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NADPH oxidase (CYBA) and FcγR polymorphisms as risk factors for aggressive periodontitis

A case–control association study

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Abstract

Introduction: Neutrophils (PMN) in aggressive periodontitis (AgP) patients have been reported to be hyperactive especially with regards to superoxide production. Polymorphisms in genes influencing PMN function have been proposed as candidate risk factors for AgP. The aim of this study was to test the association of specific gene polymorphisms affecting PMN functions with AgP.

Material and Methods: Two hundred and twenty-four patients with confirmed diagnosis of AgP and 231 subjects with healthy periodontium took part in the study. A blood sample was collected from subjects and genotypes for $p22^{phox}$ (CYBA) NADPH oxidase, FP, Fcα and Fcγ receptors were analysed in a blind fashion.

Results: The C242T $p22^{phox}$ NADPH oxidase T allele was significantly associated with AgP in a multiple logistic regression model adjusting for confounders, and this was observed for all subjects [$p = 0.002$, odds ratio (OR) = 1.87, 95% confidence interval (CI) = 1.27 – 2.83] and Caucasians ($p = 0.009$, OR = 2.07, 95% CI = 1.20–3.59). Concomitant presence of C242T $p22^{phox}$ NADPH oxidase T allele and FcγRIIIb NA1 homozygosity was associated with the generalized AgP phenotype in Caucasians ($p = 0.001$, OR = 30.35, 95% CI = 3.81 – 241.97).

Conclusions: C242T $p22^{phox}$ NADPH oxidase and FcγR polymorphisms may predispose to AgP through a modulation of neutrophil superoxide production.

Key words: aggressive periodontitis; genetic polymorphisms; NADPH oxidase; neutrophils; risk factors

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While describing early onset forms of periodontitis as a new clinical entity, Baer pointed out that in such cases the host reaction seemed to be out of proportion to the local factors (Baer 1971). Since then, evidence has confirmed that what we now call aggressive periodontitis (AgP) is an infectious disease characterized by an abnormal host response to bacterial plaque (Schenkein & Van Dyke 1994).

Neutrophils (polymorphonuclear leukocytes, PMN) and monocyte/macrophages are the first line of the cellular defence in host resistance to bacterial invasion. With this in mind, several researchers have investigated the role neutrophils play in the host inflammatory response in early onset forms of periodontitis (Van Dyke & Serhan 2003). Support for this comes from the finding of severe destructive periodontitis as a common secondary feature in subjects affected by PMN deficiencies or malfunctions, such as neutropenia or Chediak–Higashi syndrome (Deas et al. 2003). This led to the concept that AgP was due to a minor defect of the neutrophil which resulted in periodontal destruction without other systemic features. Early studies concentrated on deficiencies of PMN activity with respect to chemotaxis (Cianciola et al. 1977, Suzuki et al. 1984), phagocytosis (Suzuki et al. 1984, Van Dyke et al. 1986) or killing (Van Dyke et al. 1986, Thompson & Wilton 1991, 1992). However, more recent research supports the hypothesis

that it is the excess of activity and the release of toxic products from PMNs, and not their deficiency, that is responsible for the tissue destruction seen in AgP (Kantarci et al. 2003). PMNs of AgP patients, especially of Localized AgP, have been shown to be hyperreactive (Shapira et al. 1991, Leino et al. 1994) and there is now increasing evidence to suggest that periodontal lesions characteristic of AgP are determined by the nature of PMN and immune response (Gemmell et al. 2002, Van Dyke & Serhan 2003).

We have focused on two aspects of PMN response to bacteria. Bacterial recognition is mediated through Fc and formyl peptide receptors and polymorphisms in these genes have been studied previously (Loos et al. 2005). The role of bacterial killing has been studied functionally, but this is the first study to evaluate polymorphisms of the genes involved and their association with AgP. PMN biological activities and bacterial killing potentials are due to two different pathways: non-oxidative, through the release of proteases, and oxidative, through the so-called ''oxidative burst''. It has been widely demonstrated that the oxidative burst is increased in many localized AgP (LAgP) cases compared with healthy controls (Leino et al. 1994, Gronert et al. 2004), even if the underlying defect leading to it has escaped understanding so far. Increasing evidence suggests that specific genetic polymorphisms may influence neutrophil function and their potential for bacterial phagocytosis, superoxide production and killing (Salmon et al. 1990, Kobayashi et al. 2000b, Shimo-Nakanishi et al. 2004). Furthermore, a growing body of evidence suggests that genetic factors may increase the predisposition to develop periodontitis by modulating the immune response to bacterial pathogens (Michalowicz et al. 2000, Kinane & Hart 2003).

The aim of this study was to investigate the relationship between specific genotypes supposed to influence neutrophil activity and presence of AgP, by comparing genotype frequencies in AgP patients and healthy controls. The selected panel of genetic single nucleotide polymorphisms (SNPs) included those in Fc- and formyl peptide receptors, involved in bacterial recognition, and NADPH oxidase $p22^{phox}$ (CYBA gene), involved in neutrophil superoxide production.

Materials and Methods

Study subjects

The study was a single-blind case–control trial. A total of 455 subjects selected among patients referred to the Eastman Dental Hospital, University College London by general dental practitioners took part in the study. Two hundred and twenty-four of them had been diagnosed with AgP (AgP). Two hundred and thirty-one of them had been recruited among periodontal disease-free patients referred to other Departments of the Hospital (Oral Surgery, Conservation and Endodontic). All the patients gave written informed consent; the study had been reviewed and approved by the Eastman/UCLH joint ethics committee.

Inclusion criteria for AgP patients

Diagnosis of AgP was based on the 1999 Consensus Classification of Periodontal Diseases, in agreement with the recent Consensus report on definition of periodontitis cases (Armitage 1999, Tonetti & Claffey 2005). Our diagnostic criteria took into consideration only clinical, and not laboratory, evidence. We classified patients as having AgP, when we had evidence of:

- *Healthy status*, except from the presence of periodontitis (for example, all subjects with diabetes were excluded).
- *Rapid attachment loss and bone destruction*, proven by radiographs obtained at a few years distance. When this was not possible, severe disease at a young age was used, with patients <35 at the time of the initial diagnosis.
- *Familial aggregation*: We tried to ascertain the familial aggregation, by means of a specific questionnaire and, when possible, by examining first degree relatives. However, patients showing clear clinical signs of AgP but without a positive family history were still included (Gozalbo & Griffiths 2006).
- *Clinical and radiographic diagnosis*: All the patients with a suspected diagnosis of AgP were examined by a single experienced clinician (G.S.G.). Full mouth measures of probing pocket depth (PPD), recession (REC, measured as distance from the cement-enamel junction to the gingival margin) and lifetime cumulative attachment levels (LCAL, measured either as a direct measurement of CEJ to the base of the pocket, or as a calculation of PPD+REC) were obtained at six sites per tooth. Full mouth long cone periapical radiographs were also obtained from each patient.

Patients were diagnosed with LAgP when presenting with interproximal PPD and LCAL $\geqslant 5$ mm and radiographic bone loss of $\geqslant 30\%$ of root length on at least two permanent teeth, of which at least one was a first molar or incisor, and no more than three teeth other than first molars or incisors (Lang et al. 1999).

Patients were diagnosed with generalized AgP (GAgP) when presenting with generalized interproximal PPD and LCAL $\geqslant 5$ mm and radiographic bone loss of $\geqslant 30\%$ of root length affecting at least three permanent teeth other than first molars and incisors (Lang et al. 1999).

All individuals with dubious diagnosis (for example with retentive factors or very high plaque scores, proportionate to the amount of periodontal destruction present) were excluded. Such a measure was used to take advantage of the leeway space the new classification leaves to personal interpretation, in order to exclude patients with moderate to severe disease belonging to the ''grey area'' between AgP and chronic periodontitis.

Inclusion criteria for controls

In order to minimize recruitment bias, we enrolled control subjects who belonged to a similar socio-economic group and with similar oral health care and awareness as our AgP patients. Therefore, subjects registered with the UK National Health System and attending other Departments of the EDH were screened for inclusion. Only patients at least 25 years old were enrolled, in order to reduce the risk of including subjects who may later develop AgP. Volunteers with known specific genetic diseases or history of periodontal disease or tooth loss due to periodontal disease were not included. A single examiner performed a basic screening periodontal examination on these subjects, using the periodontal screening and recording (PSR) index and reference to the existing radiographs taken for the clinical problem that resulted in their referral. In the event of detecting codes 3, 4 or * in any sextant, further investigation was per-

formed. This consisted of pocket depth and recession estimates using a UNC 15 probe and further radiographic investigation consisting of either a panoramic view or individual periapicals. Subjects were excluded if they presented with at least one site with PPD and LCAL $\geqslant$ 4 mm or radiographic evidence of bone loss.

For both cases and controls, smoking status and ethnic origin were confirmed by a questionnaire. Ethnic origin of all grandparents were asked and recorded, and whenever they came from at least two different backgrounds, the ethnicity was considered as mixed. Subjects were divided into Caucasians, Blacks (including Black-Africans and Afro-Caribbean), Asians and others (including mixed).

DNA extraction

A 10 ml blood sample was collected through venipuncture in the ante-cubital fossa from each study subject. The blood samples were collected in sodium EDTA vacutainers and stored at -70°. DNA was extracted from peripheral blood cells using the Nucleon® BACC2 kit (Nucleon Bioscience, Coatbridge, UK) as described previously (Brett et al. 2005). The DNA concentration was estimated by measuring absorbance at 260 nm using a spectrophotometer. 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 7300/7500 Real Time PCR System. This system uses fluorescent-based PCR chemistries to provide detection of nucleic acid sequences. Most of the primers and probes (listed in Table 1) were designed using the assay-by-design service offered by Applied Biosystems (Warrington, Cheshire, UK). Two other primers and probes (last two rows in Table 1) were obtained from Applied Biosystems from their assays-on-demand products. Genotyping was performed in 25 μl reactions consisting of 10 ng of genomic DNA 12.5 μl of 2X Taqman Universal PCR Master Mix and either 0.625 μl (40 ×) or 1.25 μl (20 ×) primer/probe sets. Cycling conditions were 2 min. at 50°C; 10 min. at 95°C; 40 cycles of 95°C for 15 s; 60°C for 1 min. Real-time fluorescence detection was performed during 60°C annealing/extension step of each cycle. For each analysed sample a pair of fluorescent detectors were used, one of which matched the wild type, whereas the other was a perfect match for the mutation. The allelic discrimination assay then measured the change in fluorescence associated with the dyes (Livak et al. 1995). Dedicated sequence detection system (SDS) software (Applied Biosystems®) was used to plot the results of the allelic discrimination run on a scatter plot of allele X *versus* allele Y and automatically calls genotypes dependent on fluorescence intensities of VIC and FAM reporter dyes. Hidden duplicates were added to

Table 1. Gene polymorphism real-time probe/primer combinations designed using assay-by design service from Applied Biosystems and inventoried SNP genotyping assay products from Applied Biosystems

Gene SNP	Substitution/nucleotide position	Oligo	Sequence
Fc-gamma receptor IIIA (FcγRIIIA)	559 G-T	*Probe-G*	VIC-CCAACAAGCCCCC-MGB
		Probe-T	FAM-CCCAAAAGCCCCC-MGB
		Primer-F	GACAGCGGCTCCTACTTCTG
		Primer-R	GACAGCGGCTCCTACTTCTG
Fc-gamma receptor IIB (FcγRIIB)	695 T-C	*Probe-T*	VIC-CTACAGCAATCCCAG-MGB
		Probe-C	FAM-CTACAGCAGTCCCAG-MGB
		Primer-F	CCAAGCTCCCAGCTCTTCAC
		Primer-R	CCACTACAGCAGCAACAATGG
Fc-gamma receptor IIIB (FcγRIIIB)	141 G-C (NA antigen)	*Probe-G*	VIC-CAATGGTACAGGGTGCT-MGB
		Probe-C	FAM-CAATGGTACAGCGTGCT-MGB
		Primer-F	CCAAAGGCTGTGGTGTTCCT
		Primer-R	CTTCAGAGTCACACTGTCCTTCTC
	266 C-A (SH antigen)	*Probe-C*	VIC-CTTCATTGACGCTGCCAC-MGB
		Probe-A	FAM-TTCATTGACGATGCCAC-MGB
		Primer-F	AGCCAGGCCTCGAGCTA
		Primer-R	TGTACTCTCCACTGTCGTTGACT
Formyl peptide receptor (FPR)	301 G-C	*Probe-G*	VIC- CCTGTGCAAATTCGTCTT-MGB
		Probe-C	FAM-CTGTGCAAATTCCTCTT-MGB
		Primer-F	TGGCCTTTCGGCTGGTT
		Primer-R	AGCGGTCCAGAGCAATGAG
	546 C-A	*Probe-C*	VIC-AACTTTTCGCCCTGGAGG-MGB
		Probe-A	FAM-TTTTCGCCATGGAGG-MGB
		Primer-F	GGGACAGTAGCCTGCACTTTT
		Primer-R	TGCCTCTCACCGTCAACATG
	568 A-T	*Probe-A*	VIC-CCCTAAAGAGAGGATAAA-MGB
		Probe-T	FAM-CCCTAAAGAGTGGATAAA-MGB
		Primer-F	GGACAGTAGCCTGCACTTTTAACTT
		Primer-R	TGCCTCTCACCGTCAACATG
Fc-gamma receptor IIA (FcγRIIA)	494 A-G	National Centre for Biotechnology Information (NCBI) dbSNP ID number rs1801274	
Fc-alpha receptor (FcαR)	324 A-G	National Centre for Biotechnology Information (NCBI) dbSNP ID number rs1865096	

SNP, single nucleotide polymorphism; NA, neutrophil antigen.

each plate to test error rates. However, no detection errors were observed.

RFLP PCR detection of polymorphisms

Because of the nature of the nucleotide sequence in the proximity of the polymorphism, it was not technically possible to design a primer for real-time PCR for the NADPH oxidase $p22^{phox}$ 242 polymorphism. Therefore, in order to detect this polymorphism, the PCR product was exposed to a restriction enzyme (*RsaI*), able to digest it. After digestion, the DNA fragments were amplified by PCR, were separated by electrophoresis gel and were then visualized under UV light. Ten nanograms of DNA were used for PCR. Each PCR reaction was performed in 25 μl volume containing 1 μl of DNA in buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 500 μM of each dNTPs (dATP, dCTP, dTTP, dGTP), 2 mM $MgCl_2$ and 50 pmol of each of the primers and 1 U of taq polymerase (ABgene, Epsom, UK). Sequences of the oligonucleotide primers used for PCR amplification, the size of the predicted PCR products and the PCR amplification programme used were described previously (Inoue et al. 1998). The PCR products were digested at 37°C for 17 h using 5 μl of *RsaI* enzyme. This enzyme cut the PCR product in the presence of allele T at position 242. If the allele T was not present, the enzyme did not cut the target DNA at that point. The digested products were separated on a 3% agarose gel containing 0.5 mg/ml ethidium bromide in one TBE buffer at 100 V for 2 h. The bands were visualized using UV transilluminator. The products yielded were: 188 + 160 bp (allele T) and 348 bp (allele C).

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

Continuous, normally distributed variables were reported as means ± standard deviation (SD). Comparisons of continuous and categorical data between groups were analysed with ANOVA and χ^2 test, respectively. The α value was set at 0.05.

Two different statistical approaches were applied to investigate the associations between genetic SNPs and AgP, in order to detect both the effect of individual SNPs and combinations of neighbouring SNPs (haplotypes). The first approach involved an association analysis between each SNP and the AgP phenotype. Further analysis was performed to explore associations between genetic haplotypes and AgP.

The first approach was performed using the SPSS 12.0 package. Because of the inherent problem of multiple testing, which can lead to spurious results (Altman 1991), the α value for statistical significance for association between genetic SNPs and AgP was lowered to 0.01. χ^2 was used as a screening tool to select possible associations. Whenever a p-value of 0.2 or lower was detected, multiple logistic regression analysis adjusting for confounders (gender, ethnicity and smoking) was performed to investigate the association between genotypes in the AgP and control groups. When statistically significant results or results approaching significance were found, analyses investigating two different types of genetic models (presence of one copy or two copies of the suspected predisposing allele) were performed. The observations which resulted in the highest associations (lowest p-values) for each SNP are reported. Further analyses were performed in the subgroups of LAgP and GAgP. Because of the known strong association between ethnicity and genetic background and the risk of finding spurious associations (Pritchard & Rosenberg 1999), separate analyses were performed in Caucasians and Blacks. Considering the recognized powerful effects of smoking as a risk factor for AgP (Haber et al. 1993), and the potential for residual confounding, separate analyses were also performed on non-smokers, smokers (past and current), Caucasian smokers and Caucasians who never smoked.

The second analytic approach consisted of analysis of linkage disequilibrium (LD) between SNPs located in the same genes, and association study between haplotypes and AgP phenotype. These aimed at exploring possible interactions between 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 LD pairs programme allowed us to investigate whether any genetic polymorphisms were in LD with each other. Pairwise linkage was tested for FcγR (IIa, IIb, IIIa, IIIb NA and IIIb SH) and FPR (301, 546 and 568). Separate analysis was performed to test for LD in the whole group of subjects, and in both patients and controls separately. R^2 and D' values were investigated in order to test the degree of recombination between pairs of markers (Stram 2004). Haplotype associations were analysed in this study by use of the WHAP package (''http://pngu.mgh.harvard.edu/~purcell/whap/'') (Sham et al. 2004). This program provides a method to test haplotype associations with qualitative and quantitative traits. WHAP analysis was performed, respectively, for FcγR (IIa, IIb, IIIa, IIIb NA, IIIb SH) and FPR (301, 546, 568) polymorphisms. Interactions between all the polymorphisms studied, as pairs, triplets, quads and quintets and as single markers were tested. 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 LAgP and 167 GAgP) patients and 231 healthy controls took part in the study. Their demographic characteristics are presented in Table 2. The controls were on average older than the patients ($p < 0.001$) and the subjects diagnosed with LAgP were on average younger than GAgP ($p < 0.001$). No statistically significant differences were detected in the two groups for gender, ethnicity and smoking. Both LAgP and GAgP showed more females than males affected, with a ratio of approximately 2:1 and this was not quite as high a ratio in the controls. There was a decrease in the Black, Asian and others categories among the controls, with a concomitant increase in Caucasians. There was an increase in the number of non-smokers within the controls, but among LAgP patients the proportions of smokers was lower ($p = 0.015$).

The distribution of the genotypes for the studied genetic polymorphisms satisfied the Hardy–Weinberg equilibrium and was consistent with the lit-

Table 2. Comparison of demographic characteristics of patients and controls

	Patients		Controls		Comparisons between groups
	($n = 224$)	%	($n = 231$)	%	
Age	29.9 ± 7.2	–	38.4 ± 12.2	–	$p < 0.001$
Gender					
Male	79	*35.3*	99	*42.9*	$p = 0.103$ Fisher's exact test
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*	

erature data (Cai et al. 1999, Gardemann et al. 1999, Lehrnbecher et al. 1999, Zhang et al. 2003, Yasuda et al. 2003, Kaneko et al. 2004).

LD

Among FcγR, significant LD was detected between FcγRIIa and FcγRIIIa ($p < 0.001$, $R^2 = 0.198$); this LD was not significant in the patient group ($p = 0.09$). Significant LD was also detected between FcγRIIb and FcγRIIIb NA ($p = 0.009$, $R^2 = 0.166$ controls; $p = 0.025$, $R^2 = 0.145$ patients), FcγRIIIa and FcγRIIIb NA ($p = 0.02$, $R^2 = 0.274$ controls; $p = 0.003$, $R^2 = 0.289$ patients) and FcγRIIIb NA and FcγRIIIb SH ($p = 0.010$, $R^2 = 0.988$ controls; $p < 0.001$, $R^2 = 0.862$ patients). Among FPR, the 301, 546 and 568 polymorphisms were all in LD with each other (only 301 and 546 were not in LD in the control group, $p = 0.09$).

Comparison between AgP and controls

The distributions of all studied polymorphisms in relation to the diagnosis (AgP/healthy controls) in all subjects are reported in Table 3. This table is further sub-divided for the two main ethnic groups of the study, Caucasians and Blacks. The results of the χ^2 test to identify differences in the distributions are also reported. Although the majority of polymorphisms show no significant differences (Table 3) the distribution of genotypes are shown to allow comparison with other studies. χ^2 analysis for all subjects showed statistically significant differences for NADPH oxidase $p22^{phox}$ C242T polymorphisms ($p = 0.003$). Increases in the carriage of the NADPH oxidase $p22^{phox}$ T allele were noted in the patient group, both through an increase in the homozygous TT and heterozygous CT genotypes. The carriage of T allele was equal to 42.2% in patients and 31.3% in controls. Among Caucasians, these percentages were equal to 38.5% and 28.9%, respectively. Among Blacks, the T allele had a prevalence of 53.7% for patients and 42.2% for controls. Trends for association with the disease trait were noted for FcγRIIIb NA ($p = 0.068$) and SH ($p = 0.107$) rare alleles, although they did not reach statistical significance. The NA1 allele had a prevalence of 40.2% in Caucasian patients and 22.4% in Caucasian controls. χ^2 analysis of allele frequencies in Caucasians revealed a p-value of 0.034 for these differences.

Logistic regression revealed that the presence of the NADPH oxidase $p22^{phox}$ T allele was significantly associated with a diagnosis of AgP, adjusting for gender, smoking and ethnicity [$p = 0.002$, odds ratio (OR) = 1.87, 95% confidence interval (CI) = 1.27–2.83, see Table 4].

A similar result was observed for NADPH oxidase $p22^{phox}$ in the subgroup of Caucasians ($p = 0.017$). Table 3 shows that this difference is due primarily to an increase in the heterozygous CT genotype, with a concomitant decrease in CC homozygous in patients. Logistic regression analysis revealed that carriage of NADPH oxidase T allele was associated with diagnosis of AgP in Caucasians, adjusting for gender and smoking ($p = 0.009$, OR = 2.07, 95% CI = 1.20–3.59, see Table 4). A further analysis of Caucasians who never smoked (consisting of 38 patients and 81 controls), showed some evidence of association between a diagnosis of AgP and presence of NADPH oxidase T allele, having adjusted for gender ($p = 0.012$, OR = 3.16, 95% CI = 1.28–7.76).

Among Black subjects, NADPH oxidase $p22^{phox}$ C242T polymorphisms showed a similar statistical difference between patients and controls ($p = 0.050$) and the logistic regression having adjusted for gender and smoking further supported this ($p = 0.032$, OR = 1.93, 95% CI = 1.06–3.52, see Table 4). However, in this case these differences are primarily due to the increase in homozygous TT genotype.

Comparison between GAgP and LAgP

The distributions of all polymorphisms were compared in patients diagnosed with GAgP and LAgP. No statistically significant differences were detected.

Comparison between GAgP and controls

χ^2 analysis revealed trends for significance for the distribution of NADPH oxidase $p22^{phox}$ C242T polymorphism ($p = 0.002$ and 0.034, respectively, in mixed populations and Caucasians) and FcγRIIIb NA polymorphism ($p = 0.019$ and 0.021, respectively, in mixed populations and Caucasians; see Table 5). For the other polymorphisms there were no significant differences between GAgP and controls and these have been omitted from the table for ease of presentation.

Logistic regression analysis revealed that presence of at least one copy of $p22^{phox}$ 242T allele was significantly associated with the disease phenotype adjusting for gender, smoking and ethnicity ($p = 0.003$, OR = 1.95, 95% CI = 1.26–3.01). Association with the GAgP trait, having adjusted for gender, smoking and ethnicity, was noted for the FcγRIIIb NA1 genotype ($p = 0.013$, OR = 1.97, 95% CI = 1.15–3.37). Subanalysis on the Caucasian GAgP group also showed some evidence of association for both NADPH oxidase $p22^{phox}$ 242T allele and FcγRIIIb NA1 homozygosity with the presence of GAgP adjusting for gender and smoking ($p = 0.029$, OR = 2.94, 95% CI = 1.63–5.30 and $p = 0.013$, OR = 2.73, 95% CI = 1.24–6.04, respectively). Logistic regression analysis was performed to investigate a possible

Table 3. Distributions of all studied polymorphisms in all subjects

Poly-morphism	Geno-type	All subjects		χ^2 p =	Caucasians		χ^2 p =	Blacks		χ^2 p =
		Patients (n = 224)	Controls (n = 231)		Patients (n = 112)	Controls (n = 144)		Patients (n = 59)	Controls (n = 45)	
FcαR	AA	18 (8.2%)	15 (6.5%)	0.564	10 (9.0%)	7 (4.9%)	0.288	2 (3.5%)	0 (0%)	0.363
	AG	86 (39.3%)	84 (36.4%)		50 (45.0%)	60 (41.7%)		14 (24.6%)	9 (20.0%)	
	GG	115 (52.5%)	132 (57.1%)		51 (45.9%)	77 (53.5%)		41 (71.9%)	36 (88.0%)	
FcγRIIa	HH	55 (25.2%)	61 (26.4%)	0.957	26 (23.4%)	37 (25.7%)	0.695	14 (25.0%)	9(20.0%)	0.407
	HR	109 (50.0%)	113 (48.9%)		52 (46.8%)	71 (43.9%)		31 (55.4%)	22 (48.9%)	
	RR	54 (24.8%)	57 (24.7%)		33 (29.7%)	36 (25.0%)		11 (19.6%)	14 (31.1%)	
FcγRIIb	CC	6 (2.7%)	9 (3.9%)	0.584	1 (0.9%)	4 (2.8%)	0.555	3 (5.3%)	2 (4.4%)	0.947
	CT	61 (27.7%)	56 (24.2%)		24 (21.4%)	31 (21.5%)		19 (33.3%)	14 (31.1%)	
	TT	153 (69.5%)	166 (71.9%)		87 (77.7%)	109 (75.7%)		35 (61.4%)	29 (64.4%)	
FcγRIIIa	VV	28 (12.8%)	27 (11.8%)	0.874	15 (13.5%)	19 (13.4%)	0.981	9 (15.8%)	2 (4.4%)	0.120
	VF	95 (43.6%)	105 (45.9%)		52 (46.8%)	65 (45.8%)		21 (36.8%)	23 (51.1%)	
	FF	95 (43.6%)	97 (42.4%)		44 (39.6%)	58 (40.8%)		27 (47.4%)	20 (44.4%)	
FcγRIIIb NA	NA2/NA2	71 (32.1%)	75 (32.5%)	0.068	42 (37.5%)	47 (32.6%)	0.060	17 (29.3%)	11 (24.4%)	0.840
	NA1/NA2	104 (47.1%)	126 (54.5%)		50 (44.6%)	83 (57.6%)		28 (48.3%)	24 (53.3%)	
	NA1/NA1	46 (20.8%)	30 (13.0%)		20 (17.9%)	14(9.7%)		13 (22.4%)	10 (22.2%)	
FcγRIIIb SH	SH+/SH+	4 (1.8%)	0 (0%)	0.107	0 (0%)	0 (0%)	0.185	4 (6.9%)	0 (0%)	0.188
	SH−/SH+	24 (10.8%)	22 (9.5%)		6 (5.4%)	3 (2.1%)		14 (24.1%)	13 (28.9%)	
	SH−/SH−	194 (87.4%)	209 (90.5%)		106 (94.6%)	141 (97.9%)		40 (69.0%)	32 (71.1%)	
FPR 301	CC	21 (9.7%)	28 (12.2%)	0.555	9 (8.2%)	18 (12.7%)	0.507	4 (7.1%)	4 (8.9%)	0.684
	CG	92 (42.4%)	101 (44.1%)		52 (47.3%)	62 (43.7%)		21 (37.5%)	20 (44.4%)	
	GG	104 (47.9%)	100 (43.7%)		49 (44.5%)	62 (43.7%)		31 (55.4%)	21 (46.7%)	
FPR 546	AA	28 (12.7%)	25 (10.8%)	0.405	12 (10.7%)	15 (10.4%)	0.500	6 (10.5%)	5 (11.1%)	0.830
	AC	100 (45.5%)	95 (41.1%)		52 (46.4%)	57 (39.6%)		21 (36.8%)	19 (42.2%)	
	CC	92 (41.8%)	111 (48.1%)		48 (42.9%)	72 (50.0%)		30 (52.6%)	21 (46.7%)	
FPR 568	AA	165 (75.0%)	181 (78.4%)	0.376	82 (73.9%)	113 (79.0%)	0.427	45 (77.6%)	41 (91.1%)	0.165
	AT	54 (24.5%)	47 (20.3%)		29 (26.1%)	29 (20.3%)		12 (20.7%)	4 (8.9%)	
	TT	1 (0.5%)	3 (1.3%)		0 (0%)	1 (0.7%)		1 (1.7%)	0 (0%)	
NADPH	CC	67 (33.7%)	112 (50.0%)	0.003	34 (34.0%)	73 (52.1%)	0.017	14 (25.9%)	15 (35.7%)	0.050
	CT	96 (48.2%)	84 (37.5%)		55 (55.0%)	53 (37.9%)		22 (40.7%)	22 (52.4%)	
	TT	36 (18.1%)	28 (12.5%)		11 (11.0%)	14 (10.0%)		18 (33.3%)	5 (11.9%)	

Genotype distributions for all studied polymorphisms are presented in patients and controls of mixed ethnicity, and in the two main ethnic groups of the study, Caucasians and Blacks, with relative χ^2.
NA, neutrophil antigen.

Table 4. Results of logistic regression analysis on all subjects, Caucasian and Black subjects for presence of T allele of NADPH p22phox 242 SNP

Logistic regression analyses for the presence of NADPH oxidase $p22^{phox}$ 242 T allele (total AgP *versus* controls)				
		$p =$	OR	95% CI
All patients	Unadjusted	0.001	1.97	1.33–2.92
	Bivariate (adjusted for gender)	0.001	1.96	1.32–2.90
	Bivariate (adjusted for smoking)	0.001	1.95	1.32–2.90
	Bivariate (adjusted for ethnicity)	0.001	1.93	1.29–2.88
	Multivariate (adjusted for gender, smoking and ethnicity)	0.002	1.87	1.27–2.83
Caucasians	Unadjusted	0.006	2.11	1.24–3.59
	Bivariate (adjusted for gender)	0.009	2.05	1.12–3.51
	Bivariate (adjusted for smoking)	0.007	2.12	1.23–3.64
	Multivariate (adjusted for gender and smoking)	0.009	2.07	1.20–3.59
Blacks	Unadjusted	0.041	1.82	1.02–3.24
	Bivariate (adjusted for gender)	0.027	1.96	1.08–3.55
	Bivariate (adjusted for smoking)	0.049	1.79	1.00–3.20
	Multivariate (adjusted for gender and smoking)	0.032	1.93	1.06–3.52

Unadjusted values, bivariate and multivariate values adjusted for confounders are presented.
AgP, aggressive periodontitis.

Table 5. Distributions of FcγIIIb NA and NADPH $p22^{phox}$ polymorphisms in GAgP and controls with relative χ^2 results

Polymorphism	Genotype	All subjects		χ^2 $p =$	Caucasians		χ^2 $p =$
		GAgP ($n = 167$)	Controls ($n = 231$)		GAgP ($n = 88$)	Controls ($n = 144$)	
FcγRIIIbNA	NA2/NA2	51 (30.9%)	75 (32.5%)	0.019	31 (35.2%)	47 (32.6%)	0.021
	NA1/NA2	75 (45.5%)	126 (54.5%)		38 (43.2%)	83 (57.6%)	
	NA1/NA1	39 (23.6%)	30 (13.0%)		19 (21.6%)	14 (9.7%)	
NADPH	CC	50 (32.3%)	112 (50.0%)	0.002	28 (34.1%)	73 (52.1%)	0.034
	CT	74 (47.7%)	84 (37.5%)		43 (52.4%)	53 (37.9%)	
	TT	31 (20.0%)	28 (12.5%)		11 (13.4%)	14 (10.0%)	

Genotype distributions for all studied polymorphisms are presented in GAgP patients and controls of mixed ethnicity (all subjects) and in the Caucasians only.
GAgP, generalized aggressive periodontitis.

Table 6. Results of logistic regression analysis on Caucasian subjects for presence of T allele of NADPH p22phox 242 SNP and homozygosity for FcγRIIIb NA1

Logistic regression analyses for interaction between of NADPH oxidase $p22^{phox}$ 242 T allele and FCγRIIIB NA1 homozygosity (Caucasian GAgP *versus* Caucasian controls)				
		$p =$	OR	95% CI
Caucasians	Unadjusted	0.001	28.69	3.69-222.16
	Bivariate (adjusted for gender)	0.001	28.24	3.62-220.61
	Bivariate (adjusted for smoking)	0.001	32.14	4.02-256.88
	Multivariate (adjusted for gender and smoking)	0.001	30.35	3.81-241.97

Unadjusted values, bivariate and multivariate values adjusted for confounders are presented.
GAgP, generalized aggressive periodontitis; OR, odds ratio; CI, confidence interval.

interaction between these two polymorphisms (NADPH oxidase $p22^{phox}$ C242T and FcγRIIIb NA). In the Caucasian GAgP group, 19 patients and 14 controls were NA1 homozygous and, out of these subjects, 14 patients (74% of this subgroup of patients) and just one control (7% of this subgroup of controls) had at least one copy of the NADPH oxidase T allele. Logistic regression analysis of the interaction between these two genotypes adjusting for gender and smoking gave a relative risk of 34 for individuals with both the risk genotypes of having GAgP ($p = 0.001$, OR = 30.35, 95% CI = 3.81–241.97, Table 6).

Comparison between LAgP and controls

No polymorphism showed statistically significant different distributions between the LAgP group and the healthy controls (data not reported).

Haplotype analyses

No FPR and FcγR haplotypes displayed statistically significant associations with the AgP phenotype in Caucasians as analysed by WHAP (data not presented). In Black subjects, haplotype association between all five studied FcγR polymorphisms showed some evidence of association with AgP ($p = 0.047$ adjusting for smoking). The best haplotypic model was obtained in a constrained model with the exclusion of FcγRIIIb SH polymorphism ($p = 0.034$ adjusting for smoking). The highest LRT statistics for this haplotype association was obtained for the haplotype including FcγIIa H allele, FcγRIIb T allele, FcγRIIIa V allele and FcγRIIIb NA2 allele, which had an estimated frequency of 0.109 (11%) in patients and 0.0 in controls. FcγRIIa, FcγRIIIa and FcγRIIIb NA haplotypic combination also showed some evidence of association with AgP ($p = 0.049$ adjusting for smoking). The highest LRT statistics for these genotypes was obtained for the haplotype including FcγRIIa H allele, FcγRIIIa V allele and FcγRIIIb NA2 allele, which had an estimated frequency of 0.12 (12%) in patients and 0.0 in controls.

Discussion

By comparing a sample of 224 patients with AgP and 231 controls with healthy periodontium, we identified two polymorphisms associated with functionality of PMNs which showed association with the AgP trait: the NADPH oxidase $p22^{phox}$ C242T (CYBA gene) and the Fcγ-R IIIb NA polymorphisms.

Nadph Oxidase $p22^{phox}$ Polymorphism

The $p22^{phox}$ is one of the subunits of the NADPH of neutrophils, indispensable

for its function of catalyzing the reduction of molecular oxygen at the expense of NADPH (Ushio-Fukai et al. 1996). The resulting electrons are moved and delivered to produce O_2^- both in the phagosome and in the extracellular environment (Dahlgren & Karlsson 1999). This stimulates a cascade leading to the production of reactive oxygen species (ROS). ROS have been shown to be biologically active and involved in bacterial killing by means of activation of proteases (Reeves et al. 2002, Rada et al. 2004) and production of a cascade of anti-microbial reactive oxygen metabolites, such as hydrogen peroxide, hypoclorous acid and chloramines (Dahlgren & Karlsson 1999, Van Dyke & Serhan 2003, Rada et al. 2004) both in the intracellular and extracellular milieu. The superoxide produced in this fashion is crucial to trigger the process leading to bacterial killing, as well as to potential tissue damage (Henson & Johnston 1987). Excessive oxidative burst has been implicated in the pathogenesis of several diseases (Kantarci et al. 2003, Lee et al. 2003, Abdelrahman et al. 2005) and its potential tissue damaging effect to human periodontal ligament cells and gingival epithelial cells has been shown in vitro (Deguchi et al. 1990, Altman et al. 1992).

This is the first study, to the best of our knowledge, to investigate the relation of this C → T substitution in the gene encoding for the $p22^{phox}$ subunit of neutrophil NADPH to periodontitis. The predisposition linked with this polymorphism in our sample was seen in the whole AgP group, but especially in GAgP, and was consistently found in the Caucasian and Black subpopulations, independently from other risk factors such as smoking. The T allele was over represented in the AgP group overall, but it was interesting to note the racial differences where the increase in T allele is mainly due to an increase in CT in the Caucasians, and an increase in TT in Black subjects; this warrants further investigation. Conflicting results have been reported about possible associations between this polymorphism and the risk of developing cardiovascular disease, intracranial aneurysm and diabetes (Inoue et al. 1998, Cai et al. 1999, Gardemann et al. 1999, Cahilly et al. 2000). The C → T mutation causes an amino acid substitution at position 72 (histidine to tyrosine), involving a potential haeme-binding site, which can have as a consequence a modulation of superoxide production. Functional analyses of the effect of this polymorphism are very limited and show conflicting results (Shimo-Nakanishi et al. 2004, Wyche et al. 2004).

An increase in superoxide production has been reported in PMN isolated from AgP patients, especially with regard to LAgP (Leino et al. 1994, Gronert et al. 2004). The current concept is that PMN from LAgP patients are constitutionally hyperreactive, and this may account for the amount and rapidity of tissue destruction seen in this particular form of periodontitis (Kantarci et al. 2003). This finding has also been extended to chronic periodontitis (Fredriksson et al. 2003). The fact that no differences have been found in receptor numbers, testifies for a possible alteration in post-receptor signalling pathway (Leino et al. 1994, Fredriksson et al. 2003). Further element for speculation about the possible role of NADPH oxidase in periodontitis is given by a study on gene expression signatures in chronic and AgP (Papapanou et al. 2004). This study showed upregulated expression in periodontitis patients of a cluster of genes including NAD, involved in its biosynthesis, and activity.

In this context, we have to view the finding of this study as part of a possible explanation for the altered PMN response characteristic of AgP. The T mutation of the gene encoding for the $p22^{phox}$ subunit of NADPH of PMNs is one of the possible mechanisms which lead to the altered PMN function in AgP.

FcγR polymorphisms

Some evidence for association with the AgP phenotype emerged for the FcγRIIIb neutrophil antigen (NA), which is present in the membrane-distal IgG-like domain of the FcγRIIIb receptors. A substitution of four amino acids in the gene coding for this receptor causes differences in receptor glycosylation, which result in different IgG-binding properties (van der Pol & van der Winkel 1998, van Sorge et al. 2003). In our sample, the NA1/NA1 allotype was found to be increased in prevalence in the GAgP group (23.6% in patients compared with 13% in controls, see Table 5). The result approached the chosen threshold for statistical significance only when GAgP patients were compared with controls, and this was also true in the subgroup of GAgP Caucasian patients. In the Black subgroup, we actually observed an increased prevalence of NA2 homozygosity in patients compared with controls (see Table 3).

Another polymorphism of the FcγRIIIb gene, which is in LD with the FcγRIIIb NA, is the FcγRIIIb SH. All SH positive subjects are also NA2 positive, while SH homozygosity is very rare. This SNP showed a trend for association with AgP which in previous studies may have masked or diluted the results relative to the NA2 polymorphism (Bux et al. 1997).

The results observed in this study in relation to the NA1 allotype are in keeping with the neutrophil ''hyperactivation theory''. In particular, a possible explanation for the pathogenic role of FcγRIIIb polymorphisms in AgP lies in one of the functions transduced by these receptors on PMN: the superoxide production. The supposed higher receptor affinity of the NA1 allotype could translate to an increase in superoxide production in these patients. NA1 positive individuals have been found in increased percentages compared with NA2 subjects in studies on several autoimmune diseases, such as Wegener's granulomatosis, myasthenia gravis and thrombocytopenic purpura (van Sorge et al. 2003).

The NA polymorphism has been associated with periodontitis in a few studies mainly in Japanese and Afro-American populations (Kobayashi et al. 2000b, Fu et al. 2002). These studies show an increase, in patients with periodontitis, of the prevalence of the NA2 allotype, shown to be less efficient in phagocytosis and oxidative burst upon interaction with IgG1- and IgG3-opsonized P. gingivalis (Kobayashi et al. 2000a). However, other studies showed no association (Loos et al. 2005). Thus the release of superoxide has to be considered as a possible mechanism of AgP susceptibility mediated by the FcγRIIIb polymorphisms. The difference between our results and those previously reported by other investigators may be due to the different ethnicities of the population studied or indeed in some cases to the different diseases (AgP *versus* CP).

Further analysis was performed in order to study their possible combined effect in determining disease susceptibility between FcγRIIa H/R, FcγRIIb C/T, FcγRIIIa V/F, FcγRIIIb NA and FcγRIIIb SH polymorphisms, all located in the long arm of chromosome 1.

A haplotype determined by a combination of these polymorphisms showed limited evidence of association with AgP in Black patients. This stimulates speculation about the possible combined effect of these haplotypes or the presence of another functional polymorphism somewhere else in the gene, in LD with the polymorphisms presented here. Further functional studies are needed to investigate the role of FcγR haplotypes in relation to the handling of periodontopathogenic bacteria.

Overall conclusions

The overall results of this study point towards the importance of genetic polymorphisms regulating the immune response to periodontopathogenic bacteria in the onset of AgP. In particular, genotypes which are believed to indicate an increased activity of neutrophils (NADPH oxidase $p22^{phox}$ T allele and FcγRIIIb NA1 allele) were enriched in AgP patients compared with controls. Both the T allele of NADPH oxidase $p22^{phox}$ 242 polymorphism (CYBA gene) and the NA1 allele of FcγRIIIb polymorphism, which were enriched in patients, have been linked with increased release of superoxide from neutrophils. This supports the hypothesis that it is not the bacteria themselves, but the host reactions triggered by bacteria that causes the classical tissue damages of AgP (Gemmell et al. 2002). Therefore, consistent with the hypotheses postulated by Van Dyke & Serhan (2003), it seems that subjects whose neutrophils are hyperactive are more prone to develop AgP. The data presented here suggest that this host hyperactivity may have genetic basis. If through the presence of local factors, virulent and leukotoxic bacterial strains, or other underlying genetic factors, the resolution of the infection is not achieved, the constant presence of pathogenic bacteria will induce the establishment of a chronic inflammatory process. As a result, PMN of these subjects will be continuously called into the inflamed area to exert their defensive functions such as phagocytosis and oxidative burst. The consequent tissue-damaging cascade may be augmented in PMN carriers of the $p22^{phox}$ T allele. This excessive superoxide production might be further enhanced in subjects with the NA1 allotype for FcγRIIIb, who express a glycosylation pattern in their receptors which increases the ability to recognize and phagocytose bacteria, and activate the superoxide cascade (van Sorge et al. 2003). Therefore, in these individuals the risk of having rapid and considerable degree of irreversible periodontal destruction is increased. Among the Caucasian subjects included in this study, having at least one copy of the $p22^{phox}$ T allele gave an odds ratio of about 2 to have GAgP; similarly, the risk of having GAgP for NA1 homozygous individuals was equal to 2. The relative risk for subjects with both these risk genotypes to have GAgP was equal to 30, which suggests an additive effect due to a common mechanism of action. It is worthy to mention that the $p22^{phox}$ is a fundamental component of the NADPH oxidase in osteoclasts as well, and it has been shown to be important in bone resorption (Yang et al. 2004). Hence, together with the increased PMN activity, osteoclasts have the potential to cause more tissue damage in T carriers, and this mechanism might be responsible for the continuation of the tissue-damaging effect and lead to a more rapid and more severe bone loss in subjects with this polymorphism. These findings open interesting prospects for the future, in terms of possible specific interventions in order to modulate superoxide production in AgP patients, without compromising the protective function of PMNs (Fossati et al. 2002).

Functional studies and studies with larger sample sizes are needed to confirm the importance of the observed polymorphisms.

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Clinical Relevance

Scientific rationale for study: Genetic factors affecting neutrophil function are suspected to play an important role in the periodontal tissue damage characteristic of AgP.

Principal findings: Genetic polymorphisms in the genes coding for the NADPH oxidase and FcγR were risk factors for AgP in our sample. The risk is possibly due to an increased oxidative burst, found to be associated with NADPH oxidase polymorphism.

Practical implications: The knowledge of genetic factors predisposing to AgP opens new perspective for disease prevention and genetic therapy of such cases.