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Genetic epidemiology of periodontal diseases in the elderly

by

Lindsay M. Reynolds

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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By

Lindsay M. Reynolds

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GENETIC EPIDEMIOLOGY OF PERIODONTAL DISEASES IN THE ELDERLY

by

Lindsay M. Reynolds

ABSTRACT

Background

Previous studies have identified several risk factors for periodontal diseases. However, due to many differences between studies (i.e. study design, outcome definitions, and covariates included) the relative importance of these risk factors is unclear. The purpose of this study was to assess and compare the magnitude of many known and potential risk factors, and identify the most important risk factors for periodontal diseases.

Methods

An extensive list of risk factors was investigated with four quantitative outcomes (mean plaque index, percent bleeding on probing, extent pocket depth $\geq 5\text{mm}$, and extent attachment loss $\geq 3\text{mm}$) in three elderly populations (Health, Aging, and Body Composition (Health ABC) Study African Americans ($n = 539$), Health ABC Study Caucasians ($n = 1,010$), and Osteoporotic Fractures in Men Study (MrOS) Caucasians ($n = 686$)). Univariate linear regression was used to estimate the percentage of outcome variance accounted for by each risk factor, and backwards stepwise regression was used to estimate the effect sizes associated with the risk factors. We also surveyed the

genome for common genetic risk factors by meta-analyzing results from genome-wide association studies in two Caucasian populations (Health ABC Study and MrOS, n = 1,373). Significant signals ($p \leq 10^{-6}$) were tested for replication in two elderly populations (Atherosclerosis Risk in Communities Study Caucasians (n = 4,165) and Health ABC Study African Americans (n = 500)), and *in silico* analyses were used to evaluate the potential functional importance of the identified genomic regions.

Results

High plaque levels and inter-examiner effects explained the largest portions of variance of all outcomes. At least half of the variance of each outcome remained unexplained. In meta-analyses, each outcome was found to be associated with variants in one genomic region ($p \leq 10^{-6}$). These signals were not supported by findings from replication studies ($p > 0.05$); however, *in silico* analyses provided some support for three candidate genes (*FBLN1*, *PTPRT*, and *PPP2R2B*) for future studies.

Conclusion

Overall, plaque appears to be the largest risk factor for periodontal diseases, compared to any other risk factor or single genetic polymorphism tested in this investigation. No major genetic risk factor was identified, which may suggest these diseases are under the influence of smaller and more complex genetic effects.

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CHAPTER 1

LITERATURE REVIEW

1.1 What are periodontal diseases?

Diseases affecting the soft and hard tissues that support the teeth are broadly categorized as periodontal diseases. Over the past couple decades the definitions of periodontal diseases have changed, as we have learned more about the etiology of periodontal diseases. The most recent and widely accepted definitions of periodontal diseases were developed at the *1999 International Workshop for a Classification of Periodontal Diseases and Conditions* (Armitage 1999). The main classifications of periodontal diseases are listed in List 1.1.

List 1.1 Classification of periodontal diseases and conditions based on the “Infection/Host Response Paradigm” from the *1999 International Workshop for a Classification of Periodontal Disease and Conditions*

- I. Gingival diseases
- II. Chronic periodontitis
- III. Aggressive periodontitis
- IV. Periodontitis as a manifestation of systemic diseases
- V. Necrotizing periodontal diseases
- VI. Abscesses of the periodontium
- VII. Periodontitis associated with endodontic lesions
- VIII. Developmental or acquired deformities and conditions

1.1.1 Gingival diseases

Gingival diseases are classified into two main types: dental plaque-induced gingival diseases and non-plaque induced gingival lesions (Armitage 1999). Dental plaque-induced gingival diseases are characterized by an inflammatory response to dental plaque on the tooth surface. An estimated 200 million bacteria are contained in

1 mm³ of dental plaque. These bacteria work together to form a well-organized “society” of bacteria, known as a biofilm (Costerton et al. 1994).

Biofilms are composed of regions with high and low bacterial mass interlaced with aqueous channels (Costerton et al. 1994). These channels are believed to form a primitive circulatory system, carrying nutrients and metabolic products to different members in the biofilm community (Darveau et al. 1997). Found on both the surface of the tooth and in the gingival sulcus, biofilm can trigger an inflammatory response that can be clinically observed after three days of unobstructed growth (Lang et al. 1973). However, dental plaque-induced gingivitis can be reversed by removing the biofilm, and can be prevented by thoroughly removing dental plaque at least every second day. Non-plaque induced gingival lesions can be associated with specific bacteria, viruses, fungi, genetics, systemic conditions, and injury (Armitage 1999).

Clinical signs of gingivitis include redness, swelling, heat, and pain. The change in color of the gingival tissues is due to increased vascularity at inflamed sites. This increased vascularity leads to fluid accumulation and swelling of the gingival tissues. Additionally, inflamed gingival tissues tend to bleed upon gentle probing with a periodontal probe; whereas healthy gingival tissue does not bleed after gentle probing. Therefore, bleeding on probing is regarded as a more objective sign of gingivitis than gingival tissue color (Axelsson 2002).

1.1.2 Periodontitis

Undisturbed dental plaque not only leads to the reversible condition known as gingivitis, but can ultimately lead to irreversible damage to the periodontal tissues known as periodontitis. Clinical signs of periodontitis include recession of the gingival tissues, formation of deep pockets around the teeth, and the irreversible loss of attachment of

the periodontal ligament from the tooth to the supporting hard tissues (alveolar bone). As periodontitis progresses, alveolar bone loss can occur, which may ultimately result in tooth loss. Periodontitis can be further classified into seven different categories, based on the etiology of the disease (List 1.1).

Chronic periodontitis can develop as a result of inflammation of the gingival tissues due to dental plaque. Additionally, there are genetic (Section 1.5) and non-genetic factors (Section 1.4) that can influence the pathogenesis of chronic periodontitis (Axelsson 2002). Chronic periodontitis can further be classified by the extent of the disease, depending on how many sites in the mouth are affected. Localized periodontitis refers to periodontitis affecting 30% or fewer of sites in the mouth, while generalized periodontitis is used to describe periodontitis affecting more than 30% of the sites in the mouth (Axelsson 2002).

Aggressive periodontitis is characterized by rapid loss of periodontal support (Axelsson 2002). Therefore, it is necessary to see a patient multiple times to distinguish between chronic periodontitis and aggressive periodontitis. Aggressive periodontitis can also manifest as a part of systemic diseases such as acquired neutropenia, leukemias, and other genetic disorders including Papillon-Lefèvre syndrome and Chédiak-Higashi syndrome (Hodge and Michalowicz 2001).

Other causes of periodontitis that are independent of plaque accumulation include necrotizing periodontal diseases which may be linked to stress, poor diet, cigarette smoking, or HIV infection, as well as periodontitis resulting from developmental or acquired deformities of the teeth and gums (Armitage 1999).

1.2 Measurements of periodontal diseases

Many indices and thresholds have been developed to define periodontal diseases (Carlos et al. 1986; Borrell et al. 2005; Borrell and Papapanou 2005; Page and Eke 2007; Amir Savage et al. 2009; Preshaw 2009). The most common clinical measurements used to define periodontal diseases include bleeding on probing (BOP), pocket depth (PD), and attachment loss (AL) (Page and Eke 2007; Amir Savage et al. 2009). Additionally, indices of plaque and gingivitis are used to assess plaque accumulation and severity of gingivitis (Loe et al. 1965; Loe 1967). The following sections describe four of the most common measurements of periodontal diseases: plaque index, BOP, PD, and AL.

1.2.1 Plaque index

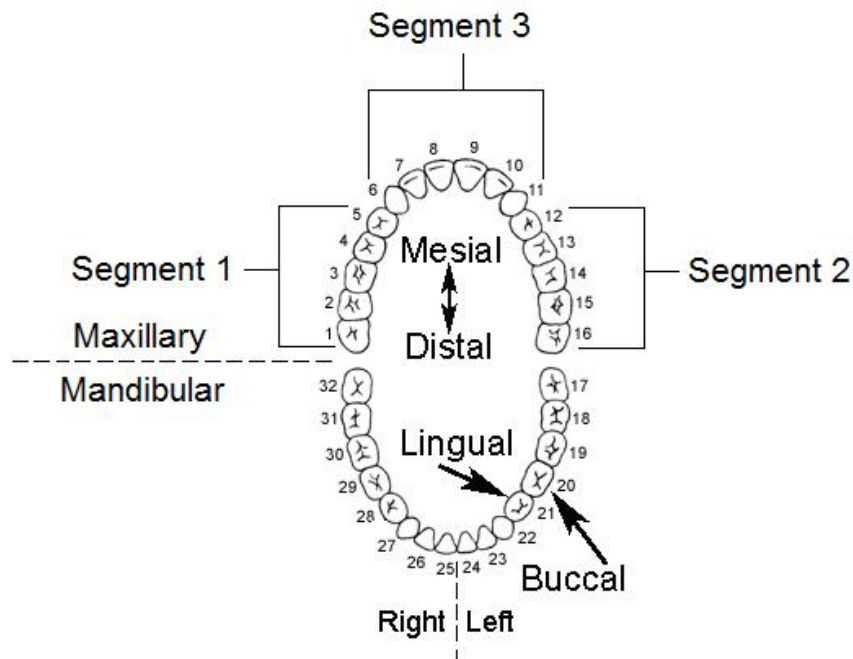
Poor oral hygiene leads to the accumulation of dental plaques on the teeth, which can lead to gingivitis, and ultimately periodontitis. There are several indices that have been developed to quantify the accumulation of plaque on the teeth. The most well known indices are the simplified oral hygiene index (OHS-I) (Greene and Vermillion 1964), and the Silness-Löe Index (Silness and Loe 1964). These plaque indices are highly associated with levels of current oral hygiene.

In 1960, Greene and Vermillion developed the oral hygiene index, which is the sum of the debris index and the calculus index. The debris and calculus indices quantify the amount of debris and calculus found on the buccal and lingual surfaces of selected teeth. To select the teeth to score for the debris and calculus indices, each dental arch (maxillary and mandibular) is divided into three segments (see Figure 1.1), and the tooth with the most debris or calculus from each segment is scored for debris accumulation, and calculus accumulation. The segments are defined as: 1) distal to the right cuspid

(teeth # 1 – 5), 2) distal to the left cuspid (teeth # 12 – 16), and 3) mesial to the right and left first bicusps (teeth # 6 – 11) (Greene 1967).

The debris score ranges from zero to three, where 0 represents no debris or stain present, 1 represents no more than 1/3 of the exposed tooth surface covered by soft debris, two represents more than 1/3 but not more than 2/3 of the exposed surface covered, and a 3 represents soft debris covering more than 2/3 of the exposed tooth surface. The debris index is then calculated by adding all of the scores from both the buccal and lingual surfaces from the teeth selected from each segment and dividing by the total number of segments scored (Greene 1967).

Figure 1.1 Diagram of the three segments of maxillary and mandibular arches used for the oral hygiene index of Greene and Vermillion



The calculus score also ranges from 0 to 3, where 0 represents no calculus present, 1 represents supragingival calculus covering no more than 1/3 of the exposed tooth surface, 2 represents supragingival calculus covering more than 1/3 but no more than 2/3 of the exposed tooth surface and/or the presence of subgingival calculus, and 3 represents supragingival calculus covering more than 2/3 of the exposed tooth surface and/or a continuous heavy band of subgingival calculus. The calculus index is then calculated by adding all of the calculus scores and dividing by the total number of segments scored. The sum of the debris index and the calculus index equals the oral hygiene index (Greene 1967).

In 1964, the simplified oral hygiene index (OHS-I) was developed by Greene and Vermillion (Greene and Vermillion 1964). The OHS-I used the same debris and calculus scoring system as the oral hygiene index; however, fewer teeth are scored (only six surfaces are scored compared to 12 scored with the oral hygiene index). To determine the OHS-I, the teeth are broken into two regions: the posterior region, and the anterior region. The posterior region is a combination of segment 1 and segment 2 (Figure 1. 1, teeth # 1 – 5 and 12 – 16), and the anterior region is composed of segment 3 (Figure 1. 1, teeth # 6 – 11). For the posterior region, the first fully erupted tooth distal to the second bicuspid (Figure 1. 1, tooth # 15), usually the first molar (Figure 1. 1, tooth # 16) is examined. The buccal surfaces of the selected upper molars and the lingual surfaces of the selected lower molars are scored for debris and calculus (Greene and Vermillion 1964).

For the anterior region, the labial surfaces of the upper right (Figure 1.1, tooth # 11) and the lower left central incisors (Figure 1.1, tooth # 31) are scored. In the absence of either of these teeth, the central incisor (Figure 1.1, tooth # 21 or 41) on the opposite side of the midline is substituted (Greene and Vermillion 1964).

Another well known plaque index is the Silness-Löe Index. This index involves scoring four surfaces (buccal, lingual, mesial and distal) of every tooth based on the accumulation of debris and calculus. To access the amount of plaque on the tooth, a pointed probe is run across the buccal surface of the tooth, and a plaque score is determined. This score ranges from 0 to 3, where a score of 0 represents no plaque; a score of 1 represents a film of plaque on the adhering to the free gingival margin; a score of 2 represents moderate accumulation of plaque within the gingival pocket, or on the tooth or gingival margin; and a score of 3 represents an abundance of plaque within the gingival pocket and/or on the tooth and gingival margin. The four scores are then added together and divided by four to determine the plaque index associated with each tooth (Silness and Loe 1964).

1.2.2 Bleeding on probing

Bleeding on probing (BOP) is a relatively objective measure of gingivitis. One technique used to access BOP involves inserting a periodontal probe into the gingival sulcus at the mesio-buccal angle of each tooth, and sweeping it along the buccal surface to the disto-buccal line angle. Any visual evidence of bleeding indicates the presence of gingivitis; if bleeding is observed after probing, BOP is present (BOP = 1), if no bleeding occurs then BOP is not present (BOP = 0) (Axelsson 2002).

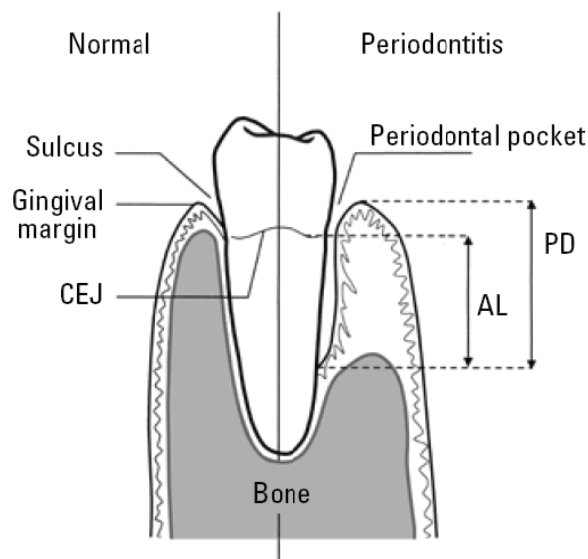
There are also many gingival indices that have been developed to measure gingivitis, which incorporate other more subjective variables to access gingivitis, such as redness and swelling. The most well known gingival index was developed by Silness and Löe, which ranges from 0 to 3. A score of 0 represents normal gingival with no inflammation, no redness, and no bleeding. A score of 1 is given when there is mild inflammation, slight color change, and no bleeding. A score of 2 is moderate

inflammation and bleeding upon probing. A score of 3 represents severe inflammation, swelling, and tendency toward spontaneous hemorrhage (Silness and Loe 1964).

1.2.3 Pocket depth

Clinically, periodontitis can be characterized by the formation of pockets between the gingival tissues and teeth. Periodontal probes are used to measure the periodontal pocket depth between the gingival tissues and the teeth, at six sites on the tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual). The distance from the gingival margin to the base of the periodontal pocket is known as the pocket depth (PD) as shown in Figure 1.2 (Katancik et al. 2005).

Figure 1.2 Clinical measurements of periodontitis



Representation of periodontal examination measurements for a normal tooth (left) and for periodontitis (right); measurements include pocket depth (PD) measured from the gingival margin to the bottom of the periodontal pocket, and attachment loss (AL) measured from the cemento-enamel junction to the bottom of the pocket (CEJ); image from (Arora et al. 2009)

Because PD is measured from the top of the gingival margin, PD measurements will be larger than the actual periodontal pocket when the gingival tissues are swollen,

and smaller than the total periodontal damage after gingival tissues have receded. Therefore, a combination of pocket depth and attachment loss measurements are often used to assess the severity of periodontitis.

1.2.4 Attachment loss

Measurements of the attachment loss (AL) of the periodontal ligament from the tooth to the supporting bone can also be used to estimate the extent and severity of periodontitis. AL is defined as the distance from a fixed point on the tooth known as the cemento-enamel junction (CEJ) to the bottom of the periodontal pocket (Axelsson 2002), as shown in Figure 1.2. Because AL is measured from a fixed point on the tooth, rather than the gingival margin, AL measurements are unaffected by gingival swelling and recession. AL measurements 3 to 4 mm are considered markers of moderate periodontitis, whereas AL measurements greater than or equal to 5 mm are considered markers of severe periodontitis (Axelsson 2002).

1.3 Prevalence of periodontal diseases

Epidemiological studies of periodontal diseases have defined periodontitis using many different thresholds of PD and AL, which has resulted in very inconsistent estimations of the prevalence of periodontitis (Amir Savage et al. 2009; Preshaw 2009). Adding further complication to the comparison of periodontitis prevalence between studies is that some studies use discrete definitions of periodontitis, while others use continuous phenotypes. However, regardless of the definition of periodontitis, the prevalence of periodontal diseases consistently varies by race, sex, and age, as described in the following sections.

1.3.1 Prevalence by race

Periodontal diseases are more prevalent among African Americans than Caucasians or Hispanics in the United States. In a study of 3,658 African American, 4,438 Caucasian, and 3,992 Hispanic adults 18 years and older, conducted from 1988 to 1994 by the National Health and Nutrition Examination Survey (NHANES) III, a periodontitis case was defined as a person who had at least 3 sites AL > 4 mm and at least 2 sites PD > 3 mm. The prevalence of periodontitis was highest among African Americans (11.4%), compared to 6.7% and 6.9% among Caucasians and Hispanics, respectively (Borrell et al. 2005). Therefore, not only does the prevalence of periodontitis depend on how it is defined, it is also dependent upon ethnicity.

Using the same definition of periodontal disease, a later study (NHANES 1999-2000) found that the prevalence of periodontitis among African Americans had dropped from 11.4% (NHANES III) to 6.8%, suggesting that the prevalence of periodontitis is decreasing. However, the prevalence of periodontitis measured by the NHANES 1999-2000 was still higher among African Americans (6.8%) than Caucasians (3.8%) or Hispanics (4.6%) (Borrell et al. 2005).

The higher prevalence of periodontal diseases seen in African Americans could be related to the decreased use of regular dental care observed among African Americans compared to Caucasians, across all age groups (Albandar 2002). It is also possible that other confounding variables such as lower education levels and a higher percentage of current smokers may contribute to the higher prevalence of periodontal diseases among African Americans. After adjusting for the potential confounding variables: age, sex, smoking, diabetes, sex, health insurance, time since last dental visit, income and education levels, the risk of periodontitis for African Americans was not significantly higher than the risk of periodontitis observed among Caucasians (OR =

1.31, 95% CI: 0.60 – 2.88) (Borrell et al. 2005). Therefore, it is possible that race represents many other socioeconomic factors, rather than an innate predisposition to periodontal diseases among African Americans compared to Caucasians.

1.3.2 Prevalence by sex

The prevalence of periodontal diseases is higher in men compared to women. In a study of 5,686 participants aged 65 years and older from the National Survey of Employed Adults and Seniors (1985 – 1986), 12.1% of men had PD $\geq$ 5mm, compared to 5.3% of women. Additionally, 64% of men had AL measurements $\geq$ 5mm, compared to only 46% of women (Albandar 2002). Furthermore, all measurements of periodontal disease were more severe in men than women, not only among seniors, but also among younger populations (Albandar 2002). It has been speculated that the differences in the prevalence of periodontal diseases between men and women may be due to higher levels of inflammation in response to infection or injury in men compared to women (Shiau and Reynolds 2010).

1.3.3 Prevalence by age

Periodontal diseases are more prevalent among older people compared to younger people. When defining a periodontitis case as someone with at least two sites with AL $\geq$ 4mm and at least one site with PD $\geq$ 4 mm, the prevalence of periodontitis was found to increase with age, regardless of ethnicity, in an analysis of the NHANES data collected 1999 to 2004. Only 1.3% of African Americans and 0.7% of Caucasians aged 18 to 34 years met the definition of periodontitis. Among 35 to 59 year olds, the prevalence of periodontitis increased to 10.7% among African Americans and 3.6% among Caucasians. The prevalence of periodontitis further increased to 15.3% of African Americans aged 60 to 85 years, and 5.6% among Caucasians (Borrell and

Crawford 2008). However, the association between age and periodontal diseases is much weaker among individuals that exercise sufficient plaque control and receive conventional periodontal therapy to remove dental plaque (Axelsson et al. 1991).

In summary, there is no consistent definition of periodontal diseases; therefore, the estimated prevalence of disease varies widely, based upon the study definition of periodontitis. However, the prevalence of periodontal diseases is consistently higher in African Americans compared to Caucasians and Hispanics, higher in men compared to women, and higher in older people compared to younger people.

1.4 Non-genetic risk factors for periodontal diseases

Many other factors, both external and internal, are also known to affect the prevalence of periodontal diseases. Examples of external factors that have been observed to modify the risk of periodontal diseases include plaque accumulation, smoking, and socioeconomic factors such as education and income level. Internal factors that modify the risk of periodontal diseases include conditions that are associated with higher levels of systemic inflammation, such as diabetes and obesity. The following sections describe non-genetic risk factors that have been associated with periodontal diseases.

1.4.1 Dental plaque

Dental plaque can induce gingivitis, which can lead to periodontitis. Therefore, factors that influence the rate of plaque formation may in turn affect the development of dental-plaque induced gingivitis and periodontitis. Inadequate salivary secretion has been seen to increase the rate of plaque formation (Axelsson 2002). Other factors that are strongly correlated with an increased rate of plaque formation are: the severity of

gingival inflammation, the volume of fluid in the gingival tissues (Axelsson 2002), and a high intake of fermentable carbohydrates such as sucrose (Carlsson and Egelberg 1965). Although a high intake of sucrose has been observed to increase the rate of plaque formation, and result in more experimental gingivitis than a low intake of sucrose (Sidi and Ashley 1984), a reasonable decrease in sucrose in the diet did not reduce the development of gingivitis (Gaengler et al. 1986). Overall, the removal of dental plaque is the most cost effective means of treatment and control of periodontal diseases (Axelsson 2002).

1.4.2 Periopathogens

Bacteria that have been strongly associated with periodontal diseases are known as periopathogens. Periopathogens have been detected in the mouths of healthy patients (Bik et al. 2010); however, the proportion of periopathogens compared to other bacteria dramatically increases in disease states (Darveau et al. 1997). Periopathogens tend to be gram-negative bacteria that rely on initial gram-positive colonizers to begin the plaque formation (Axelsson 2002). The most well studied examples of periopathogens include the gram-negative: *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Tannerella forsythia*.

Porphyromonas gingivalis (*P. gingivalis*) is the most studied periopathogen. *P. gingivalis* has been observed to produce several damaging metabolites such as collagenases, proteases, and endotoxin (Axelsson 2002). *P. gingivalis* also has the ability to reduce the innate immune response to infection by inhibiting the migration of leukocytes (Reife et al. 1995).

P. gingivalis is frequently detected with higher concentrations in periodontitis sites compared to healthy sites (Bragd et al. 1987). Additionally, this periopathogen is associated with an increased risk of systemic diseases (Inaba and Amano 2010). The

association between periopathogens, such as *P. gingivalis*, and systemic diseases is thought to arise from gingival inflammation, as well as periopathogens such as *P. gingivalis*, entering systemic circulation. Supporting this hypothesis, periopathogens have been detected in systemic circulation following periodontal probing, as well as after toothbrushing (Kinane et al. 2005). Additionally, *P. gingivalis* has been detected in heart valve lesions, atheromatous plaques, amniotic fluid of pregnant women with threatened premature labor, and the placentas from cases of preterm delivery (Inaba and Amano 2010).

Another well studied periopathogen is *Actinomyces actinomycetemcomitans* (*A. actinomycetemcomitans*), which are small, gram-negative, anaerobic, and nonmotile bacteria (Axelsson 2002). This species has been shown to invade human gingival epithelial cells cultured in vitro (Sreenivasan et al. 1993), and has also been observed to produce potentially damaging metabolites such as leukotoxin (Irving et al. 1978). Higher concentrations of *A. actinomycetemcomitans* in saliva are associated with higher levels of alveolar bone loss ($p < 0.001$) as well as a higher risk for coronary artery disease (OR: 7.47, 95% CI: 1.57 – 35.5) (Hyvarinen et al. 2012).

Tannerella forsythia (formerly *Bacteroides forsythus*) is another periopathogen that has been associated with an increased risk of periodontal diseases. In a prospective longitudinal study, subjects that harbored *T. forsythia* at baseline had a greater risk of increased pocket depth compared to those with no detectable *T. forsythia* (OR = 7.84, 95% CI: 1.74 – 35.3) (Machtei et al. 1997). Additionally, a case-control study of nonfatal myocardial infarctions (MI) found that the increased presence of *T. forsythia* subgingivally was associated with an increased risk of MI (OR = 1.62, 95% CI: 1.18 – 2.22) (Andrianakaja et al. 2011) providing another link to support the association of periodontal diseases with vascular diseases.

Other examples of periopathogens include gram-negative species such as: *Prevotella intermedia*, *Campylobacter rectus*, and *Treponema denticola* (Paju et al. 2009). However, the relative importance of individual bacterial species within dental plaque is still unclear (Axelsson 2002).

1.4.3 Smoking

Tobacco smoking has been consistently associated with an increased risk of periodontitis (Linden and Mullally 1994; Grossi et al. 1996; Tonetti 1998; Bergström 2003; Laxman and Annaji 2008). However, risk of periodontal diseases associated with smoking varies between studies, most likely due to differences between these studies such as study design, definitions of periodontal disease, as well as covariates included in association models.

One prospective study of 79 patients found that smokers had an increased risk for further attachment loss compared with non-smokers (OR = 5.41, 95% CI: 1.50 – 19.5) (Machtei et al. 1997). However, this study did not detect any associations between periodontal destruction and other known risk factors such as race or sex. The lack of association of these known risk factors may be due to the recruitment strategy employed by this study, which only selected established periodontitis cases. Additionally, race and sex may increase the likelihood of developing disease, but not play a role in the progression of disease.

Another study examined the effect of smoking on the risk of severe periodontitis by meta-analyzing six previous studies, including 2,361 subjects. This study found smokers had a increased risk of severe periodontitis (OR = 2.82, 95% CI: 2.36 – 3.39) (Papapanou 1996). The risk of periodontitis associated with smoking also appears to be dose-dependent. A previous review of the literature found that heavy smokers have a

higher risk of periodontitis compared to non-smokers with odds ratios ranging from 7.28 to 7.33, compared to the risk of periodontitis among light smokers compared to non-smokers (OR ranging from 3.15 to 3.25) (Gelskey 1999).

The increased risk of periodontal diseases observed among smokers is thought to arise from an increase in the destructive/inflammatory responses to periodontal infection, and a decrease in the protective/reparative responses (i.e. impaired fibroblast attachment and collagen synthesis) (Ryder 2007). The increased destruction of periodontal tissues observed in smokers may be due to an increase in the concentration of destructive enzymes, such as matrix metalloproteinases, cytokines, and also due to the impaired functional activities of neutrophils, such as phagocytosis and chemotaxis. Supporting this hypothesis, smoke has been observed to stimulate the migration of neutrophils into the periodontal connective tissue from the blood vessels; however, smoke has also been seen to impair neutrophil migration from the periodontal connective tissue, into the periodontal pocket (Ryder 2007).

Additionally, nicotine derivatives are known to be vasoconstrictive and affect not only peripheral blood vessels, but also gingival blood vessels (Yoshihara et al. 2005). Vasoconstriction of gingival blood vessels leads to decreased blood flow to the gingival tissues, which may explain why neutrophil activity is impaired among smokers. Vasoconstriction may also explain why lower levels of gingival inflammation and bleeding are observed in smokers, independent of dental plaque accumulation (Tonetti 1998; Bruce L. Pihlstrom 2001).

In summary, smoking has been consistently associated with a decreased risk for gingivitis, and an increased risk of periodontitis. Further, this risk appears to be dose-dependent, with heavy smokers at higher risk for periodontitis compared to light

smokers. Also, smoking appears to be one of the most important risk factors for progression of established periodontitis.

1.4.4 Socioeconomic factors

Low education level is the most important socioeconomic factor associated with an increased risk of higher plaque levels, gingivitis, and periodontitis (Albandar 2002). One longitudinal study of periodontal disease risk factors found that less than twelve years of education was associated with an increase of periodontitis (OR = 1.8, 95% CI: 1.4 – 2.4) among a population 65 years and older (Beck et al. 1997). However, other longitudinal studies and cross-sectional studies controlling for smoking habits have found mixed results regarding the association of periodontal diseases with low levels of education. Confounding the association between low education level and the risk of periodontal diseases is the increased prevalence of smokers among those with low levels of education. In summary, smoking appears to be a more important risk factor for periodontal diseases than socioeconomic factors such as low education level (Klinge and Norlund 2005).

1.4.5 Diabetes

Diabetes is a metabolic disorder characterized by hyperglycemia, which is associated with micro-vascular complications such as retinopathy, nephropathy, and neuropathy (Kim and Amar 2006). Impaired wound healing has been observed among diabetics, as well as an increased susceptibility to infection (Grossi et al. 1996), which may contribute to the higher rates of severe periodontitis observed among diabetics compared to non-diabetics (Loe 1993; Taylor et al. 1996; Collin et al. 1998).

The relationship between periodontal diseases and diabetes has been studied in prospective studies that have found consistent associations between diabetes and periodontal diseases. Diabetics were twice as likely to have severe attachment loss as those without diabetes, after controlling for other variables (Taylor et al. 1996; Taylor 2001). However, the relationship between diabetes and periodontal diseases is complicated, as many studies have also found that periodontal diseases may be a risk factor for the development of diabetes (Taylor 2001; Saito et al. 2004; Garcia 2009).

The association of periodontal diseases and diabetes is thought to arise from the formation of advanced glycation end products (AGEs) due to the chronically elevated blood glucose levels, which increases systemic inflammation (Genco et al. 2005). However, it has also been hypothesized that chronic inflammation resulting from periodontal infection may enter systemic circulation, leading to hyperglycemia (Preshaw et al. 2012). This hypothesis is supported by a longitudinal study of 2,973 non-diabetics from the Study of Health in Pomerania (SHIP) (Demmer et al. 2010). After five years, those individuals with the most severe periodontal diseases at baseline had five-fold higher changes in glycated hemoglobin (HbA1c) levels (a marker of hyperglycemia), compared to those with the lowest levels of periodontal disease at baseline ($\Delta\text{HbA1c} = 0.143\%$ vs. 0.005% , $p = 0.003$). Furthermore, a meta-analysis of 25 small pilot studies ($n = 976$) reported that periodontal treatment may result in a reduction of HbA1c levels ($0.79\% \Delta\text{HbA1c}$, $95\% \text{ CI: } 0.19 - 1.40$) (Garcia 2009); however, future longitudinal studies with larger sample sizes are required to confirm the benefit of periodontal therapy in improving glycemic control.

1.4.6 Other non-genetic risk factors

Due to the inflammatory nature of periodontal diseases, any condition that is associated with an increase in systemic inflammation, such as diabetes, may increase

the risk of periodontal diseases. Other examples of conditions associated with changes in systemic inflammation include obesity and poor diet.

Some studies have found obesity to be a risk factor for periodontal diseases. For instance, a study using the NHANES III data found that higher levels of obesity, defined as body mass index (BMI) $\geq 27 \text{ kg/m}^2$, are associated with increased inflammation, and with a higher risk of periodontal diseases (OR 1.45, 95% CI: 1.09 – 1.93) (Genco et al. 2005). However, other studies have not found significant associations between obesity and periodontal disease risk after controlling for other socioeconomic factors such as education (de Castilhos et al. 2012). Interestingly, the risk of high plaque levels has been significantly associated with obesity even after controlling for dental hygiene and socioeconomic factors ($p = 0.014$). Longitudinal studies will be required to further investigate the relationship between inflammation, obesity, and the risk of periodontal diseases (Borrell et al. 2005). However, the mixed results of past studies suggest that obesity is not a very strong predictor of periodontal disease risk.

Consumption of dietary components that have the ability to modulate systemic inflammation may also affect susceptibility to periodontal diseases. Supporting this hypothesis, changes in diet have been observed to modify the progression of periodontal diseases (Schifferle 2009). Two diet-related components that have been hypothesized to influence the risk of periodontal diseases include the consumption of alcohol and 25-hydroxyvitamin D (vitamin D).

It has been suggested that alcohol consumption may be a risk factor for periodontitis, as alcohol can damage neutrophils, macrophages, and the function of T cells, thus increasing the chances of infection (Amaral et al. 2009). However, this relationship may be complicated as other studies have found that frequent consumers of

wine had lower levels of periopathogens compared to non-frequent drinkers of wine (Signoretto et al. 2010). Future studies of the effects of alcohol consumption on systemic inflammation and the risk of periodontal diseases are necessary to further understand the relationship between alcohol consumption, inflammation, and risk of periodontal diseases.

Low serum levels of 25-hydroxyvitamin D (vitamin D) have also been associated with a higher risk of severe periodontitis. One cross-sectional study using the NHANES III data (n = 11,202) found that the lowest serum levels of vitamin D were associated with an increase in attachment loss of 0.39 mm (95% CI: 0.17 – 0.60 mm), compared with the highest levels of vitamin D (Dietrich et al. 2004). Vitamin D functions to maintain blood calcium levels and enhances the absorption of calcium from the intestines (Schifferle 2009). Vitamin D also plays a role in the innate and adaptive immune responses to infection (Kamen and Tangpricha 2010). However, longitudinal studies are required to confirm serum levels of vitamin D as a risk factor for periodontal diseases.

In summary, established risk factors of periodontal diseases include: race, sex, age, levels of dental plaque, smoking, education level, and diabetes status. Potential risk factors for periodontal diseases include: obesity, alcohol consumption, and low serum levels of vitamin D.

1.5 Genetic influences of periodontal diseases

Evidence for genetic predisposition to periodontal diseases comes from genetic studies in animals and humans. Genetic studies in animals have examined the impact of individual genes on the development of periodontal diseases using tools such as knockout mouse models, in combination with periodontal pathogens such as *P. gingivalis* (Sasaki et al. 2004). In humans, the genetic influences of periodontal

diseases have been investigated in family studies, twin studies, and population studies (Dumitrescu and Kobayashi 2010).

1.5.1 Animal studies

Genetic studies using animal models have identified a number of genes that affect the progression of periodontal diseases. Many of these genes affect the immune response to infection, such as genes that encode cytokines (i.e. interleukins) or receptors (i.e. complement receptors, Fc- γ receptors). Other genes observed to affect susceptibility to periodontal diseases are involved in the formation of the periodontal tissues (i.e. dentin matrix protein 1).

Interleukin 10 (IL-10), an anti-inflammatory cytokine, is an example of a gene involved in the host response to infection. A study investigating IL-10 function in the development of periodontitis observed significantly more severe periodontitis in *IL-10* knockout mice infected with *P. gingivalis*, compared to *P. gingivalis*-infected wild-type mice, and non-infected *IL-10* knockout mice (Sasaki et al. 2004). This study demonstrated the importance of IL-10 in the immune response to periodontal pathogens.

Interleukin 12B (IL-12B) is another example of a cytokine involved in the host response to infection. IL-12B is one of subunits of a proinflammatory cytokine, interleukin 12 (IL-12), which can stimulate the growth and function of T cells. Knockout of *IL-12B* in mice was seen to increase susceptibility to periodontitis after infection with *P. gingivalis*, compared to wild type mice (Alayan et al. 2007).

Another gene involved in the immune response to infection is the complement receptor 3 (*CR3*). A study in mice investigating periopathogen binding to CR3 in monocytes/macrophages found that binding of *P. gingivalis* to CR3 resulted in the inhibition of interleukin 12A (*IL-12A*), a subunit of IL-12 (Hajishengallis et al. 2007).

Consequently, the natural immune response to bacterial infection was down regulated, promoting pathogen virulence. Using a CR3 antagonist (XVA143), shown to block CR3 binding of *P. gingivalis* in mice, resulted in a reversal of *IL-12A* inhibition, enhanced pathogen clearance, and reduced periodontal bone loss. It is plausible that genetic variants affecting CR3 function, and genes with similar functions, may result in altered pathogen binding/recognition, and could play an important role in susceptibility to periodontal diseases.

Abnormalities in the formation of the periodontal tissues may also alter susceptibility to periodontal diseases. For instance, dentin matrix protein 1 (DMP1) is highly expressed in the periodontal tissues, and knockout of *DMP1* in mice results in abnormal periodontal tissue formation and periodontal diseases independent of bacterial infection (Ye et al. 2008). Genes such as *DMP1* that influence the integrity of periodontal tissues may harbor genetic variants that contribute to the development of periodontal diseases.

In summary, these studies highlight the importance of genes involved in the immune response to infection and those affecting the formation of periodontal tissues in modulating susceptibility to periodontal diseases. Genetic variations within genes such as these may lead to altered susceptibility to periodontal diseases, and may explain some of the heritability of periodontal diseases observed in humans.

1.5.2 Human studies

Studies of periodontal diseases in humans have found significant genetic influences to periodontal diseases. Such studies include investigations of monogenetic syndromes resulting in periodontal diseases, studies of concordance of periodontal

diseases among twins, as well as candidate gene studies investigating the risk of periodontal diseases associated with genetic variations.

1.5.2.1 Monogenic syndromes

Monogenic syndromes result from a genetic mutation at a single locus. A number of monogenic syndromes have been observed to increase susceptibility to periodontal diseases in humans, such as: Papillon-Lefèvre syndrome (PLS), Chédiak-Higashi Syndrome (CHS), and cyclic neutropenia (Hodge and Michalowicz 2001). These monogenic syndromes are thought to arise from defects in neutrophil number or function, or defects in epithelial and connective tissue formation.

Papillon-Lefèvre syndrome (PLS) (OMIM #245000) is an extensively studied monogenic syndrome resulting in rapidly progressing periodontitis, and palmoplantar hyperkeratosis (keratin growths on skin). Most children affected with PLS experience rapid destruction of the periodontium after the eruption of primary and permanent teeth, resulting in premature tooth loss. PLS patients lose most or all of their permanent teeth during the teenage years (Dhanrajani 2009). Linkage studies in families have mapped this autosomal recessive disorder to mutations within the gene encoding the lysosomal protease cathepsin C (*CTSC*) (Toomes et al. 1999). *CTSC* is a lysosomal cysteine protease involved in the activation of many serine proteases in immune and inflammatory cells. Defects in lymphocyte response to pathogens, impaired monocytic function, and a decrease in helper-to-suppressor T-cell ratio have been reported in PLS patients. These defects in immune response are thought to lead to the severe inflammatory infiltration of the subepithelial connective tissue, and rapid destruction of periodontium following tooth eruption (Dhanrajani 2009).

Lysosomal dysfunction has also been associated with another autosomal recessive disorder, Chédiak-Higashi Syndrome (CHS) (OMIM #214500). CHS results from mutations in the lysosomal trafficking regulator gene (*LYST*), and is characterized by cutaneous, ocular, neurologic, and hematologic abnormalities. Patients with CHS are prone to severe periodontitis, as well as increased susceptibility to infections of the skin and respiratory tract (Bailleul-Forestier et al. 2008). The increased susceptibility to infection of patients with CHS is thought to arise from low levels of neutrophils (neutropenia), as well as other defects in the immune system including impaired chemotactic response and cell-killing ability in natural killer cells, T-cells, granulocytes and monocytes (Rezaei et al. 2009).

Another example of a monogenic syndrome that increases susceptibility to periodontal diseases due to low levels of neutrophils is cyclic neutropenia (OMIM #162800). Cyclic neutropenia is a rare blood disorder (< 1% in US (Hsieh et al. 2007)) that can result from autosomal dominantly inherited mutations within the gene encoding neutrophil elastase (*ELA2*). *ELA2* is a serine protease exclusively expressed in neutrophils and monocytes (Rezaei et al. 2009). Cyclic neutropenia is characterized by regular oscillations in the numbers of circulating blood neutrophils (Sera et al. 2005). Children suffering from cyclic neutropenia have an increased susceptibility to infection, and experience oral ulceration, and severe periodontitis (Hodge and Michalowicz 2001).

In summary, these syndromes demonstrate how genetic variation within genes affecting immune function can alter susceptibility to periodontal diseases in humans. While these syndromes often result in severe periodontal diseases, it is plausible that there are uninvestigated genetic variants within these genes, and other genes, that could contribute to more common forms of periodontal diseases such as chronic periodontitis.

1.5.2.2 Twin studies

It is assumed that twins have very similar environments; however, the amount of genetic material shared by twins is different between monozygotic and dizygotic twins. Monozygotic twins (MZ) arise from a single fertilized ovum which results in identical genetics, while dizygotic twins (DZ) arise from separate ova and share on average half of their genetics (Gordis 2009). Therefore, by investigating the differences in concordance rates of periodontal diseases among MZ and DZ twins, it is possible to estimate the contribution of genetic factors influencing periodontal disease susceptibility, assuming the contribution of environmental factors is equal between MZ and DZ twins.

A twin study investigating the concordance of self-reported periodontal diseases among 116 MZ and 233 DZ twin pairs, found a higher concordance of periodontal diseases among MZ twins (0.38) compared to DZ twins (0.16) (Corey et al. 1993). Results from this study highlight the importance of genetics in the development of periodontal diseases.

Twin studies have also been used to estimate the heritability of periodontal diseases. Another study of periodontal diseases included 2,747 MZ and 7,831 DZ twin pairs from the Swedish Twin Registry, and estimated the heritability of self-reported periodontal disease to be 39% for women, and 33% for men (Mucci et al. 2005). Another smaller study of 64 MZ and 53 DZ twin pairs, of both Caucasian and African American ancestry, estimated the heritability of periodontal diseases to be as high as 50% (Michalowicz et al. 2000). In summary, these estimates of heritability suggest a substantial portion of the variance in periodontal diseases can be attributed to genetic variation.

1.5.2.3 Candidate gene studies

Many genes involved in the host immune response to infection have been hypothesized to harbor variants, such as single nucleotide polymorphisms (SNPs), which may predispose the host to periodontal diseases. The relationship between genetic variation and susceptibility to periodontal diseases has been most frequently investigated using genetic association studies. The most well replicated associations observed between candidate gene SNPs and periodontal diseases involve SNPs within the following genes: *IL-1*, *IL-6*, *IL-10*, and *VDR* (Dumitrescu and Kobayashi 2010; Laine et al. 2010), as shown in Table 1.1. Studies of these SNPs are summarized in the following sections, by gene.

Table 1.1 Well-studied polymorphisms associated with periodontal diseases

Gene	Polymorphisms	Genotype
Interleukin -1 (<i>IL-1</i>)	rs1800587 (<i>IL-1a</i> -889)	T vs. C
	rs1143634 (<i>IL-1b</i> +3954)	T vs. C
Interleukin -6 (<i>IL-6</i>)	rs1800795 (<i>IL-6</i> -174)	GG vs. GC and CC
	rs2069827 (<i>IL-6</i> -1363)	GG vs. GT and TT
Interleukin -10 (<i>IL-10</i>)	rs1800872 (<i>IL-10</i> -592)	CC vs. AC and AA
	rs1800872 (<i>IL-10</i> -592)	AC vs. AA
Vitamin D Receptor (<i>VDR</i>)	rs7975232	AA vs. AC and CC
	rs731236	AA vs. AG and GG

Interleukin-1

Interleukin-1 (IL-1) is a pro-inflammatory cytokine, and is involved in the destruction of extracellular matrix and periodontal tissue in response to oral infection. *IL-1* polymorphisms have been associated with experimental gingivitis, aggressive periodontitis (not significant with (Fiebig et al. 2008)) and chronic periodontitis (Ferreira et al. 2008). The *IL-1* gene cluster is composed of *IL-1a*, a regulator of intracellular events, and *IL-1b*, which is primarily an extracellular protein released from

macrophages, monocytes, and dendritic cells (Dinarello 1996). There have been many studies investigating the association of SNPs in the *IL-1a* and *IL-1b* genes with periodontal diseases (Kornman et al. 1997; Nikolopoulos et al. 2008; Struch et al. 2008) and also with IL-1 expression in the gingival crevicular fluid (Shirodaria et al. 2000; Ferreira et al. 2008).

The first investigation of SNPs within the *IL-1a* and *IL-1b* genes associating with periodontitis found the composite genotype of *IL-1a* -889 (rs1800587) and *IL-1b* +3954 (rs1143634) to be associated with severe periodontitis among 99 Caucasian non-smokers (OR = 6.80, 99.8% CI: 1.01 – 45.95) (Kornman et al. 1997). Since this initial investigation, there have been many studies trying to replicate these findings, yielding mixed results (Fiebig et al. 2008) (Li et al. 2004). Unfortunately, many studies used different definitions of periodontal diseases, making comparisons between studies difficult to interpret. Additionally, many of these studies were not powered to detect modest genetic effects because of small samples sizes.

To increase the power to detect the true effects of *IL-1* variants, a meta-analysis of 53 previous association studies of five *IL-1* SNPs with chronic and aggressive periodontitis was performed, including 4,178 cases and 4,590 controls (Nikolopoulos et al. 2008). None of the five *IL-1* SNPs investigated were significantly associated with aggressive periodontitis. However, the *IL-1b* +3954 SNP (rs1143634) was associated with chronic periodontitis in the meta-analysis of 1,470 cases and 2,328 controls from the 20 studies that investigated this SNP (OR = 1.45, 95% CI: 1.13 – 1.85). This association was seen to be stronger among the seven Asian populations included in the meta-analysis (OR = 2.19, 95% CI: 1.22 – 3.92) which consisted of 683 cases and 650 controls. The *IL-1a* -889 SNP (rs1800587) was weakly associated with chronic periodontitis (OR = 1.31, 95% CI: 0.96 – 1.79) in 11 studies that investigated this SNP,

including 1,002 cases and 878 controls. However this association was stronger within the six Caucasian populations (OR = 1.31, 95% CI: 1.03 – 1.67). The composite genotype of *IL-1a* -889 and *IL-1b* +3954 was found to be associated with chronic periodontitis (OR = 2.10, 95% CI: 1.12 – 3.92) after meta-analysis of seven studies consisting of 687 cases and 331 controls. Overall, this meta-analysis provides some evidence that the *IL-1a* -889 and *IL-1b* +3954 SNPs contribute to periodontal disease susceptibility; however, the risk of periodontal diseases associated with these variants may vary depending upon genetic background.

Interleukin-6

Another cytokine hypothesized to harbor polymorphisms associated with chronic and aggressive periodontitis is the interleukin-6 (*IL-6*) gene. *IL-6* levels increase in response to acute and chronic infections, and variants in this gene may attenuate the inflammatory response to periodontal pathogens. The most well studied *IL-6* variant is *IL-6* -174 (rs1800795), which has been associated with chronic and aggressive periodontitis. For instance, a case-control study including 124 cases and 116 controls from Germany found a significant association between this SNP and chronic periodontitis (OR = 1.90, 95% CI: 1.11 – 3.25) (Babel et al. 2006). Other studies have examined the association of this variant and other *IL-6* variants with periodontal diseases and have found mixed results (Dumitrescu and Kobayashi 2010). One of the largest studies of examining the effect of *IL-6* variants on periodontal disease risk found a moderate association between the *IL-6* -174 SNP (rs1800795) GG genotype in Caucasians with periodontal disease, in 326 aggressive periodontitis and chronic periodontitis cases combined compared with 144 healthy controls (OR = 1.8, 95% CI: 1.0 – 3.1) (Nibali et al. 2009). Also, the *IL-6* -1363 variant (rs2069827) significantly associated with periodontitis (OR = 2.7, 95% CI: 1.2 – 5.9).

A meta-analysis of the *IL-6* -174 SNP (rs1800795) was performed, including five studies consisting of 677 cases and 695 controls from different genetic backgrounds (Caucasians, Asians, and Blacks) (Nikolopoulos et al. 2008). This study did not find any significant association between the *IL-6* -174 SNP in combined analyses; however, when the study was restricted to the 380 cases and 500 controls from Caucasian populations, there was a borderline significant association with chronic periodontitis (OR = 1.28, 95% CI: 0.97 – 1.71). In summary, variants within *IL-6* may be a risk factor for periodontal diseases, but the risk associated with these variants does not appear to be substantial. This may be due to small effect sizes associated with these variants, or due to genetic or phenotypic heterogeneity among study populations.

Interleukin-10

Variants within the promoter region of interleukin-10 (*IL-10*), a potent anti-inflammatory cytokine, have been associated with decreased synthesis of IL-10 (Yilmaz et al. 2005), and have also been associated with the development of chronic periodontitis in Caucasian and East Asian populations (Scarel-Caminaga et al. 2004; Claudino et al. 2008; Cullinan et al. 2008; Zhong et al. 2012). In a study of Brazilians, including 64 chronic periodontitis cases and 43 controls, the *IL-10*-592 (rs1800872) C/C genotype was associated with a decreased risk of periodontitis compared to the C/A and A/A genotypes (OR 2.41, 95% CI: 1.08 – 5.36) (Scarel-Caminaga et al. 2004). Another study of the *IL-10* -592 SNP found a decreased risk of development of chronic periodontitis (OR = 0.33, 95% CI: 0.15 – 0.70) for the A/C genotype vs. the A/A genotype in an East Asian population including 145 cases and 126 controls (Hu et al. 2009). Further support for the association of *IL-10* variants and chronic periodontitis risk comes from a meta-analysis of 14 studies, including 1,438 patients and 1,303 controls (Zhong et al. 2012). This study found that the *IL-10* -592 SNP (A vs. C allele OR: 1.97,

95% CI: 1.36 – 3.86) and -819 SNP (T vs. C allele OR: 1.55, 95% CI: 1.07 – 2.24) were significantly associated with chronic periodontitis, particularly in Caucasians. Overall, these studies provide substantial support for the role of *IL-10* polymorphisms affecting the risk of periodontitis.

Vitamin D Receptor

Vitamin D plays an important role in bone and calcium metabolism, immunity, and cardiovascular function (Amano et al. 2009). Periodontitis is characterized by alveolar bone destruction, often resulting from immune response to infection. Therefore, it has been hypothesized that polymorphisms in the vitamin D receptor (VDR) gene may affect the risk of periodontal diseases (Krall et al. 2001; Gunes et al. 2008; Nibali et al. 2008; Wang et al. 2009; Deng et al. 2011) .

A large meta-analysis in an Asian population, including 1,338 periodontitis cases and 1,302 controls, found VDR SNP rs7975323 (AA genotype) to be associated with chronic periodontitis (OR = 2.20, 95% CI: 1.39 – 3.48) (Deng et al. 2011). Another SNP, rs731236 was seen to nominally associate with chronic periodontitis in this meta-analysis (OR = 1.86, 95% CI: 1.002 – 3.46); however, the effects did not remain significant after adjusting for multiple testing. In summary, it is possible that SNPs or other epigenetic factors affecting the expression or function of the VDR may influence host response to periodontal infection; however, additional studies are necessary to better understand the role of *VDR* polymorphisms in periodontal diseases.

1.5.2.4 Genome-wide association study

Growing knowledge of human DNA sequence variability, and the availability of new technologies allowing genotyping hundreds of thousands of single nucleotide polymorphisms (SNPs) at low cost, has enabled the genome-wide association study

(GWAS) approach to identify common genetic variants associated with disease susceptibility. This approach has been successfully applied to the identification of novel susceptibility loci in many multi-factorial diseases (Scott et al. 2007).

To date, only one published study of aggressive periodontitis has been performed using the GWAS approach. This study investigated 500,000 SNPs for association with aggressive periodontitis using a two-stage GWAS approach, in German and Dutch populations. The combined analysis included a total of 438 aggressive periodontitis cases and 1,320 controls. One SNP was identified with genome-wide significance, rs1537415 (OR = 1.59, 95% CI: 1.36 – 1.86, $P = 5.51 \times 10^{-9}$). This SNP is located within the intron of a previously uncharacterized gene, glycosyltransferase 6 domain containing 1 (*GLT6D1*), and is predicted to alter the binding affinity of transcription factor GATA binding protein 3 (GATA3) (Schaefer et al. 2009). This is the only known reported GWAS of periodontal diseases, and currently there are no known GWAS of chronic periodontitis.

In summary, these genetic association studies have provided evidence for genetic influences on periodontal diseases; however, most of them were limited in statistical power and coverage of genetic variants investigated. Additionally, these studies often use different thresholds to define periodontitis case and control status, which drastically changes the prevalence of periodontitis and impairs the ability to compare replication studies and interpret meta-analysis results. Therefore, more systematic and comprehensive investigations in search for the genetic basis of periodontal diseases, using sufficiently large and well-phenotyped populations, are needed.

CHAPTER 2

STUDY OBJECTIVES AND SIGNIFICANCE

2.1 Rationale and hypothesis

Periodontal diseases and oral biofilm-associated diseases affect a large portion of the world population, and have broad implications on systemic health. Approximately 75% of US adults are affected by gingivitis or periodontitis (Kim and Amar 2006), while severe periodontitis has been estimated to affect 5 – 20% of most adult populations worldwide (Jin et al. 2011). People affected by severe periodontitis have increased levels of systemic inflammation markers, such as IL-1, IL-6, TNF- α , and CRP, compared to those without severe periodontitis (S. Engebretson 2007; Oscarsson et al. 2008; Struch et al. 2008). Increased levels of systemic inflammation may result from the host response to periodontal infection.

Increased levels of systemic inflammation have also been associated with many other poor health outcomes such as cardiovascular diseases, diabetes, and pre-term births (Mattila et al. 1995; Geerts et al. 2004; Jin et al. 2011). The chronic presence of elevated systemic inflammation levels resulting from the host response to periodontal infection may explain the association of periodontitis with other systemic conditions, such as coronary heart disease (Mattila et al. 1995). Additionally, the increased risk of systemic diseases associated with periodontal diseases could be influenced by the presence of periopathogens that reach systemic circulation. For instance, periopathogens have been detected in systemic circulation after periodontal probing and toothbrushing (Kinane et al. 2005), and have also been detected in heart valve lesions, atheromatous plaques, and the carotid arteries of patients with cardiovascular disease (Haraszthy et al. 2000; Inaba and Amano 2010).

As periodontal diseases are a risk for many other common diseases, such as cardiovascular diseases, its prevention may have many other important public health implications, as well as financial implications. For instance, periodontal diseases, and

oral biofilm-associated diseases result in a significant financial burden on society (Beikler and Flemmig 2011). In 2006, it was estimated that the United States spent \$81 billion on oral biofilm-associated diseases (Beikler and Flemmig 2011), which was more than was estimated for heart conditions (\$78 billion), trauma-related disorders (\$68.1 billion), cancer (\$57.5 billion), mental disorders (\$57.2 billion), and pulmonary conditions (\$51.3 billion) (Beikler and Flemmig 2011).

Previous studies have identified many risk factors for periodontal diseases, including environmental risk factors, and host susceptibility. For instance, epidemiological studies have identified a number of environmental risk factors for periodontal diseases, such as poor dental hygiene (Axelsson 2002), smoking (Ryder 2007), low socioeconomic status (Albandar 2002), and poorly controlled diabetes (Salvi et al. 2008). However, due to many differences in study design, phenotype definitions, and covariates included in association models, the relative importance of these risk factors is unclear.

Additionally, twin studies have suggested that a third, to as much as half, of the variability of periodontal disease severity may be explained by genetic effects (Michalowicz et al. 2000; Mucci et al. 2005). However, only a few relatively small candidate genes studies of periodontal diseases have been published (Suzuki et al. 2004; Nikolopoulos et al. 2008; Ünür et al. 2008), and most of them were limited in statistical power, coverage of genetic variants investigated, and phenotypes examined. There has only been one published report of a systematic search across the entire genome for the susceptibility genes of aggressive periodontitis (Schaefer et al. 2009), and no reports of genome wide searches for genetic influences to more common forms of periodontal diseases, such as gingivitis and chronic periodontitis. Therefore, more

systematic and comprehensive investigations in search for the genetic basis of periodontal diseases are needed.

2.2 Specific aims

To gain a better understanding of the relative importance of environmental risks for periodontal diseases, we performed an epidemiological analysis of the risk factors for periodontal diseases. Additionally, to identify new genetic risk factors, we performed a genome-wide association study (GWAS) of periodontal diseases and related-traits including: plaque index, bleeding on probing, pocket depth, and attachment loss measurements.

Our primary study population consisted of 1,200 elderly individuals (63% Caucasians, 37% African Americans) from the Health, Aging and Body Composition (Health ABC) Study and 422 elderly Caucasian men from the Osteoporotic Fractures in Men (MrOS) Study that were genotyped for ~ 1 million single nucleotide polymorphisms (SNPs), and had comprehensive periodontal examinations.

Meta-analysis of the GWAS of periodontal disease-related traits from the Health ABC Study and MrOS Caucasian populations was performed to increase the power to detect genetic risk factors for periodontal diseases. Significant findings ($p < 10^{-6}$) were further investigated *in silico* for potential function in periodontal diseases. Additionally, replication analyses of significant findings from the Caucasian meta-analysis were performed in two populations including ~4,000 elderly Caucasians from the Dental Atherosclerosis Risk in Communities Study (ARIC) and ~500 African Americans from the Health ABC Study.

Study Aims:

1. To conduct an epidemiological analysis of potential risk factors for periodontal disease-related traits in one African American population, and two Caucasian populations, including: mean plaque index, % bleeding on probing, extent pocket depth $\geq 5\text{mm}$, and extent attachment loss $\geq 3\text{mm}$
2. To perform a GWAS of periodontal disease-related traits in two Caucasian populations including: mean plaque index, % bleeding on probing, extent pocket depth $\geq 5\text{mm}$, and extent attachment loss $\geq 3\text{mm}$, and replicate findings in a Caucasian population and an African American population
3. To conduct *in silico* analyses of the genomic regions surrounding the top signals identified by the GWAS of periodontal disease-related traits, and prioritize these identified signals based on perceived biological importance

2.3 Significance

By performing an epidemiological investigation of the risk factors for periodontal diseases, we hoped to better understand the relative importance of previously identified and potential risk factors for periodontal diseases. Additionally, as periodontal diseases are hypothesized to be largely influenced by genetics, yet only a few genetic polymorphisms have been identified that consistently associate with periodontal diseases risk, we hoped to discover new SNPs associating with periodontal diseases by performing a GWAS of periodontal disease-related traits.

Information gained from these investigations will hopefully advance our understanding of the relative contributions of environmental and genetic risk factors for periodontal diseases. Ultimately, this information may be useful for prevention or

treatment of periodontal diseases. The prevention of periodontal diseases may lead to a reduction of systemic complications associated with periodontal diseases and oral biofilm-associated diseases, as well as reduced financial cost associated with the treatment of these diseases.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study design

This study was performed using cross-sectional analysis of data collected by two cohort studies: the Health, Aging, and Body Composition (Health ABC) Study and the Osteoporotic Fractures in Men Study (MrOS). Linear regression analyses were used to estimate the risk of periodontal disease-related traits (mean plaque index, % bleeding on probing, extent pocket depth $\geq 5\text{mm}$, and extent attachment loss $\geq 3\text{mm}$) associated with known and potential risk factors for periodontal diseases in these populations. Replication analyses were performed using cross-sectional data from a third cohort study, the Atherosclerosis Risk in Communities (ARIC) Study.

3.2 Study populations

All study subjects have given consent for genetic studies and have at least one periodontal disease-related outcome available for analysis, as well as information on all covariates.

Health ABC Study

The Health ABC Study is a National Institute of Aging (NIA) sponsored prospective study of a cohort of 3,075 African American and Caucasian men (48%) and women (52%) aged 70 to 79 years at the baseline. Participants were recruited between April 1997 and June 1998, from a random sample of Caucasian residents, and all African American Medicare-eligible residents, in the Pittsburgh, Pennsylvania, and Memphis, Tennessee, metropolitan areas. Participants were eligible if they reported no difficulty walking one-fourth of a mile, climbing up ten steps or performing basic activities of daily living, were free of life-threatening illness, planned to remain in the geographic area for at least three years, and were not enrolled in any lifestyle intervention trials. All participants provided written informed consent and all protocols were approved by the

institutional review boards at both study sites. Participants in the Health ABC Study underwent extensive annual examinations for more than eight years with over 95% complete follow-up, which characterized body composition, cognitive function, bone density, and subclinical cardiovascular disease.

Participants in this study were selected from the 2,732 participants who took part in the Health ABC Study's 12 month follow-up exam. Of the 2,732 participants, 1,975 participants met with a trained Field Center examiner or a dental hygienist in Year 2 or 3 of the Health ABC Study (June 1998 – June 1999) to receive partial dental examinations and determine eligibility for rigorous dental examinations.

Exclusion criteria from periodontal probing included the following conditions based on self-reports: heart murmur, congenital heart disease, rheumatic heart disease, mitral valve prolapse, currently medicated with prednisone or any immunosuppressive medication, cardiac pacemaker, surgically implanted heart valve, shunt or artificial joint, major surgery, radiation or chemotherapy for cancer within the last 2 months, kidney dialysis, heart, kidney or other organ transplant, ever taken FenPhen to lose weight or other diet pill, no natural teeth or dental implants, or people that have been told by a dentist or a doctor that they needed to take antibiotics before every dental visit.

Participants were excluded from periodontal probing based on the above criteria due to an increased risk of cardiovascular events associated with periodontal probing without prophylactics (Dajani et al. 1997). A total of 1,443 participants were eligible for periodontal probing; however, only 1,111 eligible participants received periodontal probing, due to confusion of eligibility status at the time of the periodontal exam. We hypothesize that the exclusion of people needing prophylactics may reduce the overall effect size and significance associated with risk factors of periodontal diseases;

however, it is not expected that the direction of effect detected (for true signals) should be affected by exclusion of these people.

MrOS

The Osteoporotic Fractures in Men Study (MrOS) is a prospective study of 5,995 men aged 65 years and older, designed to determine risk factors for osteoporosis and fractures. MrOS is supported by the National Institute of Arthritis, Musculoskeletal, and Skin Disease (NIAMS), the National Institute of Aging (NIA) and the National Cancer Institute (NCI).

Participants were recruited from March 2000 through April 2002 at six clinical centers in the United States (Birmingham, Alabama; Palo Alto, California; San Diego, California; Minneapolis, Minnesota; Portland, Oregon; Pittsburgh, Pennsylvania), including self-identified Caucasian, African American, and Asian men. The institutional review board at each clinical site approved the study protocol. Recruitment strategies varied by site, including direct mailings with mailing lists generated from the Department of Motor Vehicles (DMV), voter registration and participant databases, community and senior newspaper features and advertisements, and targeted presentations.

Participants were eligible if they were: at least 65 years of age, able to consent, able to walk without the assistance of another person, had not had bilateral hip replacement, able to provide self-reported information, expected to reside near the clinical site for the duration of the study, and had no condition that in the judgment of the site investigator would make the individual unable to participate or survive the duration of the study (Blank et al. 2005).

Participants in this study also completed the MrOS baseline dental exam, which included 1,347 MrOS participants from the Birmingham and Portland clinics (September

2002 - May 2003). Thirteen per cent of the study participants required and received prophylactic antibiotics prior to the periodontal examination (Dajani et al. 1997).

ARIC Study

The Atherosclerosis Risk In Communities (ARIC) Study is a prospective cohort study of atherosclerosis, CVD risk factors, and outcomes that enrolled 15,792 community-dwelling residents aged 53 to 74 in four US communities (Jackson, Mississippi; Washington County, Maryland; suburban Minneapolis, Minnesota; and Forsyth County, North Carolina) between 1987 and 1989. Recruitment and enrollment strategies have been previously published (ARIC 1989). Subjects living in institutions, those who were not permanent U.S. residents, those with physical or mental impairment that precluded cooperation, those with language barrier, or those who had definite plans to move from the community were excluded.

During the fourth ARIC Study visit (1996 – 1998), an ancillary study was conducted known as the Dental ARIC. The Dental ARIC is a National Institutes of Dental and Craniofacial Research-funded study that included complete oral-dental examinations in a subset (n=6,017) of dentate ARIC Study participants. Persons requiring antibiotic prophylaxis for periodontal probing were excluded.

3.3 Phenotypic measurements

Clinical measurements of mean plaque index, % bleeding on probing, extent pocket depth $\geq 5\text{mm}$, and extent attachment loss $\geq 3\text{mm}$ were performed in each study population. The following sections describe the collection of periodontal phenotypes in each study population. There were some data collection differences between studies, such as the number of sites examined (full-mouth vs. half-mouth), the direction probing measurements were rounded (up vs. down), as well as different indices used to measure

plaque levels. We hypothesize these differences may bias our findings toward the null hypothesis of no effect, as they introduce “noise” and effectively reduce the signal-to-noise ratio.

Health ABC Study

Periodontal exams were performed by a periodontist or a dental examiner assisted by a data recorder. The examiners underwent an extensive training and calibration program before initiation of the study. An *a priori* level of 90% agreement on all measures was attained by all the examiners before the beginning of the study. Also, there were two additional calibration checks of the examiners during the study. The majority of the periodontal exams were conducted by seven examiners. All participants that met with an examiner received a tooth count and dental examination including measures of plaque levels (Silness and Loe 1964).

Eligible participants received a full periodontal assessment including measures of gingival inflammation (bleeding on probing), periodontal pocket depth, and attachment loss. Pocket depth and attachment loss were measured using a UNC-15 periodontal probe at six sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual). Probing depths were rounded to the next lower whole number. Patients were examined in a dental chair using a dental examination light.

MrOS

Six calibrated dentists and hygienists at the Birmingham and Portland clinics completed examinations using a dental light, mirror, chair and UNC-15 periodontal probe. Study participants requiring prophylactic antibiotics received antibiotics prior to the periodontal examination (Dajani et al. 1997).

The periodontal examination was limited to a random half-mouth (right or left) depending on the participant's MrOS ID number, and excluded 3rd molars. The periodontal examination included measurements of pocket depth and attachment loss at six sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual) rounded up to the nearest whole millimeter. Plaque levels (Silness and Loe 1964) and gingival inflammation (bleeding on probing) were also accessed, and the worst score per tooth was recorded for measurements of plaque.

ARIC Study

As part of the Dental ARIC ancillary study, participants underwent detailed oral-periodontal examinations that recorded the number of missing teeth, probing depths, attachment loss measurements and bleeding upon probing at six sites per tooth, including third molars. Probing depths were determined with a UNC-15 periodontal probe, recorded in millimeters with fractions of millimeters rounded down to the next lower millimeter. Dental ARIC clinical examiners were trained and calibrated against a standard examiner with corresponding weighted kappa statistics ranging from 0.76 to 0.86, indicating excellent agreement with the standard examiner.

3.3.1 Outcome measurements

There are four quantitative traits accessed in the study cohorts representing: plaque levels (mean plaque index), gingivitis (% bleeding on probing), and periodontitis (extent pocket depth $\geq$ 5mm and extent attachment loss $\geq$ 3mm). Descriptions of each quantitative trait are described in the following sections.

Plaque

The bacterial load was accessed by quantifying the amount of plaque buccal surface of each tooth, using a modified Silness and Loe plaque index (Silness and Loe

1964). In the Health ABC Study and ARIC Study populations, a score of 0 was given when there was no plaque or stain of the clinical crown; a score of 1 represented deposits covering less than one-third of the surface; a score of 2 was given when deposits covered less than two-thirds of the surface; and a score of 3 represented deposits covering at least two thirds of the surface.

In the MrOS population, the plaque index only ranged from 0 to 2. A score of a 0 represented no plaque in the gingival area; a score of 1 represented a film of plaque adhering to the free gingival margin and adjacent area of the tooth, which may be recognized only by running a probe across the tooth surface; and a score of a 2 represented an accumulation of soft deposits within the gingival pocket, on the gingival margin and/or adjacent tooth surface which could be seen by the naked eye.

Gingivitis

Gingivitis was measured by recording the presence of bleeding after gentle probing. In the Health ABC Study, mesiobuccal and distobuccal surfaces were examined. The periodontal probe was placed under the gingival margin at the mesiobuccal site and swept along the buccal surface to the distobuccal site. In the MrOS cohort, the probe was swept around the gingiva in the sulcus to a depth of 1-2 millimeter. The presence of bleeding was recorded as a 1, and no bleeding was recorded as a 0. In the ARIC Study, bleeding was recorded as either present (1) or absent (0) at each site in the quadrant, directly following probing of that quadrant.

Periodontitis

Periodontitis was accessed by measuring the depth of the periodontal pockets and periodontal ligament attachment loss. The probing depths were measured from the bottom of the periodontal pocket to the top of the gingival margin at six sites per tooth.

In the Health ABC Study and ARIC Study populations, probing depth measurements were rounded down to the next lower whole millimeter; in MrOS pocket depth measurements were rounded up to the nearest millimeter. Attachment loss was defined as the distance from the bottom of the periodontal pocket to the cementoenamel junction (CEJ). To measure attachment loss, the distance from the gingival margin to the CEJ was measured at six sites per tooth, rounded to the next lower whole millimeter in the Health ABC Study and the ARIC Study, and rounded up to the next millimeter in MrOS. If the gingival margin was above the CEJ, this distance was recorded as a positive number; if the gingival margin was below the CEJ (gingival recession), this distance was recorded as a negative number. Then, the distance from the gingival margin to the CEJ was then subtracted from the pocket depth measurements to determine attachment loss scores.

3.3.2 Risk factors and covariates

Health ABC Study

Each participant had a health interview and examination in Year 1 of the Health ABC Study including the following information: age, sex, race, education level (less than high school graduate, high school graduate, or more than high school), smoking habits (current smoker and pack/year, former smoker, or never smoker), and drinking habits (current drinker, former drinker, or never drinker). Physical examination included assessment of height using a balance-beam scale, and weight using a calibrated wall-mounted stadiometer and scale in a light gown with no shoes. BMI was calculated as weight divided by height squared (kg/m^2). Plasma levels of fasting glucose were measured by an automated glucose oxidase reaction (YSI 2300 Glucose Analyzer; Yellow Springs Instruments, Yellow Springs, OH). Glycated hemoglobin (HbA1c) was measured by high-performance liquid chromatography (Biorad Diamat, Richmond, CA).

In Year 2 of the Health ABC Study, serum 25-hydroxyvitamin D (vitamin D) was analyzed by a radioimmunoassay kit (DIASORIN, 25-hydroxyvitamin D ¹²⁵I RIA kit, no. 68100, Stillwater, MN, 100 tube kit). Dental habits were quantified using self-reported frequencies of flossing, brushing, and dental visits, collected during the periodontal exam, conducted between Year 2 and 3 of the Health ABC Study.

MrOS

During the MrOS baseline visit, study participants self-reported age, race, education (highest year of school completed), cigarette smoking status (current smoker and packs per year, former smoker, or never smoker), and alcoholic drinking habits (number of drinks per week). Physical examination of participants included measurement of weight using a standard balance beam, and measurement of height using a wall-mounted stadiometer, which were used to calculate BMI (kg/m²). Fasting glucose measurements were made using serum collected in the morning, which had been stored at minus 70 degrees Celsius. A hexokinase method from previously unthawed serum (Northwest Lipid Metabolism and Diabetes Research Laboratories, Seattle, WA) was used to quantify fasting glucose levels (mg/dL). Serum 25-hydroxyvitamin D (vitamin D) was quantified in 2007 using stored serum collected at the MrOS baseline visit. Both 25(OH)D₂ and 25(OH)D₃ were measured. At both visits, fasting morning blood was collected, serum was prepared immediately after phlebotomy, and then was stored at -70°C. All samples remained frozen in foil wrapped vials to prevent UV exposure until assay. Dental habits were quantified using self-reported frequencies of flossing, brushing, and dental visits, collected during the dental exam, conducted during Year 2.

ARIC Study

The following risk factors and covariates were included in our replication analyses using the ARIC Study cohort: age, sex, study examiner, education (some high school, high school diploma, bachelor's or graduate degree), smoking status and packs per year, frequency of brushing, frequency of flossing, and the time since last dental visit. These variables were collected during the ARIC Study Year 4 visit.

3.3.3 Genotyping

Health ABC Study

Genomic DNA was extracted from buffy coat collected using PUREGENE® DNA Purification Kit during the baseline exam. Genotyping was performed by the Center for Inherited Disease Research (CIDR) using the Illumina Human1M-Duo BeadChip system. Illumina BeadStudio was used to call genotypes. Samples were excluded from the dataset for the reasons of sample failure, genotypic sex mismatch, and first-degree relative of an included individual based on genotype data. Genotyping was successful for 1,151,215 SNPs in 2,802 unrelated individuals (1,139 African Americans and 1,663 Caucasians).

Imputation was performed for the autosomes using MaCH software version 1.0.16 (Li et al. 2010), including SNPs with minor allele frequency $\geq 1\%$, call rate $\geq 97\%$ and HWE $p \geq 10^{-6}$. HapMap phase II release 22 build 36 phased haplotypes were used as reference panels for imputation. For African Americans, genotypes were available on 1,007,948 high quality SNPs for imputation based on a 1:1 mixture of the CEPH:Yoruban (YRI) reference panel. For Caucasians, genotypes were available on 914,263 high quality SNPs for imputation based on the HapMap CEPH reference panel

(release 22, build 36). A total of 3,021,329 SNPs in African Americans and 2,543,887 SNPs in Caucasians resulted from imputation.

After imputation, SNPs were further cleaned to only include common SNPs (minor allele frequency $\geq 5\%$), and SNPs with acceptable imputation scores ($R^2_{\text{MaCH}} > 0.3$), resulting in 2,460,807 SNPs available for GWAS in African Americans, and 2,185,212 SNPs available for GWAS in Caucasians. Of these SNPs, 1,898,735 were available in both African Americans and Caucasians. Due to the small sample size of African Americans with periodontal exams (sample sizes ranging from 367 – 500), GWAS analyses of periodontal outcomes were performed in the Health ABC Study Caucasian population, and the Health ABC Study African American population was used for replication analyses of significant signals ($p \leq 10^{-6}$) detected among Caucasians.

MrOS

Genotyping was performed using the Illumina Human1M-Duo BeadChip system. Illumina's BeadStudio software was used to call genotypes. Samples were excluded from the dataset for the following reasons: sample failure, genotypic sex mismatch, chromosome anomalies (high BAF variances in > 5 chromosomes), relatedness (using identity by descent (IBD) analysis), and due to differences in ethnicity/population substructure (using principal component analysis (PCA)). Criteria for SNP exclusion included: $< 98\%$ call rate, duplicate sample discordance (error rate 2.26×10^{-5}), HWE $p < 1 \times 10^{-6}$, and MAF < 0.01 . Genotyping was successful for 909,059 SNPs in 5,277 unrelated Caucasian men.

Genotype phasing was performed using MaCH, and imputation was performed using Minimac. HapMap release 22 build 36 phased haplotypes were used as reference panels for imputation. Imputation analyses resulted in 3,020,516 SNPs. GWAS

analyses included 2,099,202 imputed SNPs with $MAF \geq 5\%$, $R^2_{\text{MaCH}} > 0.3$, and HWE $p \geq 10^{-6}$. Of these SNPs, 2,050,692 were available in the Health ABC Study Caucasian population for meta-analysis.

ARIC Study

DNA was extracted from blood samples drawn from an antecubital vein into tubes containing serum separator gel. Blood samples were analyzed at a central ARIC laboratory in Houston, TX. Genotyping was performed using the Affymetrix Genome-Wide Human SNP Array 6.0 chip. The quality control procedures included initial blind duplicate genotyping and identification/flagging of SNPs with kappa < 0.95 as well as reconciliation of unintentional duplicate samples (17 duplicates and one triplicate). Imputation to 2.5 million markers was performed using 669,450 SNPs and the MaCH program version 1.0.16, based on HapMap Phase II CEU build 36. The SNPs used for imputation were selected from 839,048 autosomal SNPs restricted to those with $MAF > 0.01$, HWE $p > 10^{-5}$, and call rate $> 95\%$.

Table 3.1 SNPs resulting from imputation, and used for genome-wide association studies in two primary study populations

Study Population	# SNPs after imputation	# SNPs used for GWAS
Health ABC Study Caucasians	2,543,887	2,185,212
MrOS Caucasian Men	3,020,516	2,099,202

Single nucleotide polymorphisms used for genome-wide association studies (GWAS) included those with minor allele frequency $\geq 5\%$, imputation quality score (R^2_{MaCH}) > 0.3 , and Hardy-Weinberg equilibrium $p \geq 10^{-6}$

3.4 Data analysis

The main objective of this study was to better understand the relative importance of known and potential risk factors for periodontal diseases. To assess the relative importance of risk factors, we developed quantitative definitions of periodontal disease-

related traits to capture the continuous nature of periodontal diseases. We performed an epidemiological analysis of known and potential risk factors for these outcomes using univariate linear regression and backwards stepwise regression analyses. After investigation of non-genetic risk factors, a genome-wide association study (GWAS) of periodontal disease-related traits was performed to investigate the risk of periodontal diseases associated with individual single nucleotide polymorphisms (SNPs) across the genome. All statistical testing was performed using 'R' version 2.12.0.

3.4.1 Definition of periodontal disease outcomes

For this study of periodontal diseases, we developed the following four quantitative periodontal disease outcomes to represent four clinical periodontal disease-related traits. Outcomes excluded all 3rd molar measurements; additionally, not normally distributed outcomes have been transformed to reduce skewness and kurtosis ("peakedness").

Mean plaque index

A participant's mean plaque index was calculated by averaging the measured plaque index for each tooth. The mean plaque index is a proxy for bacterial load, and is highly affected by oral hygiene; therefore, we not only investigated mean plaque index as a periodontal disease-related trait, we also included mean plaque index as a covariate for the following three periodontal disease-related traits, to adjust for the level of current oral hygiene.

Percent bleeding on probing

The participant's extent of gingivitis was quantitatively accessed by counting how many sites bleed upon probing, divided by the number of sites probed.

Extent pocket depth ≥ 5 mm

To estimate periodontitis, measured by pocket depths, the number of sites probed with pocket depths ≥ 5 mm were counted, and then divided by the total number of sites probed (extPD5). Because the distribution of the residuals resulting from linear regression of extPD5 (including all covariates) was skewed-right, extPD5 values were log transformed before regression analyses. Some of the extPD5 scores were equal to zero; therefore, extPD5 scores were shifted to the right by half of the smallest non-zero extPD5 score prior to log transformation: $\log_{\text{extPD5}} = \text{LOG}(\text{extPD5} + 0.00285)$.

Extent attachment loss ≥ 3 mm

Periodontitis was also estimated by counting the total number of sites probed with attachment loss ≥ 3 mm, and then dividing by the total number of sites probed.

3.4.2 Epidemiological analysis of risk factors

To assess the relative importance of periodontal disease risk factors, we performed an epidemiological analysis of known and potential risk factors for the four quantitative periodontal disease definitions (mean plaque index, % bleeding on probing, extent pocket depth ≥ 5 mm, and extent attachment loss ≥ 5 mm). Univariate linear regression and backwards stepwise regression analyses were performed separately for the three study populations: Health ABC Study African Americans, Health ABC Study Caucasians, and MrOS Caucasian men.

Health ABC Study

Univariate linear regression and backwards stepwise regression analyses were run on the four periodontal disease phenotypes (full-mouth examinations, excluding the 3rd molars), stratified by race (African American and Caucasian). Risk factors investigated include: periodontal examiner (7 examiners), age (years), sex (male = 1 or

female = 2), education level (less than high school = 0, high school graduate = 1, or post secondary school = 2), smoking status (never = 0, current = 1, or former = 2) and pack per year, drinking status (never = 0, current = 1, or former = 2), plasma fasting glucose levels (mg/dl), body mass index (BMI kg/m²), serum levels of 1,25 vitamin D (ng/mL), frequency of brushing (less than twice a day = 0, at least twice a day = 1), frequency of flossing (less than once a week = 0, 1 – 2 times per week = 1, more than twice a week = 3), and frequency of dental visits (less than once a year = 0, once a year = 1, and more than once a year = 2).

MrOS

Univariate linear regression and backwards stepwise regression analyses were run on the four periodontal disease phenotypes; however, these phenotypes were developed from half-mouth examinations, and excluded the 3rd molars. Risk factors investigated are the same risk factors as in Health ABC Study, except for the following risk factors that were recorded differently: education level (years of education past 8th grade), periodontal examiner (6 examiners), and drinking status (# alcoholic drinks per week).

For both the Health ABC Study and MrOS populations, serum levels of vitamin D were adjusted by the season the vitamin D was measured, due to seasonal fluctuations in vitamin D levels. To adjust serum levels of vitamin D, univariate linear regression was run with serum levels of vitamin D as the outcome, and season (December – May = 1, June – November = 2) as the risk factor. The resulting residuals were then included as a risk factor for periodontal disease-related outcomes.

Univariate linear regression

Previous studies have indicated that the risk of periodontal diseases can be modified by the known and potential risk factors listed above. However, the relative importance of these risk factors is not clear. To estimate the relative importance of these risk factors, univariate linear regression analysis was used to calculate the percent variance explained by each risk factor (correlation coefficient squared: R^2) for each of the four periodontal disease outcomes.

Backwards stepwise regression

Backwards stepwise regression analysis was also performed to create a parsimonious model, containing only the covariates that uniquely explain a significant portion of the variance of the periodontal disease outcome, holding all other covariates constant. This model initially includes all risk factors in the model, and then iteratively drops the risk factors that do not significantly explain a unique portion of the outcome variance, holding all other risk factors constant (semi-partial correlation coefficient squared: sr^2) (Cohen and Cohen 1975).

To build a parsimonious model, risk factors with the lowest sr^2 are dropped first from backwards stepwise regression analyses, until any further dropping of risk factors results in a model with a significantly lower amount of variance explained by all of the risk factors (a significantly lower R^2). After determining which risk factors significantly explain a unique portion of the variance in periodontal disease outcomes, holding all other risk factors constant, we calculated the effect size (β) of each risk factor in the parsimonious model.

3.4.3 Genome-wide association study

To investigate the genetic risk factors of periodontal diseases, we performed a genome-wide association study (GWAS) of four periodontal disease-related outcomes in the Health ABC Study Caucasians, and MrOS Caucasian men. Multiple linear regression analysis was used to test for the association between individual imputed SNP genotypes (and other periodontal disease risk factors) with periodontal disease outcomes (See 3.4.1). Primary analyses used an additive model for genetic effects. Effect of sex, a potential effect modifier, was evaluated using interaction analyses for significant findings. To reduce the genetic signal-to-noise ratio, results from our epidemiological analysis of risk factors for periodontal diseases were used to develop a GWAS linear regression model. This model included risk factors that consistently explained a significant portion of the variance of periodontal disease outcomes, including the risk factors described below.

Health ABC Study

Risk factors included in the GWAS multiple linear regression equation include: SNP (additive effect), age (years), sex (male = 1 or female = 2), education level (less than high school = 0, high school graduate = 1, or post secondary school = 2), periodontal examiner (7 examiners), smoking status (never = 0, current = 1, or former = 2) and pack per year, frequency of brushing (less than twice a day = 0, at least twice a day = 1), frequency of flossing (less than once a week = 0, 1-2 times per week = 1, more than twice a week = 3), and frequency of dental visits (less than once a year = 0, once a year = 1, and more than once a year = 2). Additionally, principal component analysis (PCA) was performed, and the first two principal components were included in the GWAS models, to correct for possible population structure.

MrOS

Other risk factors included in the GWAS of MrOS Caucasians included the same risk factors as in Health ABC Study, except for the following risk factors that were recorded differently: education level (years of education past 8th grade), periodontal examiner (6 examiners), and smoking status (never = 0, former = 1, or current = 2). Additionally, the first four principal components resulting from PCA analysis were necessary to correct for possible population structure within MrOS Caucasians.

GWAS multiple linear regression model:

Outcome ~ SNP + age + sex + education level + smoking status + packs/year + frequency of brushing + frequency of flossing + frequency of dental visits

Meta-analysis and Replication

To increase the power to detect SNPs associated with periodontal diseases, the results from Health ABC Study Caucasians and MrOS Caucasian men were meta-analyzed, using the software 'METAL' (Willer et al. 2010) assuming fixed effects. Because MrOS periodontal exams were random half-mouth exams, the periodontal outcomes for the Health ABC Study Caucasians (for GWAS analyses) were developed by randomly masking measurements on one half of the mouth by subject ID, and then creating phenotypes using half-mouth examination data.

The Bonferroni genome-wide significance threshold ($\alpha = 0.05$) for the total number of imputed SNPs in our analyses (2,217,695) is $p = 2.3 \times 10^{-8}$. However, the Bonferroni correction assumes independent multiple tests, and many SNPs are correlated. Therefore, other methods have been used to estimate genome-wide significance thresholds, such as permutation testing and simpleM (Gao et al. 2009; Johnson et al. 2010; Gao 2011). Significance thresholds of 8.5×10^{-8} were derived from permutation testing, including 10,000 random shuffles of 2.5 million imputed SNPs in

762 unrelated Caucasians (Gao 2011). Additionally, many studies use lower suggestive levels of significance as a criterion to pick SNPs to move forward for replication analyses, with $p < 10^{-6}$ being the most common threshold (Gogele et al. 2012). Because our primary GWAS sample sizes are small (~1,600 Caucasians) and this is one of the first GWAS of periodontal disease-related traits, we use a suggestive significance threshold of $p < 10^{-6}$ as a selection criterion for SNPs to carry forward for replication in ARIC Study Caucasians and the Health ABC Study African Americans.

The association model used for the ARIC Study population included the same covariates as the Health ABC Study and MrOS populations, except the frequency of dental visits was substituted with the length of time since they last went to the dentist, and ten principal components were included to adjust for population stratification. ARIC Study periodontal phenotypes were generated using full-mouth exam data. The prevalence of periodontal disease is often underestimated using half-mouth diseases (such as those used to collect MrOS phenotypes); therefore, the use of full-mouth exams in the replication analyses should more accurately reflect periodontal diseases in this population. The use of half-mouth exams to generate the phenotypes for the primary GWAS in the Health ABC Study and MrOS Caucasian populations may underestimate the prevalence of periodontal diseases relative to the ARIC Study; however, we do not expect this to result in significant differences in effect estimation for true genetic signals.

3.4.4 Power consideration

In order to compare the relative importance of different risk factors for periodontal diseases, we estimated the minimal amount of outcome variance that we are statistically powered to detect with each risk factor. Using a significance threshold $\alpha_p = 0.05$, we estimated the minimal percent variance of each periodontal outcome that we are 80%

powered to detect, given our population size, the prevalence of each binary risk factor, the standard deviation of continuous risk factors, and the mean and standard deviation of periodontal outcomes. Power calculations were performed using power calculator QUANTO v1.2 (Gauderman WJ 2006).

Power for epidemiological analysis of risk factors

We were 80% powered to detect a percent variance as small as 1.5% within the Health ABC Study African American population with many risk factors such as age, sex, number of cigarette packs per year, fasting glucose, BMI, serum vitamin D levels, and frequency of brushing. Among, Health ABC Study Caucasians, we were 80% powered to detect a percent variance as small as 0.8% with these risk factors.

We were less powered to detect the effect of other risk factors included in the analysis. For Health ABC Study African Americans, we estimate that we were 80% powered to detect at least 2.5% variance of periodontal outcomes explained by the following risk factors: education, smoking status, drinking status, frequency of flossing, and frequency of dental visits. For Health ABC Study Caucasians, we estimate we were 80% powered to detect at least 2% variance of periodontal outcomes explained by these risk factors, with the exception of drinking status and frequency of dental visits, which were 80% powered to detect ~3% variance of periodontal outcomes. We were least powered to detect variance accounted for by examiner, for both Health ABC Study African Americans (80% powered to detect ~10% variance) and Caucasians (80% powered to detect ~5% variance).

Within the MrOS Caucasian population, we estimated 80% power to detect a percent variance as small as ~1% of periodontal outcomes explained by all of the individual risk factors included in the analysis, except: examiner (~6%), smoking status,

particularly with current smokers (~2%), frequency of flossing (~2%), and frequency of dental visits (~3%).

Power analysis for GWAS

To estimate the percent variance of periodontal outcomes explained by SNPs in our GWAS analyses, we assumed additive genetic effects of alleles. We also adjusted for multiple testing ($p = 10^{-6}$) due to the high number of SNPs tested. We estimated 80% power to detect a SNP explaining 2.4% of mean plaque index, and 3.0% variance of other periodontal disease-related outcomes, from the meta-analysis of the Health ABC Study and MrOS Caucasian GWAS. The estimated minimal effect sizes detectable with 80% power are shown in Table 3.2 for MAF = 0.05 and 0.40.

Table 3.2 Minimal detectable effect size associated with risk alleles in meta-analyses of periodontal disease-related outcomes (80% power)

Outcome	β (MAF = 0.05)	β (MAF = 0.40)
Mean plaque index	0.26	0.11
Percent bleeding on probing	18.5	8.2
Extent pocket depth $\geq$ 5mm	2.6	1.2
Extent attachment loss $\geq$ 3mm	7.7	3.4

Estimation of the minimal detectable effect sizes (β) explained by risk alleles ($p = 10^{-6}$, with minor allele frequency (MAF) 0.05 and 0.40) from the meta-analysis of the Health ABC Study and MrOS Caucasian genome-wide association study of periodontal disease-related outcomes, with 80% power

3.4.5 *In silico* functional analysis

To identify and prioritize SNPs warranting further study, we investigated the genomic regions surrounding SNPs significantly associating with periodontal outcomes ($p \leq 10^{-6}$) from the meta-analysis results of the Health ABC Study Caucasians and MrOS Caucasian men. First, all SNPs in the surrounding genomic locations of significant

findings (~ 1MB) were examined for linkage disequilibrium. SNPs significantly associating with periodontal outcomes, and SNPs in high LD ($r^2 \geq 0.8$) with these SNPs, were further investigated using the following tools to examine the surrounding genomic location and look for plausible connections between the identified genomic region and periodontal diseases: SNP Function Prediction, Genevar, SCAN database, as well as using the UCSC genome browser (<http://genome.ucsc.edu>) (Kent et al. 2002; Raney et al. 2011).

SNP Function Prediction

The SNP Function Prediction tool, part of SNPinfo Web Server (Xu and Taylor 2009), predicts the potential functional impact SNPs by reporting if the SNP is a non-synonymous coding SNP, a stop codon, or if the genomic region surrounding the SNP is a potential: transcription factor binding site, intron-exon junction, or microRNA binding site. Additionally, the program reports two scores for non-coding SNPs, a conservation score and an ESPERR (Evolutionary and Sequence Pattern Extraction through Reduced Representations) Regulatory Potential (Taylor et al. 2006). The conservation score is calculated using the 17-species MultiZ alignments from the UCSC Genome Browser (Karolchik et al. 2011). The ESPERR regulatory potential (RP) score is calculated using GC content information, conservation information prepared using the 17-species MultiZ alignments from the UCSC Genome Browser, and by examining the data for weaker signals such as enhancer regions and transcriptional regulatory elements. The RP score can be used to discriminate between regulatory and neutral sites, with a reported 94% accuracy on the author's training data set. This score is on a log-scale, with positive values suggesting a regulatory potential for the SNP. Only approximately 10% of the non-coding SNPs in the author's training set were predicted to have regulatory potential (RP > 0).

Genevar

The Genevar (GENe Expression Variation) (Yang et al. 2010) tool was used to investigate cis-eQTL associations in three cell types (fibroblasts, lymphoblastoid cell lines, and T-cells) derived from umbilical cords of 75 Geneva GenCord individuals (Dimas et al. 2009) and in 156 lymphoblastoid cell lines, fat cells, and skin cells, derived from a subset of healthy female twins of the MuHTER resource (Nica et al. 2011). This tool is used to identify expression changes in genes surrounding the SNPs of interest, also known as cis-eQTLs (cis expression quantitative trait loci). Genevar uses Spearman's rank correlation coefficient to estimate the strength of the relationship between SNP alleles and gene expression intensities, and a t-statistic (with n-2 degrees of freedom) to estimate the significance of the relationship. Each probe within one megabase (Mb) of top SNPs is tested for allele specific expression changes. Therefore, the number of probes tested must be considered when interpreting the significance of the SNP-gene associations.

SCAN database

The SCAN database was also used to perform a genome-wide search for eQTLs (Gamazon et al. 2010). The SCAN database includes data collected using the Affymetrix GeneChip® Human Exon 1.0 ST Array exon array, on Epstein-Barr virus transformed lymphoblastoid cell lines (LCLs) from apparently healthy individuals from both African and European ancestry. Top SNPs, and SNPs in high LD with top SNPs are input into SCAN, and any eQTLs reaching a user specified significance threshold are output. The number of genes tested against the SNP of interest must be considered when interpreting the significance of the eQTL association. There are 28,869 well-annotated genes included on the Affymetrix exon chip, which results in a Bonferroni corrected $p = 1.7 \times 10^{-6}$; however, this threshold may be too stringent. The default

significance threshold for the SCAN database is $p \leq 10^{-4}$; also, previous association studies have used this significance threshold to identify potential eQTLs (Wen et al. 2012); therefore, we report any eQTLs that reach significance $p \leq 10^{-4}$. In sum, these tools provide information that will be used to rank and prioritize SNPs most likely to be functional for future studies.

CHAPTER 4

RESULTS

An epidemiological analysis of known and potential risk factors for periodontal diseases was performed using the following four outcomes: mean plaque index, % bleeding on probing (% BOP), extent pocket depth (PD) ≥ 5 mm, and extent attachment loss (AL) ≥ 3 mm, in three study populations (Health ABC Study African Americans, Health ABC Study Caucasians, and MrOS Caucasians). Results from the epidemiological analysis were used to develop a multiple linear regression model to use for genome-wide association studies (GWAS) of periodontal disease outcomes. GWASs were performed in the Health ABC Study Caucasians and MrOS Caucasians, and results were meta-analyzed. Significant findings ($p \leq 10^{-6}$) from the meta-analysis were further investigated for replication in the ARIC Study Caucasians, and the Health ABC Study African Americans.

4.1 Characteristics of outcomes

Table 4.1a lists the average and standard deviations for periodontal outcomes, calculated using full-mouth data collected from 1,558 subjects from the Health ABC Study. This table is stratified by race and sex, and reveals significant differences in the prevalence of periodontal disease-related outcomes between the races and sexes for all outcomes ($p < 0.0001$).

All four measures of periodontal disease were significantly higher in the Health ABC Study African Americans, compared to the Health ABC Study Caucasians ($p < 0.0001$), with extent PD ≥ 5 mm being the most significantly different. On average, African Americans had 8.8% of sites probed with periodontal pockets at least 5mm deep, compared to 2.6% of sites among Caucasians. Because the distribution of extent PD ≥ 5 mm is skewed right, the standard deviation of the outcome is larger than the mean.

Therefore, extent PD $\geq$ 5mm outcomes were log transformed for subsequent regression analyses.

There were also significant differences between the sexes for all four outcomes ($p < 0.0001$). African American and Caucasian men had higher levels of periodontal disease for all outcomes compared to the African American and Caucasian women, respectively. Generally, the differences of all outcomes between races were greater than the differences between sexes (see Figure 4.1).

MrOS periodontal data was collected using random (left or right side) half-mouth dental examinations. To make the Health ABC Study outcomes comparable to those obtained by MrOS, we created half-mouth examination data in the Health ABC Study by randomly masking either the left or the right side of the mouth scores from the full-mouth exams. Table 4.1b shows the average and standard deviations for periodontal outcomes, calculated using half-mouth data generated from full-mouth data from 1,558 subjects from the Health ABC Study. In both the Health ABC Study African American and Caucasian populations, outcomes calculated using half-mouth data (Table 4.1b) were similar to the outcomes calculated using full-mouth data (Table 4.1a), except % BOP. The percentage of sites that bleed upon probing was significantly underestimated in the Health ABC Study populations when calculated using half-mouth data, compared to using full-mouth data (see Figure 4.1). Table 4.1b also shows the average and standard deviation for outcomes collected using half mouth data in 686 Caucasian men from MrOS. Significantly higher measures of periodontal disease outcomes ($p < 0.0001$) were observed in MrOS Caucasian men, compared to the Health ABC Study Caucasian men for all outcomes, except % BOP.

Table 4.1a Outcome characteristics in the Health ABC Study participants (full-mouth examinations)

	African American		Caucasian		Racial difference t (p)
	Men	Women	Men	Women	
Sample size range	157 - 241	221 - 302	380 - 538	321 - 477	
Outcomes: average ($\pm$ SD)					
Mean plaque index	1.2 (0.7)	1.0 (0.6)	0.8 (0.5)	0.7 (0.5)	26 (+++)
% BOP	66% (32)	58% (33)	45% (30)	40% (28)	55 (+++)
Extent PD $\geq$ 5mm	11.3% (15)	7.1% (13)	3.2% (7)	2.1% (7)	105 (+++)
Extent AL $\geq$ 3mm	44% (30)	35% (29)	33% (28)	25% (23)	70 (+++)

Table 4.1b Outcome characteristics in the Health ABC Study and MrOS participants (half-mouth examinations)

	HABC African American		HABC Caucasian		MrOS Caucasian Men
	Men	Women	Men	Women	
Sample size range	157 - 241	221 - 302	380 - 538	321 - 477	686
Outcomes: average ($\pm$ SD)					
Mean plaque index	1.2 (0.7)	1.0 (0.6)	0.8 (0.5)	0.7 (0.5)	1.1 (0.5)
% BOP	36% (21)	29% (18)	21% (16)	19% (15)	24% (33)
Extent PD $\geq$ 5mm	11.1% (15)	7.1% (14)	2.9% (7)	2.2% (7)	4.6% (7)
Extent AL $\geq$ 3mm	46% (29)	35% (28)	36% (27)	26% (23)	60% (22)

Table 4.1c Outcome characteristics in the ARIC Study Caucasians (full-mouth examinations)

	ARIC	
	Men	Women
Sample size range	881 – 968	994 – 1,106
Outcomes: average ($\pm$ SD)		
Mean plaque index	0.5 (0.5)	0.4 (0.4)
% BOP	30% (23)	25% (21)
Extent PD $\geq$ 5mm	4% (8)	2% (6)
Extent AL $\geq$ 3mm	27% (24)	18% (20)

Average and standard deviation (SD) for mean plaque index, % bleeding on probing (% BOP), extent pocket depth (PD) $\geq$ 5 mm, and extent attachment loss (AL) $\geq$ 3 mm, stratified by race and sex

Table 4.1 a) Outcomes from Health ABC Study Caucasian population outcomes, calculated using full-mouth exam data; unpaired t test results between races, $p < 0.0001$ (+++)

Table 4.1 b) Outcomes from HABC and MrOS Caucasian population outcomes, calculated using half-mouth exam data

Table 4.1 c) Outcomes in ARIC Study Caucasians, stratified by sex, using full-mouth exam data

Table 4.1c shows the prevalence of periodontal disease outcomes in the ARIC Study Caucasian population. These outcomes were created using full-mouth examinations. Comparisons between ARIC Study outcomes, and outcomes generated using full-mouth examinations in the Health ABC Study Caucasians (Table 4.1a) reveal significantly lower mean plaque levels, lower % BOP, and lower extent AL $\geq$ 3mm in the ARIC Study population ($p < 0.001$).

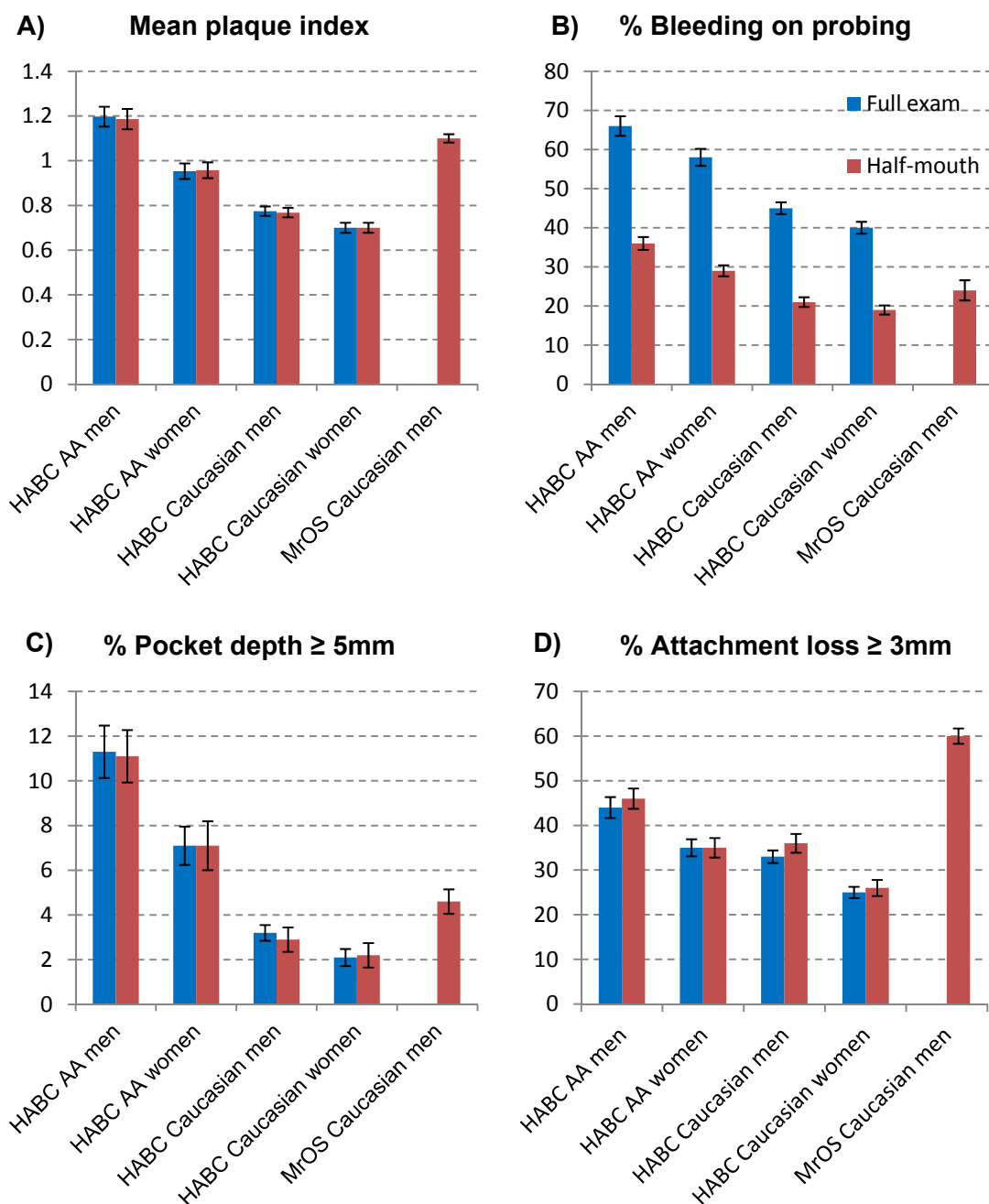
Correlation of outcomes

The four periodontal disease-related outcomes (mean plaque index, % BOP, extent PD $\geq$ 5mm, and extent AL $\geq$ 3mm) were calculated to capture different aspects of periodontal diseases. However, these outcomes were weakly correlated in all three populations. The correlations between the each of the outcomes are shown in Table 4.2 from 383 African Americans from the Health ABC Study. The correlations between periodontal-disease related outcomes for the Health ABC Study Caucasians and MrOS Caucasians are similar to the Health ABC Study African American correlations (data not shown). No strong correlations ($r > 0.6$) were observed between outcomes in any population, providing evidence that these outcomes represent related, but different aspects of periodontal diseases.

Table 4.2 Correlation of periodontal disease-related outcomes in the Health ABC Study African American population

Outcome:	Mean plaque index	% Bleeding on probing	Extent pocket depth $\geq$ 5mm
% Bleeding on probing	0.46		
Extent pocket depth $\geq$ 5mm	0.35	0.45	
Extent attachment loss $\geq$ 3mm	0.34	0.30	0.45

Figure 4.1 Comparison of outcome characteristics between the Health ABC Study and MrOS participants, stratified by race and sex



Average and 95% confidence interval for outcomes from the Health ABC Study (HABC) African American (AA) and Caucasian populations, and the MrOS Caucasian population, stratified by race and sex; outcomes were significantly different between races and sexes ($p < 0.0001$)*; Outcomes calculated using full-mouth exams (blue, left) were not significantly different from outcomes calculated using half-mouth exams (red, right)**

* No significant differences were observed between % bleeding on probing scores in HABC Caucasian men, compared to HABC Caucasian women, or MrOS Caucasian men, when outcome was calculated using half mouth exams (see panel B)

** Significant differences ($p < 0.0001$) were observed between % bleeding on probing outcome calculated using full mouth exam data, compared to half mouth exam data (see panel B)

4.2 Characteristics of risk factors

Many risk factors for periodontal diseases were included in the following regression analyses such as: age, sex, education level, smoking status and packs per year, drinking status, fasting glucose, body mass index (BMI), serum levels of 1,25 vitamin D, frequency of brushing, frequency of flossing, and frequency of dental visits. The study population characteristics for these risk factors are shown in Table 4.3 for the Health ABC Study African Americans and Caucasians, and for MrOS Caucasian men.

There were significant differences in the prevalence of most risk factors between the races and the sexes. The most significant differences were observed between the frequencies of dental visits reported by the Health ABC Study African Americans compared to the Health ABC Study Caucasians, where African Americans reported fewer dental visits than Caucasians ($p < 0.0001$). The next largest difference observed between the African Americans and Caucasians were differences in education levels and frequency of flossing. African Americans reported significantly less education and a lower frequency of flossing, compared to Caucasians ($p < 0.0001$). Additionally, significant differences ($p < 0.0001$) were seen between fasting glucose and BMI levels, where African Americans had higher fasting glucose levels and higher BMI levels, compared with Health ABC Caucasians.

Significant differences in the prevalence of risk factors for periodontal diseases were also observed between the sexes. The risk factors with the most significant differences between the sexes were: fasting glucose levels, BMI, and smoking habits. African American and Caucasian men had higher BMI and fasting glucose levels compared to African American and Caucasian women, respectively. Also, the prevalence of smoking was significantly higher, and frequency of flossing was significantly lower, in men compared to women of both races.

Table 4.3 Demographic, clinical, and lifestyle characteristics of the Health ABC Study and MrOS populations

	HABC African Americans		HABC Caucasians		MrOS Caucasians
	Men	Women	Men	Women	Men
Sample size	237	302	536	474	686
Covariates : Average ($\pm$ SD)					
Age	74 (3)	73 (3)	74 (3)	74 (3)	74 (5)
Education level*					6 (2)
Less than high school graduate	42%	30%	11%	7%	
High school graduate	29%	38%	22%	42%	
Post secondary school	29%	32%	67%	51%	
Smoking status					
Never smoker	36%	58%	34%	62%	38%
Former smoker	47%	33%	62%	32%	59%
Current smoker	17%	9%	4%	6%	3%
Pack/year	20 (23)	12 (22)	23 (31)	12 (24)	17 (24)
Drinking status*					1.5 (1.5)
Never drinker	17%	42%	15%	34%	
Former drinker	34%	25%	16%	11%	
Current drinker	49%	33%	69%	55%	
Fasting glucose (mg/dl)	110 (40)	107 (42)	105 (30)	95 (21)	106 (28)
Diabetics	20%	17%	11%	7%	13%
BMI (kg/m ²)	27 (4)	30 (6)	27 (4)	26 (4)	27 (4)
Serum 1,25 vitamin D (ng/mL)	21 (8)	21 (9)	29 (9)	29 (11)	25 (7)
Frequency of brushing					
< 2 per day	50%	27%	36%	19%	34%
≥ 2 per day	50%	73%	64%	81%	66%
Frequency of flossing					
< 1 per week	58%	38%	33%	16%	59%
1 -2 per week	19%	18%	23%	17%	10%
> 2 per week	23%	44%	44%	67%	31%
Frequency of dental visits					
< 1 per year	51%	46%	16%	11%	16%
1 per year	19%	20%	17%	15%	13%
> 1 per year	30%	35%	67%	74%	71%

HABC (Health ABC Study)

*MrOS education represent years of education after 8th grade

**MrOS drinking status represents average number of alcoholic beverages per week

4.3 Epidemiological analysis of risk factors

Univariate linear regression was used to investigate the impact of each risk factor on the periodontal disease-related outcomes, in race and sex stratified analyses. The percentage of variance explained by each risk factor was calculated; however, these calculations do not take into account any overlapping effects among risk factors. Therefore, backwards stepwise regression was used to build a parsimonious model, and to estimate the effect sizes of each risk factor in the presence of all other risk factors included in the parsimonious model, for each outcome.

The effect sizes observed in the initial race and sex stratified analyses were similar between sexes (data not shown); therefore, the sexes were combined, and univariate and stepwise regression analyses were performed stratified by race, with sex as risk factor. The race stratified results are summarized in Table 4.4 – Table 4.7, including the percentage of variance (R^2) of each outcome explained by each risk factor (from univariate analysis), as well as the effect size (β), standard error (SE), and significance (p) associated with each risk factor (from the backwards stepwise regression). Risk factors eliminated from stepwise regression models of periodontal disease-related outcomes are listed as “drop.” Risk factors associated with significant effect sizes in stepwise regression analysis are listed in Table 4.4 – Table 4.7 as follows: +++ ($p \leq 0.001$), ++ ($0.001 < p \leq 0.01$), + ($0.01 < p \leq 0.05$), “ns” ($p > 0.05$). Some risk factors included in the stepwise regression model had non-significant effect sizes because these risk factors uniquely explained a significant portion of the variance of the outcome ($p \leq 0.05$) holding all other risk factors constant, despite a non-significant effect size ($p > 0.05$) associated with the individual risk factor.

4.3.1 Mean plaque index

Univariate and backwards stepwise regression analyses for the periodontal outcome mean plaque index included 539 African Americans, and 1,010 Caucasians from the Health ABC Study, and 686 Caucasian men from MrOS (see Table 4.4). Based on backwards stepwise regression analysis of 13 potential risk factors, 27% of the variance of mean plaque levels was explained in the Health ABC Study African American population, 30% in the Health ABC Study Caucasian population, and 28% in the MrOS Caucasian population.

The dental examiner variable explained the most variance of mean plaque levels for all three populations (up to 20%) in univariate regression analyses. Other risk factors that explained a large portion of the variance of mean plaque levels include frequency of flossing (up to 9%) and frequency of dental visits (up to 9%). More frequent flossing and more frequent dental visits were associated with lower plaque levels in all three populations.

Higher education levels were associated with lower plaque levels; however, this relationship was much stronger among Health ABC African Americans compared to the Caucasian population. Sex (female) was also significantly associated with lower mean plaque level variance in the Health ABC Study African American population, but not in the Health ABC Caucasian population.

Higher serum levels of vitamin D were associated with lower plaque levels among the Health ABC African Americans and Caucasians, but this relationship was not significant in the MrOS population. Another difference observed between the Health ABC Study and MrOS populations was that the number of cigarette packs per year was

Table 4.4 Percent variance and regression coefficients for the association of covariates with mean plaque index

Outcome: mean plaque index		HABC African Americans			HABC Caucasians			MrOS Caucasian Men		
Individual covariates:		R ² (%)	β ± SE	p	R ² (%)	β ± SE	p	R ² (%)	β ± SE	p
All examiners		11.1	Not listed	+++	20.1	Not listed	+++	17.0	Not listed	+++
Age		0.4	0.015 ± 0.009	ns	0.1	0.007 ± 0.005	ns	4.9	0.014 ± 0.003	+++
Sex (ref: male)		3.3	-0.10 ± 0.05	+++	0.8		drop	NA	NA	
Education (ref: < high school graduate)		10.6	-0.22 ± 0.06 -0.26 ± 0.07	+++	1.0		drop	1.3*	-0.016 ± 0.011	ns
Smoking status (ref: never smoker)		1.1		drop	0.3		drop			drop
Pack/year		0.3		drop	0.4	0.0008 ± 0.0005	ns	2.3	0.0031 ± 0.0008	+++
Drinking status (ref: never drinker)		1.8	0.14 ± 0.06 0.12 ± 0.07	ns	0.6		drop	< 0.1*	-0.03 ± 0.01	+
Fasting glucose (mg/dl)		0.1		drop	1.1	0.0013 ± 0.0005	+	0.1		drop
BMI (kg/m ²)		0.4		drop	1.0	0.005 ± 0.004	ns	0.1		drop
Serum 1,25 vitamin D (ng/mL)		2.0	-0.007 ± 0.003	+	1.3	-0.004 ± 0.001	+	0.7		drop
Frequency of brushing (ref: < 2/day)		1.9		drop	2.0	-0.07 ± 0.03	+	2.7	-0.09 ± 0.04	+
Frequency of flossing (ref: < 1/week)		9.1	-0.18 ± 0.07 -0.25 ± 0.06	+++	4.7	-0.06 ± 0.04 -0.18 ± 0.04	+++	3.4	-0.06 ± 0.06 -0.19 ± 0.04	+++
Frequency of dental visits (ref: < 1/yr)		8.9	-0.11 ± 0.07 -0.27 ± 0.06	+++	6.1	-0.20 ± 0.05 -0.25 ± 0.04	+++	2.6	-0.13 ± 0.07 -0.14 ± 0.05	+

The percent variance (R²) explained by each risk factor is from univariate analysis; the effect size (β), standard error (SE), and significance (+++; p ≤ 0.001, ++; 0.001 < p ≤ 0.01, +; 0.01 < p ≤ 0.05, ns: p > 0.05) are from backwards stepwise regression analysis (risk factors eliminated from stepwise regression analyses are listed as “drop”) in Health ABC Study (HABC) African Americans (n = 539), HABC Caucasians (n = 1,010) and MrOS Caucasian men (n = 686); *MrOS education levels and drinking status are analyzed differently than the HABC variables

associated with higher plaque levels in MrOS; however, the relationship was not significant in the Health ABC Study populations after adjusting for other risk factors. Age also increased the risk of higher plaque levels in all populations; but this risk factor explained much more variance among the MrOS population (4.9%) than among either Health ABC Study population (0.4%).

4.3.2 Percent bleeding on probing (% BOP)

Univariate and backwards stepwise regression for the outcome % BOP included 388 African Americans and 697 Caucasians from the Health ABC Study, and 686 Caucasian men from MrOS (see Table 4.5). After backwards stepwise regression including 14 potential risk factors, 48% of the variance of % BOP was explained in the Health ABC Study African American population, 37% in the Health ABC Study Caucasian population, and 50% in MrOS Caucasians.

The dental examiner and plaque level variables explained the most variance of % BOP in univariate regression analyses (up to 33%). Higher plaque levels were associated with an increased risk of % BOP in all three populations. A large portion of the variance of % BOP was also explained by frequency of flossing (up to 6.5%), where higher frequencies of flossing resulted in a lower % BOP in all three populations.

Higher levels of education were associated with lower % BOP in all three populations. Education levels explained more variance among African Americans (6%) than in either Caucasian population (1.6 – 1.7%); however, education levels did not uniquely explain enough variance of % BOP among African Americans to be kept in the final model resulting from backwards stepwise regression. Also, age explained ~4% of the variance of % BOP in the MrOS Caucasian population, but did not uniquely explain enough variance to be included in the final model.

Table 4.5 Percent variance and regression coefficients for the association of covariates with % bleeding on probing

Outcome: % bleeding on probing	HABC African Americans			HABC Caucasians			MrOS Caucasian Men		
	R ² (%)	β ± SE	p	R ² (%)	β ± SE	p	R ² (%)	β ± SE	p
Individual covariates:									
All examiners	33.3	Not listed	+++	23.0	Not listed	+++	22.0	Not listed	+++
Age	< 0.1		drop	< 0.1		drop	4.0		drop
Sex (ref: male)	1.4		drop	0.6		drop	NA	NA	
Education (ref: < high school graduate)	6.0		drop	1.7	-4.5 ± 3.4 -7.1 ± 3.2	ns	1.6*	-1.4 ± 0.6	+
high school graduate									
post secondary school									
Smoking status (ref: never smoker)	0.4		ns	1.2		drop	0.5		drop
current smoker		-5.3 ± 3.8							
former smoker		-5.8 ± 2.6							
Pack/year	0.2		drop	0.8	0.06 ± 0.03	ns	0.9		drop
Drinking status (ref: never drinker)	0.1		drop	0.8		drop	0.5*		drop
Fasting glucose (mg/dl)	< 0.1		drop	0.3		drop	< 0.1		drop
BMI (kg/m ²)	0.1		drop	0.6	0.37 ± 0.23	ns	< 0.1		drop
Serum 1,25 vitamin D (ng/mL)	0.4		drop	0.8		drop	0.7		drop
Plaque	23.0	19 ± 2	+++	24.7	22 ± 2	+++	33.4	33 ± 2	+++
Frequency of brushing (ref: < 2/ day)	0.3		drop	0.3		drop	2.0		drop
Frequency of flossing (ref: < 1/week)	6.5	-7.3 ± 3.3 -7.6 ± 2.9	+	2.3	2.5 ± 2.8 -2.5 ± 2.3	ns	2.5	-2 ± 3 -6 ± 2	++
1-2 per week									
> 2 per week									
Frequency of dental visits (ref: < 1/yr)	1.3		drop	1.5		drop	2.3		drop

The percent variance (R²) explained by each risk factor is from univariate analysis; the effect size (β), standard error (SE), and significance (+++: p ≤ 0.001, ++: 0.001 < p ≤ 0.01, +: 0.01 < p ≤ 0.05, ns: p > 0.05) are from backwards stepwise regression analysis (risk factors eliminated from stepwise regression analyses are listed as “drop”) in Health ABC Study (HABC) African Americans (n = 388), HABC Caucasians (n = 697) and MrOS Caucasian men (n = 686); *MrOS education levels and drinking status are analyzed differently than the HABC variables

4.3.3 Extent pocket depth (PD) $\geq$ 5mm

Univariate and backwards stepwise regression for the outcome extent pocket depth (PD) $\geq$ 5mm included 377 African Americans and 699 Caucasians from the Health ABC Study, and 686 Caucasian men from MrOS (see Table 4.6). After backwards stepwise regression including 14 potential risk factors, 18% of the variance of extent PD $\geq$ 5mm was explained in the Health ABC Study African American population, 13% in the Health ABC Study Caucasian population, and 16% in the MrOS Caucasian population.

Plaque levels and the examiner variable explained the most variance in univariate regression analyses of extent PD $\geq$ 5mm (up to $\sim$ 10%). Higher plaque levels increased the risk of extent PD $\geq$ 5mm in all three populations. Smoking status also explained a significant portion of the extent PD $\geq$ 5mm variance for all three populations (up to 1.7%). Current smokers had a higher risk of extent PD $\geq$ 5mm compared to non-smokers; however, this relationship was not significant among former smokers. The number of cigarette packs per year also increased the risk of extent PD $\geq$ 5mm in MrOS, but this relationship was not significant in either Health ABC Study population.

Sex also explained a significant portion of the variance of extent PD $\geq$ 5mm among both the Health ABC Study African Americans and Caucasians, with females having a lower risk of extent PD $\geq$ 5mm compared to males.

A difference observed between study populations was that higher education levels were associated with a significant decrease in extent PD $\geq$ 5mm among African Americans, explaining 4.7% of the variance; however, this relationship was not significant in either Caucasian population (explained up to 0.1% variance). Also, the frequency of flossing and the frequency of dental visits explained much more of the

Table 4.6 Percent variance and regression coefficients for the association of covariates with extent pocket depth $\geq 5\text{mm}$

Outcome: extent pocket depth (PD) $\geq 5\text{mm}$	HABC African Americans			HABC Caucasians			MrOS Caucasian Men		
	R ² (%)	$\beta \pm \text{SE}$	p	R ² (%)	$\beta \pm \text{SE}$	p	R ² (%)	$\beta \pm \text{SE}$	p
Individual covariates:									
All examiners	5.5	Not listed	++	6.2	Not listed	+++	8.0	Not listed	+++
Age	< 0.1		drop	< 0.1		drop	0.4		drop
Sex (ref: male)	3.2	-0.18 $\pm$ 0.08	+	2.0	-0.13 $\pm$ 0.04	++	NA	NA	
Education (ref: < high school graduate) high school graduate post secondary school	4.7	-0.22 $\pm$ 0.09 -0.26 $\pm$ 0.10	+	< 0.1		drop	0.1*		drop
Smoking status (ref: never smoker) current smoker former smoker	1.5	0.04 $\pm$ 0.11 -0.17 $\pm$ 0.08	ns	1.7	0.20 $\pm$ 0.10 0.05 $\pm$ 0.05	ns	1.6	0.23 $\pm$ 0.14 -0.06 $\pm$ 0.05	+
Pack/year	0.1		drop	1.1		drop	3.4	0.004 $\pm$ 0.001	+++
Drinking status (ref: never drinker) current drinker former drinker	1.5	0.23 $\pm$ 0.09 0.10 $\pm$ 0.09	+	0.2		drop	< 0.1*		drop
Fasting glucose (mg/dl)	0.1		drop	< 0.1		drop	< 0.1		drop
BMI (kg/m ²)	0.3		drop	0.1		drop	0.1		drop
1,25 vitamin D (ng/mL)	0.5		drop	0.2		drop	< 0.1		drop
Plaque	9.6	0.38 $\pm$ 0.07	+++	5.2	0.29 $\pm$ 0.05	+++	4.5	0.23 $\pm$ 0.04	+++
Frequency of brushing (ref: < 2/day)	0.6		drop	0.1		drop	0.3		drop
Frequency of flossing (ref: < 1/week)	4.2		drop	1.2		drop	0.7		drop
Frequency of dental visits (ref: < 1/yr)	2.3		drop	0.7		drop	0.5		drop

The percent variance (R²) explained by each risk factor is from univariate analysis; the effect size (β), standard error (SE), and significance (+++: p ≤ 0.001 , ++: 0.001 < p ≤ 0.01 , +: 0.01 < p ≤ 0.05 , ns: p > 0.05) are from backwards stepwise regression analysis (risk factors eliminated from stepwise regression analyses are listed as “drop”) in Health ABC Study (HABC) African Americans (n = 377), HABC Caucasians (n = 699) and MrOS Caucasian men (n = 686); *MrOS education levels and drinking status are analyzed differently than the HABC variables

variance of extent PD $\geq$ 5mm among the African American population compared to the Caucasian populations.

4.3.4 Extent attachment loss (AL) $\geq$ 3mm

Univariate and backwards stepwise regression for the outcome extent attachment loss (AL) $\geq$ 3mm included 377 African Americans and 697 Caucasians from the Health ABC Study, and 686 Caucasian men from MrOS (see Table 4.7). After backwards stepwise regression including 14 potential risk factors, 26% of the variance of extent AL $\geq$ 3mm was explained in the Health ABC Study African American population, 29% in the Health ABC Study Caucasian population, and 27% in the MrOS Caucasian population.

The dental examiner risk factor explained the most variance of extent AL $\geq$ 3mm in univariate regression analyses for all three populations (up to ~17%). Higher plaque levels were the next largest risk factor for extent AL $\geq$ 3mm, explaining up to 10% of the variance of extent AL $\geq$ 3mm.

Higher frequencies of flossing and dental visits were two other important risk factors for extent AL $\geq$ 3mm, especially in the Health ABC Study African American population where they explained 6.3% and 5.0% of the variance of extent AL $\geq$ 3mm from univariate analysis, respectively. Smoking status (current) and higher numbers of cigarette packs per year were also significant risk factors for increased extent AL $\geq$ 3mm in all three study populations (up to 5.8% of the variance).

Sex (male) was another important risk factor in the Health ABC Study African American and Caucasian populations, explaining up to 3.5% of the variance of extent AL $\geq$ 3mm.

Table 4.7 Percent variance and regression coefficients for the association of covariates with extent attachment loss ≥ 3 mm

Individual covariates:	HABC African Americans			HABC Caucasians			MrOS Caucasian Men		
	R ² (%)	$\beta \pm SE$	p	R ² (%)	$\beta \pm SE$	p	R ² (%)	$\beta \pm SE$	p
All Examiners	12.2	Not listed	+++	14.8	Not listed	+++	16.7	Not listed	+++
Age	< 0.1		drop	0.4		drop	1.2		drop
Sex (ref: male)	3.5	-8.1 $\pm$ 2.6	++	3.3	-7.7 $\pm$ 1.8	+++	NA	NA	
Education (ref: < high school graduate) high school graduate post secondary school	1.8		drop	1.6	-3.2 $\pm$ 3.2 -7.7 $\pm$ 3.0	++	0.3		drop
Smoking status (ref: never smoker) current smoker former smoker	2.9		drop	3.1	8.8 $\pm$ 4.2 1.5 $\pm$ 2.1	ns	2.6	9.6 $\pm$ 5.3 -0.6 $\pm$ 1.8	ns
Pack/year	2.1	0.12 $\pm$ 0.06	+	3.6	0.11 $\pm$ 0.04	++	5.8	0.20 $\pm$ 0.04	+++
Drinking status (ref: never drinker) current drinker former drinker	0.8		drop	2.6	0.5 $\pm$ 2.2 7.8 $\pm$ 2.9	+	0.2		drop
Fasting glucose (mg/dl)	0.3		drop	0.2		drop	0.9		drop
BMI (kg/m ²)	0.4		drop	< 0.1		drop	0.4		drop
Serum 1,25 vitamin D (ng/mL)	0.1		drop	0.5		drop	< 0.1		drop
Plaque	9.8	15.3 $\pm$ 2.4	+++	5.3	8.4 $\pm$ 1.9	+++	6.7	7.2 $\pm$ 1.6	+++
Frequency of brushing (ref: < 2/ day)	0.8		drop	0.5		drop	0.6		drop
Frequency of flossing (ref: < 1/ week)	6.3		drop	1.7		drop	0.1		drop
Frequency of dental visits (ref: < 1/ yr) 1/ yr > 1/ yr	5.0	4.7 $\pm$ 3.4 -4.6 $\pm$ 3.0	+	2.8	-4.6 $\pm$ 3.0 -7.3 $\pm$ 2.5	+	1.5	-5.5 $\pm$ 1.8 -0.8 $\pm$ 2.1	ns

The percent variance (R²) of extent attachment loss (AL) ≥ 3 mm, explained by each risk factor is from univariate analysis; the effect size (β), standard error (SE), and significance (+++: $p \leq 0.001$, ++: $0.001 < p \leq 0.01$, +: $0.01 < p \leq 0.05$, ns: $p > 0.05$) are from backwards stepwise regression analysis (risk factors eliminated from stepwise regression are listed as "drop") in Health ABC Study (HABC) African Americans (n = 377), HABC Caucasians (n = 697), and MrOS Caucasian men (n = 686); *MrOS and HABC education and drinking status are analyzed differently

4.3.5 Summary of epidemiological analysis of risk factors

Overall, the two risk factors explained that most variance of all periodontal outcomes were the dental examiner variable and higher plaque levels. Two other risk factors that explained a large portion of the variance for periodontal disease-related outcomes were lower frequencies of flossing and lower frequencies of dental visits. Although the frequency of flossing and the frequency of dental visits explained a large portion of the variance all four periodontal disease-related outcomes in all three populations, they consistently explained more variance in the Health ABC Study African American population, compared to the Caucasian populations.

Other significant risk factors included: sex (male), lower education level, current smoking status, and higher numbers of cigarette packs per year. Risk factors that did not consistently associate with the periodontal disease-related outcomes include drinking status, fasting glucose, BMI, and serum vitamin D levels.

4.4 Genome-wide association study

Genome-wide association studies (GWAS) of periodontal disease-related outcomes were performed to detect genetic risk factors of periodontal diseases. Results from the epidemiological analysis of risk factors for periodontal diseases (Section 4.3) were used to develop a multiple linear regression model for the subsequent genome-wide association studies (see Equation 4.1). This model included significant risk factors of periodontal diseases to adjust for the effects of non-genetic risk factors (noise). The inclusion of non-genetic risk factors in the GWAS model increased the genetic signal-to-noise ratio for periodontal disease-related outcomes by effectively reducing the standard error relative to the estimated SNP effect size (data not shown).

Equation 4.1 Genome-wide association study multiple linear regression model

Periodontal disease-related outcome = SNP + age + sex + education + examiner + mean plaque index + smoking status + current pack/year + frequency of brushing + frequency of flossing + frequency of dental visits + principal components

The following outcomes were investigated using a GWAS in two primary Caucasian populations (Health ABC Study and MrOS): mean plaque index, % bleeding on probing (BOP), extent pocket depth (PD) ≥ 5 mm, and extent attachment loss (AL) ≥ 3 mm. Outcomes were calculated using half-mouth data collected from 951 Caucasians from the Health ABC Study, and 422 Caucasian men from MrOS (see Figure 4.1). After data cleaning, this study included ~2.1 million SNPs in the Health ABC Study and MrOS Caucasian populations (see 3.3.3 Genotyping). The following results are from the meta-analysis of GWAS results from the Health ABC Study Caucasians and MrOS Caucasians. SNPs associating with periodontal disease-related outcomes ($p \leq 10^{-6}$) from the Caucasian meta-analysis were further investigated for replication in the ARIC Study Caucasians, and the Health ABC Study African Americans.

Significant results from the Caucasian meta-analysis ($p \leq 10^{-6}$)

Four signals were associated with periodontal disease-related outcomes ($p \leq 10^{-6}$) from the meta-analysis of the GWAS of periodontal diseases in the Health ABC Study and MrOS Caucasians. The most significant associations detected are listed in Table 4.8. However, no significant replications were observed in either the ARIC Study Caucasians or the Health ABC Study African American populations for any of the top hits ($p < 0.05$).

Table 4.8 Significant results from meta-analysis of GWAS of periodontal disease-related outcomes in the Health ABC Study and MrOS Caucasian populations ($p \leq 10^{-6}$)

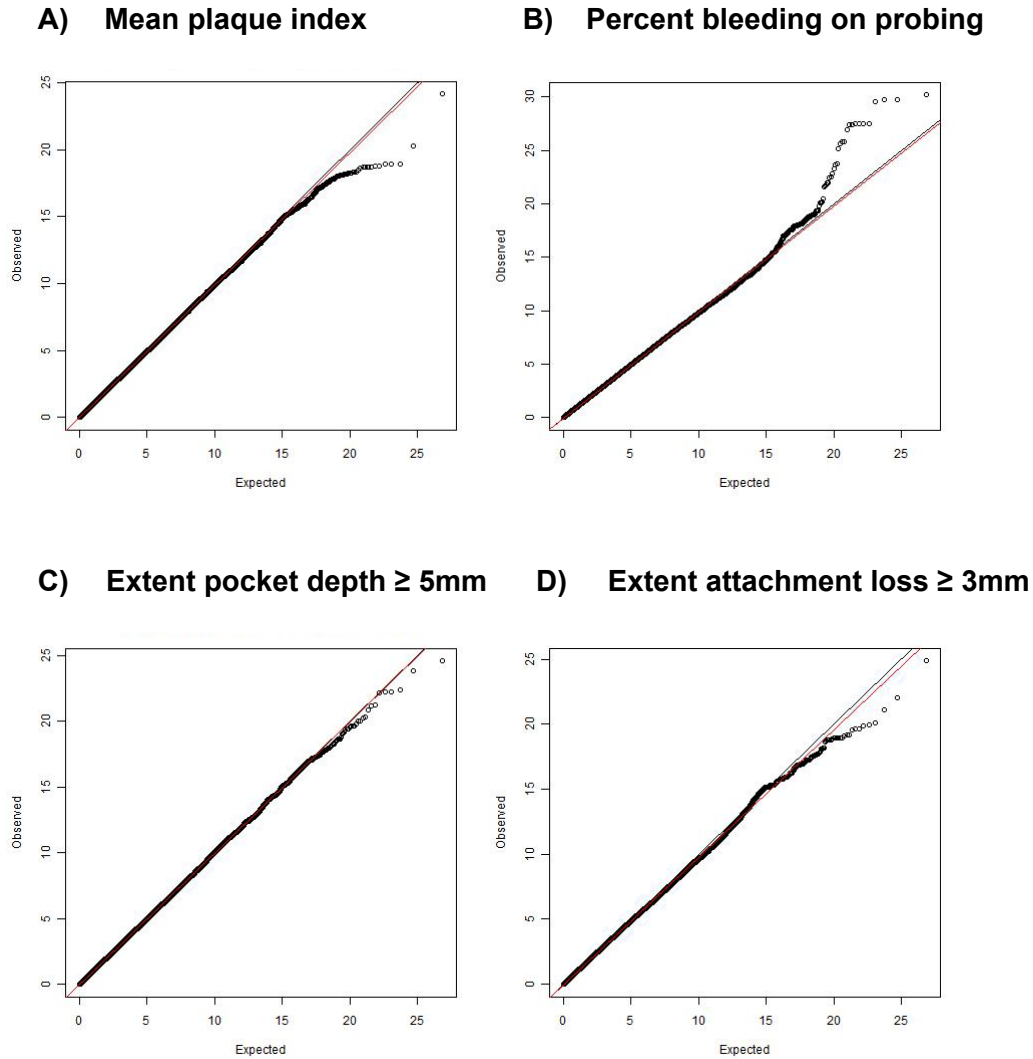
Outcome	Sample size	SNP	Chr: position	Nearby gene	P from meta-analysis
Mean plaque index	1,373	rs739270	22: 45,943,203	<i>FBLN1</i>	8.93×10^{-7}
Percent bleeding on probing	1,079	rs6072925	20: 41,618,747	<i>PTPRT</i>	3.93×10^{-8}
Extent pocket depth ≥ 5 mm	1,081	rs11957208	5: 146,048,748	<i>PPP2R2B</i>	7.20×10^{-7}
Extent attachment loss ≥ 3 mm	1,077	rs4790833	17: 1,200,693	<i>TUSC5</i>	6.16×10^{-7}

Association characteristics including: the SNP number, genomic location (chromosome (chr) and position based on Genome Reference Consortium Human Genome Build 37 (GRCh37), and significance from the most significant SNP at each locus, from meta-analysis of genome-wide association studies (GWAS) in the Health ABC Study and MrOS Caucasian populations (P from meta-analysis)

All quantile-quantile (Q-Q) plots resulting from the meta-analysis are shown in Figure 4.2. Lambda values for these plots were approximately equal to one, suggesting little residual population stratification in the study populations. The meta-analysis of GWAS results for periodontal disease-related outcomes: mean plaque index, % bleeding on probing, extent PD ≥ 5 mm, and extent AL ≥ 3 mm, are depicted as Manhattan plots in Figure 4.3, Figure 4.6, Figure 4.11, and Figure 4.14, respectively. The following sections summarize significant findings from the meta-analyses,

replication results, as well as notable findings from an *in silico* investigation of the genomic regions surrounding the identified signals.

Figure 4.2 Q-Q plots of the meta-analysis results of genome-wide association studies of periodontal disease-related traits in the Health ABC Study and MrOS Caucasian populations

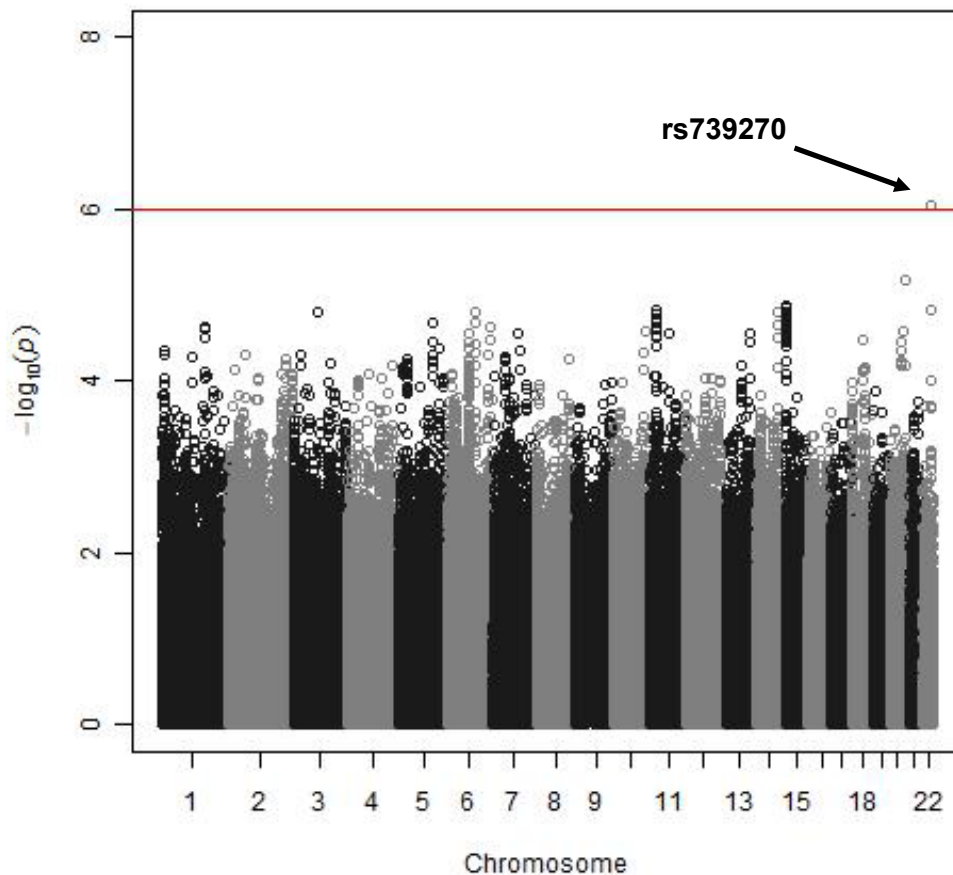


Quantile-quantile (Q-Q) plots from the meta-analysis of genome-wide association studies of periodontal disease-related outcomes in the Health ABC Study and MrOS Caucasian populations, including A) mean plaque index ($\lambda = 0.988902$), B) percent bleeding on probing ($\lambda = 0.989644$), C) extent pocket depth ≥ 5 mm ($\lambda = 0.996598$), and D) extent attachment loss ≥ 3 mm ($\lambda = 0.977663$)

4.4.1 Mean plaque index

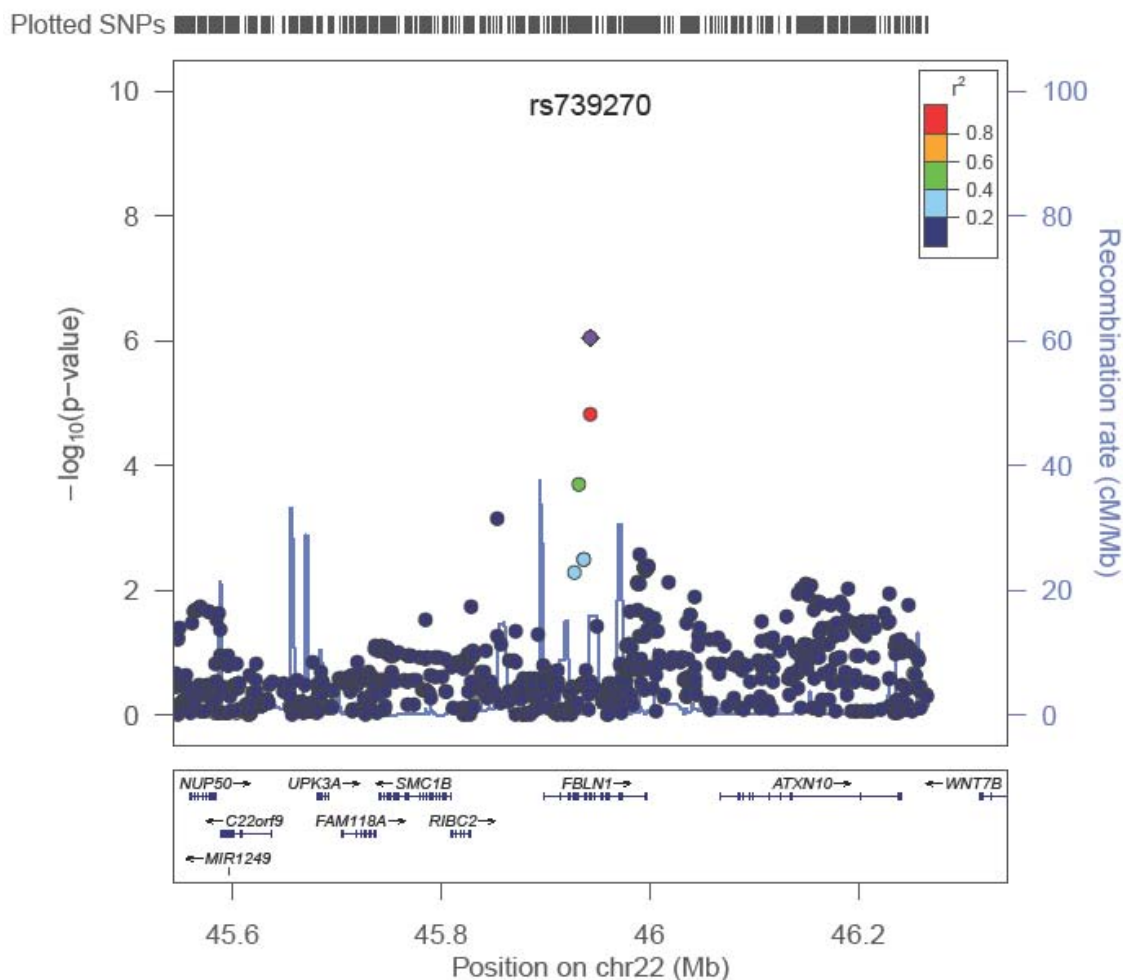
There was one significant association detected on chromosome 22q13.31 by the meta-analysis of the Health ABC Study Caucasian GWAS results ($n = 951$) and MrOS Caucasian GWAS results ($n = 422$) (rs739270: 8.93×10^{-7} , see Figure 4.3). The top SNP in the signal, rs739270, was genotyped (MAF = 0.13) in both studies, and was in high LD ($r^2 > 0.8$) with an imputed SNP rs576543 (MAF = 0.12, $p = 1.47 \times 10^{-5}$) (see Table 4.9). These two SNPs are located within the genomic region encoding *fibulin 1* (*FBLN1*).

Figure 4.3 Manhattan plot of results from meta-analysis of GWAS of mean plaque index in two Caucasian populations



Manhattan plot showing the negative log of the p values from the meta-analysis of 951 Health ABC Caucasians and 422 MrOS Caucasians, ordered by chromosomal position; red line indicates a significance (p) of 10^{-6}

Figure 4.4 Meta-analysis of mean plaque index GWAS results from two Caucasian populations for the genomic region surrounding rs739270



SNPs are plotted by chromosomal position against the association significance ($-\log_{10} p$) with mean plaque index from the meta-analysis of the Health ABC Study Caucasian population ($n = 951$) with MrOS Caucasian men ($n = 422$); the most significant association is represented as a purple diamond, and is labeled; other SNPs are colored to reflect their LD with the most significant SNP (using pairwise r^2 values from the HapMap CEU population); estimated recombination rates (from HapMap) are plotted in cyan to reflect the local LD structure; positions of genes and exons, as well as the direction of transcription, are shown below the plots (using data from the UCSC Genome Browser GRCh37); plots were generated using LocusZoom

The study specific association results of mean plaque index with rs739270 are presented in Table 4.10. The rs739270 A allele was associated with a higher mean plaque index in the Health ABC Study and MrOS Caucasian populations, compared to

the G allele; however the effect size relative to the standard error was much larger in the Health ABC population (see Table 4.10). Replication analyses in 4,165 ARIC Study Caucasians did not support the association between rs739270 and mean plaque index; additionally, no significant association was detected among 500 African Americans from the Health ABC Study.

Table 4.9 SNPs in strong LD ($r^2 > 0.8$) with rs739270 (*fibulin 1* gene region)

SNP	Chr	Position	Minor allele	MAF	LD with rs739270	Meta p	Replication p
rs739270	22	45,943,203	A	0.12	-	8.93×10^{-7}	0.42
rs5765463	22	45,942,726	G	0.13	0.95	1.47×10^{-5}	0.57

SNP characteristics including: rs number, chromosome (Chr), position (GRCh37), minor allele frequency (MAF), linkage disequilibrium (LD) with rs739270, association significance (p) with mean plaque index from the meta-analysis of the Health ABC Study Caucasian population (n = 951) with MrOS Caucasian men (n = 422) (Meta p) and from the Caucasian ARIC Study population (n = 4,165) (Replication p); sorted by genomic position

Table 4.10 Association of rs739270 with mean plaque index in four populations

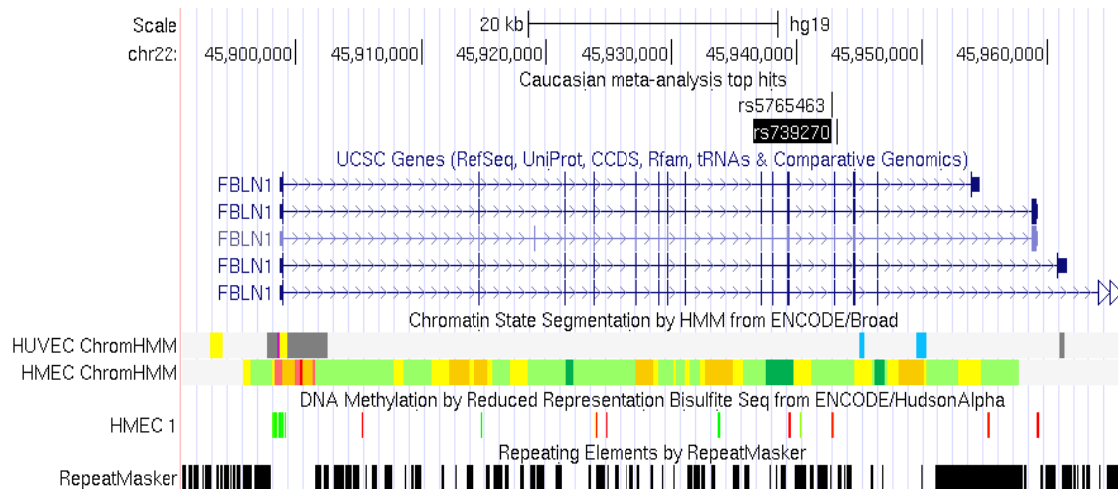
Population	MAF	N	$\beta \pm SE$	p
Health ABC Caucasians (primary)	0.12	951	0.16 ± 0.03	8.0×10^{-6}
MrOS Caucasian men (primary)	0.13	422	0.11 ± 0.05	0.03
ARIC Caucasians (replication)	0.13	4,165	0.009 ± 0.01	0.42
Health ABC AA (replication)	0.27	500	0.03 ± 0.04	0.52

Minor allele frequency (MAF), sample size (N), effect size (β), standard error (SE), and SNP association significance (p) of rs739270 with mean plaque index from the two primary GWAS cohorts (Health ABC Study and MrOS Caucasians) and two replication samples (ARIC Study Caucasians and Health ABC Study African Americans (AA))

The two SNPs identified in this signal flank the twelfth exon of *fibulin 1* transcript variants A, B, D, and E, and the thirteenth exon of fibulin 1 transcript variant C (see Figure 4.5). *Fibulin 1* is transcribed in epithelial cells (HMEC); however, transcription is repressed in endothelial cells (HUVEC), according to the Chromatin State Segmentation by the UCSC Genome Browser HMM track (ENCODE/Broad). Additionally, *in silico*

analyses revealed methylation sites near the identified signal (DNA methylation by Reduced Representation Bisulfite Seq from ENCODE/HudsonAlpha).

Figure 4.5 Genomic region directly surrounding signal identified with mean plaque index on chromosome 22q13.31

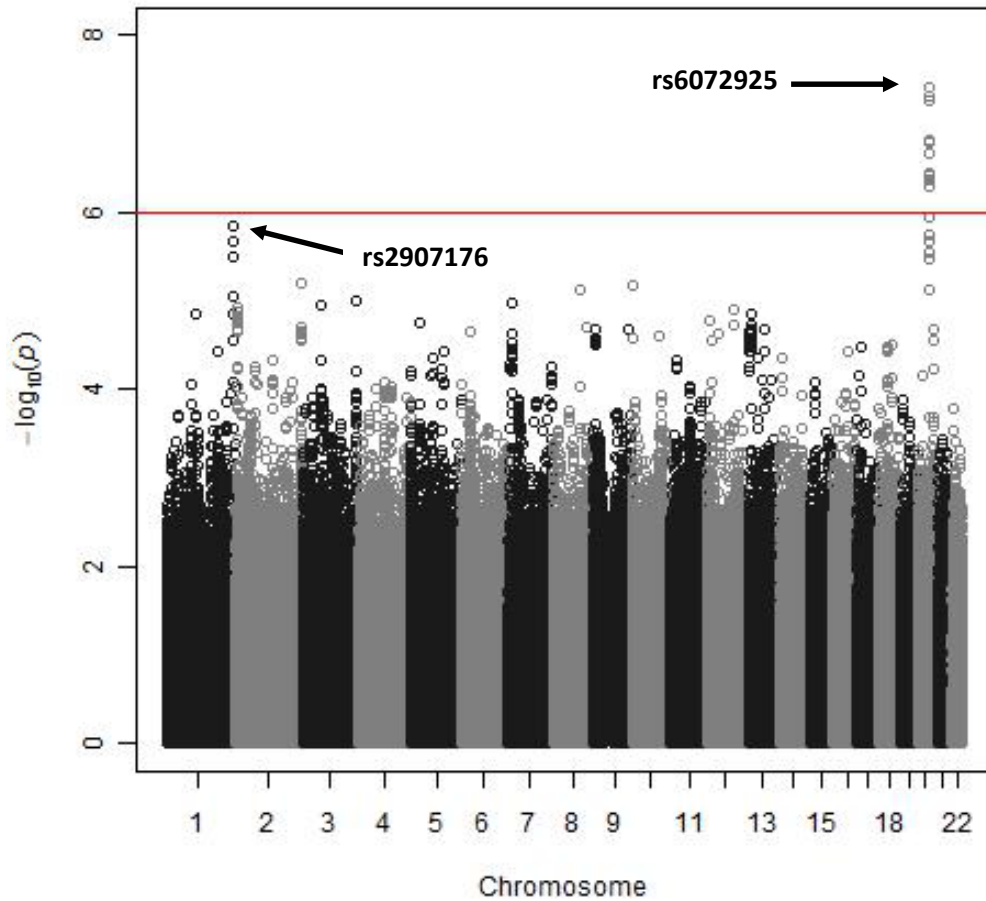


Screen shot from the UCSF Genome Browser Feb. 2009 assembly (<http://genome.ucsc.edu>) showing the genomic region directly surrounding the top hit rs739270 and one SNP in high LD with the top hit ($r^2 = 0.95$), identified by the meta-analysis of a GWAS of mean plaque index in the Health ABC Study ($n = 951$) and MrOS ($n = 422$) Caucasians (SNPs shown on top track); these SNPs flank an exon of *fibulin 1* (*FBLN1*); *fibulin 1* is transcribed (green and yellow) in epithelial cells (HMEC) but not in endothelial cells (HUVEC) (repressed DNA is colored grey and insulators are blue) (ENCODE/Broad); additionally there is a DNA methylation site observed in HMEC cells nearby (red = methylated, yellow = 50% methylated, green = unmethylated) (ENCODE/HudsonAlpha)

4.4.2 Percent bleeding on probing

A significant association between % BOP and rs6072925 ($p = 3.93 \times 10^{-8}$) was detected on chromosome 20q12, by the meta-analysis of results from the Health ABC Study Caucasians ($n = 657$) and the MrOS Caucasians ($n = 422$) (see Figure 4.6). Additionally, another signal was detected on chromosome 1p43 (rs2907176: $p = 1.39 \times 10^{-6}$) that almost reached the significance threshold of $p \leq 10^{-6}$.

Figure 4.6 Manhattan plot of results from meta-analysis of genome-wide association studies of percent bleeding on probing in two Caucasian populations



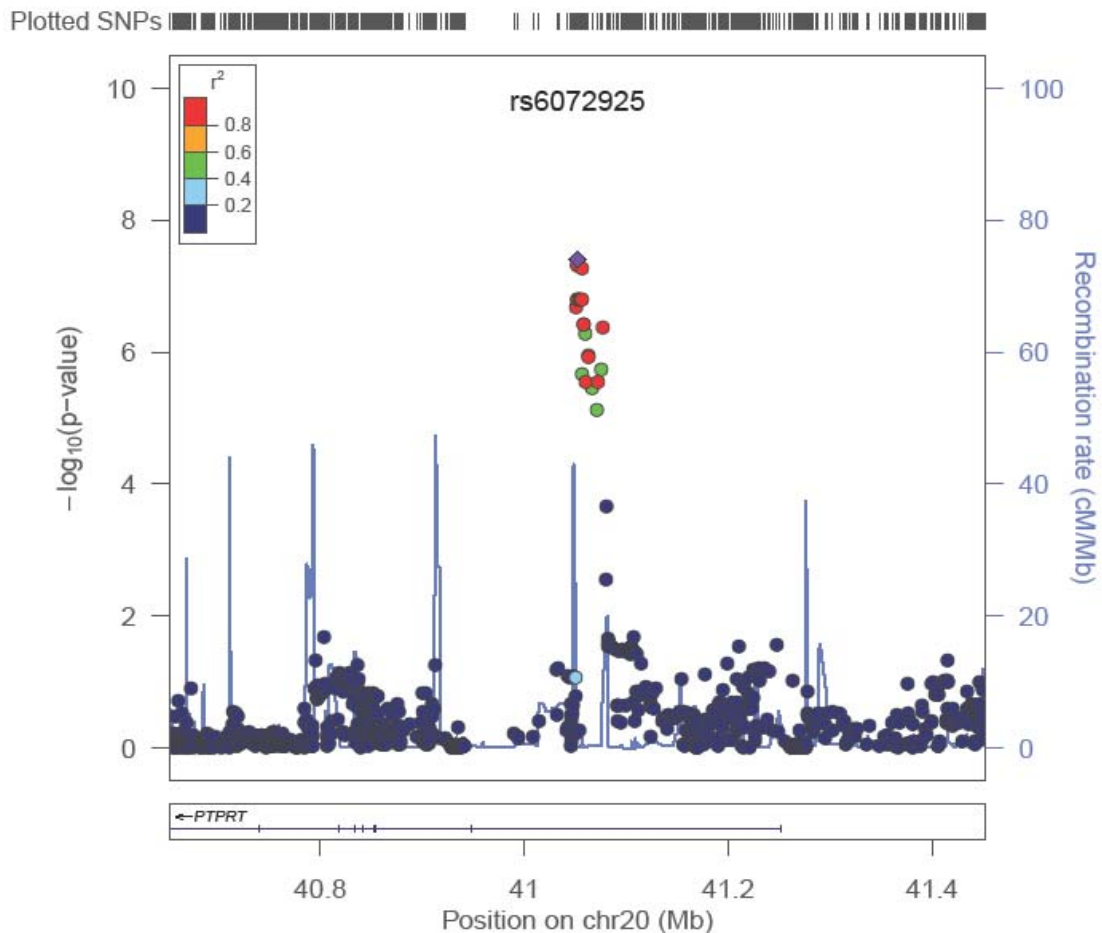
Manhattan plot showing the negative log of the p values from the meta-analysis of 657 Health ABC Caucasians and 422 MrOS Caucasians, ordered by chromosomal position; red line indicates a significance (p) of 10^{-6}

Chromosome 20q12 (rs6072925)

The most significant association detected with % BOP was with rs6072925, a genotyped SNP located within an intron of *PTPRT* (protein tyrosine phosphatase, receptor type, T) (see Figure 4.7). Seventeen SNPs were in high LD ($r^2 > 0.8$) with rs6072925 in our Caucasian populations, including three genotyped SNPs (rs6030596, rs12625239, and rs6065565) (see Table 4.11). All SNPs in high LD with rs6072925

displayed concordant effect direction with rs6072925 in the Health ABC Study and MrOS GWAS of % BOP, with p values ranging from 2.83×10^{-6} to 3.93×10^{-8} .

Figure 4.7 Meta-analysis of results from genome-wide association studies of percent bleeding on probing in two Caucasian populations for the genomic region surrounding rs6072925



SNPs are plotted by chromosomal position against the association significance ($-\log_{10} p$) with % bleeding on probing (BOP) from the meta-analysis of the Health ABC Study ($n = 657$) and MrOS ($n = 422$) Caucasian populations; the most significant association is represented as a purple diamond, and is labeled; other SNPs are colored to reflect their LD with the most significant SNP (using pairwise r^2 values from the HapMap CEU population); estimated recombination rates (from HapMap) are plotted in cyan to reflect the local LD structure; positions of genes and exons, as well as the direction of transcription, are shown below the plots (using data from the UCSC Genome Browser GRCh37); plots were generated using LocusZoom

Table 4.11 SNPs in strong LD ($r^2 > 0.8$) with rs6072925 (*PTPRT* gene region)

SNP	Chr	Position	Minor allele	MAF	LD with rs2907176	Meta p	Replication p	Conservation Score	CEU eQTL
rs6072923	20	41,617,622	A	0.23	0.91	2.09×10^{-7}	0.41	0	<i>RHOH</i>
rs6065558	20	41,618,561	G	0.22	0.99	4.92×10^{-8}	0.5	0	--
rs6065559	20	41,618,630	T	0.22	0.99	4.89×10^{-8}	0.5	0	--
rs6072924	20	41,618,733	C	0.23	0.91	1.62×10^{-7}	0.4	0	<i>RHOH</i>
rs6072925	20	41,618,747	A	0.22	--	3.93×10^{-8}	0.52	0.002	
rs6072926	20	41,618,847	C	0.23	0.91	1.62×10^{-7}	0.39	0	<i>RHOH</i>
rs6072928	20	41,620,906	G	0.23	0.91	1.58×10^{-7}	0.38	0.011	<i>RHOH</i>
rs6065560	20	41,621,072	G	0.23	0.91	1.58×10^{-7}	0.38	0.002	<i>RHOH</i>
rs16987668	20	41,622,231	G	0.23	0.91	1.56×10^{-7}	0.38	0.002	<i>RHOH</i>
rs6065561	20	41,623,001	G	0.23	0.91	1.57×10^{-7}	0.38	0	<i>RHOH</i>
rs2223558	20	41,623,138	A	0.22	0.99	5.44×10^{-8}	0.47	0	--
rs6072934	20	41,624,841	A	0.21	0.91	3.70×10^{-7}	0.41	0	--
rs6030596	20	41,625,104	T	0.21	0.91	3.79×10^{-7}	0.41	0.002	NA
rs6072936	20	41,626,988	C	0.22	0.82	2.82×10^{-6}	0.32	0.997	<i>RHOH, LTK</i>
rs6065562	20	41,629,442	G	0.21	0.9	1.12×10^{-6}	0.46	0	--
rs6072937	20	41,629,559	C	0.21	0.9	1.16×10^{-6}	0.46	0	--
rs6065565	20	41,638,464	C	0.22	0.83	2.83×10^{-6}	0.34	0.006	<i>RHOH, LTK</i>
rs12625239	20	41,643,147	G	0.21	0.9	4.20×10^{-7}	0.44	0	<i>ATPBD3</i>

Single nucleotide polymorphism (SNP) characteristics including: rs number, chromosome (Chr), position (GRCh37), minor allele frequency (MAF), linkage disequilibrium (LD) with rs6072925, association significance (p) with % sites that bleed upon probing from the meta-analysis of the Health ABC Study Caucasian population (n = 657) with MROS Caucasian men (n = 422) (Meta p) and from the Caucasian ARIC population (n = 4,069) (Replication p), and the predicted conservation score (Multiz alignment); and results from expression quantitative trait loci analysis in HapMap CEU (CEU eQTL), NA: not available for testing; *PTPRT*: protein tyrosine phosphatase, receptor type, T; sorted by genomic position

The study specific results for the top hit, rs6072925, and the most conserved SNP rs6072936, are presented in Table 4.12. The A allele of rs6072925 was associated with a lower percentage of sites that bled upon probing, compared to the G allele, in both the Health ABC Study Caucasian population ($\beta \pm SE = -3.8 \pm 0.8$; $p = 1.1 \times 10^{-5}$), and the MrOS Caucasian population ($\beta \pm SE = -7.2 \pm 2.2$; $p = 9.3 \times 10^{-4}$). The average % BOP decreased with each A allele of rs6072925, suggesting an additive effect (data not shown). Similar effect sizes and direction of effect were observed for the most conserved SNP, rs6072936.

SNP rs6072925 explained ~2.0% of the variance of % BOP among the Health ABC Study Caucasians; however, no significant replication was observed for either rs6072925, or rs6072936, among 4,069 Caucasians from the ARIC Study or 362 African Americans from the Health ABC Study.

Table 4.12a Association of rs6072925 with percent bleeding on probing in four populations

Population	MAF	N	$\beta \pm SE$	p
Health ABC Caucasians (primary)	0.22	657	-3.8 ± 0.8	1.1×10^{-5}
MrOS Caucasian men (primary)	0.23	422	-7.2 ± 2.2	9.3×10^{-4}
ARIC Caucasians (replication)	0.23	4,069	0.29 ± 0.45	0.52
Health ABC AA (replication)	0.19	362	-1.7 ± 2.3	0.48

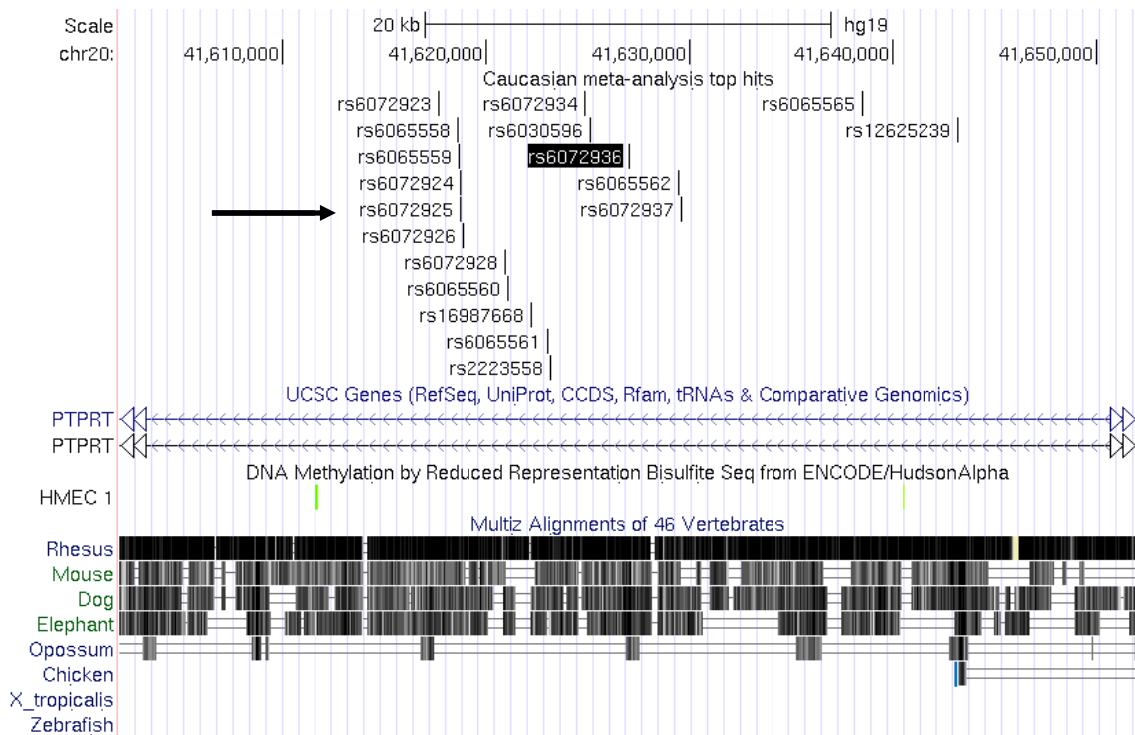
Table 4.12b Association of rs6072936 with percent bleeding on probing in four populations

Population	MAF	N	$\beta \pm SE$	p
Health ABC Caucasians (primary)	0.22	657	-2.9 ± 0.8	5.2×10^{-4}
MrOS Caucasian men (primary)	0.23	422	-6.9 ± 2.2	1.6×10^{-3}
ARIC Caucasians (replication)	0.23	4,069	0.43 ± 0.44	0.32
Health ABC AA (replication)	0.23	362	-1.5 ± 2.3	0.53

Minor allele frequency (MAF), sample size (N), effect size (β), standard error (SE), and SNP association significance (p) of rs6072925 (a) and rs6072936 (b) with % sites that bleed upon probing from the two primary GWAS populations (Health ABC Study and MrOS Caucasians) and two replication samples (ARIC Study Caucasians and Health ABC Study African Americans (AA))

Using the SCAN database to identify significant changes in gene expression levels associating with the SNPs in this signal, we identified SNPs within this signal to be eQTLs for three genes in the HapMap CEU population (see Table 4.11). For instance, a SNP in high LD with rs6072925, rs6072936 (r^2 with rs6072925 = 0.82) was identified as an eQTL for two genes in the HapMap CEU population, including *RHOH* (ras homolog family member H) ($p = 2 \times 10^{-5}$), and *LTK* (leukocyte receptor tyrosine kinase) ($p = 10^{-4}$). This SNP, rs6072936, was found to be the most conserved SNP in this signal (Vertebrate Multiz Alignment & Conservation (17 species) (see Table 4.11).

Figure 4.8 Genomic region directly surrounding signal identified with percent bleeding on probing on chromosome 20q12



Screen shot from the UCSF Genome Browser Feb. 2009 assembly (<http://genome.ucsc.edu>) showing the genomic region directly surrounding the top hit rs6072925 (indicated by black arrow), and seventeen SNPs in high LD with the top hit ($r^2 > 0.8$) (SNPs shown on top track), from the meta-analysis of a GWAS of % bleeding on probing in Health ABC Study ($n = 657$) and MrOS ($n = 422$); all SNPs in signal are located in an intron of *PTPRT* (protein tyrosine phosphatase, receptor type, T) (UCSC Genes); SNP rs6072936 (highlighted in black box) is the most well conserved SNP among 46 species of vertebrates (Multiz Alignments); also shown are two DNA methylation sites located near this signal (ENCODE/HudsonAlpha)

Additionally, a DNA methylation site was identified between two of the SNPs in this signal found to be eQTLs, rs6065565 and rs12625239 (ENCODE/HudsonAlpha) (see Figure 4.8). One of these SNPs, rs6065565, was identified as an eQTL for *RHOH* ($p = 2 \times 10^{-5}$) and *LTK* ($p = 10^{-4}$) in the HapMap CEU population. The other SNP, rs12625239, was identified as an eQTL for *ATPBD3* (ATP binding domain 3) (HapMap CEU, $p = 4 \times 10^{-5}$).

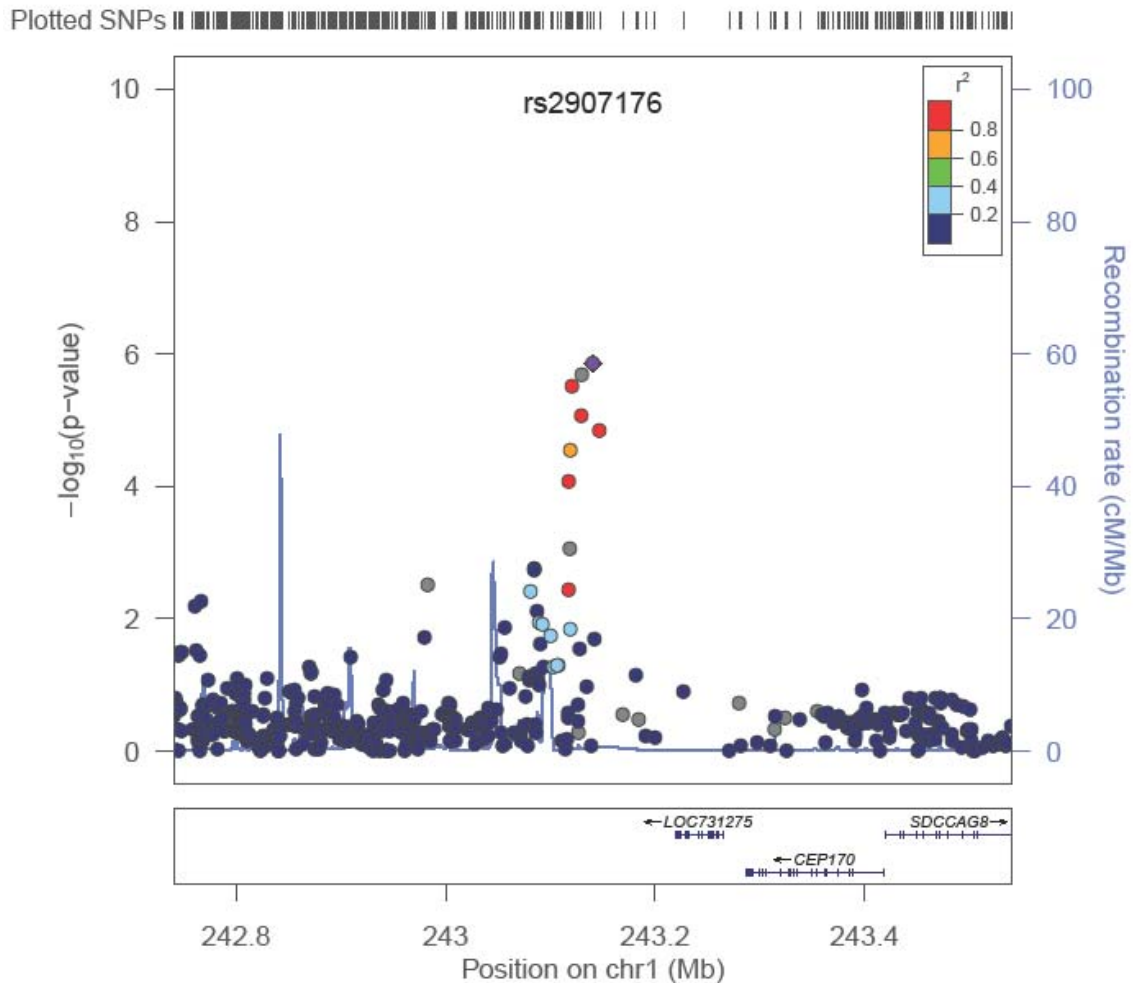
Chromosome 1p43 (rs2907176)

Another signal was detected on chromosome 1q43 that almost reached the significance threshold $p \leq 10^{-6}$ (see Figure 4.6). The most significant association in this signal was with rs2907176 ($p = 1.39 \times 10^{-6}$), located near a non-coding RNA, uncharacterized *LOC731275* (~80 kb upstream) (see Figure 4.9). Other nearby genes include *CEP170* (centrosomal protein 170kDa) (~150kb upstream from rs2907176), and *SDCCAG8* (serologically defined colon cancer antigen 8) (~280kb downstream from rs2907176).

Seven SNPs (all imputed) were found to be in high LD ($r^2 > 0.8$) with rs2907176 in our Caucasian populations (see Table 4.13). All seven SNPs showed concordant effect direction as rs2907176, and associated with % BOP, with p values ranging from 3.69×10^{-3} to 2.09×10^{-6} . The study specific GWAS of % BOP results for rs2907176 can be found in Table 4.14. The rs2907176 A allele was associated with a higher percentage of sites that bleed upon probing, compared to the G allele, in both the Health ABC Study and MrOS Caucasian populations. However the effect size relative to the standard error was much larger in the MrOS population, compared to the Health ABC Study population. Replication analyses in 4,063 ARIC Study Caucasians did not support the association

between rs2907176 and % BOP; additionally, no significant association was detected among 362 African Americans from the Health ABC Study.

Figure 4.9 Meta-analysis of results from genome-wide association studies of percent bleeding on probing in two Caucasian populations for the genomic region surrounding rs2907176



SNPs are plotted by chromosomal position against the association significance ($-\log_{10} p$) with % bleeding on probing (BOP) from the meta-analysis of the Health ABC Study Caucasian population ($n = 657$) with MrOS Caucasian men ($n = 422$); the most significant association is represented as a purple diamond, and is labeled; other SNPs are colored to reflect their LD with the most significant SNP (using pairwise r^2 values from the HapMap CEU population); estimated recombination rates (from HapMap) are plotted in cyan to reflect the local LD structure; positions of genes and exons, as well as the direction of transcription, are shown below the plots (using data from the UCSC Genome Browser GRCh37); plots were generated using LocusZoom

Table 4.13 SNPs in strong LD ($r^2 > 0.8$) with rs2907176 (*CEP170* gene region)

SNP	Chr	Position	Minor allele	MAF	LD with rs2907176	Meta p	Replication p	Conservation Score	ESPERR RP
rs3013386	1	243,117,502	T	0.25	0.91	8.27×10^{-5}	0.24	0	0.13
rs9428953	1	243,117,520	T	0.25	0.96	3.69×10^{-3}	0.27	NA	NA
rs4607871	1	243,119,539	A	0.30	0.85	2.79×10^{-5}	0.23	0	0.02
rs1081091	1	243,121,042	G	0.26	0.99	3.09×10^{-6}	0.21	0	0.05
rs10926938	1	243,129,559	G	0.29	0.83	8.72×10^{-6}	0.19	0	0.00
rs4037621	1	243,130,253	A	0.31	0.88	2.09×10^{-6}	0.20	0.004	NA
rs2907176	1	243,140,736	A	0.26	--	1.39×10^{-6}	0.26	0.98	0.14
rs12039141	1	243,147,031	G	0.29	1	1.39×10^{-5}	0.27	0	0.08

SNP characteristics including: rs number, chromosome (Chr), position (Genome build 37.3), nearby gene, minor allele frequency (MAF), linkage disequilibrium (LD) with rs2907176, association significance (p) with % sites that bleed upon probing from the meta-analysis of the Health ABC Study Caucasian population (n = 657) with MrOS Caucasian men (n = 422) (Meta p) and from the Caucasian ARIC Study population (n = 4,069) (Replication p), the predicted conservation score, and regulatory potential (ESPERR RP); *CEP170*: centrosomal protein 170kDa; sorted by genomic position

Table 4.14 Association of rs2907176 with percent bleeding on probing in four populations

Population	MAF	N	$\beta \pm SE$	p
Health ABC Study Caucasians (primary)	0.27	657	3.3 ± 1.0	1.2×10^{-3}
MrOS Caucasian men (primary)	0.26	422	8.0 ± 2.2	2.3×10^{-4}
ARIC Study Caucasians (replication)	0.28	4,063	-0.5 ± 0.4	0.26
Health ABC Study African Americans (replication)	0.23	362	-3.6 ± 2.9	0.20

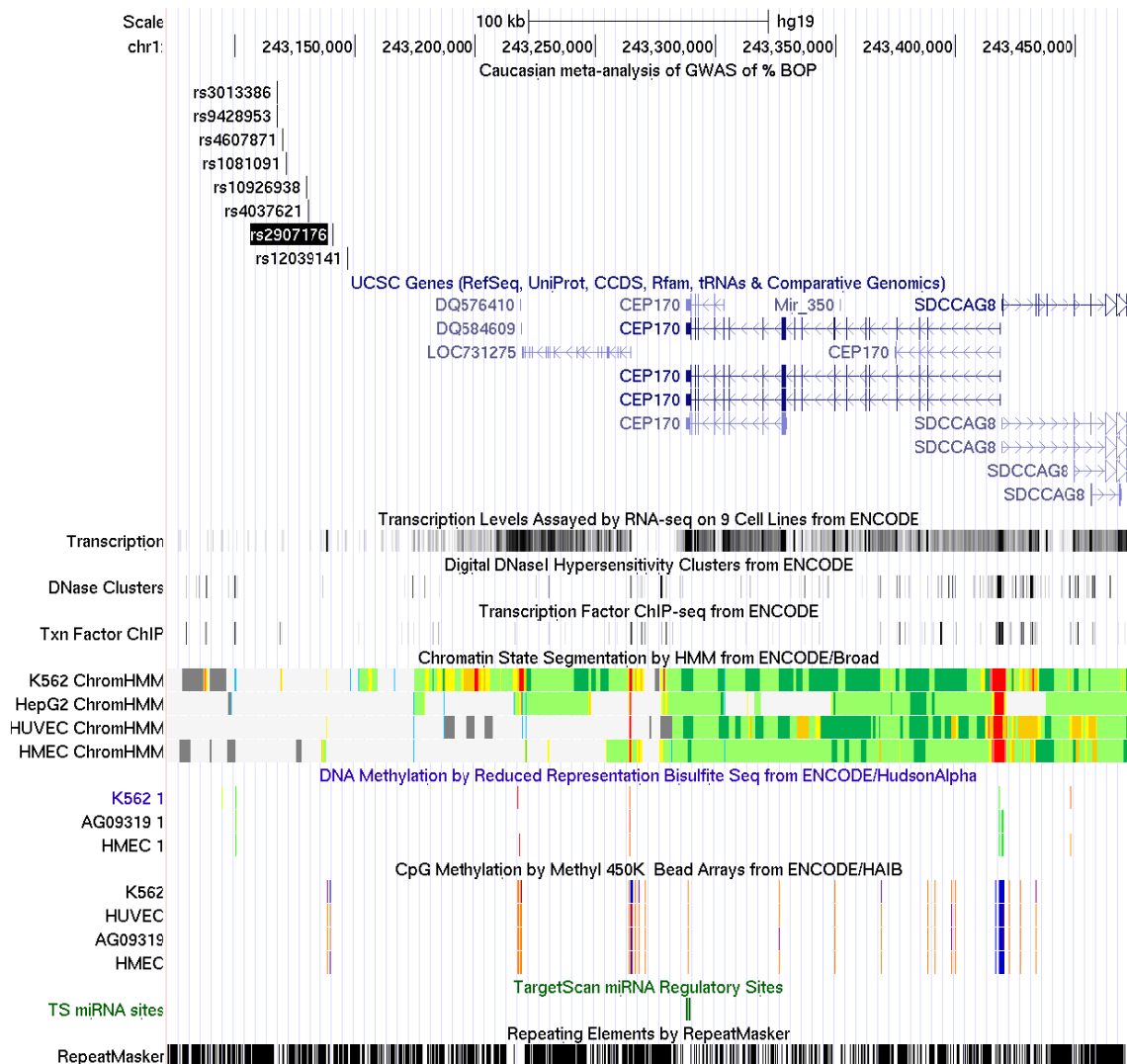
Minor allele frequency (MAF), sample size (N), effect size (β), standard error (SE), and SNP association significance (p) of rs2907176 with percentage of sites that bleed upon probing from the two primary GWAS populations (Health ABC Study and MrOS Caucasians) and two replication samples (ARIC Study Caucasians and Health ABC Study African Americans)

In silico analyses using the UCSC genome browser revealed additional genes near the identified signal including two pi-RNA genes, piR-42618 and piR-44522, and a predicted microRNA gene (Mir_350) (see Figure 4.10). A short transcribed RNA was also identified ~2 kB from rs2907176 (ENCODE RNA-seq) in a weakly conserved region. Overlapping this short RNA was a predicted weak enhancer region in endothelial cells (Chromatin State Segmentation by HMM) as well as a CpG island (Weizmann Evolutionary CpG islands). Transcription factor bindings sites were also identified nearby for transcription factors CEBPB (CCAAT/enhancer binding protein beta) and E2F6 (E2F transcription factor 6), between two SNPs in high LD with rs2907176 (rs9428953 and rs4607871). However, these bindings sites are only present in leukemia and hepatocellular carcinoma cells.

The SNP Function Prediction tool, part of SNPinfo Web Server, was used to predict the potential functional impact of these SNPs. SNP rs2907176 had the highest regulatory potential (RP) score predicted by the ESPERR Regulatory Potential score (Taylor et al. 2006) as well as the highest conservation score, generated using data from the Vertebrate Multiz Alignment & Conservation (17 species) (<http://genome.ucsc.edu/>).

Using the SCAN database, rs2907176 was identified as an eQTL for *CD84* (molecule CD84, chr 1q24, $p = 7 \times 10^{-5}$). Also, rs4607871 (LD with rs2907176 = 0.85) was identified to be an eQTL for *CD84* ($p = 1 \times 10^{-4}$). Another SNP in high LD, rs10926938 (r^2 with rs2907176 = 0.83), was identified as an eQTL for *C16orf67* (chromosome 16 open reading frame 67, $p = 1 \times 10^{-4}$).

Figure 4.10 Genomic region directly surrounding signal identified with percent bleeding on probing on chromosome 1q43

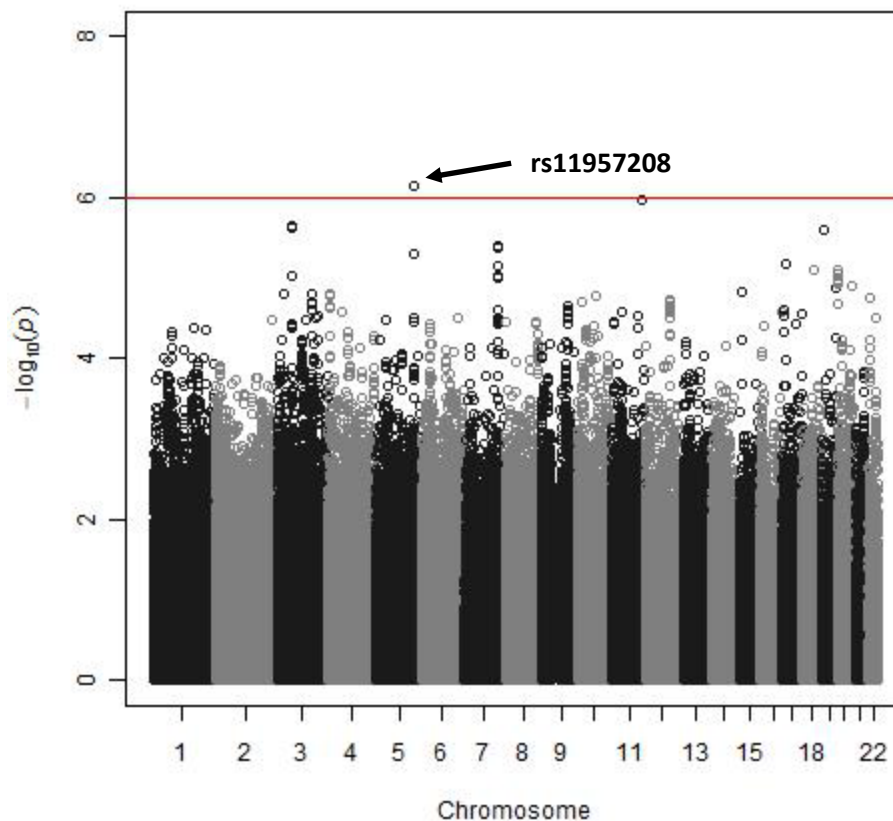


Screen shot from the UCSF Genome Browser Feb. 2009 assembly (<http://genome.ucsc.edu>) showing the genomic region directly surrounding the top hit rs2907176, and 7 SNPs in high LD with the top hit ($r^2 > 0.8$), identified by the meta-analysis of a GWAS of % bleeding on probing in the Health ABC Study ($n = 657$) and MrOS ($n = 422$) Caucasians (SNPs shown on top track); nearby features include piRNAs (DQ576410 and DQ584609), a non coding RNA (LOC731275), two centrosome-related genes (*CEP170* and *SDCCAG8*), a microRNA (Mir-350), a short transcribed region ~2kb from rs2907176 (RNA-seq), DNaseI hypersensitivity clusters, transcription factor binding sites (ChIP-seq), strong enhancers (orange), weak enhancers (yellow), insulators (blue), repressed DNA (grey) and transcribed DNA (green) in leukemia cells (K562), hepatocellular carcinoma cells (HepG2), endothelial cells (HUVEC), and epithelial cells (HMEC) (predicted by ChromHMM), histone modifications in K562, HUVEC, gingival fibroblasts (AG09319), and HMEC cells, DNA methylation (red = methylated, yellow = 50% methylated, green = unmethylated) and CpG methylation sites (orange = methylated, purple = partially methylated, bright blue = unmethylated) (ENCODE), as well as a predicted miRNA target site for *CEP170* (TargetScan)

4.4.3 Extent pocket depth (PD) $\geq 5\text{mm}$

One significant signal was detected on chromosome 5q32, by the meta-analysis of the GWAS of extent PD $\geq 5\text{mm}$ in 659 Health ABC Caucasians and 422 MrOS Caucasian men (see Figure 4.11). SNP rs11957208 was found to associate with extent PD $\geq 5\text{mm}$, $p = 7.20 \times 10^{-7}$. Additionally, there appear to be four other signals reaching similar significance on chromosomes 3 (rs1443941, $p = 2.26 \times 10^{-6}$), 7 (rs1008662, $p = 4.03 \times 10^{-6}$), 11 (rs12281864, $p = 1.05 \times 10^{-6}$) and 19 (rs3826810, $p = 2.53 \times 10^{-6}$), that did not meet the $p \leq 10^{-6}$ threshold.

Figure 4.11 Manhattan plot of results from the meta-analysis of genome-wide association studies of extent pocket depth $\geq 5\text{mm}$ in two Caucasian populations



Manhattan plot showing the negative log of p from the meta-analysis of 659 Health ABC Caucasians and 422 MrOS Caucasians, ordered by chromosomal position; red line indicates a significance (p) of 10^{-6}

The most significant SNP in the signal discovered on chromosome 5q32, rs11957208, and all four SNPs in high LD with rs11957208, are located within the genomic region encoding *PPP2R2B* (protein phosphatase 2, regulatory subunit B) (see Figure 4.12). The rs11957208 A allele was significantly associated with a higher percentage of sites probed with pocket depth ≥ 5 mm, compared to the G allele in the Health ABC Study Caucasian population ($\beta \pm SE = 0.23 \pm 0.04$; $p = 7.2 \times 10^{-8}$) (see Table 4.16). However, no significant association was observed in the MrOS Caucasian population ($\beta \pm SE = 1.35 \pm 1.11$; $p = 0.22$). Furthermore, the association between rs11957208 and extent pocket depth ≥ 5 mm was not supported by replication analyses in the ARIC Study Caucasian population ($n = 4,071$), or the Health ABC Study African American population ($n = 354$).

In silico analyses using the UCSC Genome Browser revealed the four SNPs in high LD with rs11957208 to be located within the same intron of *PPP2R2B* (see Figure 4.13). Additionally, one transcription factor binding site for CTCF was identified within the genomic region enclosed by rs11957208 and the other SNPs in high LD, in many cell types including epithelial and endothelial cells.

Table 4.15 SNPs in strong LD ($r^2 > 0.8$) with rs11957208 (*PPP2R2B* gene region)

SNP	Chr: Position	Minor allele	MAF	LD with rs11957208	Meta p	Replication p
rs1646420	5: 146,032,786	T	0.10	0.83	4.93×10^{-6}	0.9
rs460413	5: 146,034,535	A	0.10	0.83	3.19×10^{-5}	0.9
rs460781	5: 146,034,491	G	0.10	0.83	3.21×10^{-5}	0.9
rs461623	5: 146,035,556	C	0.10	0.83	3.57×10^{-5}	0.8
rs11957208	5: 146,048,748	A	0.09	--	7.20×10^{-7}	0.6

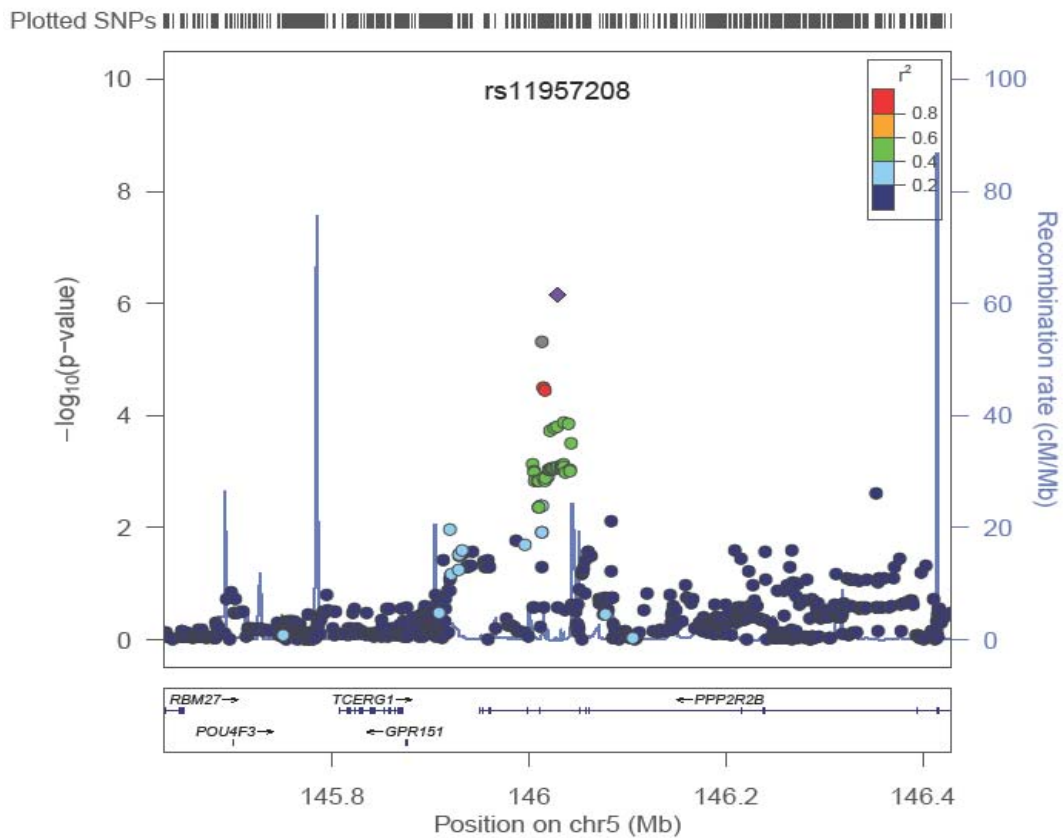
SNP characteristics including: chromosome (Chr), position (Genome build 37.3), minor allele frequency (MAF), linkage disequilibrium (LD) with rs11957208, association significance (p) with mean plaque index from the meta-analysis of the Health ABC Study Caucasian population ($n = 659$) with MrOS Caucasian men ($n = 422$) (Meta p) and from the Caucasian ARIC Study population ($n = 4,071$) (Replication p); sorted by genomic position; *PPP2R2B*: protein phosphatase 2, regulatory subunit B

Table 4.16 Association of rs11957208 with extent pocket depth ≥ 5 mm in four populations

Population	MAF	N	$\beta \pm SE$	p
Health ABC Caucasians (primary)	0.09	659	0.23 ± 0.04	7.2×10^{-8}
MrOS Caucasian men (primary)	0.09	866	1.35 ± 1.11	0.22
ARIC Caucasians (replication)	0.08	4,071	-0.01 ± 0.02	0.57
Health ABC AA (replication)	0.19	354	-0.02 ± 0.07	0.74

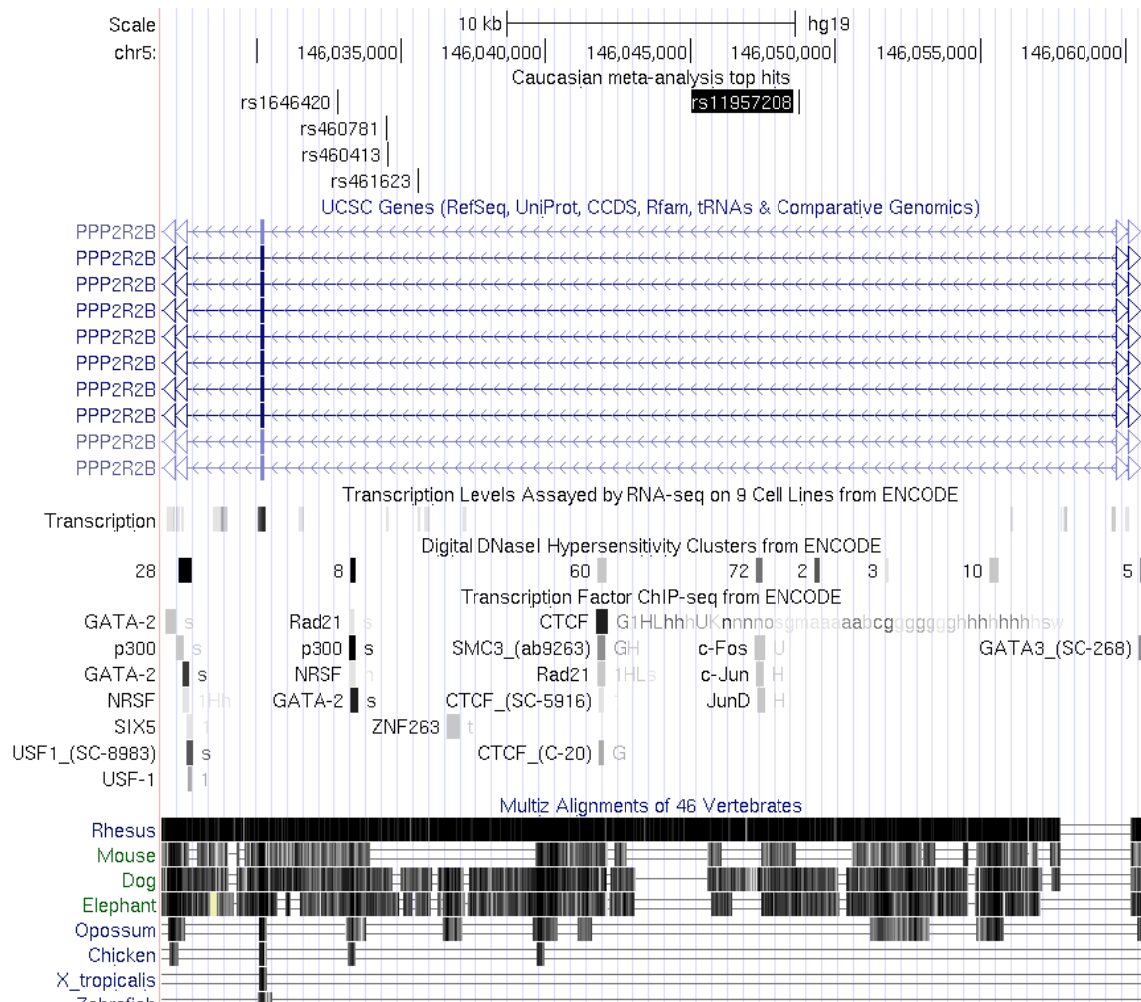
Minor allele frequency (MAF), sample size (N), effect size (β), standard error (SE), and SNP association significance (p) of rs11957208 with extent pocket depth ≥ 5 mm from the two primary GWAS cohorts (Health ABC Study and MrOS Caucasians) and two replication samples (ARIC Study Caucasians and Health ABC African Americans (AA))

Figure 4.12 Meta-analysis of extent pocket depth ≥ 5 mm from two Caucasian populations for the genomic region surrounding rs11957208



SNPs are plotted by chromosomal position against the association significance ($-\log_{10} p$) with extent pocket depth (PD) ≥ 5 mm from the meta-analysis of the Health ABC Study Caucasian population ($n = 659$) with MrOS Caucasian men ($n = 422$); the most significant association is represented as a purple diamond, and is labeled; other SNPs are colored to reflect their LD with the most significant SNP (using pairwise r^2 values from the HapMap CEU population); estimated recombination rates (from HapMap) are plotted in cyan to reflect the local LD structure; positions of genes and exons, as well as the direction of transcription, are shown below the plots (using data from the UCSC Genome Browser GRCh37); plots were generated using LocusZoom

Figure 4.13 Genomic region directly surrounding signal identified with extent pocket depth $\geq 5\text{mm}$ on chromosome 5q32



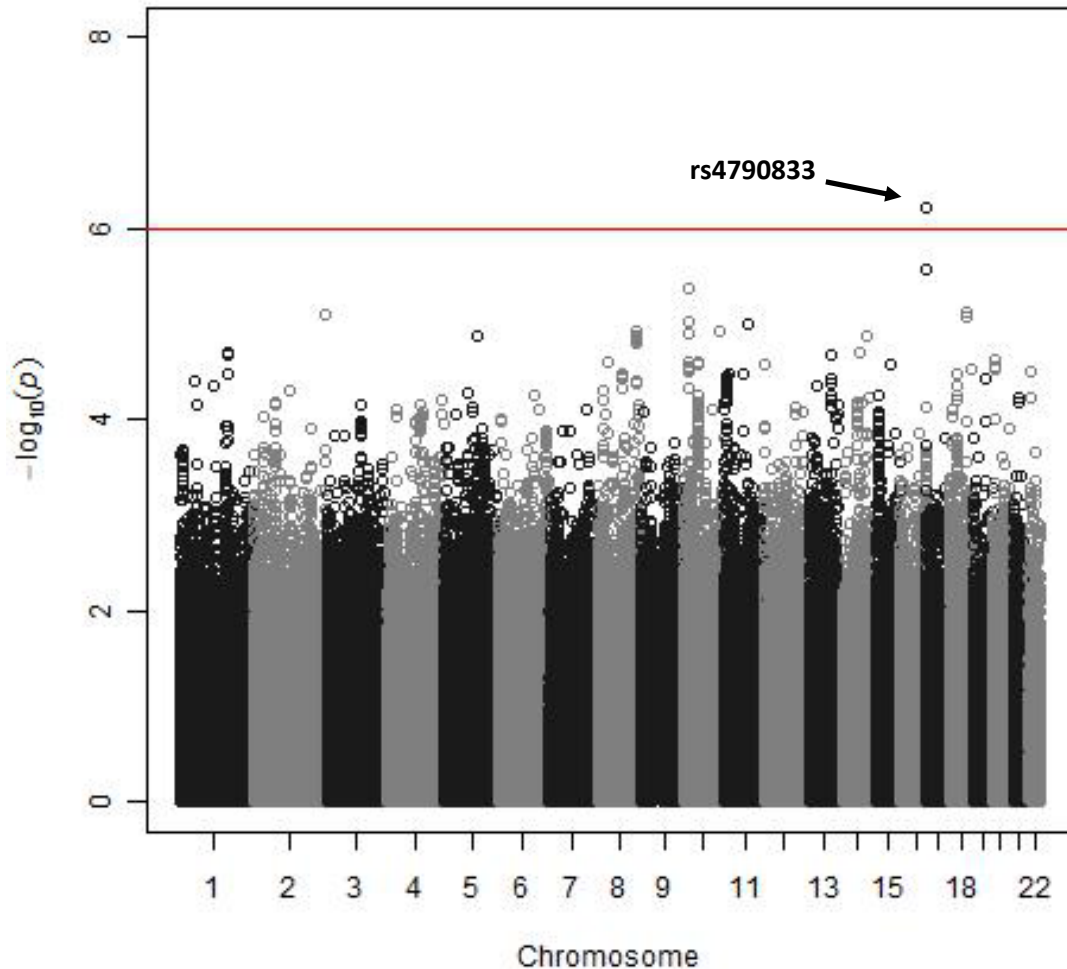
Screen shot from the UCSF Genome Browser Feb. 2009 assembly (<http://genome.ucsc.edu>) showing the genomic region directly surrounding the top hit rs11957208, and 4 SNPs in high LD with the top hit ($r^2 > 0.8$), identified by the meta-analysis of a GWAS of extent pocket depth $\geq 5\text{mm}$ in Health ABC Study ($n = 659$) and MrOS ($n = 422$) Caucasians (SNPs shown on top track); these SNPs lie within an intron of *PPP2R2B* (protein phosphatase 2, regulatory subunit B); a transcription factor binding site for CTCF in many cell types including epithelial and endothelial cells is also located between rs11957208 and rs461623

4.4.4 Extent attachment loss (AL) $\geq 3\text{mm}$

The results from the GWAS of extent AL $\geq 3\text{mm}$ are shown in Figure 4.14. One SNP on chromosome 17p13.3 is significantly associated ($p < 10^{-6}$) with extent AL $\geq 3\text{mm}$

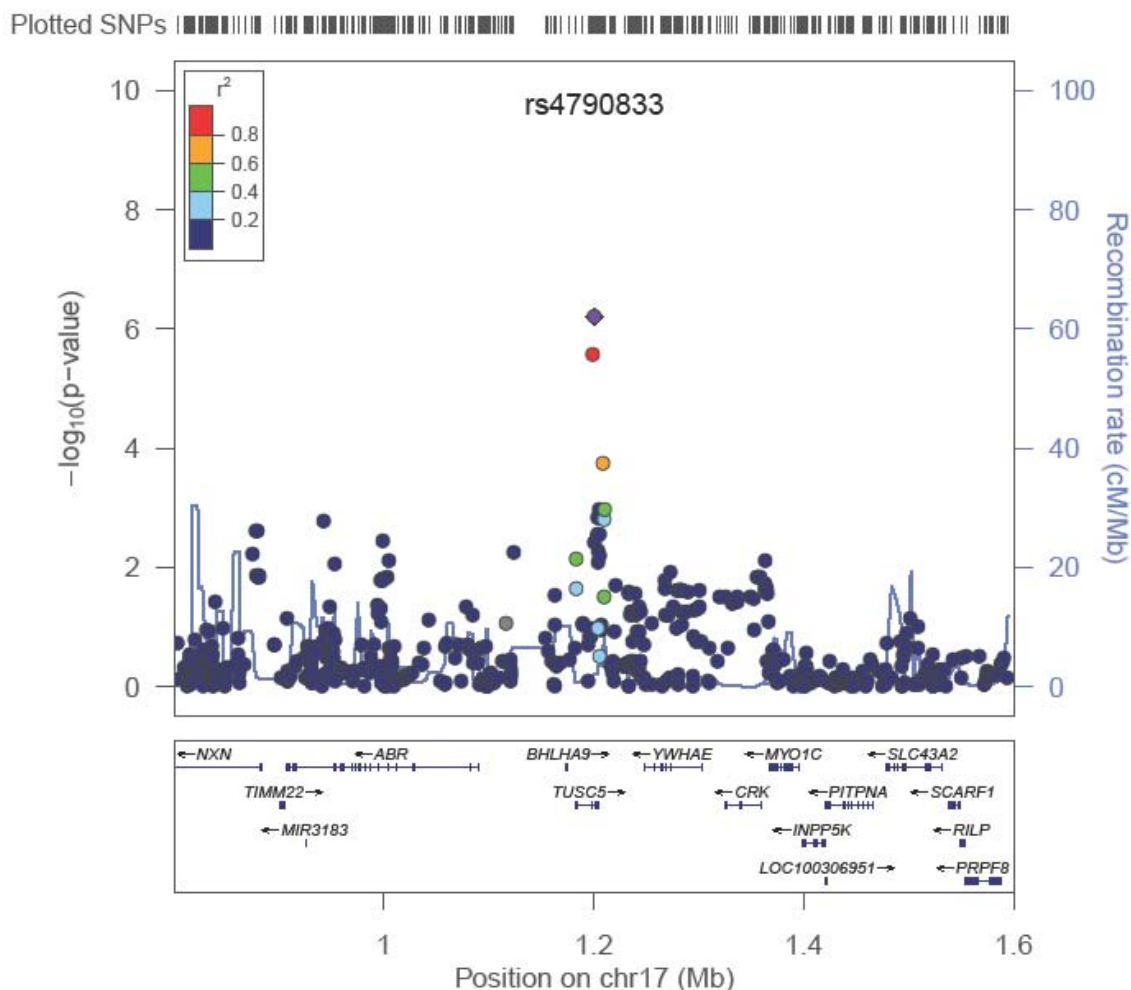
in the meta-analysis of GWAS from the Health ABC Study and MrOS Caucasian populations (rs4790833, $p = 6.16 \times 10^{-7}$). The results from the Caucasian meta-analysis for the genomic region surrounding SNP rs4790833 are presented in Figure 4.15. SNP rs4790833 is located in an intron of *TUSC5* (tumor suppressor candidate 5), and is in high LD with one other SNP, rs1472181.

Figure 4.14 Manhattan plot of results from a meta-analysis of genome-wide association studies of extent attachment loss ≥ 3 mm in two Caucasian populations



Manhattan plot showing the negative log of p from the meta-analysis of 655 Health ABC Caucasians and 422 MrOS Caucasians, ordered by chromosomal position; red line indicates a significance (p) of 10^{-6}

Figure 4.15 Meta-analysis of extent attachment loss ≥ 3 mm from two Caucasian populations for the genomic region surrounding rs4790833



SNPs are plotted by chromosomal position against the association significance ($-\log_{10} p$) with extent attachment loss ≥ 3 mm from the meta-analysis of the Health ABC Study Caucasian population ($n = 655$) with MrOS Caucasian men ($n = 422$); the most significant association is represented as a purple diamond, and is labeled; other SNPs are colored to reflect their LD with the most significant SNP (using pairwise r^2 values from the HapMap CEU population); estimated recombination rates (from HapMap) are plotted in cyan to reflect the local LD structure; positions of genes and exons, as well as the direction of transcription, are shown below the plots (using data from the UCSC Genome Browser); plots were generated using LocusZoom

The study specific results for rs4790833 are listed in Table 4.18. The A allele of rs4790833 is associated with significant effect sizes in the Health ABC Study Caucasian population ($\beta \pm SE = 6.3 \pm 1.5$, $p = 3.0 \times 10^{-5}$) and the MrOS Caucasian population ($\beta \pm$

SE = 4.3 ± 1.5 , $p = 4.7 \times 10^{-3}$). However, no significant replications were observed in either the ARIC Study Caucasian population ($n = 4,070$), or the Health ABC Study African American population ($n = 354$).

Table 4.17 SNPs in strong LD ($r^2 > 0.8$) with rs4790833 (*TUSC5* gene region)

SNP	Chr	Position	Minor allele	MAF	LD with rs4790833	Meta p	Replication p
rs1472181	17	1,198,499	C	0.29	0.84	2.63×10^{-6}	0.7
rs4790833	17	1,200,693	A	0.27	--	6.16×10^{-7}	0.9

SNP characteristics including: chromosome (Chr), position (NCBI build 36.1), minor allele frequency (MAF), LD with rs4790833, SNP association significance (Meta p) from the meta-analysis of Health ABC Caucasian population ($n = 655$) with MrOS Caucasian men ($n = 422$) for extent attachment loss ≥ 3 mm and replication results from ARIC Caucasians ($n = 4,070$) (Replication p); SNPs ordered by genomic location; *TUSC5*: tumor suppressor gene 5

Table 4.18 Association of rs4790833 with extent attachment loss ≥ 3 mm in four populations

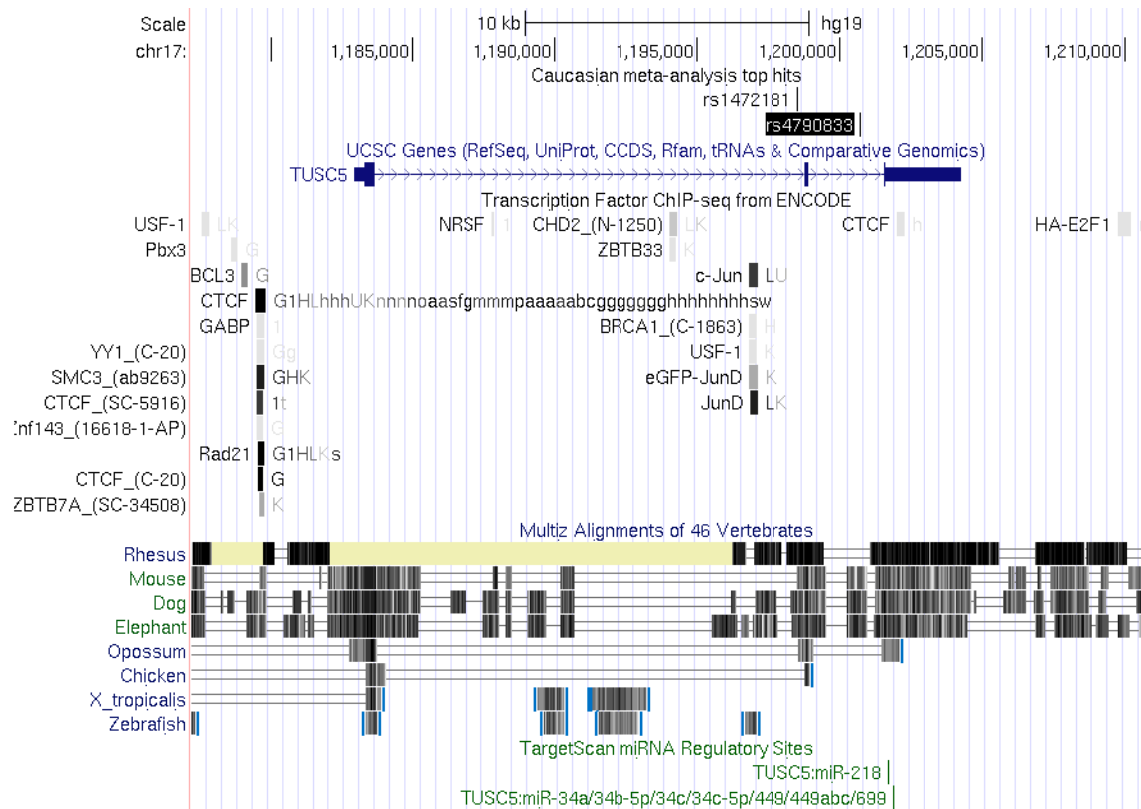
Population	MAF	N	$\beta \pm SE$	p
Health ABC Caucasians (primary)	0.27	655	6.3 ± 1.5	3.0×10^{-5}
MrOS Caucasians (primary)	0.26	422	4.3 ± 1.5	4.7×10^{-3}
ARIC Caucasians (replication)	0.25	4,070	-0.03 ± 0.58	0.96
Health ABC AA (replication)	0.24	354	2.0 ± 2.3	0.39

Minor allele frequency (MAF), sample size (N), effect size (β), standard error (SE), and rs4790833 association significance (p) from the two primary GWAS cohorts (Caucasians from the Health ABC Study (Health ABC) and MrOS) and the replication cohorts (ARIC Study Caucasians and Health ABC African Americans (AA)) with extent attachment loss ≥ 3 mm

In silico analysis using the UCSC Genome Browser to explore the identified genomic region revealed the second exon of *TUSC5* was flanked by the two SNPs in this signal, rs4790833 and rs1472181 (see Figure 4.16). Additionally, a transcription factor binding site for c-Jun (jun proto-oncogene) in endothelial cells (HUVEC) was

identified ~1.4kB away from this signal, and predicted microRNA binding sites were identified ~ 1Kb away from rs4790833 (TargetSCAN).

Figure 4.16 Genomic region directly surrounding signal identified with extent attachment loss $\geq 3\text{mm}$ on chromosome 17p13.3



Screen shot from the UCSF Genome Browser Feb. 2009 assembly (<http://genome.ucsc.edu>) showing the genomic region directly surrounding the top hit rs4790833 (boxed in black), and one SNPs in high LD with the top hit ($r^2 > 0.8$) (SNPs shown on top track), from the meta-analysis of a GWAS of extent attachment loss $\geq 3\text{mm}$ in the Health ABC Study ($n = 655$) and MrOS ($n = 422$) Caucasian populations; these SNPs are near the second exon of *TUSC5* (tumor suppressor candidate 5); other nearby features include the presence of a c-Jun transcription factor binding site in endothelial cells (L), and predicted microRNA binding sites

CHAPTER 5

DISCUSSION

5.1 Defining periodontal diseases using continuous outcomes

Throughout the past few decades, many definitions of periodontal diseases have been used in epidemiological studies (Carlos et al. 1986; Borrell and Papapanou 2005; Amir Savage et al. 2009; Preshaw 2009). Pocket depth (PD), attachment loss (AL), and bleeding on probing (BOP) are the most common clinical measures used to investigate the risk factors of periodontal diseases (Page and Eke 2007; Amir Savage et al. 2009). Many studies have used various thresholds of PD and AL to separate periodontitis cases from healthy controls, while other studies have used PD, AL, and BOP measurements as continuous outcomes (Diehl et al. 2005).

In our investigation of the risk factors for periodontal diseases, we chose to use the following four clinical measurements as continuous outcomes: plaque index, bleeding on probing, PD, and AL. The reason we chose to use continuous outcomes, rather than dichotomizing our populations into cases and controls, is because periodontal diseases progress in a continuous manner, affecting almost every individual to some degree. Therefore, continuous outcomes may better reflect the etiology of periodontal diseases. Additionally, clinical measurements are usually collected throughout the mouth during a periodontal examination; however, many of these measurements are disregarded when the population is divided based on an arbitrary threshold defining periodontitis cases or healthy controls. This transformation not only decreases the resolution of the initial clinical measurements, but can also lead to misclassification. For instance, using the latest accepted definitions of periodontal diseases, from the Centers of Disease Control (CDC) and American Academy of Periodontology (AAP), subjects are defined with either severe periodontitis (≥ 2 interproximal sites with $AL \geq 6\text{mm}$ and ≥ 1 interproximal sites with $PD \geq 5\text{mm}$), moderate periodontitis (≥ 2 interproximal sites with $AL \geq 4\text{mm}$ or ≥ 2 interproximal sites with $PD \geq$

5mm), or healthy (neither severe nor moderate disease). Using this classification system, a person with two sites with AL of 4mm will be considered to have “moderate” periodontitis, while a person with only one site with AL of 4mm will be considered “mild” or a non-case; however, these two people have very similar extent and severity of periodontitis. By using a continuous periodontal phenotype, the extent of periodontal diseases will be retained for each person, reducing the misclassification bias created using an arbitrarily defined case-control definition.

The continuous phenotypes investigated in this study representing periodontitis include extent PD $\geq$ 5mm and extent AL $\geq$ 3mm. We decided to look at the extent of disease, as opposed to the mean PD or mean AL, so that we could choose a disease threshold and observe how common the disease is in each person; as opposed to the average level of disease per person. Using the average level of disease, a person with one site with AL of 12mm will have the same average level of AL as someone with 12 sites with AL of only one millimeter. By using extent PD and extent AL, we can choose thresholds of disease (PD $\geq$ 5mm, AL $\geq$ 3mm) that reflect periodontitis. We can then calculate the extent of disease per person, as opposed to trying to classify the person as diseased or healthy.

These disease thresholds could be modified to reflect moderate or more severe periodontal diseases. In summary, rather than trying to agree on a periodontitis case definition, it may be more desirable for scientists to form a consensus on a disease threshold (i.e. PD $\geq$ 5mm; AL $\geq$ 3mm), and then use this threshold to create a continuous outcome, representing the percent of sites meeting the disease threshold.

5.2 Epidemiological analysis of risk factors

In the Health ABC Study and MrOS populations, we investigated the risk of the periodontal disease-related outcomes: mean plaque index, % bleeding on probing (BOP), extent pocket depth (PD) ≥ 5 mm, and extent attachment loss (AL) ≥ 3 mm, with many known and potential risk factors, including: dental hygiene variables, smoking habits, race, sex, age, education, fasting glucose levels, BMI, and serum levels of vitamin D.

Periodontal traits were accessed using full-mouth exams in the Health ABC Study population; however, half-mouth exams were used to collect periodontal disease-related traits in the MrOS population. Half-mouth exam scores were created in the Health ABC Study by randomly masking half of the mouth scores so that we could compare the prevalence of periodontal diseases between the Health ABC Study and MrOS populations.

Another difference in the collection of periodontal disease-related traits between the Health ABC Study and MrOS was that the Health ABC Study rounded periodontal probing depths down to the nearest lower millimeter, while MrOS periodontal probing depths were rounded up to nearest higher millimeter. Rounding probing depths down in the Health ABC Study resulted in probing depths recorded as 5mm representing actual probing depths ranging from 5mm to 6mm. While, rounding up in the MrOS population resulted in recorded probing depths of 5mm representing actual probing depths ranging from 4mm to 5mm. Therefore, it is not unexpected that higher levels of periodontal diseases are observed in the MrOS population. We also compared the extent of PD ≥ 6 mm and AL ≥ 4 mm in MrOS with the Health ABC Study Caucasian population, to try and adjust for the rounding differences; however this resulted in much lower levels of periodontal disease-related traits in MrOS Caucasian men compared to the Health ABC

Study Caucasian men (data not shown). These differences in the prevalence of periodontal diseases between studies may result from the differences in phenotype collection, as well as population differences. The Health ABC Study included men aged 70 to 80 years old, while MrOS included men aged 60 to 94 years old. Age has been observed to be a significant risk factor for periodontal diseases in other study populations (Borrell et al. 2005), which may explain why we observed significantly lower levels of periodontal disease in the Health ABC Study Caucasian men compared to MrOS Caucasian men. However, the average age of the Health ABC Study and MrOS populations were both ~ 74 years old. These differences between data collection and study populations complicate the comparison of periodontal disease-related traits (and risks) between the Health ABC Study Caucasian population and MrOS Caucasian population.

Differences in the collection of periodontal disease-related outcomes, such as those described above, are common problems complicating the comparison of epidemiological investigations of periodontal diseases (Eke et al. 2010). Different methods of collecting periodontal clinical measurements, including different periodontal probes, differences in sites probed, and differences in the direction of rounding periodontal probing depths, have been observed to impact the prevalence, and in turn the risk of periodontal diseases associated with various risk factors (Amir Savage et al. 2009). Standardizing the collection of clinical measurements for epidemiological investigations of periodontal diseases, as well as definitions of periodontal disease-related outcomes, will be important steps to minimize differences between studies, allowing for more straight-forward comparisons and interpretation of results between different studies.

The following sections discuss the results from each of the risk factors for the four periodontal disease-related outcomes (mean plaque index, % BOP, extent PD $\geq$ 5mm, and extent AL $\geq$ 3mm), investigated in the Health ABC Study African Americans, the Health ABC Study Caucasians, and the MrOS Caucasian population. The risk factors are ordered based upon perceived impact on risk of periodontal diseases from greatest to least, based on the results from this investigation as well as previous studies.

5.2.1 Mean plaque levels

High plaque levels were expected to be one of the largest risk factors for periodontal diseases, thus we thought it would be important to investigate the risk factors for plaque levels. Therefore, we investigated mean plaque levels as an outcome in our association models, and as a risk factor for % BOP (representing gingivitis) as well as for extent PD $\geq$ 5mm and extent AL $\geq$ 3mm (representing periodontitis).

As expected, higher plaque levels were the second largest risk factor (after the observer effect) for % BOP, extent PD $\geq$ 5mm, and extent AL $\geq$ 3mm in our study populations. Exposure to plaque is known to stimulate gingivitis (Lang et al. 1973), and chronic exposure to gingivitis can lead to irreversible forms of periodontal disease, characterized by deep pocket depths and attachment loss of the periodontal ligament (Axelsson 2002). The amount of plaque on the teeth explains more variance of gingivitis and periodontitis outcomes in our study populations than other known risk factors such as race, sex, smoking habits, or fasting glucose levels. Therefore, promoting good dental hygiene and the consistent removal of dental plaques appears to be the most effective way to reduce the risk of periodontal diseases.

5.2.2 Frequencies of flossing, dental visits, and brushing

Plaque levels measured at one specific time point may not accurately reflect the typical amount of plaque on the teeth. To capture plaque exposure over time, frequencies of flossing, dental visits, and brushing were included, in addition to mean plaque index, as risk factors in our analyses of gingivitis and periodontitis traits: % BOP, extent PD $\geq$ 5mm, and extent AL $\geq$ 3mm. Frequency of flossing was one of the most consistent risk factors for mean plaque levels in our study populations. Particularly, flossing more than twice a week (compared to less than once a week) was associated with lower plaque levels.

Frequency of flossing less than once a week was a significant risk factor for increased % BOP, a measure of gingivitis, even after adjusting for other risk factors, such as mean plaque index. Gingivitis is a reversible condition that is highly associated with current levels of plaque; therefore, it is not surprising that increasing the frequency of flossing would decrease the risk of gingivitis. Frequency of flossing was also a significant risk factor for extent PD $\geq$ 5mm and extent AL $\geq$ 3mm; however, after adjusting for other risk factors, such as mean plaque levels, this risk factor was dropped from the backwards stepwise regression analyses.

Visiting the dentist more than once a year was significantly associated with lower plaque levels, as well as a lower extent AL $\geq$ 3mm (compared to less than one dental visit per year). It is expected that more dental visits would be associated with lower levels of periodontal diseases, as prevention and treatment have been observed to be effective in reducing the incidence of periodontal diseases (Axelsson 2002).

It is surprising that brushing at least twice a day was not associated with a significant decrease in % BOP, extent PD $\geq$ 5mm, or extent AL $\geq$ 3mm, compared to

brushing less than twice a day. Higher frequencies of brushing were associated with lower plaque levels; however, this relationship was much weaker than the relationships between plaque levels and frequencies of flossing and dental visits.

These results suggest that frequent flossing (more than twice a week) and frequent dental visits (more than once a year) may be important to reduce plaque levels, gingivitis, and periodontitis, in addition to frequent brushing (at least twice a day). Brushing twice a day, with a fluoride toothpaste, has been observed to be very important in the prevention of caries (Kidd 2011); however, the combination of brushing twice a day and flossing more than twice a week may be a more effective strategy to reduce the prevalence of periodontal diseases (Sambunjak et al. 2011).

5.2.3 Dental examiner

Surprisingly, one of the largest “risk factors” for periodontal diseases was a covariate in our regression model representing the examiner that performed the periodontal exams. Seven examiners collected periodontal data in the Health ABC Study, and six examiners collected periodontal data for the MrOS participants. Dental examiners were trained and calibrated to attempt to minimize the differences between examiners, as past studies have observed the need for examiner calibration (Hefti and Preshaw 2012). Despite all attempts to train and calibrate the periodontal examiners, we still observed large differences between examiners.

A covariate was added to the linear regression models of periodontal disease-related outcomes to adjust for which examiner had performed the exam. This dental examiner variable explained more variance than any other risk factor for mean plaque levels, and $AL \geq 3\text{mm}$, and a similar amount of variance as other largest risk factor (mean plaque levels) for % BOP and $PD \geq 5\text{mm}$. When multiple regression analyses

were performed without the dental examiner variable, the resulting effect sizes were smaller relative to the standard error of the measurement compared to analyses including the dental examiner variable (data not presented). This finding highlights the value of including a dental examiner covariate in association analyses of periodontal diseases. Additionally, it suggests there is a need for more reliable measurements, as the large inter-examiner effects may bias the effect estimate of other risk factors on periodontal disease-related outcomes towards the null hypothesis of no effect.

5.2.4 Race

As observed in past studies (Borrell and Crawford 2008), race was a significant risk factor for periodontal disease-related traits in our study populations. African Americans had more severe measures of periodontal diseases compared to Caucasians, including: higher mean plaque levels, % BOP, extent PD $\geq$ 5mm, and extent AL $\geq$ 3mm. In general African Americans also had higher levels of risk factors for periodontal diseases, such as higher plaque levels, lower levels of education, a higher percentage of current smokers, higher fasting glucose levels, and lower frequencies of brushing, flossing, and dental visits, compared to Caucasians. These elevated risks observed in the Health ABC African American population most likely contribute to the higher levels of periodontal diseases observed in the Health ABC Study African American population, compared to the Health ABC Study Caucasian population. However, even after adjusting for these risk factors, race remained a significant risk for periodontal disease-related outcomes in race-combined analyses (data not presented). Race may represent some unobserved environmental or social factors, such as socioeconomic factors affecting access to dental care (Albandar 2002; Schrimshaw et al. 2011). Additionally, it is possible that race represents some underlying genetic

predispositions to periodontal diseases that may be more common among African Americans in our population.

5.2.5 Sex

Men had higher levels of periodontal disease-related outcomes compared to women. The largest differences observed differences between the sexes were with extent PD $\geq$ 5mm, and extent AL $\geq$ 3mm. Men also had higher levels of many periodontal disease risk factors, such as increased smoking habits, higher fasting glucose levels, and lower frequencies of brushing, flossing, and dental visits, compared to women. These risk factors likely contribute to the higher levels of periodontal diseases observed in men; however, even after adjusting for these risk factors, we still observed sex as a significant risk factor for periodontitis traits PD $\geq$ 5mm and AL $\geq$ 3mm. Sex may represent some unaccounted environmental exposures, or it may represent a biological predisposition to periodontal diseases among males. Studies have suggested that the differences in periodontal disease prevalence observed between men and women may be due to higher levels of inflammation in response to infection and injury that have been observed in men, possibly related to sex hormones (Shiau and Reynolds 2010).

5.2.6 Education

Lower levels of education were significantly associated periodontal disease-related outcomes in univariate analyses; however, after adjustment for other risk factors this relationship became much weaker in the Health ABC African American population, and became insignificant in the Caucasian populations. For instance, African Americans that did not graduate from high school had significantly higher plaque levels, and more periodontitis (measured by extent PD $\geq$ 5mm), compared to those that graduated high

school, and those that attended some post secondary school. However, these relationships were not significant in the Caucasian populations after adjusting for other risk factors.

There were a higher percentage of African Americans who did not graduate from high school (42% of men and 30% of women) compared to Caucasians (11% of men and 7% of women, from Health ABC). Therefore, it may be possible that we did not observe a significant portion of the variance of periodontal disease-related outcomes explained by low education levels in Caucasians due to the lower frequency of low education levels compared to the African American population. However, it is also possible that education represents some other social or economic risk factors that are correlated with education level, such as dental hygiene and access to dental care. Regardless, education explained a significant amount of the variance of periodontal disease-related outcomes in univariate analyses, and may be an important factor to consider when identifying individuals with an increased risk for periodontal diseases.

5.2.7 Smoking habits

Many studies have investigated the risk of periodontal diseases associated with smoking by including a variable representing current smoker, past smoker, or never smoking status, while other studies have included cigarette packs smoked per year as a variable to represent the exposure of smoke in current smokers (Tonetti 1998). We included both variables in our investigation of risk factors for periodontal diseases in order to observe the risk associated with current smoking and past smoking compared to never smoking, as well as the risks of periodontal diseases associated with increased exposure to cigarette smoking.

The numbers of cigarette packs smoked per year was significantly associated with increased periodontitis, measured by extent AL $\geq$ 3mm, in all three of the study populations, and with extent PD $\geq$ 5mm in the MrOS population. This is expected as past studies have also found a significant increase in pocket depth and attachment loss in current smokers, compared to non-smokers (Tomar and Asma 2000).

Current smoking status was a significant risk factor for decreasing % BOP, increasing extent PD $\geq$ 5mm, and increasing extent AL $\geq$ 3mm. This is expected, as smoking has been associated with an increased number of destructive enzymes, and decreased function of neutrophil migration into the periodontal tissues (Ryder 2007). Decreased neutrophil function in response to cigarette smoke may explain the decreased gingival bleeding that we observed in current smokers compared to never smokers. Additionally, a decreased immune response to periodontal infection may also lead to the increasing number of PD $\geq$ 5mm, and AL $\geq$ 3mm observed in current smokers compared to never smokers.

In summary, current smoking status and number of packs per year appear to be significant risk factors for periodontitis in our study populations. However, no significant relationships were observed between former smokers and periodontal disease-related traits in our study populations.

5.2.8 Age

Age has been observed to be a significant risk factor for periodontal diseases in other study populations (Borrell et al. 2005); however, this association may be due to the increased exposure to many risk factors over time, rather than age itself (Axelsson et al. 1991). No significant associations between older age and gingivitis (% BOP) or periodontitis outcomes (extent PD $\geq$ 5mm and extent AL $\geq$ 3mm) were detected, after

adjusting for other risk factors. Interestingly, we did detect older age as a risk factor for higher plaque levels in the MrOS Caucasian men. This relationship was not observed in either Health ABC Study population, which may be because there was a much narrower age range sampled in the Health ABC Study (70 – 80 years), compared to the MrOS population (60 – 94 years).

It is not clear why older people would have higher plaque levels. It is possible that removing plaque becomes more difficult with old age, or perhaps there are some other biological reasons why older people in the MrOS cohort tended to have higher plaque levels. For instance, there are many proteins in human saliva that can inhibit the colonization of periopathogens such as *P. gingivalis* (Stinson et al. 1992); perhaps these proteins are less functional, or produced at lower levels, in older people.

5.2.9 Serum levels of vitamin D

Lower serum levels of vitamin D were significantly associated higher plaque levels in the African American and Caucasian Health ABC Study populations, even after adjusting for all other covariates. In the Health ABC Study African American population, lower serum levels of vitamin D explained 2% of the variance of mean plaque levels in univariate analyses, more than any of the following risk factors: frequency of brushing (1.9%), drinking status (1.8%), smoking status (1.1%), age (0.4%), packs per year (0.3%), BMI (0.4%), or fasting glucose (0.1%). In the Health ABC Caucasian population, serum levels of vitamin D explained 1.3% of the variance of mean plaque levels in univariate analysis, more than any other risk factor other than: dental examiner (20.1%), frequency of dental visits (6.1%), frequency of flossing (4.7%), and frequency of brushing (2%), in univariate analyses. After adjustment for other risk factors, low serum levels of vitamin D remained significant. Low serum levels of vitamin D were also

associated with lower mean plaque levels in the MrOS population in univariate analyses ($p = 0.03$); however, after adjustment for other risk factors this relationship was no longer significant and the variable was dropped from the backwards stepwise regression analysis.

Vitamin D has been seen to modulate innate and adaptive immune responses to infection (Kamen and Tangpricha 2010). Getting enough vitamin D, which can be obtained from the diet or sun exposure, may boost the natural defenses against plaque. Lower serum levels of vitamin D have also been associated with higher levels of attachment loss (Dietrich et al. 2005); however, no significant relationships were observed between serum levels of vitamin D and gingivitis or periodontitis outcomes in our study populations.

5.2.10 Fasting glucose

Surprisingly, fasting glucose levels were not strong predictors of periodontal disease-related traits in our study populations. Past studies have observed a strong relationship between diabetes status and periodontal diseases (Taylor et al. 1996; Taylor 2001). The association is thought to arise from the formation of advanced glycation end products (AGEs) due to the chronically elevated blood glucose levels, which results in an increase in systemic inflammation (Schmidt et al. 1996). Fasting glucose levels were weakly associated with higher plaque levels in the Health ABC Study Caucasian population; however, we did not observe fasting glucose levels as a significant risk factor for gingivitis (% BOP) or periodontitis (extent PD ≥ 5 mm, extent AL ≥ 3 mm) outcomes. The lack of association between fasting glucose levels and periodontal diseases may not reflect the true risk of periodontal diseases associated with diabetes, but rather the transient nature of fasting glucose levels.

The American Diabetes Association recommended the new diagnostic measurement for diabetes to be serum glycated hemoglobin A1c (HbA1c) levels $\geq$ 6.5% (ADA 2010), as HbA1c levels reflect 2-3 months of fasting glucose levels. We performed secondary analyses including a subset of the Health ABC Study population that also had HbA1c measurements, and investigated the risk of periodontal diseases associated with HbA1c. We found that higher HbA1c levels were associated with higher levels of plaque and % BOP in both the African American and Caucasian populations, even after adjusting for all other covariates. HbA1c explained more variance than fasting glucose levels for all four periodontal disease outcomes, in univariate analyses. However, HbA1c explained more variance in the African American population, compared to the Caucasians. This is likely due to the higher percentage of diabetics in the African American population compared to the Caucasian population. This finding supports the idea that HbA1c may be a better proxy for diabetes than fasting glucose levels, as it reflects a much longer exposure to high glucose levels.

5.2.11 Drinking habits

The risks of periodontal diseases associated with drinking alcoholic beverages estimated by previous studies are inconsistent (Amaral et al. 2009). We investigated the risk of drinking alcoholic beverages in our studies; however, the different data collection methods of drinking habits between the Health ABC Study and MrOS complicate the comparison of results from the two studies. In the Health ABC Study, drinking statuses were reported as either: never drinker, former drinker, or current drinker. While in MrOS, the average number of alcoholic drinks per week was collected to assess drinking habits.

No consistent associations with drinking habits were observed in our studies of periodontal disease-related traits. Drinking alcoholic beverages may increase the risk of periodontal diseases; however, the results from this study do not provide substantial evidence indicating that drinking alcohol is a significant risk factor for periodontal diseases. It is possible that other risk factors not included in our analyses may correlate with drinking status and explain the weak association of drinking status with periodontal disease-related traits.

5.2.12 BMI

Obesity has been associated with a low-grade inflammatory state (Illan-Gomez et al. 2012). However, results from this study do not support BMI (as a measure of obesity) as a significant risk predictor for any of the periodontal disease-related traits. This is not unexpected, as past studies have seen inconsistent associations between BMI and periodontal diseases (Borrell et al. 2005; Genco et al. 2005).

5.2.13 Summary of risk factor analyses

In summary, high plaque levels appear to be the largest modifiable risk factor for gingivitis as measured by % BOP, and for periodontitis as measured by extent PD $\geq$ 5mm and extent AL $\geq$ 3mm. Less frequent flossing appears to be the largest modifiable risk factor for higher plaque levels. Not only was a higher frequency of flossing significantly associated with lower mean plaque levels, it was also significantly associated with lower levels of gingivitis, even after adjusting for mean plaque index and other risk factors.

Therefore, removing plaques, especially through flossing, may be the most effective way to reduce gingivitis, and in turn, periodontitis. Education programs and

reminders at dental visits emphasizing the importance of flossing, in addition to brushing, to remove plaque may be an effective strategy for the prevention of periodontal diseases. Reinforcing the importance of dental visits and plaque removal to smokers, especially heavy smokers, men, and African Americans will be important in the prevention of periodontal diseases, as they appear to be particularly susceptible to periodontal diseases.

Additionally, there were large inter-examiner effects on periodontal disease-related outcomes between dental examiners in our study populations. Therefore, adjusting for the dental examiner in large epidemiological studies of periodontal diseases with many dental examiners may be an important step in estimating the risk of periodontal diseases, or before investigating the risk of other outcomes associated with periodontal disease-related traits, such as cardiovascular diseases.

5.3 Genome-wide association study

Meta-analysis of GWAS results from four periodontal disease-related traits (mean plaque index, % BOP, extent PD $\geq$ 5mm, and extent AL $\geq$ 3mm) in the Health ABC Study and MrOS Caucasian populations detected four significant ($p \leq 10^{-6}$) signals. None of these signals were supported by replication studies in ~4,000 ARIC Study Caucasians or 500 Health ABC Study African Americans. However, *in silico* analyses support a potential role for three of the top signals in immune function. The following sections discuss these top signals, replication results, and results from *in-silico* analyses of the genomic regions surrounding these signals.

5.3.1 Mean plaque index: chromosome 22q13.31 (rs739270)

The signal identified on chromosome 22q13.31 (rs739270), significantly associating with mean plaque index ($p < 10^{-6}$), is supported by findings from *in silico* analyses of this genomic region. However, replication studies do not support the association between rs739270 and mean plaque index.

In silico analyses reveal that rs739270 and a SNP in high LD, rs5765463, flank an exon of *fibulin 1*. *Fibulin 1* encodes a glycoprotein important in basement membranes and elastic fibers (Argraves et al. 2003). Additionally, streptococci bacteria have been observed to bind fibulin 1 in host tissues (Courtney et al. 2009). Interactions between bacteria and extracellular matrix proteins such as fibulin 1 have been hypothesized to initiate bacterial adhesion to the extracellular matrix.

Five different splice variants of *fibulin1* have been identified. It is possible that one or more of the SNPs in the identified signal affect the splicing of *fibulin 1*, or are associated with a particular splice variant. Furthermore, different splice variants of *fibulin 1* might be associated with different rates of bacterial adhesion and biofilm formation, leading to varying degrees of risks of periodontal diseases.

Interestingly, fibulin 1 is also essential for the morphology of endothelial cells lining capillary walls and the integrity of small blood vessels, and is hypothesized to play a role in cardiovascular disease progression (Argraves et al. 2009).

The replication studies in the ARIC Study Caucasians were 80% powered to detect a change in mean plaque index as small as $\beta = 0.05$ with rs739270, and as small as $\beta = 0.12$ in the Health ABC African Americans ($p = 0.05$). It is possible that although larger effect sizes were initially detected in the Health ABC Study and MrOS Caucasian populations ($\beta \pm SE = 0.16 \pm 0.03$ and 0.11 ± 0.05 , respectively), the true effect of the

SNP is much weaker than what was detected in our primary study populations. As this identified signal is located within a promising candidate gene (*fibulin 1*), additional replication studies will be sought to verify whether genetic variants in this genomic region are indeed associated with plaque levels.

5.3.2 Percent bleeding on probing: chromosome 20q12 (rs6072925)

Although the association of rs6072925 with the percentage of sites that bled upon probing did not replicate in the ARIC Study Caucasian population or the Health ABC Study African American population ($p < 0.05$), we still consider the gene harboring this variant, *PTPRT*, to be an interesting candidate gene for further studies of gingivitis.

The signal identified on chromosome 20q12 with % BOP lies within an intron of a tyrosine phosphatase gene, *PTPRT*. The protein encoded by this gene is a transmembrane signaling molecule containing a meprin-A5 antigen-PTP (MAM) domain, an immunoglobulin domain, and four fibronectin type III-like repeats (Xu and Fisher 2012). *PTPRT* is involved in cell-cell adhesion in human epithelial cells, where it is endogenously expressed (Yu et al. 2008). Additionally *PTPRT* mutations, which are frequently observed in cancers, are hypothesized to lead to a loss of function of cell-cell adhesion that may promote tumorigenesis (Wang et al. 2004; Yu et al. 2008). It is possible that the disruption of cell-cell adhesion in the gingival epithelium may affect the integrity of the epithelial lining of the periodontal pocket, predisposing the tissue to bacterial invasion and penetration of bacterial products, which may lead to a greater tendency for bleeding on probing and breakdown of the epithelial lining.

Despite the lack of replication, *in silico* analysis of the SNPs in this signal support the potential role of these SNPs in T-cell development and function. T-cells play an important role in the immune response to periodontal diseases (Gemmell et al. 1997).

The most conserved SNP in this signal, rs6072936 is an eQTL (expression quantitative trait loci) for *RHOH*, and *LTK*. Both *RHOH* and *LTK* are primarily expressed in hematopoietic cells (Li et al. 2002), and play critical roles in receptor mediated T-cell differentiation and development (Andreotti et al. 2010; Wang et al. 2010). Therefore, we hypothesize that a SNP, such as rs6072936, may increase the risk for bacterial invasion of the periodontal pocket by altering PTPRT-mediated cell-cell adhesion within the gingival epithelium. In turn, the increased risk for bacterial invasion may lead to an increase in T-cell activation via *RHOH* and *LTK*, resulting in an increased risk for gingivitis. Further functional studies will be necessary to confirm the link between rs6072936, PTPRT function, *RHOH/LTK* expression, T-cell differentiation, and the risk of gingivitis.

5.3.3 Percent bleeding on probing: chromosome 1q43 (rs2907176)

The signal identified on chromosome 1q43 with % BOP by the Caucasian meta-analysis does not replicate in 4,063 ARIC Study Caucasians, or in 362 Health ABC Study African Americans. However, the potential role of this region in transcriptional regulation, which may be relevant to immune function and inflammation, is supported by *in silico* analyses. For instance, there are nearby CpG methylation sites, microRNA regulatory sites, and other epigenetic regulatory machinery located near this signal. Additionally, the most significant SNP identified in this signal, rs2907176, is an eQTL for a gene involved in immune function, *CD84* (molecule CD84, $p = 7 \times 10^{-5}$). Based on our findings, we hypothesize that epigenetic modifications and altered transcriptional regulation of the identified genomic region may influence the immune response to periodontal infection. Supporting evidence from *in silico* analyses are further discussed below.

CpG methylation sites

There is a large amount of evidence that this genomic region is a regulatory region. For instance, ~2kB from rs2907176 are two CpG methylation sites, cg19626044 and cg06084312. In gingival fibroblasts and endothelial cells, both sites are methylated. In epithelial cells, cg19626044 is methylated and cg06084312 is partially methylated. However, in leukemia cells and many carcinoma cells, cg19626044 is partially methylated and cg06084312 is partially methylated or unmethylated. Additionally, these CpG islands overlap a predicted weak enhancer region in endothelial cells and epithelial cells, but no enhancer region is detected in leukemia or carcinoma cells. It is possible that methylation of these CpG sites is associated with enhancer properties.

MicroRNA binding site in 3' UTR of *CEP170*

Further from the signal (~ 140 Kb), in the 3' untranslated region (UTR) of *CEP170* are predicted binding sites for several microRNA. MicroRNAs can silence gene expression post-transcriptionally, by binding to the 3' UTRs of target mRNAs (Bartel 2009). *CEP170*, the predicted target of this microRNA binding site, encodes a centrosome-associated protein (Guarguaglini et al. 2005). Interestingly, 600 bases away from *CEP170* is *SDCCAG8*, another centrosome-associated protein (Kenedy et al. 2003). *SDCCAG8* splice variants have been observed to illicit immunogenicity in many colon cancer patients (Scanlan et al. 1998), providing a possible link between this nearby gene and the immune pathway.

Nearby non-coding RNAs

Other genes of interest include two piwi-interacting RNAs (piRNAs) ~ 72 Kb from the GWAS identified signal. PiRNAs are known to be involved in silencing of transposons via methylation (Kim 2006). Additionally, there is a predicted microRNA (Mir-350) encoded in this region ~250 Kb from the signal, as well as a non-coding RNA

(LOC731275) ~ 73 Kb from the GWAS signal). Non-coding RNA, including piRNA and microRNA, are important regulators of gene expression, and it is suggested that altered expression of piRNAs and microRNAs may lead to the stem cell-like properties observed in cancers (Siddiqi and Matushansky 2012). It is likely that epigenetic modifications leading to changes in the transcription of these non-coding RNAs could lead to further downstream changes in both cis- and trans-gene expression.

Expression of *CD84* associates with rs2907176

Interestingly, rs2907176 is an eQTL for *CD84*, encoded ~ 83Mb away. *CD84* is a receptor expressed on most differentiated hematopoietic cells, such as mast cells (Álvarez-Errico et al. 2011), T lymphocytes, and B lymphocytes (Zaiss et al. 2003). *CD84* plays a role in receptor-mediated immune responses. For example, immunoglobulin heavy constant epsilon (IgE) mediated signals, degranulation, and cytokine secretion are regulated by *CD84* levels in mast cells (Álvarez-Errico et al. 2011). Additionally, high levels of *CD84* have been associated with significantly different cytokine profiles in murine macrophages, following exposure to LPS (Sintes et al. 2010). Therefore, the identification of rs2907176 as an eQTL for *CD84* provides a potential link between this genomic region and LPS-induced inflammation. It is possible that while rs2907176 may not directly affect *CD84* expression, it may be influenced by epigenetic regulation near rs2907176, such non-coding RNA transcription.

Lack of replication of rs2907176

Although replication analyses in the ARIC Study Caucasian population (n = 4,064) and in the Health ABC Study African American population (n = 362) do not support an association between rs2907176 and gingival inflammation, it is possible that the effect sizes detected in the Health ABC Study and MrOS Caucasian populations do not accurately reflect the true effect size associated with rs2907176. Replication studies

in the ARIC Study were 80% powered to detect a 1.6% increase in percentage of sites that bled upon probing. It is possible that the true effect size associated with rs2907176 is smaller than 1.6%, despite the larger effect sizes detected in the Health ABC Study ($\beta \pm \text{SE}$: 3.3 ± 1.0) and MrOS ($\beta \pm \text{SE}$: 8.0 ± 2.2) populations. It is also possible that the detected association may represent some other nearby factors affecting gingival inflammation, such as differences in methylation patterns, RNA transcription, or gene expression.

Summary

Based on our findings, we hypothesize the identified genomic location is an important regulatory region for non-coding RNA and other genes involved in regulation of gene expression. Additionally, it is possible that this regulatory region is important for a CD84-mediated response to bacteria products such as LPS, and a SNP such as rs2907176 may alter this response, leading to an increased risk for gingivitis. Future replication and functional studies investigating nearby methylation patterns, RNA transcription, and gene expression changes associated with rs2907176 and periodontal diseases will be necessary to further understand the role of this genomic region in gingival inflammation.

5.3.4 Extent pocket depth $\geq$ 5mm: chromosome 5q32 (rs11957208)

The signal detected on chromosome 5q32 is located in an intron of *PPP2R2B* (protein phosphatase 2, regulatory subunit B), which is a phosphatase expressed in T-cells (Crispín et al. 2011). *PPP2R2B* is known to respond to changes in interleukin-2 (IL-2) levels, which induce T-cell apoptosis. T-cell apoptosis is an important step in limiting the duration of immune responses; therefore, alterations in the *PPP2R2B* response to IL-2 may result in a longer T-cell half-life, and ultimately result in deeper

periodontal pockets. However, the association between rs11957208 and extent pocket depth $\geq 5\text{mm}$ did not replicate in either the ARIC Study Caucasian population or the Health ABC Study African American population. Additionally, the effect size of rs11957208 was only significant in the Health ABC Study Caucasian population, and not in our second primary GWAS population (MrOS).

In summary, the association between SNPs within this signal and deeper periodontal pockets is not supported by findings from our replication studies. However, based on the literature and our discovery of a SNP within *PPP2R2B* associating with deep periodontal pockets, *PPP2R2B* may be a good candidate gene for further investigation relating to T-cell half-life, and periodontal pocket depth.

5.3.5 Extent attachment loss $\geq 3\text{mm}$: chromosome 17p13.3 (rs4790833)

The signal detected on chromosome 17p13.3 is located within *TUSC5* (tumor suppressor candidate 5). This gene is also known as interferon-induced transmembrane protein domain containing 3. As the name suggests, this protein may play a role in the transduction of interferon-induced signals. Interferon proteins are cytokines released by the host cells in response to pathogens, such as those in periodontal infection.

However, replication analyses in the ARIC Study Caucasians or the Health ABC Study African Americans do not support the relationship between *TUSC5* SNPs and attachment loss. In summary, *TUSC5* induction by interferon proteins provides evidence that this gene may be important to the host response to periodontal pathogens, and that SNPs affecting *TUSC5* function may be important for this response. However, there is little evidence presently to link *TUSC5* and periodontitis.

5.3.6 Summary of GWAS findings

Four significant signals were detected by the meta-analysis of genome-wide association studies of periodontal disease-related traits in the Health ABC Study and MrOS Caucasian populations ($p \leq 10^{-6}$). Additionally, there was one borderline significant finding ($p = 1.39 \times 10^{-6}$). None of the signals are supported by findings from replication studies. However, there are promising candidate genes within the genomic regions identified by three of these signals, some with functional evidence implicating their roles in immune function. Firstly, we find *fibulin1* to be a good candidate gene for future studies of biofilm formation. We also consider *PTPRT* to be a good candidate for studies of susceptibility to bacterial infection and gingivitis. The third gene identified in our study that we consider as a good candidate gene for future studies is *PPP2R2B*, which may harbor genetic variants affecting T-cell half-life, and the risk of periodontitis.

The findings with rs2907176 and gingivitis may warrant further research of epigenetic regulation of this region relating to gingival inflammation. Additionally, *TUSC5* may be a good candidate gene for future studies of periodontal diseases. However, outside of this study, there is weak evidence linking the genomic regions surrounding rs2907176 and *TUSC5* SNP rs4790833 to periodontal diseases.

CHAPTER 6

CONCLUSIONS

The purpose of this study was to access and compare possible risk factors for periodontal diseases, and identify the most important risk factors. We did this by comparing the risks of known and potential risk factors, resulting from an epidemiological analysis of risk factors, as well as looking across the genome for novel genetic risk factors for periodontal diseases, using a GWAS approach.

Results from the epidemiological analysis indicate that high plaque levels are the largest modifiable risk factor for gingivitis and periodontitis. Our GWAS identified a number of potential regions of genetic susceptibility to periodontal diseases including the following genes: *fibulin1*, *PTPRT*, and *PPP2R2B*. However, none of the genetic associations detected explained as much variance of periodontal disease-related outcomes as high plaque levels, and the associations with these genetic risk factors did not replicate in our replication cohorts. Therefore, based on findings from our studies and previous studies, removing dental plaques consistently may be the most effective, as well as cost-effective, preventive treatment for periodontal diseases, regardless of genetic influences.

Additionally, there is a large portion of unexplained variance of periodontal disease-related outcomes. This variance may be due to large inter-examiner effects on periodontal measurements between dental examiners in our study populations. However, even after adjusting for the dental examiner, there is still a large portion of variance of outcomes unexplained. This unexplained variance may reflect inaccuracies in periodontal measurements, or possibly unaccounted environmental, genetic, or epigenetic risk factors of periodontal diseases.

6.1 Study strengths and limitations

Many studies of periodontal diseases have identified numerous risk factors for periodontal diseases. However, it is difficult to compare the relative importance of risk factors due to study differences in populations examined, periodontal examinations, periodontal disease definitions, and risk factors investigated. This study included a comprehensive investigation of 14 known and potential risk factors for periodontal diseases, and compared the variance of periodontal disease-related outcomes accounted for by each risk factor. Additionally, we performed these studies on three large populations with periodontal examinations and data collected on all 14 risk factors.

This study is also one of the first genome-wide association studies of periodontal diseases. Although the sample sizes in this study appear small compared to genome-wide association studies of many other complex diseases, this study utilized one of the largest available data sets with both genome-wide SNP data and extensive phenotypic data, including ~1,300 Caucasians in primary analyses as well as a larger replication cohort consisting of ~4,000 Caucasians for replication studies. Although we pay a severe multiple-testing penalty for using a GWAS approach to identify genetic risk factors for periodontal diseases, this method is effective at finding strong signals using an unbiased approach, opposed to candidate gene studies that select for specific regions of the genome.

Potential limitations to interpretation of our findings include: differences between the study populations, older ages of study participants, as well as differences in the collection of periodontal data between study populations. Age is an important factor to consider when looking at periodontal disease risk. Exposure to risk factors for periodontal diseases increases with age, making it difficult to tease apart effects from different risk factors. Our study populations consisted of older people, which although

not the ideal population, still offer valuable information as these study populations are some of the largest collected cohorts with data on many risk factors, as well as GWAS data.

Another potential limitation was the use of different methods of collecting periodontal measurements between our study populations. These differences included using half-mouth vs. full-mouth exams, rounding periodontal pocket probing scores up vs. down, or the inclusion or exclusion of people needing prophylactics. These differences may complicate the comparison of detected effect sizes, and the significance of associations between study populations; however, it is not expected that the direction of effect detected (for true signals) should be affected by differences in outcome measurement (such as full-mouth vs. half-mouth exams, inclusion or exclusion of third molars, or rounding pocket probing depths up vs. down).

6.2 Future directions

This investigation identified three genetic loci whose roles in periodontal diseases were supported by *in silico* analyses. The genomic region encoding *fibulin 1* was significantly associated with plaque levels. Future studies of the rate of plaque formation associated with *fibulin 1* SNPs and splice variants may shed additional light on the genetic influences affecting bacterial colonization and plaque formation. SNPs within *PTPRT* were significantly associated with gingivitis. This gene is involved in cell-cell adhesion of epithelial cells, and may be important for the integrity of the gingival epithelial lining. SNPs altering the function of *PTPRT* may increase the risk of bacterial infection, warranting future studies of *PTPRT* and the risk of periodontal diseases. The third signal identified was located in *PPP2R2B*, which was significantly associated with deeper periodontal pockets. *PPP2R2B* is expressed in T-cells and may play a role in

mediating T-cell half-life. Future studies of *PPP2R2B* SNPs, expression, and associated T-cell half-life will be necessary to confirm the role of this genomic locus with the increased risk of deeper periodontal pockets.

There are likely undiscovered genetic risk factors for periodontal diseases. The power of this study to detect SNPs associating with periodontal diseases was limited by the sample sizes of the available study populations with both periodontal exams and genome-wide SNP data ($n < 10,000$). Additionally, genetic effects may have been difficult to detect in these older populations, compared to a younger population. Future GWAS of periodontal diseases in younger and larger populations may reveal undiscovered SNPs associated with periodontal diseases.

These genetic studies of periodontal diseases were also limited to association studies of SNPs in Caucasian populations. We plan to perform a GWAS of periodontal disease-related outcomes in the Health ABC Study African American population, and replicate our findings in a larger African American population from the ARIC Study. It will also be important to expand studies to other ethnic groups to obtain a more comprehensive understanding of the genetic influences on periodontal diseases.

Additionally, there are many other types of variations in the human genome that were not included in this study, which likely contribute to the heritability of periodontal diseases. Future studies investigating periodontal disease-associated methylation patterns, transcription factor binding, RNA transcription, and gene expression changes will be useful to identify epigenetic risk factors for periodontal diseases. Furthermore, *fibulin 1*, *PTPRT*, and *PPP2R2B* may be good candidate genes for studies of epigenetic risk factors of periodontal diseases.

6.3 Public health implications

Plaque appears to be the largest risk factor for periodontal diseases, compared to any other known modifiable risk factor, or any single genetic polymorphism tested in this investigation. Therefore, removing plaques may be the most effective way to reduce the incidence and progression of periodontal diseases. Education programs and reminders at dental visits emphasizing the relationship between dental plaque and periodontal diseases, and the importance of removing dental plaque may be an effective strategy for the prevention of periodontal diseases. Reinforcing the importance of dental visits and plaque removal to current smokers, men, and African Americans will be important in the prevention of periodontal diseases, as they appear to be particularly susceptible to periodontal diseases.

Additionally, periodontal diseases are associated with higher levels of systemic inflammation, as well as poor health outcomes such as cardiovascular diseases. Therefore, removing plaques may not only reduce the prevalence of periodontal diseases, it may also help to reduce systemic inflammation, and possibly the risk of vascular diseases.

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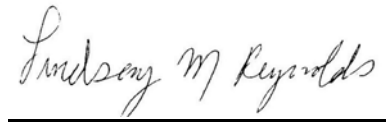
APPENDIX: LIST OF COMMON ABBREVIATIONS

AA	African American
AL	attachment loss
ARIC	Atherosclerosis Risk in Communities Study
BMI	body mass index (weight (kg) / height ² (m ²))
BOP	bleeding on probing
CEJ	cementoenamel junction
CEU	HapMap population from Utah residents with ancestry from northern and western Europe
CVD	cardiovascular disease
eQTL	expression quantitative trait loci
<i>FBLN1</i>	fibulin 1
GRCh37	Genome Reference Consortium Human Genome Build 37
GWAS	genome-wide association study
HapMap	International HapMap Project
HABC	Health, Aging, and Body Composition Study
Health ABC Study	Health, Aging, and Body Composition Study
MrOS	Osteoporotic Fractures in Men Study
PD	pocket depth
<i>PPP2R2B</i>	protein phosphatase 2, regulatory subunit B
<i>PTPRT</i>	protein tyrosine phosphatase, receptor type, T
SNP	single nucleotide polymorphism
<i>TUSC5</i>	tumor suppressor candidate 5
YRI	HapMap population from Yoruba in Ibadan, Nigeria

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9/8/12

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