To my Grandfather
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Periodontitis is an inflammatory disease of the supporting tissues of the teeth, resulting in progressive destruction of the periodontal ligament and alveolar bone with pocket formation, recession, or both. The pathogenic processes are largely a response to microbial induced destructive mechanisms. These processes are initiated by the microbial biofilm, but are undertaken by the host cells in chronic disease progression, and thus it is the host tissue itself that results in the destruction observed. The host initiates and controls the release of enzymes, including matrix metalloproteinases (MMPs), to allow the tissues to retreat from the microbial destructive lesions. Recently, there has been increasing evidence implicating MMPs as key mediators in the tissue destruction associated with the various forms of periodontal disease, including the progression from gingivitis to periodontitis.

Aggressive periodontitis (AgP) in children is a severe and rapidly progressing form of periodontitis that may cause loss of connective tissue, resorption of alveolar bone, and formation of periodontal pockets. Severe stages of the disease lead to the loosening and eventual loss of teeth if untreated. Due to its low prevalence and multiple changes in the clinical parameters used
to classify the disease, epidemiological data can be difficult to interpret; however, the prevalence of the disease is estimated to range from 0.5% to 10%, with the majority of studies focusing on patients over twenty years of age. Although there have been a number of reports concerning various MMP levels in adult periodontitis, little information is available about the levels of various MMPs associated with AgP, particularly in children. Additionally, reports on the microbial flora associated with diseased and non-diseased sites in these individuals have been limited, in terms of both a focus on AgP limited to adults, and also a limited panel of species investigated.

This study reports on the MMP levels of the collagenases (MMP-1, -8, and-13), the gelatinases (MMP-2 and -9), stromelysin (MMP-3), and macrophage elastase (MMP-12) in a cohort of children, 7 to 19 years of age, with and without AgP. Additionally, we report on the microbial composition, using a PCR-based, reverse-capture, checkerboard DNA-DNA hybridization. A total of 84 16S rRNA gene targeted probes were utilized, covering taxa that are currently cultivatable as well as several that are ‘not-yet-cultivatable.’ Gingival crevicular fluid samples and subgingival plaque samples were collected from both diseased and non-diseased sites in 23 children presenting with AgP, 9 siblings with no evidence of periodontitis, and 12 healthy, age-matched controls immediately prior to treatment. Nine of the AgP children were also sampled 6 weeks following phase I periodontal treatment, to examine the effects of treatment on both the composition of the microflora and levels of MMPs within the periodontal pocket.
CHAPTER 1
INTRODUCTION

Background and Significance

Periodontal disease is a general term used to describe specific diseases that affect the gingiva and the supporting connective tissue and alveolar bone, which anchor the teeth in the jaws. These diseases are primarily infections caused by microorganisms that colonize the tooth surface at or below the gingival margin. Periodontal diseases are separated into those that involve only the gingiva, and those that are also associated with the destruction of the underlying structures of the periodontium. An infection at the gingival margin, gingivitis, is characterized by increased redness, swelling, a change in the position of the gingival margin, and bleeding of the gingiva during brushing or periodontal probing. With an estimated prevalence of 82% among adolescent individuals and just over 50% for adults, the majority of the U.S. population experiences gingivitis at some point throughout their lifetime. While the most severe cases occur among adolescents, likely due to hormonal changes associated with puberty, gingivitis is generally benign and reversible if treated properly. (Burt, 2005, Albandar, 2005)

If left untreated, however, the gingivitis can extend below the gingival margin, and infect the underlying supporting structures, at which point it is termed periodontitis. Periodontitis is an inflammatory disease of the supporting tissues of the teeth, resulting in progressive destruction of the periodontal ligament (PDL) and alveolar bone with pocket formation, recession, or both. While both are characterized by chronic inflammation and extensive tissue damage, periodontitis can be most clearly distinguished by its characteristic loss of alveolar bone, which is irreversible without treatment. In fact, periodontitis is the disease most responsible for tooth loss in adults.
and its appropriate diagnosis and treatment are one of the greatest challenges facing dentistry today.

The pathogenic processes are largely a response to microbial-induced destructive mechanisms. These processes are initiated by the microbial biofilm, but are undertaken by the host cells in chronic disease progression, and thus it is the host tissue itself that results in the destruction observed (Williams, 2008). The resulting lesions associated with periodontitis can be highly variable, as it does not affect all teeth evenly but can present both a subject and site predilection. Additionally, pathogenesis is influenced by each individual’s unique immune and inflammatory response, in combination with a wide range of environmental and behavioral factors that include smoking, age, systemic disease, socioeconomic status, race, and gender (Offenbacher et al., 2008). In addition to the high heterogeneity of host immunological risk factors, the microbial flora responsible for the initiation and progression of disease may be equally complex; over 500 bacterial species have been detected in the subgingival biofilm, many of which are ‘not-yet-cultivatable’ (Paster et al., 2006, Paster et al., 2001). Thus, as a multifactorial and highly variable disease, the mechanisms responsible for the initiation and progression of periodontitis remain elusive.

Aggressive periodontitis (AgP) is a severe and rapidly progressing form of periodontitis that may cause loss of connective tissue, resorption of alveolar bone, and formation of periodontal pockets. Severe stages of the disease lead to the loosening and eventual loss of teeth if untreated. Under the current classification proposed by the American Academy of Periodontology, AgP occurs in patients around puberty or under the age of 30; however, it may present at various ages, including children, and can persist in older adults (Periodontology,
2000). Due to its low prevalence and multiple changes in the clinical parameters used to classify the disease, epidemiological data can be difficult to interpret; however, the prevalence of the disease in children is estimated to range from 0.1% to 0.76%, with higher prevalence among African Americans estimated from 2.1% to 2.6% (Albandar and Tinoco, 2002).

AgP most frequently presents early in the life of the individual which implies that the etiologic agents, primarily the subgingival biofilm, have been able to cause clinically detectable levels of disease over a relatively short time period. The plaque front grows with a faster speed of 3 to 5 microns a day in this disease compared to 0.2 to 1.0 uM a day in other forms of periodontitis (Oh et al., 2002). This fact remains central to the current understanding of these diseases, since it implies infection with a highly virulent microflora and/or a high level of subject susceptibility to periodontal disease. The microbial etiology of AgP affecting younger populations is difficult to discern, due to the wider classification scheme which currently does not distinguish between patient populations based on age. Descriptions of the microbial flora associated with AgP have focused primarily on older populations.

**Specific Aims**

SA 1: To determine the presence, quantitatively and qualitatively, of matrix metalloproteinases (MMPs) present within the gingival crevicular fluid (GCF) from children with AgP.

SA 2: To determine how MMPs are modulated through the progression and treatment of diseased sites in children with AgP.

SA 3: To determine the subgingival flora present within diseased sites of children with AgP.

SA 4: To determine alterations within the subgingival flora associated with AgP after phase I periodontal treatment.
CHAPTER 2
MATRIX METALLOPROTEINASE LEVELS IN CHILDREN WITH AGGRESSIVE PERIODONTITIS

Introduction

Matrix metalloproteinases (MMPs), collectively known as matrixins, form a family of highly homologous zinc- and calcium-dependent endopeptidases that regulate cell-matrix composition. This family currently includes 23 human MMPs that are commonly divided into six subgroups: collagenases, gelatinases (or type IV collagenases), stromelysins, matrilysins, membrane-type metalloproteinase, and others. Each MMP has distinct but often overlapping substrate specificities, which together can cleave virtually all components of the extracellular matrix (ECM) and basement membrane (Nagase et al., 2006). In addition to ECM degradation, MMP proteolysis can create space for cells to migrate, produce specific-cleavage fragments with independent biological activity, regulate tissue architecture through effects on the ECM and intercellular junctions, and activate, deactivate or modify the activity of signaling molecules, both directly and indirectly (Sternlicht and Werb, 2001). Additional substrates include peptide growth factors, cell surface receptors, cell-adhesion molecules, cytokines and chemokines, as well as other MMPs and unrelated proteases (Page-McCaw et al., 2007). Thus MMPs participate in a wide range of physiological processes including morphogenesis, wound healing, tissue remodeling, angiogenesis and normal immune responses to infection (Uitto et al., 2003). To prevent tissue destruction due to excessive proteolytic activity, MMP activity is tightly regulated (Leppert et al., 2001). The activities of most MMPs are normally low in steady-state tissues; however, MMP expression can be detected in repair or remodeling processes, in diseased or inflamed tissues and in cell types grown in culture (Parks et al., 2004). The same functions of
MMPs that are beneficial under normal physiological conditions turn into key mechanisms of disease pathogenesis where aberrant ECM turnover predominates, such as rheumatoid arthritis, atherosclerosis, tumor metastasis, and periodontal disease.

Periodontitis is an inflammatory disease of the supporting tissues of the teeth, resulting in progressive destruction of the periodontal ligament (PDL) and alveolar bone with pocket formation, recession, or both. The pathogenic processes are largely a response to microbial induced destructive mechanisms. These processes are initiated by the microbial biofilm, but are undertaken by the host cells in chronic disease progression, and thus it is the host tissue itself that results in the destruction observed (Kinane et al., 2001). The host initiates and controls the release of enzymes, including MMPs, to allow the tissues to retreat from the microbial destructive lesions. Recently, there has been increasing evidence implicating MMPs as key mediators in the tissue destruction associated with the various forms of periodontal disease, including the progression from gingivitis to periodontitis (Reynolds et al., 1994, Golub et al., 2001, Garlet et al., 2004). Under non-pathological conditions, controlled phagocytosis and intracellular digestion of collagen fibrils is observed at high levels in dynamic soft tissues, such as gingiva and PDL, during normal tissue remodeling. Under pathological conditions, however, the balance between collagen synthesis and degradation is disrupted, resulting in progressive tissue destruction due to excessive collagen breakdown. This condition begins in early gingivitis and if it becomes chronic, periodontitis may occur leading to breakdown of the PDL and eventually the alveolar bone. In the progression from a healthy periodontium to a diseased site, collagen breakdown switches from the well-controlled intracellular pathway to a metalloproteinase-mediated extracellular pathway (Kinane, 2000). In periodontal tissues, MMPs are expressed by
inflammatory cells (monocytes, macrophages, lymphocytes, polymorphonuclear cells) and by resident cells (fibroblasts, epithelial cells, endothelial cells) (Hannas et al., 2007). Previous studies, mostly involving chronic periodontitis patients, have demonstrated elevated levels of various MMPs associated with inflammation, as well as correlations between these levels and the extent of tissue destruction (Golub et al., 1976). In particular, active forms of MMPs in the gingival crevicular fluid (GCF) have been shown to be associated with progressive periodontal destruction (Golub et al., 1974, M. Kiili, 2002, Ilgenli et al., 2006, Hernandez et al., 2006).

Although there have been a number of reports concerning various MMP levels in adult periodontitis, little information is available about the levels of various MMPs associated with aggressive periodontitis (AgP) in children. In this study, we report on the MMP levels of the collagenases (MMP-1, -8, and-13), the gelatinases (MMP-2 and -9), stromelysin (MMP-3), and macrophage elastase (MMP-12) in a cohort of children, 7 to 19 years of age, with and without localized aggressive periodontitis. Since certain MMP levels may be higher in children due to oral tissue and bone remodeling associated with growth, we also included, for comparison purposes, MMP levels obtained for a cohort of adult subjects with chronic periodontitis (CP).

**Materials and Methods**

**Study Population**

A total of 44 participants, between the ages of 7 to 19 years, all African-American, were recruited from the Leon County Health Department, Tallahassee, Florida over a period of one year between August 2006 and July 2007. After clinical evaluations, 23 patients with AgP, diagnosed based on pocket probing depths, clinical attachment levels and radiographs; 9 siblings with no evidence of periodontitis; and 12 healthy controls, who were unrelated to the diseased subjects (healthy controls were obtained from both the Leon County clinic and the University of
Florida College of Dentistry pediatric clinics) were entered into this study. The 17 adult CP subjects were between 35 and 65 years of age, mainly Caucasian, mostly non-smokers, and in general good health with no known underlying systemic diseases. The CP subjects were drawn from patients in the general dental clinics and the periodontics clinic.

Pertinent information concerning the study protocol was explained to each patient and informed consent was obtained from all participants, or their parents in the case of underage patients, as required in the study protocol approved by the University of Florida Institutional Review Board. Complete medical and dental histories were taken from all participants. All patients were in good general health, non-obese, and were considered to be free of any underlying systemic diseases. Exclusion criteria included: any history of systemic disease that could interfere with the clinical characteristics, incidence, or progression of periodontal disease; periodontal treatment within the previous 6 months; chronic treatment with any medication known to affect periodontal status within the previous 3 months (i.e., antibiotics, NSAIDS, contraceptives). Clinical diagnosis and selection of patients was based on the clinical and radiographic criteria proposed by the American Academy of Periodontology (Armitage, 1999).

Sample Collection

GCF samples were collected from two deep sites (PD \( \geq 4 \) mm), almost always from the 1st molars, and one shallow site (PD \( \leq 2 \) mm), usually a pre-molar, from each of the AgP subjects. Samples, usually from the 1st molars, were collected from 2 sites (PD \( \leq 2 \) mm) in each subject in the sibling group and the healthy control group. In addition, for comparison purposes, GCF samples were collected from 2 sites (PD \( \geq 4 \) mm) in each subject diagnosed with adult CP. GCF samples were collected first, to avoid any tendency the site may have for bleeding on/after
plaque sampling. Samples with any gingival bleeding were excluded. The sampling area was isolated with cotton rolls to avoid contact with saliva, and supragingival plaque was removed to eliminate the risk of plaque contamination. After gentle air-drying, GCF samples were collected using Periopaper (ProFlow, Inc., Amityville, NY) by inserting the strip 1 mm into the sulcus and allowing the GCF to wick up the strip. The volume of GCF collected was immediately measured chair side with a calibrated Periotron 8000 (ProFlow, Inc., Amityville, NY). The strip was placed in a dry microcentrifuge tube and kept on ice. Upon return to the laboratory, the samples were stored at -80°C until used for measurement of the MMPs. The GCF readings were converted to volume based on standards curves generated for the instrument used.

**Gingival Crevicular Fluid Processing and MMP Enzymatic Assays**

The GCF absorbed to the strip was eluted with 50 µL of MMP buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM CaCl2, pH 7.5) followed by centrifugation to minimize retention of proteins on the filter paper. The activities of MMP- 1, 2, 3, 8, 9, 12, and 13 were assessed using Enzolyte 520 fluorimetric MMP kits specific for each MMP(AnaSpec, San Jose, CA), in accordance with the manufacturer’s instructions. The detection limits of these kits are given by the manufacturer as 0.1 to 1.0 ng depending on the particular assay kit. Briefly, each specific MMP substrate (50 µL) was diluted in 5 ml of assay buffer (provided with the kit) and 50 µL of diluted substrate was added to the desired well of a black fluorimetric 96-well microtiter plate (Fisher Scientific, Ocala, FL). Two µL of the sample was added to each well and mixed by gently shaking for 15 minutes in the dark and then incubation for an additional 45 minutes in the dark. Each of the 7 MMP substrates was arranged on each plate in rows and the samples were applied in columns, with the first two wells of each row serving as controls for background fluorescence.
Fluorescence intensity was measured at Ex/Em = 490 nm/520 nm using a Synergy HT fluorimetric reader and associated software (BioTek, Winooski, VT). The data, initially expressed as relative fluorescence units, were converted to ng based on standard curves (R2 >0.99) generated with the purified recombinant MMP (AnaSpec, San Jose, CA) and its specific substrate. This value was adjusted to concentration (ng/µL) based on the volume of the GCF collected.

The MMPs were assayed without activating the pro-enzyme MMP form for all of the samples collected from the children groups. The MMP activity previously obtained for the adult periodontitis group was measured following incubation for one hour with 1 mM 4-aminophenylmercuric acetate (APMA).

**Statistical Analysis**

Due to the distribution of MMP values obtained, non-parametric statistics were applied to avoid the effect that outliers might exert. Statistical differences among the 5 groups were sought using the Kruskal-Wallis test. Statistical differences between the diseased group and each of the other groups were detected using the Mann-Whitney (a non-parametric version of the unpaired t-test). We chose to consider the samples from the deep sites and the shallow sites within the children with AgP to be unpaired since GCF was collected from sites that were clearly clinically different even though they were from the same subjects. Based on the data obtained, we treated each site as being independent rather than considering the subject to be independent. Statistical significance was considered to be a p-value ≤0.05. All statistical analyses were performed using the StatView software program (SAS Institute, Inc, Cary, NC).
Results

A summary of the subjects included in the 5 groups is given in Table 2-1. The deep sites in the AgP group presented with PD from 4 to 11 mm. Pocket depth was not strongly correlated to age ($R^2=0.318$). Children as young as 7 years and as old as 18 presented with 4 and 5 mm pockets while some of the deepest pockets (PD >8 mm) were found in children age 12 to 14.

A graphic summary of the MMP levels (mean ± SD) is presented in Figure 2-1. Comparisons were only made within each MMP. As is apparent in Fig. 2-1, the MMP levels associated with the deep sites from the AgP group are consistently elevated relative to the other 4 groups. For statistical purposes, the deep (PD $\geq$4 mm) and the shallow ($\leq$2 mm) sites within the children with AgP were considered as two separate groups relative to MMP activity (Table 2-2). Statistical differences ($p<0.025$) were found among the 5 groups for all MMPs tested, with the exception of MMP-13. Highly significant differences ($p<0.0001$) in MMP-8 were detected between the deep sites from the AgP children relative to all of the other groups with the exception of the shallow sites in these same AgP children ($p=0.02$) Statistical significant differences between the AgP deep sites and the healthy unrelated children were detected for each of the MMPs. With the exception of MMP-1, significant differences were also detected between the AgP deep sites and the sites from the adults with chronic periodontitis.

Although no significant differences were detected between the shallow sites in the AgP children relative to the siblings ($p>0.35$), MMP levels associated with both the shallow sites and the siblings were higher than those obtained from the healthy unrelated children and statistical significant differences ($p\leq0.05$) were found for MMP-3, -9, and 12. A trend toward significance
was detected for MMP-1 (p=0.054) and possibly for MMP-2 (p=0.076), but not for MMP-8 (p>0.10) or MMP-13 (p>0.61).

The sites sampled in the AgP group, including both the deep and the shallow sites, were examined to determine if differences were apparent in the MMP levels based on pocket depth. The pockets were separated into deep (PD 7-11 mm), moderate (PD 4-6 mm), and shallow (PD ≤2 mm) (Figure 2-2). The deepest pockets (PD 7-11 mm) had slightly higher mean levels of the MMPs with the exception of MMP-8. The latter was slightly elevated in the moderate pockets (PD 4-6 mm) relative to the deeper pockets. The mean MMP levels associated with the shallow pockets (PD <2 mm) were consistently the lowest relative to both the deeper and the moderate pockets. Statistical significant differences in the MMP levels associated with these three PD groups were detected for MMP-3 (p=0.0458), MMP-8 (p=0.0143), and MMP-12 (p=0.0015). No differences were detected between the deeper and the moderate sites.

A coefficient correlation matrix was run to determine if any positive relationships existed between PD and MMP levels or between individual MMP levels for the AgP sites with PD 4-11 mm (Table 2-3). No significant correlations were detected between PD and any particular MMP level. However, strong positive correlations were found between the levels associated with certain MMPs. The collagenases (MMP-1, -8, and -13) were strongly correlated (R² >0.90) with each other, as were the gelatinases (MMP-2 and -9) (R² = 0.928). MMP-3 (stromelysin) yielded a positive correlation (R² >0.80) with each of the two gelatinases.

**Discussion**

This paper presents two interesting findings. One is the presence of AgP in a cohort of children as young as 7 years of age. The second is the relative high levels of MMPs present in
the GCF from the diseased sites of these AgP children relative to non-diseased sites within the same children, to their siblings, and to healthy unrelated children as well as subjects with chronic periodontitis. AgP in children, particularly young children, is considered extremely rare and there is very limited information regarding the disease process. AgP in older children, e.g. localized juvenile periodontitis or LJP, has been more completely investigated. However, even with the latter, there is little information concerning MMP levels. Almost all of the data on MMP levels in periodontal diseases has been derived from adult subjects with chronic periodontitis. Tissue extracts and cultured tissue explants of inflamed gingiva have been shown to contain more collagenase activity than extracts from healthy human gingival (Korostoff et al., 2000, Sarment et al., 1999, Ejeil et al., 2003). Collagenease activity in the GCF has been reported to be increased and to show correlation with the severity of periodontal disease (Sorsa et al., 2004). MMP-8, in particular, has been implicated with the destruction associated with periodontitis. This proteolytic enzyme has been reported to be elevated, relative to healthy tissues, in adult periodontitis (Chen et al., 2000, Haerian et al., 1995, Kinane et al., 2003, Kumar et al., 2006), in diseased peri-implant sulcular fluid (Kivela-Rajamaki et al., 2003), and in localized juvenile periodontitis (Tervahartiala et al., 2000). The latter study by Tervahartiala, et al. reported higher numbers of MMP-8 and -13 positive cells in the gingiva of diseased sites in both adults and juveniles. However, in the latter only 7 subjects, age range 18-22 years of age, were examined.

In this study, the active levels of seven MMPs (1, 2, 3, 8, 9, 12 and 13) in GCF were determined in a cohort of 44 African American children with and without AgP. To our knowledge, this is the first study to examine a wide range of MMPs in the GCF of such children.
The principal finding of this study was that active MMPs in the GCF of diseased sites of this group were significantly higher than healthy subjects within the same population. In addition, these sites also contain significantly higher MMP levels, with the exception of MMP-1, relative to adult CP diseased sites, despite the fact that the adult MMPs were activated with APMA before the assay. A previous study utilizing a fluorogenic MMP assay noted a 3-fold increase in fluorescent substrate activity following APMA activation (Bhide et al., 2000). Thus, MMP levels in our CP group were likely elevated due to activation. Although this study, as well as several others, suggest MMP activity may be associated with the severity of periodontitis, it is not clear whether the elevation of these enzymes exacerbates the disease process or is elevated as a result of the disease.

Although the principal findings of this study are mostly congruent with previous studies, the most significant results may be the similarities of MMP activity between the shallow sites of AgP patients and their siblings; in fact, no statistically significant differences were found for any of the MMPs between these sites. In addition, the shallow sites of the AgP and sibling groups were significantly higher compared with the healthy controls for MMPs-3, 9, and 12, including a strong trend towards significance for MMP-1. Interestingly, MMPs-3, 9, and 12 have all been suspected of roles in bone resorption. MMP-3 can be produced by osteoblasts and its synthesis has been shown to be up-regulated in estrogen-deficient mouse osteoblasts, leading to the implication it may have a role in early stages during the pathogenesis of osteoporosis (Breckon et al., 1999). Additionally, MMP-3 production by osteoblasts and osteocytes of human neonatal rib has been proposed to facilitate cellular migration and expansion of bone (Zeitler et al., 2004). MMP-3 activation of proMMP-1, also produced by osteoblasts, and their coordinated up-
regulation upon osteoblast stimulation during bone formation has been shown to be key in the processing of collagen on bone surface, allowing osteoclast recruitment leading to bone resorption during bone morphogenesis (Sasaki et al., 2007). Both MMP-9 and MMP-12 have been shown to be produced by osteoclasts and are believed to play key roles in osteoclastic bone resorption by facilitating migration of osteoclastic cells toward bone surfaces through matrices (Hou et al., 2004). Osteoclasts express MMP-9 at very high levels and its inhibition has been shown to have an inhibitory effect on osteoclastic bone resorption (Ishibashi et al., 2006).

Osteoclast-derived MMP-12 is believed to play a role in osteoclast attachment, spreading, and resorption; however, gene knockout experiments have demonstrated that its activity is not critical in osteoclastic bone resorption (Hou et al., 2004). However, MMP-12 is primarily produced by macrophages and studies examining its in vivo substrates suggest strong roles in osteoclast recruitment (Chen, 2004). In vivo, MMP-12 has been shown to be important in activating other MMPs such as MMP-2 and MMP-3, by which MMP-12 exaggerates the cascade of proteolytic processes (Chen, 2004).

Our study represents a contribution aimed at better characterization of the hyper-inflammatory response associated with localized sites in children with AgP. GCF is an inflammatory exudate that drains the infected pocket and is believed to provide insight into the disease process currently operating in adjacent tissue (Lamster and Ahlo, 2007). It should be emphasized that our assay measured the active forms of MMPs. MMPs can exist in latent or active forms, but the active form is more tightly related to progressive periodontal disease while the latent form is more strongly associated with gingival inflammation alone (Sorsa et al., 2004). In addition, we chose to evaluate the MMP levels in GCF since this fluid provides a non-invasive
means to investigate disease activity. However, due to the episodic nature of disease activity associated with CP, it is very likely that many of the CP sites samples were in remission. This would be in clear contrast to our AgP group, which reflects a more consistently active disease state, and may skew differences between the groups. In addition, in order to assay such a wide range of MMPs we utilized a fluorimetric assay. Thus, substrate differences make it difficult, if not impossible, to compare our absolute values with previous studies or even between MMP types within this study. However, our results consistently demonstrate significant differences between groups for each MMP.

Although all of MMPs assayed were elevated in the AgP diseased sites relative to shallow sites within the same subjects and to the siblings, the levels associated with these two latter groups tended to be elevated relative to the unrelated healthy children. The elevated levels of MMP activity found within shallow sites in the both the AgP children and their siblings may provide a unique opportunity to further evaluate the potential prognostic capability of MMPs. Although previous research points to high heterogeneity of host immunological risk factors in patients with periodontitis (Takahashi et al., 2001), in addition to the hormonal variations that likely exist, such differences would be expected to be lower between siblings if disease development follows a similar pattern. In addition, environmental and behavioral characteristics are likely to be more similar versus non-related patients. Previous studies indicate that parents, offspring, and siblings of children diagnosed with AgP, have a 50% chance of either having had or of developing this disease (Takahashi et al., 2001, Kinane et al., 2001, Albandar and Tinoco, 2002). Although, the clinical utility of GCF-based diagnostics in adult CP has not been fully implemented, the potential sensitivity in assessing treatment responses of AgP in children, as
well as possible indicating sites at risk, may provide a more appropriate stage to assess their clinical utility.

AgP in children is a supposedly rare form of periodontal disease with prevalence rates reported under 1% in North American populations (Albandar and Tinoco, 2002). Although the cohort reported here is drawn from one rural area in north Florida, there are indications that other such areas may have similar AgP cohorts, particularly among African-Americans in rural, dentally under-served areas. We think that AgP in children in these rural areas is vastly under-diagnosed and deserves further study.
Table 2-1. Characteristics of the five subject groups.

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<thead>
<tr>
<th>Group</th>
<th>PD (mm; mean ± SD)</th>
<th>Age (years; mean ± SD)</th>
<th>Subjects (n)</th>
<th>Sites (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgP deep sites</td>
<td>6.2 ± 1.7</td>
<td>14.1 ± 4.0</td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>AgP shallow sites</td>
<td>2.0</td>
<td>14.1 ± 4.0</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Siblings of AgP</td>
<td>2.0</td>
<td>12.6 ± 5.2</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>2.0</td>
<td>13.6 ± 4.4</td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>Adult periodontitis</td>
<td>5.2 ± 0.6</td>
<td>Not available</td>
<td>17</td>
<td>34</td>
</tr>
</tbody>
</table>
Figure 2-1. Matrix metalloproteinase (MMP) levels in gingival crevicular fluid (GCF) from the five subject groups. The length of each bar represents the concentration of active enzyme present (mean ± SD) for each group: AgP deep sites (purple), AgP shallow sites (white), siblings (green), healthy controls (light blue), and adults with chronic periodontitis (blue).
Table 2-2. Statistically significant differences in Matrix metalloproteinase (MMP) levels among the 5 subject groups and differences between the Aggressive Periodontitis (AgP) sites relative to each of the other groups.

<table>
<thead>
<tr>
<th></th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-3</th>
<th>MMP-8</th>
<th>MMP-9</th>
<th>MMP-12</th>
<th>MMP-13</th>
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<tbody>
<tr>
<td>Among groups</td>
<td>0.0027</td>
<td>0.0210</td>
<td>0.0210</td>
<td>&lt;0.0001</td>
<td>0.0016</td>
<td>&lt;0.0001</td>
<td>0.0621</td>
</tr>
<tr>
<td>Deep vs shallow</td>
<td>0.0870</td>
<td>0.2585</td>
<td>0.0298</td>
<td>0.0020</td>
<td>0.1395</td>
<td>0.0014</td>
<td>0.1180</td>
</tr>
<tr>
<td>Deep vs sibs</td>
<td>0.0115</td>
<td>0.2367</td>
<td>0.0534</td>
<td>&lt;0.0001</td>
<td>0.1157</td>
<td>0.0056</td>
<td>0.2031</td>
</tr>
<tr>
<td>Deep vs controls</td>
<td>0.0005</td>
<td>0.0043</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0003</td>
<td>&lt;0.0001</td>
<td>0.0280</td>
</tr>
<tr>
<td>Deep vs adults</td>
<td>0.0889</td>
<td>0.0143</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0056</td>
<td>&lt;0.0001</td>
<td>0.0132</td>
</tr>
</tbody>
</table>

Deep = sites in AgP children with PD≥4 mm; shallow= sites in AgP children with PD ≤2 mm; Sibs = siblings of AgP children, with no evidence of disease; controls = healthy unrelated children; Adults = sites with PD≥4 mm from adult patients with chronic periodontitis.
Figure 2-2. MMP levels in deep, moderate, and shallow pockets of Aggressive Periodontitis (AgP) subjects. The length of each bar represents the concentration of active enzyme present (mean ± SD) in deep (PD 7-11 mm; purple), moderate (PD 4-6 mm; light blue), and shallow (PD ≤2 mm; blue) pockets in the AgP group.
<table>
<thead>
<tr>
<th></th>
<th>PD</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-3</th>
<th>MMP-8</th>
<th>MMP-9</th>
<th>MMP-12</th>
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<tr>
<td>PD</td>
<td>1.0000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td>-0.0391</td>
<td>1.0000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>-0.0382</td>
<td>0.7410</td>
<td>1.0000</td>
<td></td>
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<tr>
<td>MMP-3</td>
<td>-0.0084</td>
<td>0.6325</td>
<td>0.8085</td>
<td>1.0000</td>
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<tr>
<td>MMP-8</td>
<td>-0.1046</td>
<td>0.9295</td>
<td>0.6646</td>
<td>0.5590</td>
<td>1.0000</td>
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<tr>
<td>MMP-9</td>
<td>0.0433</td>
<td>0.7039</td>
<td>0.9277</td>
<td>0.8517</td>
<td>0.6106</td>
<td>1.0000</td>
<td></td>
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</tr>
<tr>
<td>MMP-12</td>
<td>-0.0121</td>
<td>0.5264</td>
<td>0.6334</td>
<td>0.7509</td>
<td>0.4750</td>
<td>0.7590</td>
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<tr>
<td>MMP-13</td>
<td>-0.0628</td>
<td>0.9504</td>
<td>0.7959</td>
<td>0.5905</td>
<td>0.9188</td>
<td>0.6893</td>
<td>0.4636</td>
<td>1.0000</td>
</tr>
</tbody>
</table>
CHAPTER 3
EFFECTS OF PHASE I PERIODONTAL THERAPY ON THE SUBGINGIVAL MICROBIOTA IN AGGRESSIVE PERIODONTITIS

Introduction

Aggressive periodontitis (AgP), formerly classified as Juvenile periodontitis, comprises a group of rare, often severe, rapidly progressive forms of periodontitis. It is characterized by a non-contributory medical history, rapid attachment loss and bone destruction, and a familial aggregation of cases. Under the current classification proposed by the American Academy of Periodontology, AgP occurs in patients around puberty or under the age of 30; however, it may present at various ages, including children, and can persist in older adults (Periodontology, 2000). Due to its low prevalence and multiple changes in the clinical parameters used to classify the disease, epidemiological data can be difficult to interpret; however, the prevalence of the disease in children is estimated to range from 0.1% to 15%, with higher prevalence rates among African Americans (Fine et al., 2007).

The microflora associated with AgP has been described by culture, immunological, and molecular methods and is believed to mainly compose of Gram-negative, capnophilic, and anaerobic rods (Darby and Curtis, 2001). A large number of studies have associated Aggregatibacter actinomycetemcomitans (A.a) with early-onset periodontitis. These studies have demonstrated an increased prevalence in early-onset periodontitis sites/subjects, increased levels in active/progressing sites, decreased levels in health or gingivitis, its presence as an increased risk for disease development, and the elimination/reduction of this species with successful treatment (Tonetti and Mombelli, 1999, Slots et al., 1980, Fine et al., 2007, Haubek et al., 2008, Yuan et al., 2001). While the overwhelming majority of data support the association of A.a. with
AgP, several studies have also presented conflicting data ranging from the absence of this species in disease to its detection in periodontal health (Loesche et al., 1985, Christersson et al., 1992, Yang et al., 2005). Additionally, several other taxa including \textit{Campylobacter}, \textit{Capnocytophaga}, \textit{Eubacterium}, \textit{Fusobacterium nucleatum}, \textit{Eikenella corrodens}, \textit{Porphyromonas gingivalis}, and \textit{Prevotella intermedia} have been suspected as possible pathogens in AgP (Darby and Curtis, 2001, Oh et al., 2002, Moore et al., 1985, Kamma et al., 2004).

The perceived importance of A.a. as the dominant pathogen in AgP, may in part reflect the more extensive investigation and cultivation of this organism. However, culturing is not representative of the composition of a microbial community, since it is often too selective, especially for fastidious and ‘not-yet-cultivatable’ species. Recent investigations of the human subgingival microflora based on ribosomal 16S cloning and sequencing have estimated that approximately 50% of bacterial species present to be novel species or phylotypes (Paster et al., 2001). Several of these not-yet-cultivable species have recently been identified as potential periodontal pathogens (Kumar et al., 2003).

The purpose of the present study was to describe the association of newly identified bacterial species or phylotypes with AgP, as well as species previously linked to periodontitis. Additionally, patients were examined before and 6-weeks following phase I periodontal therapy to determine its effect on the subgingival flora.

\textbf{Materials and Methods}

\textbf{Study Population}

Subjects in this study were a subset of 44 African American children, between the ages of 7 and 19 years, for whom cross-sectional baseline clinical data were reported previously (Alfant et
al., 2008). Study subjects were recruited from the Leon County Health Department, Tallahassee, Florida, over a period of 1-year between August 2006 and July 2007.

Pertinent information concerning the study protocol was explained to each patient and informed consent was obtained from all participants, or their parents in the case of underage patients, as required in the study protocol approved by the University of Florida Institutional Review Board. Complete medical and dental histories were taken from all participants. All patients were in good general health, non-obese, and were considered to be free of any underlying systemic diseases. Exclusion criteria included: any systemic condition that could interfere with the clinical characteristics, incidence, progression of periodontal disease, or the nature of the therapy and if they needed to be pre-medicated for dental treatment and monitoring; periodontal treatment within the previous 6 months; chronic treatment with any medication known to affect periodontal status within the previous 3 months (i.e., antibiotics, NSAIDS, contraceptives). Clinical diagnosis and selection of patients was based on the clinical and radiographic criteria proposed by the American Academy of Periodontology (Armitage, 1999).

Sample Collection

Subgingival plaque samples were collected from two deep sites (PD ≥5mm), almost always from the 1st molars, from each of the AgP subjects using a paper-point technique. Samples were collected by inserting a sterile absorbent paper point to the depth of the sulcus and moving it laterally along the surface of the tooth and the sulcular epithelial lining. The paper-point sample was placed in a dry microcentrifuge tube and kept on ice. Upon return to the laboratory, the samples were stored at -80°C until further processing. Each site was treated independently and samples were not pooled. Samples were collected at baseline and 6-weeks
following non-surgical periodontal therapy, consisting of scaling and root planning (SRP) without the use of antibiotics.

**Reverse Capture Checkerboard Hybridization**

The general procedure of polymerase chain reaction (PCR) ‘reverse-capture, checkerboard’ hybridization was performed as originally described by Paster, et al., (Paster et al., 1998) with minor modifications. In brief, the procedure is summarized as follows: species-specific ‘capture probes’ are synthesized with 19-22 thymidines (T) at the 5’ end of the oligonucleotide. Each probe is loaded onto a nylon membrane through individual lanes of a minislot device, and after absorption, the poly-T tails are preferentially cross-linked to the membrane via UV irradiation, leaving the specific probe available for hybridization. The 16S rRNA genes of DNA isolated from subgingival biofilm samples are amplified using universal primers (the forward primer is 5’-labeled with biotin). The biotin-labeled amplicon is hybridized to the capture probe bound to the membrane. The hybridized biotin-labelled amplicons are detected with the Phototope-Star Detection Kit. The hybridization spots are visualized by exposure to Kodak BioMax MR-1 film.

Specifically, 58 pmole of capture probe was resuspended and mixed in 994.2 µL of TE (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0). Using a Minislot-30 apparatus, each capture probe was applied into individual lanes over a positively-charged nylon membrane and allowed to absorb to the nylon surface. The membrane was then placed on TE soaked blotting paper, and the probes were cross-linked by UV irradiation; during which, the thymidine tails preferentially bind, allowing the ‘capture-sequence’ to be available for subsequent hybridization. The membrane was then washed in 5X SSPE, 0.5% SDS for 30 min at 55 ºC with shaking, followed by rinsing in
distilled water. The membrane was prehybridized for 1 h at 55 °C in 60 mL of appropriately prepared prehybridization solution, after which it was placed in a Miniblotter-45 apparatus for loading of PCR amplicons, prepared as described below.

**DNA extraction and amplification of subgingival plaque samples**

Subgingival plaque samples were pelleted and DNA extracted using the MO BIO UltraClean Microbial DNA Isolation Kit. The UltraClean DNA Isolation kit utilizes bead beating tubes combined with heat, detergent, and mechanical force against specialized beads and provides a sensitive, rapid, and reproducible means of obtaining high quality genomic DNA, free of PCR inhibitors. The resulting DNA was eluted in 65 µL 10 mM Tris. The concentration was then measured with Invitrogen’s Qubit fluorimetric dsDNA assay; 10 µL of each sample was used for the 16S rRNA gene PCR/labeling step.

The PCR/labeling step is carried out using GoTaq Hot Start Polymerase. A Master Mix is utilized, comprising final concentrations of the following: 1X Green GoTaq Flexi Buffer, 2.5 mM MgCl₂, 200 µM of each dNTP, 0.5 µM final concentrations of each primer (Forward 5-biotin labeled primer, Reverse primer), 2.5 Units of Taq polymerase, 10 µL of extracted DNA sample, in a 100 µL reaction volume. The PCR reaction is carried out using a Bio-Rad thermal cycler under the following conditions: initial denaturation step at 94 °C for 1 min, denaturation at 94 °C for 45 s, annealing at 50 °C for 45 sec, and elongation at 72 °C for 90 sec with an additional 5 sec added for each cycle. 30 cycles are run, followed by a final elongation step at 72 °C for 15 min. PCR products were confirmed by gel electrophoresis before application for hybridization.
**Hybridization and detection**

9.6 µL of denaturing solution (400 mM NaOH, 10 mM EDTA) was added to 9.6 µL of PCR product. 108.1 µL of heated hybridization buffer was added and the sample heated to 55 ºC; immediately before loading 7.7 µL of neutralizing solution (0.5N HCl, 500 mM Tris HCl, pH 8.3) was added and briefly mixed. The prehybridized membrane, containing the cross-linked capture-probes, was drained of prehybridization solution, placed upon a foam pad, positioned with lanes perpendicular to the original loading orientation, before clamping of the Miniblot-45 apparatus. Up to 45 samples were loaded into their respective lanes of the Miniblot-45, with hybridization buffer loaded into empty lanes. The apparatus was wrapped in saran wrap, placed in a ziplock bag with two damp paper towels to maintain moisture, and hybridized at 55 ºC for 2 to 4 h. Following hybridization, the membrane is washed under appropriate stringency before detection. The hybridized biotin-labeled amplicons are detected with New England BioLabs Phototope-Star Detection Kit, according to the manufacturer’s recommended procedure, followed by exposure to Kodak BioMax MR-1 film.

Not all samples were available for microbial analysis because there was not enough plaque collected. Probe species included periodontally related species and phylotypes from PCR-cloning studies of human dental plaque. Reactions were scored as each species present or absent per site. The detection limit for the assay was approximately 10^4 cells.

**Statistical Analysis**

The detection frequency of target species were statistically analyzed across sites within each group, responders and non-responders. The detection frequencies for each species were compared among groups by Fisher’s exact test. Significance of differences in mean prevalence (% sites colonized) of species observed before (BT) and 6-weeks after treatment (AT) was
determined using the Wilcoxon signed ranks test. A $P$-value $\leq 0.05$ was considered statistically significant. Significance levels were not adjusted for multiple comparisons. All statistical analyses were performed using GraphPad Prism version 5.00 for Macintosh (GraphPad Software, San Diego, California).

**Results**

The prevalence of 81 bacterial species examined at baseline and 6-weeks are listed in Table 3-1. The data were derived from a total of 26 subgingival plaque samples (13 matched sites at each time point) in 7 subjects. The most prevalent species before therapy (>40%) are presented in Figure 3-1, in descending order of detection frequency. *A. actinomycetemcomitans* serotypes a, b, and c were commonly detected, however, several other suspected periopathogens were detected with higher frequency. *Actinomyces naeslundii* I, *Eubacterium saphenum*, *Filibacter alcocis*, *Fusobacterium nucleatum* ss nucleatum, *Fusobacterium nucleatum* ss polymorphum, *Porphyromonas endodontalis*, *Streptococcus anginosus*, and TM7 clone AH040 were detected from >90% of sites before therapy. In general, SRP did not have a major effect on the subgingival microbiota 6-weeks post-therapy.

At the 6-week reevaluation visit, patients were grouped according to their response to treatment based on mean clinical parameters. The ‘responding’ group consisted of 3 patients that demonstrated a positive response to therapy, while the ‘non-responding’ group consisted of 4 subjects that either did not respond well to therapy or whose condition worsened. Figure 3-2 shows the prevalence (% positive sites) of each group before and 6-weeks post-therapy for the subgingival complexes as originally described by Socransky (Socransky et al., 1998). Figure 3-3 shows additional species, including many not-yet-cultivatable and under-investigated species.
There were differences in species detection among the responding and non-responding subgingival samples before treatment. *Aggregatibacter actinomycetemcomitans* serotypes a/b (p = 0.005), *Aggregatibacter actinomycetemcomitans* serotype c (p = 0.021) and *Streptococcus salivarius* (p = 0.032) were detected more frequently in the non-responding group. *Eubacterium nodatum* (p = 0.007), *Selenomonas* sp. clones EW084/DS071 (p = 0.007), *Selenomonas infelix* (p = 0.032), *Synergistes* sp. clone D084 (p = 0.035), and *Treponema socranskii* (p = 0.035) were detected more frequently in the responding group.

After treatment, *Aggregatibacter actinomycetemcomitans* serotypes a/b (p = 0.007) and *Aggregatibacter actinomycetemcomitans* serotype c (p < 0.001) was more frequently detected in non-responders relative to responders. In the responding group, *Eubacterium nodatum* (p = 0.035), *Selenomonas* sp. clones EW084/DS071 (p = 0.032), and *Selenomonas infelix* (p = 0.005) remained elevated relative to the non-responders. Additionally, *Bacteroidetes* sp. clone AU126 (p = 0.007), *Dialister pneumosintes* (p = 0.007), *Eubacterium infirmum* (p = 0.007), *Eubacterium yurii* (p = 0.021), *Prevotella oralis* (p = 0.035), *Selenomonas sputigena* (p =0.032), and *Streptococcus constellatus* (p < 0.001) became significantly elevated in the non-responders relative to the responders after treatment, despite no significant differences being detected before treatment. The clearest difference between the two groups, both before and after therapy, was the very high frequency of *Aggregatibacter actinomycetemcomitans* in the non-responding group which was virtually absent from the responding sites.

**Discussion**

AgP most frequently presents early in the life of the individual which implies that the etiologic agents, primarily the subgingival biofilm, have been able to cause clinically detectable
levels of disease over a relatively short time period. The plaque front grows with a faster speed of 3 to 5 microns a day in this disease compared to 0.2 to 1.0 uM a day in other forms of periodontitis. This fact remains central to the current understanding of these diseases, since it implies infection with a highly virulent microflora and/or a high level of subject susceptibility to periodontal disease. The microbial etiology of AgP affecting younger populations is difficult to discern, due to the wider classification scheme which currently does not distinguish between patient populations based on age (van der Velden, 2005, Periodontology, 2000). Descriptions of the microbial flora associated with AgP have focused primarily on older populations.

*Aggregatibacter actinomycetemcomitans* was detected in four of the seven patients presented in this study at baseline. It is generally accepted that failure to eradicate this organism and its continued presence post-therapy exposes the patient to an enhanced risk for further periodontal deterioration (Takamatsu et al., 1999). Non-surgical periodontal therapy, consisting of scaling and root planning without the use of antibiotics, did not successfully suppress the levels of this pathogen. These patients consisted of the ‘non-responder’ group, and while other suspected periodontal pathogens were also detected, the presence or absence of *Aggregatibacter actinomycetemcomitans* was the clearest distinction between the two groups. The label ‘non-responder,’ may be somewhat of an understatement since these patients not only did not respond to therapy, but in fact, demonstrated continued periodontal deterioration. Thus, the failure to eradicate *Aggregatibacter actinomycetemcomitans* through mechanical debridement alone, along with the poor clinical response observed, is in agreement with similar findings of several other studies (Drisko, 2001, Slots and Ting, 1999). This strongly suggests that the use of antibiotics may be necessary in order to effectively treat this cohort of patients infected with A.a.
While *Tannerella forsythia* was detected in five of the seven patients, *Porphyromonas gingivalis* and *Prevotella intermedia* were not detected in any of the patients. It should be emphasized that the detection limit of our assay was approximately $10^4$ cells. Although, conventional PCR may have been more sensitive, we used the reverse-capture oligonucleotide probe assay in order to examine the simultaneous presence of multiple species. Thus, although we did not detect the former two species at this level, we cannot conclude that they were not present below our level of detection. Furthermore, a recent study using a similar 16S reverse-capture assay detected *Porphyromonas gingivalis* with fairly low frequency, only to find it present at higher levels using a more sensitive conventional PCR technique (Tanner et al., 2006). This may be due to primer differences, with conventional PCR better able to specifically target individual species, a lower threshold of detection compared to the hybridization assay, or both.

*Porphyromonas gingivalis* has been suspected as having a role in AgP (Kamma et al., 2004), although we could not detect it at any sites. Although suspected as a possible pathogen in AgP, *Porphyromonas gingivalis* is believed to be more prevalent in asian and older populations (Rylev and Kilian, 2008, Slots and Ting, 1999, Takeuchi et al., 2003). This species may simply not be present in this relatively small and isolated community or these patients may have been too young for horizontal transmission of the bug to spread (Asikainen and Chen, 1999). It was interesting, however, that we detected the closely related species *Porphyromonas endodontalis* in all subjects, both pre- and post-therapy. This may reflect the extent of destruction from many of the sites sampled, that were often the deepest pockets of each patient with probing depths greater than 6 mm. *Porphyromonas endodontalis* has been reported to be commonly associated with endodontic infections, where it thrives in conditions that reflect abscess-like conditions, as
opposed to the comparatively more open environment of the periodontal pocket (Zerr et al., 2001). However, more recent studies have detected this species from deep periodontitis sites using molecular methods (Kumar et al., 2003). Since none of these pockets would be considered early/initial periodontal pockets, but instead greatly deteriorated sites, it may be assumed that many of the species present are simply the opportunists; most adapted for survival in the altered environmental conditions that prevail at sites of destructive disease.

While we did not detect *Prevotella intermedia*, we did detect *Prevotella nigresecens* in two of the seven patients. Previous studies using culture-based analysis, generally unable to differentiate between the two species, have detected this complex in AgP. Additionally, *Prevotella loeschii* and *Prevotella oris* were detected at similar frequencies. Interestingly, these species were detected at much higher frequencies post-therapy. However, there was not a clear pattern between the two patient groups, making these results difficult to interpret.

*Eubacterium saphenum, Filibacter alcocis,* and *Fusobacterium nucleatum ss polymorphum* were detected at every site at baseline, with treatment having virtually no effects on these species. Additionally, several ‘not-yet-cultivatable’ species were detected with relatively high frequency, these included: *Bacteroidetes* sp. clone AU126, the *Capnocytophaga* clones: (BM058/BU084/DZ074), *Eubacterium* sp. clone BB124, *Selenomonas* sp. clone EW076, *Synergistes spcs.*, and TM7 clone AH040. While more thorough longitudinal investigations are needed to better define what role these ‘not-yet-cultivatable’ phylotypes may have, it is interesting that in addition to many of the ‘not-yet-cultivatables’ that were detected at high frequency, so were many fastidious species that also may have been overlooked by previous investigations relying solely on culture-based analysis.
Although SRP did not have a significant effect on the composition of the biofilm, it does appear that the responders retained a greater degree of microbial stability. This can best be illustrated in Figure 3-4, where the responders have a much more balanced profile of species changes after treatment, whereas the non-responders appear to be dominated post-therapy by a relatively small number of species. Additionally, the responders retained more of the Actinos, Purple, Yellow, and Green complexes which are generally associated with health; or at the least, with a pattern of formation for the biofilm more likely associated with health (Haffajee et al., 2006). While the responders appear to be improving at 6-weeks post-therapy, it will be interesting to see if they continue to do so for a longer duration and what, if any, further microbial changes accompany this improved clinical response. For the non-responders, it appears SRP may have actually worsened their condition, both clinically, and from the increased prevalence of A.a post-therapy; therefore, our initial results indicate that the use of subjunctive antibiotics may be necessary for successful treatment (Walker and Karpinia, 2002).
Table 3-1. Prevalence of 81 bacterial species/phylotypes at baseline and 6-weeks post-therapy in 13 sites from 7 AgP subjects.

<table>
<thead>
<tr>
<th>Species</th>
<th>% Sites n = 13</th>
<th>% Subjects n = 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 6-weeks</td>
<td>Baseline 6-weeks</td>
</tr>
<tr>
<td><em>Aggregatibacter actinomycetemcomitans</em> serotype c</td>
<td>46  62</td>
<td>31  31</td>
</tr>
<tr>
<td><em>Aggregatibacter actinomycetemcomitans</em> serotypes a, b</td>
<td>54  69</td>
<td>31  38</td>
</tr>
<tr>
<td><em>Actinomyces naeslundii</em> serotype I</td>
<td>92  92</td>
<td>46  54</td>
</tr>
<tr>
<td><em>Actinomyces naeslundii</em> serotype II</td>
<td>77  46</td>
<td>38  38</td>
</tr>
<tr>
<td><em>Actinomyces naeslundii</em> serotype III</td>
<td>54  23</td>
<td>38  23</td>
</tr>
<tr>
<td><em>Actinomyces odontolyticus</em></td>
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<td>23  15</td>
</tr>
<tr>
<td><em>Actinomyces</em> sp. strain B19SC</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><em>Actinomyces israelii</em></td>
<td>46  31</td>
<td>31  23</td>
</tr>
<tr>
<td><em>Bacteroidetes</em> sp. clone AU126</td>
<td>62  31</td>
<td>46  15</td>
</tr>
<tr>
<td><em>Bifidobacterium</em> (Genus-specific)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bifidobacterium dentium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Campylobacter concisus</em></td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td><em>Campyloacter gracilis</em></td>
<td>69</td>
<td>85</td>
</tr>
<tr>
<td><em>Campylobacter rectus/concisus</em></td>
<td>77</td>
<td>62</td>
</tr>
<tr>
<td><em>Campylobacter cluster: (rectus/showae/curvus)</em></td>
<td>62</td>
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Figure 3-1. The most prevalent (>40%) species detected in 13 sites from 7 AgP subjects. Species are ordered in decreasing order of site prevalence.
Figure 3-2. Subgingival complexes in AgP before and after therapy. The chart represents the prevalence of 42 taxa at baseline and 6-weeks post-therapy in 3 responders (5 sites) and 4 non-responders (8 sites). The length of each bar stack indicates the % of sites colonized by different levels of each species. The species are grouped according to the microbial complexes described by Socransky et al. (1998).
Figure 3-3. ‘Not-yet-cultivable’ and other taxa in AgP before and after therapy. The chart represents the prevalence of 39 taxa at baseline and 6-weeks post-therapy in 3 responders (5 sites) and 4 non-responders (8 sites). The length of each bar stack indicates the % of sites colonized by different levels of each species.
Figure 3-4. Changes in species prevalence following periodontal therapy between the AgP responders and non-responders. Each bar chart represents mean % change in prevalence of 81 taxa from baseline to 6-weeks post-therapy in 3 responders (5 sites; blue) and 4 non-responders (8 sites; green). The species are grouped according to the microbial complexes as described by Socransky et al. (1998), with the addition of several ‘not-yet-cultivatables’ and under-investigated taxa.
CHAPTER 4
EFFECTS OF PHASE I PERIODONTAL THERAPY ON MATRIX METALLOPROTEINASE LEVELS IN AGGRESSIVE PERIODONTITIS

Introduction

Periodontitis is an inflammatory disease of the supporting tissues of the teeth, resulting in progressive destruction of the periodontal ligament (PDL) and alveolar bone with pocket formation, recession, or both. The pathogenic processes are largely a response to microbial induced destructive mechanisms. These processes are initiated by the microbial biofilm, but are undertaken by the host cells in chronic disease progression, and thus it is the host tissue itself that results in the destruction observed (Kinane et al., 2001). The host initiates and controls the release of enzymes, including matrix metalloproteinases (MMPs), to allow the tissues to retreat from the microbial destructive lesions. Recently, there has been increasing evidence implicating MMPs as key mediators in the tissue destruction associated with the various forms of periodontal disease, including the progression from gingivitis to periodontitis. (Reynolds et al., 1994, Golub et al., 2001, Garlet et al., 2004) In periodontal tissues, MMPs are expressed by inflammatory cells (monocytes, macrophages, lymphocytes, polymorphonuclear cells) and by resident cells (fibroblasts, epithelial cells, endothelial cells). (Hannas et al., 2007)

Previous studies, mostly involving chronic periodontitis (CP) patients, have demonstrated elevated levels of various MMPs associated with inflammation, as well as correlations between these levels and the extent of tissue destruction. (Golub et al., 1976) In particular, active forms of MMPs in the gingival crevicular fluid (GCF) have been shown to reflect progressive clinical attachment loss (CAL) and disease severity (Golub et al., 1974, Kiili et al., 2002, Ilgenli et al., 2006, Hernandez et al., 2006). Additionally, these enzymes have been proposed as possible
prognostic indicators of periodontal disease progression that may be more sensitive than traditional clinical indices, which may provide a much needed advancement in the planning of periodontal treatment.

We have previously reported on the MMP levels of the collagenases (MMP-1, -8, and-13), the gelatinases (MMP-2 and -9), stromelysin (MMP-3), and macrophage elastase (MMP-12) in a cohort of children, 7 to 19 years of age, with and without localized aggressive periodontitis (Alfant et al., 2008). MMP levels were elevated in AgP sites relative to non-diseased sites within the same subjects, in siblings, and in unrelated controls. Additionally, the MMPs associated with the AgP sites in children were generally elevated compared to an adult cohort with a history of chronic periodontitis. In this study, we investigated the effect phase I periodontal therapy would have on the MMP levels in GCF from these AgP children.

**Materials and Methods**

Eight patients aged 13-18 previously diagnosed with AgP were included in this study. GCF samples were collected from two deep sites (PPD ≥ 4mm), almost always from the 1\textsuperscript{st} molars. The sampling area was isolated with cotton rolls to avoid contact with saliva, and supragingival plaque was removed to eliminate the risk of plaque contamination. After gentle air-drying, GCF was collected using Periopaper (ProFlow, Inc., Amityville, NY) by inserting the strip 1 mm into the sulcus and allowing the GCF to wick up the strip. The volume of GCF collected was immediately measured chair side with a calibrated Periotron 8000 (ProFlow, Inc., Amityville, NY). The strip was placed in a dry microcentrifuge tube and kept on ice.

Upon return to the laboratory, the samples were stored at -80\textdegree C until used for measurement of the MMPs. The GCF readings were converted to volume based on standards curves generated
for the instrument used. The activities of MMP-1, 2, 3, 8, 9, 12, and 13 were assessed using Enzolyte 520 fluorimetric MMP kits (AnaSpec, San Jose, CA) specific for each MMP, in accordance with the manufacturer’s instructions, as described previously. Fluorescence intensity was measured at Ex/Em = 490 nm/520 nm using a Synergy HT fluorimetric reader (BioTek, Winooski, VT) and associated software.

The data, initially expressed as relative fluorescence units, were converted to ng based on standard curves ($R^2 >0.99$) generated with the purified recombinant MMP and its specific substrate. This value was adjusted to concentration (ng/µL) based on the volume of the GCF collected. Due the distribution of MMP values obtained, non-parametric statistics were applied to avoid the effect that outliers might exert. Statistical differences among the 3 groups were sought using the Kruskal-Wallis test. Statistical differences for the MMP levels between pre- and post-treatment were analyzed using the Wilcoxon signed rank-test. The Mann-Whitney test was used to determine the significance of the differences between baseline versus control and post-treatment versus control for MMP levels. Based on the data obtained, we treated each site as being independent rather than considering the subject to be independent. Statistical significance was considered to be a p-value $\leq 0.05$. Significance levels were not adjusted for multiple comparisons. All statistical analyses were performed using GraphPad Prism version 5.00 for Macintosh (GraphPad Software, San Diego, California).

**Results and Discussion**

A graphic summary of the MMP levels (mean ± SD) is presented in Figure 4-1. Comparisons were only made within each MMP. As is apparent in Figure 4-1, the MMP levels associated with the diseased sites from the AgP group are consistently elevated relative to the
controls, both before and 6-weeks post-therapy. Table 4-1 lists the significant differences among
the groups. Statistical differences (p<0.01) were found among the 3 groups for all MMPs tested.
Highly significant differences (p<0.0001) for all MMPs, with the exception of MMP-12, were
detected between the AgP sites and healthy controls. After treatment, statistical significant
differences between the AgP deep sites and the controls were detected for each of the MMPs.
Significant differences were also detected between the AgP deep sites at baseline and 6-weeks
post-therapy for MMPs-1, 2, 8, 9, and 12.

Based upon clinical mean patient clinical parameters taken at the 6-week reevaluation visit,
patients were grouped according to their response to treatment, as described in the microbial
analysis. The ‘responding’ group consisted of 4 patients that demonstrated a positive response to
therapy, while the ‘non-responding’ group consisted of 4 subjects that either did not respond well
to therapy or demonstrated continued periodontal deterioration. These subjects were the same
seven patients from the microbial analysis in the previous chapter, with an additional patient for
whom plaque samples were not available. It should be emphasized that while the responders
have demonstrated some improvement at 6 weeks post-therapy, they are still far from
‘successfully’ treated, and due to the aggressive nature of AgP, may still regress. Furthermore,
the MMP levels of the responders and non-responders were not statistically significantly
different between the two groups; there were no differences at baseline, after treatment, or
relative to controls at either point. Thus, because there were no differences in MMP levels
between the responders and non-responders, they were combined to represent the ‘diseased AgP’
group.
Despite the limited size of our study population and the relatively short time period following treatment, we detected statistically significant reduction of the collagenases (MMPs-1, -8, and -13) and gelatinases (MMPs-2 and -9) 6 weeks after periodontal therapy. Also, a general reduction in gingival bleeding index (data not shown) was observed post-therapy, indicating reductions in gingival inflammation. The decreases observed in collagenases and gelatinases are similar to previous studies investigating MMP changes associated with treatment in chronic periodontitis (CP) (Buduneli et al., 2002, Tuter et al., 2002, Sorsa et al., 2006). Decreasing the levels of these MMP groups, in particular, has been associated with an accompanying reduction in gingival inflammation and improved clinical prognosis (Uitto et al., 2003, Sorsa et al., 2004a, Ryan and Golub, 2000). Studies reporting successful treatment of CP subjects have not only reported reduction of MMPs post-therapy, but also to similar levels relative to those of healthy controls. Interestingly, despite half of our patients showing no response to treatment, it does appear that phase I periodontal therapy led to reductions in gingival inflammation within 6 weeks, based on the reduced gingival bleeding and the accompanied lowering of collagenase and gelatinase levels.

Although a reduction in the collagenase and gelatinase levels was observed, they remained significantly higher relative to healthy controls. Thus, it is not clear whether the suspected modulation of gingival inflammation observed at 6 weeks is a temporary phenomenon or a trend that will continue along the healing process. Additionally, differences in MMP-12 (macrophage elastase) levels between AgP subjects and healthy controls were not statistically significant at baseline, but the AgP sites increased to levels significantly higher post-therapy. While there may be some indication that initial treatment may have dampened the inflammation observed in the
soft tissues, the destruction of the underlying alveolar bone may have been unaffected. Further clinical evaluations, however, that are able to determine if alveolar bone loss is progressing are required.

A longer re-evaluation period, most likely requiring periodontal maintenance, if not further treatment, are needed to determine the true effects of phase I periodontal treatment. A reduction in inflammation is a start, but considering the severe and aggressive nature of this form of disease, it is imperative that these patients are monitored more frequently in order to potentially prevent further deterioration and immediately enforce the appropriate changes in treatment strategy. AgP is characterized by a hyper-inflammatory response, resulting in rapid and severe bone lose, which implies the involvement of MMP-12 and other mediators involved in bone destruction. Although this study, as well as several others, suggest MMP activity may be associated with the severity of periodontitis, it is not clear whether the elevation of these enzymes exacerbates the disease process or is elevated as a result of the disease, further making the biological and clinical significance of these MMP reductions difficult to interpret.

MMP-3, which is also suspected of involvement with bone resorption, was the only other MMP assayed that did not decrease at statistically significant levels. Additionally, AgP tends to run in families and in a recent study we reported similarities between the MMP profiles of AgP subjects and their periodontal-healthy related siblings. Interestingly, non-diseased AgP sites and their siblings did not differ in MMP-3, -9, and -12 levels and were all significantly elevated relative to healthy controls. Considering that phase I periodontal therapy, which is generally considered ineffective against AgP, did not lower the levels of MMP-3 and -9, makes their potential role in the pathogenesis of AgP intriguing. Longitudinal studies, monitoring a larger
study group over longer time periods, are currently underway that will enable us to more thoroughly investigate the involvement of MMPs during the development, progression, and treatment of AgP.
Figure 4-1. MMP levels at baseline and 6-weeks following periodontal therapy. The length of each bar stack represents the concentration of active enzyme present (mean ± SD) for: AgP sites at baseline (blue), AgP sites 6 weeks after treatment (green), and healthy controls (orange).
Table 4-1. Statistical significant differences in MMP levels following periodontal therapy.

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BT = sites from AgP subjects before treatment; AT = sites from AgP subjects 6 weeks after treatment; C = sites from healthy unrelated controls.
CHAPTER 5
CONCLUSIONS

In 1923, Gottlieb described a 22-year-old man as having “diffuse atrophy of alveolar bone.” The case he would characterize, described without any contributing gingival enlargement as a distinctive clinical feature, would later become considered the first case report of Aggressive Periodontitis (AgP) (Guzeldemir and Toygar, 2006). Five-years later, Gottlieb described the rapid destruction of this disease as resulting in disorganized and irregular periodontal ligament fibrils. Ten-years later, Wannenmacher described the incisor and premolar pattern of the disease (Saxen, 1980), a feature that has remained distinctive ever since. While descriptions of the disease still remain dominated by individual case reports, along with limited data regarding its pathogenesis, the classification of AgP continues to evolve. The terms periodontosis, juvenile periodontitis, and early-onset periodontitis have all been used, but they generally have referred to a severe and rapidly progressing form of periodontitis, often with a distinctive bilateral molar and incisor presentation, and usually limited to young adults under 30-years of age.

Problems, inconsistencies, and deficiencies with previous classification systems, have led to several workshops in order to establish a widely accepted scheme for the classification of periodontal diseases (Armitage, 2002). While disease occurrence may be perceived more frequently in younger individuals, this may be due to its observed frequency relative to other forms of periodontitis within this age group. However, AgP may present at various ages, including children and can persist in older adults (Periodontology, 2000). Most recently, the 1999 international workshop on the classification of periodontal diseases reclassified ‘Prepubertal Periodontitis’ as ‘Aggressive Periodontitis,’ along with the removal of its age-dependent description (Armitage, 1999).
Due to its low prevalence and multiple changes in the clinical parameters used to classify the disease, epidemiological data can be difficult to interpret; however, the prevalence of the disease in children is estimated to range from 0.1% to 0.76%, with higher prevalence among African Americans estimated from 2.1% to 2.6% (Albandar, 2002). However, what makes these data difficult to interpret are the multiple changes in classification over the past 30 years, which has stirred considerable debate, and has made interpreting older data difficult (Armitage, 2002).

We have recently identified a cohort of African-American children (aged 7-19 years) in Tallahassee, Florida. Almost all of our current understanding of the pathogenesis of AgP comes from older subjects, generally >30 years of age and not uncommonly up to 50 years. AgP, like other forms of periodontal disease, is generally believed to be a chronic inflammatory reaction initiated by the oral biofilm. A microbial induced inflammatory disease between a 7-year-old child and a 50-year-old adult likely have similar overlapping pathogenic properties for their conditions, however, they are more likely to result from distinct pathogenetic features, in terms of the immunologic, microbiologic, genetic, and environmental factors that likely alter the presentation, duration, severity, and progression of these disease. For example, if one assumes the periodontal disease is initiated by a specific subset of pathogens (in combination with a susceptible host) then the older individual, assuming equal immunological susceptibility, would be at a far greater risk of exposure and acquisition of the species, groups of species, or different combinations of the infectious microbes, compared with the 7-year-old.

In these studies, we investigated the subgingival flora and MMPs associated with AgP in children. We were able to investigate both variables cross-sectionally and also longitudinally to determine how initial periodontal therapy may modulate these factors. The microflora associated
with AgP in children has focused almost exclusively on Aggregatibacter actinomycetemcomitans, although taxa including Campylobacter, Capnocytophaga, Eubacterium, Fusobacterium nucleatum, Eikenella corrodens, Porphyromonas gingivalis, and Prevotella intermedia have also been suspected as potential pathogens (Darby and Curtis, 2001, Oh et al., 2002, Moore et al., 1985, Kamma et al., 2004). With the exception of the cultural studies by Moore and Holdeman on localized juvenile periodontitis (LJP) in 1985 (Moore et al., 1985), a comprehensive study of AgP in children has not been done. Additionally, it has not been done using advanced molecular techniques that permit a more thorough microbial investigation, which can more effectively examine fastidious and ‘not-yet-cultivatable’ species. Our investigation was able to identify 81 specific taxa; many suspected periodontal pathogens, several whose role is less clear, and others, such as the ‘not-yet-cultivatable’ phylotypes that have never before been investigated.

Our initial studies were based on a whole-genomic ‘Checkerboard’ DNA-DNA hybridization assay. This procedure allows the simultaneous quantitative analysis of 40 microbial species against 28 patient samples, on a single solid support membrane (~15x15 cm). As originally described by Socransky, the ‘checkerboard’ assay utilized whole genomic probes to study the subgingival microflora associated with periodontitis, and has since proven to be a rapid, sensitive (10^5-10^6 cells), and inexpensive method relative to traditional culture-based approaches. One major advantage of the ‘checkerboard’ hybridization assay is that clinical samples can be analyzed directly, without the need for cultivation or delicate sample handling, thus providing a practical approach in conducting large-scale clinical studies needed to elucidate the role of the oral microbiome in health and disease. However, traditional ‘checkerboard’ assays...
based on whole genomic probes, are limited to cultivatable species, which require in vitro
cultivation of these species for the development and validation of species-specific probes. While
suitable in certain contexts, whole-genomic probes face challenges in maintaining specificity
among closely related species and precision between assays; although these challenges can
generally be overcome, the resources and maintenance required are challenging for smaller
research laboratories.

In contrast, methods employing rRNA-based oligonucleotide probes have been developed
that can differentiate between closely related species or even sub-species, while also
circumventing the need for in vitro bacterial cultivation, thus permitting the use of probes
targeting not-yet-cultivable species. 16S rRNA-based molecular analysis of the subgingival
flora has recently revealed a much broader bacterial diversity than previously realized, with
estimates upwards of 500 different species identified from the periodontal pocket, with each
individual harboring 100-200 species at any particular site; and a growing percentage classified
among the not-yet-cultivated species. PCR-based approaches have shown that many of these not-
yet-cultivated species may be specifically associated with either health or disease, but were likely
neglected due to technical constraints. Utilizing probes that target the 16S rRNA gene, Paster
described a “PCR-based, reverse-capture, checkerboard hybridization” assay capable of
monitoring not-yet-cultivated species, in addition to the use of ‘universal’ and ‘genus-specific’
probes that can be used as reference standards for a more thorough analysis of the microbial
composition of clinical samples (e.g., percentage of total bacteria that are Streptococci, including
species not represented in a limited panel of species-specific probes). The ‘reverse-capture’ limit
of detection is about $10^3$ cells, practically eliminates probe cross-reaction with closely related
species, and allows shortened hybridization times of between 2 to 4 h (compared to overnight for whole-genomic). Oligonucleotide capture-probes, manufactured commercially, also permit the construction of ‘stock’ membranes that can be preserved indefinitely, which further streamlines the time required to carry out the extensive hybridization assay. Additionally, only about 10% of each clinical sample’s extracted DNA may be sufficient for up to 10 reverse-capture membranes allowing an expanded array of probe panels (potentially allowing specific detection for up to 300 separate probes), while allowing the remaining 90% of total sample to be archived for future use.

The adaptation of this technique to work in our laboratories has greatly expanded our capability to characterize not only the subgingival flora of AgP in these studies, but also can be extended to any future investigations of the oral microbiome. This system is more sensitive, cheaper, faster, and generally robust compared with our previously used whole-genomic approach. Our initial results have revealed a much broader spectrum of microbial flora associated with diseased sites of AgP in children than has been previously reported. To the best of our knowledge, this represents the most comprehensive study of the subgingival flora associated with diseased sites in children with AgP. Additionally, several species were detected in our study population, particularly many of the ‘not-yet-cultivable’ phylotypes, which have never before been described in this form of disease.

Our initial results have hopefully laid the foundation for future longitudinal studies, which may better characterize and elucidate the role of the subgingival flora during the pathogenesis of AgP. This may eventually lead to improvements in the prevention, detection, and treatment of AgP. Due to the limited research available of AgP in children, current clinical procedures adapt
from and rely heavily on those typically implemented with chronic periodontitis, for which the scientific and clinical research is much more extensive.

While the pathogenic processes are largely a response to microbial induced destructive mechanisms. These processes are initiated by the microbial biofilm, but are undertaken by the host cells in chronic disease progression, and thus it is the host tissue itself that results much of the destruction observed (Kinane et al., 2001). The host initiates and controls the release of enzymes, including matrix metalloproteinases (MMPs), to allow the tissues to retreat from the microbial destructive lesions. The host initiates and controls the release of enzymes, including MMPs, to allow the tissues to retreat from the microbial destructive lesions. Recently, there has been increasing evidence implicating MMPs as key mediators in the tissue destruction associated with the various forms of periodontal disease, including the progression from gingivitis to periodontitis (Reynolds et al., 1994, Golub et al., 2001, Garlet et al., 2004). Previous studies, mostly involving chronic periodontitis patients, have demonstrated elevated levels of various MMPs associated with inflammation, as well as correlations between these levels and the extent of tissue destruction (Golub et al., 1976). In particular, active forms of MMPs in the gingival crevicular fluid (GCF) have been shown to be associated with progressive periodontal destruction (Golub et al., 1974, Kiili et al., 2002, Ilgenli et al., 2006, Hernandez et al., 2006).

We have demonstrated that certain MMP levels are elevated in diseased sites of AgP children relative to non-diseased sites in the same children, to their siblings, and to diseased sites associated with chronic periodontitis. Additionally, we have shown the effects of phase I periodontal therapy on these levels 6 weeks post-therapy. While studies involving chronic periodontitis (CP) patients have shown reductions in MMP levels to the levels found in healthy
controls (Tuter et al., 2005, Tuter et al., 2002), we have demonstrated significant reductions, but not as low as the levels detected in healthy control children. Since phase I periodontal therapy without the use of antibiotics in AgP is generally unsuccessful, our results indicate MMPs may be intriguing targets for future research, including investigating their potential prognostic value and/or as drug targets.

Similar to our study on the subgingival flora, this may represent the most comprehensive analysis of MMPs of AgP in children. While previous studies of MMPs have focused almost exclusively on chronic periodontitis or adult populations, there is virtually no data concerning MMP levels of AgP in children. Also, the use of a relatively wide panel of 7 MMPs, in addition to an analysis consisting of non-diseased sites within the same individual, their siblings, healthy controls and CP distinguish our cross-sectional study from what is typically reported.

AgP in children may be very rare, but it tends to run in families, giving siblings a 50% chance of also having or of developing the disease. Additionally, these families were generally fairly large, commonly with 3-4 children per family. A retrospective longitudinal investigation of the healthy siblings, as well as the AgP children as they continue with treatment, should provide some valuable insight into the mechanisms responsible for causing this disease. This can hopefully lead to a better understanding the pathogenesis of AgP, and in turn, result in more effective screening, prevention, and treatment of this disease.
LIST OF REFERENCES


Barnett Alfant was born in 1982 in Gainesville, Florida. Shortly thereafter, his family moved to Melbourne, Florida, where his father worked as a periodontist and his mother raised him and his brother, Zachary. After receiving his high school degree from Satellite Beach High School, Barnett began his undergraduate career at the University of Miami. In 2005, he received his bachelor degree in biology. After graduation, he worked in the lab of Dr. Clay Walker at the University of Florida, College of Dentistry, Department of Oral Biology. After his acceptance into the College of Medicine, M.S. Program in Medical Sciences, Barnett was given the opportunity to continue his work under the guidance of Dr. Walker. Barnett completed his Master of Science degree in May 2009 and applied to dental school.