the inflammation process but not when the disease becomes to be extensive.

To identify susceptible individuals in the early stages of the periodontitis, when bone loss is limited, it might be useful to the clinician to perform better preventive therapy and periodontal treatment.

Strengths and limitation of the study: The strengths of this study include a deep and detailed periodontal examination and that patients originated from geographically isolated towns. In fact, within the limitations of small sample size (for a GWAS), the advantage of conducting a GWAS in isolated populations is that it could allow the detection of genetic variation, which could require a much larger sample size. Moreover, this study provides a potential link between an inflammatory pathology, such as periodontal disease, and a still poorly studied protein. However, this study needs to be confirmed in other populations where both periodontal status and genetic data are available to enable replication.

In conclusion, even if it is not possible at this point to use the present results to diagnose chronic periodontitis, to the best of our knowledge the present investigation for the first time reported an association between SNPs in the EFCAB4B gene and chronic localized periodontitis. It suggests a possible role of this gene in the inflammatory response underlying pathogenesis of periodontal disease and contributes to the lack of knowledge in its complex pathogenetic pathway. Moreover, further investigations are required to understand better the role in periodontal disease of identified SNPs and of EFCAB4B gene, as well as its link with the complex mechanism of the inflammatory response.

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SUPPORTING INFORMATION

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