

# **A Comprehensive Metagenomic Approach to Determine the Relationship between Periodontal Disease and Cardiovascular Disease in Young Adults**

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## **ABSTRACT:**

Periodontal Disease (PD) is shown to be associated with an increased the risk of a number of systemic diseases, including atherosclerosis. We are investigating a possible causal relationship in a young population of individuals with PD (ages 20-30) to determine if they show early signs of atherosclerosis. Using powerful new culture-independent molecular approaches based on pyrosequencing technology, we are conducting a comprehensive metagenomic analysis to identify, classify, and quantify bacterial species-associated PD and atherosclerosis (both pre- and post- treatment for PD). This survey of microbial diversity is expected to cover between 300,000 and 500,000 16S sequences for 300 samples from 40 patients. The bioinformatics pipeline will include 1) databasing data sets of unique 100-250-base 16SrRNA sequences per species, 2) sequence alignment and phylogenetic analysis, and 3) computing distance metrics for differences between microbial communities (samples) at different stages of PD and with or without changes in brachial artery flow dynamics. Multivariate analyses, such as Principle Coordinates Analysis (PCoA), will be used to show the distribution of 16S community samples. Subsequently, functional annotation of the genes combined with relative abundance data for each species will allow for the prediction of important functional relationships among potential subgroups of species that support the persistence of PD *and* that could be involved in the mechanism of action leading to atherosclerosis. Ultimately, this knowledge could be used to develop an effective probiotic treatment for PD.

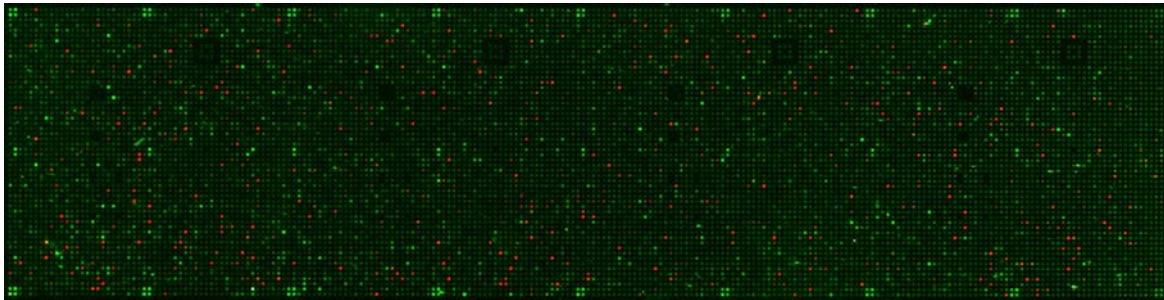
## **BACKGROUND AND SIGNIFICANCE:**

Epidemiologic studies have shown a significant association between periodontal disease (PD) and atherosclerosis, but a causal relationship has not been established. If periodontal disease contributes in a significant way to the initiation and/or progression of heart disease, then educating children and families about oral hygiene and providing routine preventive dentistry would represent a relatively low-cost intervention to reduce the incidence of atherosclerosis. Endothelial function (a sensitive and reliable indicator of vascular health) has improved in middle-aged men after their periodontal disease was aggressively treated (Tonetti et al. 2007). PD is accompanied by a local inflammatory response, with invasion of neutrophils and lymphocytes, which may chronically progresses to systemic inflammation. Patients have elevated levels of TNF $\alpha$ , IL-1 $\beta$ , C-reactive protein (CRP), IL-6, and monocyte chemotactic protein-1 (MCP-1) (Amar et al. 2003), which could be due to either an unchecked response to chronic local infection or to the presence of occasional bacteria (or bacterial fragments) in the circulation that triggers a systemic response. The magnitude of inflammatory burden is related to the progression of atherosclerotic calcifications in patients with advanced disease (Li et al. 2007). In PD bacteria form calculus through calcium deposition in a complex porous matrix on the tooth's surface, resulting in local inflammation, and swollen, tender gums

that bleed easily upon brushing or flossing. The most well-known bacteria are *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans*. Two studies implicated *P. gingivalis* as a particular risk factor for atherosclerosis (Chou et al. 2005; Yamazaki et al. 2007). Some bacteria secrete factors that limit growth of other species, likely substantially altering the entire oral ecosystem. Little has been done to comprehensively characterize the oral microbial ecosystem, which includes anaerobic species and those never before cultured (Lepp et al. 2004). A non-biased metagenomic approach to identifying the diverse oral flora is essential. Identifying those strongly associated with PD, as well as oral flora specifically associated with atherosclerosis (as reflected by endothelial dysfunction) is highly probable.

## RESEARCH DESIGN AND METHODS:

### Preliminary Results:

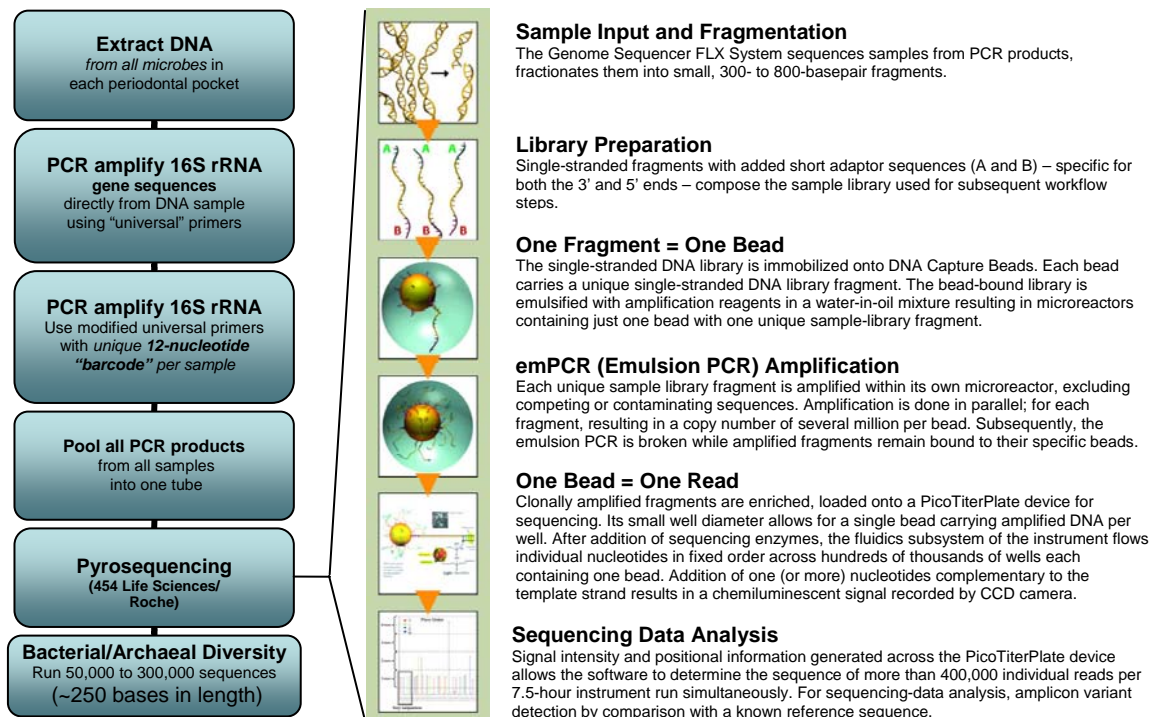


**Figure 1. Microarray Analysis**

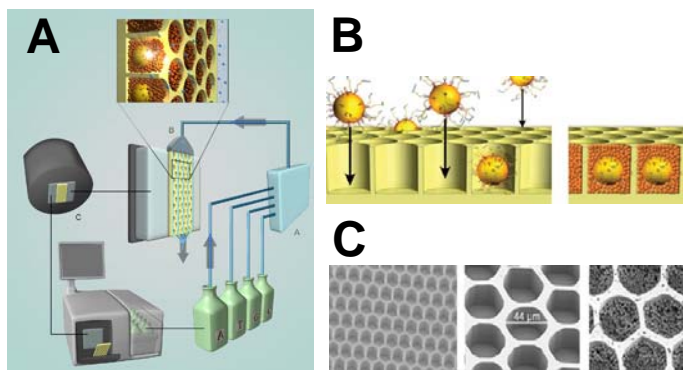
This CustomArray chip (Combinatrix) demonstrates the ability to rapidly detect the relative presence or absence of 16S rRNA transcripts uniquely representing known bacterial species as well as specific toxin and antibiotic-resistance genes in two environmental samples (communities). Due to the diversity of bacterial cells, it is much more challenging to quantify their relative amounts in this way. However, PCR combined with new pyrosequencing technology (diagram below) allows us to identify and quantify the relative abundance of 1000's of species per sample. (courtesy of Lesley Lee, Kelley Lab, Dept of Biology)

### Culture-Independent Molecular and Microbiological Methods:

Culture-independent molecular techniques, based on PCR and molecular cloning of small subunit ribosomal (16S rRNA) gene sequences, revolutionized our ability to study previously uncultured microbial organisms. The 16S rRNA gene is the “gold-standard” of markers for culture-independent microbial studies because: (1) It is found in all forms of cellular life: bacterial, archaeal and eukaryal; (2) Enormous databases of 16S sequences from cultured and uncultured microbes, allow for easy identification and phylogenetic analyses; (3) This molecule contains both highly conserved regions necessary for universal primer design, and highly variable regions necessary for identifying species and phylogenetic relationships (Pace 1997; Hugenholtz et al. 1998). Our lab has studied microbial communities in sheep respiratory tracts (Safaei et al. 2006), acidic hot springs (Mathur et al. 2007), contaminated human environments (McManus and Kelley 2005; Lee et al. 2007) and oceanic waters (Breitbart et al. 2004; Angly et al. 2006). Although we cannot completely rule out bias, particularly PCR bias, culture-independent diversity estimates are considerably less biased than culturing.

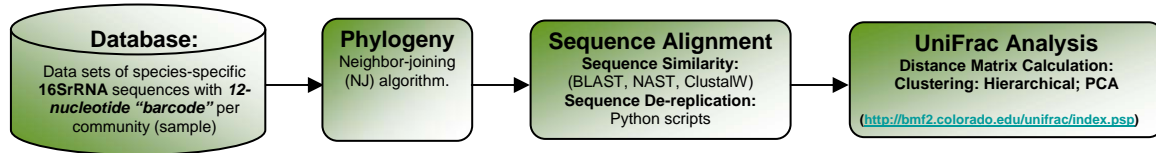


The molecular-based pyrosequencing approach (above) is an especially tractable method that increases data generation by several orders of magnitude, while eliminating the tedious and error-prone step of cloning each PCR product and picking colonies for sequencing.



**Figure 2:** 454 pyrosequencing technology allows for recording the signal corresponding to addition of each nucleotide while the fragment is amplified in parallel on a single bead. (A) Nucleotides flow sequentially in fixed order across the PicoTiterPlate (B) device during a sequencing run. (C) Tiny enzyme covered packing beads surround each bead and catalyze the chemiluminescence reaction during the emulsion PCR reaction.

## Bioinformatics and Data Analysis Pipeline:



**Figure 3: Flow of Analysis of Sequence Data**

**Database:** Large public, as well as local, databases of 16S sequences from cultured and uncultured microbes allow for easy identification and phylogenetic analyses.

**Phylogeny:** Due to the size of the trees, the simple and rapid Neighbor-joining (NJ) algorithm will be used to determine relationships among individual sequences.

**Alignment:** Python programming scripts will be used to sort sequences by barcode and determine basic similarity (e.g. BLAST) to known sequences in the database. The scripts will also be used to de-replicate the sequences (1% divergence) to quantify how often particular microbes are present in the samples. Conditions for alignment (e.g. % identity and coverage) are yet to be determined.

**UniFrac software** measures the fraction of total branch length in a phylogenetic tree that leads to sequences from one community or the other but not both in each pair-wise comparison. A distance matrix of values determined for all pairs of environments is used to either hierarchically cluster them using an Unweighted Pair-Group Method with Arithmetic mean (UPGMA) or to perform dimensionality reduction using Principle Components Analysis (PCA). Samples will be clustered by microbial community, by disease state, treatment status, and by other clinical parameters, in terms of phylogeny.

### Expected outcomes

This will be the first study to evaluate endothelial function in relation to periodontal disease in young adults aged 20-30. If periodontal disease contributes to vascular dysfunction and the development of atherosclerosis, the follow-up study will reveal whether vascular dysfunction is reversible in this age group and may provide insight into the role of poor oral hygiene as a contributing factor to health disparity.

The most important information derived from this work will be the evaluation of the complex oral microbial flora in this patient population. Previously uncharacterized species of bacteria may be identified, some of which are expected to correlate with periodontal disease. We hypothesize that some organisms may correlate strongly with endothelial dysfunction. We also hypothesize that some organisms will correlate with good oral (and/or cardiovascular) health. Such organisms may secrete factors that potentially suppress the overgrowth of oral pathogens and could be beneficial.

This study, which will demonstrate whether treating periodontal disease in young adults can normalize endothelial function, represents an important step towards demonstrating causality and will furthermore establish the characteristics of a microbial ecosystem that may correlate more strongly with vascular dysfunction than the presence or absence of a single species.

### Future Directions:

Since phage can determine bacterial population dynamics, our collaborators in Dr. Forest Rohwer's lab will be investigating the bacterial phage populations in the same bacterial communities collected from the periodontal patient samples. In addition, the 3-D

structure of communities (biofilms) may be a next step in determining physical/chemical interactive relationships among key species involved in PD and endothelial dysfunction.

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