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Analysis of genetic polymorphisms as risk factors for Aggressive Periodontitis

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ABSTRACT

This PhD consisted of a series of studies aiming at detecting genetic risk factors for Aggressive Periodontitis (AgP). AgP is a destructive disease of the periodontium affecting around 1% of the population and leading to early tooth loss. Microbiological and environmental factors are thought to act on a genetically susceptible host to determine AgP.

We conducted a case- control association study on 224 AgP patients (both Generalised AgP and Localised AgP) and 231 healthy controls to detect differences in genotype distributions of 13 single nucleotide polymorphisms (SNPs). The selected SNPs included FcR and FPR, NADPH oxidase, IL-6, TNF-α and VDR polymorphisms. Further studies on subsets of patients were conducted to detect associations between these SNPs and classical features of AgP: disease severity, familial aggregation, presence of periodontopathogenic bacteria and neutrophil hyperactivity.

The NADPH $p22^{phox}$ 242 polymorphism was associated with the AgP trait and with disease severity. The IL-6 -174 SNP was associated with LAgP and with increased detection of periodontopathogenic bacteria. The FcγRIIIb NA polymorphism was associated with GAgP, while FcγR haplotypes were linked with AgP in Blacks and FcγRIIa was associated with familial aggregation of the AgP phenotype. The VDR Taq-I polymorphism showed a trend for association with AgP in smokers.

The overall results of the study provide two possible pathogenic pathways leading to AgP: one is mediated through an excessive inflammatory response triggered by the presence of specific bacteria in individuals with hyper-responsive genotypes (NADPH p22phox 242 T allele, FcγRIIIb NA1 homozygosity, IL-6 -174 G homozygosity); the second is initiated by an increased susceptibility to bacterial colonization (FcγRIIa R homozygosity).

In conclusion, this study supports the importance of genetic factors in Aggressive Periodontitis and hypothesizes possible pathogenic mechanisms.

CHAPTER 1

1. INTRODUCTION

1.1. Definition of Aggressive Periodontitis

In 1971 Baer (1971) described a form of periodontal disease affecting young individuals. At the time this pathological entity was called 'Periodontosis', and was not considered an inflammatory, but a degenerative process of the periodontium. In his paper Baer defines the criteria for the classification of Periodontosis as:

- occurring in healthy individuals
- characterized by rapid loss of alveolar bone affecting more than one tooth of the permanent dentition
- amount of destruction not commensurate with amount of local irritants present.

Baer also reports the strong familial association of these cases and suggests the possible importance of a genetic predisposition.

More than 30 years on, while the concept of periodontitis as a non-inflammatory process seems now prehistoric, it is necessary to credit Baer with having identified and described the main characteristics of this clinical entity which has subsequently been called 'Early onset Periodontitis' and which we now call 'Aggressive Periodontitis'.

The term 'Aggressive Periodontitis' (AgP) was introduced in the classification by the 1999 Consensus Report on Classification of Periodontal Diseases by the American Academy of Periodontology (Armitage 1999). The group discussing the classification concluded that AgP is a specific type of periodontitis with clearly identifiable clinical and laboratory findings which make it sufficiently different from Chronic Periodontitis (CP), which is the most common form of periodontal diseases. The consensus report also distinguishes common and secondary features that render AgP a distinct entity.

The common features of AgP are:

- Except for the presence of periodontitis, patients are otherwise clinically healthy
- Rapid attachment loss and bone destruction
- Familial aggregation

The secondary features are:

- Amounts of microbial deposits are inconsistent with the severity of periodontal tissue destruction
- Elevated proportions of *Actinobacillus actinomycetemcomitans* and, in some populations, *Porphyromonas gingivalis*
- Phagocyte abnormalities
- Hyper-responsive macrophage phenotype, including elevated levels of PGE2 and IL-1β
- Progression of attachment loss and bone loss may be self-arresting

Not all these features, however, must be present to assign a diagnosis of AgP. The emphasis of this new classification is on the fact that it is not considered correct to use the term 'Early Onset' because it is too restrictive, since not all cases of this form of disease occur in very young individuals.

Curiously, despite the substantial understanding of AgP as an inflammatory process, the hinges of our classification are still those described by Baer: health, rapid progression, familial aggregation and a small amount of supra- and sub-gingival deposits. However, it has to be admitted that the new classification is not universally accepted, and in some cases the differences between AgP and CP are not so clear-cut, and leave scope for individual interpretations. This is due to the difficulty of establishing the rate of progression of the disease (hardly possible without having previous clinical records of the patient), of establishing the familial aggregation (which makes examining relatives necessary), and to the fact that the secondary characteristics are not always present nor are they easily detected.

1.1.1. SUBCLASSIFICATION

AgP cases are sub classified into Localised (LAgP) and Generalised (GAgP). The first includes patients with a molar/incisor pattern of bone and attachment loss, previously classified as Localised Juvenile Periodontitis (LJP) or Localised Early Onset Periodontitis (LEOP). GAgP roughly merges the old terms of Generalised Juvenile Periodontitis (GJP), Generalised Early Onset Periodontitis (GEOP), post-juvenile Periodontitis and rapidly progressive Periodontitis (RPP). Throughout this thesis, we will preferably refer to LAgP and GAgP even with regards to old studies. It is also worthy of mention that most of the literature in this field relates to LAgP, rather than the generalised form.

In more detail, the characteristics of LAgP and GAgP following the current classification are as follows:

- **LAgP**

- circumpubertal onset
- robust serum antibody response to infecting agents
- localized first molar/incisor presentation with interproximal attachment loss on at least two permanent teeth, one of which is a first molar, and involving no more than two teeth other than first molars and incisors

- **GAgP**

- usually affecting persons under 30 years of age
- poor serum antibody response to infecting agents
- pronounced episodic nature of the destruction of attachment and alveolar bone
- generalized interproximal attachment loss affecting at least three permanent teeth other than first molars and incisors

This thesis focuses on the genetic aspects of AgP, but in order to understand the importance of genetics, it is necessary to understand the evidence of the role that the other factors play in its onset and development.

1.2. Epidemiology

A few epidemiologic studies have been conducted throughout the world, trying to establish the incidence rate of AgP. Drawing conclusions from these surveys is not very easy because of several factors:

1. Classification bias: the classifications of disease used by the different studies are very heterogeneous (Baer's criteria, Early Onset, AgP, or even non standard individual devised classifications are used)
2. Selection bias: due to the lack of random selection of samples in some surveys
3. Screening bias: different methods of screening are used and some can lead to underestimation of the disease
4. Age criteria: most studies focused on adolescents (15-16 years old), which leaves us very little understanding of the disease prevalence in other age groups
5. Population differences: very variable rates are described in developed vs. undeveloped countries and in populations of different ethnic backgrounds
6. Lack of power: most of the studies are based on a very small number of subjects.

The biggest published survey is from Löe & Brown (1991) on 11007 adolescents in the United States of America, and reports a total prevalence of about 0.66%, with 0.17% of Caucasians and 2.64% of Blacks affected. The overall prevalence of LAgP and GAgP was 0.53% and 0.13% respectively. Saxby (1987), in an UK survey, found a prevalence of 0.1% in the age range from 15 to 19 years old. The prevalence was higher for Blacks (0.8%) and Asians (0.2%) than for Caucasians (0.01%). Higher prevalence is reported in studies conducted in developing countries (Albandar et al. 2002, Albandar & Tinoco 2002).

Whilst we should be aware of the limitations of past studies, we can conclude from these that AgP seems to affect about 0.1% of Caucasian, and 1-2.5% of Black adolescents.

The reported sex ratios vary greatly between different studies and different populations, but the supposed higher prevalence in females does not seem to be proven (Hart et al. 1991). As emphasised above, we can only guess that AgP prevalence in young adults (in their twenties or early thirties) is somehow higher than these figures.

1.3. Pathogenesis

Periodontal disease is an infectious disease, with a multifactorial aetiopathogenesis. It affects the surrounding and supporting apparatus of the teeth, including periodontal ligament and alveolar bone. The destruction of the periodontal structures leaves the teeth with reduced support, causing mobility and eventually tooth exfoliation. Understanding the reason why this happens has always represented a challenge for any clinician who comes across such cases, especially when this occurs, as in the case of AgP, during adolescence or early adulthood (Tonetti & Mombelli 1999).

The complex interaction of a variety of microbial, environmental, behavioural, and systemic factors with innate and acquired host factors determines the outcome of periodontal destruction. In healthy conditions, a balance exists between oral bacteria, host response and local factors. If this balance is somehow disturbed and shifted by one of these factors or by a combination of them, the net result is severe destruction of the supporting apparatus of the teeth. The attention of periodontal research is therefore directed towards the study of highly virulent periodontal pathogens, interfering local and environmental factors and, of course, host response (Kornman et al. 1997a).

From an aetiopathogenetic standpoint, AgP has been distinguished from CP because of the supposed different importance that the various factors play in their onset and development. In fact, CP is believed to be due mainly to the cumulative effect of local and environmental factors, such as defective plaque control, smoking, in combination with pathogenic bacteria and a degree of genetic susceptibility. On the other hand, in AgP the effect of genetic and probably microbiological factors, seems to be prevalent, and this causes a very early onset and a rapid progression of disease in the absence of clear local or environmental factors (Tonetti & Mombelli 1999).

1.3.1. MICROBIOLOGICAL FACTORS

Periodontal disease is an infectious disease, so it is naturally important to underline the importance of microbiological factors. Going back to the 1999 classification, the presence of "elevated proportions of *Actinobacillus actinomycetemcomitans* (*A.a.*) and, in some populations, of *Porphyromonas gingivalis* (*P.g.*)", is mentioned within the secondary features which can help distinguish AgP from CP cases (Armitage 1999).

The first studies looking at the microbiological factors in AgP are from Newman et al. (1976) and Listgarten (1976). The lack of grossly noticeable tooth surface deposits in AgP cases was widely recognized at the time. Electron microscopy studies of plaque samples collected from early-onset cases revealed the presence of a cuticle covering the root surfaces, composed of "globules of amorphous material, forming an irregular-shaped covering containing small clumps of Gram-negative coccoid bacteria and polymorphonuclear leukocytes (PMN) in varying stages of disintegration" (Listgarten 1976). Fewer morphologic forms were detected as compared to CP samples, and Newman at el. (1976) distinguished 5 groups of Gram-negative anaerobic rods associated with early-onset lesions. Many studies based on new techniques which allow detection and counts of periodontal pathogens collected from periodontal pockets have followed. A metanalysis of all the different studies is very difficult, because of the heterogeneity of classification systems and the methodologies used (PCR, cultures, DNA probes, immunofluorescence, antibodies).

Mombelli et al. (2002) recently studied the evidence produced by numerous bacterial association studies for AgP and CP. They concluded that the presence or absence of known or supposed periodontal pathogens could not discriminate between subjects with AgP from those with CP. A trend of association was present between AgP and certain periodontal bacteria, such as *Actinobacillus actinomycetemcomitans* (*A.a.*), *Porphyromonas gingivalis* (*P.g.*), *Tannerella forsythensis* (*T.f.*), *Prevotella intermedia* (*P.i.*), *Treponema denticola* (*T.d.*), *Campylobacter rectus* (*C.r.*), *Fusobacterium*

nucleatum (*F.n.*). The first three were designated as periodontal pathogens in the World Workshop in Periodontology in 1996 (Consensus Report 1996) and deserve more detailed discussion in this context.

1.3.1.1. Actinobacillus actinomycetemcomitans (A.a.)

A.actinomycetemcomitans is a small, non motile, Gram-negative, saccharolytic, capnophilic, round- ended rod. It is considered particularly important in LAgP (Socransky & Haffajee 1992), where its detection in different studies varies form 20-25% up to 90%, which was consistently more than the percentage of detection in GAgP and CP lesions. It has been demonstrated that most localized early-onset cases produce antibodies locally and have elevated serum antibodies against *A. actinomycetemcomitans* (Listgarten et al. 1981, Tew et al. 1985). Among its virulence factors, leukotoxins, collagenases and endotoxins have been described as heavily involved in periodontal disease pathogenesis (Haffajee & Socransky 1994). *A. actinomycetemcomitans* has also been shown to stimulate cytokine production and neutrophil response (Belibasakis et al. 2005). Furthermore, its elimination from infected periodontal pockets has been associated with a good response to treatment (Kornman & Robertson 1985). However, *A. actinomycetemcomitans* has also been detected in relatively high prevalence in CP and healthy subjects (Mcnabb et al. 1992), while cases of AgP, even localized, where *A. actinomycetemcomitans* was not detected, are often reported (Ishikawa et al. 2002). Contrasting results come from serotype studies. A highly leukotoxic variant (JP2) has been implicated in a small percentage of subjects with AgP and especially in some ethnic subgroups (Haubek et al. 2004). There also seems to be a relation between age and presence of *A. actinomycetemcomitans*, the highly leukotoxic variant being detected mainly at a very early age with carriage at a later age restricted to less virulent serotypes (Haraszthy et al. 2000).

1.3.1.2. Porphyromonas gingivalis (P.g.)

P.gingivalis is a Gram-negative, anaerobic, non-motile, asaccharolytic rod. It belongs to the family of the black- pigmented *Bacteroides* and is one of the most prevalent bacteria in AgP lesions, detected in a range of about 60-90% of generalized cases, and consistently less in LAgP and CP cases (Mombelli et al. 2002). *Porphyromonas gingivalis* produces virulence factors including proteases, collagenases, endotoxins and is able to determine a strong immune response in the host both locally and systemically (Haffajee & Socransky 1994). Moreover, *P.gingivalis* seems to have the ability to affect and influence the phagocytic response in humans (Wilton et al. 1992) and it has been shown to be capable of invading the host's epithelial cells *in vitro* (Papapanou et al. 1994). In addition, clinical studies have observed a persistence of *P.gingivalis* in periodontal lesions which did not respond well to treatment (Van Winkelhoff et al. 1988).

1.3.1.3. Tannerella forsythensis (T.f.)

Previously known as *Bacteroides forsythus*, *T. forsythensis* is a Gram-negative, anaerobic rod, detected in about 70-100% of AgP cases, both in Generalised and in Localised cases (Mullally et al. 2000, Mombelli et al. 2002). *T. forsythensis* was shown to be detected more frequently in 'active' periodontal lesions (Dzink et al. 1988) and in sites showing breakdown after periodontal therapy (Shiloah et al. 1998). Individuals affected by refractory forms of periodontitis have been shown to have elevated detection rates and elevated antibodies against *T. forsythensis* (Taubman et al. 1992).

Summarizing, we have evidence of an increased prevalence of certain bacteria in AgP cases when compared with healthy subjects. However, we don't know how much of the difference can be due to the fact that some of the suspected pathogens may be a consequence rather than a cause of the disease. This difference is less marked when we compare AgP to CP, and not enough to distinguish between these two forms of periodontitis (Mombelli et al. 2002). Further studies on the presence of toxic strains of *A.actinomycetemcomitans* and *P.gingivalis* may give increased evidence for their pathogenic effects.

We can hence conclude that, with our present knowledge, microbiological factors alone are not enough to explain the difference between AgP and CP and indeed between health and disease.

1.3.2. GENETIC FACTORS

In the last 10-15 years the focus of research in the field of periodontitis has shifted more towards a genetic approach in an attempt to identify individual risk factors for the onset and development of periodontal diseases. This section will discuss the evidence of the importance of genetics for AgP and explore the ways genetic markers can be identified and confirmed as risk factors.

1.3.2.1. Identification of genetic markers for disease

There are up to 50,000 different genes in the human genome (Parra et al. 2003). Genes can be polymorphic at several sites, meaning that they can exist in different forms (alleles). When an allele is expressed in at least 1% of the population it is conventionally called a polymorphism. If, on the other hand, the frequency is lower, it is called a mutation. Some mutations or polymorphisms are 'silent' or 'synonymous', which means the variation in the allele does not change the coded amino acid. Others are functional, and they change the coded amino acid and subsequently the resulting protein (Collins et al. 1999). If the affected protein is involved in a biological process, that mutation or polymorphism can therefore change the predisposition to a specific phenotype or disease through the effect of the coded protein. In addition to this, other polymorphisms located in the promoter region of the gene exert a functional effect by changing the transcriptional levels of the gene itself, therefore increasing the numbers of the coded protein.

The power of these effects can either be deterministic or just predisposing to a disease. In this latter case, it is the interaction between the genetic predisposition and other environmental factors which may cause the onset of the disease.

There are just few examples of diseases caused by either a genetic or by an environmental factor alone (haematophilia A, for example, is caused by a mutation in a gene coding for a coagulation factor). For many diseases, such as for example cancer or cardiovascular disease, the human population show different susceptibilities, determined by the combination of both genetic and environmental components (Kinane et al. 2005).

Recent studies on periodontitis suggest a cumulative high-susceptibility profile model, given by the combination of the effects resulting from a variety of high- susceptibility genotypes. The contribution of an allelic variant can either be deterministic or subtle, with only a modifying effect on aetiology (Kinane & Hart, 2003). Increasing evidence suggests that periodontitis may be a complex genetic disease, meaning that its traits are the result of the interaction of multiple different loci. The genetic variants etiologically important are not rare mutations, but common polymorphisms, which occur in unaffected as well as affected individuals (Kinane & Hart, 2003). A combination of polymorphisms or their association with environmental factors can give rise to an increased susceptibility to periodontitis.

Several approaches are available in order to detect genes responsible for disease susceptibility:

1. Familial aggregation. The study of the relatives of the affected patient can show a genetic predisposition to the disease. However, environmental factors will still contribute to disease susceptibility.
2. Twin studies. Especially studying monozygous twins reared apart, enormous insight into the relevance of genetic factors to disease predisposition can be gained.
3. Segregation analyses. Allows studying the pattern of transmission of a disease, by means of examining relatives of the affected patients.
4. Linkage analyses. It is a complex genetic analysis which aims at identifying a specific chromosomal location where the suspected gene responsible for the disease predisposition is situated.

5. Association studies. The aim of this kind of study is to identify possible predisposing genotypes, by a case-control design. Genetic polymorphism analysis is performed in a group of patients and in a group of healthy subjects for specific suspected genes and the distributions of the studied genotypes are compared between the two groups (Kinane & Hart 2003).

1.3.2.2. Evidence for genetic factors in Aggressive Periodontitis

Back in 1971 Baer suspected a possible role for genetic factors in the predisposition to early onset periodontitis. Several reports have followed, showing increased rates of diagnosis of periodontitis in families with at least one individual affected by AgP (Butler et al. 1969, Melnick et al. 1976, Spektor et al. 1985, Stabholz et al. 1998).

Michalowicz et al. (1991, 2000) studied a group of monozygous and dizygous twins, both reared apart and reared together. They concluded, trying to adjust for environmental factors, that approximately half of the variance in periodontitis in the population is attributable to genetic factors.

Different theories have been hypothesized about the transmission of the AgP trait. A pattern of transmission consistent with Mendelian inheritance of a gene of major effect has been suggested (Beaty et al. 1987, Marazita et al. 1994). Segregation analyses have shown conflicting results, with some studies, mainly in Black African populations, suggesting a possible autosomal dominant mode of inheritance (Marazita et al. 1994), and other studies suggesting a recessive mode of inheritance (Boughman et al. 1988). Even reports of an X-linked transmission exist (Melnick et al. 1976). The biggest study so far available in the literature was performed in the United States (Marazita et al. 1994) on 104 affected individuals and their families. They performed a segregation analysis on these subjects and proposed a dominant mode of inheritance with 70% of penetrance. Thus there is a lack of agreement regarding the transmission of periodontal diseases and there may be differences between the results seen in a black North American populations and white Caucasian populations. These differences may be due to the fact that periodontitis in general and AgP in particular, are probably

heterogeneous pathogenically and genetically, and therefore there may be different defects and different genes predisposing to AgP in different families. These genes may be of major effect or just predisposing, and this may vary depending on the cases. Therefore, AgP may be transmitted in different ways in each family, and different loci may be capable of causing the disease in both dominant and recessive manners.

A few studies have reported linkage analyses on AgP families. Boughman et al. (1987) suspected linkage to chromosome 4 in a large family where LAgP and dentinogenesis imperfecta were segregating as dominant traits. Wang et al. (1996) suggested two chromosome loci, respectively located on chromosome 6 and 9, to be of interest for AgP, while Li et al. (2004) identified by linkage analysis of 4 LAgP families, an AgP locus on chromosome 1q25.

1.3.2.2.1 Association studies

This approach is the best to study modifying genes, which exert a limited effect on disease predisposition and may not be detected in linkage analyses. Therefore, association mapping is the method of choice to study complex- trait loci (Risch & Merikangas 1996). It also allows for study of the interaction between different genetic markers and environmental factors.

In association studies, the distribution of a suspected predisposing allele or genotype is studied in a group of diseased and a group of healthy subjects (Pritchard & Rosenberg 1999). If differences in the allelic or genotypic distribution emerge, there are at least four possible explanations: i) the allele is predisposing to the disease; ii) the allele is in linkage-disequilibrium with the real predisposing allele; iii) the association is due to population stratification, iv) the association is due to a statistical artefact. In order to reduce the risk of falling into one of the last three categories, an accurate choice of the control group has to be made, and functional studies of the suspected genotype should be performed (Hodge 1994).

Several aspects have to be considered in relation to genetic association studies for periodontitis:

1. Selection of controls
    - Definition of healthy status. Periodontitis is a relatively common human disease and usually affects people late in life. Therefore, association studies on Chronic Periodontitis should not include patients younger than at least 40- 50 years of age. Without doing so, the risk of including individuals susceptible to develop CP later in life may dilute or mask any possible association. With regards to AgP, patients are usually affected in their teens, twenties or early thirties. Hence, the age limit for controls can be significantly lowered. The definition of periodontal health is also particularly difficult to set, and it may vary with age and in relation to local factors.
    - Stratification bias. Controls, as well as patients, should represent the 'normal population' as much as possible and they should not be recruited from a restricted subgroup of subjects. Moreover, confounders such as ethnic origin, known environmental and socioeconomic factors have to be controlled in the two groups of subjects (cases and controls). This is due to the powerful effect of factors such as smoking and ethnicity in the predisposition to periodontitis. Ethnic factors, which have strong associations with genetic SNPs, have to be controlled for in order to avoid stratification bias. Otherwise, spurious associations may emerge due to linkage of a SNP with a difference in ethnicity rather than with a difference in disease susceptibility (Pritchard & Rosenberg 1999). Socioeconomic factors are probably slightly less important in AgP than in CP. This is because of the supposed stronger genetic background and weaker relation to local factors, such as oral hygiene, which might be influenced by belonging to different socioeconomic groups.

2. Power. Large numbers of subjects have to be included in order to test hypotheses of association, and to verify the possible interactions between genes and environmental

factors. This is particularly difficult in the case of AgP, considering its low prevalence (Löe & Brown 1991).

3. External validity. Apparently significant associations may reflect the choice of the patient group, and may not be generalised to other populations, different for ethnicity or environmental characteristics.

4. Definition of disease. Considerable confusion took place in the past with regards to the definition of early onset cases, and an attempt has been made to homogenize it through the introduction of the new classification (Armitage 1999). However, this is far from being ideal, and still leaves some scope for individual interpretation.

5. Choice of genetic marker. Association studies should be based on functional evidence to support the possible linkage of a certain gene with the disease (Hodge 1994). In reality, this very rarely happens, and most of the time a hypothesis of association leads to a study to test the hypothesis. If the association is confirmed, then an effort is made to try and understand the functional mechanism behind it.

These are just some of the possible reasons why, despite several association studies having been conducted in recent years in relation to CP and AgP, most studies report contrasting results.

1.3.2.3. Evidence for risk assessment

The confirmation of a genetic, microbiological, systemic or environmental factor as a true risk factor requires the fulfilment of a series of requisites (Beck et al. 1994):

- Identification, i.e. odds ratio >1.0 in cross- sectional investigations
- Retention of significance and direction of effect in a multi-factorial model including other exposures as confounders
- Assessment, meaning external validation through studies on independent populations

- Targeting step, i.e. proof of reduced incidence of the disease after intervention strategies targeted at the factor.

Genetic polymorphisms, which are currently non-modifiable, cannot fulfil the last step. The 5th European workshop in periodontology recently concluded that, out of an overabundance of putative risk factor, only three have currently been shown to fulfil all criteria: smoking, diabetes and certain bacteria (Tonetti & Claffey 2005).

1.3.2.4. Putative genetic risk factors

A range of different genetic deficiencies or variations in the host response have been studied as potential modifiers of the response to bacterial plaque, and therefore are among the plethora of putative predisposing factors to periodontitis. Several genetic polymorphisms have been analyzed and associated to susceptibility to infections, to inflammatory diseases and especially to periodontitis (Kinane et al. 2005). Restricting our considerations to AgP, because of the several contrasting results as mentioned earlier, we have little evidence to confirm with certainty any genetic polymorphisms as clear risk factors for AgP. Three different broad categories of candidate gene modifiers for periodontitis exist:

1. Innate response : cytokine markers and neutrophil markers
2. Adaptive response: immunological markers
3. Structural factors of periodontal tissues

The main attention of AgP genetic research so far has been focused mainly on the first category. The next chapter will include a brief discussion about the pathogenic mechanisms of the innate response and will present an overview of the most important known candidate genetic risk factors for AgP.

CHAPTER 2

2. INNATE RESPONSE IN PERIODONTAL DISEASE

Healthy gingiva features a protein-rich inflammatory infiltrate, which exudes through the vessels and creates the gingival crevicular fluid (Brill & Krasse 1958). In normal healthy gingiva, this inflammatory infiltrate contributes to maintaining the balance with the microbial challenge, through specific and non-specific mechanisms, thus preserving the physiological homeostasis between bacterial presence and host response (Griffiths 2003). When the normal balance is somehow disrupted by bacterial overload due to plaque accumulation and/or by impaired host response, the first signs of gingival inflammation appear and the innate response is activated. The innate (or non-specific) response includes mechanisms that operate without any previous contact with the disease-causing organism. The activation of the innate response leads to inflammation, which may lead to resolution of the infection or to establishment of a chronic lesion. The classical cardinal points, which characterize the inflammatory lesion, are *rubor, tumor, calor, dolor* and *functio lesa*, which mean the area becomes red, swollen, hot, painful and loses its function. These factors are common in periodontal lesions, which present with gingival redness and swelling due to vasodilatation and vascular permeability; the heat is less easy to evaluate and the pain less common in the periodontium, while loss of function caused by tooth mobility is common in advanced periodontitis. Thus, in gingivitis, alterations in the vascular network occur, many capillaries dilate, and the inflammatory infiltrate is produced in increased quantity. This infiltrate is initially characterized by the presence of monocytes/macrophages, lymphocytes and neutrophils. The junctional epithelium proliferates, and the immune (specific) response also gets activated, with the release in the infiltrate of increasing number of plasma cells. When this overall host response is not able to effectively oppose the microbial challenge, gingivitis may evolve into periodontitis, with destruction of periodontal fibers and connective tissue and apical migration of the junctional epithelium. Both the plaque and the inflammatory infiltrate subsequently move apically (Bosshardt & Lang 2005).

In this process, the inflammatory response is crucial and the inability to successfully deal with the bacterial challenge and therefore the persistence of the inflammatory

infiltrate can represent the basis for the pathogenesis of periodontitis (Van Dyke et al. 2003).

As anticipated, the cells involved in the inflammatory process are mainly monocytes/macrophages, lymphocytes and neutrophils (PMN). These cells are activated and regulated thanks to a complex network of interactions of cytokines. Cytokines are soluble proteins, secreted by cells, which act as messengers transmitting signals to other cells. A member of the cytokine group is the interleukin subclass, responsible for regulation of immune and inflammatory responses. Cytokines, depending on their function, can be divided into proinflammatory (such as interleukin-1 and 6 and tumor necrosis factor), chemotactic (such as interleukin-8) and lymphocyte signaling cytokines (such as interleukin-4, 5, 10 and 13). Each of these cytokines is coded by a specific gene, and several genetic variants (polymorphisms) have been identified in these genes. Some of these polymorphisms are probably responsible for gain or loss of function of the coded cytokines, and therefore may determine a change in the capacity and effectiveness of the inflammatory response. This theory has led to the hypothesis that polymorphisms of some of these cytokines may predispose to chronic inflammatory diseases, such as periodontitis (Kinane et al. 2005).

2.1. INTERLEUKIN 1

2.1.1. Function

Interleukin 1 (IL-1) is one of the main pro-inflammatory cytokines. It is synthesized by a variety of cells including activated macrophages, keratinocytes, stimulated B lymphocytes and fibroblasts, and it elicits a wide range of biological activities that initiate and promote the host response to injury and infection (Kinane et al. 2005). There are two different structural forms of IL-1: IL1α, which is the acidic form, and IL-1β, which is the neutral form. Both have very similar activities. Lord et al. (1991) demonstrated that IL-1α, and to an even greater extent IL-1β, are transcribed in PMN stimulated with bacterial lypopolysaccharide. Furthermore, both forms stimulate osteoclast activity and are potent bone resorbing factors in vivo. These processes are mediated through stimulation of the production of IL-1 itself, IL-6, TNFα, prostaglandin E2 and matrix metalloproteinases (MMPs). There are two primary cell surface receptors for IL-1. The IL-1 type I receptor transduces a signal, whereas the type II receptor binds IL-1 without transducing a signal.

2.1.2. Polymorphisms

The genes for IL-1 (IL-A, IL-B, IL-RN) are located on chromosome 2q14, and encode respectively for IL-1α, IL-1β and IL-1 Ra (receptor antagonist). The latter is structurally similar to IL-1 β, but lacks agonist activity, being a competitive inhibitor of the IL-1B effect. Danis et al. (1995) supported the theory that polymorphisms in regions of cytokine genes that affect transcription may contribute to interindividual variations in cytokine production. Therefore, it is possible to speculate on an increased IL-1 production followed by an increased risk of developing chronic inflammatory conditions depending on IL-1 polymorphisms. Particular attention has been attracted by an IL1A -889 polymorphism, by two ILB polymorphisms (-511 and +3954) and by a VNTR 86-pair repeat IL1 RN polymorphism. Pociot et al. (1992) revealed that the presence of the uncommon allele ('2') of ILB +3954 polymorphism was associated with increased monocyte production of ILβ in vitro. Contrasting results have been obtained more recently by Santtila et al. (1998) and Mark et al. (2000). Shirodaria et al. (2000) found that allele 2 of IL1A -889 was associated with a 4-fold increase in IL-1α.

Linkage of genes of the IL-1 cluster has been suggested for rheumatoid arthritis and Alzheimer disease (McGeer et al. 2001).

2.1.3. Relation to periodontitis

Because of its proinflammatory function and its ability to induce bone resorption, a role for IL-1 in periodontal disease has been suspected. Stashenko et al. (1991) reported higher levels of ILβ in periodontitis; moreover, increased IL-1 in gingival crevicular fluid has been correlated with onset of gingivitis and severe periodontal disease (Kinane et al. 1992, Ebersole 1993). Ishihara et al. (1997) suggested that the amounts of crevicular IL-1 are closely associated with periodontal disease severity, while Masada et al. (1990) showed reduction of IL-1 in gingival tissues after periodontal treatment. Kornman et al. (1997b) identified an increased risk of developing periodontitis in non smokers aged 40 to 60 who carried the '2' allele for IL-1A -889 and IL-1B +3954 (defined as 'IL-1 combined genotype'). Gore et al. (1998) and Rogers et al. (2002) also found increased prevalence of IL-1B+3954 allele 2 in advanced Chronic Periodontitis patients compared to healthy or mild diseased subjects. Studies from Papapanou et al. (2001) and Meisel et al. (2003), however, did not confirm these results. Recently, we observed an increased tendency for periodontal progression in Chronic Periodontitis patients heterozygous for IL-1B +3954 (Nibali et al. 2005). Diehl et al. (1999) obtained evidence of linkage disequilibrium for Generalised Early Onset Periodontitis and IL1A -889 and IL1B +3954 polymorphisms, suspecting a predisposing role for allele '1' of IL1β +3954. However, they were not able to attribute it a major effect. Conversely, Quappe et al. (2004) in a Chilean population, observed an association between heterozygosity for IL1B +3954 and AgP, while Parkhill et al. (2000) found an increased prevalence of allele 2 in Caucasian AgP patients. Hodge et al. (2001), in a group of 56 Caucasians with Generalized Early Onset Periodontitis, observed no associations for any of the described IL-1 polymorphisms. Walker et al. (2000), examining a population of 37 African Americans with Early Onset Periodontitis, concluded that, given the high prevalence of allele 1 in this population, the +3954 polymorphism would provide little diagnostic or predictive information on the disease. Danis et al. (1995) associated a

VNTR 86- pair repeat IL-1 RN polymorphism with increased production of IL-1 RN protein, and reduced production of IL1α by monocytes. This polymorphism was later associated by Langdhal et al. (2000) with increased risk of osteoporotic fractures. Tai et al. (2002), in a case-control study on 47 Japanese Generalised Early Onset Periodontitis and 97 healthy individuals, observed an association between this IL-1 RN polymorphism and the periodontitis group, while no differences in IL-1A -889 and IL-1B +3954 polymorphisms were detected.

Kinane & Hart (2003) recently stated that in the context of the high redundancy in the cytokine system, IL-1 is so highly regulated that any polymorphism coding for increased production of this molecule could be controlled by feedback mechanisms associated with its regulation, therefore excluding a major contribution to periodontal disease pathogenesis.

We can conclude that there is inconclusive evidence of a role for IL-1 polymorphisms in AgP. If a relation exists, it may be limited and variable depending on different populations.

2.2. INTERLEUKIN 6

2.2.1. Function

Interleukin 6 (IL-6) is a multifunctional cytokine with a central role in host defence (Terry et al. 2000). It exerts its functions activating a cell-surface signaling assembly composed of IL-6, IL-6RA, and the shared signaling receptor gp 130. Its functions vary from stimulation of the hepatic acute phase response to infection and injury, to stimulation of osteoclasts, differentiation and activation of macrophages and T cells (Lotz et al. 1988), stimulation of neutrophils, growth and differentiation of B cells and stimulation of hematopoiesis (Revel 1989). Its great importance in the acute phase response, partially induced by regulation of fibrinogen, is proven by the compromised response to infections and tissue damage in IL-6 knockout mice (Fattori et al. 1994). IL-6 is not constitutively expressed, but its production is induced by a number of inflammatory stimuli such as IL-1, platelet-derived growth factor, tumor necrosis factor α (TNF-α), bacterial products such as endotoxin, and viral infections (Terry et al. 2000). Many cells are able to produce IL-6; among them monocytes/macrophages, fibroblasts, neutrophils, endothelial cells, adipocytes, T cells and mast cells. Aberrant production of IL-6 by neoplastic cells has been implicated as a contributory factor to the growth of B cell dyscrasias, T-cell lymphoma, renal and ovarian cell carcinomas, and Kaposi sarcoma (Kawano et al. 1988, Foster et al. 2000). Dysregulation of IL-6 production has also been associated with a number of systemic diseases such as plasmacytosis, juvenile chronic arthritis, cardiovascular disease, rheumatoid arthritis, osteoporosis, Paget disease and Alzheimer disease (Terry et al. 2000, Shibata et al. 2002, Antonicelli et al. 2005). Furthermore, serum IL-6 levels are considered to be a predictor of postmenopausal bone loss (Scheidt- Nave 2001).

2.2.2. Polymorphisms

The IL-6 gene is located on chromosome 7 (locus 7p21). The IL-6 locus showed evidence of linkage to osteopenia (Ota et al. 1999) and association with bone mineral density (Garnero et al. 2002, Nordstrom et al. 2004). Several polymorphisms have been detected on this gene, among which the following substitutions exist: -597 G to A, -572 G to C, -373 An to Tn and -174 G to C. There is increasing evidence about

interindividual variation in transcription and expression of the IL-6 gene due to the haplotype determined by combinations of these polymorphisms (Terry et al. 2000, Fife et al. 2005). Fishman et al. (1998) reported a statistically lower frequency of allele C at position -174 in 92 patients with systemic juvenile rheumatoid arthritis, compared to 383 healthy subjects. In the same study, the C allele was also associated with significantly lower levels of plasma IL-6: after stimulation with LPS or IL-1, only the -174G construct expression significantly increased compared with the unstimulated levels. On the other hand, expression from the -174C construct did not change after 24 hours (Fishman et al. 1998). Homozygosity for -174G has been associated with increased IL-6 production, Kaposi sarcoma in HIV-infected men (Foster et al. 2000) and with mortality rates after acute coronary syndrome (Antonicelli et al. 2005).

2.2.3. Relation to periodontitis

Reports showing a link between IL-6 levels and periodontal infections are becoming more and more frequent. Takahashi et al. (1994) showed the presence of IL-6 in endothelial cells, fibroblasts and macrophages of subjects affected by periodontitis, while they did not detect it in cells from healthy individuals. Increased levels of IL-6 in gingival crevicular fluid have been demonstrated in AgP patients (Kamma 2004). Moreover, we showed a sharp increase in serum IL-6 levels at day 1 after intensive periodontal treatment of patients with advanced Chronic Periodontitis (D'Aiuto et al. 2004). We also observed in a group of severe Chronic Periodontitis patients treated with intensive treatment plus locally-delivered tetracyclines, reduction at 2 months post-treatment of serum IL-6 levels compared to untreated controls (D'Aiuto et al. 2005).

A few investigators have tried to evaluate the distribution of polymorphisms in the IL-6 gene in periodontitis patients: Trevilatto et al. (2003) showed an association between -174 G allele and susceptibility to chronic periodontitis in Brazilian Caucasians; Holla et al. (2004) studying 148 chronic periodontitis patients and 107 healthy controls, did not detect any associations for the -174 polymorphism, while they did detect significant differences in the -572 polymorphism, with increased presence of allele C in the controls; in a study of 112 Japanese chronic periodontitis and 77 healthy subjects, all

individuals (patients and controls) were homozygous for -174 G, while a significant association with periodontal health was noted for the -373 A9T11 genotype (Komatsu et al. 2005). Although the association between allele G and increased levels of IL-6 seems consistent in most studies, Terry et al. (2000) recently concluded that more than one of the IL-6 polymorphic sites are functional, and IL-6 transcription is influenced not only by a simple additive mechanism but rather through complex interactions determined by the haplotype.

From these preliminary data, although no association studies have been published looking at the distribution of IL-6 polymorphisms in AgP, we can conclude that there is a functional basis for a possible association.

2.3. TUMOR NECROSIS FACTOR (TNF)

2.3.1. Function

TNF is a multifunctional pro inflammatory cytokine, with effects on lipid metabolism, coagulation, insulin resistance, and endothelial function. Two forms of TNF exist (TNF-α and TNF-β), which have similar biologic activities and share 30% amino acid homology (Aggarwal et al. 1985). Numerous cells can produce TNF, mainly activated macrophages, but also monocytes, natural killer (NK) cells, B and T cells, basophils, eosinophils, neutrophils (PMN), fibroblast and osteoclasts (Vassalli 1992). TNF production by macrophages is stimulated by lipopolysaccharide (LPS), and suppressed by PGE2, Interferon-α and –β, IL-4, IL-6 and IL-10. The effects of TNF production include induction of acute phase proteins, mobilization of PMN, release of antibody and complement, T and B cell activation, stimulation of bone resorption, increased platelet adhesion to blood vessel walls, increased extravascularization of lymphocytes and macrophages (Ruuls & Sedgwick 1999). The sum of these effects stimulates phagocytosis; however, excessive levels of TNF may cause alterations of cellular metabolism and harmful effects for the host, such as systemic oedema, hypoproteinemia and neutropenia. Overproduction of TNF has been implicated in the onset of autoimmune diseases, Crohn's disease, as well as cancer (Mocellin et al. 2005, Russo & Polosa 2005).

2.3.2. Polymorphisms

The TNF-α gene is located on chromosome 6 (locus 6p21). The TNF-α locus showed evidence for linkage with osteoporosis (Ota et al. 2000). The possible functional effect may be due to its effect on bone resorption. Other reports suggest a possible association of this locus with systemic lupus erythematosus, Crohn's disease and rheumatoid arthritis (Mulcahi et al. 1996, Fowler et al. 2005). Several TNF-α polymorphisms have been identified and studied. TNF-α production seems to be affected by a -308 A-G polymorphism. Wilson et al. (1997) and Kroeger et al. (1997) showed higher transcriptional levels in the presence of allele A rather than G at this position. Koss et al. (2000) and Sashio et al. (2002) found an association between -308 G-A genotype and ulcerative colitis.

2.3.3. Relation to periodontitis

The information related to TNF-α polymorphisms and periodontitis is limited: Kinane et al. (1999a) found no association between TNF -308 polymorphism and AgP; Galbraith et al. (1998) found an increased prevalence of G homozygosity at position -308 in 20 severe CP patients in a small case-control study; while Craandjiik et al. (2002) found no associations between periodontitis and any of the studied TNF polymorphisms (-238, -308, -376, +489). Likewise, Donati et al. (2005) reported a lack of association between TNF- α -308 polymorphism and severe Chronic Periodontitis in a population consisting of 60 affected individuals and 39 controls. Conversely, Soga et al. (2003) studied the distribution of polymorphisms at -238, -308, -857, -863 and -1031 in 64 CP and 64 healthy Japanese subjects. They concluded that carriers of at least one variant allele of TNF-α -1031, -863 and -857 tended to have higher production of TNF-α from circulating monocytes/macrophages and appeared to have higher risk of having CP.

We can therefore conclude that there are very limited results available to allow conclusions to be made regarding the relevance of a relationship between TNF-α polymorphisms and AgP.

2.4. VITAMIN D RECEPTOR (VDR)

2.4.1. Function

The vitamin D receptor (VDR) is part of the vitamin D endocrine system, along with the active form of vitamin D and its metabolizing enzymes. It is a nuclear receptor, and modulates gene expression once complexed with the active form of vitamin D. The vitamin D system is involved in several endocrine pathways such as calcium metabolism, immune modulation, regulation of cell growth and differentiation and induction of apoptosis of osteoblasts, keratinocytes, T-cells and cancer cells (Dusso et al. 2005). Animal studies suggest a pivotal role for VDR in growth, bone formation and immune functions (Yoshizawa 1997). The synthesis of osteocalcin, the most abundant non collageneous protein in bone, is induced by calcitriol, the active hormonal form of vitamin D. This occurs through VDR and a specific VDR-responsive element in the osteocalcin gene promoter.

2.4.2. Polymorphisms

The VDR gene is located on chromosome 12 (locus 12q12-14). Three allelic variants in the VDR gene, which are recognized by the restriction enzymes BsmI, ApaI and TaqI, have been identified and showed to predict differences in bone density, accounting for normal physiologic variations in osteocalcin levels and up to 75% of the total genetic effect on bone density in healthy persons (Morrison et al. 1994, 1995, 2004). Other more recent reports show conflicting results in the different populations studied (Eisman et al. 1996, Zee et al. 2000), and calcium and vitamin D intake have been suggested to play a role in the relation between genotype and function (Ferrari et al. 1998). Moreover, the VDR gene has been found to be associated with osteoarthritis, hyperparathyroidism, cancer and Crohn's disease, but with conflicting results (Uitterlinden et al. 2004). Recently, the TaqI- T allele of VDR polymorphisms has been linked to susceptibility to tuberculosis and hepatitis B (Bellamy et al. 1999).

2.4.3. Relation to periodontitis

Tachi et al. (2003) found VDR Taq-I TT genotype to be associated with CP. No association was found from other investigators (Yoshihara et al. 2001, Sun et al. 2002), but their control groups included younger subjects, that could develop CP as they grow older. Inagaki et al. (2003) reported an association between the VDR Apa-I AA genotype to periodontal disease progression over 23 years in a group of healthy or mild periodontitis subjects, while no association was found for Taq-I polymorphism. We recently showed an association of the TaqI T homozygosis with extent of CP and an effect on periodontal disease progression attributable to the interaction between this polymorphism and smoking (Nibali et al. 2005).

Hennig et al. (1999) reported increased risk of developing L-AgP in t allele carriers in a small case-control study, while no association was observed for G-AgP. It is worthy of mention that this restriction fragment length polymorphism (RFLP) of the VDR gene, called Taq-I, is present in the coding sequence (exon 9) but is 'synonymous': its variation does not change the amino acid sequence of the encoded protein. Nonetheless, it may be in linkage disequilibrium with an unknown functional sequence elsewhere in the gene.

Speculations about the effect of VDR on AgP interaction may involve a combinatory effect on the immune response and/or on the bone metabolism, but this needs to be elucidated further.

2.5. NEUTROPHILS

Phagocytes such as neutrophils (polymorphonuclear leukocytes, PMN) and monocyte/macrophages are the first part of the cellular defence in host resistance to bacterial invasion. PMN are produced in the bone marrow and released into the bloodstream, where they respond to a variety of stimuli via nonspecific and specific interactions (Van Dyke et al. 2003). A series of chemoattractans and environmental factors modulate PMN margination, adhesion, diapedesis and then migration from the vessel walls to the extravascular spaces. PMN directional migration is called chemotaxis, and is activated by chemoattractans like IL-8, platelet-activating factor, leukotrienes and N-formyl-methionine-leucyl-phenylalanine (FMLP). All these ligands interact with PMN through specific surface receptors, and activate signal transduction through a complex mechanism of cytoskeleton organization. In the extracellular spaces PMN are ready to exert their killing activity following bacterial phagocytosis. Compelling evidence gives PMN a prominent role in periodontal tissues defence against microbial pathogens, with particular regards to AgP (Hart et al. 1994, Van Dyke et al 2003). It is widely recognized that whenever this first PMN defensive step is somehow defective, the net result is a shift in the physiologic homeostasis between bacteria and host response, resulting in periodontal disease. 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-Higaghi syndrome (Deas et al. 2003).

Studies conducted in the past two decades (Suzuki et al. 1984, Van Dyke et al. 1986, Gronert et al. 2004) demonstrated chemotactic and phagocytic defects in AgP patients. Conversely with what these reports suggest, 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 hyper reactive (Leino et al. 1994, Gronert et al. 2004) 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 et al. 2003).

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'. The role of the oxidative burst in inducing bacterial killing, but also tissue damage, has been widely recognized for many years (Henson et al. 1987, Weiss et al. 1989), and implicated in the pathogenesis of rheumatoid arthritis, diabetes, reperfusion injury, ulcerative colitis, pre-eclampsia, haemorrhagic shock (Kantarci et al. 2003, Abdelrahman et al. 2005). This pathogenic potential due to the PMN activity is also evident on human periodontal ligament *in vitro* (Deguchi 1990). Altman et al. (1992) demonstrated the specific tissue damaging effect of the release of reactive oxygen species (ROS) on gingival epithelial cells. ROS have been shown to be biologically active and involved in bacterial killing both directly and indirectly, thanks to the following mechanisms: i) production of a cascade of anti-microbial reactive oxygen metabolites, such as hydrogen peroxide, hypoclorous acid and chloramines (Dahlgren et al. 1999, Van Dyke et al. 2003, Rada et al. 2004), both in the intracellular and extracellular milieu, ii) activation of proteases through a depolarization compensatory mechanism mediated by K+ flux subsequent to the reduction of O_2 (Reeves et al. 2002, Rada et al. 2004).

It has been widely demonstrated that the oxidative burst which leads to ROS production is increased in many 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. Gronert et al. (2004) suspected a role of PMN diacylglycerol-kinase α signalling in the pathogenesis of LAgP.

The research which forms the basis for this PhD has focused on two aspects of PMN function: bacterial recognition through specific receptors and superoxide production through NADPH oxidase activity.

2.5.1. BACTERIAL RECOGNITION

Bacterial recognition is an important step towards PMN activation. PMN recognise bacteria opsonised to immunoglobulins (Ig) through specific Fc receptors. They are also able to recognise bacteria which are not bound to Ig through formyl-peptide receptors (FPR), which bind bacterial formyl peptide. These two different classes of receptors will be treated separately in the next sections.

2.5.1.1. Fc receptors

2.5.1.1.1 Function

PMN receptors for the Fc, or constant part of Immunoglobulins are considered to act as a bridge between cellular and humoral immunity through their binding to bacterial-bound Ig, which activates important effector functions. The Fc receptors expressed on PMN consist of a heterogeneous family of membrane bound and soluble proteins, differing in structure and ligand affinity and specific receptors exist for IgA, IgD, IgE, IgG and IgM (Ravetch & Bolland 2001, van Sorge et al. 2003). Particular interest has been attracted by Fc-α and Fc-γ receptors, which bind respectively IgA and IgG. Increased concentrations of both these Ig antibodies against periodontopathic bacteria have been detected in gingival crevicular fluid and in the periodontal pocket epithelium of periodontitis patients (Tew et al. 1985, Yuan et al. 2000).

Fc-α receptors (CD89) are present on PMN, monocytes/macrophages and eosinophils. Once they interact with IgA coated on the surface of invading bacteria, they mediate several immunologic defense processes such as phagocytosis, antibody-dependent cell-mediated cytotoxicity, and release of inflammatory mediators (Monteiro & van de Winkel 2003).

Fc-γ receptors are expressed in leukocytes and interact with bacteria-bound IgG, triggering phagocytosis, superoxide production, cellular cytotoxicity, antigen presentation, cytokine release, degranulation and regulation of antibody synthesis (van der Pol & van de Winkel 1998). They fall into three categories: Fc-γRI (CD64), Fc-γRII (CD32) and Fc-γRIII (CD16). Fc-γRI has been shown to be particularly effective in

antigen presentation, while Fc-γRII is more effective in inducing phagocytosis, and FcγRIII is involved in phagocytosis and superoxide production by PMN (van Sorge et al. 2003). A diagram showing activation mechanisms for FcγRIII is represented in figure 2.1. There seems to be a very high redundancy in the Fc receptor functions, meaning that their overlapping functions are usually able to guarantee compensation for diminished or absent function of one of these receptors. It has been shown that Fc-γRI and Fc-γRIII deficient individuals do not have higher incidence of infectious or autoimmune diseases. Nonetheless, interindividual differences in Fc-γ receptor genes may affect receptor functions at a more subtle level (van der Pol & Van de Winkel 1998).

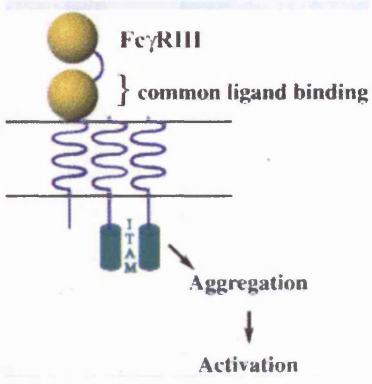

Figure 2.1. Representation of an FcRIIIB with activation functions (taken from Ravetch & Bolland 2001). A cytoplasmic ITAM sequence is associated with the receptor and allows signal transduction.

2.5.1.1.2 Polymorphisms

Fc-α receptor gene is located on chromosome 19 (locus 19q13.4). Two polymorphisms have been identified in its functional promoter region (Shimokawa et al.2000) and one coding for an IgG-binding domain (Kaneko et al. 2004).

Fc-γ receptor genes are located on the long arm of chromosome 1 (locus 1q21-24). A schematic representation of chromosome 1 is presented in figure 2.2. Fc-γRI class is encoded by three genes (Fc-γRIa, Fc-γRIb and Fc-γRIc). Three genes also encode for

Fc-γRII (a, b and c), while two encode for Fc-γRIII (a and b). Mutations and polymorphisms in the Fc-γRII and Fc-γRIII are suspected to affect ligand ability and effector functions, such as phagocytosis and killing of bacteria (van Sorge et al. 2003).

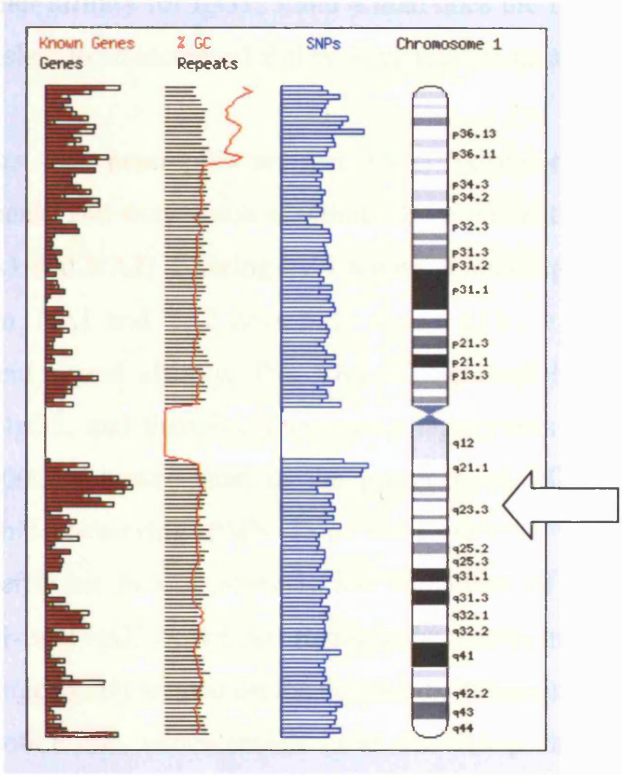

Figure 2.2. Schematic representation of chromosome 1, indicating the regions rich in genes and SNPs within the chromosome. The FcγR genes are located in one of these regions (1q21.24, indicated by the arrow) (image taken from the Human Genome Project at the Sanger Institute website).

Fc-γRIIa is polymorphic in at least two sites, one of which (at position 494 in the gene) codifies for amino acids (at position 131) located in spots involved in IgG-binding. The histidine (H) at position 131 has been shown to increase receptor affinity and specificity, while the presence of an arginine (R) results in PMN with reduced ability to phagocytose IgG2-opsonized particles in humans (Bredius et al. 1994). Eleven polymorphisms, three of which have been shown to be non synonymous, have been detected in the Fc-γRIIb gene in a population of Japanese subjects (Yasuda et al. 2003).

Fc-γRIII is the only PMN receptor without an intracellular part (see figure 2.1). It is anchored in the outer leaflet of the membrane via a glycosyl-phosphatidylinositol molecule. Both a and b genes are polymorphic. A G-T substitution at nucleotide 559 in the gene results in a valine (V) instead of a phenylalanine (F) at position 176. The V allotype exhibits higher affinity for IgG1, 3 and 4 than does the F allotype (Koene et al. 1997). This may translate to an increased ability of phagocytosis and killing of bacteria.

The Fc-γRIIIb bears the neutrophil antigen (NA) polymorphism, implicated in autoimmune neutropenia and transfusion reactions. Five nucleotide substitutions result in two isoforms (NA1 and NA2) differing by 4 amino acids (at position 36, 65, 82 and 106) in an Ig domain. NA1 and NA2 have been shown to have different glycosilation and therefore different ligand affinity. The NA1 allotype exhibits higher affinity for immune-complexed IgG3, and therefore increased phagocytosis (Salmon et al. 1990). Kobayashi et al. (2000b) showed that, in the presence of CD32-blocking antibody fragments, Fc-γRIIIbNA2-carrying PMN from both patients with CP and healthy controls were less efficient in phagocytosis and induction of oxidative burst upon interaction with IgG1- and IgG3-opsonized *P.gingivalis*. Furthermore, Bux et al. (1997) identified another antigen (SH) located on the Fc-γRIIIb, determined by a single base C-A mutation at nucleotide 266, which results in an Ala78Asp amino acid substitution. SH+ individuals are rare (usually <5%) and seem to be always accompanied by presence of the NA2 allotype, either because of close homology or because of linkage. This new antigen may have functional influences, still to be elucidated (Koene et al. 1998).

Considering the supposed importance of Fc-γ receptors in handling bacterial infections, the possible role of FcγR polymorphism in relation to disease susceptibility has been the subject of several studies. In particular, the ability to interact with IgG2 conferred by the Fc-γRIIa H131 allotype has caused speculation about its possible clinical significance. The 131 Fc-γRIIa polymorphism has been associated with risk of upper respiratory tract infections and meningococcal septic shock (Bredius et al. 1994, Sanders et al. 1994). Homozygosity for R131 was also reported to increase the susceptibility to immune-complexed autoimmune diseases such as systemic lupus erythematosus (SLE) (Blasini et

al. 1993). A combined Fc-γRIIa-R/R131- Fc-γRIIIb-NA2/NA2 genotype has been associated with increased frequency of meningococcal disease (Fijen et al. 1999, Platonov et al. 1998).

2.5.1.1.3 Relation to periodontitis

Dense infiltrates of neutrophils with high expression of Fc-α receptors have been detected in the apical part of diseased periodontal pockets (Yuan et al. 2000). Kaneko et al. (2004) investigated a population of 46 AgP patients and 90 healthy Japanese controls for the distribution of a -324 A-G transition. They found a statistically significant increase in prevalence of allele A in the patient group. This polymorphism does not change the coded amino acid, therefore the supposed effect might be due to another polymorphism in linkage disequilibrium with the -324 (Kaneko et al. 2004). PMN from allele A homozygous individuals also exhibited decreased phagocytosis of *Porphyromonas gingivalis*.

Periodontitis is among the most widely studied bacterial diseases in relation to Fc receptor polymorphisms. Kobayashi et al. (1997) suggested some years ago the possible role of Fc-γRIIIb NA2 allotype in inducing recurrence of periodontitis in a group of CP patients. They then extended their observation to Early Onset periodontitis and proposed the importance of the same allele in disease susceptibility (Kobayashi et al. 2000a). Yoshihara et al. (2001) confirmed this result analyzing Fc-γRIIIb genotype distribution in a Japanese population, albeit with small numbers of subjects (only 42 AgP). Fu et al. (2002), studying 48 LAgP and 67 controls, all of Afro-American descent, gave support to the previous findings, identifying the NA2 allele as a risk susceptibility factor. Chung et al. (2003) could not confirm this finding in 30 GAgP patients from Taiwan, and neither could studies performed on Caucasians with CP (Colombo et al. 1998, Loos et al. 2003). Loos et al. (2003), performing a study of 12 GAgP, 56 CP and 61 Caucasian controls, concluded that homozygosity for Fc-γRIIa 131H may be a risk factor for AgP. Yamamoto et al. (2004), in a study of 422 Caucasian adults observed an increase in the HH genotype in CP patients. Yasuda et al. (2003) found increased prevalence of allele T at nucleotide 232 of Fc-γRIIb in a Japanese AgP population. Looking at all these reports

as a whole, the evident lack of a study including large numbers of subjects reduces the external validity of these results. Nonetheless, AgP seems to be consistently associated to the Fc-γRIIIb NA2 allotype in Japanese populations. Furthermore, this finding is supported by the functional results in relation to handling of periodontal bacteria (Kobayashi et al. 2000b). However, data on AgP Caucasians are lacking. Association with other Fc-γR polymorphism has shown conflicting results, maybe because of the different populations studied.

2.5.1.2. FPR receptors

2.5.1.2.1 Function

The formyl peptide receptor (FPR) is a transmembrane receptor expressed in PMN and monocytes. The FPR has 7 transmembrane domains, connected by intra- and extra-cellular loops, which are coupled to intracellular effectors through guanine nucleotide regulatory proteins (G proteins) (Quehenberger et al. 1993, Seifert et al. 2001). It recognizes and binds N-formyl peptide, a chemotactic factor derived from human mitochondrial proteins and bacterial products, such as FMLP. When recognition occurs, PMN are driven to sites of bacterial invasion or tissue damage. Signaling events include cell adhesion, phagocytosis, stimulation of oxidative burst and production of inflammatory cytokines (Cui et al. 2002). Multiple domains of the FPR seem to be required for high-affinity ligand binding. Major determinants are probably located on the first extracellular loop and its adjacent transmembrane domain. An additional important site for determination of ability to bind the ligands is located in the second extracellular loop. Its substitution, as well as substitutions of the fourth and fifth transmembrane domains, has been associated with a 27-fold decrease in ligand binding affinity (Quehenberger et al. 1993). An FPR variant with lower affinity is called FPR-like 1, and interacts with high concentrations of FMLP.

2.5.1.2.2 Polymorphisms

FPR is encoded by the FPR1 gene, located on chromosome 19 and organized into 3 exons and 2 introns. Several gene polymorphisms in the gene encoding for different components of the receptor have been detected (Zhang et al. 2003).

2.5.1.2.3 Relation to periodontitis

Reduced chemotaxis of PMN is now a confirmed feature of LAgP (Cianciola et al. 1977, Genco et al. 1980, VanDyke et al. 1985). Given the importance of FPR in stimulating chemotaxis, several investigators have suspected the FPR1 gene as being responsible for chemotactic defects in AgP. Gwinn et al. (1999), in a study of 30 LAgP, 5 GAgP, 30 CP patients and 20 healthy controls, found a remarkable association between two single base substitutions at position 329 (C to T) and 378 (C to G) and the presence of LAgP: 29 out of 30 African American LAgP patients, and none of the other subjects included in the study exhibited at least one of these two substitutions. Both these polymorphisms result in amino acid changes in the second intracellular loop of the receptor and may be linked to difference in ligand affinity (Seifert et al. 2001). Further research, however, has not supported these findings and what looked like a remarkable association, may actually be due to population stratification. Zhang et al. (2003) performed sequencing analysis of the FPR1 gene spanning from nucleotides 250 to 615, on 111 AgP patients and 115 healthy controls of mixed ethnicity. None of these subjects presented any of the nucleotide substitutions reported by Gwinn et al (1999). Nonetheless, they identified six single nucleotide polymorphisms (301 G-C, 306 T-C, 348 T-C, 546 C-A, 568 A-T and 576 T-C-G), two of which were associated with the AgP phenotype in their study group of African Americans. These two polymorphisms (at nucleotide 568 and 576) are non-synonymous, determining a change in the coded amino acid in the second extracellular loop of the receptor. In particular, the 568 T to A substitution determines a change from a basic to an acid amino acid (arginine to tripsine) at codon 190 (Zhang et al. 2003). Considering the above mentioned importance of this second extracellular loop in ligand affinity, this change may have a reflection on receptor function. The polymorphism at position 301 is also functional, although affecting a site not shown to be important for ligand affinity. Maney et al. (2005) studying the FPR-1 genetic profile of 12 African American AgP patients and 12 controls, identified the 348 polymorphism as a possible risk indicator for AgP, and found only weak associations for the other polymorphisms described by Zhang et al. (2003). They also did not find any of the polymorphisms described by Gwinn et al.

(1999). Therefore, the basis for a possible association between FPR polymorphisms and AgP exists and deserves further study.

2.5.2. SUPEROXIDE PRODUCTION

2.5.2.1. NADPH oxidase

2.5.2.1.1 Function

The NADPH (nicotinamide adenine dinucleotide phosphate) oxidase is the enzyme responsible for the activation of the oxidative cascade in PMN, which through the release of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) leads to the production of reactive oxygen species (ROS). The NADPH oxidase (also known as phagocyte oxidase or respiratory burst oxidase) is a terminal electron carrier from NADPH to molecular oxygen (Robinson et al. 2004). The resulting electrons are moved and delivered to produce O_2^- both in the phagosome and in the extracellular environment (Dahlgren & Karlsson 1999) with the following stoichiometry:

$NADPH + 2\ O_2 \rightarrow NADP+ + 2O_2^- + H+.$

Then superoxide undergoes a dismutation reaction to form peroxide:

$O_2^- + O_2^- + 2H+ \rightarrow H_2O_2 + O_2.$

The NADPH system is a multicomponent enzyme consisting of six subunits (see figure 2.3). Two of them, $gp91^{phox}$ and $p22^{phox}$, located in the membrane, form flavocytochrome b558, the catalytic core of the enzyme. The $gp91^{phox}$ (also termed Nox2 or subunit β) contains the NADPH-binding site, a flavin adenine dinucleotide (FAD) and two haeme groups coordinated by two pairs of histidine residues (Werner et al. 2004). The 22-kDa α subunit ($p22^{phox}$) has a regulatory role and is essential for oxidase activation and recruitment of cytosolic factors. Three of the other subunits, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ form a complex located in the cytosolic component of resting neutrophils. The fourth component is the small GTP-binding protein known as Rac2 ($p21^{rac}$), also located in the cytoplasm.

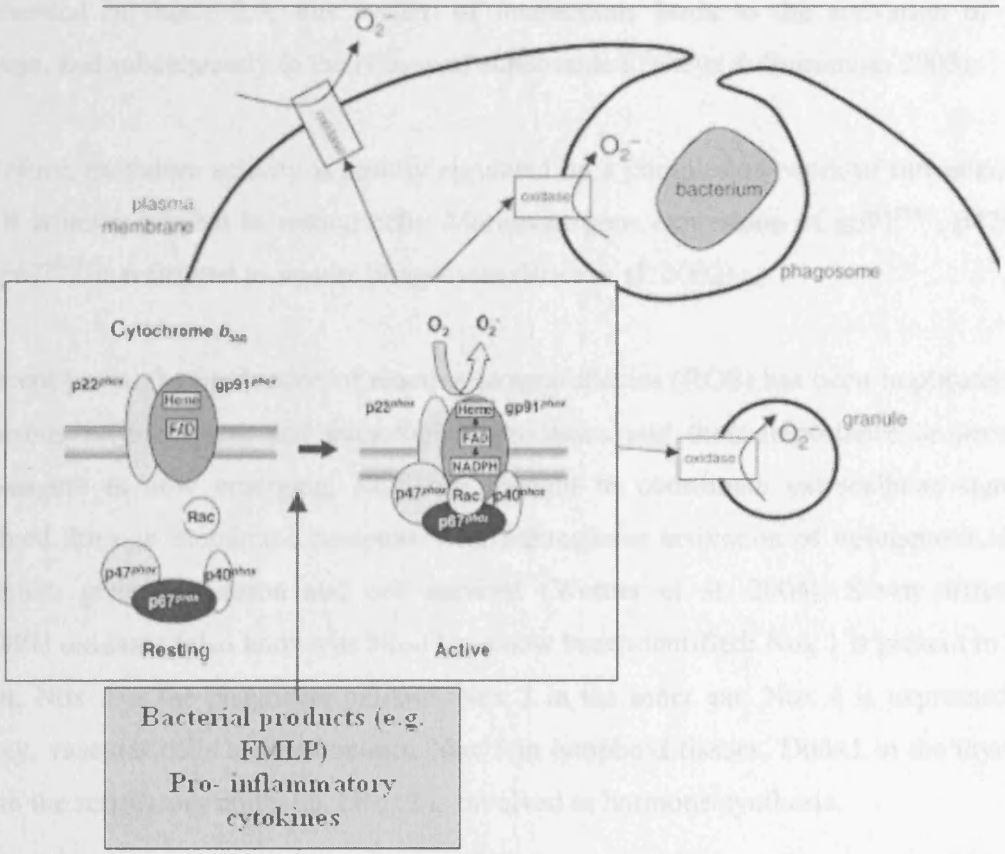

Figure 2.3. Schematic representation of the neutrophil NADPH oxidase. Upon stimulation by bacterial products or pro- inflammatory cytokines, the four cytosolic components (p40phox, p47phox, p67phox and Rac) migrate towards the membrane. In the membrane they interact with the two membrane components (p22phox and gp91phox) thus activating the enzymatic functions and determining production and release of O_2^- both in the phagosomes and in the extracellular environment (images adapted from Dahlgren & Karlsson 1999 and Takeya & Sumimoto 2003).

The NADPH oxidase is normally dormant. Upon stimulation by bacterial products, pro-inflammatory cytokines or by appropriate agents such as the protein kinase C activator PMA (4B-phorbol 12-mystrate 13-acetate), the cytosolic components migrate to the membrane, where they interact with cytochrome b558, and thus activate the enzymatic reactions. In more detail, stimulation of the neutrophils activates $p40^{phox}$, which regulates the recruitment to the membrane of $p67^{phox}$ and $p47^{phox}$. Simultaneously, upon activation $p47^{phox}$ becomes phosphorylated at multiple serine sites, and its SH3 domains become accessible to a proline-rich region of $p22^{phox}$, which activates $gp91^{phox}$. In addition, Rac2 is independently activated and binds to $p67^{phox}$ and $gp91^{phox}$. As

represented in figure 2.3, this system of interactions leads to the activation of the enzyme, and subsequently to the release of superoxide (Takeya & Sumimoto 2003).

Therefore, oxidative activity is strictly regulated by a complex network of interactions, and it is never present in resting cells. Moreover, gene expression of $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$ is restricted to mature phagocytes (Price et al. 2002).

In recent years, the production of reactive oxygen species (ROS) has been implicated in numerous physiological and pathological processes and their importance as second messengers is now emerging. ROS are thought to coordinate extracellular signals received through membrane receptors with intracellular activation of mitogenesis, cell adhesion, gene expression and cell survival (Werner et al. 2004). Seven different NADPH oxidases (also known as Nox) have now been identified: Nox 1 is present in the colon, Nox 2 is the phagocyte oxidase, Nox 3 in the inner ear, Nox 4 is expressed in kidney, vascular cells and osteoclasts, Nox 5 in lymphoid tissues, Duox1 in the thyroid and in the respiratory epithelia, Duox2 is involved in hormone synthesis.

The possible role of the osteoclastic Nox 4 in relation to periodontal disease is particularly intriguing. Several studies suggest that it is involved in activation and formation of osteoclasts, and is responsible for osteoclastic superoxide production, therefore playing a role in bone resorption (Yang et al. 2001). Osteoclasts have been shown to secrete hydrogen ions, proteinases and superoxide in order to excavate a resorption pit or lacuna in the bone surface. Superoxide may stimulate bone resorption by activating collagenases and by participating in fragmentation of matrix proteins. Yang et al. (2001) showed a decrease in bone resorption following inhibition of the superoxide production by the oxidase. Furthermore, defects in superoxide generation have been found in patients suffering from osteopetrosis, a disease characterized by excessive bone formation. In this context, it is necessary to underline the importance of the $p22^{phox}$ subunit, shown to be indispensable in the osteoclastic Nox 4 superoxide production (Yang et al. 2004).

2.5.2.2. Polymorphisms

The gene encoding for the $p22^{phox}$ subunit of flavocytochrome b558 is located on chromosome 16 and known as CYBA (for subunit α or $p22^{phox}$). Defects in this gene, as well as in the genes coding for $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$, are associated with chronic granulomatous disease (CGD), an inherited disorder characterized by a severe defect in host defense against bacteria and fungi (Dinauer et al. 1993). In CGD, the activity of the NADPH oxidase enzyme is greatly diminished or absent. In myeloid cells, the absence of $p22^{phox}$ protein because of genetic defects also results in the loss of $gp91^{phox}$ expression and vice versa, indicating that each of these proteins requires the other for mutual stability (Rae et al. 2000). Furthermore, UshioFukai et al. (1996) provided evidence that the $p22^{phox}$ is critical for the function of the NADPH oxidase. They showed that the transfection of antisense $p22^{phox}$ cDNA decreased superoxide production in rats.

Parkos et al. (1988) described a 242C-T polymorphism of the CYBA gene causing a histidine to tyrosine change at position 72. This substitution involves a potential haem-binding site, which can have as a consequence an increase or decrease of superoxide production. Several groups have studied the possible relation between this polymorphism and the risk of developing cardiovascular disease, intracranial aneurysm and diabetes, with conflicting results. The T allele has first been linked to a protective role on coronary risk (Inoue et al. 1998). However, this result was not confirmed by subsequent studies, which either found no associations (Li et al. 1999, Gardemann et al. 1999, Renner et al. 2000), or tendency to increased cardiovascular risk in young Caucasians (Cai et al. 1999) and progression of coronary atherosclerosis (Cahilly et al. 2000). Existing data suggest that the vascular and phagocytic $p22^{phox}$ are very similar, even though the latter has much higher activity (De Keulenaer et al.1998). In order to investigate the functional importance of this amino acid substitution, Shimo-Nakanishi et al. (2004) measured the PMN oxidative burst on a subset of Japanese patients with thrombotic cerebral infarction, and related it to the C242T $p22^{phox}$ polymorphism. They showed that, although basal superoxide production was not different, subjects with the T allele had significantly higher oxidative burst than CC subjects under stimulation by

PMA. In contrast, Wyche et al. (2004) in a study on 90 healthy Caucasians detected an increase in superoxide production in CC positive individuals.

2.5.2.3. Relation to periodontitis

There are no published studies in the literature investigating a possible role of any NADPH oxidase polymorphisms in periodontal disease. However, we can hypothesize that given the importance of superoxide production in the defense against bacterial infections, its tissue-damaging potential and considering the tight regulation of the NADPH oxidase activity, a variation in its activity, due to genetic polymorphisms, may predispose to the onset of AgP. The increased superoxide production seen in AgP patients may therefore be linked with polymorphism in the $p22^{phox}$ component of NADPH oxidase, both in PMNs and osteoclasts, and could account for the considerable destruction of periodontal attachment and alveolar bone.

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 aggressive periodontitis (Papapanou et al. 2004). They found up-regulated expression in periodontitis patients of a cluster of genes including NAD, including the PBEF gene, involved in its biosynthesis, and the NAD+ isocitrate dehydrogenase β, NDUFB8, NDUFC1 and NDUFB2 genes, involved in NADH dehydrogenization.

CHAPTER 3

3. AIMS OF THE STUDY

The overall main points outlined in the preceding chapters, may be summarized as follows:

- AgP affects a small but significant percentage of the population (at least around 1%) and leads to tooth loss at a young age
- It is an infectious disease, associated with specific bacteria
- Genetically determined host response affects susceptibility to AgP by modifying the response to bacterial plaque

However, despite the conspicuous amount of knowledge developed in recent years, we are still a long way from having a complete understanding of AgP aetiopathogenesis. Furthermore, patients are often not very well informed about the characteristics and consequences of AgP, and the same applies to some general dentists. This is due to the fact that periodontitis is not usually accompanied by painful symptoms: this means that the patients are not always able to imagine that the bleeding they see when they brush their teeth is the manifestation of a severe gingival disease, which if not treated early may put the future of their teeth at risk. All these aspects taken together mean that when such a patient gets to the attention of a periodontist, the disease is usually at an advanced stage.

The management of AgP patients is therefore extremely difficult and delicate, considering the young age of the patients and the advanced degree of periodontal destruction. The treatment usually consists of oral hygiene instruction, supra- and sub-gingival debridement, which may also involve the use of antimicrobials, and extraction of the hopeless teeth. These are the teeth whose attachment and bone loss is too advanced and therefore cannot respond adequately to the treatment. This first phase of treatment is usually followed by sessions of surgical treatment, aimed at correcting the tissue damage provoked by AgP. The loss of a number of teeth, usually first molars and incisors, at a young age means that AgP patients face a lifetime of complex dentistry in order to replace them, over 50 years or more.

Several improvements have recently been made in the ability to treat AgP patients, and to replace missing teeth, by means of bridges or osseointegrated implants. However, a better understanding of the disease process is still needed, in order to inform more specific and more effective prevention and treatment regimes. A specific area which requires further research is the genetically determined host response of AgP patients. As mentioned in the previous chapter, several studies have been conducted in the last 5-10 years but, because of lack of power or other problems, have left us with more doubts than certainties.

The aim of my PhD has been to take advantage of the large number of AgP patients referred to our Department to gain a better insight into the genetic susceptibility factors leading to AgP. General aims are:

- Detecting genetic polymorphisms which are risk factors for the onset of AgP
- Exploring the familial aggregation of AgP
- Correlating genetic risk factors with the severity of AgP
- Exploring possible mechanisms of association between genetic risk factors and onset of AgP

Specific aims will be discussed within each individual chapter. The next chapter will describe the general materials and methods and the procedures adopted in order to achieve these aims.

CHAPTER 4

4. GENERAL MATERIALS AND METHODS

The research on which this PhD is based took place entirely at the Eastman Dental Institute and Hospital (EDI/EDH). Patient examinations were performed at the main clinic of the Department of Periodontology and at the Eastman Clinical Investigation Centre (ECIC). Control subjects were examined at the Departments of Oral Surgery, Conservation, Endodontics and ECIC. The laboratory work was performed in the laboratories of the Eastman Dental Institute and Hospital.

The EDH receives referrals for specialist consultations and treatment from general dental practitioners (GDPs). Referrals regarding periodontal problems are directed to the Department of Periodontology, and several consultants are responsible for screening the patients through an initial consultation. Once a patient is examined clinically and radiographically, provisional diagnosis is made and patients are either discharged back to their GDP or allocated for treatment within the Hospital.

In the last 8 years, a special clinic has been arranged for patients suffering from Early Onset forms of Periodontitis (EOP, now AgP). Therefore, patients with a suspect diagnosis of AgP are referred by the examining consultant to this clinic, where a more detailed examination will allow the diagnosis to be confirmed (see flow chart in figure 4.1). Examination in the 'Early Onset clinic' consists of full mouth periodontal clinical measurements, full mouth long cone periapical radiographs with parallel technique, discussion about medical and family history, clinical photographs and case presentation to the patient.

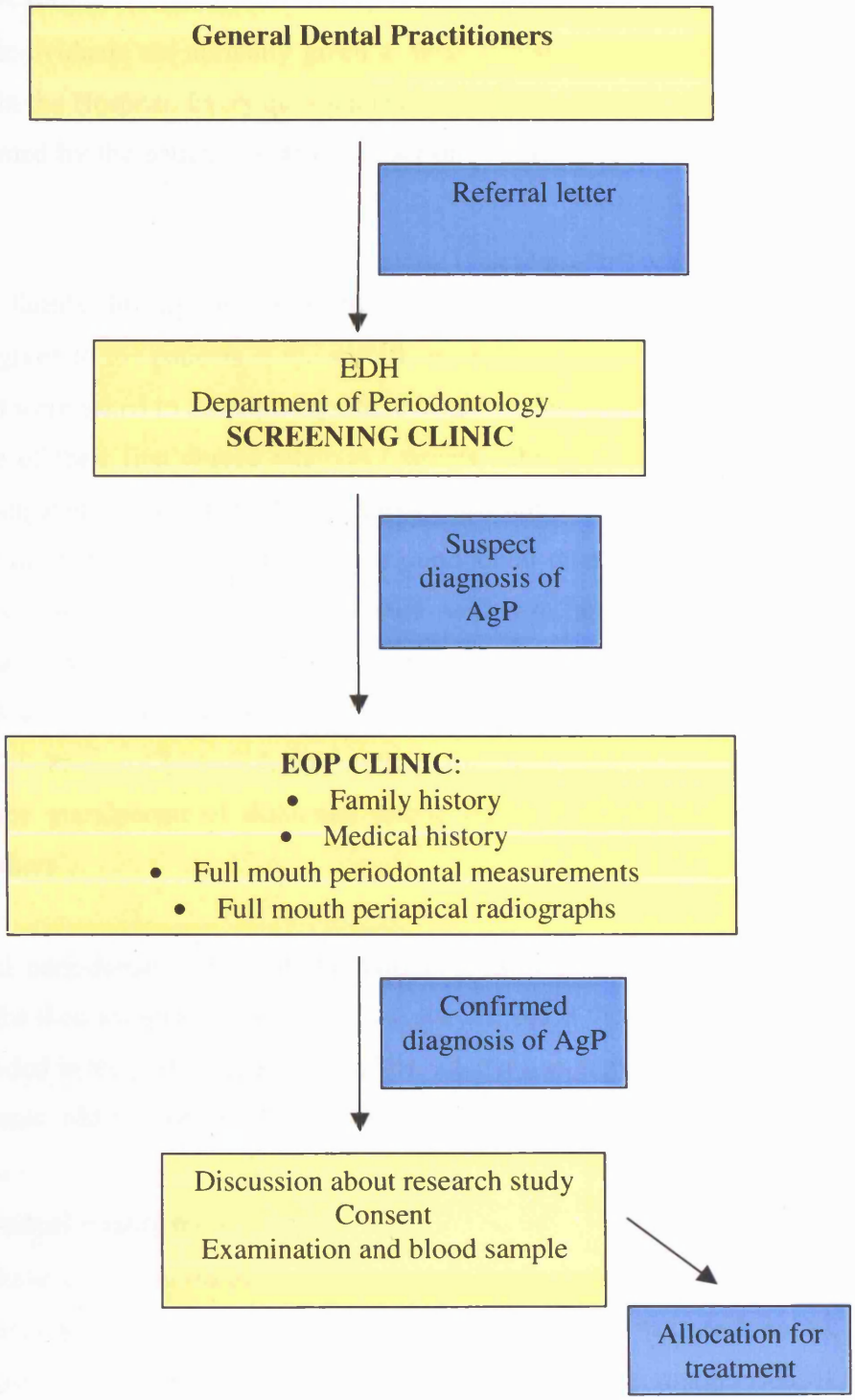

Figure 4.1. Chart showing the flow of patients from their General Dental Practice to inclusion in the study.

4.1. Anamnesis

All examined individuals are normally given a medical history questionnaire to fill at their first visit in the Hospital. Every questionnaire was checked by the examiner and the answers confirmed by the patient. Long term past or present usage of medications was recorded.

Ethnicity and family history of periodontal disease were recorded in a specific questionnaire, given to the patients at the specific examination visit in the 'Early Onset clinic'. Patients were asked to describe the ethnic origin of their parents, and to write the details and age of their first degree relatives (parents, siblings and children); specific questions investigated whether any of the relatives had any missing teeth, loose teeth, bleeding gums or if they had been treated for periodontal disease. Patients were also invited to investigate whether their relatives were willing to be examined at the EDH. The patients were divided into 3 main ethnic groups: Caucasian, Black (including Black-Africans and Black-Caribbeans) and Asian (including all different Asian ethnic groups, such as Indian, Pakistani, Chinese, Japanese etc.). All other subjects and subjects who had at least one grandparent of dissimilar ethnic origin than the other three were classified as 'others'.

The dental and periodontal history of the patients was also investigated. They were asked to describe their symptoms, when they had started, and if they had any periodontal treatment provided in the past. Whenever possible, a letter was sent to their GDPs asking to provide patients' old records (probing pocket depths, radiographs).

4.2. Periodontal examination

Single pass, whole mouth measures of the distance from the free gingival margin to the base of the sulcus (probing pocket depth, PPD, rounded up to the next millimetre), the distance from the cement-enamel junction (CEJ) to the free gingival margin (FGM) and bleeding on probing (BOP, positive or negative) were collected using a manual, incremental UNC-15 periodontal probe. Both PPD and recessions (REC= CEJ-FGM) measurements were used to determine the lifetime cumulative attachment loss (LCAL=

PPD+ REC). Six sites were measured for each natural tooth, one each at the mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual sites encircling the tooth. The measurements were made at the corresponding contact points or their equivalent in case of a missing tooth, and at the midpoint of buccal and lingual surfaces. An example of a probing sheet used for the study is presented in appendix I.

Scores of full mouth bleeding on probing (FMBS) and plaque (FMPS) were also recorded. Both were measured as dichotomous variables: if a site bled within 15 seconds after probing the score was positive (1), otherwise it was negative (0). FMBS represents the percentage of bleeding sites in the whole mouth. FMPS was calculated as the percentage of sites with plaque detected by moving a periodontal probe around the cement-enamel junction of each tooth.

4.3. Diagnosis

Anamnestic, radiographic and clinical data collected were studied in order to confirm a diagnosis of AgP. Diagnosis of AgP was based on the 1999 Consensus Classification of Periodontal Diseases (see chapter 1). Our diagnostic criteria took into consideration only clinical, and not laboratory, evidence. A single experienced clinician confirmed the diagnosis. Patients were classified as having AgP when they had evidence of:

- *Healthy status*, except for the presence of periodontitis. All patients with possible contributory medical history, such as specific recognized genetic diseases with periodontal manifestations (i.e. Papillon-Lefevre syndrome), diabetes, or prolonged use of anti-inflammatory or immunosuppressive medications were excluded.
- *Rapid attachment loss and bone destruction*, proven by X-rays 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.* Patients were preferably included in the study if they had positive family history. However, given the difficulty of ascertaining accurate family history and of examining all their first- degree relatives, patients showing

clear clinical signs of AgP were still included even without a proven positive family history.

- *Clinical and radiographic diagnosis.* All individuals who fulfilled the above mentioned criteria, but who had retentive factors or high plaque scores, proportionate to the amount of periodontal destruction present, were excluded. Such a measure was used to take advantage of the lee-way space the new classification leaves to personal interpretation, in order to exclude patients belonging to the 'grey area' between AgP and Chronic periodontitis. Patients were classified accordingly to the 1999 classification of Aggressive Periodontitis (Armitage et al. 1999) with Localised AgP (LAgP) when presenting with interproximal PPD and LCAL $\geq$ 5 mm and radiographic bone loss of $\geq$ 30% of root length on at least two permanent teeth, of which at least one was a first molar or incisor, and including no more than two teeth other than first molars or incisors. Patients were diagnosed with Generalised AgP (GAgP) when presenting with generalised interproximal PPD and LCAL $\geq$ 5 mm and radiographic bone loss of $\geq$ 30% of root length affecting at least three permanent teeth other than first molars and incisors.

4.4. Blood sampling

Once a diagnosis of Aggressive Periodontitis was confirmed, patients were asked to give their consent to have a blood sample taken for routine testing and genetic investigation. The blood samples were drawn by the examiner from the antecubital vein of the patient sitting in the dental chair in a semi-reclined position. After applying a tourniquet on the bicep area and disinfecting with an alcohol swab, a vacutainer tube system with a 19 or 21 gauge butterfly needle was used. The first blood sample was used to measure full blood count (purple tube). Two EDTA tubes (7ml- pink) were then used for storage of blood for DNA extraction. A vacutainer tube (3.5ml - gold) containing serum separator gel additive was used for cholesterol and lipids screening; a fluoride oxalate tube (light grey) was used to quantify glucose levels. Additionally a red cup vacutainer for serum collection, and a citrate vacutainer (light blue) for plasma separation were used. Within one hour of collection these samples were centrifuged at 3000 rpm for 15 minutes.

Serum and plasma collected were then placed into aliquots of 1ml in plastic cryovials and stored at –70°C.

The next chapters will describe one by one the different studies which, taken together, form this PhD. In the first one (chapter 5), the clinical data collected in our patient sample will be analyzed and discussed. The following chapter (6) reports the methods and results of a genetic association study performed on our AgP patients and on 231 healthy controls. Chapter 7 describes the relations detected between genetic markers and disease severity. The eighth chapter relates to the family study performed by examining the relatives of our AgP patients. The ninth chapter describes a study we have performed on a subset of our patient sample, in order to investigate the prevalence of specific bacterial pathogens and the microbe-genetic interactions. The tenth chapter reports about a functional study performed on the neutrophils (PMN) of a subset of our patients.

All the results of these different studies will then be discussed as a whole and conclusions will be drawn in the last chapter.

CHAPTER 5

5. CLINICAL DATA

5.1. BACKGROUND

Early onset forms of periodontitis have always been broadly distinguished into Localised and Generalised, with the Localised characterised by an incisor-molar pattern of bone loss, and the Generalised affecting a larger number of teeth. Several attempts have been made to identify different patterns of disease within these two broad categories. Manson and Lehner (1974) subdivided the Localised form into typical and atypical. The first was symmetrical with involvement of incisors and first molars in both jaws; the atypical form was asymmetrical, with one arch involved to a greater extent than the other. Another sub classification identified three forms of disease: class I, involving first molars and/or incisors; class II, involving first molars, incisors and some other teeth, with a total of fewer than 14 other teeth affected; class III, with generalised involvement of more than 14 teeth (Bial & Mellonig 1987). The current classification distinguishes just LAgP and GAgP (see chapter 1). The clinical differential diagnosis is based on the number of teeth affected other than first molars and incisors: in LAgP no more than two other teeth are affected. If more teeth are affected, the case falls into the GAgP classification. LAgP usually has an earlier onset, around puberty, but cases of primary dentition involvement have also been described (Sjodin et al. 1993, Hilgers et al. 2004). Some investigators reported progression from LAgP to GAgP (Brown et al. 1996), so doubts remain whether some cases of GAgP may represent the evolution of untreated LAgP cases.

LAgP cases classically consist of vertical bone loss affecting first molars and incisors symmetrically in both sides. The lesions usually present radiographically as an arch-shaped image of radiolucency surrounding maxillary or mandibular first molars and incisors. The reason for increased susceptibility of these teeth to LAgP is not clear. One possible explanation is that first molars and incisors are the first teeth to erupt in the permanent dentition. Therefore, they reflect more than other teeth the cumulative effect of disease pathogenesis, being present in the mouth for longer. An alternative explanation is that the disease may be the manifestation of an aetiologic factor present in

the early phases of puberty or before that, when these are the only teeth fully erupted (Lindskog & Blomlof 1983).

GAgP usually presents a more widespread pattern of bone loss with multiple teeth affected in both jaws and both arches.

The aim of this chapter was to:

1. Describe the clinical characteristics of the AgP patients described in this thesis
2. Examine the subgroups of Localised and Generalised Aggressive Periodontitis to determine differences in clinical presentation, some of which would be predetermined by the definitions set in the classification
3. Examine the effect of patient factors such as age, gender, ethnicity and smoking on the clinical parameters

5.2. MATERIALS AND METHODS

Clinical data from 224 consecutive AgP patients referred for care to the Department of Periodontology were studied. The data collected included probing pocket depths (PPD), recessions (REC), lifetime cumulative attachment loss (LCAL), full mouth plaque scores (FMPS) and full mouth bleeding on probing scores (FMBS) (see paragraph 4.2 for details). Recession and therefore LCAL measure were recorded only in 184 patients. Three different examiners collected the clinical measurements throughout the study. Although they underwent exercises of intraexaminer calibration and they had been trained to perform clinical measurements with the same technique, no interexaminer calibration was performed between them. Radiographic records, including long cone periapical radiographs with parallel technique and/or orthopantomograph were available for all subjects. Clinical and radiographic data and clinical photographs are presented for two example cases for both GAgP and LAgP.

5.2.1. CASE 1 Generalised Aggressive Periodontitis

This patient was referred by her General Dental Practitioner (GDP), who thought she might be affected by Early Onset Periodontitis. She presented as a 31-year old

Caucasian, with clear medical history and a past history of smoking (15 cigarettes/day for 10 years, had given up for 1 year). She was complaining about mobility and bleeding on brushing, which started a few years before and got worse recently. She reported a family history of periodontal problems and tooth loss at a young age in her parents. Clinical examination revealed good oral hygiene, 28 teeth fully erupted plus two lower third molars partially erupted, localised recessions with localised loss of interdental papillae and no evident marginal inflammation (see figure 5.1). Clinical measurements revealed:

- Full mouth plaque score of 23%
- Full mouth bleeding score of 82%
- 158 probing pocket depths $\geq$ 5 mm (94% of all sites)
- Average probing pocket depth of 7.34 mm
- Average recessions of 0.72 mm
- Average lifetime cumulative attachment loss of 8.06 mm

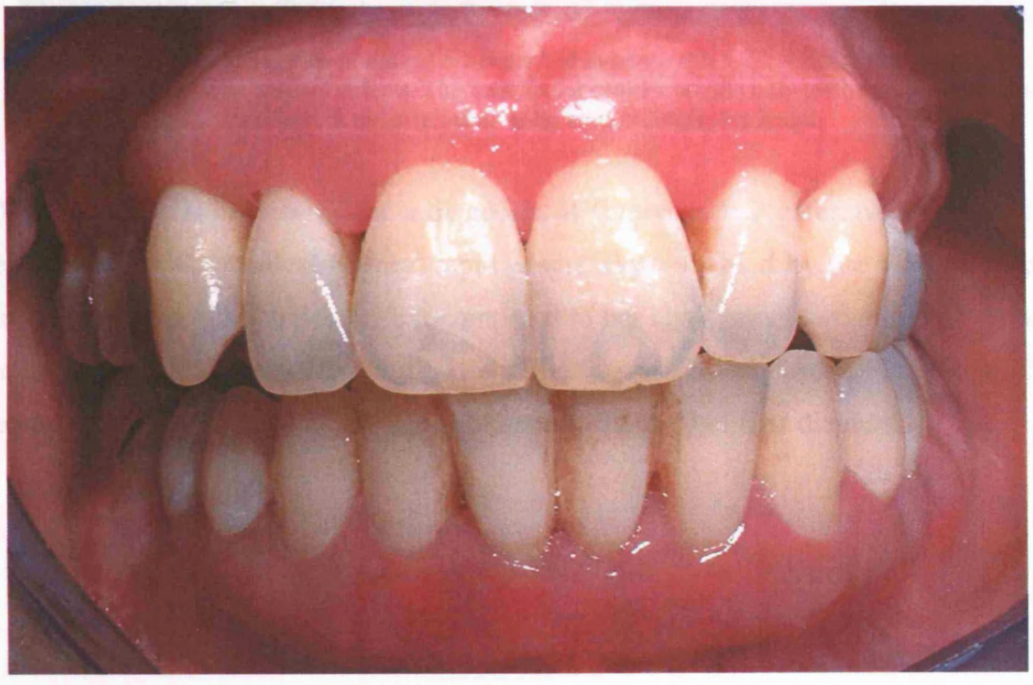

Figure 5.1. Frontal intraoral view of the 31-year old Caucasian former smoker female patient described in paragraph 5.2.1. Localised recessions and loss of interdental papillae are evident. Small amounts of localised deposits of plaque are also present.

Radiographic examination revealed:

- Generalised horizontal bone loss > 30% of root length on all teeth (ranging from 30 to 80%)
- Localised vertical bone loss (see figure 5.2)

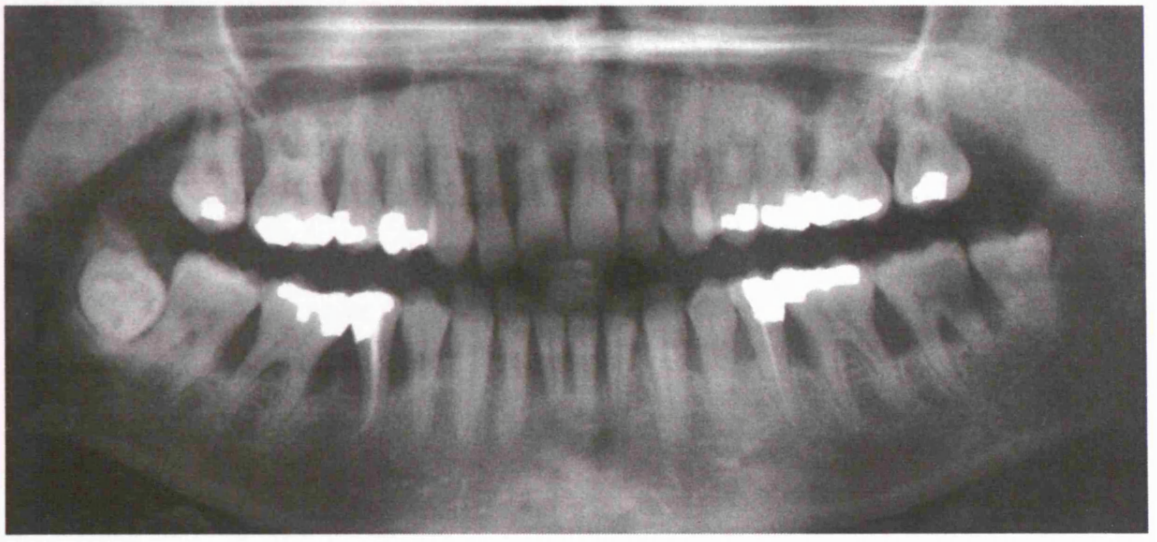

Figure 5.2. Orthopantomograph of the patient described in paragraph 5.2.1. Generalised bone loss is present, with localised vertical defects. The mandibular canines are the only teeth still preserving at least half of the original alveolar bone radiographic length.

All the characteristic features for a diagnosis of Generalised Aggressive Periodontitis were present: clear medical history, rapid progression (testified by the very advanced attachment loss and bone destruction compared to her age), possible family history and amount of local factors not proportionate with the periodontal destruction present. No laboratory diagnostic criteria had been obtained at the time of first diagnosis.

5.2.2. CASE 2 Localised Aggressive Periodontitis

This patient presented as a Black Caribbean 14-year old, referred by her GDP, complaining of occasional bleeding on brushing and soreness around some of the back teeth. She was a student, born in Jamaica, and had recently moved to London. Her medical history was clear and she was not a smoker. She referred to a positive family history of gum disease; she had 5 brothers and sisters; only one sister lived in the UK and she had some gum problems. Clinical examination revealed a moderate standard of oral hygiene, with a lack of gross deposits of plaque and calculus, and a minimally restored dentition (see figure 5.3). All teeth were present, with the exception of third molars which were still unerupted. Clinical measurements revealed:

- Full mouth plaque score of 25%
- Full mouth bleeding score of 15%
- 10 probing pocket depths of $\geq$ 5 mm (6% of all sites)
- Average probing pocket depth of 3.01 mm
- No recessions

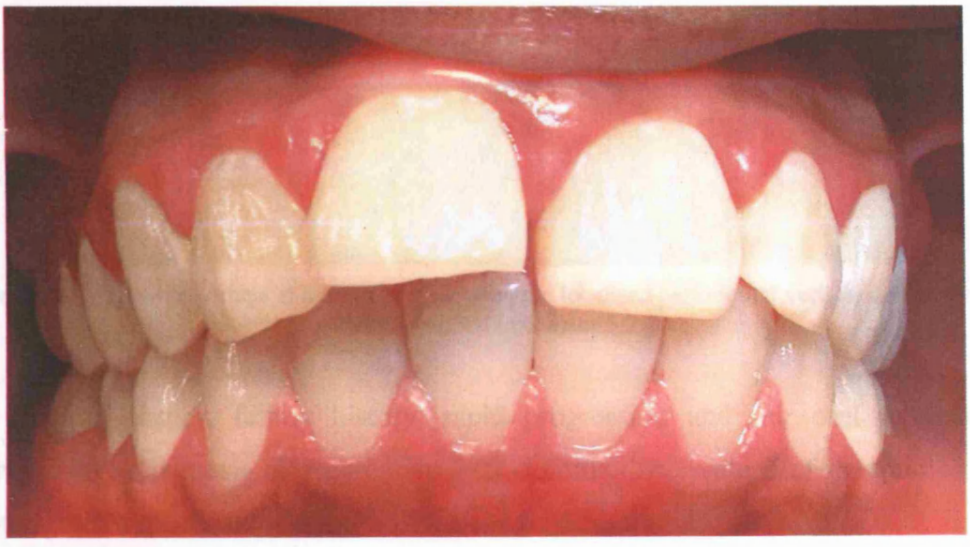

Figure 5.3. Frontal intraoral view of the 14-year old Black Caribbean female patient described in paragraph 5.2.2. Localised marginal inflammation is present, with very small amounts of local deposits.

Radiographic examination revealed:

- Localised vertical bone loss affecting all first molars and upper right central incisor (see figures 5.4 and 5.5).

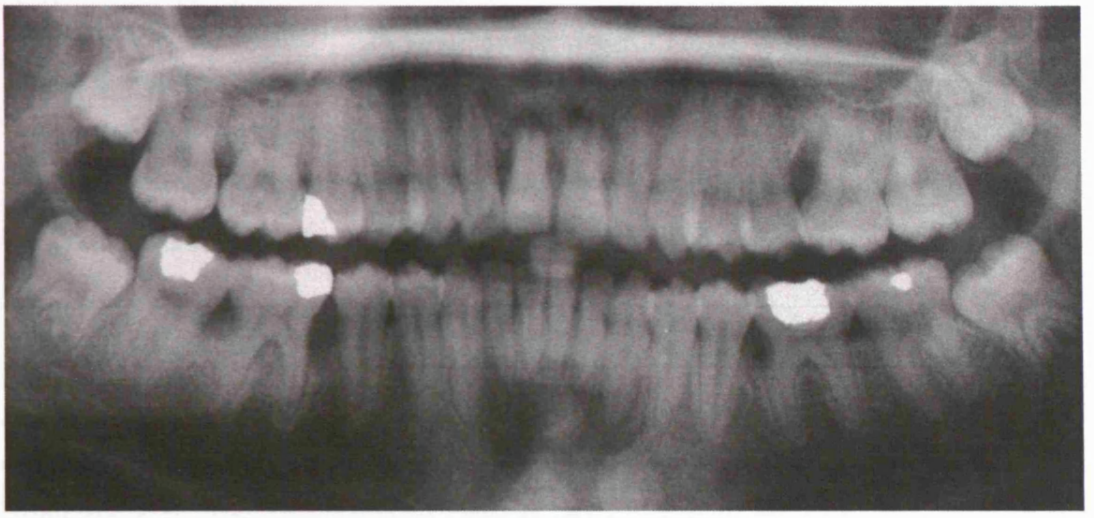

Figure 5.4. Orthopantomograph of the patient described in paragraph 5.2.2. Localised bone loss is present around 16 (UR6), 11 (UR1), 26 (UL6), 36 (LL6) and 46 (LR6).

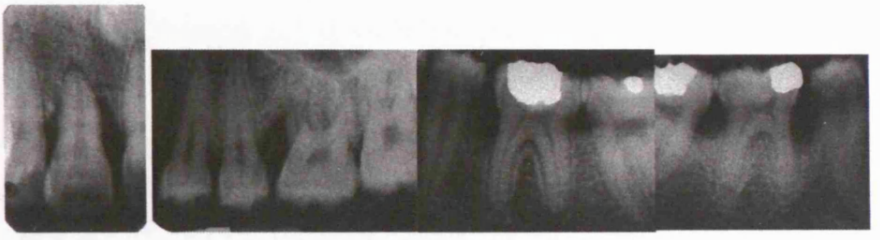

Figure 5.5. Long cone periapical radiographs showing the localised periodontal vertical defects of the patient described in chapter 5.2.2.

Clear medical history, family history, rapid progression and fair oral hygiene all contributed to a diagnosis of Aggressive Periodontitis. The pattern of destruction was consistent with a diagnosis of LAgP.

5.2.3. Statistical analysis

Clinical data from all patients were entered in an SPSS 12.0 file and proofed for entry errors. Continuous, normally distributed variables are reported as means ± standard

deviations (SD). The normality of distribution of the clinical parameters (av. PPD, av. LCAL, number of PPD $\geq$ 5 mm, number of PPD $\geq$ 10 mm and FMBS) was plotted and tested by Kolgomorov- Smirnov and Shapiro-Wilk tests. Since average PPD and average LCAL did not show normal distribution, they were log-transformed for the analysis. Comparisons of continuous and categorical data between groups were analyzed with ANOVA and Chi-square test, respectively. Multivariate analysis was used to detect the possible influence of smoking, ethnicity, age and gender on clinical parameters, separately in GAgP (whole group and Caucasians) and LAgP cases. The α value was set at 0.05. The normality of distribution of each of the residuals was then confirmed by Q-Q plots and by Kolgomorov- Smirnov and Shapiro-Wilk tests.

5.3. RESULTS

Demographic and clinical data on all patients are reported in table 5.1, while table 5.2 shows the subdivisions in GAgP and LAgP. Average FMBS, PPD, LCAL and PPD $\geq$ 5 mm and $\geq$ 10 mm are presented. Patients had an average age just short of 30 years old, half of them were Caucasians, and about half of them had never smoked in their life. The female: male ratio was almost 2: 1. Average clinical measurements reveal that FMPS was 42% on average, and about half of the sites bled on probing. The patients had an average of 28 teeth each including third molars, so they had lost just a small percentage of their teeth. The mean probing pocket depth (excluding third molars) approached 4 mm per site, and the mean LCAL was 4.5 mm. Fifty-three sites per patient on average had $\geq$ 5 mm PPD and about 50% of the patients had at least 1 site with 10 mm PPD, with an average of 2.9 such sites in each patient. As expected, GAgP patients were older than GAgP patients and had statistically significant increased averages of clinical measurements.

		PATIENTS	
		(n=224)	%
AGE		29.9 ± 7.2	-
GENDER	***Male***	79	*35.7*
	Female	145	*64.3*
ETHNICITY	***Caucasian***	112	*50.0*
	Afro-Caribbean	59	*26.3*
	Asian	34	*15.2*
	other	19	*8.5*
SMOKING	***Non smokers***	118	*52.7*
	Former smokers	52	*23.2*
	Light smokers (<20/day)	38	*17.0*
	Heavy smokers (≥20/day)	16	*7.1*
FULL MOUTH PLAQUE SCORES		-	41.9 ± 24.9
FULL MOUTH BLEEDING SCORES		-	49.7 ± 24.1
NUMBER OF TEETH		28.0 ± 2.8	-
Av. PPD		3.9 ± 1.1	-
Av. REC		0.5 ± 0.8	-
Av. LCAL		4.5 ± 1.6	-
NUMBER OF PERIODONTAL POCKETS ≥ 5 mm		53.4 ± 34.0	*34.5* ± 22.3
NUMBER OF PERIODONTAL POCKETS ≥ 10 mm		2.9 ± 5.3	1.9 ± 3.4

Table 5.1: Demographic and clinical characteristics of the Aggressive Periodontitis (AgP) patients. PPD indicates probing pocket depths, REC indicates recession and LCAL stands for lifetime cumulative attachment loss

		GAgP		LAgP		Comparison between groups
		(n=167)	%	(n=57)	%	
AGE		31.4 ± 6.0	-	25.6 ± 8.4	-	*p< 0.000*
GENDER	***Male***	59	*35.3*	20	*35.1*	*p= 1.000 Pearson Chi-square*
	Female	108	*64.7*	37	*64.9*	
ETHNICITY	***Caucasian***	88	*52.7*	24	*42.1*	*p= 0.226 Pearson Chi-square*
	Afro-Caribbean	45	*26.9*	14	*24.6*	
	Asian	21	*12.6*	13	*22.8*	
	Other	13	*7.8*	6	*10.5*	
SMOKING	***Non smokers***	79	*47.3*	39	*68.4*	*p= 0.015 Pearson Chi-square*
	Former smokers	42	*25.1*	10	*17.5*	
	Light smokers (< 20/day)	30	*18.0*	8	*14.0*	
	Heavy smokers (≥ 20/day)	16	*9.6*	0	*0*	
FULL MOUTH PLAQUE SCORES		-	*44.5 ± 25.1*	-	*32.0 ± 21.7*	*p= 0.007*
FULL MOUTH BLEEDING SCORES		-	*49.7 ± 24.1*	-	*33.8 ± 23.0*	*p< 0.001*
NUMBER OF TEETH		28.0 ± 2.9	-	28.2 ± 2.5	-	*p= 0.647*
AVERAGE PPD		4.2 ± 1.0	-	2.8 ± 0.6	-	*p< 0.001*
AVERAGE REC		0.7 ± 0.8	-	0.1 ± 0.2	-	*p< 0.001*
AVERAGE LCAL		4.9 ± 1.5	-	3.0 ± 0.8	-	*p< 0.001*
NUMBER OF PERIODONTAL POCKETS ≥ 5 mm		63.9 ± 31.8	*46.1 ± 13.8*	21.8 ± 15.6	*13.8± 9.8*	*p< 0.001*
NUMBER OF PERIODONTAL POCKETS ≥ 10 mm		3.3 ± 5.8	*2.1 ± 3.8*	2.0 ± 3.7	*1.2 ± 2.2*	*p= 0.117*

Table 5.2: demographic and clinical characteristics of AgP divided by diagnosis: GAgP and LAgP

5.3.1. Disease distribution

Teeth exhibiting at least one site with 5 mm PPD were defined as 'affected'. The choice of PPD rather than LCAL was made because LCAL data were not available for all patients. Figure 5.6 reports the number of missing teeth and the percentage of affected teeth in the mixed AgP group. Excluding third molars, 6.8% of 6272 teeth of all AgP

patients were missing. More teeth were missing in the maxilla (7.5%) rather than in the mandible (6.1%). First premolars were the most commonly missing teeth (12.5%), followed by first molars (11.5%) and second premolars (8.7%). Almost six per cent of second molars, 4.7% of incisors and just 2.3% of canines were missing. The same pattern maxilla-mandible was present for percentage of teeth affected by periodontal disease (62.3 versus 57%). In terms of tooth type, 86% of first molars were affected, followed by their adjacent teeth: 74% of second molars and 58% of first premolars. Fifty-one per cent of incisors, 48% of first premolars and canines were affected. The difference between upper and lower jaw was most evident for percentages of affected incisors (respectively 55.5% versus 47.4%) and first premolars (respectively 52.8% versus 43.6%).

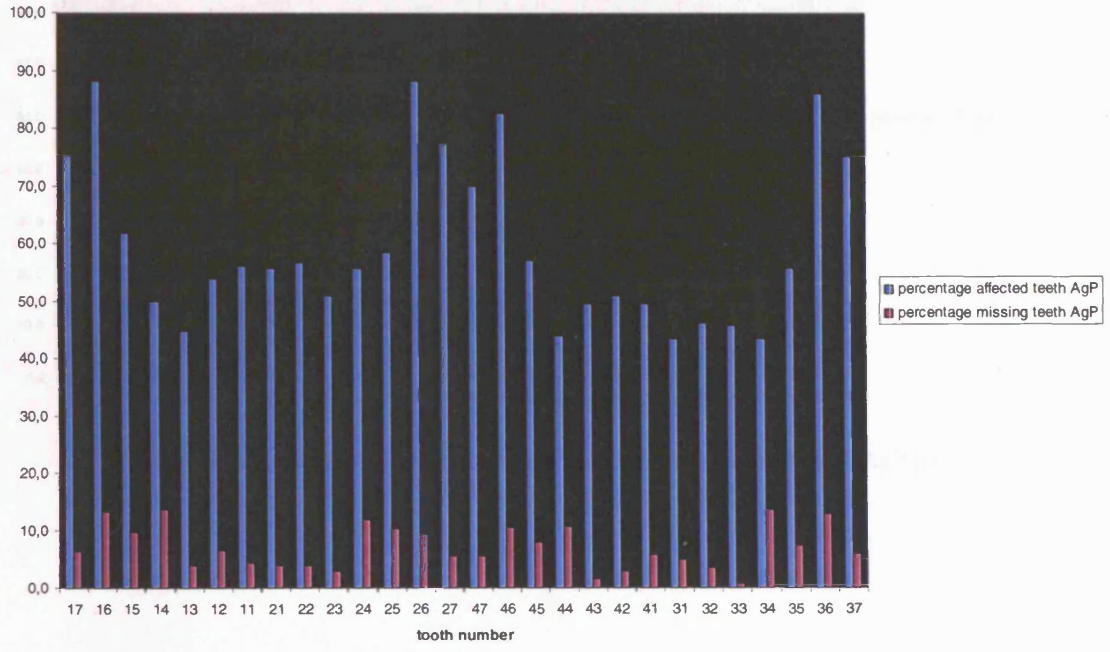

Figure 5.6: percentages of missing and affected (at least one PPD $\geq$ 5 mm) teeth in AgP patients.

Figures 5.7 and 5.8 show the percentages of affected teeth separately in the GAgP and LAgP groups. Clear differences appeared comparing the groups, with dramatic increases in all percentages in the GAgP group. These patients showed a more even distribution of affected teeth, with peaks around the molars and especially in the maxilla. The maxillary first molars were the most frequently affected (90%), whereas the least affected

maxillary tooth was 13 (UR3), which was still involved in >60% of subjects. In the mandible the least affected was 31 (LL1), which was involved in 50% of subjects. A more classical incisor-molar pattern was evident in the LAgP group. In LAgP cases, all percentages were considerably lower. First molars were the only teeth consistently affected (74%), followed by second molars (37%), second premolars (25%), incisors (20%), first premolars (12%) and canines (11%). Respectively 29 and 26% of teeth were affected in the maxilla and mandible.

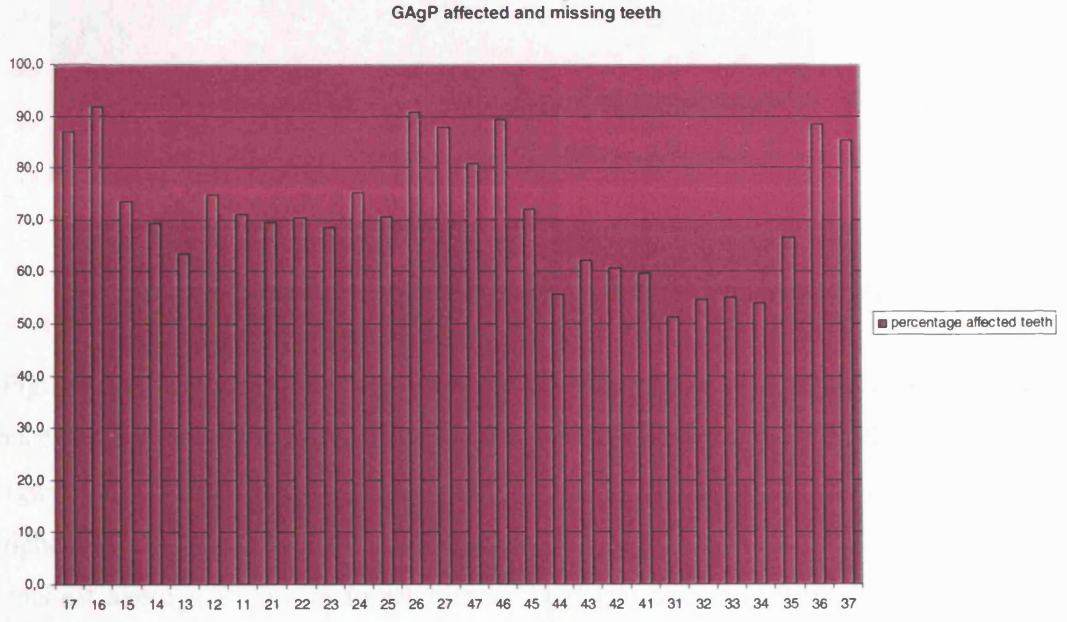

Figure 5.7: percentages of affected (at least one PPD ≥ 5 mm) teeth in GAgP patients

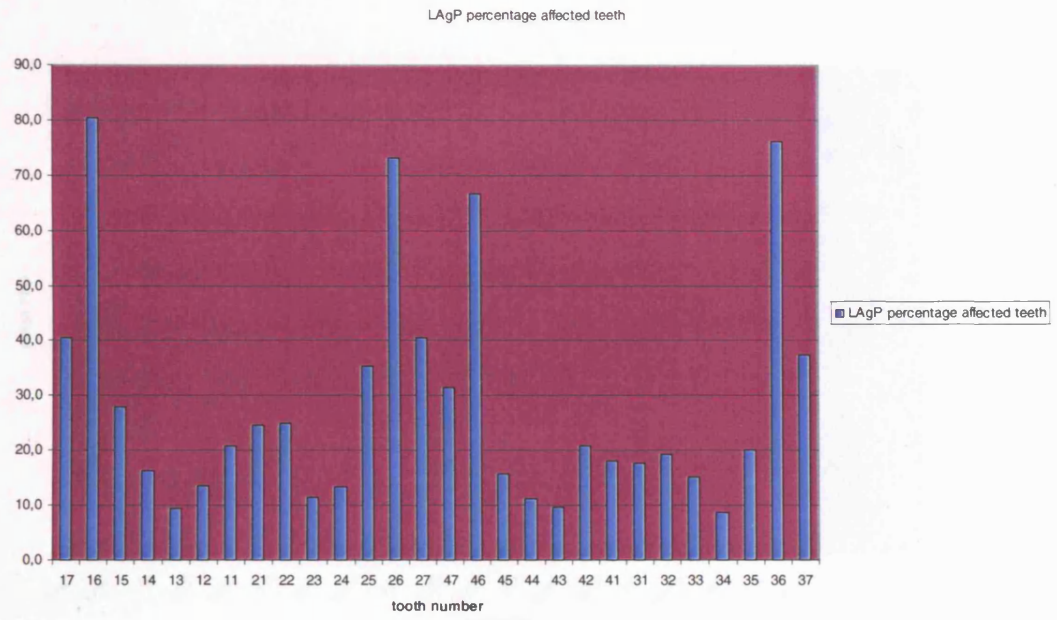

Figure 5.8: percentages of affected (at least one PPD ≥ 5 mm) teeth in LAgP patients

Figures 5.9, 5.10, 5.11 and 5.12 show the distribution for all AgP patients of mean PPD buccally and palatally in the maxilla, and buccally and lingually in the mandible. The figures show the evidently higher probing pocket depth around molars. The site with highest PPD was 36 (LL6) disto-lingual, which had an average PPD of 6.02 mm. The smallest average PPD was in relation to 34 (LL4) buccal (1.9mm). Distal surfaces of second premolars were consistently found to have a higher average PPD than the correspondent mesial surface of the same teeth. The same was observed for mesial surfaces of second molars in the maxilla, as compared to their distal surfaces. Interproximal surfaces had higher mean PPD compared to mid surfaces. Maxillary teeth had increased mean PPD compared to mandibular teeth (data reported in appendix II).

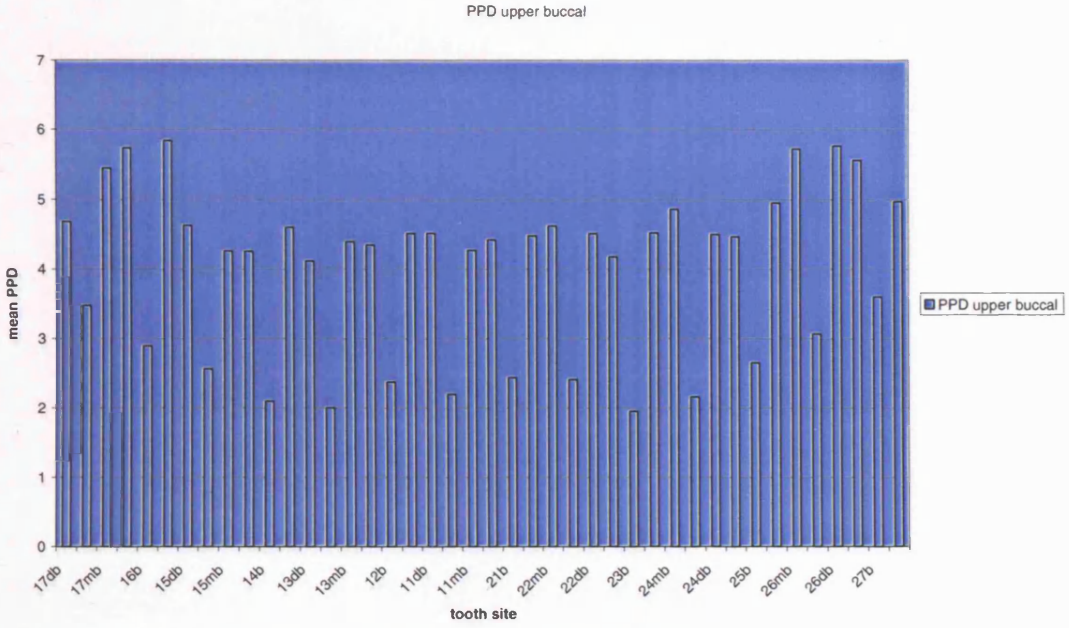

Figure 5.9: average probing pocket depths on buccal surfaces of the maxilla in AgP patients

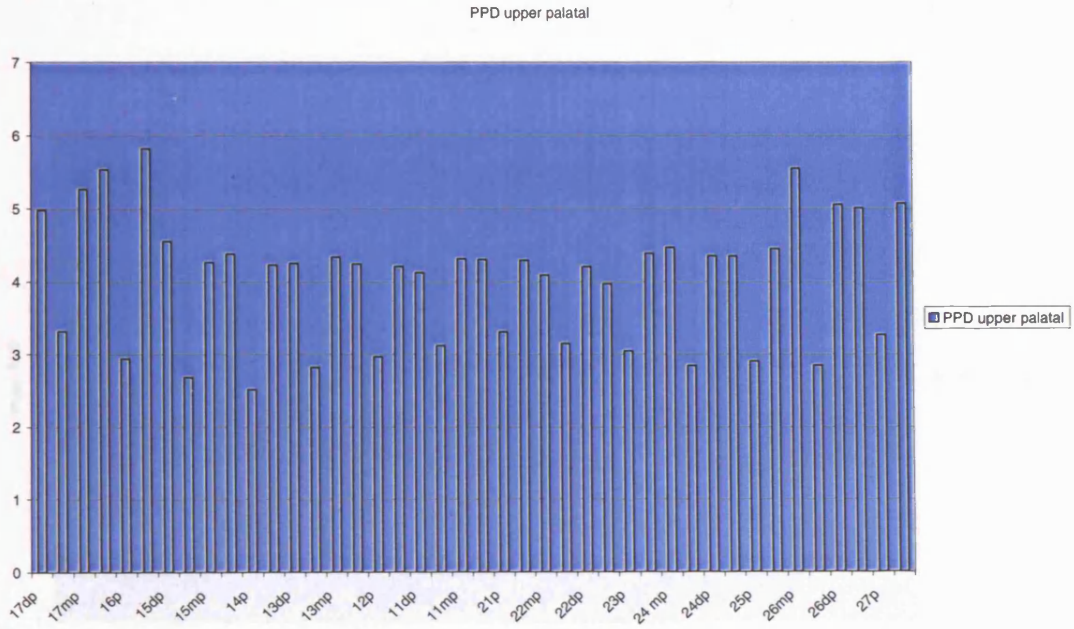

Figure 5.10: average probing pocket depths on palatal surfaces of the maxilla in AgP

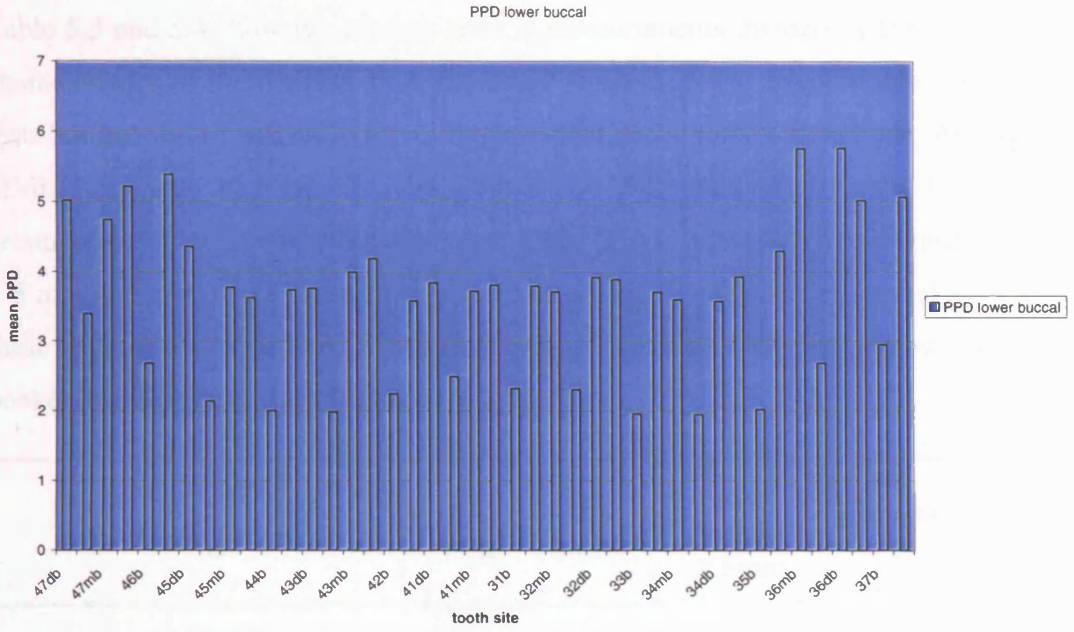

Figure 5.11: average probing pocket depths on buccal surfaces of the mandible in AgP patients

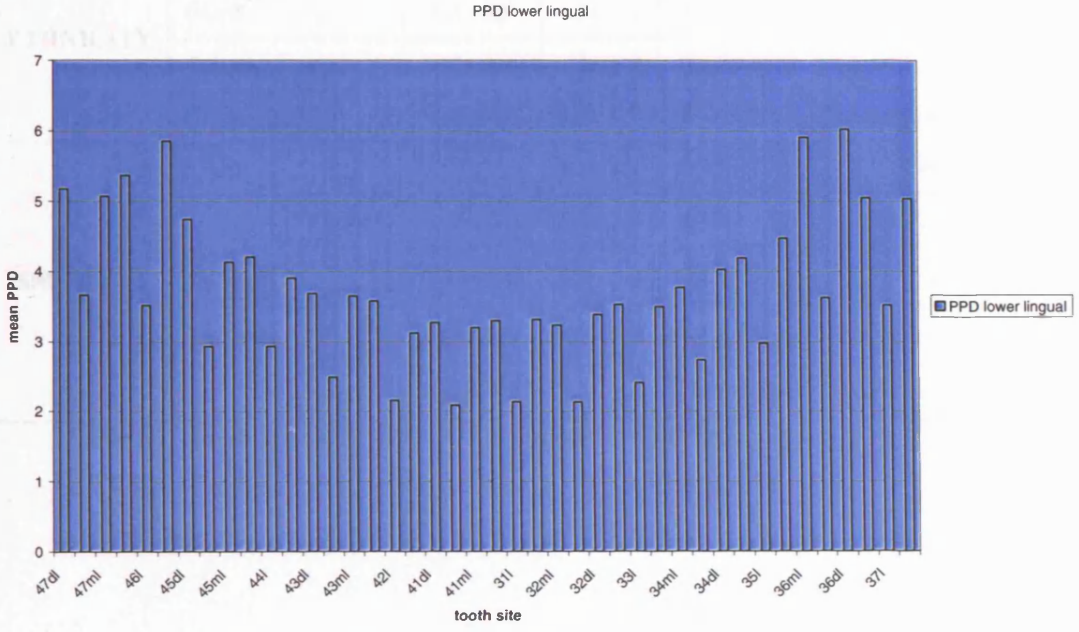

Figure 5.12. Average probing pocket depths on lingual surfaces of the mandible in AgP patients

5.3.2. Gender differences

Table 5.3 and 5.4 show the average clinical measurements divided by the demographic characteristics of the patients, both for GAgP (table 5.3) and LAgP (table 5.4). Gender distributions were constant in the different ethnic and smoking subgroups. Average age of diagnosis was 30.2 years old for women and 29.2 years old for men. In the GAgP group, men had an increase in the average PPD, LCAL, as well as in the number of PPD $\geq$ 5 mm and $\geq$ 10 mm compared to women (see table 5.3). Multivariate analysis showed these differences to be border line statistically significant only for number of probing pocket depths $\geq$ 10 mm (p=0.050).

GAgP			**Av. PPD**	**Av LCAL**	**no. pockets ≥ 5mm**	**no. pockets ≥ 10 mm**	**FMBS**
GENDER	*Female*		4.1± 0.9	4.7± 1.3	61.7± 28.8	2.3± 5.1	52.9± 52.9
	Male		4.4± 1.1	5.2± 1.8	67.9± 36.6	4.9± 6.6	55.3± 55.3
ETHNICITY	*Caucasian*		4.3± 1.1	5.1± 1.7	67.2± 34.8	3.2± 6.4	51.4± 24.3
	Black		4.3± 0.8	4.9± 1.1	63.4± 25.2	4.5± 5.8	59.0± 22.9
	Asian		3.8± 0.9	4.2± 1.2	50.7± 31.2	2.1± 3.9	44.1± 19.0
	Other		4.2± 0.9	4.8± 1.7	65.0± 30.8	1.1± 1.6	65.2± 18.7
SMOKING	*Never*		3.99± 0.9	4.5± 1.3	55.5± 27.7	3.2± 5.6	52.1± 23.3
	Former or current	*Former*	4.26± 1.1	5.2± 1.8	67.4± 35.2	4.14± 7.2	53.5± 23.3
		Current light	4.48± 1.0	5.0± 1.4	71.3± 34.1	3.00± 5.0	56.9± 24.2
		Current heavy	4.75± 0.9	5.7± 1.6	83.1± 26.6	1.81± 3.3	56.3± 24.3
		total	4.43± 1.0	5.2± 1.6	71.6± 33.6	3.3± 6.0	55.2± 23.5

Table 5.3: clinical characteristics of GAgP patients divided by gender, ethnicity and smoking

LAgP			Av. PPD	Av LCAL	no. pockets ≥ 5 mm	no. pockets ≥ 10 mm	FMBS
GENDER	*Female*		2.7± 0.6	2.9± 0.7	19.4± 13.7	1.5± 3.2	30.3± 19.1
	Male		3.0± 0.7	3.3± 0.9	26.2± 18.4	2.8± 4.4	40.2± 20.8
ETHNICITY	*Caucasian*		2.9± 0.8	3.1± 0.9	25.2± 19.9	1.9± 4.0	51.4± 18.0
	Black		2.9± 0.6	3.2± 0.8	22.8± 12.3	3.2± 4.6	59.0± 12.3
	Asian		2.6± 0.4	2.8± 0.5	16.7± 12.1	1.1± 2.0	44.1± 25.1
	Other		2.6± 0.1	2.7± 0.2	16.3± 6.4	1.0± 1.7	65.2± 47.3
SMOKING	*Never*		2.8± 0.6	3.0± 0.7	20.3± 13.0	1.9± 3.4	34.1± 20.0
	Former or current	*Former*	3.2± 0.9	3.5± 1.1	32.1± 24.8	3.3± 5.4	25.0± 21.3
		Current light	2.6± 0.3	2.8± 0.3	16.0± 5.9	0.7± 0.9	41.4± 19.3
		Current heavy	-	-	-	-	-
		total	2.9± 0.7	3.3± 0.9	24.9± 20.2	2.2± 4.2	33.2± 21.0

Table 5.4: clinical characteristics of LAgP patients divided by gender, ethnicity and smoking

5.3.3. Ethnic differences

There were higher percentages of Asian subjects affected by LAgP rather than GAgP. Asians also had a decrease in all clinical parameters when compared to Blacks and Caucasians (table 5.3), although these differences did not reach statistical significance. Multivariate analysis revealed that ethnicity was associated with higher FMBS (p=0.013).

5.3.4. Effect of smoking

In the GAgP group, smokers were found to have increased averages of PPD, LCAL and number of PPD ≥ 5 mm (table 5.3). Multivariate analysis revealed that these differences were not statistically significant in the whole group of GAgP patients. However, in the Caucasian GAgP subgroup, smoking was associated with average PPD (p=0.002), average LCAL (p=0.001) and number of PPD ≥ 5 mm (p=0.009). A similar tendency of increased disease severity was noted in the LAgP group (table 5.4), but no statistical significance was observed (probably because of the small sample size).

5.3.5. Age differences

Older subjects had higher average PPD, LCAL and number of PPD ≥ 5 mm (data not presented in tables). Multivariate analysis revealed that age was associated with increased average PPD (p=0.042), LCAL (p=0.020) and number of PPD ≥ 5 mm (p=0.042) in GAgP patients. In the Caucasian GAgP subgroup age was associated with average PPD (p=0.029) and average LCAL (p=0.003). No statistically significant associations were noted in the LAgP group.

5.4. DISCUSSION

Clinical characteristics of 224 patients diagnosed with AgP were examined. Age, gender and smoking were found to have an effect on disease severity. Older subjects, males and smokers had increased clinical parameters. The effect of age on AgP severity has been described previously (Hormand and Frandsen 1979) and is linked to the cumulative effect of aetiologic factors throughout the years. The observation that smokers have more severe disease is in agreement with the known effect of smoking on periodontitis (Haber et al. 1993) and confirms previous reports in relation to AgP (Schenkein et al. 1995, Mullally et al. 1999). The data from our patients point towards a more important effect of smoking in Caucasians (with regards to average PPD, average LCAL and number of ≥ 5 mm PPD). Nonetheless, since this study was not performed on a random population, but on patients referred by their general dentists, these results may be a reflection of health awareness and care of subgroups of individuals. For example, males and smokers may be less concerned about their oral health and consequently delay their attendance at their dental practice until the problem becomes very severe. This ascertainment bias in the supposed female preponderance in AgP was postulated by Hart et al. (1991) and confirmed by the increased percentage of women in our sample.

In agreement with previous reports, patients diagnosed with LAgP were significantly younger than GAgP subjects (Hormand and Frandsen 1979, Bial and Mellonig 1987), and a smaller percentage of them were smokers (Schenkein et al. 1995). This might mean that the effect of smoking is more evident on GAgP, and that in some LAgP cases

the disease may spread with age, especially in smokers, and evolve to become GAgP (Brown et al. 1996).

Interesting data were observed with regard to disease distribution. These data have to be interpreted carefully, because they are derived from analysis of probing pocket depths, which do not reflect exactly attachment and bone loss, and because they were collected by three different examiners. Nonetheless, periodontal pocketing is an earlier sign of periodontal disease than radiographic bone loss, which depends on the degree of demineralization of the bone and may occur only at a more advanced stage of periodontal destruction. The maxilla was confirmed to be more affected by AgP (Hormand and Frandsen 1979), with an increase in the percentage of teeth with at least one $\geq$ 5 mm periodontal pocket. First molars were the most commonly affected teeth, and also the ones with higher average probing pocket depths in both GAgP and LAgP. Among all other teeth, Hormand and Frandsen (1979) and Bial and Mellonig (1986) found incisors to be more frequently affected. We also know from the current classification that LAgP cases have an incisor-molar pattern of attachment and bone loss, with no more than 3 other teeth involved. In this report, teeth adjacent to first molars were found to be more frequently affected than incisors, not only in the whole group of patients, but also in the LAgP group. This is likely to be secondary to the presence of periodontal disease in the adjacent first molars and is confirmed by the fact that distal sites of second premolars and mesial sites of second molars show more severe disease. Currently, the possibility of spread of the periodontal pathogenic process to the surfaces adjacent to first molars has been underestimated in the classification. In practical terms, we might encounter a case of AgP where first molars and incisors are affected and, as a reflection of the first molar involvement, second premolars may also show attachment loss. Based on the current classification, we would have to classify this as a GAgP, even if the pattern is genuinely that of LAgP.

In conclusion, data from the AgP group presented here can be summarized as follows:

- Age, male gender and smoking are associated with periodontal disease severity. LAgP may evolve into GAgP with age, especially in smokers.
- Severe periodontal pockets in AgP are more common in the maxilla and affect mainly first molars and their adjacent teeth, followed by incisors.
- More accurate clinical diagnosis is required to distinguish clear cases of LAgP from GAgP.

Further studies should be conducted in order to compare the disease distribution in large cohorts of GAgP, LAgP and CP patients. Differences in extent, severity and type of affected teeth may contribute to a better differential diagnosis between these different disease entities.

CHAPTER 6

6. GENETIC ASSOCIATION STUDY

6.1. Background

The genetic background of Aggressive Periodontitis was discussed in the introductory chapter of this thesis. AgP is commonly thought to be a multifactorial polygenic disease, determined by the interaction of environmental and genetic factors. A very small percentage of individuals seem to exhibit a strong predisposition to the periodontal damage characteristic of AgP. Environmental factors such as cigarette smoking and the presence of specific bacteria in the subgingival plaque are thought to trigger the onset of AgP in susceptible individuals (Kinane et al. 2005). Early onset periodontitis represents a common feature of a few single gene disorders, such as Papillon- Lefevre syndrome and some genes able to determine early onset periodontitis have been identified (Kinane et al. 2005). These single gene disorders, however, can explain only a minimal subset of early onset periodontitis cases. All the others seem to be consistent with a polygenic pathogenesis. However, despite increases in the knowledge of the human genome and of the role of up to 50,000 human genes, we still do not know which genetic risk factors are able to determine a subtle variation in the immune response, which manifests itself only through a modification of the AgP risk, with no other consequences elsewhere in the body.

While in single gene defects a single mutation is able to cause the onset of a disease, in polygenic diseases, a range of different genetic variants in several genes are responsible for determining the predisposition to the disease. Millions of these genetic variants, which are called polymorphisms, exist in the human genome. Two types of genetic variants are distinguished: diallelic polymorphisms (SNPs, single nucleotide polymorphisms) and multiallelic markers (Collins et al. 1999). In diallelic SNPs, the two alleles give three possible combinations (genotypes): homozygosity for the common allele (wild type), homozygosity for the rare allele or heterozygosity (presence of one copy of each allele).

Approaches to the detection of genetic risk factors for the onset of a disease include family studies (including twin studies, linkage and segregation studies) and case-control

association studies. In these latter studies genetic polymorphisms are studied in two cohorts of subjects, one affected by the disease and one diagnosed as not affected by that particular disease. Several polymorphisms have been suspected as potential susceptibility factors for AgP and have been tested in case-control association studies. Conflicting results have emerged from most studies, and still no genetic polymorphisms have been confirmed as risk factors for AgP. In chapter 2, an overview of the most promising polymorphisms was presented.

The aim of this study was to detect genetic polymorphisms as risk factors for the onset of AgP in a large cohort of subjects of mixed races. Considering the close relation between ethnicity and genetic polymorphisms, subgroup analyses were conducted for the different ethnic groups. The null hypothesis tested was that there was no difference in the distribution of the studied genotypes between the groups of patients and healthy controls.

6.2. MATERIALS AND METHODS

In chapter 1, several important aspects in relation to the design of genetic association studies were discussed. These aspects concern the definition of disease and healthy status, the selection of subjects and the selection of a panel of genetic polymorphisms. The next subparagraphs deal with the ways we attempted to overcome the challenge represented by all these factors.

6.2.1. STUDY DESIGN

6.2.1.1. Definition of disease

Confusion reigns in the AgP genetic literature in terms of the definition of disease, because of the recent introduction of the new classification (Armitage 1999), and because of the scope it leaves to personal interpretation, especially with regard to the differential diagnosis between AgP and the more severe cases of Chronic Periodontitis. The diagnosis of AgP applied to this study was very strict. Only patients whose degree of periodontal destruction could not be correlated with the cumulative effect of local factors were included in the study (see chapter 4 for exact inclusion and exclusion

criteria). By doing so, we aimed to include only subjects with severe periodontal destruction, not explicable other than by the presence of a highly predisposing genotype. This aimed at reducing the risk of including false positives in the study.

6.2.1.2. Definition of controls

6.2.1.2.1 Definition of healthy status

According to the new classification, age no longer represents one of the criteria for diagnosis of AgP. However, it is assumed that the onset of AgP is usually in adolescence or early adulthood. Therefore, it is highly unlikely that young adults who are examined and found to have no periodontal destruction would have a genotype showing susceptibility to AgP. Therefore, in order to reduce the risk of including subjects who may later develop AgP (false negatives), only patients at least 25 years old were enrolled. Volunteers with known genetic diseases, infections or severe medical conditions (e.g. AIDS) or history of periodontal disease or tooth loss due to periodontal disease were not included. Clinical exclusion criteria were different for individuals younger than 35 years of age compared to those older than 35 years (more stringent criteria were applied to younger subjects):

- $\leq$ 35 years old subjects were excluded if they presented with at least one site with PPD and LCAL $\geq$ 4 mm.
- > 35 years old subjects were excluded if they presented with one or more sites with PPD and LCAL $\geq$ 5 mm.

6.2.1.2.2 Stratification bias

One of the objectives of the control recruitment was to obtain a group of subjects as similar as possible to our patient group, the only constant difference being their periodontal status. In order to achieve this aim, we recruited subjects referred to other Departments of the Eastman Dental Hospital. Therefore, these two groups of subjects (AgP patients and controls) belong to a similar socio-economic group of subjects registered with the National Health System in the United Kingdom, and referred to the EDH for care. All control subjects had some form of dental problems, but no periodontal

disease. No individual matching was attempted in terms of smoking, ethnicity and gender.

The degree of non-response to participation in the control group was low, with about 90% of the suitable individuals who were asked to participate in the study agreeing. An even higher percentage of responders (subjects who agreed to have a blood sample taken for genetic analysis) was noted in the patient group (only 6 patients out of 230 declined). We can therefore suppose that any bias produced by non-responders in the two groups was minimal, and was likely to be balanced in the two groups.

6.2.1.3. Power

No large genetic association studies have been published so far in the Early Onset / Aggressive periodontal literature. Most published studies include less than 100 subjects in each group (cases/controls). These numbers might not be enough to detect subtle differences in genotype distributions, such as the ones we expect in a multifactorial disease such as AgP. We based our power calculation on a pilot study (Brett et al. 2003) performed on a total of 90 AgP (50 of whom were Caucasians), 82 CP patients (55 of whom were Caucasians), and 100 Caucasian blood donors. Frequencies of IL-1A -889, IL-1 B -511 and +3954, IL-6 -174, IL-10 -627 and -1082, TNF-α -308, TLR4 299 and 399 and VDR Taq-I (1056) polymorphisms were studied in patients and controls. Statistical analysis performed by Clump software revealed that IL-1 +3954 was associated with AgP, while IL-6 -174, VDR and TLR4 399 were associated with the periodontitis trait. The limitations of this pilot study, apart from a small sample size, include the failure to take account of environmental factors in the analysis and also the lack of knowledge regarding the periodontal conditions of the controls, some of whom may have had periodontitis. The sample size calculation was based on the results relative to the IL-6 genotype, which showed no statistically significant association with the AgP trait, although a trend was present for an increase in prevalence of G allele in patients, and an increase of C homozygosity in controls. N-Query advisor® software was used in order to calculate the sample size for this study. The preliminary analysis indicated a prevalence of 10% in AgP Caucasians and 25% in Caucasian controls for IL-6 C

homozygosity. A total sample size of 250 cases and 250 controls would give an overall study power of 99% to detect an odds ratio of 3 of belonging to the AgP group. Assuming 65% of our patients were Caucasians, within this subgroup the power would be 93% to detect a similar odds ratio. Since the preliminary analysis was undertaken using a control group of unknown periodontal status, the actual odds ratios are likely to be greater than those indicated in that analysis. Therefore, these power calculations represented a conservative estimate of the study power. The number of subjects recruited for this study falls slightly short of 250 in each group. This still represents a >90% power to detect significant associations.

6.2.1.4. External validity

Our group of patients and controls belongs to a very heterogeneous community of subjects living in London. This heterogeneity may represent a problem in terms of the interpretation of the genetic data; on the other hand, it also makes it easier to generalise our results, because they are not obtained in a small secluded population. Even restricting the analysis to the subgroup of Caucasians, the same consideration can be applied.

6.2.1.5. Choice of genetic marker

At least one million SNPs exist in coding regions of the human genome, all theoretically able to have a role in disease susceptibility (Collins et al. 1999). Based on the existing literature on the pathogenesis and genetics of AgP, the task of selecting a panel of candidate genetic loci to be tested is not easy. A whole range of genetic polymorphisms have been suspected to play an important role in the susceptibility to AgP: Interleukin-1 (Diehl et al. 1999, Parkhill et al. 2000, Hodge et al. 2001, Tai et al. 2002, Quappe et al. 2004), Interleukin-4 (Michel et al. 2001, Gonzales et al. 2004), Interleukin-10 (Kinane et al. 1999a), TNF-α (Kinane et al. 1999a), Vitamin D receptor (Hennig et al. 1999, Yoshihara et al. 2001), Fcα receptor (Kaneko et al. 2004), Fcγ receptors (Fu et al. 2002, Chung et al. 2003, Loos et al. 2003, Yasuda et al. 2003, Yamamoto et al. 2004), FP receptors (Gwinn et al. 1999, Zhang et al. 2003), estrogen receptor α (Zhang et al. 2004), matrix metalloproteinase 1 and 3 (Itagaki et al. 2004),

matrix metalloproteinase 9, cathepsin G, collagen and many others (Suzuki et al. 2004). These markers and others have also been studied in relation to Chronic Periodontitis (Kinane & Hart 2003).

Considering the evidence in the literature with regard to neutrophil function in AgP (Kantarci et al. 2003), it was decided to focus this association study mainly on the role genetic polymorphisms regulating PMN function may have in predisposing to AgP. Therefore, the panel of SNPs chosen for investigation in this study was as follows: Fcα -324 (Kaneko et al. 2004), FcγRIIa 494 (Carlsson et al. 1998), FcγRIIb 232 (Yasuda et al. 2001), FcγRIIIa 559 (Wu et al. 1997), FcγRIIIb NA (Bux et al. 1997), FcγRIIIb SH (Bux et al. 1997), FPR 301 (Zhang et al. 2003), FPR 546 (Zhang et al. 2003), FPR 568 (Zhang et al. 2003) (see paragraph 6.3.3 for selection of FPR markers), $p22^{phox}$ 242 NADPH oxidase (Parkos et al. 1988). In addition to these genetic markers of PMN function, two genetic polymorphisms of cytokines involved in periodontal disease pathogenesis [Interleukin-6 -174 (Fishman et al. 1998), TNF- α -308 (Wilson et al. 1997)] and one involved in the immune response and in bone metabolism [VDR Taq-I (Ensrud et al. 1999)] were also selected. Therefore, a total of 13 candidate SNPs were selected. All these polymorphisms are SNPs caused by a single nucleotide substitution. Each individual presents two alleles for the correspondent nucleotide. Therefore, for each polymorphism, three possible genotypes exist: two copies of the common allele (homozygosity for the common allele), two copies of the rare allele (homozygosity for the rare allele) or one copy of each allele (heterozygosity). A summary of the chosen SNPs, their gene location, and their supposed function is represented in table 6.1.

Molecule	Chromosome	Function	Polymorphism	Amino acid change	Suspected effect
IL-6	7p21	Pro-inflammatory cytokine Stimulation of osteoclasts	-174 G-C promoter region (negative regulatory domain)	NO (regulatory gene)	Allele C: inhibition of production of IL-6
TNF-α	6p21	Pro-inflammatory cytokine Effect on lipid metabolism, insulin resistance	-308 A-G promoter region	NO (effect on transcription al activity)	Allele A: higher transcription levels of TNF-α
VDR	12q12-14	Ca metabolism Immune modulation Regulation of osteoblasts	Taq-I (1056)	synonymous	Linkage disequilibriu m with another SNP (?)
Fc-αR	19p13.4	Bacterial recognition (IgA- PMN)	324 A-G IgG-like domain (ligand-binding site)	synonymous	Allele A: decreased phagocytosis of *P.gingivalis*
Fc-γR IIa	1q21-24	Bacterial recognition (IgG2-PMN)	494 A-G	131 H-R in IgG binding site	Allele H: > receptor affinity and specificity, and phagocytosis of IgG-2 opsonized particles
Fc-γR IIb	1q21-24	Bacterial recognition (IgG- lymphocytes)	695 C-T	232 I-T in exon 5	?? unknown
Fc-γR IIIa	1q21-24	Bacterial recognition (IgG-PMN)	559 G-T	176 V-F	Amino acid V: > affinity for IgG1,3,4
Fc-γR IIIb	1q21-24	Bacterial recognition (IgG-PMN)	141 C-G (NA1/NA2)	4 amino acid substitutions first extracellular domain	NA1: different glycosilation, different ligand activity, > phagocytosis of *P.gingivalis*

Molecule	Chromosome	Function	Polymorphism	Amino acid change	Suspected effect
Fc-γR IIIb	1q21-24	Bacterial recognition (IgG-PMN)	266 C-A	78 Ala-Asp 4 amino acid substitutions first extracellular domain (SH antigen)	?? unknown
FPR	19 (FPR-1 gene)	Bacterial recognition, chemotaxis, phagocytosis, oxidative burst	301 G-C	101 Val-Lys	Similar amino acids (small change)
FPR	19 (FPR-1 gene)	Bacterial recognition, chemotaxis, phagocytosis, oxidative burst	546 C-A	synonymous	-
FPR	19 (FPR-1 gene)	Bacterial recognition, chemotaxis, phagocytosis, oxidative burst	568 A-T	190 Arg-Tryps (2^{nd} extracell. loop, important for ligand affinity)	Different ligand affinity
P22phox NADPH oxidase	16 (CYBA gene)	Superoxide production in PMN and osteoclasts	242 C-T	72 Hys- Tim (heme-binding site)	Modulation of superoxide production

Table 6.1: Schematic representation of the panel of SNPs selected for the study, giving name of the molecule, relative gene, chromosome location, function, details of the polymorphisms and their suscepted effects.

6.2.2. SELECTION OF SUBJECTS

Consecutive AgP patients referred to the Department of Periodontology of the Eastman Dental Hospital and Institute were recruited as described in chapter 4. Healthy controls were recruited from subjects referred to the Departments of Oral Surgery, Conservation, Endodontics and Staff Dental Service. Additional subjects were selected among patients referred for assessment to the implant clinic of the Periodontal Department, and from subjects screened for participation in the IQE (International Qualifying Examination); these consisted mainly of students and staff at University College London, who needed dental examination or treatment. A total of 100 subjects were selected from Oral

Surgery, 52 from Endodontics, 50 from the IQE screening, 16 from the Conservation Department, 11 from Staff Dental Service and 2 from the Periodontal Department.

Control subjects were approached when they attended one of these clinics at the EDH. The purpose of the study was explained to them and they were asked to participate. Suitable subjects at least 25 years old who volunteered underwent a specific periodontal examination, performed by a single examiner. The examination consisted of assessment of oral hygiene, gingival appearance and recording of Community Periodontal Index for Treatment Needs (CPITN). Panoramic radiographs were also obtained. Their smoking history and their parents' ethnic origins were recorded. Individuals who at the examination were found to satisfy the clinical entry criteria indicated in paragraph 6.2.1.2.1 had a blood sample taken for DNA extraction, in the same fashion as for the AgP patients.

6.2.3. GENETIC ANALYSIS

6.2.3.1. DNA extraction

DNA was extracted from leukocytes using Nucleon® BACC2 kit (Nucleon Bioscience, Coatbridge, UK) according to manufacturer's instructions. Briefly, blood samples collected in EDTA tubes were thawed at room temperature and 30 ml of reagent A (red cell lysis buffer; 10 mM Tris-HCL pH 8; 0.32 M sucrose; 5 mM $MgCl_2$; 1% Triton X-100) were added to 10 ml of blood in a 50 ml polypropylene centrifuge tube. The contents were mixed by inverting several times and then centrifuged at 1300g for 5 min to sediment the leukocytes. The supernatant was discarded and 2.0 ml of reagent B (white cell lysis buffer) and 300 μg of proteinase K (Invitrogen, Paisley, UK) were added to the cell pellet. After vortexing, the tubes were incubated at 37° C for 30 min and 0.5 ml sodium perchlorate (0.5 M) was added, the contents mixed by inversion, then 2.0 ml chloroform added and mixed again. 300 μl of Nucleon® resin was added and tubes centrifuged at 1300g for 3 min to separate the phases; this was followed by the removal of the upper aqueous phase containing the DNA which was transferred to a fresh tube. Two volumes of cold ethanol were added to precipitate the DNA and the

DNA resuspended in 250 µl of sterile water. The DNA concentration was estimated by measuring absorbance at a 260 nm wavelength using a spectrophotometer. 10 ng of DNA were subsequently used for polymerase chain reaction (PCR) analysis.

6.2.3.2. Genetic polymorphism analysis

Allelic discrimination assays were performed using the Applied Biosystems 7300/7500 Real Time PCR System. Some of the primers and probes (listed in table 6.2) were designed using the Assay-by-Design service offered by Applied Biosystems (Warrington, Cheshire, UK). The other primers and probes (listed in table 6.3) were obtained from Applied Biosystems from their Assays-on-Demand products. Genotyping was performed in 25µl reactions consisting of 10ng of genomic DNA, 12.5µl of 2X Taqman Universal PCR Master Mix, either 0.625µl (40X) or 1.25µl (20X) primer/probe sets, with the addition of purified water. Cycling conditions were 2 m at 50°C; 10 m at 95°C; 40 cycles of 95°C for 15 s; 60°C for 1 m. Real-time fluorescence detection was performed during 60°C annealing/extension step of each cycle. The allelic discrimination assay is a multiplexed, end-point assay which detects variants of a single nucleic acid sequence. The presence of two primer/probe pairs in each reaction allows genotyping of the two possible variants at the SNP site in a target template sequence. Two fluorescent dye detectors are used, one being a perfect match to the wild type, the other one to the mutation. The allelic discrimination measures the change in fluorescence of the dyes associated with the probes. 7300/7500 SDS software plots 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. Examples of the scatter plots showing the distribution of the genotypes for each SNP are shown in appendix III to XIV. Hidden duplicates were added to each plate to test error rates. However, no detection errors were observed.

Gene SNP	Substitution	Oligo	Sequence
IL-6-174	G to C	*Probe-G*	VIC-CCTTTAGCATCGCAAGAC-MGB
		Probe-A	FAM-CTTTAGCATGGCAAGAC-MGB
		Primer-F	GACGACCTAAGCTGCACTTTTC
		Primer-R	GGGCTGATTGGAAACCTTATTAAGATTG
FCγRIIB	T to C	*Probe-T*	VIC-CTACAGCAATCCCAG-MGB
		Probe-C	FAM-CTACAGCAGTCCCAG-MGB
		Primer-F	CCAAGCTCCCAGCTCTTCAC
		Primer-R	CCACTACAGCAGCAACAATGG
FCγRIIIB NA	G to C	*Probe-G*	VIC-CAATGGTACAGGGTGCT-MGB
		Probe-C	FAM-CAATGGTACAGCGTGCT-MGB
		Primer-F	CCAAAGGCTGTGGTGTTCCT
		Primer-R	CTTCAGAGTCACACTGTCCTTCTC
FCγRIIIB SH	C to A	*Probe-C*	VIC-CTTCATTGACGCTGCCAC-MGB
		Probe-A	FAM-TTCATTGACGATGCCAC-MGB
		Primer-F	AGCCAGGCCTCGAGCTA
		Primer-R	TGTACTCTCCACTGTCGTTGACT
FPR-301	G to C	*Probe-G*	VIC- CCTGTGCAAATTCGTCTT-MGB
		Probe-C	FAM-CTGTGCAAATTCCTCTT-MGB
		Primer-F	TGGCCTTTCGGCTGGTT
		Primer-R	AGCGGTCCAGAGCAATGAG
FPR-546	C to A	*Probe-C*	VIC-AACTTTTCGCCCTGGAGG-MGB
		Probe-A	FAM-TTTTCGCCATGGAGG-MGB
		Primer-F	GGGACAGTAGCCTGCACTTTT
		Primer-R	TGCCTCTCACCGTCAACATG
FPR-568	A to T	*Probe-A*	VIC-CCCTAAAGAGAGGATAAA-MGB
		Probe-T	FAM-CCCTAAAGAGTGGATAAA-MGB
		Primer-F	GGACAGTAGCCTGCACTTTTAACTT
		Primer-R	TGCCTCTCACCGTCAACATG
FCγRIIIA	G to T	*Probe-G*	VIC-CCAACAAGCCCCC-MGB
		Probe-T	FAM-CCCAAAAGCCCCC-MGB
		Primer-F	GACAGCGGCTCCTACTTCTG
		Primer-R	GACAGCGGCTCCTACTTCTG

Table 6.2: Gene polymorphism real-time probe/primer combinations designed using Assay-by Design service from Applied Biosystems

Gene SNP	Substitution	National Centre for Biotechnology Information (NCBI) dbSNP ID number
FCγRIIA 494	A to G	rs1801274
FCαR 324	A to G	rs1865096
TNFα (-308)	A to G	rs1800629
VDR Taq-I	T to C	rs731236

Table 6.3: Inventoried SNP genotyping assay products from Applied Biosystems

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 amplified by PCR were run on an electrophoretic gel and then visualized under UV light. 10 ng of DNA were used for polymerase chain reaction (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), 2mM $MgCl_2$ and 50 pmol of each of the primers and 1U of taq polymerase (Abgene, 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) and are given below: sense primer 5' TGCTTGTGGGTAAACCAAGGCCGGTG 3' and antisense primer 5' AACACTGAGGTAAGTGGGGGTGGTCCTGT 3'; cycling conditions: 95° C for 5 min, 35 cycles of 95° C for 30 sec., 62° C for 30 sec., 72° C for 1 min. The PCR products were digested at 37° C for 17 h using 5 u 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 1 TBE buffer at 100 V for 2 h. The bands were visualised using UV transilluminator. The products yielded were: 188bp + 160 bp (allele T) and 348bp (allele C). An example of one of the results for this polymorphism as obtained by gel electrophoresis is reported in appendix XV.

All the results were confirmed by a blind examiner, unaware of the origin of the genomic DNA. 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. The total numbers of detected alleles for each genotype are reported in table 6.11. Out of a total of 5785 genotypes to be detected, only 0.01% was not scored. Therefore, this should not affect the reliability of our results.

6.2.3.3. Sequencing analysis

Sequencing of the region of the FPR gene containing candidate SNPs (Zhang et al. 2003) was performed prior to SNPs analysis in a subset of subjects in order to detect possible informative SNPs in the gene. Polymorphisms at nucleotide 301, 306, 329, 348, 378, 546, 568 and 576 were analyzed in 31 LAgP patients and 28 healthy controls. DNA purification was performed before the sequencing reaction. In brief, 15 µl of H_2O were added to 5 µl of DNA and transferred to a 0.5 ml tube on ice. Then 2 µl 3M sodium acetate and 50 µl of 95% ETOH were added, and the tubes were incubated for 10-15 minutes, after which they were centrifuged at 14 k for 20 minutes at 4° C. The liquid was then removed, and 250 µl of 75% ETOH were added. The tubes were centrifuged again at 14 k for 15 minutes at 4° C and the ETOH removed. The samples were then dried for a few seconds at 95° C and resuspended in 15-20 µl of TSR and vortex mixed. Subsequently, the samples were heated at 95° C for 2-3 minutes, vortex mixed and placed on ice. The reagents used were 2 µl of 1:4 buffer- diluted ABI stock®, 10-30 pmoles of primer and 200-400 ng of DNA in a final volume of 5-7 µl. The sequencing reaction followed this protocol: rapid thermal ramp to 95° C, 95° C for 10 seconds, rapid thermal ramp to 50° C, 50° C for 5 seconds, rapid thermal ramp to 60° C, 60° C for 4 minutes, followed by rapid thermal ramp to 4° C and hold. ABI 310® genetic analyzer was used for sequencing. The resulting peaks, corresponding to the DNA bases, were analyzed by Chromas® software, which allowed to read the DNA sequence.

6.2.3.4. Hardy-Weinberg equilibrium

Nearly one century ago, two scientists (Godfrey Hardy and Wilhelm Weinberg) independently developed a formula to calculate the distribution of genotypes in a population throughout generations (Crow 1999). Until then, it was believed that rare alleles would decrease in frequency with time, and eventually disappear. They concluded, based on probability considerations, that genotype frequencies are stable and wouldn't change if there was no evolution of the species. Hardy and Weinberg developed an equation that can be used to discover the expected genotype frequencies in a population. This formula is now known as the Hardy-Weinberg equilibrium. In this equation $(p^2+ 2pq+ q^2) = 1$, p is the frequency of the dominant allele and q is the frequency of the recessive allele for a trait (Crow 1999). The Hardy-Weinberg law fails to apply in cases of mutations, considerable introduction in a population of individuals from other communities, non random mating and natural selection. In this last event, if a particular genotype makes individuals less able to produce offspring or less successful to survive until the end of their reproductive period, that genotype is likely to undergo a reduction in population frequency. The Hardy-Weinberg equilibrium was tested in all the studied polymorphisms in our sample.

6.2.4. STATISTICAL ANALYSIS

The GC utilities package was used for linkage disequilibrium (LD) analysis between genetic markers. The LD pairs programme allowed us to investigate if any genetic polymorphisms were in linkage disequilibrium with each other. Pair-wise 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 linkage disequilibrium in the whole group of subjects, and in both patients and controls separately. R squared 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).

Statistical analysis was performed using the SPSS 12.0 package. Continuous, normally distributed variables were reported as means ± standard deviation (SD). Comparisons of continuous and categorical data between groups were analyzed with ANOVA and Chi-

square test, respectively. For these analyses, the alpha value was set at 0.05. However, because of the inherent problem of multiple testing, which can lead to spurious results (Altman 1991), the alpha value for association between genetic SNPs and AgP was set at 0.01. Chi- square 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 as well as presence or absence of alleles (for example, supposing the two alleles were C and G, GG versus CG and CC, and CC versus CG and GG) in the AgP and control groups. These analyses allowed investigation of different types of genetic models. Only the results 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 (Schenkein et al. 1995, Tonetti & Mombelli 1999), 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.

Logistic regression analysis considers each SNP as an independent variable, without taking into account interactions between polymorphisms, even if these are located in the same gene (Sham et al. 2004). Haplotype associations were analyzed in this study by use of the WHAP package. This program provides a method to test haplotype associations with qualitative and quantitative traits (ref. www.genome.wi.mit.edu/~shaun/whap/). Haplotypes with a frequency of less than 1% are automatically excluded from analysis. Different thresholds can be chosen in order to exclude rare haplotypes (< 2% or <5% for example). 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. For example, FPR 301 polymorphism association with the disease trait was tested alone, in combination with 546, in combination with 568, and with both 546 and 568

together. 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).

6.3. RESULTS

A total of 224 AgP patients and 231 healthy controls took part in the study. Their demographic characteristics are presented in table 6.4. Due to our age criteria for recruitment, controls were on average older than the patients (controls average age: 38.4 range 25-77; patients average age: 29.9 range 10-45). No statistically significant differences were detected in the two groups for gender, smoking, and ethnicity. However, a higher percentage of males, Caucasians and non smokers were detected in the control compared to the patient group.

		PATIENTS		CONTROLS		Comparisons between groups
		(n=224)	%	(n=231)	%	
AGE		29.9 ± 7.2	-	38.4 ± 12.2	-	-
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 Chi-square*
	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 Chi-square*
	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*	

Table 6.4: Comparison of demographic characteristics of patients and controls

Table 6.5 and 6.6 report the demographic characteristics of subjects belonging to the two biggest ethnic subpopulations of the study, respectively Caucasians and Blacks. Among

Caucasians, a statistically significant imbalance was noted for gender and smoking, with higher proportions of women and smokers present in the Caucasian patient group when compared to controls. In the Black subgroup the opposite tendency was observed: fewer women and fewer smokers were present in the patient group compared to the control group.

CAUCASIANS		PATIENTS		CONTROLS		Comparisons between groups
		(n=106)	%	(n=144)	%	
AGE		29.0 ± 7.2	-	39.0 ± 13.5	-	-
GENDER	*Male*	36	*32.1*	70	*48.6*	*P=0.010 Fisher's exact test*
	Female	76	*67.9*	74	*51.4*	
SMOKING	*Non smokers*	38	*33.9*	81	*56.3*	*P=0.002 Pearson's Chi-square*
	Former smokers	37	*33.0*	30	*20.8*	
	Light smokers (< 20/day)	23	*20.5*	26	*18.1*	
	Heavy smokers (≥ 20/day)	14	*12.5*	7	*4.9*	

Table 6.5: Comparison of demographic characteristics of Caucasian patients and controls

BLACKS		PATIENTS		CONTROLS		Comparisons between groups
		(n=59)	%	(n=45)	%	
AGE		29.2 ± 8.6	-	38.6 ± 9.5	-	-
GENDER	*Male*	25	*42.4*	12	*26.7*	*P=0.105 Fisher's exact test*
	Female	34	*57.6*	33	*73.7*	
SMOKING	*Non smokers*	43	*72.9*	26	*57.8*	*P=0.277 Pearson'sChi-square*
	Former smokers	5	*8.5*	6	*13.3*	
	Light smokers (< 20/day)	10	*16.9*	13	*28.9*	
	Heavy smokers (≥ 20/day)	1	*1.7*	0	*0*	

Table 6.6: Comparison of demographic characteristics of Black patients and controls

Table 6.7 represents the demographic characteristics in the group of GAgP and LAgP patients. The subjects diagnosed with Localised AgP were on average younger. No gender differences were detected between the groups, with both LAgP and GAgP showing more females than males affected, with a ratio of approximately 2:1. Among LAgP patients the proportions of smokers was lower (p= 0.015).

		GAgP		LAgP		Comparison between groups
		(n=167)	%	(n=57)	%	
AGE		31.4 ± 6.0		25.6 ± 8.4		*p< 0.001*
GENDER	***Male***	59	35.3	20	35.1	*p= 1.000 Pearson Chi-square*
	Female	108	64.7	37	64.9	
ETHNICITY	***Caucasian***	88	52.7	24	42.1	*p= 0.226 Pearson Chi-square*
	Afro-Caribbean	45	26.9	14	24.6	
	Asian	21	12.6	13	22.8	
	other	13	7.8	6	10.5	
SMOKING	***Non smokers***	79	47.3	39	68.4	*p= 0.015 Pearson Chi-square*
	Former smokers	42	25.1	10	17.5	
	Light smokers (< 20/day)	30	18.0	8	14.0	
	Heavy smokers (≥ 20/day)	16	9.6	0	0	

Table 6.7: Comparison of demographic characteristics of GAgP and LAgP patients

6.3.1. FPR gene sequencing

During the sequencing analysis performed on 31 LAgP patients and 28 controls, two of the reactions did not give clear results for the first SNPs in the selected coding region of the FPR gene. Therefore, the markers from 301 to 378 were assessed only in 12 of the 28 controls and in 31 of the patients. None of the mutations described by Gwinn et al. (1999) in a LAgP Black population were detected in any of the samples included in this study: T homozygosity and C homozygosity were always found respectively at position 329 and 378. At position 306, the T mutation was detected only in 1 AgP patient,

confirming the rarity of this allele as observed by Zhang et al. (2003), which did not make it a realistic candidate risk indicator for AgP. Chi-square analysis revealed that the 301 polymorphism had a tendency for association with the AgP trait ($p<0.001$, but this result was not reliable because of the small sample size), while no associations were found for the 306 ($p=1.000$), 348 ($p=0.804$), 546 ($p=0.247$) and 568 ($p=0.799$) polymorphisms. The polymorphism at position 576 is three-allelic, therefore either a thymine, a cytosine or a guanine can be present. Being three-allelic, it is not suitable for real time PCR analysis. In our sample, the percentages of C, G or T allele frequencies were respectively 17, 35 and 48% in patients and 14, 48 and 38% in controls, and the genotype distribution was not statistically different ($p=0.470$). All these results have to be taken with caution, because of the small sample size and of the imbalance in ethnicities between patients and controls, and were used only as a screening tool to detect the most promising association and exclude the unlikely ones (as for the 329 and 378 polymorphisms). Based on these results, the 301 (which showed a statistically significant association with the disease) and 546 polymorphisms (which showed a trend for association) were included in the panel of SNPs to study in the whole group of patients and controls. Based on the observations about the possible functionality of the different SNPs (Zhang et al. 2003, see chapter 2), the 568 SNP was also included in the chosen panel of polymorphisms.

6.3.2. Hardy-Weinberg equilibrium

The Hardy-Weinberg formula was applied to the genotype frequencies in our subject sample to test for any deviations from the expected genotype equilibrium (a web site was used to run the analysis: http://www.kursus.kvl.dk/shares/vetgen/_Popgen/genetik/applets/kitest.htm). No significant differences were noted when frequencies of genotypes of all individuals (cases and controls pooled together) and controls alone were computed. When genotypes from AgP patients alone were analyzed, significant variations from the expected equilibrium were detected for FcγIIIb SH and IL-6 -174 polymorphisms.

6.3.3. Linkage disequilibrium between polymorphisms

Tables 6.8 and 6.9 show the p values relative to the pair-wise linkage disequilibrium (LD) between markers respectively for FcγR and FPR. The top right elements of the first table show R-squared measures of linkage disequilibrium. In parenthesis D' measures are shown, taking into account differences in allele frequencies. R-squared and D' values approaching 1 indicate higher LD. The bottom left elements of the table shows the respective p values for the LD. Among FcγR, significant linkage disequilibrium was detected between FcγRIIa and FcγRIIIa ($p<0.001$); this LD was not significant in the patient group ($p=0.09$). Significant LD was also detected between FcγRIIb and FcγRIIIb NA ($p=0.009$ controls; $p= 0.025$ patients), FcγRIIIa and FcγRIIIb NA ($p=0.02$ controls; $p= 0.003$ patients), and FcγRIIIb NA and FcγRIIIb SH ($p=0.010$ controls; $p<0.001$ 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, $p=0.09$).

Fcγ receptor polymorphisms		IIa	IIb	IIIa	IIIb NA	IIIb SH
IIa	*All subjects*	-	0.016 (0.037)	0.198 (0.273)	0.081 (0.096)	0.046 (0.182)
	Controls		0.038 (0.088)	0.265 (0.368)	0.022 (0.028)	0.082 (0.363)
	Patients		0.067 (0.149)	0.115 (0.157)	0.129 (0.146)	0.030 (0.107)
IIb	*All subjects*	0.70827	-	0.074 (0.228)	0.156 (0.414)	0.076 (0.134)
	Controls	0.54851		0.104 (0.326)	0.166 (0.463)	0.096 (0.188)
	Patients	0.28992		0.037 (0.112)	0.145 (0.366)	0.057 (0.092)
IIIa	*All subjects*	0.00003	0.10960	-	0.176 (0.284)	0.001 (0.002)
	Controls	0.00003	0.09545		0.164 (0.274)	0.058 (0.355)
	Patients	0.09310	0.57160		0.186 (0.289)	0.037 (0.099)
IIIb NA	*All subjects*	0.08123	0.00067	0.00016	-	0.190 (0.889)
	Controls	0.68918	0.00912	0.02207		0.182 (0.988)
	Patients	0.04496	0.02506	0.00256		0.211 (0.862)
IIIb SH	*All subjects*	0.33750	0.10035	1.00000	0.00001	-
	Controls	0.30783	0.13801	0.38333	0.00975	
	Patients	0.61011	0.38966	0.52708	0.00024	

Table 6.8: R-squared and D' values (in parenthesis) for Linkage Disequilibrium with relative p values for FcγR. The top right elements show R-squared values of linkage disequilibrium. In parenthesis D' values are shown. The bottom left elements show the respective p values.

FP receptor polymorphisms		301	546	568
301	*All subjects*	-	0.152 (0.155)	0.217 (0.843)
	Controls		0.108 (0.115)	0.195 (0.764)
	Patients		0.204 (0.226)	0.256 (0.995)
546	*All subjects*	0.00085	-	0.214 (0.820)
	Controls	0.09194		0.190 (0.792)
	Patients	0.00163		0.247 (0.865)
568	*All subjects*	0.00000	0.00000	-
	Controls	0.00059	0.00021	
	Patients	0.00030	0.00016	

Table 6.9 R-squared and D' values (in parenthesis) for Linkage Disequilibrium with relative p values for FPR. The top right elements show R-squared values of linkage disequilibrium. In parenthesis D' values are shown. The bottom left elements show the respective p values.

6.3.4. 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 6.10. This table is further sub divided for the two main ethnic groups of the study, Caucasians and Blacks. The results of the Chi-square test to identify differences in the distributions are also reported. Chi-squared analysis for all subjects showed statistically significant differences for IL-6 -174 and NADPH oxidase $p22^{phox}$ C242 T polymorphisms (p=0.005 and p=0.003 respectively). Increases in the frequency of IL-6 -174 G homozygosity and in the carriage of the NADPH oxidase $p22^{phox}$ T allele were noted in the patient group. 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.

Poly-morphism	Geno-type	All subjects		Chi square p=	Caucasians		Chi square p=	Blacks		Chi square 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%)	

Table 6.10: Distributions of all studied polymorphisms in all subjects. 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 Chi-squared. (continues in the next page)

Poly-morphism	Geno-type	All subjects		Chi square p=	Caucasians		Chi square p=	Blacks		Chi square p=
		Patients (n=224)	Controls (n=231)		Patients (n=112)	Controls (n=144)		Patients (n=59)	Controls (n=45)	
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%)	
VDR	tt	32 (14.3%)	45 (19.5%)	0.328	14 (12.5%)	28 (19.4%)	0.328	10 (16.9%)	7 (15.6%)	0.974
	Tt	97 (43.3%)	96 (41.6%)		54 (48.2%)	63 (43.8%)		20 (33.9%)	15 (33.3%)	
	TT	95 (42.4%)	90 (39.0%)		44 (39.3%)	53 (36.8%)		29 (49.2%)	23 (51.1%)	
TNF-α	AA	157 (70.7%)	157 (68.3%)	0.839	68 (60.7%)	90 (62.9%)	0.519	41 (70.7%)	31 (68.9%)	0.265
	AG	61 (27.5%)	69 (30.0%)		40 (35.7%)	51 (35.7%)		17 (29.3%)	12 (26.7%)	
	GG	4 (1.8%)	4 (1.7%)		4 (3.6%)	2 (1.4%)		0 (0%)	2 (4.4%)	
IL-6	CC	17 (7.7%)	30 (13.0%)	0.005	14 (12.5%)	28 (19.4%)	0.041	0 (0%)	0 (0%)	0.554
	CG	66 (29.7%)	91 (39.4%)		49 (43.8%)	74 (51.4%)		6 (10.5%)	7 (15.6%)	
	GG	139 (62.6%)	110 (47.6%)		49 (43.8%)	42 (29.2%)		51 (89.5%)	38 (84.4%)	
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%)	

Table 6.10 continued.

Logistic regression revealed that the presence of the NADPH oxidase $p22^{phox}$ T allele was associated with a diagnosis of AgP, adjusting for gender, smoking and ethnicity (p=0.002, O.R. =1.87, 95% C.I. =1.27-2.83) (see table 6.11). The association of IL-6 G homozygosity with diagnosis of AgP did not reach statistical significance, having adjusted for gender, smoking and ethnicity (p=0.016, O.R. =1.68, 95% C.I. =1.10-2.57).

Logistic regression analysis for presence of T allele (tot.AgP vs. controls)			
	p=	O.R.	95% C.I.
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

Table 6.11 Results of logistic regression analysis on all subjects for presence of T allele of NADPH p22phox 242 SNP. Unadjusted values, bivariate and multivariate values adjusted for confounders are presented.

Chi-squared analysis in the subgroup of Caucasians showed some evidence of association with the AgP trait for IL-6 (p=0.041) and NADPH oxidase (p=0.017), with GG and TT respectively being increased in patients compared to controls. Logistic regression analysis revealed that carriage of NADPH oxidase T allele was associated with diagnosis of AgP, adjusting for gender and smoking (p=0.009, O.R. =2.07, 95% C.I. =1.20-3.59 respectively) (see table 6.12).

Logistic regression analysis for presence of T allele (tot. AgP vs. controls)			
	p=	O.R.	95% C.I.
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

Table 6.12 Results of logistic regression analysis on Caucasian subjects for presence of T allele of NADPH p22phox 242 SNP. Unadjusted values, bivariate and multivariate values adjusted for confounders are presented.

IL-6 G homozygosity showed a trend for association with diagnosis of AgP, adjusting for gender and smoking (p=0.032, O.R. =1.80, 95% C.I. =1.05-3.10). Similarly, in Caucasians who never smoked (consisting of 38 patients and 81 controls), NADPH oxidase T allele showed some evidence of association with a diagnosis of AgP, adjusting for gender (p=0.012, O.R. =3.16, 95% C.I. =1.28-7.76).

Among smokers (current and past: 106 AgP patients and 94 controls) of mixed ethnicity, both homozygosity for FcγRIIIb NA1 homozygosity and presence of VDR T allele showed tendencies for association with AgP (p= 0.027, O.R.= 2.46, 95% C.I.= 1.10-5.50 and p= 0.032, O.R.= 2.35, 95% C.I.= 1.07- 5.15 respectively). Both FcγRIIIb NA and VDR Taq-I polymorphisms and smoking were shown to be independent variables (p=0.910 and p=0.998 respectively). Figure 6.1 shows the genotype distribution for the Taq-I VDR polymorphism in controls and patients who never smoked, and figure 6.2 shows the same data in the smokers.

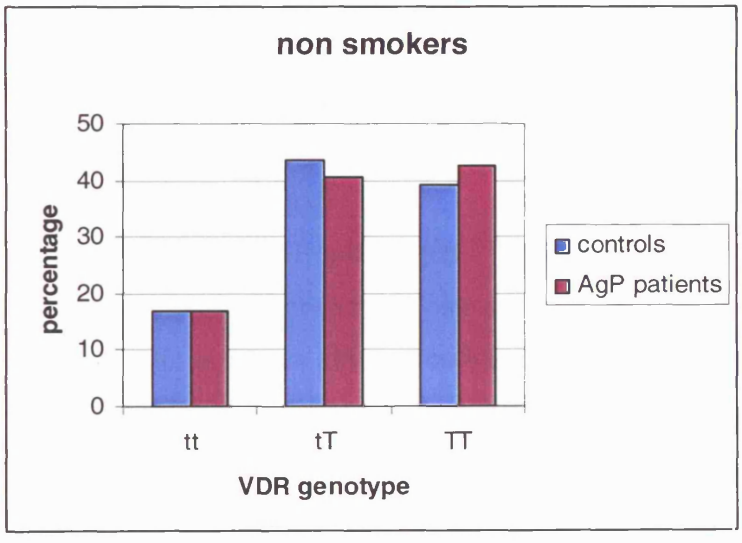

Figure 6.1 VDR Taq-I genotype distribution in subjects who never smoked

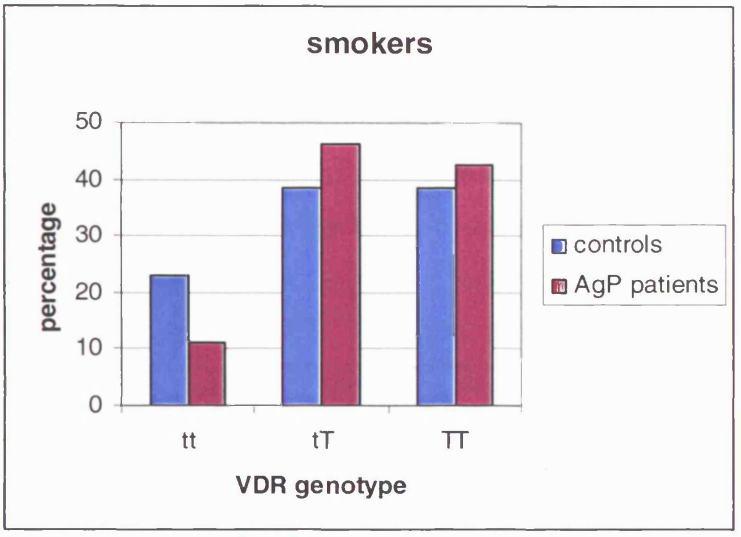

Figure 6.2 VDR Taq-I genotype distribution in smokers (current and past)

While in non smokers the distribution between controls and patients is very similar, among smokers there is an evident enrichment of T allele presence. Logistic regression analysis on the whole group of subjects in order to investigate a possible interaction between smoking (current and past) and VDR polymorphism revealed that neither of these two factors was independently associated with AgP (p=0.348 and p=0.898 respectively). However, the interaction VDR/smoking showed a trend for association (p= 0.066).

Among Black subjects, only NADPH oxidase $p22^{phox}$ C242 T polymorphisms did not exhibit a homogeneous distribution between patients and controls (p=0.050). Tendencies for not equivalent distributions, which did not reach statistical significance, were noted for FcγRIIIa (p=0.120), FcγRIIIb SH (p=0.188) and FPR 568 (p=0.165) polymorphisms. Logistic regression revealed limited evidence of association between NADPH oxidase $p22^{phox}$ polymorphism and diagnosis of AgP having adjusted for gender, smoking and ethnicity (p=0.032, O.R. =1.93, 95% C.I. =1.06-3.52). No statistical analysis was performed on allele distributions. However, appendix XVI shows allele frequencies in patients and controls for all studied polymorphisms.

6.3.5. Comparison between GAgP and LAgP.

The distributions of all polymorphisms in the GAgP and LAgP groups are reported in table 6.13. No statistically significant differences were detected, although there was an increased prevalence of G homozygosity for IL-6 -174 in the LAgP group (73% versus 59%, p=0.165).

Polymorphism	Genotype	All subjects		Chi square p=
		LAgP (n=57)	GAgP (n=167)	
FcαR	AA	4 (7.3%)	14 (8.5%)	0.956
	AG	22 (40.0%)	64 (39.0%)	
	GG	29 (52.7%)	86 (52.4%)	
FcγRIIa	HH	14 (25.5%)	41 (25.2%)	0.389
	HR	31 (56.4%)	78 (47.9%)	
	RR	10 (18.2%)	44 (27.0%)	
FcγRIIb	CC	2 (3.6%)	4 (2.4%)	0.850
	CT	16 (29.1%)	45 (27.3%)	
	TT	37 (67.3%)	116 (70.3%)	
FcγRIIIa	GG	7 (12.7%)	21 (12.9%)	0.800
	GT	26 (47.3%)	69 (42.3%)	
	TT	22 (40.0%)	73 (44.8%)	
FcγRIIIbNA	NA2/NA2	20 (35.7%)	51 (30.9%)	0.207
	NA1/NA2	29 (51.8%)	75 (45.5%)	
	NA1/NA1	7 (12.5%)	39 (23.6%)	
FcγRIIIbSH	SH+/SH+	1 (1.8%)	3 (1.8%)	1.000
	SH-/SH+	6 (10.9%)	18 (10.8%)	
	SH-/SH-	48 (87.3%)	146 (87.4%)	
FPR 301	CC	5 (9.3%)	16 (9.8%)	0.797
	CG	25 (46.3%)	67 (41.1%)	
	GG	24 (44.4%)	80 (49.1%)	

Polymorphism	Genotype	All subjects		Chi square p=
		LAgP	GAgP	
FPR 546	AA	9 (16.4%)	19 (11.5%)	0.165
	AC	19 (34.5%)	81 (49.1%)	
	CC	27 (49.1%)	65 (39.4%)	
FPR 568	AA	40 (72.7%)	125 (75.8%)	0.739
	AT	15 (27.3%)	39 (23.6%)	
	TT	0 (0%)	1 (0.6%)	
VDR	tt	9 (15.8%)	23 (13.8%)	0.855
	Tt	23 (40.4%)	74 (44.3%)	
	TT	25 (43.9%)	70 (41.9%)	
TNF-α	AA	42 (73.7%)	115 (69.7%)	0.469
	AG	15 (26.3%)	46 (27.9%)	
	GG	0 (0.0%)	4 (2.4%)	
IL-6	CC	3 (5.4%)	14 (8.4%)	0.165
	CG	12 (21.4%)	54 (32.5%)	
	GG	41 (73.2%)	98 (59.0%)	
NADPH	CC	17 (38.6%)	50 (32.3%)	0.394
	CT	22 (50.0%)	74 (47.7%)	
	TT	5 (11.4%)	31 (20.0%)	

Table 6.13 Distributions of all studied polymorphisms in LAgP and GAgP subjects with relative Chi-squared referring to differences in genotype distributions between patients with LAgP and patients with GAgP.

6.3.6. Comparison between GAgP and controls.

Table 6.14 shows the distributions of all polymorphisms in the patients diagnosed with GAgP and in the healthy controls both in the whole group of subjects and in Caucasians. Chi- squared analysis revealed border-line significance for the distribution of FcγRIIIb NA polymorphism (p=0.019 and p=0.021 respectively in mixed populations and Caucasians) and association with the disease trait for NADPH oxidase $p22^{phox}$ C242 T

polymorphism (p=0.002 and p=0.034 respectively in mixed populations and Caucasians).

Poly-morphism	Geno-type	All subjects		Chi square p=	Caucasians		Chi square p=
		GAgP (n=167)	Controls (n=231)		GAgP (n=88)	Controls (n=144)	
FcαR	AA	14 (8.5%)	15 (6.5%)	0.574	8 (9.2%)	7 (4.9%)	0.359
	AG	64 (39.0%)	84 (36.4%)		38 (43.7%)	60 (41.7%)	
	GG	86 (52.4%)	132 (57.1%)		41 (47.1%)	77 (53.5%)	
FcγRIIa	HH	41 (25.2%)	61 (26.4%)	0.869	21 (24.1%)	37 (25.7%)	0.382
	HR	78 (47.9%)	113 (48.9%)		37 (42.5%)	71 (43.9%)	
	RR	44 (27.0%)	57 (24.7%)		29 (33.3%)	36 (25.0%)	
FcγRIIb	CC	4 (2.4%)	9 (3.9%)	0.602	0 (0%)	4 (2.8%)	0.287
	CT	45 (27.3%)	56 (24.2%)		19 (21.6%)	31 (21.5%)	
	TT	116 (70.3%)	166 (71.9%)		69 (78.4%)	109 (75.7%)	
FcγRIIIa	GG	21 (12.9%)	27 (11.8%)	0.783	12 (13.8%)	19 (13.4%)	0.966
	GT	69 (42.3%)	105 (45.9%)		41 (47.1%)	65 (45.8%)	
	TT	73 (44.8%)	97 (42.4%)		34 (39.1%)	58 (40.8%)	
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%)	
FcγRIIIbSH	SH+/SH+	3 (1.8%)	0 (0%)	0.111	0 (0%)	0 (0%)	0.431
	SH-/SH+	18 (10.8%)	22 (9.5%)		4 (4.5%)	3 (2.1%)	
	SH-/SH-	146 (87.4%)	209 (90.5%)		84 (95.5%)	141 (97.9%)	
FPR 301	CC	16 (9.8%)	28 (12.2%)	0.522	7 (8.0%)	18 (12.7%)	0.543
	CG	67 (41.1%)	101 (44.1%)		39 (44.8%)	62 (43.7%)	
	GG	80 (49.1%)	100 (43.7%)		41 (47.1%)	62 (43.7%)	

Poly-morphism	Geno-type	All subjects		Chi square p=	Caucasians		Chi square p=
		GAgP (n=167)	Controls (n=231)		GAgP (n=88)	Controls (n=144)	
FPR 546	AA	19 (11.5%)	25 (10.8%)	0.219	8 (9.1%)	15 (10.4%)	0.164
	AC	81 (49.1%)	95 (41.1%)		46 (52.3%)	57 (39.6%)	
	CC	65 (39.4%)	111 (48.1%)		34 (38.6%)	72 (50.0%)	
FPR 568	AA	125 (75.8%)	181 (78.4%)	0.600	63 (72.4%)	113 (79.0%)	0.382
	AT	39 (23.6%)	47 (20.3%)		24 (27.6%)	29 (20.3%)	
	TT	1 (0.6%)	3 (1.3%)		0 (0%)	1 (0.7%)	
VDR	tt	23 (13.8%)	45 (19.5%)	0.328	12 (13.6%)	28 (19.4%)	0.499
	Tt	74 (44.3%)	96 (41.6%)		43 (48.9%)	63 (43.8%)	
	TT	70 (41.9%)	90 (39.0%)		33 (37.5%)	53 (36.8%)	
TNF-α	AA	115 (69.7%)	157 (68.3%)	0.819	53 (60.2%)	90 (62.9%)	0.342
	AG	46 (27.9%)	69 (30.0%)		31 (35.2%)	51 (35.7%)	
	GG	4 (2.4%)	4 (1.7%)		4 (4.5%)	2 (1.4%)	
IL-6	CC	14 (8.4%)	30 (13.0%)	0.065	11 (12.5%)	28 (19.4%)	0.168
	CG	54 (32.6%)	91 (39.4%)		42 (47.7%)	74 (51.4%)	
	GG	98 (59.0%)	110 (47.6%)		35 (39.8%)	42 (29.2%)	
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%)	

Table 6.14: Distributions of all studied polymorphisms in GAgP and controls with relative Chi-squared results. Genotype distributions for all studied polymorphisms are presented in GAgP patients and controls of mixed ethnicity (all subjects) and in the Caucasians only.

Logistic regression analysis revealed that carriage of at least one copy of $p22^{phox}$ 242 T allele was associated with the disease phenotype adjusting for gender, smoking and ethnicity (p=0.003, O.R. =1.95, 95% C.I. =1.26-3.01) (see table 6.15). A trend for association with the GAgP trait, having adjusted for gender, smoking and ethnicity, was noted for the FcγRIIIb NA polymorphism (p=0.013, O.R. =1.97, 95% C.I. =1.15-3.37), FcγRIIIb SH polymorphisms (p=0.111) and IL-6 GG (p=0.065).

Logistic regression analysis for presence of T allele (GAgP vs. controls)

	p=	O.R.	95% C.I.
Unadjusted	0.001	2.10	1.37-3.22
Bivariate (adjusted for gender)	0.001	2.08	1.35-3.18
Bivariate (adjusted for smoking)	0.001	2.06	1.34-3.17
Bivariate (adjusted for ethnicity)	0.001	2.02	1.31-3.11
Multivariate (adjusted for gender, smoking and ethnicity)	0.003	1.95	1.26-3.01

Table 6.15 Results of logistic regression analysis on GAgP patients compared with controls for presence of T allele of NADPH p22phox 242 SNP. Unadjusted values, bivariate and multivariate values adjusted for confounders are presented.

Sub analysis on the Caucasian GAgP group also showed border-line association for FcγRIIIb NA1 homozygosity and NADPH oxidase $p22^{phox}$ 242 T allele with the presence of GAgP adjusting for gender and smoking (p=0.013, O.R. =2.73, 95% C.I. =1.24-6.04 and p=0.029, O.R. =2.94, 95% C.I. =1.63-5.30 respectively). Logistic regression analysis was performed to investigate a possible 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 1 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=0.005, O.R. = 33.9, 95% C.I.: 2.9-390.5).

6.3.7. Comparison between LAgP and controls.

The distributions of all polymorphisms in the patients diagnosed with LAgP and in the healthy controls are reported in table 6.16. Only the IL-6 -174 polymorphism showed a statistically significant different distribution between the LAgP group and the healthy controls (p=0.003).

Poly-morphism	Geno-type	All subjects		Chi square p=	Caucasians		Chi square p=
		LAgP (n=57)	Controls (n=231)		LAgP (n=24)	Controls (n=144)	
FcαR	AA	4 (7.3%)	15 (6.5%)	0.838	2 (8.3%)	7 (4.9%)	0.509
	AG	22 (40.0%)	84 (36.4%)		12 (50.0%)	60 (41.7%)	
	GG	29 (52.7%)	132 (57.1%)		10 (41.7%)	77 (53.5%)	
FcγRIIa	HH	14 (25.5%)	61 (26.4%)	0.521	5 (20.8%)	37 (25.7%)	0.474
	HR	31 (56.4%)	113 (48.9%)		15 (62.5%)	71 (43.9%)	
	RR	10 (18.2%)	57 (24.7%)		4 (16.7%)	36 (25.0%)	
FcγRIIb	CC	2 (3.6%)	9 (3.9%)	0.758	1 (4.2%)	4 (2.8%)	0.933
	CT	16 (29.1%)	56 (24.2%)		5 (20.8%)	31 (21.5%)	
	TT	37 (67.3%)	166 (71.9%)		18 (75.0%)	109 (75.7%)	
FcγRIIIa	GG	7 (12.7%)	27 (11.8%)	0.946	3 (12.5%)	19 (13.4%)	0.992
	GT	26 (47.3%)	105 (45.9%)		11 (45.8%)	65 (45.8%)	
	TT	22 (40.0%)	97 (42.4%)		11 (41.7%)	58 (40.8%)	
FcγRIIIbNA	NA2/ NA2	20 (35.7%)	75 (32.5%)	0.898	11 (45.8%)	47 (32.6%)	0.375
	NA1/ NA2	29 (51.8%)	126 (54.5%)		12 (50.0%)	83 (57.6%)	
	NA1/ NA1	7 (12.5%)	30 (13.0%)		1 (4.2%)	14 (9.7%)	
FcγRIIIbSH	SH+/ SH+	1 (1.8%)	0 (0%)	0.114	0 (0%)	0 (0%)	0.149
	SH-/ SH+	6 (10.9%)	22 (9.5%)		2 (8.3%)	3 (2.1%)	
	SH-/ SH-	48 (87.3%)	209 (90.5%)		22 (91.7%)	141 (97.9%)	

Poly-morphism	Geno-type	All subjects		Chi square p=	Caucasians		Chi square p=
		LAgP (n=57)	Controls (n=231)		LAgP (n=24)	Controls (n=144)	
FPR 301	CC	5 (9.3%)	28 (12.2%)	0.826	2 (8.7%)	18 (12.7%)	0.510
	CG	25 (46.3%)	101 (44.1%)		13 (56.5%)	62 (43.7%)	
	GG	24 (44.4%)	100 (43.7%)		8 (34.8%)	62 (43.7%)	
FPR 546	AA	9 (16.4%)	25 (10.8%)	0.440	4 (16.7%)	15 (10.4%)	0.340
	AC	19 (34.5%)	95 (41.1%)		6 (25.0%)	57 (39.6%)	
	CC	27 (49.1%)	111 (48.1%)		14 (58.3%)	72 (50.0%)	
FPR 568	AA	40 (72.7%)	181 (78.4%)	0.391	19 (79.2%)	113 (78.5%)	0.919
	AT	15 (27.3%)	47 (20.3%)		5 (20.8%)	29 (25.9%)	
	TT	0 (0%)	3 (1.3%)		0 (0%)	1 (0.7%)	
VDR	tt	9 (15.8%)	45 (19.5%)	0.732	2 (8.3%)	28 (19.4%)	0.390
	Tt	23 (40.4%)	96 (41.6%)		11 (45.8%)	63 (43.8%)	
	TT	25 (43.9%)	90 (39.0%)		11 (45.8%)	53 (36.8%)	
TNF-α	AA	42 (73.7%)	157 (68.3%)	0.497	15 (62.5%)	90 (62.9%)	0.837
	AG	15 (26.3%)	69 (30.0%)		9 (37.5%)	51 (35.7%)	
	GG	0 (0%)	4 (1.7%)		0 (0%)	2 (1.4%)	
IL-6	CC	3 (5.4%)	30 (13.0%)	0.003	3 (12.5%)	28 (19.4%)	0.019
	CG	12 (21.4%)	91 (39.4%)		7 (29.2%)	74 (51.4%)	
	GG	41 (73.2%)	110 (47.6%)		14 (58.3%)	42 (29.2%)	
NADPH	CC	17 (38.6%)	112 (50.0%)	0.290	5 (27.8%)	73 (52.1%)	0.065
	CT	22 (50.0%)	84 (37.5%)		12 (66.7%)	53 (37.9%)	
	TT	5 (11.4%)	28 (12.5%)		1 (5.6%)	14 (10.0%)	

Table 6.16: Distributions of all studied polymorphisms in LAgP and controls. Genotype distributions for all studied polymorphisms are presented in LAgP patients and controls of mixed ethnicity (all subjects) and in Caucasians only. Relative Chi-squared results are presented.

Logistic regression analysis revealed that G homozygosity was significantly associated with LAgP, having adjusted for gender, smoking and ethnicity (p=0.006, O.R. =2.71, 95% C.I. =1.33-5.54) (see table 6.17).

Logistic regression analysis for IL-6 homozygosity (tot. LAgP vs. controls)

	p=	O.R.	95% C.I.
Unadjusted	0.001	3.01	1.58-5.73
Bivariate (adjusted for gender)	0.001	2.98	1.56-5.68
Bivariate (adjusted for smoking)	0.001	2.98	1.56-5.68
Bivariate (adjusted for ethnicity)	0.007	2.65	1.30-5.39
Multivariate (adjusted for gender, smoking and ethnicity)	0.006	2.71	1.33-5.54

Table 6.17 Results of logistic regression analysis on LAgP patients compared with controls for G homozygosity for IL-6 -174 SNP. Unadjusted values, bivariate and multivariate values adjusted for confounders are presented.

Even among the small group of Caucasians with LAgP (n=24), G homozygosity showed an association with LAgP, having adjusted for gender and smoking (p=0.008, O.R. =3.37, 95% C.I. =1.38-8.25) (see table 6.18). FcγRIIIb SH (p=0.114 in all subjects and p=0.149 in Caucasians) and NADPH oxidase (p=0.065 in Caucasians) showed tendencies for association with LAgP.

Logistic regression analysis for presence of T allele (tot. Caucasian LAgP vs. controls)

	p=	O.R.	95% C.I.
Unadjusted	0.007	3.40	1.40-8.26
Bivariate (adjusted for gender)	0.007	3.39	1.39-8.29
Bivariate (adjusted for smoking)	0.007	3.39	1.39-8.23
Multivariate (adjusted for gender and smoking)	0.008	3.37	1.38-8.25

Table 6.18 Results of logistic regression analysis on LAgP Caucasian patients compared with Caucasian controls for G homozygosity for IL-6 -174 SNP. Unadjusted values, bivariate and multivariate values adjusted for confounders are presented.

6.3.8. Haplotype analyses

No FPR and FcγR haplotypes displayed statistically significant associations with the AgP phenotype in Caucasians as analyzed by WHAP (pair- wise results are presented in tables 6.19 and 6.20).

FcγR	IIa	IIb	IIIa	IIIb NA	IIIb SH
IIa	-	0.644	0.607	0.445	0.117
IIb	-	-	0.876	0.892	0.121
IIIa	-	-	-	0.975	0.290
IIIb NA	-	-	-	-	0.132
IIIb SH	-	-	-	-	-

Table 6.19: p values (adjusted for smoking) obtained by WHAP analysis for pair-wise haplotype effects on AgP in Caucasian subjects

FPR	301	546	568
301	-	0.563	0.584
546	-	-	0.331
568	-	-	-

Table 6.20: p values (adjusted for smoking) obtained by WHAP analysis for pair-wise haplotype effects on AgP in Caucasian subjects

Table 6.21 shows the pair-wise results of haplotype associations for FcγR in relation to AgP in the group of Black patients. No statistically significant results were observed.

FcγR	IIa	IIb	IIIa	IIIb NA	IIIb SH
IIa	-	0.444	0.651	0.195	0.578
IIb	-	-	0.94	0.177	0.669
IIIa	-	-	-	0.147	0.428
IIIb NA	-	-	-	-	0.932
IIIb SH	-	-	-	-	-

Table 6.21: p values (adjusted for smoking) obtained by WHAP analysis for haplotype effect on AgP in Black subjects

However, haplotype association between all five studied FcγR polymorphisms showed some evidence of association with AgP in Black individuals (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 estimated frequencies of the possible haplotypes with the four remaining FcγR as analyzed by RunGC are presented in table 6.22. The highest likelihood- ratio test (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%) and 0.0 respectively in patients and controls.

Allele combinations	Estimated frequencies	LRT

FcγIIa	FcγIIb	FcγIIIa	FcγIIIb	Controls	Patients	Combined	mean
H	C	V	NA2	0.000	0.012	0.010	1.34
H	C	V	NA1	0.000	0.035	0.007	3.68
H	C	F	NA2	0.061	0.016	0.045	2.33
H	C	F	NA1	0.000	0.000	0.003	0.01
H	T	V	NA2	0.000	0.109	0.064	11.55
H	T	V	NA1	0.232	0.102	0.165	4.20
H	T	F	NA2	0.063	0.175	0.100	4.31
H	T	F	NA1	0.087	0.076	0.094	0.06
R	C	V	NA2	0.038	0.042	0.041	0.02
R	C	V	NA1	0.000	0.000	0.001	0.01
R	C	F	NA2	0.100	0.088	0.080	0.06
R	C	F	NA1	0.000	0.028	0.024	3.03
R	T	V	NA2	0.024	0.024	0.027	0.00
R	T	V	NA1	0.005	0.022	0.010	0.83
R	T	F	NA2	0.224	0.068	0.157	7.20
R	T	F	NA1	0.163	0.199	0.169	0.28

Table 6.22: FcγR haplotype frequencies in Black subjects as estimated by RunGC software. All the possible allele combinations between FcγRIIa, IIb, IIIa and IIIb NA polymorphisms are presented. Estimated frequencies for each haplotype combination between FcγRIIa, IIb, IIIa and IIIb NA in controls and patients and all subjects are presented. In the last column, Likelihhod- Ratio Test (LRT) values are reported.

Single marker analysis on FPR haplotypes in Black subjects revealed a tendency for association for FPR 568 (p=0.029). The extent of this association decreased in the haplotype analysis (see table 6.23 for pair- wise results).

FPR	301	546	568
301	-	0.449	0.064
546	-	-	0.091
568	-	-	-

Table 6.23: p values (adjusted by smoking) obtained by Whap analysis for haplotype effect on AgP in Black subjects

6.4. DISCUSSION

6.4.1. Polymorphisms distribution

The distribution of the genotypes for the studied genetic polymorphisms satisfied the Hardy-Weinberg equilibrium and was consistent with the literature data. In more detail, the distributions of FcαR 324 genotype and FcγRIIb 695 in controls were consistent with those reported respectively by Kaneko et al. (2004) and Yasuda et al. (2003), although these were observations made in Japanese subjects. Similarly, VDR Taq-I genotype distribution was comparable with previous studies (Carling et al. 1997), as were TNF-α (Parks et al. 2004, Donati et al. 2005) and the FPR polymorphisms (Zhang et al. 2003). Genotype distributions for FcγRIIa, IIIa and IIIb were compared with those reported in a recent review of association studies conducted on different populations (Lehrnbecher et al. 1999). The two alleles (H and R) for FcγRIIa 494 polymorphism have been reported as having an almost equal distribution in Caucasian populations, while HH homozygosity is more frequent in Asians. Similarly, among our Caucasian controls (see table 6.10) H and R were almost equally prevalent. FcγRIIIa 559 genotypes also had a distribution in our healthy controls consistent with literature data (Koene et al. 1997, Lehrnbecher et al. 1999) (see table 6.10). Similarly, for the FcγRIIIb NA polymorphism

we found a percentage of 13, 54 and 32 % respectively for NA1/NA1, NA1/NA2 and NA2/NA2 genotypes, which is as described in previous studies (Lehrnbecher et al. 1999), with a slight increase of frequency of NA2 homozygosity in Blacks. Lehrnbecher et al. (1999) also observed a prevalence of around 4% and 28% for the FcγRIIIb SH positive variant respectively in Caucasians and Blacks. These frequencies are mirrored by our data, where 2% of Caucasian and 29% of Black controls were found to be SH positive. With regard to IL-6 -174 polymorphism, Fishman et al. (1998) in a North-London population, found a genotype distribution of 38% GG, 44% GC and 18% CC in Caucasians and 91% GG, 9% GC and 0% CC in Afro-Caribbeans. The corresponding figures for our controls were 29% GG, 51% GC and 19% CC in Caucasians, and 84% GG, 16% GC and 0% CC in Blacks, showing a slightly higher prevalence of the C allele in our sample. However, in a study on 2751 middle-aged healthy U.K. men, the –174C allele frequency was found to be 43% (Humphries et al. 2001), higher than the 33% in our controls (see appendix XVI). Therefore these results, although showing some small differences with our frequencies, are still comparable. Gardemann et al. (1999), examining a population of 2205 German Caucasians found a prevalence of 66% for frequency of the C allele at position 242 of the CYBA gene coding for NADPH oxidase $p22^{phox}$, while Cai et al. (1999) also found a prevalence of 66% in 689 Australian Caucasians. The same allele had a similar but slightly higher prevalence in our Caucasian controls (69%) (see appendix XVI), and a smaller one in our Caucasian patients (58%). Obviously, having included only subjects with healthy periodontium as controls, this may account for small differences in genotype distribution with these various literature data, which did not take into account periodontal conditions, and may have included a small percentage of subjects suffering from severe periodontitis.

The main finding of this study is that IL-6 -174 and NADPH oxidase $p22^{phox}$ C242 T polymorphisms were risk indicators for the presence of Aggressive Periodontitis in our sample. FcγRIIIb NA polymorphism, FcγR haplotypes and VDR polymorphism in smokers also showed some evidence of association with the AgP trait. This finding is in agreement with the supposed importance of genetic factors in the pathogenesis of AgP.

The polymorphisms which showed association with the disease trait will now be discussed in separate paragraphs.

6.4.2. IL-6 polymorphism

Homozygosity for allele G of IL-6 was a significant risk indicator for Localised AgP, with a relative risk of around 3. In the Caucasian subgroup for example, 29% of controls and 58% of LAgP subjects were GG homozygous (see table 6.16). The presence of the G allele at position -174 has been linked to increased promoter activity of the IL-6 gene, with consequent increased production of IL-6. Fishman et al. (1998) demonstrated increased activity linked with the G allele after stimulation with lipopolysaccharide or Interleukin-1 and suspected a protective role for the presence of allele C towards juvenile rheumatoid arthritis. However, conflicting reports also exist which describe no relationship between this polymorphism and IL-6 concentrations or IL-6 increase related to C homozygosity (Endler et al. 2004), probably because of tissue- specific regulation of IL-6 expression (Fife et al. 2005). Since Fishman's paper, the IL-6 -174 polymorphism has been associated with several different diseases and conditions, such as systemic arthritis, coronary heart disease, Alzheimer disease and Kaposi sarcoma (Humphries et al. 2001, Faltraco et al. 2003, Ogilvie et al. 2003). Trevilatto and co-workers recently found an association between G homozygosis and chronic periodontitis in a Caucasian Brazilian population (Trevilatto et al. 2003). The suspected mechanism of periodontal disease susceptibility due to this polymorphism may be mediated by an increased release and activity of IL-6, which is crucial in mounting an effective inflammatory response. IL-6 takes part in the complex cytokine network responsible for the effectiveness and amplification of the inflammatory process. In particular, IL-6 has a potent role in inducing osteoclasts and determining bone resorption. Increased levels of IL-6 are characteristic features of diseases such as rheumatoid arthritis, otseoporosis and psoriasis (Terry et al. 2000). The presence of IL-6 in inflamed periodontal tissues has been widely demonstrated, in particular in gingival crevicular fluid (Kamma et al. 2004); moreover, serum concentrations of IL-6 seem to be associated with the presence of periodontal disease (D'Aiuto et al. 2005). Therefore, a plausible explanation of our results is that homozygosity for IL-6 -174 G allele predisposes to an increased risk of

developing AgP, especially the localised form, by means of an excessive stimulation of the IL-6 pro-inflammatory function and, as a consequence, of the inflammatory and destructive processes. This hyperstimulation, especially if combined with the presence of specific bacteria, leads to the considerable tissue damage observed in LAgP patients. However, it remains to be explained why, in the complex and to some extent redundant network of cytokines, there is no feedback mechanism in place to counterbalance the hyperactivity of IL-6 in these subjects and also why the effect of this genotype seems more important towards LAgP rather than GAgP. A possible explanation is that in LAgP especially and in AgP in general, this possible feedback regulatory system is somewhat deficient, or that it is inhibited by the initiation of periodontal inflammation due to specific pathogenic bacteria. Thus, in a GG subject under normal circumstances the supposed hyperactivity of IL-6 should be balanced by a negative loop. Whenever specific bacterial stimuli initiate periodontal inflammation, the negative feedback is overwhelmed by the pro-inflammatory messages triggered by the bacteria and IL-6 is therefore excessively stimulated, with the establishment of a chronic inflammatory process and a persistent pro-inflammatory stimulation. This is a very simplistic scenario, reflecting a much more complex reality, but might partially explain the significance of this polymorphism. Of course, functional data are needed in relation to the effect of this polymorphism on IL-6 release both systemically and locally and both in healthy and diseased subjects. Additional information may also be gained by the study of IL-6 haplotype, given by the combination of the -174 with other polymorphisms in the same gene which seem to act together in enhancing IL-6 promoter activity (Terry et al. 2000, Fife et al. 2005, Komatsu et al. 2005). It is also interesting to notice that the increased risk due to G homozygosity was not found in the group of solely Black subjects. The reason might be the extreme rarity of allele C in these subjects (see table 6.10) and to the fact that because of the small sample size in Blacks, the study might be underpowered to detect such an association.

6.4.3. NADPH oxidase polymorphism

This is the first study to report a role for NADPH oxidase $p22^{phox}$ C242 T polymorphism in the susceptibility to Aggressive Periodontitis. The predisposition linked with this

polymorphism in our sample was seen in the whole AgP group, but it is most striking in Generalised AgP, and was consistently found in the Caucasian and Black subpopulations, with the limitation of smaller sample sizes (see table 6.13). Analysis of the subgroup of Caucasians who never smoked confirmed this tendency for association, which was not found in smokers. The T allele was over represented in the AgP group, and in particular the TT genotype was strongly associated with GAgP. The risk attributable to this polymorphism was independent from other risk factors such as smoking and ethnic origin.

The NADPH of neutrophils consists of two membrane components, the $gp91^{phox}$ and the $p22^{phox}$ subunit, and four cytosolic components. Upon activation of PMN, all the components get assembled, forming a system which catalyzes the reduction of molecular oxygen at the expense of NADPH. The resulting electrons are moved and delivered to produce O_2^- both in the phagosome and in the extracellular environment (Dahlgren 1999). UshioFukai et al. (1996) provided evidence of the functional importance of $p22^{phox}$ in superoxide production in animal models.

The role of the oxidative burst in inducing bacterial killing, but also tissue damage, has been widely recognized for many years (Henson 1986, Weiss 1989), and implicated in the pathogenesis of rheumatoid arthritis, diabetes, reperfusion injury, ulcerative colitis, pre-eclampsia, hemorrhagic shock (Kantarci et al. 2003, Lee et al. 2003, Abdelrahman et al. 2005). The potential tissue damaging effect to human periodontal ligament cells by PMN activity has been shown *in vitro* some years ago (Deguchi 1990) and the specific effect of release of ROS on gingival epithelial cells has also been demonstrated (Altman et al.1992). ROS have been shown to be biologically active and involved in bacterial killing by means of two different mechanisms: i) activation of proteases through a depolarization compensatory mechanism mediated by K+ flux subsequent to the reduction of O_2 (Reeves et al. 2002, Rada et al. 2004); ii) production of a cascade of anti-microbial reactive oxygen metabolites, such as hydrogen peroxide, hypoclorous acid and chloramines (Dahlgren et al. 1999, Van Dyke et al. 2003, Rada et al. 2004), both in the intracellular and extracellular milieu.

This is the first study, to the best of our knowledge, which investigated the relation of this C $\rightarrow$T substitution in the gene encoding for the $p22^{phox}$ subunit of neutrophil NADPH to periodontitis. 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) showed a protective role on coronary risk linked with the T allele. However, some other studies found no associations (Li et al. 1999, Gardemann et al. 1999, Renner et al. 2000) or association between the T allele and a tendency to increased cardiovascular risk in young Caucasians (Cai et al. 1999) and progression of coronary atherosclerosis (Cahilly et al. 2000).

The C $\rightarrow$T mutation causes an amino acid substitution at position 72 (histidine to tyrosine). This substitution involves a potential haeme-binding site, which can have as a consequence an increase or decrease of superoxide production. Functional analyses of the effect of this polymorphism are very limited. Shimo-Nakanishi et al. (2004), in order to assess the functional relevance of the T substitution, measured the PMN oxidative burst on a subset of Japanese patients with thrombotic cerebral infarction and Japanese healthy subjects, in relation to the C242T $p22^{phox}$ polymorphism. They showed that, under stimulation by PMA (4B-phorbol 12-mystrate 13-acetate), subjects with the T allele had significantly higher oxidative burst than CC subjects. On the other hand, Wyche et al. (2004), in a study on 90 healthy Caucasians, observed an increased superoxide production linked with the presence of the C allele. In this context it is important to underline that, despite the fact that the vascular and phagocytic $p22^{phox}$ are very similar, the latter has much higher activity (De Keulanauer et al.1998). The possible functional changes due to the mutation are still to be elucidated. However, we can speculate on a possible effect on the susceptibility to periodontal disease through its modulation of the oxidative burst.

The presence of PMN defects in LAgP patients has been suggested for many years (Suzuki et al. 1984, Van Dyke et al. 1985). Several studies have reported a decreased chemotaxis (Cianciola et al. 1977), increased superoxide production (Leino et al. 1994, Gronert et al. 2004) and phagocytic defects in PMN isolated from LAgP patients (Suzuki et al. 1984, Van Dyke et al. 1985, Shapira et al. 1991). The current concept, therefore, 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 other forms of periodontitis: Fredriksson et al. (2003), investigating the superoxide production in 15 Chronic Periodontitis patients and 15 controls, found that not only the intracellular, but also the extracellular radical generation was significantly increased in patients compared to controls, justifying the possible ROS-related tissue damage. The fact that no differences have been found in receptor numbers, testifies for a possible alteration in post receptor signaling pathway (Leino et al. 1994, Fredriksson et al. 2003).

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 aggressive periodontitis. 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. It is interesting to note that, despite the fact that our patient group was heterogeneous in terms of ethnic background and smoking, even restricting the analysis to Caucasians, and even excluding all the current and former smokers, the T allele was still a significant risk factor for AgP.

We can only speculate, given the lack of robust evidence of functional effect of this polymorphism, that the T mutation may lead to an increased extracellular release of reactive oxygen intermediates which, through direct and indirect mechanisms, can increase the potential damage on the periodontal tissues. Therefore, this genetic polymorphism may represent a mechanism for PMN hyperactivity, independent from receptor numbers and DNA expression. Further studies are needed in order to ascertain the functional significance of the C242T mutation in healthy and periodontitis patients,

and to relate it also to superoxide production and phagocytic activity of known periodontal pathogens. This finding opens also interesting prospectives for the future, in terms of possible genetic therapy in order to modulate superoxide production in AgP patients, without compromising the protective function of PMNs (Fossati et al. 2002).

6.4.4. FcγRIIIb polymorphism

The other polymorphism which showed a trend for association with the AgP phenotype is the FcγRIIIb neutrophil antigen (NA). In our sample, the NA1/NA1 allotype was found to be increased in prevalence in the GAgP group (23.6% in patients compared to 13% in controls, see table 6.14). The result approached statistical significance only when GAgP patients were compared to controls, and this was also true in the subgroup of GAgP Caucasian patients and in the subgroup of smokers of mixed ethnicity. In the Black subgroup, we actually observed an increased prevalence of NA2 homozygosity in patients compared with controls (see table 6.10).

The neutrophil antigen (NA) is present in the membrane-distal IgG-like domain of the FcγIIIb receptors. A substitution of 4 amino acids in the gene coding for this receptor causes differences in receptor glycosylation, which result in different IgG-binding properties (van Sorge et al. 2003). The NA polymorphism has been associated with periodontitis in a few studies mainly in Japanese populations (Kobayashi et al. 2000a, Yoshihara et al. 2001). These studies show an increased prevalence of the NA2 allotype in patients with periodontitis. The same allele represented a risk indicator for AgP in a Black population (Fu et al. 2002). Functional results supported these finding, showing FcγRIIIb-NA2-carrying PMN from both patients with chronic periodontitis and healthy controls to be less efficient in phagocytosis upon interaction with IgG1- and IgG3-opsonized *P. gingivalis* (Kobayashi et al. 2000). These literature results are in conflict with the results observed in our study of an increase of NA1 homozygosity in patients. 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 vs. CP). We also investigated another polymorphism of the FcγRIIIb gene, which is in linkage disequilibrium with the FcγRIIIb NA (see table

6.8). This polymorphism is characterized by a nucleotide substitution at position 266 and gives origin to the SH allotype, associated with neonatal neutropenia (van der Pol et al. 1998). All SH positive subjects are also NA2 positive. Only 4 subjects in total were SH homozygous, they were all Black and they all had AgP (see table 6.10); in the Caucasian group, 5.4% of patients versus 2.1% of controls exhibited one copy of the SH allele. These results were not statistically significant, but they show that SH+ individuals might have an increased risk of having AgP. Therefore, the observed increased risk of having AgP observed in relation to NA2 in previous studies may be masked or diluted by a subset of NA2 subjects who present the SH allotype (Bux et al. 1997). However, the results observed in this study in relation to the NA1 allotype remain difficult to explain with the functional evidence which now exists. 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 to NA2 subjects in studies on several autoimmune diseases, such as Wegener's granulomatosis, myasthenia gravis and thrombocytopenic purpura (van Sorge et al. 2003). Kobayashi et al. (2000b) also found increased superoxide production upon interaction with IgG1 and IgG3- opsonized *P.gingivalis* in NA1 compared to NA2-carrying individuals. Considering the importance of superoxide production in exacerbating extracellular damage and given the interesting results observed in relation to the NADPH polymorphism in this study, the release of superoxide has to be considered as a possible mechanism of AgP susceptibility mediated by the FcγRIIIb polymorphisms. Indeed, the association NA1 homozygosis/ NADPH oxidase $p22^{phox}$ 242 T allele seems to have an additive effect in determining disease susceptibility to GAgP, with a relative risk equal to 34 for Caucasian subjects with both risk genotypes. This additive effect may be mediated by a stimulation of superoxide production.

6.4.5. Vitamin D receptor Taq-I polymorphism

The t allele of VDR Taq-I polymorphism was suspected as a susceptibility factor for LAgP in a small group of Caucasians (Hennig et al. 1999). On the other hand, the T

allele has been associated with a risk of CP (Tachi et al. 2003). In our sample, no associations were detected between VDR polymorphism and the whole group of AgP. However, in the group of smokers the presence of the T allele showed a trend for association with the AgP phenotype. This observation confirms our previous report (Nibali et al. 2005) of an interaction between VDR polymorphisms and smoking. Following 61 CP patients for 1 year, we found that smokers who were carriers of the T allele for this polymorphism had an increased chance of severe progression of attachment loss. Therefore, it seems that while in subjects who do not smoke the T allele does not contribute to the periodontitis risk, in smokers the interaction smoking/VDR Taq-I T allele may have a role in the pathogenesis of AgP and probably periodontitis in general. Speculations about the VDR- smoking interaction may involve a combination effect on the immune response or on bone metabolism. Larger sample sizes are needed to detect an association between this polymorphism and AgP in smokers.

6.4.6. FcγR haplotype

The association between FcγRIIIb and AgP was discussed in paragraph 6.4.4. Possible trends for association were also observed for FcγRIIa 494 and FcγRIIIa 559 polymorphisms. FcγRIIa are the main receptors involved in interaction with IgG2, and the presence of an arginine (R) instead of a histidine (H) at position 131, determined by a single nucleotide polymorphism at position 494, has been shown to reduce the binding efficiency of the receptor and therefore its effector functions. The R allele therefore is supposed to predispose to infections by IgG2-opsonized bacteria (van Sorge et al. 2003, Bredius et al. 1994). Yamamoto et al. (2004) found an enrichment of H homozygosis in moderate to severe Caucasians with periodontitis. In this study, we observed increased prevalence of R allele and especially R homozygosity in Caucasian subjects (30% vs. 25% RR homozygous in patients vs. controls), although this did not reach statistical significance. This imbalance was more evident in the Caucasian GAgP group (33% vs. 25%). Surprisingly, however, in Black subjects an opposite tendency was observed (20% vs. 31% RR subjects in patients vs. controls).

A G-T substitution at nucleotide 559 in the FcγRIIIa gene results in the presence of a valine (V) instead of a phenylalanine (F) at position 176, supposedly reducing the affinity for IgG1, 3 and 4 (Koene et al. 1997). This polymorphism has been reported as a possible risk factor for AgP and CP in different populations and with conflicting results (Kobayashi et al. 2000a, Kobayashi et al. 2001, Loos et al. 2003). Almost identical percentages of genotype distributions were detected in our sample between patients and controls. However, in the group of Black GAgP subjects, it was noted that 18% of patients vs. 4% of controls were V homozygous.

FcγRIIa H/R, FcγRIIb C/T, FcγRIIIa V/F, FcγRIIIb NA and FcγRIIIb SH polymorphisms are located in different genes all in the long arm of chromosome 1. The detection of linkage disequilibrium between some of these markers confirms the close relationship between them, and stimulated the formulation of hypotheses about a possible common role in determining disease susceptibility. A haplotype determined by a combination of these polymorphisms showed some evidence of association with AgP in the subset of patients of Black ethnicity. The strength of the association increased when the FcγRIIIb SH polymorphism was not included in the analysis. No significant association was found in the Caucasian subgroup for the same haplotype. Therefore it seems that, in this racial subgroup, the FcγR haplotypes have an important disease-predisposing effect, resulting from the additive effect of each single polymorphism. This effect might be mediated by the effect on IgG binding, PMN phagocytosis or superoxide production. In fact, the signal transduction from FcγRIIIb is supposed to involve interaction with FcγRIIa (van Sorge et al. 2002), and stimulation of PMN through FcγRIIIb was shown to enhance FcγRIIa- mediated phagocytosis (Pricop et al. 1999). On the other hand, it might also be a reflection of the presence of another functional polymorphism somewhere else in the gene, in LD with the polymorphisms presented here. This result reinforces the importance of the combined effect of different polymorphisms located closely in a chromosome in determining disease susceptibility. Further functional studies are needed to investigate the role of FcγR haplotypes in relation to the handling of periodontopathogenic bacteria.

6.4.7. Other polymorphisms

As far as the other studied polymorphisms were concerned, none of them showed statistically significant associations with the AgP trait. Nonetheless, some of them showed trends for association, and deserve a comment.

We observed an increase in the carriage of at least one copy of the allele A at position 324 in the Fc-α receptor gene in patients compared with controls (47% versus 43% in the overall population, 54 versus 46% in Caucasians, and 28 versus 20% in Blacks, see table 6.10). Similarly more frequent were A homozygous subjects (8 vs. 6%, 9 vs. 5% and 3 vs. 0% respectively in all subjects, Caucasians and Blacks). These differences did not reach statistical significance, probably due to a lack of power. Kaneko et al. (2004) found an increased prevalence of homozygosity for allele A at position 324 in the Fc-α receptor gene in Japanese AgP patients. Individuals homozygous for this allele also exhibited decreased phagocytosis of *P.gingivalis*. Since this FcαR 324 polymorphism is synonymous, we conclude that it may be in LD with a polymorphism predisposing to AgP. However, this effect is probably limited and larger sample sizes are needed to detect its power in the different ethnic groups.

A slight increase in G homozygosity was noted in the FPR 301 polymorphism, especially in the Black subgroup (see table 6.10). However, these differences were not marked. The frequency of allele A at position 546 in the FPR gene was increased in the AgP patient group. In the Caucasian GAgP subgroup (see table 6.14), in particular, 61% of patients and 50% of controls carried at least one copy of the A allele. The C-A substitution at nucleotide 546 in the FPR gene is synonymous, and does not change the coded amino acid and only slight differences in allele distributions were noted from a previous study (Zhang et al. 2003). On the other hand, Zhang et al. (2003) also observed a statistically significant increase in the homozygosity for allele A of 568 A-T FPR polymorphism in a group of African Americans with AgP. Thirty-seven out of their 38 AgP subjects were homozygous for A. In our subgroup of Black subjects, 22% of patients vs. 9% of controls exhibited at least one copy of the rare allele T (see table

6.10). This genotype distribution failed to show any statistically significant differences with AgP when logistic regression analysis was used, but approached statistical significance using WHAP analysis. These observations raise doubts over the supposed importance of this polymorphism as a risk factor for AgP, given the conflicting evidence present.

No substantial differences in genotype distributions were noted for TNF-α -308 polymorphism.

6.5. CONCLUSIONS

The main finding of this study lies in having detected two genetic polymorphisms which seem to modulate the risk to develop Aggressive Periodontitis. As it was discussed in chapter 1, whenever a genotype or an allele is found to be associated to a disease in case-control genetic association studies there are four possible reasons: i) the genotype really increases the risk of developing the disease, ii) the genotype is in linkage-disequilibrium with the real predisposing allele, iii) the result is due to population stratification or iv) to statistical artefact. With our study design we tried to reduce the risks of falling in the last two categories, therefore we have to believe in one of the first two options in order to explain our results. The two polymorphisms found to be significantly associated with AgP (IL-6 -174 and NADPH oxidase $p22^{phox}$ C242T) are functional. IL-6 -174 is thought to increase the IL-6 gene promoter activity, while NADPH oxidase $p22^{phox}$ change the coded amino acids. Therefore, every chance exists that these polymorphisms may be really predisposing to AgP. Further limited evidence for association with AgP was found for FcγRIIIb NA polymorphism in GAgP, FcγR haplotype in relation to AgP in Black subjects, and VDR polymorphism in smokers. FcγRIIIb NA polymorphisms is functional and changes the coded amino acid, with suspected effect on receptor function; FcγR haplotype may have a functional effect too, although the presence of another functional SNP in LD cannot be ruled out. The only polymorphism we found associated with AgP (in smokers) which is not functional is the VDR Taq-I polymorphism, suspected to be in LD with a functional SNP in the gene, still to be discovered.

This study has a larger sample size than any of the previously published case-control association studies on Aggressive Periodontitis, and failed to confirm many of the results published so far. We embarked on this study with very limited evidence of the importance of genetic factors in AgP. Interpreting these results based on the existing literature, we can say that we identified a novel genetic risk factor for AgP (242 T allele of NADPH oxidase $p22^{phox}$ CYBA gene) and we confirmed two genetic risk factors that have already been shown to be candidates in CP (homozygosity for G of IL-6 -174 and VDR Taq-I T allele in smokers). We also produced some evidence in conflict with previous reports, but new in Caucasians for the FcγRIIIb NA polymorphism. It is also interesting to notice how we identified different risk polymorphisms for different forms of disease (GAgP and LAgP). However, in the interpretation of these results we have to acknowledge that only the first two of four possible steps for the inclusion of these SNPs as real risk factors for AgP have been accomplished (see paragraph 1.3.2.3). While we obtained identification of supposed risk factors, and retention of significance and direction of effect in a multi-factorial model, the validation through studies on independent populations and the targeting step have not been achieved. This concept and the possible effect of chance in isolated reports remind us to be cautious in generalising these results (Ioannidis 2003, Ioannidis et al. 2003). Furthermore, some other genetic polymorphisms, such as FcαR, FcγRIIIa, FcγRIIIb SH may have some importance in increasing the AgP risk. Paradoxically, the sample size of this study was probably enough to reduce the effect of chance, but at the same time not large enough to identify clear relative risks in polymorphisms that have a subtle but still consistent effect in AgP risk.

Functional studies, haplotype analysis and studies with larger sample sizes are desperately needed in order to confirm the importance of the observed polymorphisms.

CHAPTER 7

7. GENETIC INFLUENCE ON DISEASE SEVERITY

7.1. BACKGROUND

It is not clear whether factors which predispose to periodontitis also affect its severity. Most previous studies have either focused on the possible effect of a factor in the initiation of AgP, CP, or on its effect on severity. In fact, in the particular case of AgP, some factors might play a role in the pathogenic mechanism that leads to its initiation, while other factors may then intervene in determining its extent and severity. Equally unclear and linked to disease severity is the relationship with disease progression (Tonetti & Claffey 2005). However, it is reasonable to suspect that if a genetic factor predisposes to AgP, subjects who present with this factor should also be prone to have more severe disease than others. Based on this concept, we analyzed the clinical data described in chapter 5, relating them to the genotypic data we detected in the case-control study (see chapter 6). Possible associations between all genetic polymorphisms studied in our sample and clinical parameters indicating disease extent and severity were investigated. No formal power calculation was performed to identify the number of subjects needed to detect if any of the studied SNPs had an effect on disease severity. Therefore, we have to consider the analyses presented in this chapter as exploratory analyses. Nonetheless, data derived from this chapter might be useful to design more focused studies which address the question "Do genetic factors have an effect on disease severity?".

7.2. MATERIALS AND METHODS

All 224 subjects diagnosed with AgP, whose clinical characteristics were presented in chapter 5 and whose genetic profile was presented in chapter 6, were included in this study (see chapter 4 for inclusion and exclusion criteria). From all the clinical information collected the following were deemed to have relevance to the severity of disease at subject level: mean probing pocket depth (PPD) and average lifetime cumulative attachment loss (LCAL) (data on LCAL were not available for all patients as highlighted in chapter 5) and number of pockets $\geq$ 5 mm. Because of the difficulty in comparing the severity of LAgP and GAgP cases, the two forms of disease were

analyzed separately. Furthermore, measurements of average PPD or LCAL and number of pockets above a certain threshold are probably not very good parameters to evaluate the severity or extent of periodontitis in general, but especially in a case of LAgP.

7.2.1. Statistical analysis

SPSS 12.0 package was used for statistical analysis. Because of the fact that multiple testing can lead to spurious results (Altman 1991), the alpha value was set at 0.01. The normality of distribution of the clinical parameters (av. PPD, av. LCAL and number of PPD $\geq$ 5 mm) was plotted and tested by Kolgomorov- Smirnov and Shapiro-Wilk tests. Since average PPD and average LCAL did not show normal distribution, they were log-transformed for the analysis. Multivariate analysis was then performed to evaluate the association between genotypes and clinical parameters (log av. PPD, log av. LCAL and number of PPD $\geq$ 5 mm) both in the whole group of GAgP and LAgP, in Caucasian GAgP and in Black GAgP. Smoking history, ethnicity, gender and age were entered as covariates. Pair wise comparisons tested the magnitude of the estimated effects associated with each genotype (estimated differences adjusted for confounders are reported). Box's M test of the homogeneity of the covariance matrices of the dependent variables and Levene's test for homogeneity of variance were run to test for homogeneity of data. The normality of distribution of each of the residuals was then confirmed by Q-Q plots and by Kolgomorov- Smirnov and Shapiro-Wilk tests.

7.3. RESULTS

The mean of the clinical measurements subdivided according to the various genotypes for each SNP are presented in appendix XVII. Tables 7.1, 7.2 and 7.3 show the results of multivariate analysis for the polymorphisms showing some evidence of association with clinical parameters. No statistically significant association with the clinical parameters ($p < 0.01$) were detected. However, some polymorphisms (FcαR, FPR 301 and NADPH oxidase $p22^{phox}$) revealed border-line associations ($p<0.05$) with disease severity.

Subjects homozygous for both A and G allele of FcαR polymorphism had more severe disease compared to heterozygous individuals (see appendix XVII). Multivariate

analysis showed that FcαR polymorphism was associated with differences in average PPD (p=0.037) and number of ≥ 5 mm PPD (p=0.015) (see table 7.1). The general models, containing confounders, were statistically significant (p<0.01), but explained only a small part of the overall variation (see model p value and adjusted R squared values in table 7.1).

Fcα		All GAgP	Caucasian GAgP
Average PPD	*Model p*	0.001	0.001
	Model F	3.437	4.956
	Adjusted R squared	0.124	0.213
	Fcα p	0.037	0.035
Average LCAL	*Model p*	0.001	0.000
	Model F	3.482	5.919
	Adjusted R squared	0.126	0.252
	Fcα p	0.124	0.064
Number of pockets ≥ 5 mm	*Model p*	0.005	0.002
	Model F	2.938	4.143
	Adjusted R squared	0.101	0.177
	Fcα p	0.015	0.019

Table 7.1 Results of multivariate analysis to analyze the effect of genetic polymorphisms on disease severity in all GAgP and Caucasian GAgP patients. The p value of the model, the F value (indicating the ratio of the 2 mean squares of the analyses) and the adjusted R squared (indicating how much of the variability is explained by the model) are presented, together with the p value for each polymorphism.

The largest inter- genotype differences adjusted for confounders (data not presented in tables) were noted between G homozygous subjects and heterozygous individuals for number of ≥ 5 mm PPD (p=0.019, estimated difference = 13.7, 95% C.I.= 2.3- 25.0) and between A homozygous and heterozygous subjects for average PPD (p= 0.030, estimated difference = 1.2 mm, 95% C.I.= 1.0- 1.3) and number of ≥ 5 mm PPD (p=0.022, estimated difference = 22.6, 95% C.I.= 3.2- 42.0). Among GAgP Caucasians, FcαR polymorphism showed some evidence of association with differences in average PPD (p=0.035) and number of ≥ 5 mm PPD (p=0.019) (see table 7.1). The largest inter-genotype differences adjusted for confounders (data not presented in tables) were noted between G homozygous subjects and heterozygous individuals for average PPD (p=0.028, estimated difference = 1.13 mm, 95% C.I.= 1.0- 1.3) and number of ≥ 5 mm PPD (p=0.014, estimated difference = 19.6, 95% C.I.= 4.1- 35.1) and between A homozygous and heterozygous subjects for average PPD (p= 0.046, estimated difference

= 1.2 mm, 95% C.I.= 1.0- 1.5), average LCAL (p=0.027, estimated difference = 1.3 mm, 95% C.I.= 1.0- 1.6) and number of ≥ 5 mm PPD (p=0.036, estimated difference = 28.1, 95% C.I.= 1.8- 54.4).

FPR 301 polymorphism was associated with differences in average PPD (p=0.049) (see table 7.2). Although the general models, containing confounders, were statistically significant, they explained only a small part of the overall variation (see model p and R value in table 7.2). The largest inter- genotype differences adjusted for confounders (data not presented in tables) were noted between G homozygous subjects and heterozygous individuals for average PPD (p=0.016, estimated difference = 1.1 mm, 95% C.I.= 1.0- 1.1) and number of ≥ 5 mm PPD (p=0.020, estimated difference = 13.5, 95% C.I.= 2.1- 24.7). Among GAgP Caucasians, FPR 301 polymorphism did not show evidence of association with any of the studied clinical parameters (see table 7.2). In the subgroup of Black individuals, FPR 301 was also associated with average PPD (p=0.042), although no marked pair wise differences between genotypes were detected.

FPR 301		All GAgP	Caucasian GAgP
Average PPD	*Model p*	0.001	0.003
	Model F	3.437	3.979
	Adjusted R squared	0.125	0.169
	FPR 301 p	0.049	0.222
Average LCAL	*Model p*	0.003	0.001
	Model F	3.094	4.703
	Adjusted R squared	0.109	0.202
	FPR 301 p	0.477	0.575
Number of pockets ≥ 5 mm	*Model p*	0.013	0.014
	Model F	2.502	3.088
	Adjusted R squared	0.084	0.125
	FPR 301 p	0.061	0.150

Table 7.2 Results of multivariate analysis to analyze the relationships between genetic polymorphisms on disease severity in all GAgP and Caucasian GAgP patients. The p value of the model, the F value (indicating the ratio of the 2 mean squares of the analyses) and the adjusted R squared (indicating how much of the variability is explained by the model) are presented, together with the p value for each polymorphism.

In the whole group of subjects of mixed ethnicities, NADPH oxidase 22^{phox} polymorphism showed a moderate trend of association with the clinical parameters (see table 7.3). The largest differences were noted comparing T homozygous subjects and CC subjects for average PPD (p=0.076) and number of ≥ 5 mm PPD (p=0.078). In Caucasians with GAgP, multivariate analysis revealed that NADPH oxidase 22^{phox} polymorphism was associated with number of ≥ 5 mm PPD (p=0.038) (see table 7.3). Comparison between genotypes (data not presented in tables) showed that when T homozygous were compared to C homozygous subjects they showed an increase in average PPD (p= 0.039, estimated difference = 1.2 mm, 95% C.I.= 1.1- 1.5), average LCAL (p=0.021, estimated difference = 1.3 mm, 95% C.I.= 1.0- 1.6) and number of PPD ≥ 5 mm (p=0.017, estimated difference = 32.0, 95% C.I. = 6.0- 58.0). T homozygous subjects had increased average LCAL also when compared to heterozygous subjects (p=0.047, estimated difference = 1.2 mm, 95% C.I.= 1.0- 1.5).

No results approaching statistically significance for any polymorphisms were noted in the LAgP group.

NADPH oxidase $p22^{phox}$		All GAgP	Caucasian GAgP
Average PPD	*Model p*	0.007	0.003
	Model F	2.800	4.115
	Adjusted R squared	0.100	0.186
	NADPH oxidase p	0.196	0.108
Average LCAL	*Model p*	0.004	0.000
	Model F	3.037	5.641
	Adjusted R squared	0.111	0.254
	NADPH oxidase p	0.419	0.066
Number of pockets ≥ 5 mm	*Model p*	0.032	0.006
	Model F	2.200	3.677
	Adjusted R squared	0.069	0.164
	NADPH oxidase p	0.146	0.038

Table 7.3 Results of multivariate analysis to analyze the relationship between genetic polymorphisms on disease severity in all GAgP and Caucasian GAgP patients. The p value of the model, the F value (indicating the ratio of the 2 mean squares of the analyses) and the adjusted R squared (indicating how much of the variability is explained by the model) are presented, together with the p value for each polymorphism.

7.4. DISCUSSION

FcαR A324G, FPR G301C and NADPH oxidase 22^{phox} C242T polymorphisms showed a relation with disease severity in the Generalised Aggressive Periodontitis patients included in this study. None of these differences reached the level of statistical significance set for this study ($p<0.01$). However, we presented here an exploratory, hypothesis- generating analysis. The observed results, which neared statistical significance, may be used to inform a power calculation and allow us to conduct a new study to test the hypotheses of association between these polymorphisms and disease severity.

FcαR 324 A homozygous GAgP patients had more severe disease in our sample than heterozygous individuals. Consistent with this observation, subjects homozygous for the A allele have been shown previously to have decreased phagocytosis of *P.gingivalis* (Kaneko et al. 2004) and in the association study presented in the previous chapter there was a higher frequency of this genotype in patients compared to controls (see table 6.10). We can therefore speculate that, due to an increased susceptibility to periodontopathogenic bacteria, AA individuals may have more severe disease. However, we have to bear in mind that we did not observe any statistical relevance when the clinical features of A and G homozygous subjects were compared with each other, but only when they were compared to the heterozygous subjects. Hence, these results have to be interpreted with caution and require further studies with larger sample size in order to be confirmed or rejected.

A relation with disease severity was also noted for individuals homozygous for the FPR 301 G allele. These subjects had increased average PPD and number of pockets $\geq$ 5 mm compared to the other genotypes, and a gradient effect was observed between G homozygous, heterozygous and C homozygous, which suggests a dominant role for allele G. This effect did not reach statistical significance in the Caucasian GAgP subgroup, but was significant for average PPD in Blacks. Consistent with this was the observed increase in G homozygosity in the patient group compared to controls, especially in Blacks, in the association study presented in the previous chapter (see table

6.10). Speculation about a role of this polymorphism in GAgP severity in Blacks should be supported by functional data on the effect of this polymorphism.

Presence of allele T of NADPH oxidase 22^{phox} C242T polymorphism was previously associated in our sample with presence of AgP (see chapter 6). T homozygosity was particularly enriched in GAgP patients, and in the subgroup of GAgP Caucasians. In relation to disease severity, we noted an effect due to T homozygosity rather than to carriage of the T allele. In fact, CC and CT individuals had very similar clinical parameters, in terms of average probing pocket depth (PPD), average lifetime cumulative attachment loss (LCAL) and number of probing pocket depths equal or above the 5 mm threshold. However, TT individuals had a considerable increase in PPD, which approached statistical significance in the Caucasian GAgP group. In particular, T homozygous subjects exhibited an increase in LCAL equal to 1.3 mm on average, and in number of PPD $\geq$ 5 mm equal to 32 when compared to CC individuals. We can therefore suspect that while carriage of the T allele may predispose to GAgP, within subjects with this disease, homozygosity for the T allele, probably through an increase in the oxidative burst (Shimo-Nakanishi et al. 2004), may be able to predispose to more advanced destruction. This is reflected by the increased number of sites with severe pocket depths, and by the increased averages of both probing pocket depth and lifetime cumulative attachment loss. This result is consistent with our previous findings and points to the fact that once GAgP is initiated, T homozygosity may be involved in its severity and probably in its progression.

In conclusion, this chapter shows a possible influence of genetic polymorphisms in disease severity. These observations, coupled with the evidence gathered from the association study previously described, may shed light into the meaning and importance of genetic polymorphisms in AgP susceptibility.

CHAPTER 8

8. FAMILY STUDY

8.1. BACKGROUND

As outlined in the general introduction to this thesis (see chapter 1), the observation of a familial association of Early Onset Periodontitis dates back many decades (Baer 1971). Given the importance attributed to the familial aggregation in the new classification, and the possible use of family screening as a preventive tool, it is worth trying to clarify the evidence we have to say that early onset cases of periodontitis aggregate in families. The studies from Michalowicz et al. (1991, 2000) can be considered milestones of the periodontal literature in relation to familial aggregation. They studied monozygous and dizygous twins, both reared apart and reared together, and concluded that approximately half of the variance in periodontitis in the population is attributable to genetic factors. However, there is a considerable lack of systematic studies aimed at ascertaining the prevalence of EOP in families and the mode of transmission.

8.1.1. Disease prevalence in families

A literature search on the familial aggregation of EOP identifies some studies based on large pedigrees with high prevalence (Melnick et al. 1976, Hodge et al. 2000, Trevilatto et al. 2002), and a few studies on multiple families with results ranging from moderate (Saxen et al.1980) or very elevated prevalence of EOP in families of affected individuals (Marazita et al. 1994). The largest family study available so far in the periodontal literature was conducted by Marazita et al. (1994). They examined relatives from 100 families (mainly of African - American origin) with at least one individual affected by EOP. These families yielded 463 first degree blood-related individuals, of which 118 (25%) were found positive for a diagnosis of EOP. This means that, according to their data, one in every four relatives of an affected proband also has EOP. However, these data are likely to represent a considerable overestimation of the real disease prevalence, due to the disease definition adopted. Individuals were diagnosed with EOP based on age between 13 and 35 years, and $\geq$ 4 mm attachment loss on at least two first molars or one first molar and one incisor (LJP), or $\geq$ 5 mm attachment loss on at least eight permanent teeth (GEOP). This definition can be criticised as it does not take into account medical history or presence of local factors. However, what is more striking is

that the attachment loss threshold was lowered to ≥ 2 mm for diagnosis of EOP to be made in the relatives. In practical terms, this means that if a relative presented as a diabetic 35 years old with 2 mm of attachment loss on two first molars, he would be classified as an EOP case, whereas now we would consider this to be Chronic Periodontitis.

An additional difficulty of studies ascertaining the family prevalence of AgP arises from the necessity of diagnosing older subjects with multiple missing teeth, without having any previous dental records. In these cases, it becomes necessary to rely only on the patients' memory of past dental problems and treatments and on the clinical examination at the time of presentation (Hodge et al. 2000). Therefore, a real measure of the prevalence of AgP in a large cohort of a considerable number of families is still lacking.

8.1.2. Mode of transmission

Diseases associated to genes of major effect are transmitted following Mendelian inheritance. This is distinguished into X-linked, which means due to the transmission of a gene located in the X chromosome, or autosomal. X-linked transmission can be recessive (e.g. haematophilia) or dominant (e.g. some inherited forms of rickets). In the first case the disease phenotype occurs in homozygous females and males with one copy of the allele, and the disease can skip generations thanks to carrier heterozygous females, who do not express the disease. X-linked dominant transmission is very easy to identify: only one copy of the allele in the X chromosome is necessary to express the disease; therefore it is transmitted from affected fathers to all daughters and from mothers to sons.

Similarly, autosomal transmission can be either recessive or dominant. In case of an autosomal recessive mode of transmission (e.g. cystic fibrosis), the phenotype is present only in homozygous subjects; conversely, in case of dominant transmission, even heterozygous subjects express the disease (e.g. Marfan syndrome).

These are however very simplistic models; in reality, things are complicated by the different penetrance of diseases (probability of phenotypic expression of a gene). If for example 70% of allele carriers manifest the disease, it means the penetrance is 70%.

Furthermore, not all hereditary phenotypes are due to a single gene. Most diseases are controlled by the combined influence of several genes (polygenic heredity), which interact between them and with environmental factors to determine the disease phenotype.

Different theories have been hypothesized about the transmission of Early Onset forms of periodontal disease. A pattern of transmission consistent with Mendelian inheritance of a gene of major effect has been suggested (Saxen et al. 1980, Beaty et al. 1987, Boughman et al. 1988, Marazita et al. 1994). X-linked dominant pattern was hypothesized (Melnick et al. 1976, Spektor et al. 1985), but later dismissed because of probable ascertainment bias (Hart et al. 1991). Segregation analyses show conflicting results, some suggesting a possible autosomal dominant mode of inheritance (Boughman et al. 1988, Marazita et al.1994), mainly in black African-American populations, and some others a recessive one (Saxen 1980). Marazita et al. (1994) performed a segregation analysis on the families reported in the previous paragraph and proposed a dominant mode of inheritance with 70% of penetrance. These different conclusions may be due to different disease definitions, different population and to the heterogeneity of AgP. However, they show the lack of evidence on the subject of transmission of AgP.

8.1.3. Specific aims

Specific objectives of this study were:

- Estimating the percentage of affected first degree blood relatives of AgP patients
- Identifying environmental, demographic or genetic factors able to predict familial aggregation of AgP
- Establishing the type of AgP (Localised or Generalised) present in the relatives and relating it to the proband diagnosis

These specific aims underlie two more clinically-based general questions:

- Is the familial aggregation of AgP strong enough to justify its inclusion as a principal characteristic of the diagnosis?
- Does the prevalence of AgP in families justify the need to examine first degree blood relatives of our AgP patients? Or is this needed just in subgroups of subjects?

8.2. MATERIALS AND METHODS

All patients diagnosed with AgP in our Department were given a case presentation and the suspected genetic background of their condition was explained to them. In this context, the importance of examining their first degree blood relatives was highlighted and patients were given a questionnaire to complete. In this questionnaire patients were invited to write their relatives' names and if they knew they had missing teeth, loose teeth, bleeding gums or if they had been treated for gum disease. All available first degree blood relatives were invited for a specific assessment at the Eastman Dental Hospital. The study protocol was approved by the Eastman Joint Research and Ethics Committee. The proband and the relatives who were examined provided signed informed consent.

No formal power calculation was performed in the design of this study. Out of a total of 230 AgP patients examined, relatives from only 54 of them were able to attend for examination at the EDH. Many patients excluded themselves because of poor family histories (no contact with family, family living abroad, adopted). Many other relatives refused to come for an assessment because they were not interested or did not have the possibility to attend. Therefore, no random selection was used to select this population of probands and relatives. Hence, we have to consider the analyses presented in this chapter as exploratory analyses and be cautious with the interpretation of the results. Nonetheless, data derived from this chapter might be useful to design more focused studies in order to answer the question: "Do genetic factors have an effect on susceptibility to AgP?".

8.2.1. Relatives' examination

Relatives who attended for examination were given a medical history form to complete and were asked questions about their dental health and their previous dental treatments, with particular regards to periodontal treatment. Clinical screening involved assessment of gingival appearance and oral hygiene and a Community Periodontal Index of Treatment Needs (CPITN) examination, using a WHO probe. Whenever probing pocket depths (PPD) or lifetime cumulative attachment loss (LCAL) $\geq$ 4 mm were noted, full mouth measures of PPD and LCAL were obtained using a UNC 15 periodontal probe. Radiographic assessment included panoramic or long cone periapical radiographs. Once a diagnosis was made, a letter describing the diagnosis and, if necessary, the treatment plan was sent to their general dentists and copied to the patient. If the patients consented, a blood sample was collected and stored at -70° C.

8.2.2. Relatives' diagnosis

Authors of previous reports investigating the family history of AgP adopted heterogeneous disease criteria, sometimes different even within the same study between probands and relatives (Marazita et al. 1994, Hodge et al. 2000). We chose to apply the same diagnostic criteria to our probands and relatives, and the 1999 Consensus Classification was used (see chapter 1). All subjects were diagnosed as healthy or with either gingivitis, chronic periodontitis (CP) or AgP. The only difference between the diagnosis of probands and relatives was that at the time of examination, for the relatives we already had certainty of their family history of AgP.

All available fathers, mothers and siblings of our probands, above the age limit of 12 years old were invited for examination. We are aware that patients as young as 13 years old may still be too young to manifest the disease phenotype. However, only 5 subjects (5.2%) were younger than 20 years old. In instances where patients had experienced multiple tooth loss, an attempt was made to understand the cause for tooth loss, and to obtain old dental records. Whenever, because of the patient age and the lack of previous records, there were uncertainties, a diagnosis of AgP was dismissed.

8.2.3. Statistical analysis

SPSS 12.0 package was used for statistical analysis. Continuous, normally distributed variables are reported as means ± standard deviations (SD). The percentage of relatives affected by CP and AgP was calculated considering the total of relatives examined. As we had not managed to examine all the possible relatives, another percentage was calculated to reflect all the possible relatives of the 54 probands, assuming that none of the relatives we had not examined would actually have AgP. Comparisons of continuous and categorical data between groups were analyzed with ANOVA and Chi-square test respectively. In an exploratory analysis, the demographic characteristics of each relative (gender, age, smoking, and ethnicity) and the genetic polymorphisms of the correspondent probands were entered as variables, in order to detect possible risk indicators for positive diagnosis.

WHAP analysis (see chapter 6) was performed in order to detect if any FcγR and FPR haplotypes were able to predict the familial aggregation of AgP.

8.3. RESULTS

Fifty four probands had at least one relative who volunteered for examination. Table 8.1 reports the demographic and clinical characteristics of the probands. Most of them were females, Caucasians, and almost half of them had never smoked. These characteristics mirror the gender, ethnic and smoking status of the whole group of 224 AgP included in the association study (see table 6.4). These 54 patients had a total of 224 first degree blood relatives, including fathers, mothers, brothers and sisters. Ninety nine of them (almost two per proband) agreed to take part in the study and attended for examination. Most probands (53.7%) yielded just one relative each; two relatives each were examined from 14 probands, 3 relatives each from 7 probands, four from 1, five from 2 and seven relatives were examined from 1 single proband. Table 8.2 reports the characteristics of the 99 relatives examined. As it is evident from the table, the majority of relatives who attended were females: more mothers than fathers, and more sisters than brothers were examined. Ten subjects (10.1%) were diagnosed with AgP (7 GAgP and 3 LAgP), 40 (40.4%) with CP, 29 (29.3%) presented with gingivitis, while 20 (20.2%) were

periodontally healthy. A conservative estimate of the prevalence of AgP in these families, including the total of relatives not-examined (assuming none of them to be affected by AgP) was 4.5%.

PROBANDS (n=54) **PARAMETER**		**MEAN**
AGE (Mean ± S.D.)		28.1 ± 6.7
FEMALES (percentage)		39 (72.2%)
SMOKERS (percentage)	*Current heavy (≥ 20 cigarettes/day)*	6 (11.1%)
	Current light	8 (14.8%)
	Former	16 (29.6%)
	Never	24 (44.4%)
ETHNICITY (percentage)	*Caucasians*	32 (59.3%)
	Blacks	12 (22.2%)
	Asians	6 (11.1%)
	Others	4 (7.4%)
TEETH AT BASELINE (Mean ± S.D.)		27.9 ± 2.7
NUMBER OF POCKETS ≥ 5 mm (Mean ± S.D.)		54.3 ± 33.6
PROBING POCKET DEPTH (mm) **(Mean ± S.D.)**		3.9 ± 1.1
LIFETIME CUMULATIVE ATTACHMENT LOSS (LCAL) (mm) **(Mean ± S.D.)**		4.5 ± 1.4

Table 8.1: Demographic and clinical characteristics of AgP patients who had at least one relative examined as part of the study

RELATIVES (n=99) PARAMETER		MEAN
AGE (Mean ± S.D.)		38.7 ± 15.2
FEMALES (percentage)		61 (61.6%)
SMOKERS (percentage)	*Current heavy (≥ 20 cigarettes/day)*	5 (5.0%)
	Current light	16 (16.1%)
	Former	12 (12.1%)
	Never	66 (66.7%)
ETHNICITY (percentage)	*Caucasians*	66 (66.7%)
	Blacks	15 (15.2%)
	Asians	13 (13.1%)
	Others	5 (5.1%)
RELATION TO PROBAND (percentage)	*Fathers*	13 (13.1%)
	Mothers	23 (23.2%)
	Brothers	26 (26.3%)
	Sisters	37 (37.4%)
DIAGNOSIS (percentage)	*Healthy*	20 (20.2%)
	Gingivitis	29 (29.3%)
	Chronic Periodontitis	40 (40.4%)
	Aggressive Periodontitis	10 (10.1%)

Table 8.2: Demographic characteristics and diagnosis of relatives of AgP patients examined

8.3.1. Association between diagnosis and relative characteristics

Table 8.3 shows that out of the 10 relatives diagnosed with AgP, 1 was a father, 1 a mother, 4 were brothers and 4 sisters of the original probands. Gender, ethnicity and smoking were not associated with the diagnosis. The relation to the proband was not associated with the diagnosis of AgP but had an effect on the diagnosis of destructive periodontitis (AgP+ CP): respectively 85%, 56%, 38% and 43% of fathers, mothers, brothers and sisters were diagnosed with CP (Pearson's Chi square p=0.034).

RELATIVES		No AgP	AgP	PEARSON'S CHI SQUARE	AgP+ CP	PEARSON' S CHI SQUARE
GENDER	*Female (n= 61)*	56 (91.8%)	5 (8.2%)	0.426	30 (49.2%)	0.837
	Male (n=38)	33 (86.8%)	5 (13.2%)		20 (52.6%)	
ETHNICITY	*Caucasian (n= 66)*	61 (92.4%)	5 (7.6%)	0.665	32 (48.5%)	0.819
	Black (n= 15)	13 (86.7%)	2 (13.3%)		9 (60.0%)	
	Asian (n= 13)	11 (84.6%)	2 (15.4%)		6 (46.2%)	
	Others (n= 5)	4 (80.0%)	1 (20.0%)		3 (60.0%)	
SMOKING	*Current heavy (n= 5)*	4 (80.0%)	1 (20.0%)	0.560	4 (80.0%)	0.566
	Current light (n=16)	14 (87.5%)	2 (12.5%)		7 (43.8%)	
	Former (n= 12)	12 (100.0%)	0		6 (50.0%)	
	Never (n= 66)	59 (89.4%)	7 (10.6%)		33 (52.4%)	
RELATION TO PROBAND	*Fathers (n= 13)*	12 (92.3%)	1 (7.7%)	0.628	11 (84.6%)	0.034
	Mothers (n= 23)	22 (95.7%)	1 (4.3%)		13 (56.5%)	
	Brothers (n= 26)	22 (84.6%)	4 (15.4%)		10 (38.5%)	
	Sisters (n= 36)	33 (89.2%)	4 (10.8%)		16 (43.2%)	
TOTAL (n= 99)		89 (89.9%)	10 (10.1%)		50 (50.5%)	

Table 8.3: Relationships between demographic characteristics of relatives and periodontal diagnosis with relative Chi-squared results

8.3.2. Association between diagnosis and proband characteristics

Ten out of 54 probands (18.5%) had at least one blood relative affected by AgP. In 35 of the examined families (64.8%) there was at least one relative with destructive periodontitis (CP or AgP).

Table 8.4 shows that each relative diagnosed with AgP had the same diagnosis as his related proband: all probands of the 7 GAgP relatives were diagnosed with GAgP and

all probands of the 3 LAgP relatives were diagnosed with LAgP. Clinical and radiographic images of two of the probands and their affected relative with AgP are presented here.

PROBAND					RELATIVE				
Gender	**Age Dx**	**Smoking**	**Ethnicity**	**Dx**	**Rel. to proband**	**Gender**	**Age Dx**	**Smoking**	**Dx**
F	29	Former	Cauc.	GAgP	Mother	F	50	Current	GAgP
F	34	Former	Other	GAgP	Brother	M	29	Never	GAgP
F	31	Current	Cauc.	GAgP	Twin sister	F	31	Never	GAgP
M	14	Current	Cauc.	LAgP	Brother	M	14	Never	LAgP
F	27	Never	Asian	GAgP	Sister	F	33	Never	GAgP
F	36	Current	Cauc.	GAgP	Father	M	40	Current	GAgP
F	31	Never	Asian	GAgP	Sister	F	29	Never	GAgP
F	18	Current	Black	LAgP	Brother	M	20	Never	LAgP
M	31	Current	Cauc.	GAgP	Brother	M	39	Never	GAgP
F	14	Never	Black	LAgP	Sister	F	15	Never	LAgP

Table 8.4: Demographic characteristics and diagnosis of probands and correspondent relatives affected by AgP

Figure 8.1 represents the case of a 14-year old Black Caribbean (already described in paragraph 5.2.2), while figure 8.2 relates to her 15-year old sister. They both had a similar pattern of localised disease, although the sister only had the left first molars affected.

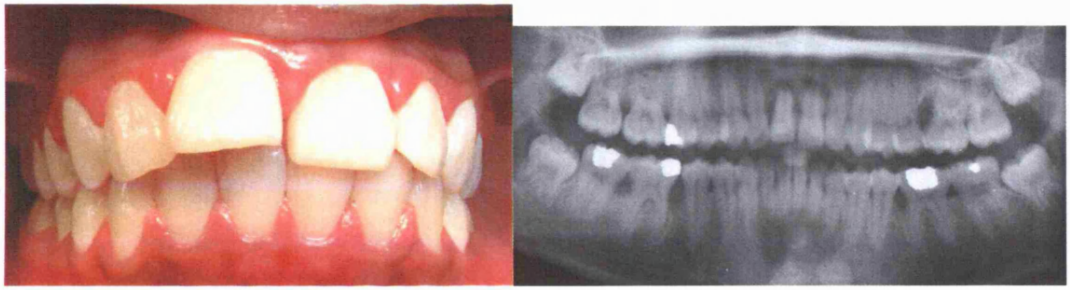

Figure 8.1. Frontal intraoral picture and orthopantomograph of the 14-year old Black- Caribbean female patient described in paragraph 8.4.2. Localised vertical bone defects are present around all first molars and 11 (UR1). A diagnosis of LAgP was made.

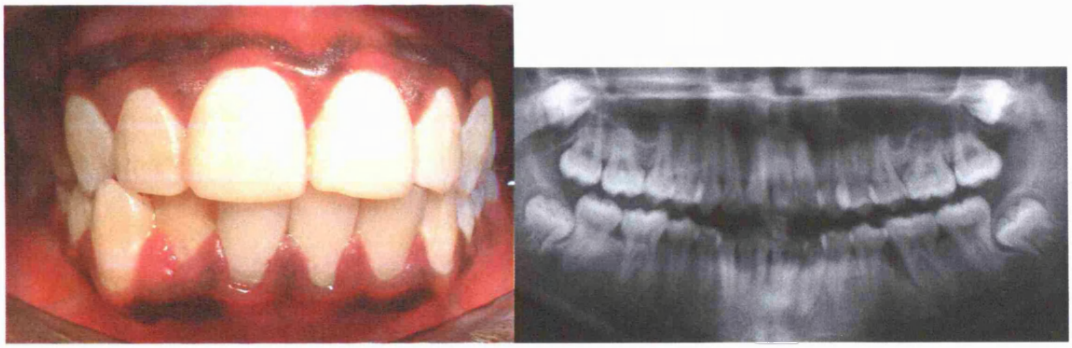

Figure 8.2. Frontal intraoral picture and orthopantomograph of the 15-year old Black- Caribbean female patient, sister of the patient shown in figure 8.1. Localised vertical bone defects are present around 26 (UL6) and 36 (LL6). A diagnosis of LAgP was made.

Figures 8.3 and 8.4 represent orthopantomographs of a female Caucasian patient (the proband) and of her father. The proband (figure 8.3) was 30 years old at the time of diagnosis and was a light smokers (10 cigarettes/day for 16 years). She presented with excellent standards of oral hygiene, generalised attachment and bone loss and was classified as GAgP. Her father was a 50 year-old former smoker. He reported a history of periodontal problems since his early twenties, with extraction of a number of teeth due to excessive mobility. Clinical examination revealed moderate amounts of plaque,

localised marginal inflammation and moderate to severe periodontal probing pocket depths. He had attended for a visit at the EDH 10 years before, therefore we were able to retrieve previous radiographic records (see figure 8.4), which show generalised bone loss, affecting most of the remaining dentition. Anamnestic, clinical and radiographic findings were consistent with a diagnosis of GAgP.

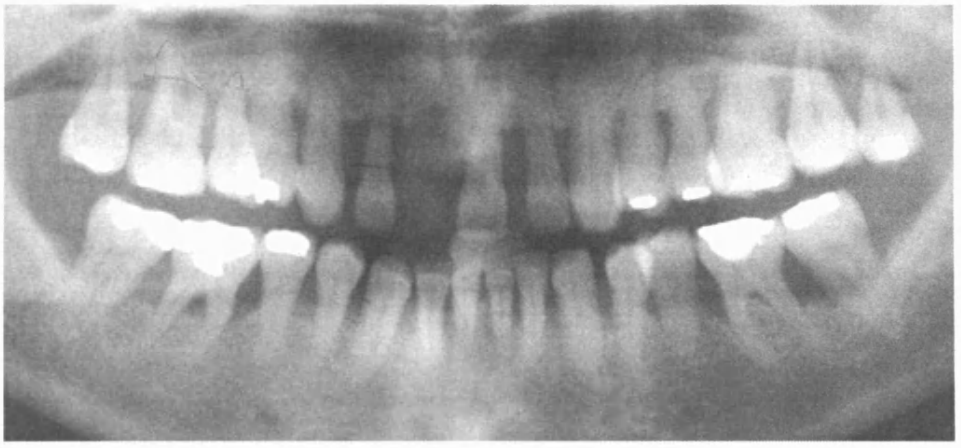

Figure 8.3. Orthopantomograph of the 30-year old Caucasian female patient described in paragraph 8.4.2. A generalised pattern of alveolar bone loss is present both in the maxilla and in the mandible.

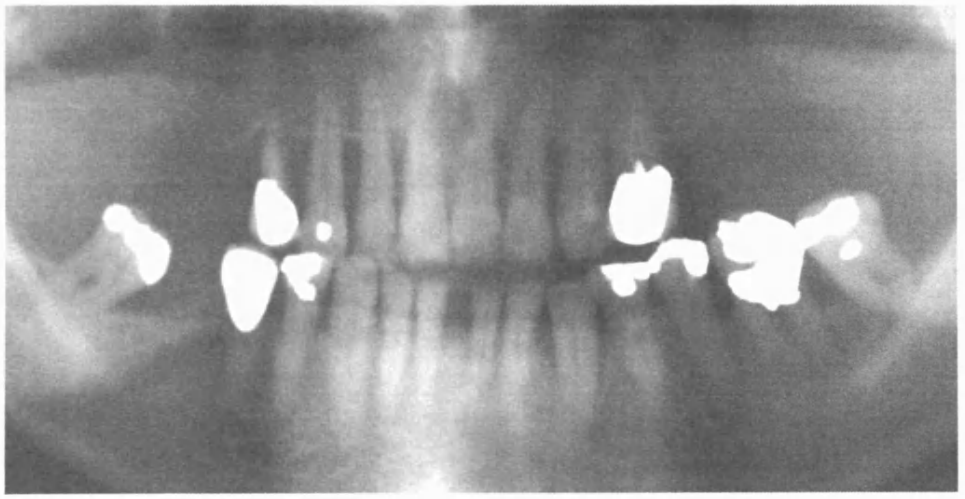

Figure 8.4. Ortpantomograph of the 50-year old father of the patient shown in figure 8.3 (the radiograph was taken when the patient was 40 years old). A generalised pattern of alveolar bone loss is present both in the maxilla and in the mandible.

Table 8.5 reports the relatives' diagnosis in relation to proband characteristics, such as gender, diagnosis, ethnicity and genotypes (described in chapter 6). The proband of one

of the 10 affected relatives is among the 6 out of 230 probands who did not have a blood sample taken, therefore genetic SNP results are available from just 9 probands of AgP relatives. Probands' gender, ethnicity and age of diagnosis were not associated with relatives' diagnosis. The proband genotype for Fcγ IIa was significantly associated with diagnosis of AgP in the correspondent relative (Pearson's Chi square p=0.003). However, some cells of the Chi-squared table would have expected values less than 5 - which make this result not very reliable, probably for a lack of power. Tendencies for association were also noted for FcγRIIIa (which is in linkage disequilibrium with the FcγRIIa gene, as assessed in chapter 6) and IL-6 polymorphisms.

RELATIVES / **Proband characteristics**		**no AgP**	**AgP**	**PEARSON'S CHI SQUARE**
Gender	Female (n= 61)	56 (91.8%)	5 (8.2%)	0.426
	Male (n=38)	33 (86.8%)	5 (13.2%)	
Diagnosis	LAgP (n= 33)	30 (90.9%)	3 (9.1%)	1.000
	GAgP (n= 66)	59 (89.4%)	7 (10.6%)	
Ethnicity	Caucasian (n=66)	61 (92.4%)	5 (7.6%)	0.665
	Black (n=15)	13 (86.7%)	2 (13.3%)	
	Asian (n=13)	11 (84.6%)	2 (15.4%)	
	Others (n=5)	4 (80.0%)	1 (20.0%)	
Fcα -324	AA (n=7)	6 (85.7%)	1 (14.3%)	0.890
	AG (n=39)	35 (89.7%)	4 (10.3%)	
	GG (n=46)	42 (91.3%)	4 (8.7%)	
FcγIIa 494	HH (n=18)	17 (94.4%)	1 (5.6%)	0.003
	HR (n=54)	52 (96.3%)	2 (3.7%)	
	RR (n=20)	14 (70.0%)	6 (30.0%)	
FcγIIb 232	CC (n=6)	6 (100%)	0	0.430
	CT (n=18)	15 (83.3%)	3 (16.7%)	
	TT (n=68)	62 (91.2%)	6 (8.8%)	
FcγIIIa 559	VV (n=10)	9 (90.0%)	1 (10.0%)	0.176
	VF (n=46)	44 (95.7%)	2 (4.3%)	
	FF (n=36)	30 (83.3%)	6 (16.7%)	

Table 8.5: Relatives' diagnosis in relation to correspondent probands' demographic characteristics and genetic profile

RELATIVES Proband characteristics		no AgP	AgP	PEARSON'S CHI SQUARE
FcγIIIb NA	NA2/NA2 (n=43)	38 (88.4%)	5 (11.6%)	0.617
	NA1/NA2 (n=34)	32 (94.1%)	2 (5.9%)	
	NA1/NA1 (n=15)	13 (86.7%)	2 (13.3%)	
FcγIIIb SH	SH+/SH+ (n=2)	2 (100.0%)	0	0.663
	SH-/SH+ (n=5)	5 (100.0%)	0	
	SH-/SH- (n=85)	76 (89.4%)	9 (10.6%)	
FPR 301	CC (n=9)	8 (88.9%)	1 (11.1%)	0.647
	CG (n=43)	40 (93.0%)	3 (7.0%)	
	GG (n=38)	33 (86.8%)	5 (13.2%)	
FPR 546	AA (n=15)	14 (93.3%)	1 (6.7%)	0.771
	AC (n=36)	33 (91.7%)	3 (8.3%)	
	CC (n=41)	36 (87.8%)	5 (12.2%)	
FPR 568	AA (n=71)	63 (88.7%)	8 (11.3%)	0.679
	AT (n=21)	20 (95.2%)	1 (4.8%)	
	TT (n=0)	0	0	
VDR Taq-I	tt (n=2)	2 (100.0%)	0	0.288
	Tt (n=60)	52 (86.7%)	8 (13.3%)	
	TT (n=30)	29 (96.7%)	1 (3.3%)	
TNF- α -308	AA (n=68)	61 (89.7%)	7 (10.3%)	1.000
	AG (n=24)	22 (91.7%)	2 (8.3%)	
	GG (n=0)	0	0	
IL-6 -174	CC (n=7)	6 (85.7%)	1 (14.3%)	0.268
	CG (n=19)	19 (100.0%)	0	
	GG (n=66)	58 (87.9%)	8 (12.1%)	
NADPH p22phox 242	CC (n=19)	18 (94.7%)	1 (5.3%)	0.580
	CT (n=61)	54 (88.5%)	7 (11.5%)	
	TT (n=4)	4 (100.0%)	0	
Total (n= 99)		89 (89.9%)	10 (10.1%)	

Table 8.5 continued

8.3.3. Haplotype analysis

WHAP analysis confirmed the association between FcRγIIa and the presence of AgP in the family (p=0.001, adjusting for gender, ethnicity and smoking). The haplotype association between all five studied Fc receptor polymorphisms was still associated with AgP (p=0.014), but the extent of this association was smaller than for FcRγIIa only.

8.4. DISCUSSION

A small subset of AgP patients examined in our Department provided relatives who lived in the UK and were willing to volunteer for a specific periodontal examination. The demographics of this group with respect to ethnic origin, gender and smoking status mirrored the characteristics of the 224 AgP patients included in the case-control genetic association study. Therefore, although no data regarding the socio-economic status of these patients have been examined, it seems reasonable to assume that they broadly represent the whole group of AgP patients described in this thesis.

Eighteen per cent of the families of AgP patients included in the study had at least one additional individual affected. The prevalence of AgP in first degree blood relatives of AgP patients that we managed to examine was found to be equal to 10%. This prevalence is considerably lower than has been reported in most previous studies (Spektor et al. 1985, Marazita et al. 1994, Trevilatto et al. 2002). This discrepancy is likely to be due to the fact that most studies focused just on one family with multiple subjects affected (Trevilatto et al. 2002), or to the excessively lenient definition often adopted for the diagnosis of AgP in relatives (Marazita et al. 1994). However, our findings are more in agreement with the report from Saxen (1980), who examining 127 subjects from 33 Finnish families of AgP patients found AgP in 11 siblings (9%) from 8 families (24%). In Saxen's study, most of the existing relatives (94%) were examined. In our study, on the other hand, only 99 out of 224 possible relatives were examined (44%), which carries the risk of possible self- selection bias. In other words, less than half of the relatives accepted our invitation to be examined. Out of the 224 first degree blood relatives, some were deceased, some lived abroad, and some of them were odontophobic or simply did not care to have a dental examination. This has led to an increased female/male ratio in relatives, as expected from previous similar studies (Hart et al. 1991) and might also have led to over- or under- estimation of AgP prevalence in these families. Considering the total number of relatives of our probands, we can say that at least 10 out of 224 (4.5%) were affected by AgP. Even this conservative prevalence is higher than the prevalence of AgP in the population as described in epidemiological studies (see chapter 1). Moreover, 65% of the examined relatives were found to be

affected by Chronic Periodontitis, a higher percentage than what would be expected in a random adult population (Kelly et al. 1999). Therefore, this study supports the concept of familial aggregation in AgP and suggests the employment of routine examination of first degree blood relatives of AgP patients as a useful preventive tool. Moreover, an observation on a subset of our patients revealed that the proband report about their relatives' periodontal condition was reasonably reliable (Gozalbo & Griffiths 2005), and effective in the selection of risk families to examine. Nonetheless, the extent of this familial aggregation is probably not large enough to justify its inclusion criteria as one of the principal features of the diagnosis.

Hart et al. (1991) reported that the supposed increased prevalence of AgP in female relatives was probably due to ascertainment bias. Our data seem to support this statement: 13% of male relatives and 8% of female relatives had AgP. Ethnicity and smoking habits were not associated with AgP in the relatives. More siblings than parents were found to be affected by AgP.

Further analysis was conducted to detect if any proband characteristic (gender, ethnicity, genetic polymorphisms) could represent a risk predictor for familial aggregation. Within the limitations of a small sample size, Fcγ IIa polymorphism was a significant risk predictor for presence of AgP in the family. 30% of relatives of RR homozygous probands and only 4% of heterozygous and HH homozygous had AgP. Considering the families rather the number of examined relatives as the unit for statistical analysis, AgP was found in 60% of families of Fcγ IIa R homozygous and 7% of heterozygous and H homozygous subjects. Interestingly, other risk polymorphisms observed in the association study (IL-6 and NADPH oxidase) showed trends of association with familial aggregation. Of the relatives of either IL-6 -174 C homozygous and heterozygous probands, only 1/26 (4%) had AgP, compared to 8/66 (12%) of the relatives of G homozygous probands. Similarly for the NADPH p22phox polymorphism, 1/19 (5%) of relatives of C homozygous probands and 7/61 (11%) of relatives of T allele carriers were found to have AgP. These preliminary data point towards the importance of genetic predisposition to AgP in families. The same genotypes predisposing the probands to

AgP, may also be present in the relatives of these probands, thus predisposing them to AgP also. Only a genetic polymorphism study on affected and non affected relatives, and a larger sample size could clarify and confirm these observations.

It is interesting to observe the exact correspondence of diagnosis between probands and relatives affected by AgP. A diagnosis of LAgP was made in 3 relatives of LAgP patients, and GAgP was diagnosed in 7 relatives of GAgP patients. A similar observation was noted by Saxen (1980) and suggests that LAgP and GAgP might have different genetic predisposing factors.

We have not tested any hypothesis for the mode of inheritance in our sample. However, according to our female/male ratios and to the observation of the absence of periodontitis in some of the daughters of an affected father, the likelihood of an X-linked transmission seems to be small. In autosomal dominant mode of transmission, the disease will tend to appear in successive generations. Although we managed to examine only 9 complete couples of parents in this study, none of them were affected by AgP. Furthermore, only 2 out of the other 18 parents were affected. This does not seem to support an autosomal dominant mode of transmission. The remaining possibilities are autosomal recessive mode of inheritance, supposing the existence of a gene of major effect, or complex polygenic and multifactorial inheritance. In an autosomal recessive mode of inheritance, the phenotype is present only in homozygous subjects. The parents are usually carriers (heterozygous) and there is 25% chance for the offspring to be affected.

On the other hand, AgP may behave like many common acquired diseases, such as diabetes, asthma, Parkinson disease, rheumatoid arthritis, which do not conform to any recognized pattern of Mendelian inheritance, and show multifactorial inheritance (Hansen & Pedersen 2005). This means that several genes contribute to disease susceptibility, each exerting additive effects, which interact with environmental factors to determine disease onset. For example, type I diabetes is now recognized as a multifactorial disease with polygenic susceptibility consisting of one major locus and up

to 20 modifying loci. The extent of familial heritability of multifactorial diseases is calculated by dividing the incidence in families of affected individuals by the incidence in the population (ratio = λ) (Khouri et al. 1993). Type I diabetes has an incidence of 0.4% in UK population and about 6% in affected families. The λ for type I diabetes is therefore equal to 15. The corresponding figure for AgP, assuming that the overall prevalence in the population is about 1% (see chapter 1) and the family prevalence is 10% would be equal to 10.

Considering the importance of environmental factors such as bacteria and smoking, and the supposed importance of multiple genes as risk factors for periodontitis, this polygenic model seems a more likely mode of inheritance for AgP. Nonetheless, the very strong familial aggregation encountered in some cases (Spektor et al. 1985), raises doubts about a polygenic nature of transmission.

A consistent finding of most studies, confirmed by this report, is the increased prevalence of destructive periodontitis in families, irrespective of the difference in diagnosis between CP and AgP. This has probably led to the concept of autosomal dominant transmission, but may be masked by the rather high prevalence of CP in the normal population. This concept points in two directions: i) AgP and CP may indeed have similar pathogenesis, or be different manifestations of the same disease, which would not justify a subdivision in the classification; ii) there might be similar polygenic background to CP and AgP, and some genes may have an additive effect resulting in the more severe form of the disease. Alternatively there may be environmental factors which can increase the susceptibility of a subject who has a genetic background which is already prone to AgP.

The overall impression, taking together literature reports and data from the present investigation, is that AgP is genetically very heterogeneous and has a whole spectrum of different possible modes of transmission. It may be the result of various pathogenic pathways, and susceptibility to AgP may lie in different defects and different genes in each population and even in each family. These genes may be of major effect (capable

of causing the disease in both dominant and recessive manners) or just predisposing, and lead all to a similar disease phenotype, which we are not able to differentiate.

In conclusion, this study suggests the employment of routine examination of first degree blood relatives of AgP patients as a useful preventive tool and supports the use of family history as an easily accessible genetic tool for individual disease prevention in AgP (Guttmacher et al. 2004).

CHAPTER 9

9. MICROBE-GENETIC INTERACTIONS

9.1. INTRODUCTION

Response to infection varies enormously between individuals and genetic factors may explain these individual variations (Wang 2005). This concept has led to a growing interest in the genetic predisposing factors for life-threatening infectious diseases such as malaria, tuberculosis, hepatitis and AIDS. It has been shown recently that genetic variants in the promoter region of the CC chemokine receptor 5 gene confer protection against HIV infection (Cooke & Hill 2001). A recent review (Schroder & Schumann 2005) underlined how SNPs in Toll-like receptors (TLR) loci may affect the ability to respond to infectious agents. Moreover, genetic polymorphisms of inflammatory markers have recently been associated with the prevalence of bacterial infections in patients in intensive care units (Sutherland et al. 2005), and polymorphisms in Fcγ receptors have been suspected to have an effect on host defence against malaria infection (Omi et al. 2002). Understanding the molecular basis for these different responses may improve our understanding of infectious disease pathogenesis and help in the treatment and control of these infections (Cooke & Hill 2001).

The pathogenesis of AgP is considered to be due to a shift in the homeostasis existing between the bacteria present in the subgingival plaque and the host response. Thus in AgP, host responses (genetically determined) and microbiological factors seem to be deterministic components which, even in the absence of conspicuous amounts of subgingival plaque or other local factors, may trigger or cause the onset of this disease (Kinane & Hart 2003).

Actinobacillus actinomycetemcomitans, *Tannerella forsythensis* (previously *Bacteroides forsythus*) and *Porphyromonas gingivalis* were officially designated as being among the aetiological agents of periodontitis in 1996 (Consensus Report 1996) and in the majority of populations, AgP has been associated with a mixed infection with these microorganisms (Mombelli et al. 2002). The presence of elevated proportions of *A. actinomycetemcomitans* and, in some populations, of *P. gingivalis,* is now considered one of the laboratory diagnostic features of AgP (Armitage 1999). *A.*

actinomycetemcomitans is a small, non-motile, Gram-negative, saccharolytic, capnophilic, round-ended rod. The proportion of subjects who carry this bacterium varies from 20% to 90% in AgP cases (Mombelli et al. 2002). It has also been demonstrated that most AgP cases have elevated serum antibodies against *A. actinomycetemcomitans* (Kinane et al. 1999b). *P. gingivalis* is a Gram-negative, anaerobic, non-motile, asaccharolytic rod. It is one of the most prevalent bacteria isolated from AgP lesions and may be detected from 60-90% of generalised cases. *T. forsythensis* is a Gram-negative, anaerobic rod, which may be detected in 70-100% of patients with Generalised AgP (GAgP) (Mombelli et al. 2002).

A range of different genetic deficiencies or variations in the host response are considered to predispose to AgP and several genetic polymorphisms have been associated with susceptibility to this infection, with particular regards to neutrophil receptors and cytokines (Kinane & Hart 2003). In the study presented in chapter 6 we have detected associations between AgP and two genetic polymorphisms (IL-6 -174 and NADPH oxidase $p22^{phox}$ C 242 T) and tendencies for association with AgP for two other polymorphisms (FcγRIIIb and VDR Taq-I) and a haplotype (FcγR). The mechanisms of this supposed genetic predisposition are still not clearly elucidated, and may involve interactions with periodontal bacteria.

In the present study, we hypothesized that the host genotype can influence the composition of the subgingival microbiota. Evidence which supports the hypothesis that a particular genotype could render host defence mechanisms more selectively active against specific periodontal pathogens is very limited (Socransky et al. 2000), and there are no previous studies investigating these possible interactions in patients diagnosed with GAgP. The aim of this study was to investigate whether genetic polymorphisms of inflammatory markers may influence the presence of *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis* in a population diagnosed with GAgP.

9.2. MATERIALS AND METHODS

9.2.1. Study population

Out of all the subjects included in the genetic association study, 45 consecutive subjects diagnosed with Generalised AgP were identified and included in the study. All of the patients gave written informed consent and the study had been reviewed and approved by the Eastman/UCLH joint ethics committee.

Inclusion and exclusion criteria were described in chapter 4. Furthermore, subjects were excluded from the study if: i) they were pregnant or lactating females, ii) they suffered from any other systemic diseases (cardiovascular, pulmonary, liver, cerebral diseases or diabetes); iii) they had received systemic or local antibiotic treatment in the previous 3 months; iv) they were taking long-term anti-inflammatory or immunosuppressive drugs; v) they had received a course of periodontal treatment within the last 6 months. The demographic and clinical characteristics of the 45 patients are presented in table 9.1.

PARAMETER		**MEAN**
AGE (Mean ± S.D.)		29.9 ± 5.5
FEMALES (percentage)		30 (66.7%)
SMOKERS (percentage)		9 (20.0%)
ETHNICITY (percentage)	*Caucasians*	27 (60.0%)
	Blacks	7 (15.6%)
	Asians	10 (22.2%)
	Others	1 (2.2%)
TEETH AT BASELINE (Mean ± S.D.)		28.5 ± 2.7
NUMBER OF POCKETS ≥ 5 mm (Mean ± S.D.)		64.0 ± 30.8
PROBING POCKET DEPTH (mm) (Mean ± S.D.)		4.2 ± 1.0
LIFETIME CUMULATIVE ATTACHMENT LOSS (LCAL) (mm) (Mean ± S.D.)		4.8 ± 1.3

Table 9.1. Demographic characteristics of the patients

9.2.2. Clinical examination

All of the patients with a suspected diagnosis of AgP were examined by a single experienced clinician. As described in chapter 4, full mouth measures of probing pocket depth (PPD), recession and lifetime cumulative attachment levels (LCAL) were obtained. Six sites were measured for each natural tooth, one each at the mesiobuccal, mid-buccal, distobuccal, distolingual, mid-lingual and mesiolingual sites encircling the tooth. Full mouth long cone periapical radiographs were also obtained for each patient.

Patients were diagnosed with Generalized AgP (GAgP) when presenting with generalized interproximal PPD and LCAL $\geq$ 5 mm and radiographic bone loss of $\geq$ 30% of root length affecting at least three permanent teeth other than first molars and incisors. Once the eligibility criteria had been fulfilled, consent was taken for blood and subgingival plaque sampling and patients were entered into the study. The blood samples were collected via venipuncture and stored at -70° C until the genetic analysis was performed.

9.2.3. Plaque sampling

The plaque samples were collected from the 4 deepest sites in each quadrant (Mombelli et al. 1991). The supragingival portion of the root surface of the site was carefully cleaned with a curette and isolated from saliva by a gentle air spray and by positioning a cotton roll in proximity of the tooth. A sterile curette was then inserted to the bottom of the pocket and the microbiological sample was collected with a single apical-coronal stroke. Samples were then immediately placed in a sterile container with 1 ml of reduced transport fluid (Syed & Loesche 1972) before periodontal probing.

9.2.4. DNA extraction and genotyping

Genetic procedures applied in this study have been described in chapter 6.

9.2.5. Microbiological analysis

The samples were processed immediately on arrival at the Department of Microbiology of the Eastman Dental Hospital. Samples were vortex mixed for 30 seconds (or until the plaque had been dispersed) and subsequently diluted in reduced transport fluid to a dilution factor of 10^{-6}. Duplicate 50µl aliquots of the sample at each dilution were then plated on the following media: (a) Fastidious anaerobic agar (FAA) containing 5% defibrinated horse blood (for the total anaerobic count) and all black-pigmented colonies were counted and subcultured for identification of *P. gingivalis*, (b) *A. actinomycetemcomitans* was isolated on tryptone soya agar (Oxoid Ltd, Basingstoke, UK) containing 0.1 % yeast extract, 10 % horse serum (E & O Laboratories, Bonnybridge, UK), bacitracin (75 mg/L; Sigma, Poole, UK) and vancomycin (5mg/L; Sigma; Slots, 1982) and incubated at 37°C for 5 days in air supplemented with 5% CO_2. (c) FAA containing 7% defibrinated horse blood and 10 mg/L N-acetyl muramic acid (colonies with the appropriate morphology were counted and subcultured for identification of *T. forsythensis*). All FAA plates were incubated in an anaerobic environment (10 % hydrogen, 10 % carbon dioxide, balanced with nitrogen) in an anaerobic cabinet at 37^0C for 5 days. The identity of isolates was determined by partial sequencing of the 16S rRNA gene amplified by PCR. The whole genomic DNA was extracted from cultures of presumptive *A. actinomycetemcomitans, P. gingivalis* and *T. forsythensis* using the Puregene^{TM} DNA isolation Kit, (Gentra Systems, Minneapolis, USA). The extracted DNA was then used as a template for species confirmation via PCR, which targeted specific regions within the 16S rRNA gene. The nucleotide sequences for the primers were as follows: *P. gingivalis* specific forward primer (PgF), 5'-TGTAGATGACTGATGGTGAAAACC-3'; *A. actinomycetemcomitans* specific forward primer (AaF), 5'-ATTGGGGTTTAGCCCTGGTG-3'; *T. forsythiensis* specific forward primer (TfF), 5'-TACAGGGGAATAAAATGAGATACG-3' and a conserved reverse primer (ConR), 5'-ACGTCATCCCCACCTTCCTC-3' (Genosys, Cambridgeshire, UK). The expected product lengths were 197 bp for *P. gingivalis*, 360 bp for *A. actinomycetemcomitans* and 745 bp for *T. forsythensis* (13). The PCR results were confirmed by DNA sequencing of the amplicons which was carried out using an ABI310 Genetic Analyser (PE Biosystems, Warrington, U.K.). Sequences were analysed

using the Ribosomal Database project II (Maidak et al. 2000) and BLAST at the National Centre for Biotechnological Information (Altschul et al. 1997). The detection limit for each of the studied bacteria was 10 cells/ml.

9.2.6. Statistical analysis

The SPSS 12.0 package was used for statistical analysis and the alpha value was set at 0.01, because of the problem of multiple variable testing (Altman 1991). Continuous, normally distributed variables are reported as means ± standard deviations (SD). Comparisons of continuous and categorical data between groups were analyzed with ANOVA and Chi-square test respectively, on all tested polymorphisms. Associations between genotype and the presence of periodontopathogenic bacteria (*A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis*) were screened by Chi-square test. Whenever p values < 0.2 were detected, multiple logistic regression analysis was performed. A backward likelihood- ratio elimination algorithm was used entering age, smoking, ethnicity, number of periodontal pockets ≥ 5 mm and genotype as variables. SLE (significance level for entry into the model) e SLS (significance level to stay in the model) were set at 0.05 and 0.1 respectively. The results of the final models are reported. Further analysis was performed to investigate the prevalence of concomitant detection of *A. actinomycetemcomitans* and *P. gingivalis*, and *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis* together. Multivariate analysis was performed in positive subjects for each of the studied bacteria in order to investigate relation between genotypes and log counts of detected bacteria. WHAP analysis (see chapter 6) was performed to detect possible FcR and FPR haplotype associations able to predict the presence of the studied bacteria or the concomitant presence of the different species.

9.3. RESULTS

The allele distributions of all studied polymorphisms satisfied the Hardy-Weinberg equilibrium. *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis* were detected in 23 (51.1%), 23 (51.1%) and 29 (64.4%) subjects respectively. *A. actinomycetemcomitans* and *P. gingivalis* were concomitantly detected in 16 subjects

(35.6%), while all three bacteria were present together in 10 subjects (22.2%). No statistically significant differences were observed in bacterial detection for age, gender, ethnicity, smoking and disease severity, indicated by the number of probing pocket depths ≥ 5 mm (data not presented). Average log counts for *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis* were respectively 4.43 ± 1.18, 6.02 ± 1.34 and 6.23 ± 0.48 cfu/ml. Out of all the candidate polymorphisms studied, FcγIIIb receptor and IL-6 polymorphisms showed association with the presence of *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis*. Distributions of all polymorphisms in relation to detection rates of the above mentioned bacteria are reported in table 9.2.

Poly-morphism	Geno-type	patient #	A.a. detected	P.g. detected	T.f. detected	A.a. + P.g. detected	A.a. + P.g. + T.f. detected
FcαR	AA	6	2 (33.3%)	3 (50.0%)	4 (66.7%)	1 (16.7%)	1 (16.7%)
	AG	18	10 (55.6%)	9 (50.9%)	14 (77.8%)	7 (38.9%)	4 (22.2%)
	GG	21	10 (47.6%)	10 (47.6%)	11 (52.4%)	8 (38.1%)	5 (23.8%)
FcγRIIa	HH	13	3 (23.1%)	5 (38.5%)	9 (69.2%)	3 (23.1%)	1 (7.7%)
	HR	18	10 (55.6%)	8 (44.4%)	10 (55.6%)	6 (33.3%)	13 (16.7%)
	RR	13	8 (61.5%)	8 (24.7%)	9 (69.2%)	6 (46.2%)	5 (38.5%)
FcγRIIb	CC	1	0	0	0	0	0
	CT	12	6 (50.0%)	6 (50.0%)	7 (58.3%)	4 (33.3%)	3 (25.0%)
	TT	32	16 (50.0%)	16 (50.0%)	22 (68.8%)	12 (37.5%)	7 (21.9%)
FcγRIIIa	VV	6	1 (16.7%)	1 (16.7%)	2 (33.3%)	1 (16.7%)	0 (0%)
	VF	19	9 (47.4%)	9 (47.4%)	11 (57.9%)	7 (36.8%)	3 (15.8%)
	FF	20	12 (60.0%)	12 (60.0%)	16 (80.0%)	8 (40.0%)	7 (35.0%)
FcγRIIIbNA	NA2/ NA2	17	6 (35.3%)	7 (41.2%)	8 (47.1%)	4 (23.5%)	1 (5.9%)
	NA1/ NA2	18	9 (50.0%)	9 (50.0%)	14 (77.8%)	7 (38.9%)	6 (33.3%)
	NA1/ NA1	10	7 (70.0%)	6 (60.0%)	7 (70.0%)	5 (50.0%)	3 (30.0%)

Table 9.2 Detection rates of periodontopathogenic bacteria by genotype

Poly-morphism	Geno-type	patient #	A.a. detected	P.g. detected	T.f. detected	A.a. + P.g. detected	A.a. + P.g. + T.f. detected
FcγIIIbSH	SH+/ SH+	0	0	0	0	0	0
	SH-/ SH+	8	7 (87.5%)	6 (75.0%)	5 (62.5%)	6 (75.0%)	4 (50.0%)
	SH-/ SH-	37	15 (40.5%)	16 (43.2%)	24 (64.9%)	10 (27.0%)	6 (16.2%)
FPR 301	CC	3	1 (33.3%)	1 (33.3%)	2 (66.7%)	0 (0%)	0 (0%)
	CG	15	8 (53.3%)	7 (46.7%)	8 (53.3%)	6 (40.0%)	3 (20.0%)
	GG	25	13 (52.0%)	13 (52.0%)	17 (68.0%)	10 (40.0%)	7 (28.0%)
FPR 546	AA	5	2 (40.0%)	5 (100.0%)	4 (80.0)	2 (40.0%)	1 (20.0%)
	AC	25	12 (48.0%)	9 (36.0%)	18 (72.0%)	8 (32.0%)	7 (28.0%)
	CC	14	7 (50.0%)	7 (50.0%)	7 (50.0%)	5 (35.7%)	2 (14.3%)
FPR 568	AA	33	15 (45.5%)	14 (42.4%)	20 (60.6%)	11 (33.3%)	7 (21.2%)
	AT	12	7 (58.3%)	8 (66.7%)	9 (75.0%)	5 (41.7%)	3 (25.0%)
	TT	0	0	0	0	0	0
VDR	tt	5	3 (60.0%)	3 (60.0%)	2 (40.0%)	3 (60.0%)	2 (40.0%)
	Tt	24	12 (50.0%)	9 (37.5%)	15 (62.5%)	6 (25.0%)	3 (12.5%)
	TT	15	7 (46.7%)	10 (66.7%)	11 (73.3%)	7 (46.7%)	5 (33.3%)
TNF-α	AA	34	18 (52.9%)	19 (55.9%)	22 (64.7%)	14 (41.2%)	9 (26.5%)
	AG	9	4 (44.4%)	3 (33.3%)	6 (66.7%)	2 (22.2%)	1 (11.1%)
	GG	2	0	0	1 (50.0%)	0	0
IL-6	CC	7	3 (42.9%)	3 (42.9%)	3 (42.9%)	2 (28.6%)	1 (14.3%)
	CG	12	2 (16.7%)	4 (33.3%)	7 (58.3%)	1 (8.3%)	0
	GG	26	17 (65.4%)	15 (57.7%)	19 (73.1%)	13 (50.0%)	9 (34.6%)
NADPH	CC	22	10 (45.5%)	8 (36.4%)	13 (59.1%)	6 (27.3%)	4 (18.2%)
	CT	17	10 (58.8%)	11 (64.7%)	10 (58.8%)	8 (47.1%)	4 (23.5%)
	TT	5	2 (40.0%)	3 (60.0%)	5 (100.0%)	2 (40.0%)	2 (40.0%)

Logistic regression revealed that homozygosity for G allele of IL-6 -174 polymorphism was associated with detection of *A. actinomycetemcomitans* (p=0.003, O.R. =13.7, 95% C.I. =2.4-77.1 adjusting for age and smoking) (see table 9.3), and approached statistical significance for combined detection of *A. actinomycetemcomitans* and *P. gingivalis* (p=0.019, O.R. = 7.86, 95% C.I. =1.4- 44.1 adjusting for age and ethnicity) and combined detection of all three studied pathogens (p=0.042, O.R. = 9.53, 95% C.I. =1.1-83.4).

		B	S.E.	Wald	df	Sig.	O.R.	95,0% C.I.for EXP(B)	
								Lower	Upper
Step 1	*IL-6GG*	2.604	0.938	7.703	1	0.006	13.522	2.149	85.073
	Age	0.168	0.094	3.196	1	0.074	1.183	0.984	1.423
	Ethnicity			2.763	3	0.430			
	Ethnicity(1)	2.246	1.458	2.373	1	0.123	9.446	0.543	164.468
	Ethnicity(2)	1.020	0.946	1.163	1	0.281	2.773	0.435	17.691
	Ethnicity(3)	-19.500	40192.97	0.000	1	1.000	0.000	0.000	
	Smoking(1)	1.942	1.135	2.930	1	0.087	6.976	0.754	64.502
	5mm PPD	0.009	0.012	0.508	1	0.476	1.009	0.985	1.034
	Constant	-8.086	3.545	5.203	1	0.023	0.000		
Step 2	*IL-6GG*	2.538	0.922	7.570	1	0.006	12.650	2.075	77.116
	Age	0.166	0.094	3.125	1	0.077	1.180	0.982	1.419
	Ethnicity			2.581	3	0.461			
	Ethnicity(1)	2.153	1.420	2.300	1	0.129	8.613	0.533	139.257
	Ethnicity(2)	.896	0.921	0.946	1	0.331	2.449	0.403	14.887
	Ethnicity(3)	-19.653	40192.97	0.000	1	1.000	0.000	0.000	
	Smoking(1)	2.095	1.109	3.568	1	0.059	8.128	0.924	71.482
	Constant	-7.404	3.370	4.827	1	0.028	0.001		
Step 3	*IL-6GG*	2.617	0.882	8.812	1	0.003	13.695	2.433	77.086
	Age	0.138	0.082	2.817	1	0.093	1.148	0.977	1.348
	Smoking(1)	1.800	0.973	3.426	1	0.064	6.051	0.899	40.709
	Constant	-6.098	2.868	4.522	1	0.033	0.002		

Table 9.3. Results of backwards logistic regression analysis for detection of *A.actinomycetemcomitans* The variables entered on step 1 were IL6GG, age, ethnicity, smoking, and number of PPD≥ 5 mm. B indicates the set of coefficients estimated for the model, S.E. is the standard error, Wald's test indicates the significance of the parameters, df indicates the degrees of freedom and O.R. indicated the odds ratios for each parameter. The last two columns show the 95% confidence intervals. Step 3 is the most significant one, indicating the importance of IL-6 genotype, age and smoking.

FcγIIIb NA polymorphism showed some evidence for association with detection rates of *A. actinomycetemcomitans* (p=0.038, O.R. = 6.84, 95% C.I. =1.1- 42.0 adjusting for ethnicity). Tendencies for association, although not statistically significant, were also observed for FcγIIIb SH antigen and FcγIIa polymorphisms (see table 9.2).

WHAP analysis revealed that FcγR haplotypes were associated with presence of *A.actinomycetemcomitans* (p=0.010 adjusting for gender, smoking and ethnicity). The highest p values were observed for constrained models including FcγIIa and IIIb NA (p= 0.013 and 0.016 respectively for *A. actinomycetemcomitans* and combined presence of all three bacteria), FcγIIa, IIIb NA and IIIb SH (p=0.003 and 0.006 respectively for *A. actinomycetemcomitans* and combined presence of all three bacteria) and IIIb NA and IIIb SH (p=0.008, 0.006 and 0.02 respectively for *A.a.*, *A.a.* and *P.g.* and all three studied bacteria).

Logarithmic conversions of counts of anaerobic and aerobic bacteria, and *A.actinomycetemcomitans*, *P.gingivalis* and *T.forsythensis* obtained by the cultural method described above in positive individuals for each of these bacteria were analyzed by genotypes. No statistically significant associations were detected between total log counts and genetic polymorphisms (data not presented). This may be partially due to the small number of positive subjects for each of the three bacteria, which reduced the power to detect any association. Appendix XVIII shows the total of each log count of studied bacteria computed in the table by different genotypes.

9.4. DISCUSSION

This is the first study to investigate the association between neutrophil receptors, NADPH oxidase $p22^{phox}$, IL-6, TNF-α and VDR single nucleotide polymorphisms and the detection of periodontopathogenic bacteria in patients with AgP. Socransky et al. (2000) suggested that differences in genetic background of the host may influence the composition of the subgingival microbiota. They observed an association between the

IL-1 genotype and the levels of suspected periodontal pathogens in a population with Chronic Periodontitis.

The results of this study show that polymorphisms of genes encoding for neutrophil receptors (FcγIIIb) and proinflammatory cytokines (IL-6) are associated with the presence of pathogenic bacteria in the periodontal pockets of Aggressive Periodontitis subjects. This finding was independent of other risk factors such as age, smoking, ethnic origin and disease severity. Therefore, these results reinforce the hypothesis that the host genotype can influence the composition of the subgingival microbiota and support data from previous studies which suggest that individual genetic susceptibility may influence the host response to infections (Sutherland et al. 2005).

In particular, our findings suggest that Interleukin-6 -174 polymorphism may be important in determining susceptibility to colonization with periodontopathogenic bacteria, especially *A.actinomycetemcomitans*. Interleukin 6 (IL-6) is a multifunctional cytokine with a central role in host defence (Terry et al. 2000), produced by monocytes/macrophages, fibroblasts, endothelial cells, adipocytes, T cells and mast cells. Dysregulation of IL-6 production has been associated with a number of systemic diseases such as plasmacytosis, juvenile chronic arthritis, rheumatoid arthritis, osteoporosis, Paget disease and psoriasis (Terry et al. 2000). IL-6 has been shown to have a crucial role in the inflammatory response to infectious agents (especially Gram-negative bacteria), which is necessary in order to control the infection (Dalrymple et al. 1996, Remick et al. 2005). Homozygosity for the G allele at position -174 in the promoter region has been linked to increased serum concentration of IL-6 and increased construct expression upon stimulation with LPS (Fishman et al. 1998, Terry et al. 2000) and suspected as a susceptibility factor for periodontitis (Trevilatto et al. 2003). Furthermore, we have shown in the case-control study described in chapter 6, an association between G homozygosity and AgP. Recently, a complex regulatory haplotype has been discovered in a region extending upstream of the previously known promoter region. This haplotype might have a more pronounced functional effect than the -174 polymorphism alone (Fife et al. 2005). In our study, -174 G homozygous

subjects exhibited higher detection rates of periodontopathogenic bacteria. If the supposed increased activity associated with the G allele is confirmed, the reason why patients with increased IL-6 activity are more prone to carriage of the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* remains to be explained. A possible explanation could be that G homozygosity may predispose to periodontal disease through stimulation of an excessive inflammatory cascade, which increases the risk for tissue destruction. Bacteria such as *A. actinomycetemcomitans* and *P. gingivalis* are known to stimulate the inflammatory cascade and the production of IL-6 from gingival fibroblasts (Belibasakis et al. 2005, Bodet et al. 2005). Therefore, the presence of periodontopathogenic bacteria in IL-6 -174 G homozygous individuals may act over an already 'primed' substrate and excessively amplify the local inflammatory response in these subjects, and this may result in the characteristic tissue destruction seen in AgP. Consequently, detection rates of these pathogenic bacteria and G homozygosity cluster in the same group of AgP patients.

The other finding of the study is in relation to Fc-γ receptor polymorphisms. Neutrophils recognize bacteria opsonized by Immunoglobulins (Ig) through specific Fc receptors and their binding activates important effector functions such as phagocytosis, superoxide production, cellular cytotoxicity, antigen presentation, cytokine release, degranulation and regulation of antibody synthesis (van Sorge et al. 2003). The Fc-γIIIb bears the neutrophil antigen (NA) polymorphism, implicated in autoimmune neutropenia and transfusion reactions. This antigen is determined by five nucleotide substitutions, which result in two isoforms (NA1 and NA2) differing by 4 amino acids (at position 36, 65, 82 and 106) in an Ig domain. NA1 and NA2 have been shown to have different glycosylation and therefore different ligand affinity. The NA1 allotype exhibits higher affinity for immune-complexed IgG3, and therefore increased phagocytosis (Salmon et al. 1990). Kobayashi et al. (2000b) showed Fc-γIIIb-NA2-carrying PMN from both patients with chronic periodontitis and healthy controls to be less efficient in phagocytosis upon interaction with IgG1- and IgG3-opsonized P. gingivalis. The Fc-γIIIb NA2 allotype has been shown to be involved in susceptibility to periodontitis by several investigators (Kobayashi et al. 2000a, Fu et al. 2002, Yasuda et al. 2003).

However, in the case-control study reported in chapter 6 we showed an association between the NA1 homozygous allotype and Generalised AgP in Caucasians. Bux et al. (1997) identified another antigen (SH) located on the Fc-γIIIB, determined by a single base C-A mutation at nucleotide 266, which results in an Ala78Asp amino acid substitution. SH+ individuals are rare and seem to be always accompanied by the NA2 allotype, either because of close homology or because of linkage. This new antigen may have functional influences, still to be elucidated (Koene et al. 1998). Fc-γIIa is polymorphic in at least two sites, one of which (at position 131) codes for amino acids located in spots involved in IgG-binding. The histidine (H) at position 131 has been shown to increase receptor affinity and specificity, while the presence of an arginine (R) results in PMN with reduced ability to phagocytose IgG2-opsonized particles (Bredius et al. 1994). In conflict with the literature data, in this report Fc-γIIIb NA1 positive individuals had higher detection rates of *A. actinomycetemcomitans* than NA2 subjects. However, among NA2 subjects, SH positive individuals exhibited very high detection rates of the studied bacteria. Therefore in our sample, while results from Fc-γIIa R confirmed a decreased capacity for phagocytosis and killing linked to this genotype, the same did not apply to the Fc-γIIIb NA2 allele. Nonetheless, as already hypothesized (Koene et al. 1998), the SH antigen might have masked some of the functional results previously observed in relation to the NA2 allotype. Furthermore, the highest association with carriage of *A.a.*, *A.a.* and *P.gingivalis* and all three bacteria were observed for haplotype combinations between Fcγ IIa, IIIb NA and IIIb SH, pointing towards the importance of the interaction between these three FcR loci.

The same considerations proposed for the IL-6 polymorphism may apply to the interpretation of this finding for the Fc-γIIIb NA1 allele. NA1 homozygous individuals are likely to have a neutrophil hyperactivity (Kobayashi et al. 2000b); therefore, NA1 homozygosity and presence of *A. actinomycetemcomitans* may cluster together in AgP patients, and their combination may trigger the excessive immune response leading to AgP onset.

The results of this study suggest microbe-genetic interactions in Aggressive Periodontitis. Fc-γIIIb receptor and IL-6 polymorphisms were found to be associated with the presence of periodontopathogenic bacteria. This stimulates speculation about the possible role of these polymorphisms both in the defence against bacterial infections and in the pathogenesis of periodontal disease. Further studies with larger sample size are now required to test these hypotheses. Periodontal disease, being one of the most common multibacterial diseases in developed countries, may represent a useful model to study pathways and mechanisms of microbe-genetic interactions.

CHAPTER 10

10. SUPEROXIDE PRODUCTION

10.1. BACKGROUND

The results described in chapter 6 show a skewed distribution for NADPH oxidase $p22^{phox}$ C242T polymorphism between our patient and control groups. This SNP is supposed to be involved in superoxide production within neutrophils (Shimo-Nakanishi et al. 2004). The results we observed led to the hypothesis that this polymorphism might increase the risk of developing AgP through an excessive production of superoxide by neutrophils. Moreover, it has been widely demonstrated that PMN from AgP individuals, especially LAgP, release under stimulation more superoxide than matched controls (Shapira et al. 1991, Leino et al. 1994). A neutrophil hyperactivity has therefore been suspected as a possible pathogenic mechanism leading to AgP (Kantarci et al. 2003).

The aim of this study was to measure the superoxide production from PMNs in a subset of AgP patients and relate it to their NADPH oxidase $p22^{phox}$ C242T polymorphism. The null hypothesis is that NADPH oxidase $p22^{phox}$ C242T polymorphism has no effect on PMN superoxide production.

10.2. MATERIALS AND METHODS

10.2.1. Patient recruitment and examination

A subset of patients with AgP who took part in the genetic case-control association study described in chapter 6 (see chapter 4 for inclusion and exclusion criteria) was recruited to have an assessment of their superoxide production. In order to have an ethnically homogeneous group of subjects, only Caucasians were recruited. All Caucasian subjects scheduled to attend a recall examination were asked to participate in this study. Twenty-eight AgP patients attended the Early Onset clinic at the Department of Periodontology, EDH, during the two months when the study took place; only one of the patients declined participation in the study.

Therefore, a total of 27 Caucasians diagnosed with AgP agreed to have a blood sample taken for functional analysis of superoxide production. Their demographic characteristics are presented in table 10.1. Only one of these patients had been diagnosed with LAgP; all the others were affected by GAgP. None of these subjects were using concomitant medications at the time of examination.

10.2.2. Blood sample

A blood sample was drawn from each subject as described in chapter 4. Twenty millilitres of blood were collected in one 10 ml- EDTA tube and transferred to the laboratory for examination immediately after collection.

10.2.3. Neutrophil isolation

One ml of dextran was added to 10ml of blood in a universal. The tube was inverted and left to stand at room temperature for 30-45 minutes. During this time the erythrocytes settled to the bottom of the universal. Using a pipette, the supernatant was removed and gently added on the top of 10ml of Ficoll-Paque in a labelled tube, which was then centrifuged for 10 minutes at 2000rpm, 20°C. The remaining supernatant after centrifugation was removed, leaving the whitish/reddish pellet (mainly neutrophils) at the bottom of the tube. In order to lyse the red blood cells, first 10 ml of water and then immediately 10 ml 2x saline were added to the cell pellet. The cells were centrifuged again at 1200rpm for 7 minutes. The supernatant was then again discarded, leaving a white neutrophil pellet at the bottom, which was re-suspended in 5ml of PBS/glucose buffer. A cell count was performed using a haematocytometer. A concentration of $1x10^6$ cells/ ml of PBS/glucose buffer was required; therefore, if the estimated concentration was higher, additional PBS/glucose buffer was added until the right concentration was reached. If, on the other hand, the measured concentration was lower, the tube containing the neutrophils was centrifuged again and the pellets re-suspended in the appropriate volume of PBF/glucose buffer.

10.2.4. Superoxide measurement from neutrophils

Several different methodologies are used to measure the production of superoxide, including cytochrome c reduction, adrenochrome formation, cyanide-resistant oxygen consumption, hydroethidine oxidation, electrochemical detection, chemiluminescence reactions, aconitase inhibition, hydrogen peroxide production (Tarpey & Fridovich 2001). All these methods have advantages and disadvantages. The method used in this study was the cytochrome c reduction assay. This method uses the reduction rates of ferricytochrome c to evaluate the amount of oxygen released by PMN following stimulation with 4B-phorbol 12-mystrate 13-acetate (PMA). The reduction reaction happens mainly thanks to oxygen, but also to compounds such as ascorbate and glutathione. This reaction produces a colour change that can be measured by a spectrophotometer. Suda et al. (2005) reported a maximum level of O_2^- release within the first five minutes of stimulation. Therefore, in order to be able to detect the immediate response to PMA and its time pattern, we did not incubate and centrifuge the samples after stimulation with PMA, as described previously by Shimo-Nakanishi et al. (2004). However, we incubated some duplicate samples and did not notice significant variations in O_2^- production compared to the non incubated ones (data not presented). For technical reasons, we also did not use superoxide dismutase (SOD) as a negative control. SOD can be added to increase the specificity of the reaction since it catalyses the conversion of superoxide to oxygen and hydrogen peroxide allowing the estimation of reduction due to superoxide alone and correcting for the effects of other reductants.

In brief, the spectrophotometer was switched on and set at 550nm. Two cuvettes for each subject were filled with 1 ml of PBS/glucose buffer containing $1x10^6$ cells and 100 µl of cytochrome C. Each cuvette was then positioned in the spectrometer for at least 1 minute, and the absorbance rate before the addition of PMA was read, and used as a baseline reference. Respectively 1 and 2 µg/ml of PMA were added to each of the two cuvettes and the absorbance rates were read and recorded every 15 seconds up to 5 minutes. The Beer-Lambert law (Sassaroli & Fantini 2004) was used to calculate the number nmol of superoxide produced by $1x10^6$ cells:

$$A = 21.1 \times C \times D,$$

where:

A is the measured absorbance, 21.1 is the absorption coefficient, C is the concentration in M, and D the light path of the cuvette in cm. Considering this last measure was equal to 1 cm, the molarity was calculated as follows:

C (mMol) = A x1000/21.1.

10.2.5. Statistical analysis

The SPSS 12.0 package was used for statistical analysis. All recorded measures were entered in a spreadsheet and proofed for entry errors. The normality of distribution of the superoxide production measures was plotted and tested by Kolgomorov- Smirnov and Shapiro-Wilk tests. Since they did not show normal distribution, they were log-transformed for the analysis. Repeated ANOVA analysis was used to account for the differences in superoxide production between the different genotypes in time. The residuals of log- transformed values were again plotted (Q-Q plot) and showed a better pattern of distribution (see figure 10.1), although Kolgomorov- Smirnov and Shapiro-Wilk test still showed non normal distribution, albeit relating to extreme measures only.

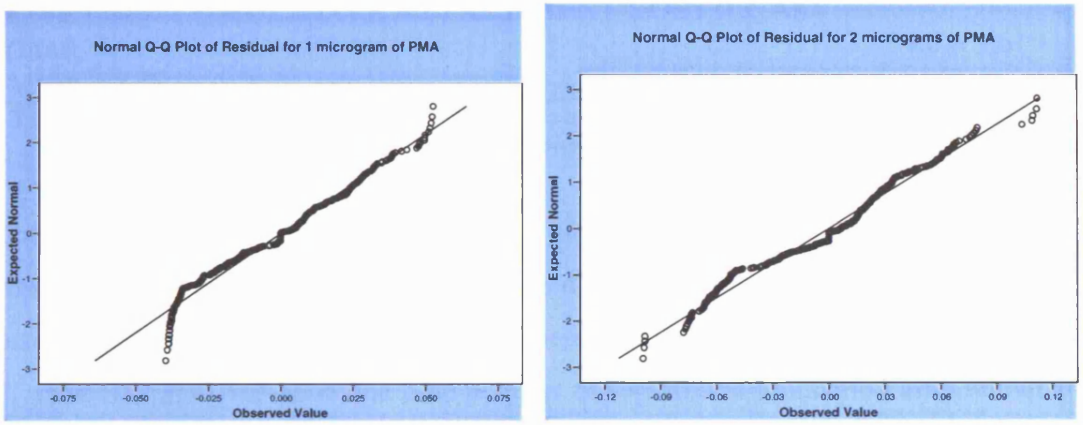

Figure 10.1: Q-Q plot of residuals of repeated ANOVA analysis for NADPH genotypes after stimulation respectively with 1 and 2 µg of PMA

In order to assess clear-cut genetic-driven effects, data from homozygous subjects for the suspected risk allele (T allele of NADPH oxidase $p22^{phox}$ 242 polymorphism) were

compared to data from patients carrying at least one copy of the other allele. The effects of confounders such as smoking and gender were also assessed.

10.3. RESULTS

The patients' demographic and clinical characteristics were comparable with those of the whole AgP population (see table 10.1 and 6.5).

AgP PATIENTS (n=27)		**MEAN**
AGE (Mean ± S.D.)		31.6 ± 6.5
FEMALES (percentage)		16 (59.3%)
SMOKERS (percentage)	***Current smokers***	4 (14.8%)
	Non smokers	23 (85.2%)
TEETH AT BASELINE (Mean ± S.D.)		28.3 ± 2.7
NUMBER OF POCKETS ≥ 5 mm Mean ± S.D.		63.2 ± 38.7
PROBING POCKET DEPTH (mm) Mean ± S.D.		4.2 ± 1.2
LIFETIME CUMULATIVE ATTACHMENT LOSS (LCAL) (mm) Mean ± S.D.		4.8 ± 1.6

Table 10.1: Demographic and clinical characteristics of patients included in the study on superoxide production

The addition of PMA was followed by a considerable increase in the production of superoxide by PMNs. This increase reached its climax in most patients at the first reading (after 15 seconds), after which there was a continuous very slow decrease. Figures 10.2 and 10.3 show the time patterns of superoxide production after stimulation respectively with 1 and 2 μg of PMA. In the x axis, fifteen time points from 0 (stimulation with PMA) to 5 minutes (300 seconds) are shown. The time points from 0 to 2 minutes are separated by 15 seconds intervals, while from 2 to 5 minutes the intervals are 30 seconds. In the y axis, log- transformed estimated marginal means of O_2^- production are shown (with standard errors), as assessed by repeated ANOVA analysis.

The time patterns of superoxide production were similar for both PMA concentrations, but the magnitude of the response was approximately 3x with 2 μg compared to 1 μg.

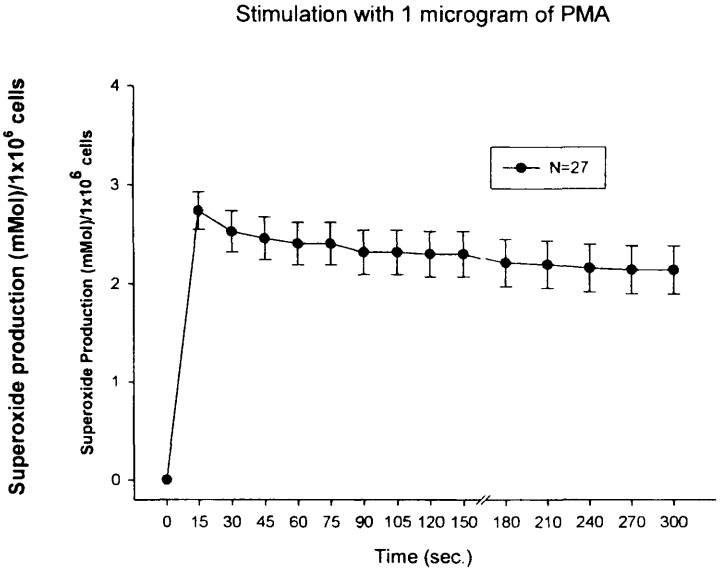

Figure 10.2: Average superoxide production (mMol) on all patients after stimulation with 1 μg of PMA up to 5 minutes

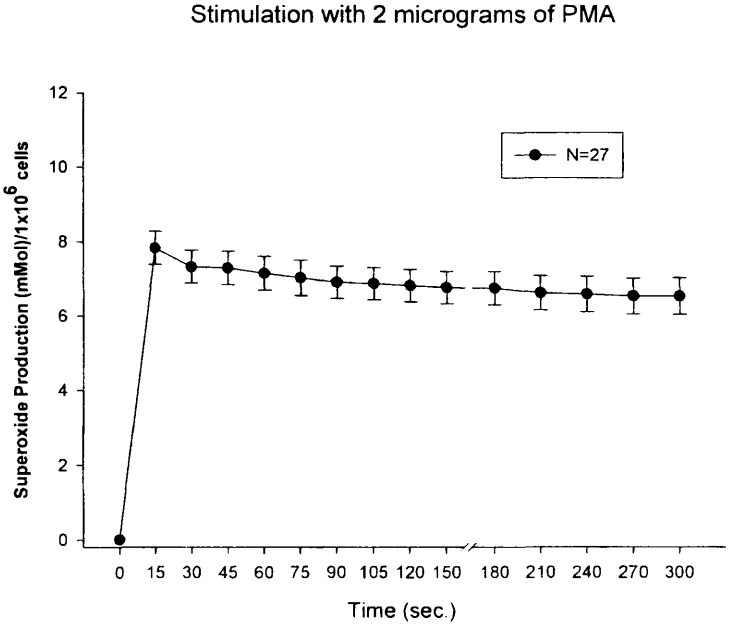

Figure 10.3: Average superoxide production (mMol) on all patients after stimulation with 2 μg of PMA up to 5 minutes

Repeated ANOVA analysis revealed that smoking and gender did not have a significant effect on superoxide production (data not presented). Therefore, these two parameters were not included as covariates in the analysis.

Figures 10.4 and 10.5 report the time patterns of O_2^- production after stimulation respectively with 1 and 2 μg of PMA when patients are clustered by their NADPH oxidase $p22^{phox}$ C242T genotypes. Subjects are divided into T homozygous and C allele positive individuals (including heterozygous and C homozygous). No statistically significant differences were detected after addition of 1 μg of PMA for NADPH oxidase $p22^{phox}$ C242T genotypes (p= 0.477) (figure 10.4).

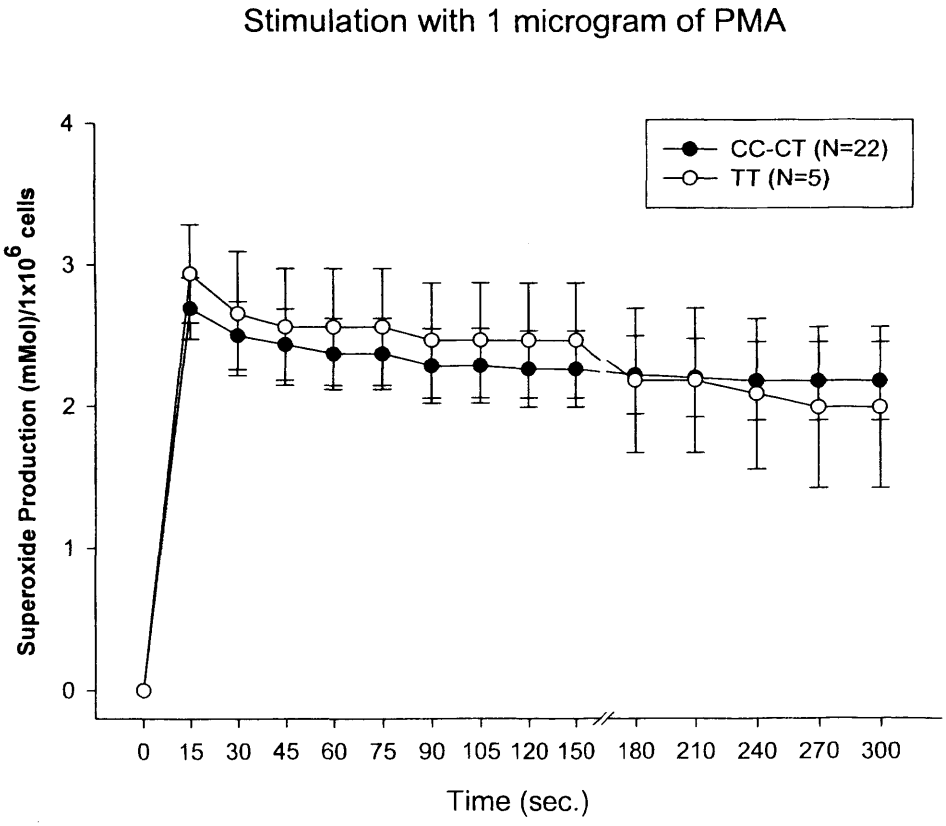

Figure 10.4: Average superoxide production (mMol) on all patients by NADPH genotypes after stimulation with 1 μg of PMA up to 5 minutes

Upon stimulation with 2 μg of PMA, however, the model was statistically significant ($p<0.001$, adjusted R squared = 0.404) and the TT genotype was significantly associated with an increase in superoxide production ($p<0.001$). This increase appeared at the first time point after stimulation and remained constant up to the last measurement (5 minutes).

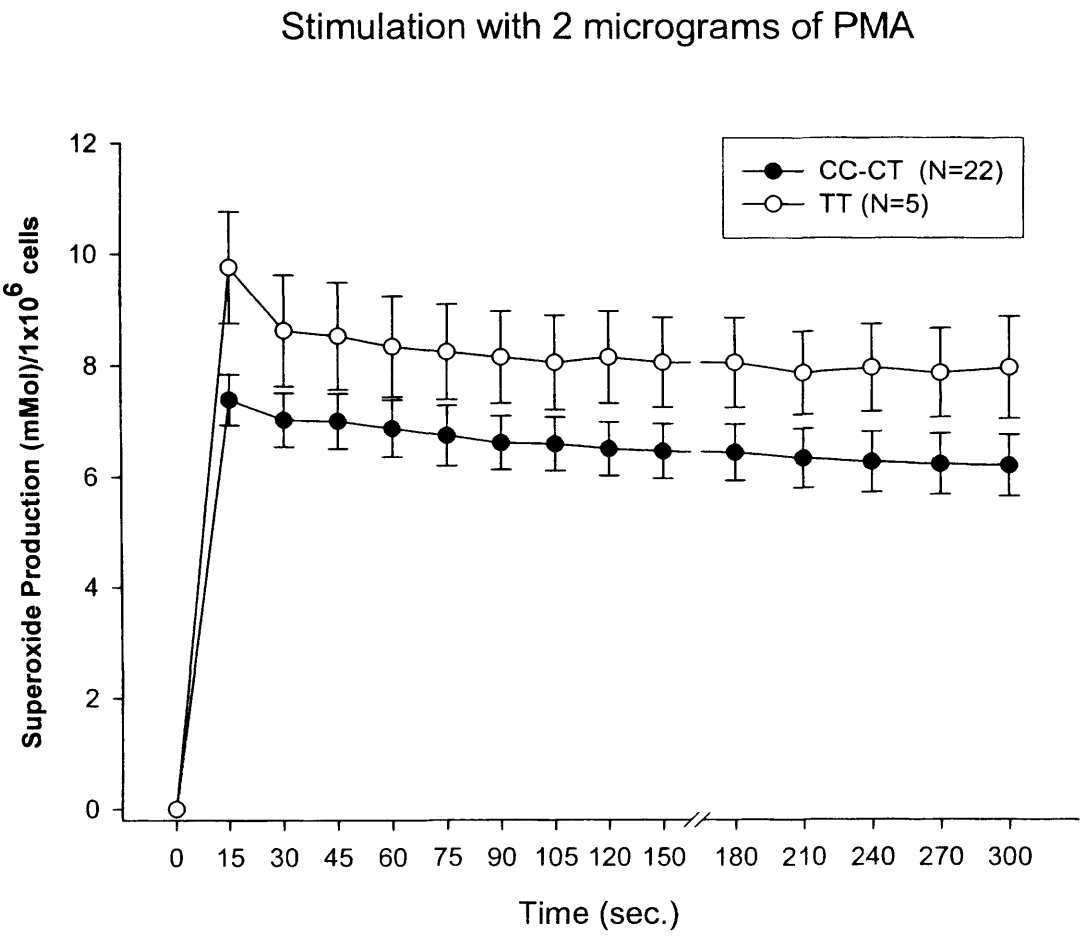

Figure 10.5: Average superoxide production (mMol) on all patients by NADPH genotypes after stimulation with 1 μg of PMA up to 5 minutes

10.4. DISCUSSION

The results observed in this study show that NADPH oxidase $p22^{phox}$ C242T polymorphism was associated with superoxide generation from neutrophils in our patients. PMNs from T homozygous individuals for the NADPH oxidase polymorphism exhibited an increase in the release of O_2^- after stimulation with 2 μg of PMA. Stimulation with a smaller concentration of PMA (1 μg) did not show any differences.

The observations relative to the NADPH oxidase $p22^{phox}$ C242T polymorphism are in agreement with a report from Japanese individuals (Shimo-Nakanishi et al. 2004). They observed an increase in PMA-stimulated superoxide production in CT compared to CC individuals, suggesting an effect given by the presence of the T allele. No T homozygous subjects were included in their study. In our study, the observed increase in superoxide production was linked to T homozygosity, while no difference was present between CT and CC individuals. On the other hand, a study conducted on Caucasian subjects detected the opposite result (Wyche et al. 2004), showing a reduction in superoxide production in T homozygous individuals. These differences in the outcome are likely to be due to the different methods used, to the small number of participants and maybe to the different types of patients included. Our methods were similar to those described by Shimo-Nakanishi et al. (2004). However, we did not compare basal oxidative burst between subjects, but just increases relative to a stable basal level, using individual resting values as baselines. Our results lack the inhibitory effect given by the use of superoxide dismutase (SOD) on superoxide production. This means we have measured the total amount of superoxide produced by PMNs after stimulation with PMA. A percentage of this oxidative burst may be due to other factors (such as ascorbate and glutathione) other than NADPH oxidase. However, independently of the source, all produced O_2^- initiates and promotes a series of reactions which can lead to tissue damage. TT individuals in our study had an increase in the total oxidative burst upon stimulation with PMA, therefore are likely to be predisposed to more tissue damage than the other subjects. Having observed differences between genotypes for the whole amount of superoxide produced, it is likely to mean that excluding the effect of

other oxidative sources will increase the magnitude of the effect on NADPH oxidase production.

The $p22^{phox}$ is one of the subunits forming the NADPH oxidase system. As described in chapter 7, the C to T mutation at position 242 in the gene coding for the $p22^{phox}$ causes an amino acid substitution at position 72 (histidine to tyrosine). This substitution involves a potential heme-binding site, which can have as a consequence an increase in superoxide production. Moreover, the presence of the T allele is also supposed to be involved in up-regulation of $p22^{phox}$ mRNA and protein (Shimo-Nakanishi et al. 2004). It is still not clear whether this polymorphism has a direct effect on NADPH oxidase function or is in linkage disequilibrium with a functional SNP elsewhere in the gene. However, this is the first study to show an effect of this polymorphism on oxidative bursts in patients with Aggressive Periodontitis. This effect was consistent with time but was observed only upon a strong stimulation (high concentrations of PMA). It is difficult to relate the used concentration of PMA to *in vivo* reality, as PMA is an artificial system not naturally present in inflamed periodontal tissues. In the case-control study described in chapter 6 we observed an increase in the prevalence of carriage of the T allele in AgP patients compared to controls. T homozygous subjects had a particularly high frequency in the GAgP group, and were found to have more severe disease (see chapter 7). The finding of increased oxidative burst in T homozygous subjects confirms that the pathogenic mechanism of tissue damage in these subjects is likely to be mediated by a hyper activation of neutrophils upon stimulation.

Further studies with larger sample sizes and including also healthy subjects as controls are needed to confirm the role of this polymorphism on neutrophil function.

CHAPTER 11

11. GENERAL DISCUSSIONS

The series of studies presented in this PhD thesis aimed at detecting genetic factors associated with Aggressive Periodontitis. Despite the efforts made in recent years, which led to the conclusion of a strong role of genetics in AgP onset, the genetic nature of AgP is still somehow mysterious. Despite numerous efforts and dozens of genetic polymorphisms being studied, none have been singled out as real risk factors for AgP (Kinane et al. 2005). This PhD provided a complete analysis of the largest group of AgP patients so far described in the literature. Stringent inclusion criteria and the dedication of years of research resulted in the identification of 224 subjects suffering from a disease with severe signs of periodontal attachment and bone loss at early age, which we now call Aggressive Periodontitis (Armitage 1999). We applied stringent inclusion criteria, which allowed us to select a population of subjects suffering from very advanced disease at an early age, highlighted by the numbers of sites affected and the average age of the subjects (see table 5.1 and 5.2 for description of clinical parameters). Many of these subjects were 'dentally-crippled' because through this aggressive disease they had lost or were about to lose several teeth at a very early age, with substantial negative repercussions on their lives. The severity and apparent lack of a clear aetiologic factor led years ago to the understanding of this disease as a degenerative condition of the periodontium (Baer et al. 1971). However, we now know that Aggressive Periodontitis is an infectious disease, due to microbes present in the bacterial plaque, which take advantage of the unique anatomy of the periodontium to invade the host. The teeth are the only mineralised tissues directly in contact with both the outside environment (oral cavity) and the most inner tissue of the human body (the bone). This atypical anatomic structure makes the tissues surrounding the teeth (the periodontium) an attractive way for pathogenic bacteria to colonize and develop in the human host. While this infectious process, which starts from the crevice between teeth and gingival epithelium, takes decades to cause minimal damage in most of the human population, a subset of subjects (those suffering from AgP) show a dramatic predisposition to it. Therefore, they exhibit considerable periodontal attachment loss and bone damage which lead to tooth mobility and eventually tooth exfoliation. This process is accompanied by the development of an inflammatory infiltrate in the periodontal crevice, which is probably to be held

responsible for the characteristic tissue damage of AgP (Gemmell et al. 2002, Bosshardt & Lang 2005).

This PhD consisted of a study on the effect of several genetic polymorphisms (SNPs) regulating the host response on the Aggressive Periodontitis (AgP) trait. A specific panel of SNPs was selected based on the literature and on their possible functional importance. The relation between these SNPs and AgP was investigated in a case-control association study (chapter 6). The associations between the studied SNPs and common features of AgP, as defined by the current classification (see chapter 1) were then examined: disease severity (chapter 7), familial aggregation (chapter 8), presence of known periodontopathogenic bacteria (chapter 9) and phagocyte abnormalities (chapter 10). Most previous association studies on AgP included relatively small numbers of subjects and investigated one or two SNPs (Kinane et al. 1999a, Kobayashi et al. 2000a). We included in this study 224 AgP patients and 231 controls, and investigated a large range of SNPs (13). This gives the advantage of being able to look at relative effects and interactions between different SNPs; on the other hand, because of the multiple testing, looking at many SNPs has an effect on the confidence of the observed results (Altman 1991). The conclusions drawn from the studies described in the previous chapters will be discussed as a whole in this chapter, and related to the literature evidence. First of all, each of the different SNPs will be discussed separately, and then an overview and general discussion and conclusions will be presented.

11.1. FcRα -324 polymorphism

The FcRα gene codes for Fc receptors α (CD89), present on PMN, monocytes/macrophages and eosinophils. These receptors bind IgA coated on the surface of invading bacteria, and mediate several immunologic defence processes such as phagocytosis, antibody-dependent cell-mediated cytotoxicity, and release of inflammatory mediators (Monteiro & van de Winkel 2003). IgA– mediated phagocytosis in periodontitis is thought to be more effective than that mediated by IgG (Mazengera & Kerr 1990) and PMN in the apical portion of periodontal pockets express a large number of Fcα recptors for interaction with IgA- coated bacteria (Yuan et al. 2000). An A-G

substitution at position 324 in the Fcα gene, 114 base pairs upstream of the transcriptory region, is suspected of having a bearing on the activity of the receptors. In particular, Kaneko et al. (2004) observed decreased phagocytosis of *Actinobacillus Actinomycetemcomitans* in vitro in PMN from subjects homozygous for the rare allele (A). The A homozygous genotype was also increased in prevalence in Japanese AgP patients compared with controls (Kaneko et al. 2004). In our sample, we observed a slightly increased carriage of the A allele and of A homozygosity in Caucasians and Blacks with AgP compared with controls (see table 6.10). Furthermore, A homozygous AgP patients had more severe disease than heterozygous individuals (see table 7.1), while no differences were noted for presence of periodontopathogenic bacteria. Among relatives of allele A- carrying AgP a slight increase in AgP prevalence was found (see table 8.5). Taking all these findings as a whole, a role for the A allele of this SNP in the expression of the AgP phenotype seems to emerge. The 324 polymorphism may be in linkage disequilibrium with a predisposing gene, whose effects translate into a less effective PMN response. Therefore, further studies should aim at detecting this supposed predisposing polymorphism and elucidate its functions, and studies on larger samples should be conducted before we can draw definitive conclusions on the susceptibility effect of this SNP towards AgP.

11.2. FcγR polymorphisms

The Fc γ receptors include a family of receptors for IgG. These immunoglobulins play an important role in the binding of periodontopathogenic bacteria and have been found at increased levels in diseased periodontium (Tew et al. 1985). IgG-bound bacteria are recognized by Fcγ receptors, stimulating a series of reactions aimed at eliminating the invading parasites (van Sorge et al. 2003). Specific Fcγ receptors exist for different classes of IgG: FcγRI (CD64), FcγRII (CD32, subdivided in IIa and IIb), FcγRIII (CD16, subdivided in IIIa and IIIb). FcγRI are expressed in myeloid cells and neutrophils, FcγRIIa in myeloid cells, neutrophils, platelets and T cells, FcγRIIb in B cells, monocytes, macrophages and neutrophils, FcγRIII in neutrophils and eosinophils. All these classes have different affinities to IgG, diverse signal transduction activity and are present in different cells. Their genes have a common location on the long arm of

chromosome 1. SNPs in these genes have been associated with influence on receptor function, which might predispose to autoimmune and infectious diseases. The analysis of five different FcγR SNPs in this study revealed considerable linkage disequilibrium between them. In other words, the interpretation of the results relative to association with AgP in our study cannot leave aside the relationship between all these polymorphisms and the fact that they are closely associated with each other. Moreover, there are strong suggestions for an interplay and overlap in the functions of Fcγ receptors. FcγRIIIb, for example, lacks intrinsic signalling capacity, and is likely to interact with FcγRIIa to determine leukocyte responses. Indeed, even a synergistic activity of these two receptor types has been hypothesized (van der Pol & van de Winkel 1998). In our sample, a possible role emerged for FcγRIIa R/H and FcγRIIIb NA polymorphisms in susceptibility to AgP.

A G to A nucleotide substitution at position 494 in the gene coding for FcγRIIa determines an arginine (R) to histidine (H) amino acid change in a IgG- ligand binding domain. The presence of this arginine has been shown to reduce ligand interaction. Since interaction with IgG2 depends almost entirely on FcγRIIa activity, R homozygosity results in deficient phagocytosis of IgG2- opsonized particles (van Sorge et al. 2003). In our group of Caucasians, we found an increase of R homozygosity among patients (especially GAgP) (see tables 6.10 and 6.14). R homozygous subjects also had increased detection rates of *Actinobacillus actinomycetemcomitans* in their periodontal pockets (see table 9.2). However, whilst showing interesting trends, neither of these results reached statistical significance. The most striking result with this polymorphism was in relation to familial aggregation: relatives of R homozygous subjects were found to have an increased risk of having AgP compared to H- carrying individuals (see table 8.5). Taken together, these results suggest that R homozygosity may have a predisposing effect on AgP, probably through an impaired bactericidal activity versus *A.actinomycetemcomitans.* We can speculate that relatives of R homozygous subjects, being more likely to be R homozygous themselves, have an increased risk of having AgP probably through the same mechanism of increased susceptibility to periodontopathogenic bacteria. It is interesting to note how most previous studies

detected no associations between this polymorphism and periodontitis (Colombo et al. 1998, Kobayashi et al. 2000a, Fu et al. 2002), while some studies showed increased risk for disease onset and severity linked with the H allele, mainly in Caucasians with CP (Loos et al. 2003, Yamamoto et al. 2004). Generalising our results to other ethnic groups is complicated if not impossible, considering that in our sample of AgP Black patients the opposite tendency towards increased H homozygosis was noted.

A polymorphism in the FcγRIIIb gene determines four amino acid substitutions, responsible for allelic differences in receptor glycosylation. The two deriving allotypes are called NA (neutrophil antigen) 1 and 2. The NA2 allotype has been shown to be less effective in binding IgG3 and phagocytosing IgG3-coated particles (Salmon et al. 1990). A decrease in ability to phagocytose *P.gingivalis* has been shown more recently (Kobayashi et al. 2000b). A C-A substitution located at nucleotide 266 in the same gene determines an amino acid change responsible for the SH antigen (Bux et al. 1997), in linkage disequilibrium with the NA allotype (all SH positive individuals are also NA2 positive). In our sample, the NA1 allotype showed some evidence of association with the GAgP trait (see table 6.14) and increased detection of periodontopathogenic bacteria (see table 9.2). This is in conflict with previous studies, where the NA2 antigen was associated with AgP (Kobayashi et al. 2000a, Fu et al. 2002), or where no association with AgP or CP was detected (Colombo et al. 1998, Meisel et al. 2001, Chung et al. 2003). Hence, this study supports the theory of the NA1 allotype as a genotype predisposing to GAgP, especially in Caucasians. A possible mechanism of action involves an excessive superoxide production, which would be responsible for the periodontal tissue damage. This would also explain why the NA1/NA1 genotype was associated with detection of *A.actinomycetemcomitans* in our sample. Intuitively, based on its supposed reduced phagocytic activity, we would expect the NA2 to be associated with increased bacterial presence in the periodontal pockets. However, more NA1 than NA2 GAgP subject are positive to *A.a.*, probably because the persistence of this bacterial insult in these subjects stimulates an excessive neutrophil response and leads to AgP. Therefore, NA1 and *A.a.* cluster in the same group of AgP patients. In fact, among *A.actinomycetemcomitans* positive individuals, slightly increased counts of *A.a.* were

detected in NA2 patients (see appendix XVIII), suggesting that when *A.a.* infects the periodontal pockets, the increased effiency of NA1 individuals may be able to improve phagocytosis and limit the numbers of this bacterium, but at the same time stimulates host defensive tissue-damaging reactions leading to AgP. The role of the SH antigen also has to be investigated further. This was the first study to report frequencies of this polymorphism in a periodontitis population. Despite the fact that SH homozygous individuals were found only among AgP patients, their number was too small to draw any conclusions. However, some of the observed results related to the NA2 allotype in other studies on non Caucasian population may be influenced by the potential effects of this allotype (Koene et al. 1998). Further studies about FcγRIIIb NA polymorphism have to include haplotype analysis including the IIa and IIb SH loci, in order to clarify possible mechanisms of action.

Fc-γ IIb receptors are present in B cells, monocytes, macrophages, and neutrophils (Ravetch & Bolland 2001). Since they are involved in IgG interactions and in the regulation of Fc-γ R inflammatory responses, they have been studied in relation to periodontal disease. Yasuda et al. (2003) identified the T allele at position 695 in the Fc-γ RIIb gene as a risk indicator for AgP in Japanese individuals. No association was noted in our study between this polymorphism at position 695 (Yasuda et al. 2003) and the presence or severity of AgP, presence of periodontopathogens and familiarity of AgP. Furthermore, significant linkage disequilibrium was noted with the Fc-γRIIIb NA locus, which might have caused a positive association in Yasuda's study.

A similar LD phenomenon may be at the basis of the supposed importance of a 559 SNP in the Fc-γRIIIa gene. This polymorphism has been studied in relation to periodontitis, because of the higher affinity of the V allotype (determined by the presence of allele G) compared to the F allotype, for IgG 1, 3 and 4. In a study of Caucasians, Loos et al. (2003) associated the V allele to periodontitis risk, while Meisel et al. (2001) associated it with disease severity. We observed decreased detection of periodontopathogenic bacteria in the pockets of VV homozygous subjects (see table 9.2), while no difference was observed in terms of polymorphism distribution in the whole group of patients and

controls. Interestingly, however, Black AgP patients had an increased prevalence of V homozygosity compared with Black controls (see table 6.10). Any conclusion about the possible effect of this polymorphism has to take into account the very strong linkage disequilibrium shown with the Fc-γIIa HR and Fc-γR IIIb polymorphisms, which might influence the magnitude of Fc-γR IIIa disease-association (van Sorge et al. 2003). If an effect on disease susceptibility exists, it is at best very limited and probably acting just in some populations.

11.3. Formyl-peptide receptors (FPR) polymorphisms

The constant finding of reduced chemotaxis in patients suffering from AgP (Van Dyke et al. 1985) has led to the search for a genetic reason for it. The main culprit is the gene coding the formyl peptide receptors (FPR), which are expressed in PMN and recognize and bind N-formyl peptide, a chemotactic factor derived from human mitochondrial proteins and bacterial products. Multiple domains of the FPR seem to be required for high-affinity ligand binding. FPR is encoded by the FPR1 gene, located on chromosome 19. We screened 31 LAgP patients and 28 controls, by DNA sequencing for a region of the FPR1 gene encompassing all the SNPs described in the periodontal literature. Our results confirm that the associations reported by Gwinn et al. (1999) were probably due to population stratification and that the polymorphisms at position 329, 378 and 306, cannot be realistic markers of disease because of extreme rarity of the uncommon allele. Three out of the other SNPs described by Zhang et al. (2003) appeared to have promising relationships with the AgP trait and were analyzed on all patients.

The 301 G-C polymorphism determines a substitution of a valine with a lysine in a site not known to be important for ligand affinity. We detected a slight, not statistically significant, increase in the prevalence of the G allele in patients compared with controls (see table 6.10). In addition, G homozygous AgP patients had more severe disease when compared with C homozygous patients (see table 7.2).

The allele A at nucleotide 546, and A homozygosity were increased in prevalence in the patient group (see table 6.10). A homozygous subjects showed a tendency for increased

attachment loss (see appendix XVII) and for increased detection of *Porphyromonas gingivalis* (see table 9.2). None of these observations reached statistical significance, but showed a consistent trend. Functionally, however, there is no reason to believe that this polymorphism is a real marker of disease, since it is synonymous, and does not change the coded protein. The observed tendencies may be the reflection of linkage disequilibrium with a functional gene and stimulates further research in order to detect the functional gene.

The 568 polymorphism is determined by a A-T nucleotide substitution, resulting in a change from a basic to an acid amino acid (Arginine to Tripsine) at codon 190 (Zhang et al. 2003) in the second extracellular loop of the receptor, believed to have functional relevance on ligand affinity. Zhang et al. (2003) detected an increased prevalence of A homozygosity in African Americans with LAgP. On the other hand, we found the T allele increased in prevalence in Black patients (see table 6.10), although this did not reach statistical significance. Subjects with the T allele showed slight increases in the detection of all studied bacteria (see table 9.2).

Considering the close genetic linkage between FPR polymorphisms, we cannot exclude the possibility that the tendencies for association seen in this study may reflect a real effect on disease susceptibility. Speculation points towards a minor effect linked to the 568 polymorphism, mainly in Blacks, but conflicting literature evidence and lack of a large sample of Black AgP subjects, leave doubts about the interpretation of these data. The real polymorphism able to modify AgP susceptibility may lie somewhere else in the gene, and may be just in LD with the studied polymorphisms.

11.4. NADPH p22phox polymorphism

One of the main findings of this PhD is the effect of this polymorphism on AgP phenotype. A C-T substitution at position 242 in the CYBA gene, coding for the $p22^{phox}$ subunit of the NADPH oxidase has recently been identified and studied, with conflicting results, in relation to cardiovascular disease susceptibility (Inoue et al. 1998). This was the first study to aim at investigating a possible effect of this SNP on AgP risk. In our

sample the T allele was associated with the AgP phenotype, with particular enrichment of T homozygosity in GAgP Caucasians (see tables 6.10 and 6.14). T homozygosity was also associated with increased disease severity in GAgP (see table 7.3) and with increased release of superoxide from neutrophils (see figure 10.5). The family study revealed a slight increase in family predisposition in T allele carriers (see table 9.2). These results taken together indicate that the C242T $p22^{phox}$ polymorphism is a novel genetic risk factor for Aggressive Periodontitis. This is a functional polymorphism, causing a histidine to tyrosine change at position 72 in the $p22^{phox}$ protein. This substitution involves a potential haem-binding site, which can have an effect on superoxide production. Consistent with data from Shimo-Nakanishi et al. (2004), our results support the hypothesis that T homozygosity increases the release of superoxide, determining a cascade of oxygen metabolites and the activation of proteases. This leads to increased tissue damage on the periodontium, where the effect of the neutrophil in response to periodontal bacteria is particularly important. Therefore, if the bacterial stimulus is not eliminated, the excessive neutrophil stimulation becomes chronic and it leads to a constant release of toxic metabolites and to continuous chronic damage. This response is probably hyperactivated in T positive subjects and even more so in TT homozygous subjects. From our data, it seems that the T allele is important in determining disease susceptibility and, among AgP patients, having two copies of this allele increases the susceptibility to more severe disease.

However, the effect of the increased superoxide production may not be due only to neutrophils, but also to osteoclasts, which use a NADPH oxidase system to produce ROS, thought to be important in bone resorption (Yang et al. 2001). Osteoclasts have been shown to secrete hydrogen ions, proteinases and supeoxide in order to excavate a resorption pit or lacuna in the bone surface. Superoxide may stimulate bone resorption by activating collagenases and by participating in fragmentation of matrix proteins. The $p22^{phox}$ subunit has been shown to be indispensable in the osteoclastic Nox 4 superoxide production (Yang et al. 2004). Therefore, we can hypothesise that the combined hyperactivity of neutrophils and osteoclasts linked with the T allele or T homozygosity interact to predispose to periodontal destruction and, as a consequence, to AgP.

Neutrophils and osteoclasts can play a role in different steps of disease pathogenesis: while neutrophils-derived ROS may be more important in the initiation of the disease, osteoclasts-driven superoxide production may be involved in the progression and in the chronic damage.

The C242T $p22^{phox}$ polymorphism has also been linked with increased lipid oxidation, which may have importance as a risk factor for cardiovascular disease. Furthermore, Nakano et al. (2003) found that circulating concentrations of HDL were 1.5-fold lower and oxidized LDL were higher in diabetes patients with T allele compared to CC homozygous subjects. Considering the hyper inflammatory metabolic status of severe periodontitis patients (Nishimura & Murayama 2003, D'Aiuto et al. 2005), the possible role of C242T $p22^{phox}$ polymorphism as a link between periodontitis and cardiovascular disease deserves further attention and investigation.

11.5. Interleukin-6 polymorphism

A C-G polymorphism at position -174 in the promoter region of the Interleukin-6 gene has been linked to regulation of IL-6 activity (Fishman et al. 1998). This has been implicated in several diseases and conditions, such as juvenile rheumatoid arthritis, systemic arthritis and cardiovascular disease (Fife at al. 2005). The effect seems to be mediated by an excessive IL-6 release linked with G homozygosity, which would amplify the pro-inflammatory functions of this cytokine. Among other functions, IL-6 stimulates the hepatic acute phase response to infection and injury, stimulates the osteoclasts, activates macrophages, neutrophils and T cells along with the growth and differentiation of B cells (Lotz et al. 1988, Revel 1989). IL-6 knockout mice (Terry et al. 2000) have a compromised response to infections and tissue damage. IL-6 production is induced by a number of inflammatory stimuli such as IL-1, platelet-derived growth factor, tumor necrosis factor α (TNF-α), bacterial products such as endotoxin, and viral infections (Terry et al. 2000). Monocytes/macrophages, fibroblasts, endothelial cells, adipocytes, T cells and mast cells are among the cells able to produce IL-6. The presence and supposed importance of IL-6 in periodontal disease, especially in stimulating bone resorption (Ota et al. 1999, Todhunter et al. 2005), has led to an interest in this -174

polymorphism. G homozygosity was suspected as a risk factor for periodontitis in a Brazilian population (Trevilatto et al. 2003). The results presented in the context of this PhD show that -174 G homozygous was a risk indicator for AgP in our population, with a very strong relation with LAgP (see table 6.10 and 6.16). Moreover, G homozygous subjects had increased detection rates of periodontopathogenic bacteria (see table 9.2) and increased familial aggregation of AgP (see table 8.5). These findings strongly support a genuine role for this polymorphism in inducing AgP. Given the lack of robust data and the conflicting evidence regarding the effect of this polymorphism on IL-6 concentrations in serum (Fishman et al. 1998, Endler et al. 2004), we can only speculate that the GG genotype is associated with an increased IL-6 release and function in the periodontal tissues. G homozygous subjects might have an increased IL-6 function and osteoclastic activity. This would justify the increased detection of periodontopathogenic bacteria, whose presence, coupled with the 'hyperstimulated' genotype, may predispose to an excessive and continuous IL-6 production with consequent tissue damage. Alternatively, G homozygosity, for some reason yet to be made clear, may actually decrease the ability to face the microbial challenge and therefore predispose to AgP. However, given the similar observations on the FcγRIIIb NA polymorphism and bacterial detection, the first supposed mechanism (site-specific increased IL-6 activity) seems a more feasible explanation. As observed for the FcγRIIa genotype, the increased familial aggregation may reflect the increased likelihood of relatives of G homozygous individuals to be homozygous themselves, and to have a similar disease pathogenesis. Moreover, considering that relatives are also more likely to share the same subgingival microflora (Petit et al. 1994, Haubek et al. 2005), the pathogenic combination of IL-6 G homozygosity and periodontopathogenic bacteria seems to offer a very reasonable explanation for the pathogenic pathway leading to AgP in our probands and relatives.

11.6. Vitamin D receptor polymorphism

We examined in our sample Taq-I, one of the known polymorphism of the VDR gene. VDR is a nuclear receptor, and modulates gene expression once complexed with the active form of vitamin D. Because of its role in bone metabolism, immune modulation, regulation of cell growth, differentiation and induction of apoptosis of a variety of cells,

such as osteoblasts (Dusso et al. 2005), it has been suspected of playing a role in periodontal disease pathogenesis. However, conflicting results arise from studies on the relationship between TaqI, BsmI and FokI polymorphisms of the VDR gene and periodontal disease (Hennig et al. 1999, Yoshihara et al. 2001, Tachi et al. 2003, Nibali et al. 2005). Results from this study (see figures 6.1 and 6.2) confirm the importance of the interaction between Taq-I polymorphism and smoking in the pathogenesis of periodontitis, that we had described previously (Nibali et al. 2005). Due to a possible common mechanism of action on immune modulation or bone metabolism, this VDR polymorphism and smoking seem to have an additive effect in increasing the AgP burden. However, functional studies are needed to explore the mechanisms of this association, considering that the Taq-I polymorphism is synonymous, and does not change the coded amino acid. Linkage disequilibrium with a functional polymorphism somewhere else in the gene is suspected. No effects on carriage of periodontopathogenic bacteria and familial aggregation were detected.

11.7. Tumor necrosis factor α polymorphism

TNF-α production has been described as being dependent upon a polymorphism in the TNF- α gene at nucleotide -308. Higher transcriptional levels are associated with the presence of allele A rather than G (Wilson et al. 1997) and supposed to contribute to the periodontitis burden. No evidence for any effect of this polymorphism on AgP risk has been produced so far (Kinane et al. 1999a, Soga et al. 2003). No associations were noted in our study with presence of AgP, disease severity, detection of periodontopathogenic bacteria and familial aggregation.

11.8. Overall conclusions

We can summarise the results presented in the different chapters of this study as follows:

- Smoking, age and male gender are associated with more advanced disease
- Periodontal lesions in AgP tend to affect more molars and maxillary teeth
- The current diagnosis based on the number of affected teeth is probably not accurate in distinguishing localised and generalised cases
- IL-6 -174 GG genotype and carriage of NADPH oxidase $p22^{phox}$ 242 T allele are associated with AgP
- FcγRIIIb NA1/NA1 genotype is associated with GAgP
- FcγR haplotypes are associated with AgP in Blacks
- The vitamin D receptor Taq-I polymorphism is associated with AgP in smokers
- FcαR, NADPH oxidase $p22^{phox}$ 242 TT and FPR 301 GG genotypes showed tendencies for association with disease severity in GAgP
- 10% of examined first-degree blood relatives of AgP patients (at least 4.5% of the total relatives) suffer from AgP
- FcγRIIa RR genotype is associated with familial aggregation of AgP
- FcγR haplotypes and IL-6 -174 GG genotype are associated with detection of periodontopathogenic bacteria
- NADPH oxidase $p22^{phox}$ 242 TT genotype is associated with increased neutrophil oxidative burst.

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 $p22^{phox}$ and FcγR) and cytokines (IL-6) were enriched in AgP patients compared with controls. 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). Both the T allele of NADPH oxidase $p22^{phox}$ 242 polymorphism and the NA1 allele of FcγRIIIb polymorphism, which were enriched in patients, have been linked with increased release of superoxide from neutrophils.

Kobayashi et al. (2000b) showed increased phagocytosis of *P.gingivalis* in PMN from NA1 positive individuals, while Shimo-Nakanishi et al. (2004) found an increase in superoxide production in T allele positive subjects. This increase was also observed in the AgP patients included in our study: T homozygous patients showed increased oxidative burst upon stimulation with 2 ηg of PMA. Therefore, it seems that subjects whose neutrophils are hyperactive are more prone to develop AgP. This is consistent with the hypotheses postulated by Van Dyke et al. (2003), who supports the idea that hyperactive neutrophils may determine AgP damage. It is known that impaired PMN function, characteristic of diseases such as cyclic neutropenia or Chediak-Higashi syndrome have as a consequence increased susceptibility to infections and one of the sites affected is the periodontium. Patients affected by these diseases present with severe periodontal destruction at a very early age and healthy periodontal conditions are very difficult to achieve and maintain in such cases. Hence, the concept arose that AgP could be caused by more subtle PMN defects and impairments, whose only manifestation was at periodontal level. However, research into the function of neutrophils of AgP subjects has produced evidence that PMN from these subjects are not hypo- but hyper-active, and an excessive activity, coupled with the presence of specific microbes, as well an impaired activity, may lead to periodontal tissues damage (Kantarci et al. 2003). Several investigators have demonstrated an amplified PMN superoxide production in AgP patients (Van Dyke et al. 1985, Leino et al. 1994) compared with healthy subjects. Nonetheless, the mechanisms leading to this hyperactivity remain unknown. The data presented in this PhD thesis suggest that the host genetic background leads to this hyperactivity. In particular, PMN hyperactivation may be mediated by NADPH oxidase $p22^{phox}$ and FcγR polymorphisms. This excessive PMN activity may be balanced by feedback mechanisms in normal conditions. On the other hand, bacteria such as *A.actinomycetemcomitans* in the periodontal crevice stimulate PMN recruitment and migration towards the periodontium, where they act as the first line of defence against the invading organisms. Through the presence of local factors, particularly virulent and leukotoxic bacterial strains, or other underlying genetic factors, the resolution of the infection may not be achieved. In this event, the constant presence of *A. actinomycetemcomitans* induces the establishment of a chronic inflammatory process,

with the involvement of the immune system and the production of antibodies. 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. It is known that the superoxide anion (O_2^-) is released both inside PMN and outside, leading to possible tissue damage through a cascade of oxidative species and the stimulation of the release of proteases (Rada et al. 2004). In PMN carriers of the $p22^{phox}$ T allele, the potential to cause tissue damage may be augmented, because this polymorphism codes for an amino acid in a haem-binding region of the NADPH oxidase. Therefore, in these individuals the risk of evolving from a simple gingival inflammation (gingivitis) to a more serious irreversible periodontal destruction is increased. One of the stimulatory mechanisms for superoxide production comes from the recognition of the presence of bacteria. In PMN this happens, among other mechanisms, thanks to a family of transmembranous receptors, called Fc receptors, which recognize bacteria bound to Immunoglobulins (IgG). Once the recognition has taken place, bacteria are internalized in the phagocytes and the killing mechanism starts. Polymorphisms in the genes encoding for these receptors determine different receptor abilities and therefore different signal transmission. Individuals with the NA1 allotype for FcγRIIIb express a glycosylation pattern in their receptors which increases the ability to recognize and phagocytose bacteria, including the periodontopathogenic ones (van Sorge et al. 2003). One of the increased activities in NA1 subjects is again the oxidative burst, with consequent risk of tissue damage. Among the Caucasian subjects described in this thesis, having at least one copy of the $p22^{phox}$ T allele gave a relative risk 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 34, which suggests an additive effect due to a common mechanism of action. It is worthy to mention that the measure of superoxide production is just an indicative measure of a hyperactive status of PMN and may not be the only pathway through which these polymorphisms predispose to AgP. Equally important to mention is 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. 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 severe bone loss in subjects with this polymorphism. In fact, T homozygous GAgP Caucasian patients had considerably more advanced attachment loss (1.2 mm more than C allele carriers, see paragraph 7.3), which is likely to be associated with alveolar bone loss.

This scenario describing a primed, 'aggressive' PMN defensive phenotype becomes even more suggestive when the other important finding of this study is considered: the role of Interleukin-6. The cytokine network has a determinant function in the coordination of the inflammatory process, and IL-6 is one of its pro-inflammatory players. Among IL-6 activities, bone resorption and neutrophil stimulation stand out. The finding that G homozygous individuals for the -174 polymorphism had increased risk of having AgP in our sample, especially the Localised form, and also had more *A.actinomycetemcomitans* detected and more relatives affected by AgP, leads to speculation about its pathogenic activity. The particular enrichment of G homozygosity in LAgP suggests that the effect of this polymorphism is particularly linked with *A.actinomycetemcomitans*, the bacterium most consistently associated with LAgP (Mombelli et al. 2002). This is indeed confirmed by the findings from the family study presented in chapter 8, which shows that siblings, who share the same genetic background and most likely a similar bacterial microflora (Zambon et al. 1983, Petit et al. 1994, Haubek et al. 2005), end up having a similar disease. As observed in relation to the neutrophil polymorphisms, a possible explanation is that the presence of certain bacteria such as *A.actinomycetemcomitans* in the periodontium of G homozygous subjects leads to the establishment of a chronic inflammatory lesion, which results in an enhanced and rapid bone resorption and AgP onset at an early age. Since we have evidence to say that *A.a.* stimulates gingival fibroblast to produce IL-6 (Belibasakis et al. 2005), we can speculate that in G homozygous subjects, this increase brings an excessive tissue-damaging inflammatory response mediated by fibroblasts and/or other cell types (including PMN). This PhD thesis therefore shifts the attention of periodontal cytokine research onto the role of IL-6, which could reveal much more functional significance than most of the other cytokines extensively studied so far. New research

should be designed to study the effect of IL-6 genotypes on the presence of periodontopathogenic bacteria, and emphasis has to be placed on the role IL-6 genetic haplotype may play in periodontal pathogenesis. As a matter of fact, different research groups have already pointed out the importance of haplotype analysis in association studies for other diseases, especially with regards to IL-6 (Terry et al. 2000, Fife et al. 2005). Figure 11.1 reports a schematic diagram of the complex interactions that may exist between Interleukin-6, NADPH oxidase and Fc receptors of neutrophils and periodontopathogenic bacteria. These interactions form the basis of the excessive response seen in AgP, especially with regard to the effect on bone resorption.

The hypothesis regarding the 'aggressive' status of the host response in AgP patients is however complicated by some other findings of this study. The results relative to the FcγRIIa polymorphism point towards the 'impaired' response theory, because R homozygous individuals were more common among patients and, similar to the IL-6 polymorphism, they had more bacteria detected and more relatives affected. Since R homozygosity has been shown to confer reduced ability to recognize and phagocytose IgG2-opsonized particles (van Sorge et al. 2003), it looks in this case as though not being able to face the bacterial challenge can also result in increased disease predisposition. This is of course plausible, if we consider that FcγRIIa are the only receptors able to bind IgG2, which are the predominant Immunoglobulins in the periodontium. An impaired function of these receptors probably has a more evident repercussion on the disease risk than reduced function of the FcγRIIIb might have. Furthermore, FcγRIIIb are the most numerous receptors expressed in PMN, so they may have more potential than others to stimulate tissue damage. Moreover, in certain populations other hypoactive genotypes, such as FcγRIIIb NA2/NA2 or FcγRIIIa FF have been associated with periodontitis (Loos et al. 2005). In our sample, for example, a supposedly hypoactive genotype (Kaneko et al. 2004), FcαR -324 A homozygosity, showed a tendency towards increased risk of AgP. Moreover, in the Black subpopulations, FcγRIIIb NA2 homozygosity was enriched in patients. In general, a consistent difference was noted between Caucasians and Blacks: among Caucasians, FcγRIIa R and FcγRIIIb NA1 alleles were more frequent in patients, whilst the opposite

was observed in the Black subgroup, which is more in agreement with the previous literature, predominantly based in Black and Japanese populations (Loos et al. 2005).

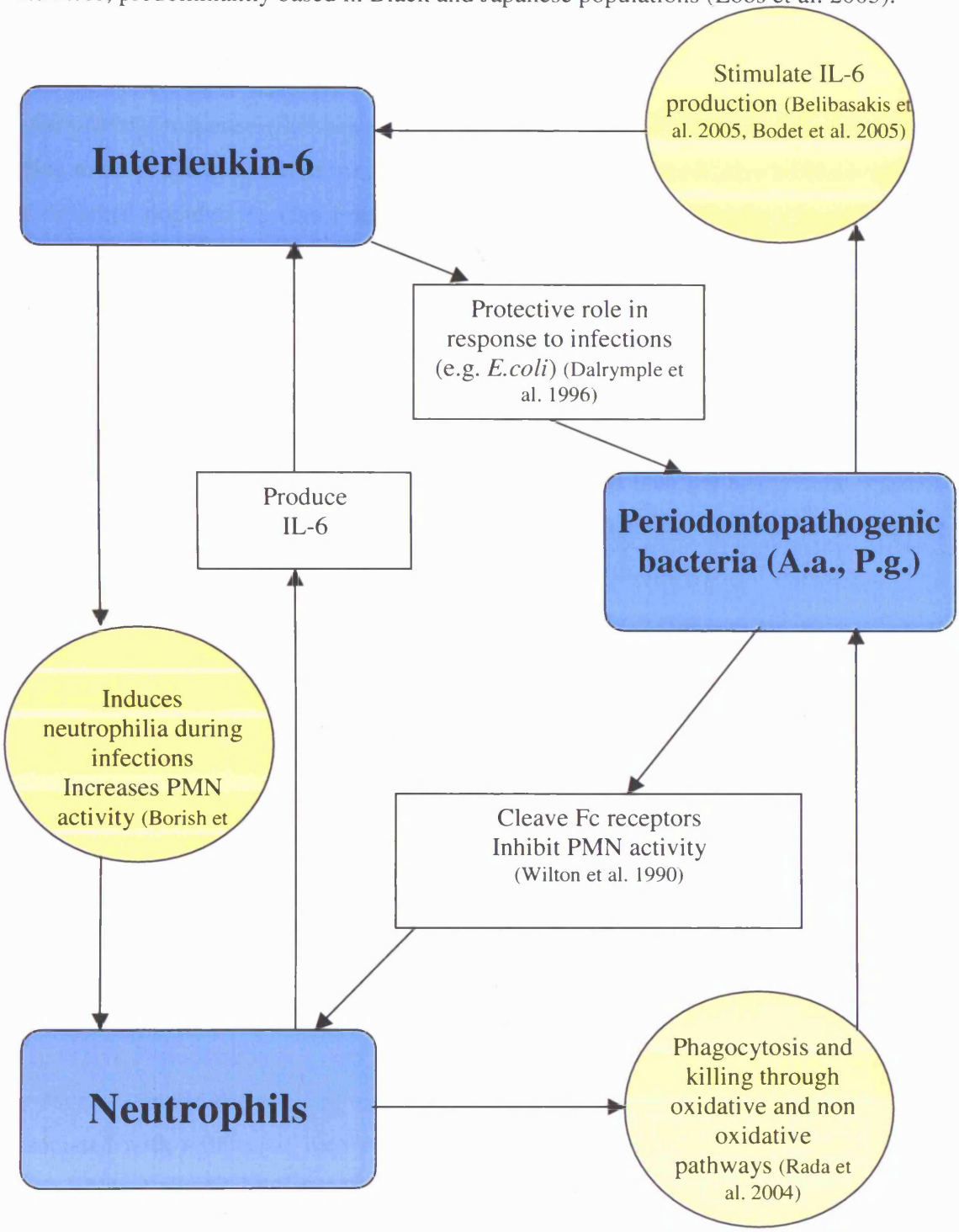

Figure 11.1 Relationships between IL-6, PMN and periodontopathogenic bacteria, with a potential role on periodontal disease pathogenesis.

Therefore, the impression is that two completely diverse pathways may lead to AgP: one characterized by the primed or 'aggressive' genotype, with hyper activation of the host response, and another one characterized by hypo functional ('impaired') genotypes. Probably they can both predispose to AgP, the first by mounting an excessive chronic inflammatory response (this seems to be the most common pathway), the second by being more prone to bacterial toxic products. These two pathways may be characteristic of different populations (the primed genotype seems to fit better for Caucasians) or could indeed coexist in the same subpopulations or even in the same subject. As a matter of fact, paradoxically both Fcα A and G homozygous subjects, and FcγIIa H and R homozygous subjects had more advanced disease compared with heterozygous subjects for the same polymorphisms. Both pathways converge to the hypothesis that AgP originates from an inability of the host to resolve the inflammatory processes triggered by the presence of bacteria (Van Dyke et al. 2003). This inability has genetic reasons, either because the host is not able to eliminate the insulting bacteria, or because it excessively amplifies the inflammatory response with consequent tissue damage.

Parallel to these findings, great attention has to be attributed to the importance of detecting of an interaction between Vitamin D receptor polymorphism and smoking in the pathogenesis of AgP. Speculation about a functional role of this polymorphism are hampered by the lack of understanding about its functions. However, combined effects on bone metabolism or on the immune system can be suspected. The confirmation of the role of this polymorphism in determining AgP in smokers may have important repercussions in prevention strategies in smokers with the Taq-I risk allele.

All the evidence produced by this study and by previous studies suggests that Aggressive Periodontitis is a very complex, heterogeneous disease which can be caused by several genetic defects. For example, cases of pre-pubertal periodontitis have been associated with a defect in cathepsin C (CTSC), a neutrophil protease, responsible for the Papillon-Lefevre syndrome (PLS) (Hart et al. 2000, Hewitt et al 2004). Mutations in the CTSC gene were detected in families with pre-pubertal periodontitis; although the affected subjects suffered from periodontitis, they did not have the palmo-plantar

keratosis typical of PLS. Subjects affected by these mutations have minimal or undetectable activity of the cathepsin C protease, and develop severe forms of periodontitis in their deciduous and permanent dentition (Hewitt et al. 2004). Other single gene defects may exist which can cause a clinical phenotype which we classify as AgP, either alone or as part of a syndrome. Subtle differences in genetic polymorphisms regulating Fc receptors or cytokines are probably irrelevant in such cases. This example testifies the genetic heterogeneity of AgP which can probably be due to single gene defects or to a combined genetic profile including for instance T homozygosity for NADPH $p22^{phox}$ or G homozygosity fro IL-6 -174 polymorphisms. With routine clinical diagnostic tools we would not be able to discriminate different phenotypes caused by different multiple or single genetic defects. Indeed, this thesis shows how we are currently not even able to accurately distinguish some cases of LAgP from GAgP.

The genetic polymorphisms detected as risk factors for AgP in this thesis deserve further investigation to elucidate mechanisms of action and to test and confirm their importance in different populations. This 'aggressive' genotype we propose as a possible genetic background of AgP cases may have developed through the years by a mechanism of selective pressure. AgP of course is not a life-threatening disease able to exert selection in the population. However, other lethal diseases might have helped selecting this hyper-responsive genotype throughout centuries (Cooke & Hill 2001). In other words, diseases which have a significant effect on mortality before reproductive age cause a natural selection in the population. This is the case of some infectious diseases, resistance to which may be genetically determined (Cooke & Hill 2001). Resistant subjects, who have a decreased risk of contracting the infectious disease in question and of dying from it, will have more chance of leaving offspring and their genetic patrimony is more likely to be transmitted throughout generations. At the same time, however, such genetic background might predispose to other diseases. An explicative example is represented by malaria, an infectious disease caused by *Plasmodium falciparum*. Individuals heterozygous for the HbS variant of haemoglobin are protected against *P.falciparum* infection and have a decreased risk of contracting malaria, because their erythrocytes offer a less suitable growth environment for this parasite. Therefore, selective pressure

has occurred throughout generations and led to a very high prevalence of this HbS variant in areas where malaria was endemic. As a consequence, HbS homozygosity, which leads to thalassaemia, is dramatically increased in prevalence in those areas. Selective pressure is also suspected to be able to represent the basis for diffusion not only of single-gene disorders, but also of complex diseases, such as Crohn's disease. The florid inflammatory status which characterizes this gastrointestinal disorder might in fact represent a protective factor against certain infections (Cooke & Hill 2001). Recently, infectious diseases such as Black Death plague and smallpox epidemics have been suspected as possible selective pressure agents, especially in European populations (Galvani & Slatkin 2003). Plague epidemics have been very frequent in Europe for at least 400 years and had a mortality rate of up to 40%. Smallpox, on the other hand, was endemic in Europe for hundreds of years and its mortality rates, although smaller than plague, were very high among children, removing a large number of individuals of reproductive potential (Galvani & Slatkin 2003). Both these infectious diseases resulted in a selection of resistant individuals, who survived because of specific genetically-driven host response mechanisms and produced offspring. A mutation in the chemokine receptor CCR5, which confers protection against HIV infection, is thought to have developed from one of these epidemics, most likely smallpox. This is reinforced by the fact that this mutation is virtually absent in extra-European populations, where no smallpox epidemic was recorded (Galvani & Slatkin 2003). In this context, the 'aggressive' genotype we observed in the AgP patients included in this study may have been the result of selective pressure from some infectious agents. In particular, considering the very high prevalence of the NADPH p22phox oxidase 242 T allele and IL-6 -174 G allele in Black populations, infectious diseases relevant especially in Black African populations might have selected this hyper-responsive genotype. These alleles could have been protective versus life-threatening infectious diseases because of the strong oxidative burst and inflammatory response linked with them, but at the same time might give origin to significant tissue damage, for example in the periodontium. This is coherent with the fact that AgP seems to be more common in Black populations, who have increased frequencies of these risk alleles.

In conclusion, this thesis produces evidence consistent with a multifactorial polygenic nature of AgP, even if some cases may be caused by single gene defects. Thus, similar to the situation for other chronic diseases, like diabetes or cardiovascular disease, there might be some main modifying genes and several others with a small effect on disease susceptibility (Hansen & Pedersen 2005). The combination of main and minor risk alleles with environmental factors and, most of all, periodontopathogenic bacteria able to trigger an inflammatory response, may lead to AgP. The example of another oral disease, Amelogenesis imperfecta (AI), can be used to examine the prospective benefit of a better understanding of AgP genetics. The AI trait can be acquired, X-linked, autosomal dominant or autosomal recessive. Clinical characteristics have been detected, which allow a sub classification of AI into pitted hypoplastic, rough hypoplastic, hypocalcified etc. (Aldred et al. 2003). All these forms correspond to different known genetic defects. In AgP, a similar ability to clinically discern phenotypic characteristics due to specific genotypic factors would help achieve a better diagnosis. Some clinical features could for example be linked with increased familial aggregation, or with a different response to conventional treatment. While the prospect of a better clinical diagnosis seems difficult to achieve in AgP, the possibility of a genetic diagnosis with an impact on management may soon become a reality.

The work included in this PhD identified genetic polymorphisms as risk factors for onset, severity and familial aggregation of AgP. It also suggested possible mechanisms of association between genetic risk factors and AgP. I hope this thesis can contribute to the continuous research aimed at understanding genetic risk factors for AgP. The identification of the risk genotypes we performed should be followed by haplotype studies, family studies, functional analyses and studies on the effects of these genotypes on disease progression. Once clear mechanisms of disease susceptibility are confirmed in different populations, the management of AgP can enter a new dimension, with the prospect for more specific treatments. Being able to detect the different defects leading to AgP in each family and each individual, will help inform preventive strategies and new treatment modalities directed at modulating the activity of the hypo- or hyper-functioning genes (Van Dyke et al. 2003). New techniques which are rapidly becoming

available, such as wide genome screening and gene therapy, may change in the near future the approach to AgP.

LIST OF ABBREVIATIONS

AgP: Aggressive Periodontitis

GAgP: Generalised Aggressive Periodontitis

LAgP: Localised Aggressive Periodontitis

CP: Chronic Periodontitis

LJP: Localised Juvenile Periodontitis

GJP: Generalised Juvenile Periodontitis

L-EOP: Localised Early Onset Periodontitis

G-EOP: Generalised Early Onset Periodontitis

RPP: Rapidly Progressive Periodontitis

PPD: Probing Pocket Depth

REC: recession

LCAL: Lifetime Cumulative Attachment Loss

CEJ: cement- enamel junction

FGM: free gingival margin

FMBS: full mouth bleeding score

FMPS: full mouth plaque score

CPITN: Community Periodontal Index for Treatment Needs

WHO: World Health Organization

GDP: General Dental Practitioner

EDH: Eastman Dental Hospital

Aa: *Actinobacillus actinomycetemcomitans*

Pg: *Porphyromonas gingivalis*

Tf: Tannerella forsythensis

PGE: Prostaglandin E

IL: Interleukin

TNF: Tumor Necrosis Factor

TLR: Toll-like receptor

FPR: formyl peptide receptor

FMLP: N-formyl peptide formyl-methionine-leucyl-phenylalanine

NA: neutrophil antigen

PMN: polymorphonuclear leukocytes (neutrophils)

MMP: matrix metalloproteinases

LPS: lipopolysaccharide

ROS: reactive oxygen species

NADPH: nicotinamide adenine dinucleotide phosphate

NOX: NADPH oxidase

PMA: 4B-phorbol 12-mystrate 13-acetate

SOD: superoxide dismutase

Ig: Immunoglobulin

DNA: deoxyribonucleic acid

RNA: ribonucleic acid

SNP: single nucleotide polymorphism

RFLP: restriction fragment length polymorphism

ANOVA: analysis of variance

LD: linkage disequilibrium

PCR: polymerase chain reaction

LRT: likelihood- ratio test

ETOH: ethanol

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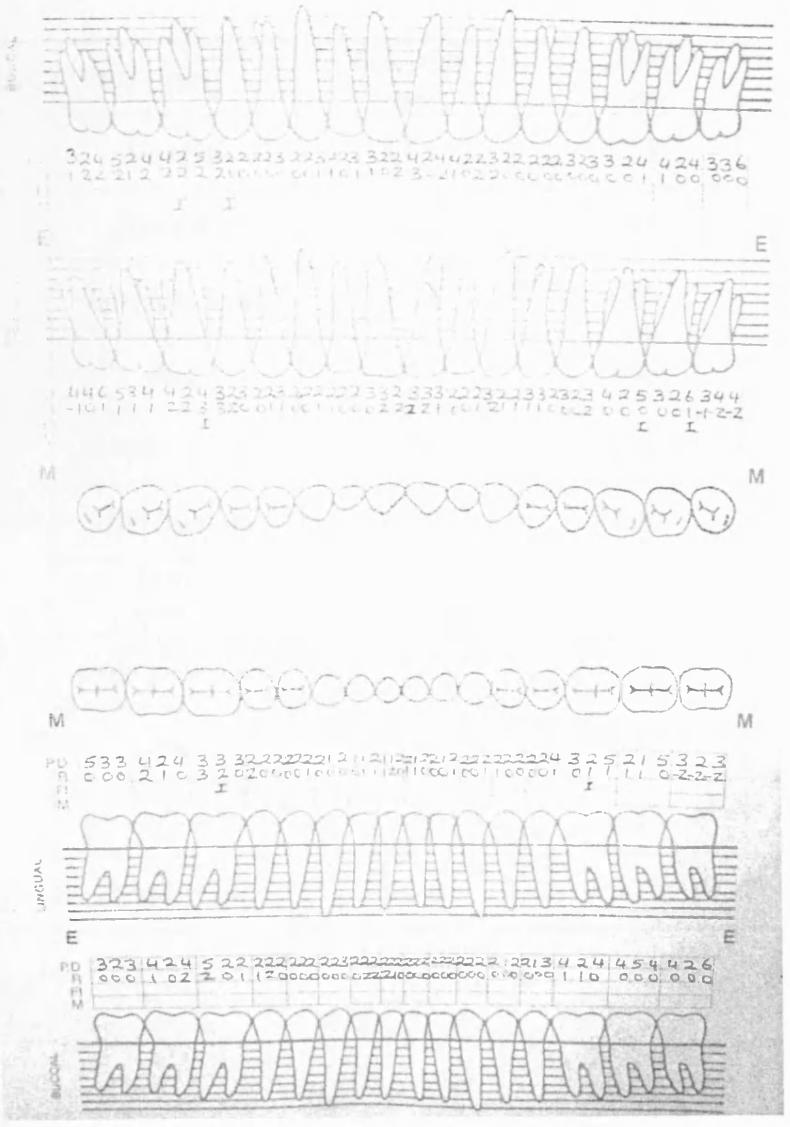
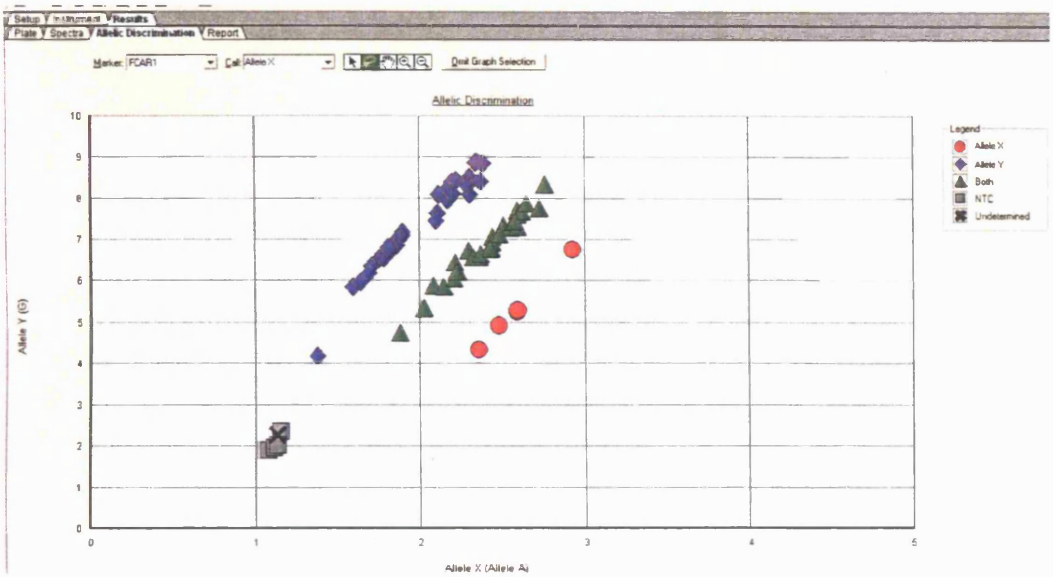
APPENDIX I. Example of probing sheet used to collect the clinical measurement. PD indicates probing pocket depth, R indicates recession, FI stands for furcation involvement and M for mobility



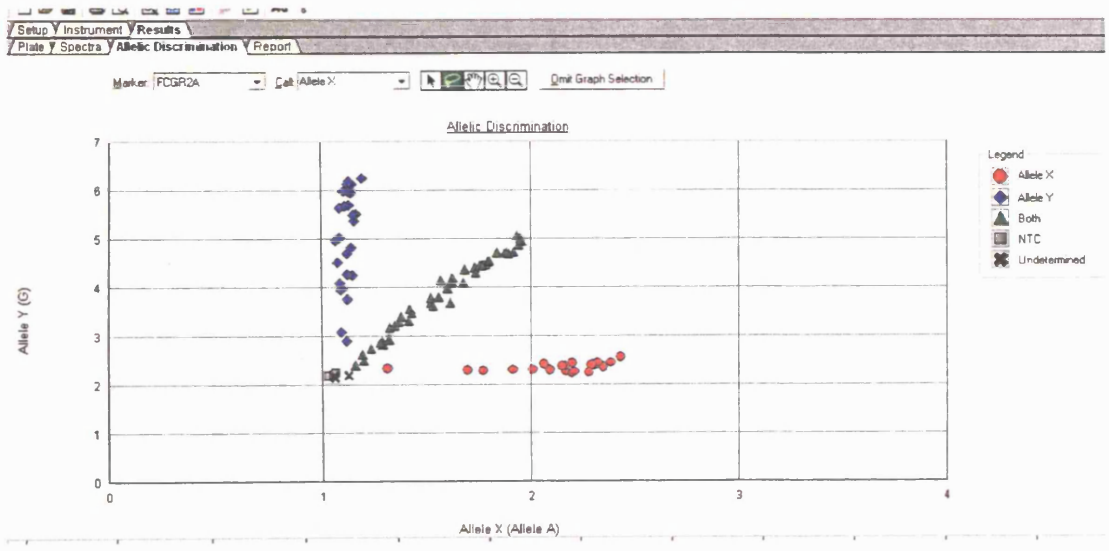
APPENDIX II. Average probing pocket depths reported for jaw, tooth surface and tooth type

AVERAGE PPD (mm)		Tot. AgP (mm)	GAgP (mm)	LAgP (mm)
JAW	*Maxilla*	4.03	4.39	2.94
	Mandible	3.68	3.98	2.74
SURFACE	*Interproximal*	4.44	4.83	3.24
	Mid	2.75	2.96	2.11
TOOTH TYPE	*Second molars*	4.49	4.86	3.37
	First molars	4.70	4.90	4.16
	Second premolars	3.78	4.11	2.80
	First premolars	3.55	3.90	2.41
	Canines	3.44	3.82	2.24
	Second incisors	3.46	3.82	2.36
	First incisors	3.46	3.79	2.44

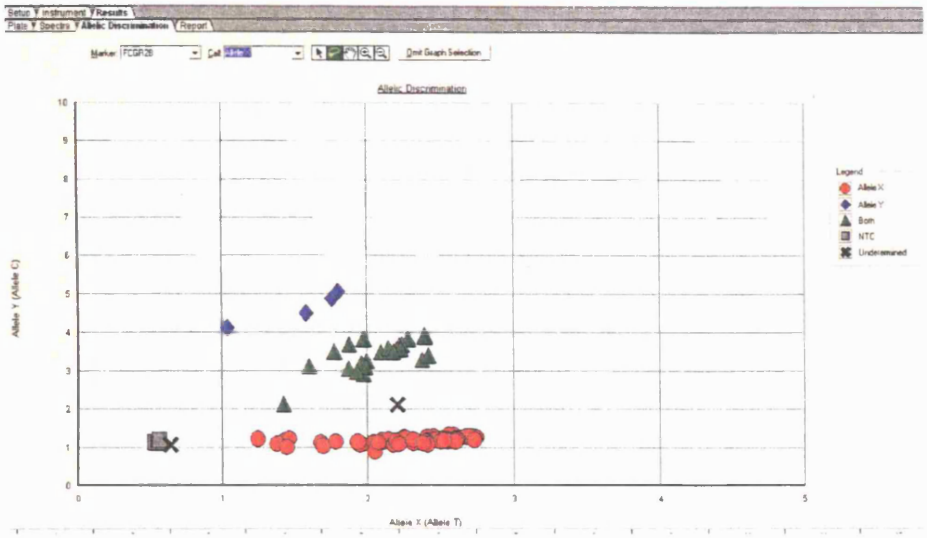
APPENDIX III. Scatter plots showing the distribution of the FcαR 324 genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele A, the green triangles heterozygous samples and the blue rhombi samples homozygous for the G allele. The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



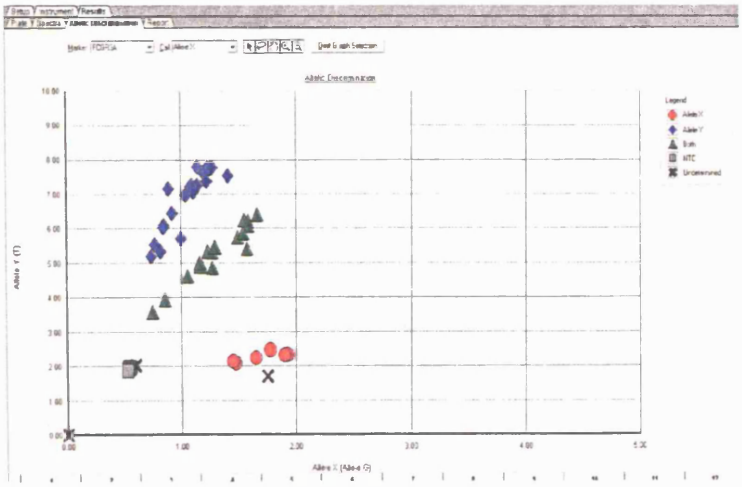
APPENDIX IV. Scatter plots showing the distribution of the FcγRIIa 494 genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele A, the green triangles heterozygous samples and the blue rhombi samples homozygous for the G allele. The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



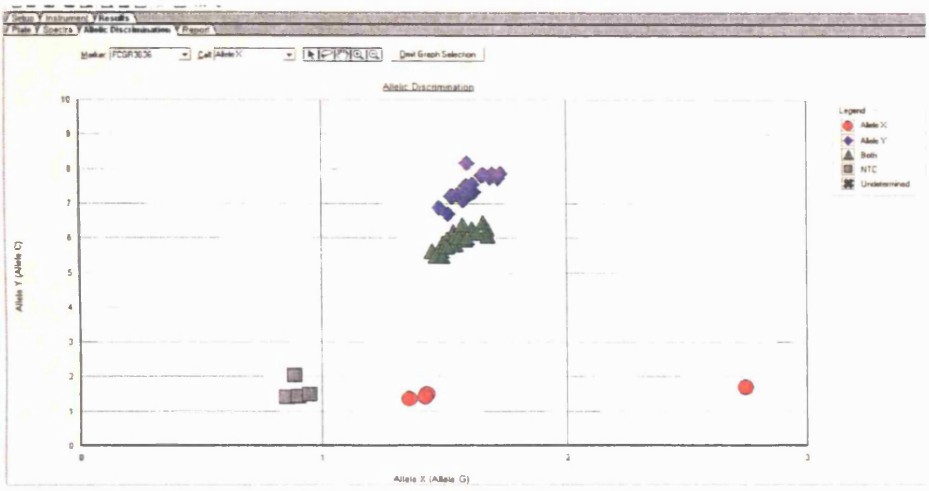
APPENDIX V. Scatter plots showing the distribution of the FcγRIIa 494 genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele T, the green triangles heterozygous samples and the blue rhombi samples homozygous for the C allele. The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



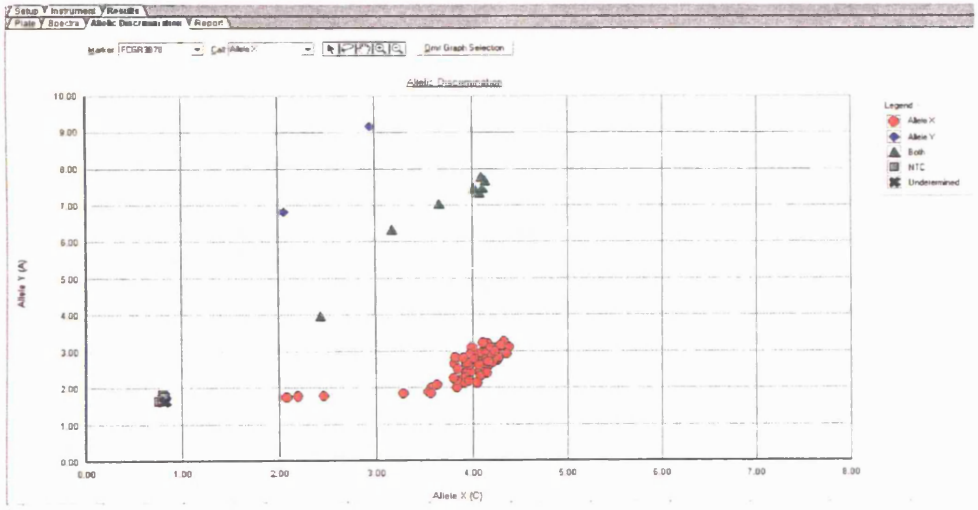
APPENDIX VI. Scatter plots showing the distribution of the FcγRIIb 695 genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele G, the green triangles heterozygous samples and the blue rhombi samples homozygous for the T allele. The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



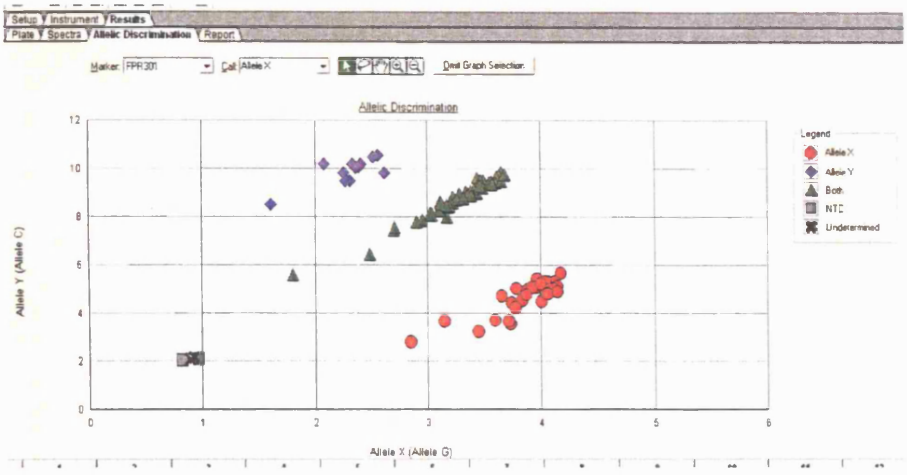
APPENDIX VII. Scatter plots showing the distribution of the FcγRIIIb NA (141) genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele G (NA2), the green triangles heterozygous samples and the blue rhombi samples homozygous for the C allele (NA1). The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



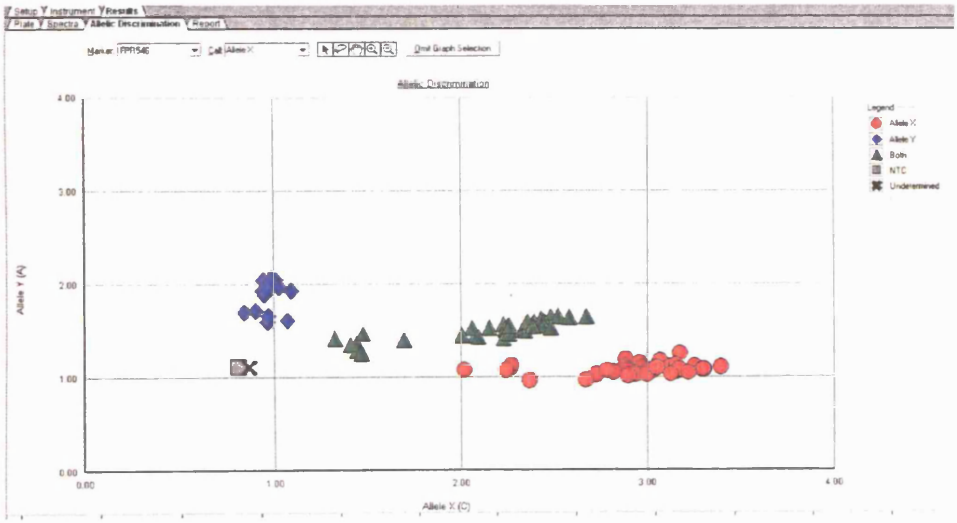
APPENDIX VIII. Scatter plots showing the distribution of the FcγRIIIb SH (266) genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele A (SH+), the green triangles heterozygous samples and the blue rhombi samples homozygous for the C allele (SH-). The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



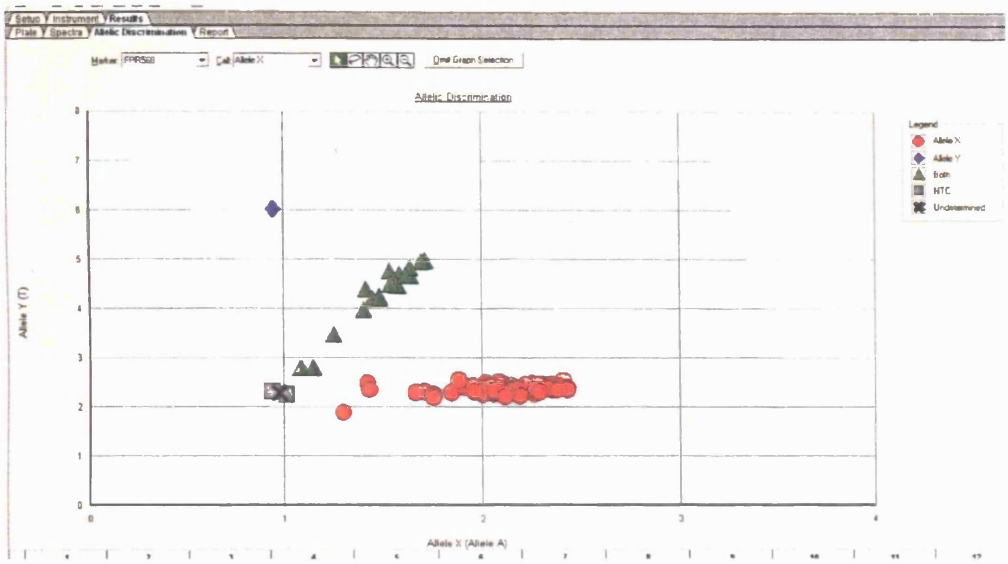
APPENDIX IX. Scatter plots showing the distribution of the FPR 301 genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele G, the green triangles heterozygous samples and the blue rhombi samples homozygous for the C allele. The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



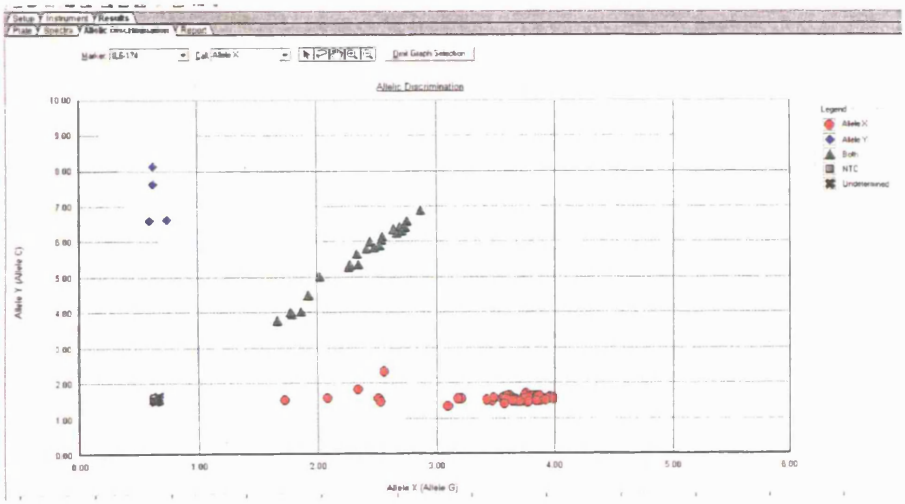
APPENDIX X. Scatter plots showing the distribution of the FPR 546 genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele C, the green triangles heterozygous samples and the blue rhombi samples homozygous for A allele. The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



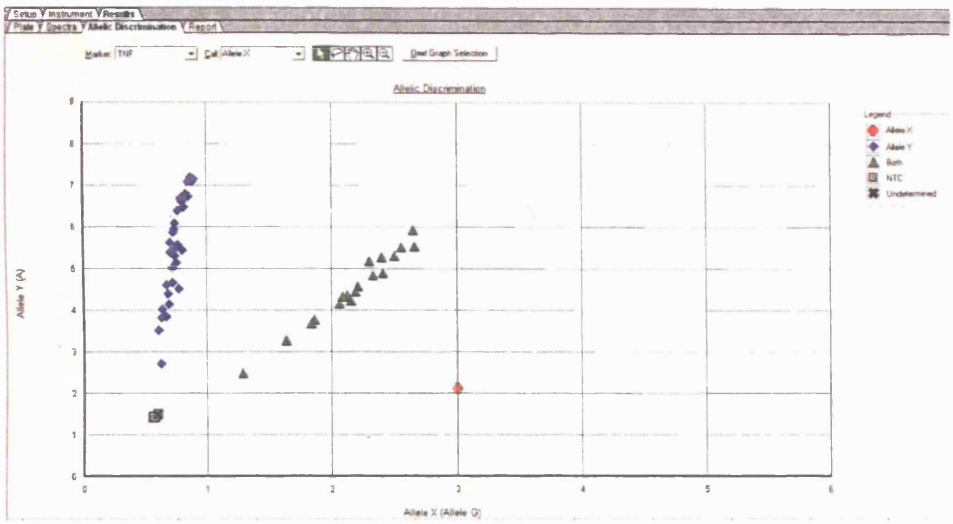
APPENDIX XI. Scatter plots showing the distribution of the FPR 568 genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele A, the green triangles heterozygous samples and the blue rhombi samples homozygous for T allele. The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



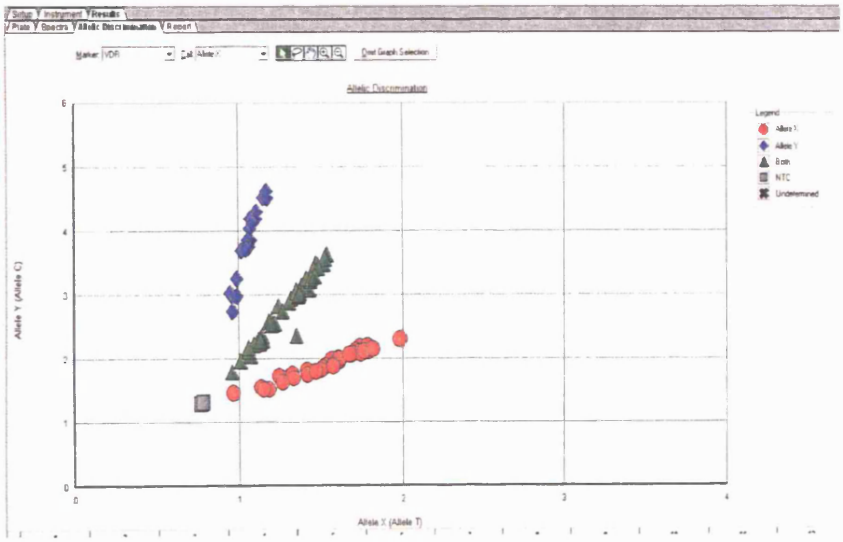
APPENDIX XII. Scatter plots showing the distribution of the IL-6 -174 genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele G, the green triangles heterozygous samples and the blue rhombi samples homozygous for allele C. The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



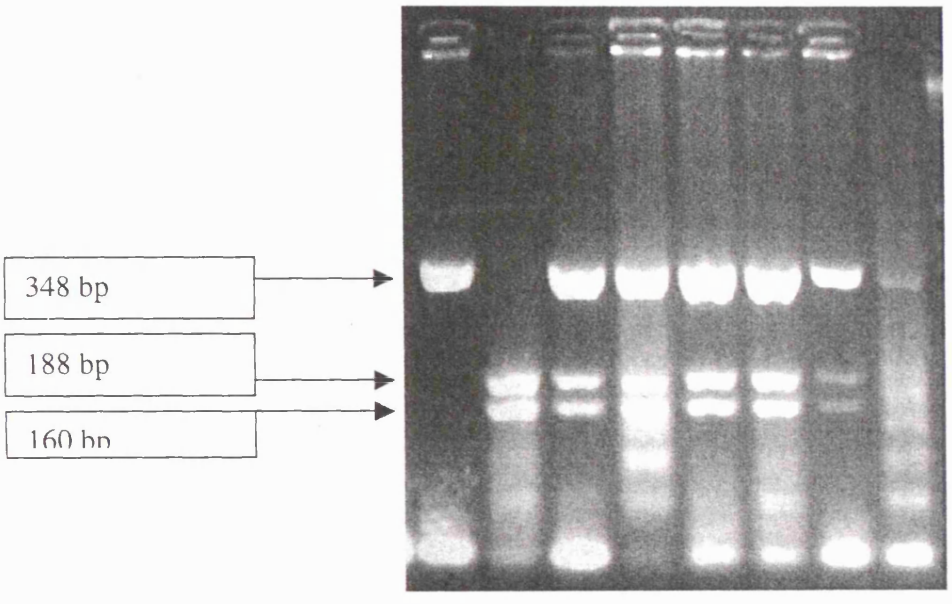
APPENDIX XIII. Scatter plots showing the distribution of the TNF-α -308 genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele G, the green triangles heterozygous samples and the blue rhombi samples homozygous for allele A. The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



APPENDIX XIV. Scatter plots showing the distribution of the VDR Taq-I (1056) genotypes as plotted by the 7300/7500 SDS software. The red balls indicate samples homozygosous for allele T (TT), the green triangles heterozygous samples and the blue rhombi samples homozygous for allele C (tt). The squares indicate controls which do not contain DNA. Crosses indicate samples whose genotype was undetermined.



Appendix XV. Visualization under UV light of the PCR products of the NADPH p22phox C242T polymorphism. Digestion of the PCR product (348 bp) by RsaI enzyme makes 160-bp and 188-bp fragments in the T mutation, while the same enzyme does not cut the PCR product in the wild type (C allele). The 7 samples shown in the picture were respectively classified as follows starting from the first on the left: CC, TT, CT, CT, CT, CT, CT. The last column shows a size marker.



Appendix XVI. Allele distributions in patients and controls for all studied SNPs.

Polymorphism	Allele	All subjects		Caucasians		Blacks	
		Patients (n=224)	Controls (n=231)	Patients (n=106)	Controls (n=144)	Patients (n=59)	Controls (n=45)
Fcα	*A*	122 (27.8%)	114 (24.7%)	70 (31.5%)	74 (25.7%)	18 (15.8%)	9 (10.0%)
	G	316 (72.1%)	348 (75.3%)	152 (69.5%)	214 (74.3%)	96 (84.2%)	81 (90.0%)
	tot	438	462	222	288	114	90
FcγIIa	*H*	219 (50.2%)	235 (50.8%)	104 (46.8%)	145 (50.3%)	59 (52.7%)	40 (44.4%)
	R	217 (40.8%)	227 (49.2%)	118 (53.2%)	143 (49.7%)	53 (47.3%)	50 (55.6%)
	tot	436	462	222	288	112	90
FcγIIb	*C*	73 (16.6%)	74 (16.0%)	26 (11.6%)	39 (13.5%)	25 (21.9%)	18 (20.0%)
	T	367 (83.4%)	388 (84.0%)	198 (88.4%)	249 (86.5%)	89 (78.1%)	72 (80.0%)
	tot	440	462	224	288	114	90
FcγIIIa	*V*	151 (34.6%)	159 (34.7%)	82 (36.9%)	103 (36.2%)	39 (34.2%)	27 (30.0%)
	F	285 (63.4%)	299 (65.3%)	140 (63.1%)	181 (63.8%)	75 (65.8%)	63 (70.0%)
	tot	436	458	222	284	114	90
FcγIIIbNA	*NA2*	246 (55.6%)	276 (59.7%)	134 (59.8%)	177 (77.6%)	62 (53.4%)	46 (51.1%)
	NA1	196 (44.4%)	186 (40.3%)	90 (40.2%)	111 (22.4%)	54 (46.6%)	44 (48.9%)
	tot	442	462	224	288	116	90
FcγIIIbSH	*SH+*	32 (7.2%)	22 (4.7%)	6 (2.7%)	3 (1.0%)	22 (18.9%)	13 (14.4%)
	SH-	412 (92.8%)	440 (95.3%)	218 (87.3%)	285 (99.0%)	94 (81.1%)	77 (85.6%)
	tot	444	462	224	288	116	90

Appendix XVI continued.

Poly-morphism	Allele	All subjects		Caucasians		Blacks	
		Patients (n=224)	Controls (n=231)	Patients (n=106)	Controls (n=144)	Patients (n=59)	Controls (n=45)
FPR 301	*C*	134 (31.6%)	157 (34.2%)	70 (31.8%)	98 (34.5%)	29 (25.4%)	28 (31.1%)
	G	290 (68.4%)	301 (65.8%)	150 (68.2%)	186 (65.5%)	83 (74.6%)	62 (68.9%)
	tot	424	458	220	284	114	90
FPR 546	*A*	156 (35.4%)	145 (31.4%)	76 (33.9%)	87 (30.2%)	33 (28.9%)	29 (32.2%)
	C	284 (64.6%)	317 (68.6%)	148 (66.1%)	201 (69.8%)	81 (81.1%)	61 (67.8%)
	tot	440	462	224	288	114	90
FPR 568	*A*	384 (87.2%)	409 (88.5%)	193 (86.9%)	255 (89.1%)	102 (87.9%)	86 (95.5%)
	T	56 (12.8%)	53 (11.5%)	29 (13.1%)	31 (10.9%)	14 (12.1%)	4 (4.5%)
	tot	440	462	222	286	116	90
VDR	*t*	161 (35.9%)	186 (40.2%)	82 (36.6%)	119 (41.3%)	40 (33.9%)	29 (32.2%)
	T	287 (64.1%)	276 (59.8%)	142 (63.4%)	169 (58.7%)	78 (66.1%)	61 (67.8%)
	tot	448	462	224	288	118	90
TNF-α	*A*	375 (84.4%)	383 (83.2%)	176 (78.6%)	231 (80.8%)	99 (85.3%)	74 (82.2%)
	G	69 (15.6%)	77 (16.8%)	48 (21.4%)	55 (19.2%)	17 (14.7%)	16 (17.8%)
	tot	444	460	224	286	116	90
IL-6	*C*	100 (22.5%)	151 (32.7%)	77 (34.4%)	130 (45.1%)	6 (5.3%)	7 (7.7%)
	G	344 (77.5%)	311 (67.3%)	147 (65.6%)	158 (54.9%)	108 (94.7%)	83 (92.3%)
	tot	444	462	224	288	114	90
NADPH	*C*	230 (57.8%)	308 (68.7%)	123 (61.5%)	199 (71.1%)	50 (46.3%)	52 (57.8%)
	T	168 (42.2%)	140 (31.3%)	77 (38.5%)	81 (28.9%)	58 (53.7%)	32 (42.2%)
	tot	398	448	200	280	108	84

Appendix XVII. Clinical data recorded in GAgP patients, divided by genotypes for all polymorphisms.

All patients GAgP	Genotype	no.	Av. PPD	Av. LCAL	no. ≥ 5 mm PPD	no. ≥ 10 mm PPD
Fcα	AA	14	4.4±1.4	5.4±2.0	70.5±38.7	5.2±8.0
	AG	64	4.0±1.0	4.6±1.5	55.3±29.6	2.7±6.0
	GG	84	4.3±1.0	5.0±1.4	69.4±31.6	3.3±5.0
FcγIIa	HH	41	4.3±0.9	5.0±1.4	68.7±33.6	3.0±4.2
	HR	78	4.1±0.9	4.7±1.3	60.0±29.8	3.0±6.0
	RR	42	4.3±1.2	5.1±1.8	65.9±34.5	3.8±6.6
FcγIIb	CC	4	3.8±0.5	4.4±1.2	52.0±21.5	1.2±1.5
	CT	45	4.3±1.2	5.1±1.6	67.2±37.0	4.0±7.2
	TT	113	4.2±0.9	4.9±1.5	61.7±34.3	2.9±5.1
FcγIIIa	VV	20	4.2±1.0	4.9±1.5	61.7±34.3	2.9±4.2
	VF	69	4.2±1.2	5.1±1.7	64.8±34.3	3.9±7.1
	FF	72	4.1±0.9	4.7±1.3	63.4±29.7	2.7±4.6
FcγIIIbNA	NA2/NA2	51	4.2±1.0	4.8±1.5	62.6±32.1	3.8±7.1
	NA1/NA2	74	4.2±1.1	4.9±1.6	64.8±34.6	3.2±5.5
	NA1/NA1	37	4.3±0.8	4.9±1.4	64.0±27.1	2.3±3.8
FcγIIIbSH	SH+/SH+	3	3.3±0.9	3.7±0.4	34.3±4.9	0.7±1.1
	SH-/SH+	17	4.4±1.1	5.2±1.6	71.3±34.6	4.2±6.5
	SH-/SH-	144	4.2±1.0	4.9±1.5	63.7±31.6	3.1±5.7
FPR 301	CC	16	3.9±0.9	4.6±1.6	55.5±33.6	2.2±4.6
	CG	60	4.0±1.1	4.8±1.5	58.7±35.0	3.1±6.5
	GG	79	4.4±0.9	5.0±1.5	70.0±28.8	3.5±5.3
FPR 546	AA	19	4.5±1.4	5.3±1.7	71.7±40.6	6.0±10.6
	AC	79	4.0±0.9	4.8±1.6	58.6±30.6	2.5±4.1
	CC	64	4.4±1.0	4.8±1.3	68.2±30.4	3.3±5.3
FPR 568	AA	123	4.2±1.0	4.8±1.5	62.4±33.2	3.2±6.1
	AT	39	4.3±0.9	5.1±1.7	68.4±28.0	3.4±4.8
	TT	1	4.7	4.5	76.0	5.0
VDR	tt	23	4.3±1.1	4.9±1.7	65.1±36.4	4.3±7.2
	Tt	72	4.2±1.0	4.9±1.6	65.9±30.7	2.7±5.5
	TT	69	4.1±1.0	4.9±1.4	61.5±31.8	3.4±5.6
TNF-α	AA	113	4.3±1.0	4.8±1.5	64.4±32.4	3.3±0.5
	AG	45	4.2±1.0	5.1±1.5	65.9±31.5	3.1±0.9
	GG	4	3.3±0.3	3.8±1.0	39.2±13.1	0
IL-6	CC	14	4.4±1.2	4.7±1.7	68.6±37.3	4.3±7.7
	CG	53	4.2±1.0	5.0±1.7	65.0±31.0	2.6±5.4
	GG	96	4.2±1.0	4.9±1.4	62.8±32.0	3.5±5.7
NADPH	CC	50	4.2±1.0	4.8±1.6	61.7±32.6	3.4±5.4
	CT	73	4.1±0.9	4.8±1.3	63.0±30.7	3.0±6.0
	TT	31	4.4±1.2	5.2±1.8	67.1±33.7	3.7±6.5

Appendix XVIII. Log counts of periodontopathogenic bacteria by genotype

Log counts of bacteria by genotypes		Tot	Tot an-aerobes log	Tot aerobes log	A.a. log	P.g. log	T.f. log
Fcα	AA	6	6.7±0.4	5.7±0.4	4.8±1.2	5.6±0.5	5.9±0.4
	AG	18	7.4±0.7	6.2±0.8	4.5±1.3	6.6±0.8	6.4±0.4
	GG	21	7.1±0.5	6.1±0.8	4.3±1.1	6.1±0.6	6.1±0.5
FcγIIa	HH	13	7.1±0.5	6.3±0.6	4.5±0.6	5.7±0.4	6.1±0.4
	HR	18	7.2±0.7	6.0±0.9	4.4±1.2	6.3±0.9	6.4±0.6
	RR	13	7.1±0.6	6.1±0.8	4.4±1.5	6.4±0.8	6.1±0.3
FcγIIb	CC	1	6.8	4.1	0	0	0
	CT	12	7.1±0.5	6.0±0.8	4.1±1.2	6.0±0.6	6.2±0.3
	TT	32	7.2±0.7	6.1±0.7	4.5±1.2	6.4±0.8	6.2±0.5
FcγIIIa	VV	6	7.1±0.7	6.1±1.1	4.7	5.7	5.9±0.5
	VF	19	7.0±0.5	6.0±0.6	4.4±1.1	6.0±0.6	6.3±0.3
	FF	20	7.3±0.7	6.1±0.8	4.4±1.3	6.5±0.8	6.2±0.6
FcγIIIbNA	NA2/NA2	17	7.1±0.6	5.9±1.0	4.8±1.4	6.3±0.8	6.1±0.4
	NA1/NA2	18	7.2±0.7	6.2±0.6	4.5±1.3	6.2±0.6	6.2±0.4
	NA1/NA1	10	7.2±0.5	6.1±0.5	4.0±0.9	6.4±1.0	6.4±0.7
FcγIIIbSH	SH+/SH+	0	0	0	0	0	0
	SH-/SH+	8	6.9±0.4	5.6±0.6	4.6±1.2	5.9±0.6	6.1±0.3
	SH-/SH-	37	7.2±0.6	6.1±0.8	4.3±1.2	6.4±0.8	6.2±0.5
FPR 301	CC	3	6.9±0.5	4.9±0.7	5.6	5.7	5.7±0.6
	CG	15	7.2±0.8	6.1±0.7	4.1±1.2	6.0±0.7	6.1±0.3
	GG	25	7.2±0.5	6.2±0.8	4.6±1.2	6.4±0.8	6.4±0.5
FPR 546	AA	5	7.0±0.6	6.1±1.1	4.4±0.6	6.0±1.1	6.0±0.5
	AC	25	7.1±0.5	6.1±0.6	4.6±1.3	6.2±0.6	6.1±0.4
	CC	14	7.3±0.8	6.1±1.0	4.0±1.0	6.4±0.8	6.5±0.6
FPR 568	AA	33	7.1±0.5	6.0±0.8	4.4±1.1	6.2±0.9	6.2±0.5
	AT	12	7.2±0.8	6.4±0.7	4.4±1.3	6.3±0.6	6.3±0.4
	TT	0	0	0	0	0	0
VDR	tt	5	7.2±0.7	5.9±0.3	4.2±2.3	7.0±0.7	7.0±0.8
	Tt	24	7.1±0.6	5.9±0.7	4.6±1.0	6.0±0.6	6.2±0.4
	TT	15	7.2±0.6	6.4±0.2	4.3±1.1	6.2±0.8	6.2±0.3
TNF-α	AA	34	7.2±0.6	6.1±0.8	4.2±1.2	6.3±0.8	6.2±0.5
	AG	9	7.1±0.4	6.1±0.6	5.2±0.7	6.1±0.4	6.1±0.4
	GG	2	6.8±1.1	5.9±0.7	0	0	6.3
IL-6	CC	7	7.1±0.4	5.8±0.4	3.9±1.1	6.1±0.9	5.9±0.7
	CG	12	7.1±0.6	6.1±1.0	4.1±1.1	6.3±1.0	6.2±0.4
	GG	26	7.2±0.7	6.2±0.7	4.5±1.2	6.3±0.7	6.3±0.5
NADPH	CC	22	7.3±0.7	6.1±0.8	4.0±1.1	6.6±0.4	6.3±0.3
	CT	17	7.0±0.6	6.0±0.9	5.0±1.1	6.2±0.9	6.3±0.6
	TT	5	6.9±0.5	6.3±0.5	3.5±0.3	5.5±0.3	6.1±0.6