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Association between interleukin-6 promoter haplotypes and aggressive periodontitis

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Abstract

Background: Interleukin-6 (IL-6) polymorphisms have been shown to affect IL-6 promoter activity. This study investigated the possible role of IL-6 genetic polymorphisms and haplotypes in the predisposition to aggressive periodontitis (AgP).

Material and Methods: A case–control association study on 224 AgP patients and 231 healthy controls was performed in order to detect differences in genotype distributions of five single nucleotide polymorphisms (SNPs) located in the promoter region of the IL-6 gene.

Results: The IL-6 − 1363 polymorphism was associated with a diagnosis of AgP in subjects of all ethnicities ($p = 0.006$, adjusted logistic regression). The − 1480 SNP was associated with LAgP in subjects of all ethnicities ($p = 0.003$). The − 1480 and − 6106 polymorphisms were associated with Localized AgP in Caucasians ($n = 24$) ($p = 0.007$ and 0.010, respectively). Haplotypes determined by the − 1363 and − 1480 polymorphisms were also associated with LAgP ($p = 0.001$) in Caucasians.

Conclusions: This study supports the hypothesis of a link between IL-6 genetic factors and AgP and highlights the importance of two IL-6 polymorphisms (– 1363 and – 1480) in modulating disease phenotype and susceptibility.

Key words: aggressive periodontitis; genetic; haplotypes; interleukin-6

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A plethora of genetic factors, ranging from single gene defects to more subtle combinations of single nucleotide polymorphisms (SNPs), have been suggested as predisposing to aggressive periodontitis (AgP) (Kinane et al. 2005). SNPs with a supposed functional effect have been identified in the gene coding for the proinflammatory cytokine interleukin-6 (IL-6) (Fishman et al. 1998, Bennermo et al. 2004) and an association with periodontitis and its treatment outcomes has been reported for the − 174 and − 572 SNPs in Caucasians (Trevilatto et al. 2003, Holla et al. 2004, Brett et al. 2005, D'Aiuto et al. 2005). We recently reported an association between IL-6 − 174 SNP and subgingival presence of *Actinobacillus actinomycetemcomitans* (Nibali et al. 2007). Recent reports suggest that more than one of the IL-6 polymorphic sites are functional, and IL-6 transcription is influenced by complex interactions determined by these haplotypes (Fife et al. 2005, Qi et al. 2006).

The aim of this study was to investigate the relationship between five polymorphisms in the IL-6 gene promoter and their haplotypes and the presence of AgP, by comparing genotype frequencies in AgP patients and healthy controls.

Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests.

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Material and Methods

Study subjects

The study had a case–control design and the subject sample has already been described previously (Nibali et al. 2006). A total of 455 persons, selected among patients referred to the Eastman Dental Hospital (EDH), University College London, by general dental practitioners participated in the study. The 224 cases had been diagnosed with AgP, whereas the 231 controls were recruited among patients referred to other departments of the Hospital. Sixty-eight of the cases were included in a previous report (Brett et al. 2005), where only the IL-6 − 174 SNP and not the full haplotype had been analysed. All patients gave written informed consent; the study had been reviewed and

approved by the Eastman/UCLH joint ethics committee.

Inclusion criteria for AgP patients

- The clinical diagnosis of AgP was based on the 1999 Consensus Classification of Periodontal Diseases (Lang et al. 1999), using only clinical, and not laboratory, evidence, as described previously (Nibali et al. 2006).

The presence of interproximal PPD and LCAL $\geqslant$5 mm and radiographic bone loss of $\geqslant$30% of root length on at least three teeth other than first molars or incisors was used to distinguish generalized from localized AgP cases clinically (Lang et al. 1999).

Inclusion criteria for controls

Controls had to be at least 25 years old, in order to reduce the risk of including subjects who may develop AgP later. Volunteers with known specific genetic diseases or a history of periodontal disease or tooth loss due to periodontal disease were not included. A single calibrated examiner (L. N.) performed a basic screening periodontal examination on these subjects, using the Periodontal Screening and Recording (PSR) index and existing radiographs. In the event of detecting codes 3, 4 or * in any sextant, further investigation was performed. Subjects were excluded if they presented with at least one site with PPD and LCAL $\geqslant$4 mm and radiographic evidence of bone loss (Nibali et al. 2006).

For both cases and controls, smoking status and ethnic origin were confirmed by a questionnaire.

DNA extraction

DNA was extracted from peripheral blood cells from a 10 ml blood sample using the Nucleon® BACC2 kit (Nucleon Bioscience, Coatbridge, UK) as described previously (Brett et al. 2005). Ten nanograms of DNA were subsequently used for polymerase chain reaction (PCR) analysis.

Real-time PCR allele discrimination

Allelic discrimination assays were performed using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Warrington, UK). Five polymorphisms in the IL-6 gene promoter region were selected, including SNPs at positions -174 (CCTTTAGCAT[C-G]GCAAGAC, rs 1800795), -572 (CAACAGCC[C-G]CTCACAG, rs 1800796), -1363 (CACTGTTTTATC[G-T]GATCTTG, rs 2069827) and -6106 (forward primer CATCAGGCTTTTGGGCTTTCAA, reverse primer AGTCTACTGTTAATGGGACTACAGGAA, SNP sequence TCTCTACA[A-T]TAAGAAATAC), and a base-pair deletion at position -1480 (forward primer CCCCATTTTCATTTTCACACCAAAGA, reverse primer GGTCATCCATTCTTCACCGATTGT, SNP sequence ACCGTCTCT[C-G]TGTTTAG). The -174, -572, -1363 and -1480 polymorphisms belong to haplotypes studied previously and shown to influence IL-6 production (Terry et al. 2000, Fife et al. 2005), while the -6106 is a novel polymorphism further upstream in the gene promoter region. All the primers and probes were designed using the Assay-by-Design service offered by Applied Biosystems (Warrington, Cheshire, UK). Genotyping was performed in 25 μl reactions as previously described (Nibali et al. 2006). Hidden duplicates were added to each plate to test error rates. However, no detection errors were observed. All genotyping was performed blindly with respect to clinical diagnosis by a single investigator (L. N.). Whenever the results were not clear, the analysis was repeated. If, after repetition, the result was still uncertain, no result was recorded for that polymorphism.

Statistical analysis

The Hardy–Weinberg formula was applied to the genotype frequencies in our subject sample to test for any deviations from the expected genotype equilibrium.

The SPSS 12.0 package was used to detect differences in distributions for each SNP between groups. Categorical data between groups were analysed with the χ^2 test. In order to adjust for multiple testing, the α value for statistical significance for association between genetic SNPs and AgP was set at 0.01. A binary independent variable (AgP or healthy control) was used to ascertain the association between each SNP and the diagnosis of AgP. Subanalyses were performed among GAgP, LAgP and controls. χ^2 analyses were performed to detect differences in allele prevalence, while multiple logistic regression analysis adjusting for confounders (gender, ethnicity and smoking) was performed to detect differences in genotype frequency. Owing to the risk of finding spurious associations (Pritchard & Rosenberg 1999), separate analyses were performed in the two largest ethnic subpopulations of the study: Caucasians and Blacks.

A second analytic approach consisted of analysis of linkage disequilibrium (LD) between SNPs, and an association study between haplotypes and AgP phenotype. These aimed at exploring the possible effect of combinations of polymorphisms located in the same gene in determining disease susceptibility. The GC utilities package (''http://www.smd.qmul.ac.uk/statgen/dcurtis/software.html'') (Curtis et al. 2006) was used for LD analysis between genetic markers. The LDpairs programme allowed us to investigate LD between polymorphisms. R^2 and D' values are reported as measures of LD; the maximum value of 1 indicates that no recombination exists between pairs of markers (Stram 2004). Haplotype associations were analysed in this study using the WHAP package (''http://pngu.mgh.harvard.edu/ $\sim$ purcell/whap/'') (Sham et al. 2004). The RunGC programme was used for separate analysis of haplotype frequencies in both cases and controls, and to test for significant differences between these two groups using a likelihood-ratio test (LRT).

Results

A total of 224 AgP (57 localized AgP and 167 generalized AgP) patients and 231 controls took part in the study. Their demographic characteristics are presented in Table 1. The distribution of the genotypes for all the polymorphisms studied satisfied Hardy–Weinberg equilibrium in controls and patients of each ethnic subgroup. A total of 18 genotypes out of 2225 (0.8%) across all subjects for all studied SNPs were not scored because they were unclear after repeated analysis.

LD

Significant LD was detected between most polymorphisms. The strongest LDs were noted between -174 and -1480 (R^2 0.914 and D' 0.975), -174 and -572 (R^2 0.222, D' 1.0), -572 and -1480 (R^2 0.208, D' 1.0) and -1480 and -6106 (R^2 0.308, D' 1.0) (see Fig. 1).

Table 1. Comparison of demographic characteristics of patients and controls

	Patients		Controls		Comparisons between groups
	($n = 224$)	%	($n = 231$)	%	
Age	29.9 ± 7.2 (10–45)	–	38.4 ± 12.2 (25–77)	–	–
Gender					
Male	79	35.3	99	42.9	$p = 0.097$ Pearson's χ^2
Female	145	64.7	132	57.1	
Ethnicity					
Caucasian	112	50.0	144	62.3	$p = 0.063$ Pearson's χ^2
Black	59	26.3	45	19.5	
Asian	34	15.2	29	12.6	
Other	19	8.5	13	5.6	
Smoking					
No smokers	118	52.7	135	58.4	$p = 0.168$ Pearson's χ^2
Former smokers	52	23.2	41	17.7	
Light smokers (<20/day)	38	17.0	46	19.9	
Heavy smokers (⩾20/day)	16	7.1	9	3.9	

Standard deviation and range are reported for continuous variables (age).

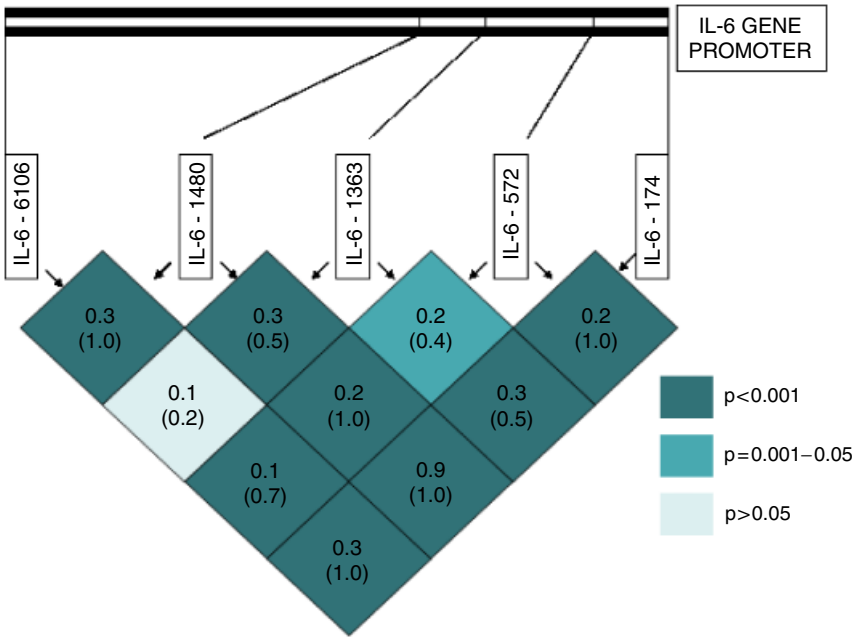

Fig. 1. Schematic representation of LD between the five analysed IL-6 polymorphisms. R^2 and D' values (in parenthesis) for LD between each marker are reported. The different-colour shades represent *p*-values (see legend).

Comparison between AgP and controls

The distributions of the polymorphisms in relation to the diagnosis (AgP/healthy controls) in all subjects are reported in Table 2. Increases in allele distribution in the patient group (GAgP and LAgP) were detected for − 174 G, − 1363 G and − 1480 C alleles (no deletion). These allelic differences between AgP and healthy controls were marked (although not statistically significant) in the Caucasian population (χ^2 $p =$ 0.014 for − 174, 0.013 for − 1363 and 0.019 for − 1480, respectively, not presented in the table), but not in the Black population.

Among Caucasians, logistic regression analysis adjusted for confounders revealed limited evidence of association with a diagnosis of AgP for − 174 [$p = 0.036$, odds ratio (OR) = 1.50, 95% confidence interval (CI) = 1.03–2.20], − 1363 ($p = 0.013$, OR = 2.35, 95% CI = 1.19–4.63) and − 1480 ($p =$ 0.037, OR = 1.49, 95% CI = 1.02–2.19). No significant associations were detected in the Black subgroup. When AgP patients of all ethnicities were compared with all controls adjusting for gender, smoking and ethnicity, logistic regression revealed a statistically significant association with a diagnosis of AgP for the − 1363 polymorphism ($p = 0.006$, OR = 2.27, 95% CI = 1.26–4.07).

When looking at the different disease entities (LAgP and GAgP), significant associations were detected between LAgP and healthy controls. In the Caucasian group (144 healthy controls and 24 LAgP patients, see Table 3), logistic regression analysis revealed statistically significant associations for − 1480 ($p =$ 0.007, OR = 3.09, 95% CI = 1.37–6.97), and − 6106 ($p = 0.010$, OR = 2.27, 95% CI = 1.21–4.24) polymorphisms having adjusted for gender and smoking. When the analysis was extended to subjects of all ethnicities, logistic regression analysis revealed statistically significant associations with LAgP for − 1480 ($p = 0.003$, OR = 2.71, 95% CI = 1.39–5.28), having adjusted for gender, smoking and ethnicity.

Haplotype analyses

Among Caucasians, haplotypes formed by all five studied IL-6 SNPs displayed no statistically significant associations with the AgP phenotype and with the GAgP group. However, remarkable associations were detected in the LAgP Caucasian subgroup: $p = 0.01$ for the haplotype made up of all five SNPs and $p = 0.001$ for a constrained model including only − 1363 and − 1480. Table 4 shows the

Table 2. Distributions of all studied polymorphisms in AgP and controls

Polymorphism	Genotype	All subjects		Logistic regression (adjusted) *p*	Caucasians		Logistic regression (adjusted) *p*	Blacks		Logistic regression (adjusted) *p*
		patients (*n* = 224)	controls (*n* = 231)		patients (*n* = 112)	controls (*n* = 144)		patients (*n* = 59)	controls (*n* = 45)	
IL-6 − 174	CC	17 (7.7%)	30 (13.0%)		14 (12.5%)	28 (19.4%)		0 (0%)	0 (0%)	
	CG	66 (29.7%)	91 (39.4%)	0.018	49 (43.8%)	74 (51.4%)	0.036	6 (10.5%)	7 (15.6%)	0.630
	GG	139 (62.6%)	110 (47.6%)		49 (43.8%)	42 (29.2%)		51 (89.5%)	38 (84.4%)	
IL-6 − 572	CC	166 (75.1%)	194 (84.0%)		97 (87.4%)	133 (92.4%)		46 (80.7%)	39 (86.7%)	
	CG	46 (20.8%)	34 (14.7%)	0.022	14 (12.6%)	11 (7.6%)	0.083	8 (14.0%)	6 (13.3%)	0.208
	GG	9 (4.1%)	3 (1.3%)		0	0		3 (5.3%)	0 (0%)	
	TT	1 (0.5%)	4 (1.7%)		1 (0.9%)	4 (2.8%)		0 (0%)	0 (0%)	
IL-6 − 1363	TG	15 (6.8%)	35 (15.2%)	0.006	11 (10.0%)	28 (19.4%)	0.013	1 (1.7%)	2 (4.4%)	0.503
	GG	203 (92.7%)	192 (83.1%)		98 (89.1%)	112 (77.8%)		57 (98.3%)	43 (95.6%)	
IL-6 − 1480	CC	147 (66.8%)	117 (51.1%)		53 (48.6%)	48 (33.8%)		54 (91.5%)	38 (84.4%)	
	CG	59 (26.8%)	87 (38.0%)	0.015	43 (39.4%)	69 (48.6%)	0.037	5 (8.5%)	7 (15.6%)	0.409
	GG	14 (6.4%)	25 (10.9%)		13 (11.9%)	25 (17.6%)		0 (0%)	0 (0%)	
IL-6 − 6106	AA	139 (62.6%)	141 (61.0%)		67 (60.4%)	91 (63.2%)		39 (66.1%)	28 (62.2%)	
	AT	71 (32.0%)	73 (31.6%)	0.548	35 (31.5%)	44 (30.6%)	0.516	17 (28.8%)	12 (26.7%)	0.511
	TT	12 (5.4%)	17 (7.4%)		9 (8.1%)	9 (6.3%)		3 (5.1%)	5 (11.1%)	

Genotype distributions for all studied polymorphisms are presented in AgP patients and controls of mixed ethnicity (all subjects), and in the subgroups of Caucasians and Blacks. Relative *p*-values from logistic regression analyses are presented (adjusted for gender, ethnicity and smoking).
Aggressive periodontitis, AgP; IL-6, interleukin-6.

Table 3. Distributions of all studied polymorphisms in LAgP and controls

Polymorphism	Geno-type	All subjects		Logistic regression (adjusted) *p*	Caucasians		Logistic regression (adjusted) *p*
		LAgP (*n* = 57)	controls (*n* = 231)		LAgP (*n* = 24)	controls (*n* = 144)	
IL-6 − 174	CC	3 (5.4%)	30 (13.0%)		3 (12.5%)	28 (19.4%)	
	CG	12 (21.4%)	91 (39.4%)	0.011	7 (29.2%)	74 (51.4%)	0.027
	GG	41 (73.2%)	110 (47.6%)		14 (58.3%)	42 (29.2%)	
	CC	39 (68.4%)	194 (84.0%)		23 (95.8%)	133 (92.4%)	
IL-6 − 572	CG	16 (28.1%)	34 (14.7%)	0.140	1 (4.2%)	11 (7.6%)	0.659
	GG	2 (3.5%)	3 (1.3%)		0	0	
	TT	0	4 (1.7%)		0	4 (2.8%)	
IL-6 − 1363	TG	1 (1.9%)	35 (15.2%)	0.031	0	28 (19.4%)	0.998
	GG	53 (98.1%)	192 (83.1%)		23 (100%)	112 (77.8%)	
	CC	44 (81.5%)	117 (51.1%)		16 (72.7%)	48 (33.8%)	
IL-6 − 1480	CG	8 (14.8%)	87 (38.0%)	0.003	4 (18.2%)	69 (48.6%)	0.007
	GG	2 (3.7%)	25 (10.9%)		2 (9.1%)	25 (17.6%)	
	AA	30 (54.5%)	141 (61.0%)		10 (43.5%)	91 (63.2%)	
IL-6 − 6106	AT	19 (34.5%)	73 (31.6%)	0.290	7 (30.4%)	44 (30.6%)	0.010
	TT	6 (10.9%)	17 (7.4%)		6 (26.1%)	9 (6.3%)	

Genotype distributions for all studied polymorphisms are presented in LAgP patients and controls of mixed ethnicity (all subjects) and in Caucasians only. Relative *p*-values from logistic regression analyses are presented (adjusted for gender, ethnicity and smoking).
Aggressive periodontitis, AgP; IL-6, interleukin-6.

haplotype combinations between all five IL-6 polymorphisms in Caucasians, giving estimated frequencies for controls, LAgP and GAgP subjects. The highest Likelihood-Ratio test value is obtained for a combination of alleles, including IL-6 − 1363 T and − 1480 G, with a supposedly protective function towards AgP (estimated frequencies = 34% in controls, 26% in GAgP and only 9% in LAgP).

In subjects of all ethnicities, a constrained model including only − 1363 and − 1480 SNPs showed an association with AgP (adjusted $p = 0.004$). In LAgP patients, the lowest *p*-value for association with the disease phenotype was also found for the − 1363 and − 1480 haplotypes (adjusted $p = 0.002$).

Discussion

A previous study by our group showed an association between IL-6 − 174 SNP and chronic and AgP (Brett et al. 2005). It has been suggested that the positive associations seen between this polymorphism and other diseases are due to LD across the promoter region (Fife et al. 2005). In order to address this possibility, further analysis of SNPs and haplotypes in the promoter region of the IL-6 gene, on a larger sample of AgP patients and healthy controls, was carried out.

We found that: (i) IL-6 gene polymorphisms were associated with LAgP, (ii) the − 1480 deletion showed a stronger association with the disease trait than the previously described − 174 polymorphism and (iii) haplotype combinations between − 1363 and − 1480 SNPs consistently showed the strongest association with the disease trait.

Table 4. IL-6 haplotype estimated frequencies (as analysed by RunGC software) in Caucasian controls ($n = 144$), LAgP ($n = 24$) and GAgP ($n = 88$) subjects

Allele combinations					Estimated frequencies			LRT mean LAgP *versus* controls	LRT mean GAgP *versus* controls
− 174	− 572	− 1363	− 1480	− 6106	controls	LAgP	GAgP		
C	C	T	C	A	0.01	0.03	0.03	0.62	1.05
G	C	G	C	A	0.15	0.16	0.18	0.02	0.56
G	C	G	C	T	0.12	0.20	0.08	1.45	1.02
G	G	G	C	A	0.03	0.01	0.07	0.44	3.42
C	C	T	G	A	0.34	0.09	0.26	8.02	2.10
C	C	G	C	A	0.18	0.03	0.00	0.33	3.64

Different haplotype combinations are shown. In the last columns, Likelihood-ratio test values are reported. A higher value indicates higher association. Aggressive periodontitis, AgP; IL-6, interleukin-6.

The results reported here might serve to explain the conflicting reports linking the IL-6 − 174 G allele with increased promoter activity (Fishman et al. 1998, Endler et al. 2004) and with IL-6 production (Burzotta et al. 2001, Bennermo et al. 2004). An increase in the prevalence of the − 174 G allele was found in periodontitis patients compared with healthy controls (Trevilatto et al. 2003, Brett et al. 2005). This finding was confirmed in the present study. However, differences in genotype distributions in the Localized Aggressive group were particularly marked for the − 1480 and − 6106 polymorphisms. This leads to the speculation that the previous association noted between the − 174 SNP and periodontitis (Trevilatto et al. 2003, Brett et al. 2005) might be a function of its LD with the − 1480, − 1363 or − 6106 polymorphisms or with yet another functional SNP in LD in the IL-6 gene (Qi et al. 2006). The haplotype analysis in this investigation led to the conclusion that haplotype combinations (especially between the − 1363 and − 1480 polymorphisms) revealed a skewed distribution between LAgP and healthy subjects, pointing towards possible additive or synergistic effects of these SNPs.

Limited evidence exists on the functional effect of IL-6 − 1363 and − 1480 SNPs (Fife et al. 2005). However, we can speculate about the possible role of these polymorphisms in the pathogenesis of AgP, based on their LD with IL-6 − 174 SNP, for which several functional studies have been perfomed in the literature (Fishman et al. 1998, Terry et al. 2000). One possible explanation links these SNPs with an increased inflammatory response, specifically in the presence of periodontopathogenic bacteria. In particular, we observed an association between IL-6 − 174 SNP and *A. actinomycetemcomitans* (Nibali et al. 2007), one of the recognized periodontopathogens associated with AgP (AAP 1996). Our group also showed that the same polymorphism was associated with greater serum IL-6 and C-reactive protein concentrations (D'Aiuto et al. 2004) and seems to modulate the clinical response to periodontal treatment (D'Aiuto et al. 2005). The presence of certain bacteria such as *A. actinomycetemcomitans* in the periodontium of supposedly IL-6 ''hyperproducers'' (such as − 174 G homozygous or − 1480 C homozygous) (Fishman et al. 1998, Burzotta et al. 2001, Bennermo et al. 2004) may trigger the establishment of a chronic inflammatory lesion. This may result in enhanced and rapid bone resorption and AgP onset at an early age. Because bacteria such as *A. actinomycetemcomitans* can stimulate IL-6 production (Belibasakis et al. 2005), we can speculate that in genetically predisposed subjects, this increase may lead to an excessive tissue-damaging inflammatory response mediated by fibroblasts and/or other cell types. Alternatively, an enhanced IL-6 response could act to limit the extent of periodontal involvement to incisors and first molars (typical of LAgP) in otherwise susceptible individuals. At the same time, some subjects, such as those carrying IL-6 − 1363 T and − 1480 G alleles, might have a reduced inflammatory response that could make them less susceptible to AgP. However, in interpreting these results, we have to acknowledge the limited evidence on the functional effect of the SNPs and haplotypes studied and the relatively small sample size of the study (especially with regard to LAgP Caucasians), with the residual possibility of a spurious association (Cooper et al. 2002).

In conclusion, this study supports the theory that IL-6 polymorphisms might play a role in the genetic profile predisposing to LAgP. Interestingly, in the same population, we previously detected an association between SNPs regulating neutrophil function and the generalized form of disease (GAgP) (Nibali et al. 2006). Because these polymorphisms and haplotypes are not rare in the general population, it could be suggested that they might modify the underlying susceptibility to periodontal disease to produce the AgP phenotype. Functional analyses of the role of these IL-6 polymorphisms and haplotypes are now needed in order to gain a better understanding of the pathogenic process.

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Clinical Relevance

Scientific rationale for the study: Genetic factors affecting the inflammatory response may play a part in the pathogenesis of aggressive periodontitis.
Principal findings: Genetic polymorphisms in the genes coding for the IL-6 polymorphisms and their combinations (haplotypes) were associated with aggressive periodontitis in our sample.
Practical implications: A combination of IL-6 genetic polymorphisms may be important in periodontal disease pathogenesis. The knowledge of inflammatory pathways determined by these genetic factors may help in disease prevention and treatment.