THE IMMUNOGENETICS OF DENTAL CARIES

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ABSTRACT

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Background: Bacterial adherence to the acquired dental pellicle, important in caries, is mediated by receptor-adhesin interactions such as *Streptococcus mutans* antigen I/II (I/II). Ten I/II epitopes from the A, V, P and C regions were chosen to determine their reactivity in human saliva. Underlying the body's ability to immunologically respond to bacteria that lead to caries are the human leukocyte antigen (HLA) genes, specifically HLA class II (HLA-II) genes that control antigen presentation. Previous studies suggested that a specific HLA biomarker group (HLA-DRB1*04) may have differential control of immune responses to I/II. However, it was not known whether secretory IgA (SIgA) responses to the selected epitopes from HLA-DRB1*04 positive subjects were different compared to their non-biomarker counterparts (negative), or across other caries factors, since no study to date had thus assessed these questions.

Methods: Per IRB approval, the study population was divided into age, sex and race matched DRB1*04 positive (n=16) and negative groups (n=16). SIgA-epitope (and whole cell) reactivity was determined using ELISA. Other caries factors were measured. Subjects received a clinical exam by a trained examiner.

Differences between DRB1*04 positive and negative groups were examined using a two-sided, two-sample t-test.

Results: DRB1*04 positive subjects had numerically, but not statistically, higher reactivity to 9 out of 10 epitopes, the exception being residues 834-853 from the V and P regions of I/II across multiple measures. Though statistically insignificant, DRB1*04 positive subjects also exhibited 25-30 $\mu g\ mL^{-1}$ less total IgA (TIgA) than negative counterparts. All clinical caries data proved inconclusive when comparing groups, likely due to exogenous factors and sample size.

Conclusion: DRB1*04 positive subjects showed a trend toward lower TIgA. Moreover, they also showed a lower SIgA response across multiple measures to 834-853, the I/II V and P region epitope. This region forms a sort of functional epicenter involved in collaboration between domains along the entire I/II antigen, and governs the region involved in initial attachment to the acquired dental pellicle. This region may be involved in an in vivo discontinuous conformationally specific immunogenic epitope that serves as an HLA-II binding motif which remains elusive.

Richard L. Gregory, PhD, Chair

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CHAPTER ONE

Dental Caries and Human Genetics

Dental caries (caries) is a multifactorial oral disease that ubiquitously, albeit unevenly, affects the world population. Caries often produces severe "consequences of pain and dysfunction," which interfere with the "most basic functions of eating, sleeping, speaking, being productive and enjoying general health" (Edelstein, 2006). Although caries has been thought to be a predominantly environmentally-related disease, the full range of this infectious disease cannot be explained by environmental variables. Four major factors which directly contribute to caries development include the host, bacteria in the oral biofilm, diet (quality, quantity, and frequency), and time (Harris et al., 2009; Pinkham, 2005; Selwitz et al., 2007). Except for time, all of these factors are modified by host genes. Tooth anatomy and morphology as well as dentin and enamel mineralization, for example, are genetically controlled (Haruyama et al., 2009; Stephanopoulos et al., 2005; Townsend et al., 2003). In animal and human studies, differences in genetic background produce divergent immunomodulatory effects against bacterial antigens involved in caries (Robinette et al., 2009; Wallengren et al., 2005). Recent genetic evidence also points to the interesting fact that the types and levels of oral bacteria colonized in humans (i.e., the human oral microbiome) are genetically regulated by the host (Corby et al., 2007; Corby et al., 2005). Though the host and the oral microbiome are genetically modulated, so too is the diet.

Research has demonstrated that the proverbial sweet tooth is not as far-fetched as initially thought, and that a nuanced view reveals genetic background significant to sucrose sweetness preference (Bretz et al., 2006; Fushan et al., 2009). We now understand that a variety of behaviors are under significant genomic

control such as our sleep patterns or when we do things during the day (Brown et al., 2008), which would include eating and snacking. Oral hygiene would be included in this framework as it is predominatly a behavioral phenomenon, and as such, its genetic regulation cannot be underestimated. One study that has important implications for oral hygiene was done in the context of social reward (Caldu and Dreher, 2007). Those who had a particular variant of a reward gene were much more likely to accomplish a novel task, for our purposes brushing correctly, in the face of a social reward. Alternatively, those who had a different reward gene variant were much more likely to lack motivation altogether to complete the task at hand (e.g., brushing). What have traditionally been considered purely environmental and personal variables are, therefore, also genetically regulated.

Thus, the caries process is under more genetic control than is provided for in the traditional caries paradigm. However, it is very important to understand that when we say caries is genetically regulated, we are not saying that genetics explains 100 percent of the disease. In other words, caries is a multifactorial disease because it has a significant environmental component, there are multiple genes involved, there are gene-environment interactions at play, and the risk runs in families in a non-Mendelian manner. This simply means that the disease process "arises from the complex interaction of environmental and host genetic factors" (Burgner et al., 2006). The epigenetics of caries has not been discussed, but provides almost an infinite number of possibilities since the heritable changes that are passed on control how genes (e.g., associated with caries) are expressed, and therefore how proteins downstream function (Rodenhiser and Mann, 2006). It is important to understand that environmental insults interacting with host genes can produce "epigenetic patterns and thereby effect changes in gene activation and cell phenotype" (Barros and Offenbacher, 2009) over

generations. It would make sense, then, that genes and environment are inextricably tied to each other. Epigenetics, therefore, should provide the nexus for a new frontier which bridges the gap between genetics, oral disease, and environmental variables. This multifactorial nature of caries should not surprise us since the preponderance of evidence in both animal and human models demonstrate that caries susceptibility has a significant genetic component and that multiple genes are at work (Boraas et al., 1988; Conry et al., 1993; Finn and Caldwell, 1963; Horowitz et al., 1958; Hunt et al., 1944; Jackson et al., 1973).

The genomic puzzle of caries is just beginning to be unraveled, however, and the pieces are just beginning to be put in place. Researchers have now published the first genome-wide scan for caries, which demonstrated that chromosome 6 housing the Human Leukocyte Antigen (HLA) complex was among 5 chromosomes revealing unique and significant results with regards to caries susceptibility in a non-parametric linkage analysis (Vieira et al., 2008). This research attempts to focus on one piece of the genomic puzzle—HLA class II genes and the caries process.

The link between HLA Class II Genes and the Caries Process

Almost a half century ago it was discovered that HLA genes play a major role in modulating immunity. Interchangeably called the human major histocompatibility complex (MHC) or HLA (Shiina et al., 2009), this region is one of the most vital genomic areas with regard to infectious disease (Horton et al., 2004). A link has been suggested between HLA class II (HLA-II) alleles and this region because the molecules encoded by these alleles are inextricably tied to inducing how humans fight against bacterial threats (by presenting antigen efficiently, inefficiently or not at all).

The connection between HLA-II alleles—via HLA-encoded proteins and bacterial antigens—and caries has become an area of tremendous possibility. One

of the chief causative bacterial pathogens in caries is a member of the mutans streptococci family, *Streptococcus mutans* (Loesche, 1986; Mitchell, 2003), whose immunopathogenesis has been associated with specific HLA-II alleles. Similarly, recent studies suggest that other members of the streptococcal family, including *Streptococcus pneumonia* (Boyton et al., 2008) and *Streptococcus pyogenes* (Kotb et al., 2002), are also immunopathogenically associated with specific HLA-II alleles. Current studies have found associations between other infectious bacteria (e.g., *Mycobacterium leprae*), the diseases they cause, and specific HLA-II alleles (Vanderborght et al., 2007).

In the case of caries, it is unclear if there are significant associations between specific HLA-II alleles and the disease itself, although the HLA-II association is clearer with regard to the organisms implicated in the caries process, including and especially mutans streptococci. Nontheless, the preponderance of evidence suggests that there is a significant association between specific HLA-II alleles and caries. The common denominator across all caries factors are bacteria (i.e., the cariogenic agent), which colonize the human oral microbiome and attach themselves to the tooth structure. If we take bacteria out of the equation, caries does not develop (Casanova and Abel, 2004), as was shown more than 5 decades ago using gnotobiotic rats (Orland et al., 1954). How the host deals with cariogenic bacteria is one of the most important questions in understanding how the biological system produces resistant or susceptible disease outcomes, including caries. Focusing on HLA-II alleles makes sense because their proteins are responsible for presenting antigen to T cells in mucosal immunity, ultimately (assuming no immunogenetic dysfunction) providing appropriate mucosal immunity against bacterial cariogens such as mutans streptococci. By doing so, we may unravel some of the complex relationships between host HLA genomics, immunity, and the infectious disease of caries.

HLA Genetics and Caries

Researchers have suggested significant and important associations between HLA-II genes, HLA-II proteins and Antigen I/II (I/II), which is found on the cell surface of most strains of *S. mutans* and several other species that are potentially cariogenic (Tsuha et al., 2004). Antigen I/II is an immunodominant antigen of *S. mutans* with respect to activating antibody responses. Although a variety of researchers have termed I/II differently, including P1, the term I/II will be used throughout this dissertation. This surface adhesin allows bacteria to attach to salivary agglutinin (SAG) found in the layer of salivary glycoproteins deposited on teeth called the dental pellicle, which then forms dental plaque (or biofilm). While it is becoming increasingly clear that other bacterial species should be explored when considering caries, "no other species is yet a serious contender to *S. mutans* for a role as a worthwhile marker organism" for caries (Russell, 2008). Thus, *S. mutans* has been, and continues to be, the model organism for caries research. Those interested in the effect of HLA-II genes and their proteins on caries have thus used it (and its associated antigens) as the pathogenic model in animals and humans.

Animal and human studies suggest that HLA-II genes play a role in the development of caries. Rat Major Histocompatability Complex (MHC) genes clearly regulate immunity against *S. mutans* (Niiyama et al., 1987). One set of researchers showed that murine MHC genes, found in the H-2 region, controlled serum IgG response to I/II A-region epitopes (Takahashi et al., 1992). The murine H-2 region, corresponding to the HLA region in humans, has also a marked effect on clinical caries (measured by the modified Keyes method) susceptibility (Suzuki and Kurihara, 1998). Thus, animal and human studies suggest that these genes control immunity against the bacteria, and more specifically its associated antigens that are, in large measure, responsible for the caries process.

A foundational paper by Lehner et al. in England, involving human subjects, suggested an immune gene effect on caries by demonstrating that 10 patients who expressed HLA-DR4 (i.e., HLA-DRB1*04) antigens tended to be caries susceptible and failed to mount an immune response to I/II, measured by T-cell helper function via an antibody-forming cell assay, until the concentration of I/II was increased 1000 times (Lehner et al., 1981). The other 14 subjects who were typed as DR1, DR2, DR3, and DRw6 [i.e., DRB1*01, DRB1*02, DRB1*03, and DRB1*13 or 14 (Burmester et al., 2003)] were caries resistant and mounted immune responses to low doses of I/II. These results were statistically and dramatically significant, and the evidence demonstrated a link between a common clinical measure of caries, DMFS (decayed, missing due to caries, and filled tooth surfaces), and HLA-DR4-gene products. Another study found that DR4 patients exhibited more than double the suppressive regulatory T cell (Treg) activity than their DRw6 counterparts at a low concentration of I/II, further suggesting that DR4 subjects, under certain conditions, respond differently to a specific *S. mutans* antigen (Lehner, 1982).

One set of Swedish researchers has therefore tested the purported hypothesis (Lehner, 1982) that those patients who are DR4 positive are more likely to have higher levels of oral mutans streptococci. This group has found on two separate occasions a trend that supports this hypothesis. One study of 76 renal transplant patients demonstrated that all DR4 positive patients exhibited high levels of mutans streptococci, whereas those who were DR4 negative were more likely to have statistically significantly lower levels of mutans streptococci (Wallengren et al., 1991). The same study demonstrated a similar trend in healthy subjects although it did not reach statistical significance. This same trend in healthy subjects was also observed in a follow-up study, and a side note mentioned by the authors is that one participant, who was homozygous for DR4,

exhibited the highest levels of mutans streptococci correlating with a very high DMFS index (Wallengren et al., 1997), DMFS being an indicator of the extent of caries in a subject.

Although previous studies suggested a DR4-caries connection, none of these studies provided high resolution genotyping as the antigens tested were determined by immunological reactions and not direct DNA sequencing. Knowledge of HLA genetics changes and advances by the day, and methodologies and understandings have dramatically advanced and changed since these studies were done.

The first set of researchers, led by Acton, to use an updated nomenclature in testing various HLA-II alleles against levels of *S. mutans* and caries (measured by DMFS) indicated that African-American women demonstrated similar trends (up to this point only Caucasian populations had been studied), but the association to caries did not reach statistical significance (Acton et al., 1999). However, there were associations between HLA-DRB1*04 and *03 alleles and high levels of *S. mutans* (depending on the inclusion criteria). Of interest is that when the authors excluded those who had no signs of clinical disease, they found that the DRB1*04 association with bacterial counts gained tremendous statistical strength (Odds Ratio = 11.8, 95% CI = 2.09-66.2, $p = 0.005$), although the *03 association lost significance altogether. This supports the complex nature of this disease process, where host factors may interact with bacterial factors to increase the susceptibility of the host, although the actual pathological process is likely to be influenced by other factors as well.

In another non-Caucasian Asian cohort, a variety of alleles from three loci, including HLA-DQA1, DQB1, and DRB1, as well as a variety of haplotypes, were examined (Ozawa et al., 2001). The authors found that the DRB1*04 association with mutans streptococci was not significant and hypothesized that the

difference might be explained by population or ethnic differences. This is a distinct possibility. One set of authors recently observed that it is extraordinary, but nonetheless a fact that identical diseases, such as an autoimmune disease, may be associated with different HLA-II alleles "in different ethnic or racial populations" (Bondinas et al., 2007). The researchers examining an Asian population did find that mutans streptococci levels were weakly associated with a different allele, specifically HLA-DQB1*0601. Even so, the authors admitted that if they had a larger sample size they might have been able find more robust associations between important HLA-II alleles and oral microorganisms. This is a common limitation when examining a specific HLA-II allelic variant and its association with disease. For instance, of the total sample size of 106 in their study, only 3 subjects were DRB1*0401 positive, whereas they had approximately 30 subjects who were positive for DQB1*0601, the variant they found to be weakly associated with mutans streptococci. These limitations have typically led to observable trends not reaching statistical significance.

Another study in a Caucasian sample demonstrated that a particular allelic variant of DRB1*04, *0401, was found to be statistically associated with salivary IgA activity, those who were DRB1*04 positive were significantly different from those who were *04 negative (Wallengren et al., 2001). As is well known, antibodies play a major role in immunity and are linked to HLA genetics. The predominant secretory antibody in saliva is secretory IgA (SIgA), with the IgA:IgG ratio in oral secretions from the parotid gland being 500 times greater than that of serum (Brandtzaeg, 2007). In the protease-laden environment of the oral cavity, SIgA is remarkably stable, and active against *S. mutans*. A majority of the evidence indicates that a higher level of salivary IgA in the oral cavity is related to a lower level of caries (Gregory et al., 1990). In a 2005 follow-up study, with a much larger sample size (n=58 DRB1*04 positive; in contrast to

n=19), these results were confirmed. For example, those subjects who exhibited immunodeficiency—as recently conceptualized (Casanova and Abel, 2004)—against whole cell extracts of *S. mutans* and *S. sobrinus*, and I/II, were all DRB1*04 positive. These data demonstrated that they were either entirely unable to mount a detectable IgA response or their response was significantly lower than DRB1*04 negative subjects (Wallengren et al., 2005). Of the DRB1*04 allelic variants analyzed, only DRB1*0401 was found to reach statistical significance although it is clear from the raw data that it cannot be concluded that the other allelic variants did not lead to the same outcome (after analyzing *0401, *0404, *0402, *0403, *0405, *0407, *0408, *0414, and *04 negative persons). Virtually everyone that did not mount IgA responses against *S. mutans* were DRB1*04 positive. Of 3 human samples that demonstrated no detectable immune response against I/II, all were DRB1*0401.

In an Iranian cohort of children published very recently, HLA-DRB1*04 positive children were 10 times more likely to have early childhood caries (ECC) (Bagherian et al., 2008). This is interesting from the standpoint that all 79 subjects were between the ages of 12 and 72 months, which has significance with regard to both the natural history of caries and the development of the salivary immune response. Although colonized with certain types of oral streptococci (i.e., *Streptococcus salivarius* and *Streptococcus mitis*) as newborns, children are likely not permanently infected with mutans streptococci, meaning predominantly *S. mutans* and *S. sobrinus*, which are the main cariogenic agents in caries, until anywhere from 18 to 36 months (Taubman and Nash, 2006). Permanent infection with some of the mutans group does not happen until tooth eruption begins, instead of only the 18-36 month range, although we now know this can happen earlier, but colonizing numbers are lower and harder to detect until the primary molars start erupting. Newborns lack significant levels of SIgA, but by 12 to 24

months most children have SIgA levels similar to those found in adults. Unfortunately, the Iranian study did not analyze levels of mutans streptococci colonization or SIgA. The significance in knowing whether someone has an HLA-II susceptibility allele for caries is apparent if HLA-II-mediated immunodeficiency or immune dysfunction with regard to IgA immunity leads to the initial colonization of mutans streptococci, and the development of caries. Of 35 children who did not have ECC, 34 of them, or 97 percent, were DRB1*04 negative.

The preponderance of the evidence, albeit uneven, suggests an association between specific HLA-II alleles, especially DRB1*04 in Caucasians, and caries. That said, human studies conducted heretofore have had limitations that must be remedied, especially with regard to sample size and allele frequency. This purported association needs further validation studies to advance the science of HLA genetics and caries. Furthermore, the HLA-II association and its ultimate significance with regard to understanding caries are certainly not one-to-one. A specific HLA-II allele, or some combination of them, will surely not explain all of the variation seen in caries. To underscore an earlier point, caries is a complex disease, and as such, develops amidst multiple genetic and environmental factors. Amidst the genetic puzzle, this research only focuses on one of the genetic pieces, albeit potentially more important than we now understand. By understanding how particular HLA-II alleles make one susceptible or resistant to the clinical infectious disease of caries, we will unravel part of the genetic puzzle. Our lack of detailed understanding with regard to how HLA-II alleles make one susceptible or resistant to caries is a general problem that has yet to be solved across a spectrum of diseases. This problem will not be solved anytime soon, but the more we know the closer we are.

A Functional Immunogenetic Framework: ***Mechanisms connecting HLA-II to Caries***

The mechanisms involved in how specific HLA-II alleles may make one susceptible to caries are essentially unknown or unproven. Although the general points of connection between genes and immunity are fairly clear, the details remain murky. As is well known, antibodies play a major role in immunity and are linked to HLA genetics. From the immunity standpoint, a majority of the evidence indicates that a higher level of salivary IgA antibodies against *S. mutans* antigens in the oral cavity is related to a lower level of caries (Brandtzaeg, 2007). High salivary IgA antibody levels relate to low levels of *S. mutans* during early oral colonization, and low levels of salivary IgA correlate with high levels of *S. mutans* and caries experience (Nogueira et al., 2005). The working hypothesis of how this happens is that salivary IgA interacts with critical epitopes of adhesins of cariogenic bacteria (i.e., I/II, GTFs [glucosyltransferases], or GBPs [glucan-binding proteins]), blocking these adhesins and thus inhibiting their function, or alternatively, priming *S. mutans* to become opsonized, phagocytosed, and destroyed by gingival neutrophils, thus depriving *S. mutans* (or similar cariogenic bacteria) of its ability to attach to the tooth surface and produce disease. Any type of immunity against caries must inhibit bacterial adhesion or facilitate clearance of mutans streptococci (via aggregation), thus preventing lesion formation (Fontana et al., 2000). An immunodeficiency or some level of immune dysfunction with regard to cariogenic bacteria (e.g., lack of, or low levels of IgA) will lead to caries.

There are seemingly 3 major putative ways that immunodeficiency may occur in relation to caries. First, SIgA antibody response to cariogenic bacteria may be, under specific biological conditions, greatly suppressed by Tregs. It has been suggested that pathogen survival and persistence may be a homeostatic

compromise on the heels of excessive Treg regulation (Belkaid and Rouse, 2005). In an earlier study, when little was known about Tregs, DR4 patients exhibited more than double the suppressive regulatory T cell (Treg) activity than their DRw6 counterparts at a low concentration of I/II, further suggesting that DR4 subjects, under certain conditions, respond differently to a specific *S. mutans* antigen (Lehner, 1982). This is certainly a possibility, and a similar mechanism has been shown to occur in the immune response to herpes simplex virus (HSV), hepatitis C virus, and HIV (Belkaid and Rouse, 2005). Mice that have been depleted of their Treg repertoire have demonstrated increased immunity to HSV. This phenomenon occurs across a spectrum of immune responses including CD4+ and CD8+ T cell activity, "as well as mucosal antibody concentration." Thus, Tregs might see specific bacterial peptides bound to DRB1*0401-encoded molecules (or other specific *04 allelic variants) and suppress host immune responses against cariogenic bacteria, producing deleterious levels of bacteria within the oral cavity. Some Tregs are not peptide specific but instead see class II molecules generally. How this would regulate caries response is less clear. Of note is that there is also evidence that Tregs can regulate the secondary responses to infections such as listeria, HSV or leishmania. How this occurs remains ambiguous.

Second, HLA-II alleles (i.e., DR, DP & DQ or their variants) may compete for disease related peptides, thus the DRB1*0401 (or other)-encoded protein variant might bind a peptide, albeit inefficiently, that in the context of another class II molecule (DP or DQ) might drive an efficient and robust immune response. It is becoming clearer that both HLA-I and -II allelic variants provide significantly different biological responses. For example, T-cell mediated immunity increases by 10-fold against an Epstein-Barr virus epitope when a particular HLA-I allelic variant, differing by only one amino acid from other

related alleles, is present (Archbold et al., 2009). In this case, the outcome is beneficial. Early on it was shown that HLA restriction was important with regard to cariogenic bacteria. Thus, the capture of bacterial epitopes by specific class II alleles may promote or thwart protective immunity.

Third, the DRB1*04 allelic variant peptide complex might activate a distinct type of CD4 T cells, such as interleukin-17 (IL-17) producing cells, which promote inflammation but not necessarily beneficial bacterial clearance or antibody responses. These cells work within what has been called the Th17 pathway (Steinman, 2007), which ties us back to our first mechanistic idea of Tregs, as Th17 cells can modulate Tregs and produce inflammatory IL-17. Th17 cells can produce sustained tissue damage (in brain, heart, joint, lung, bone, and intestinal models) leading to autoimmunity, while antagonizing Th1 cells, which have now been shown under the revised Th1/Th2 hypothesis to provide more of a protective role in immunity to pathogens. Th17 can be protective for some bacterial pathogens but also more likely to promote autoimmunity.

There are multiple avenues in which we might direct our efforts in elucidating every nuanced step from the HLA-II allelic variant itself to the biomolecular pathways intervening to produce caries. It is a complicated and complex task, but one in which opportunities abound. The more we learn about how HLA-II genes affect antibody response to critical epitopes, and the immune response in general, the better position we will be in to understand why some are more susceptible to caries than others, and the mechanism(s) by which caries may be inhibited. It is important to know that some allelic variants will predispose to caries and others will protect against it. In time, it will be important to identify those alleles that are protective (Xavier and Rioux, 2008), and employ a multidimensional model (Chang et al., 2008), including one that better approximates the oral microbiome (Filoche et al., 2010). A significant

opportunity ahead of us is to characterize the functional outcomes from polymorphisms that are associated with caries, either from previous literature or recent genome-wide association studies. Studying allelic variants within a specific diseased population is an excellent way to determine functional immunogenetic pathways (Xavier and Rioux, 2008).

Conclusion

Complex immunogenetic pathways involved in the caries disease process itself are not only crucial to understanding the fundamental nature of this infectious disease, but also essential in paving the way for new algorithms of diagnosis and detection (e.g., using biomarkers in lab-on-a-chip technologies to distinguish caries-susceptible and caries-resistant patients), vital for developing new therapeutic approaches (e.g., vaccines), and crucial in revealing common susceptibility alleles (i.e., shared genomics) across a range of distinct but mechanistically related (i.e., shared pathogenesis (Zhernakova et al., 2009)) infectious diseases. Although HLA-II pathways account for only one piece of the caries genomic puzzle, they may prove even more important than we have suggested.

Dissertation Outline

This dissertation is divided into five chapters. Chapter one (the current chapter) provides a general introduction describing caries and human genetics and lays significant groundwork to understand the HLA-II gene and caries connection. Chapter two describes our current understanding with regard to how bacterial cariogens produce disease and how mucosal immunity responds to cariogenic bacteria as well as the link between type 1 diabetes and caries. Chapter three describes and explains the materials and methods used for three studies (1 laboratory study and 2 clinical studies) we have conducted. Chapter four describes the results, in separate sections, of the 3 studies. Chapter five

discusses the findings of the 3 studies, in separate sections, and what they might mean with regard to HLA-II genes and caries. It also describes future directions for continuing research in this significant area.

CHAPTER TWO

The Symbiosis of the Human Microbiome

All organisms function in the face of constant pathogenic threat, yet most continue to live and are protected from a variety of pathologies. Part of this protection in nature comes from symbiotic relationships. Take microrhizae, a fungal group that lives in symbiosis with the roots of plants, as it increases a root's capacity to uptake water. In humans, the host-microbe symbiosis has produced the relative homeostasis we observe in host-commensal relationships. For example, we are born into the world germ free, yet thereafter grow and develop while 100 trillion microbes populate our intestinal tract (one of several microbiomes in our body) and we remain healthy (Ley et al., 2006). One experimentally corroborated instance of symbiosis in the human intestine involves *Bacteroides fragilis*, which not only regulates its multiple capsular polysaccharides, but is also able to thereby conduct the cellular and physical maturation of the developing immune system making it critical for host–bacterial symbiosis (Liu et al., 2008). One set of authors describe the overall picture of host-commensal symbiosis in the intestinal tract accordingly:

> These resident bacterial populations make a number of key contributions to host health, including enhancing digestive efficiency, promoting proper immune system development, and limiting pathogen colonization. In return, resident microorganisms derive benefit from association with their hosts by inhabiting a protected, nutrient-rich environment. Thus, these host-microbial associations constitute a mutually beneficial symbiosis (Duerkop et al., 2009).

The notion of symbiosis is not novel, but was postulated over a century ago (Carroll et al., 2009; Metchnikoff and Mitchell, 1908) by the 1908 Nobel Laureate in Physiology or Medicine, Ilya Ilyich Mechnikov. The host-pathogen

relationship is different in that deleterious outcomes follow, and under particular conditions host-opportunistic relationships will also lead to infection or disease (Virgin, 2007).

Despite the benefits and fact of symbiosis, it is thought that the microbiomes associated with humans are relatively narrow in scope when compared to all earthly possibilities. Of the total divisions (deep evolutionary lineages) of bacteria that exist, only 4 percent of those divisions populate the gastrointestinal microbiome. Additionally, only 8 percent of the Archaea divisions are naturally harbored, indicating that there are definite limits to symbiosis (Ley et al., 2006). Still, the numbers here are perhaps not quite as important as the fact that symbiotic relationships naturally exist. Only 1 out of every 10 cells in our body is eukaryotic. The other nine are prokaryotic, helping us to understand that the human microbiome is something we cannot overlook.

There is a continuing debate over how microbes that colonize the host become pathogenic or remain symbiotic. Many believe the microbial niche is altered by environmental cues enabling new colonizers access; or that these cues somehow stimulate changes in the biological activity of commensals, leading to infection or disease (Avila et al., 2009). Due to its abundance, others hold that the commensal microbiota protects the human microbiome from pathogenic organisms that are actually living among the commensals, but at such low thresholds that their ability to infect is thwarted. Both of these scenarios most certainly play out to one degree or another in both pathogenesis and symbiosis, but also fundamental to these questions is the influence of host genetics on the microbiome.

Human Genetics Regulate Composition of Microbiome

It has now been demonstrated that host genetics play a major role in determining the composition of the intestinal microbiome (Khachatryan et al.,

2008). Moreover, it has also been shown that host genetics influence the gastrointestinal microbiome of mice (Toivanen et al., 2001). Interestingly enough, the murine H-2 region, corresponding to the HLA region in humans, is what was pinpointed as having a predominant affect on the intestinal microbiome of mice. Twin studies in humans also demonstrate the strong influence host genetics has on the intestinal microbiome (Stewart et al., 2005). A much older study has also suggested that the nasal microbiome is genetically regulated (Hoeksma and Winkler, 1963). More recently, others have demonstrated that the oral microbiome is also genetically modulated (Corby et al., 2007; Corby et al., 2005). Significantly, a number of studies suggest that HLA-II genes (as well as other HLA and host genes), are involved in determining the composition of the human microbiome, especially perhaps with regard to tolerance of commensal bacteria or activation against pathogenic bacteria (Azuma et al., 1994; Cario, 2008; De Palma et al., 2010; Klein et al., 2009; Mohammadi et al., 1996; O'Mahony et al., 2008), a point that we will return to.

Diversity of the Oral Microbiome

Although the human gastrointestinal microbiome has been of great interest, so too have the ocular (Miller and Iovieno, 2009), nasal, oral (Filoche et al., 2010), urogenital, and skin (Gao et al., 2007) microbiomes become areas of focus (McGuire et al., 2008). Researchers in all of these areas have begun sequencing the existent microorganisms and much work is being done to understand how biofilms protect and predispose to disease. Actually, it was the microorganisms in the oral microbiome that were most readily accessible and first studied in 1683 (Avila et al., 2009). Anthony van Leeuwenhoek, a self-made scientist who was not bound by the traditions of the academy, wrote to the Royal Society describing some of the first observations ever recorded on living bacteria. Leeuwenhoek observed *Streptococcus* commensals, pathogens and opportunists.

In the case of caries, the prototypic pathogen is *S. mutans* (Ajdić et al., 2002; Nakano et al., 2007; Ooshima et al., 1981; Vinogradov et al., 2004), although it is certainly not the only one (Russell, 2008). Indeed, Leeuwenhoek beheld an array of microorganisms. In fact, the oral micriobiome is probably the most diverse and unique microbiome in the human body (Kreth et al., 2009) as it is the gateway to the rest of the body and faces more extreme perturbations than any other human microbiome (Avila et al., 2009).

For instance, the number of species-level phylotypes (phylogenetic type) or species in the oral cavity has now been determined to be almost 20,000 (Keijser et al., 2008) using a well established standard. This standard indicates that when "clusters of related 16S rRNA gene sequences" are "characterized by levels of pairwise sequence identity" at or above 97 percent, they represent the same species (Ley et al., 2006). Otherwise, they represent distinct phylogenetic types or species. Additionally, according to other well respected standards (Sogin et al., 2006), the number of species in the oral cavity may be closer to 26,000, and that only accounts for perhaps 50 percent of the overall microbiota (Keijser et al., 2008). Either of these numbers exceeds the total numbers of commensal species populating the skin (hundreds) and gut (thousands; although these figures are likely higher), and the larger number represents more than 65 percent of what others have found in the deep marine biosphere (Sogin et al., 2006), where we would expect biodiversity to far outstrip the more narrow diversity in humans.

All of these facts highlight the incredible diversity of the oral microbiome despite the fact that one recent article incorrectly equates the oral microbiome to the salivary microbiome (Carroll et al., 2009), thus vastly underestimating by at least 5-fold (Keijser et al., 2008), the total number of resident microorgisms found there. For example, the oral microbiome may be divided into more specialized microbiomes (Aas et al., 2005; Kreth et al., 2009), such as the tooth surface

microbiome, the salivary microbiome (Nasidze et al., 2009), and the soft tissue microbiome. By not including the soft tissue and tooth surface microbiomes, one would greatly underestimate the composition and diversity of the oral microbiome. Whatever the divisions, the principles of symbiosis or pathogenesis are similar.

Commensals and *Streptococcus mutans* in Oral Microbiome

In plaque samples alone (i.e., tooth surface microbiome), 10,000 different species have been identified. Nonetheless, 1,000 of these species reportedly make up at least 90 percent of the total population (Keijser et al., 2008). It is important to note that although the previous numbers are very large, a vast number of these species remain clinically insignificant (even taking into account the theoretical framework of the microbiome) and are found in only trace amounts. Thus, the field of significant oral microbial players narrows substantially. More than 80 percent of early colonizers in the plaque and mucosa (Avila et al., 2009) are oral streptococci (Kreth et al., 2009). The oral streptococci are very important (either symbiotically or pathogenically) in the oral microbiome and can be divided into 5 groups, including Mutans, Salivarius, Anginosus, Sanguinis, and Mitis. In humans, the Mutans group, otherwise called mutans streptococci, is made up of *Streptococcus mutans* and *Streptococcus sobrinus*, both of which have been associated with caries (this group also includes *S. downei* (macaques), *S. ratti* (rats), and *S. criceti* (hamsters), which are all associated with caries); the former continues to remain the prototypic caries pathogen, and no other species within the oral microbiome "is yet a serious contender to *S. mutans* for a role as a worthwhile marker organism" in the caries process (Russell, 2008).

***Streptococcus mutans* in the Ecological Pathogenesis of Caries**

In order for a caries pathogen to lead to the disease, four broad categories must be met. The tooth biofilm must begin to form (e.g., after cleaning the tooth

surface), which occurs via adhesin-receptor interactions. An accumulation or aggregation of pathogenic microbes must reach a particular deleterious threshold. These microbes must be acidogenic, meaning that they will metabolically produce acid. Finally, they must also be aciduric, meaning that they will grow and metabolize under very low pH. These essential requirements must occur within the framework of the biofilm staying at an acidic pH for enough time to alter the composition of the flora in favor of aciduric species, since those species that are less acid-resistant typically produce a more alkaline dynamic. *S. mutans* can and does fulfill all of these requirements as it interacts with other members of the oral microbiota.

Streptococcus sanguinis and *Streptococcus gordonii* of the Sanguinis group are some of the first oral streptococcal species to colonize the native tooth structure (Kreth et al., 2009). It is interesting to note that both of these species express adhesins with virtually identical binding profiles, thus creating a competitive environment for the same host receptors at the same time. Although *S. sanguinis* is more prevalent in plaque and saliva than *S. gordonii*, *S. gordonii* competes more effectively with *S. sanguinis* for tooth structure than any other oral streptococci that have been tested. Thus, both of these early colonizers have a significant impact on biofilm formation. One study demonstrated that these commensals (*S. sanguinis*, statistically significant; *S. gordonii*, high but not statistically significant) are intimately involved in host-symbiotic relationships (Becker et al., 2002). In caries-free hosts, the oral microbiome was dominated by *S. sanguinis* in relation to *S. mutans*, which protected these individuals from these and other pathogenic microbiota gaining advantage. In hosts that were susceptible (manifesting a severe form of caries), the oral microbiome was virtually erased of *S. sanguinis* and *S. mutans* dominated the landscape.

How might this happen? *S. mutans* must attach to the host tissue in earnest by multiplying and metabolizing nutrients available, thereby lowering the pH of the biofilm. Thus displacing acid-susceptible species until the biofilm has achieved an acidic state, which leads to demineralization of the tooth structure. In an immunogenetically deficient host, one example of how *S. mutans* does this is to produce bacteriocins (mutacin I and mutacin IV), which inhibit the growth of both *S. sanguinis* and *S. gordonii* (Kreth et al., 2009). Bacteriocins of *S. mutans* may act on other genera as well although their activity is typically confined to Gram-positive bacteria (Corr et al., 2007). Neither streptocin nor sanguicin (the bacteriocins respectively produced by *S. gordonii* and *S. sanguinis*) are capable of targeting *S. mutans*. Thus, in vying for a niche in the oral microbiome, *S. mutans* proves a formidable foe. These commensals, however, do have somewhat of an advantage during intial colonization. They produce hydrogen peroxide (H_2O_2), which is oxygen (O_2) dependent. Because the biofilm's density early on in colonization is low, the O_2 tension is sufficient to allow aerobic respiration, thereby preventing *S. mutans* from invading. However, once the microbial density reaches a particular threshold, *S. mutans* bacteriocin genes are triggered and expressed while the bacteriocins from these other commensals are ineffective against their major foe. Since bacteriocins are most effective in a mature biofilm, *S. mutans* has the competitive advantage over *S. gordonii* and *S. sanguinis* and is one reason this formidable pathogen can begin to initiate changes in the microflora. Almost 90 percent of *S. mutans* isolates produce mutacins, suggesting that this is evolutionarily important to the species.

Another example of how *S. mutans* can penetrate the oral microbiome is its mutualistic relationship with a gram-negative early colonizer, *Veillonella atypica* (Kreth et al., 2009). These two seem to do better when the other is around. *V. atypica* uses lactic acid (an organic acid), which *S. mutans* produces

prodigiously, as an energy source since it cannot metabolize fermentable carbohydrates. Moreover, *V. atypica* seems to detoxify the environment for *S. mutans*. But there actually seems to be a more significant reason. Together, they become much more resistant to antimicrobial attack (thus nullifying the antimicrobial protein-effect in the context of mucosal immunity, which will be discussed later in this chapter) and the transcription profile of *S. mutans* changes significantly to accomplish presumably very important survival functions within the oral microbiome.

A final theme of the most important step in penetrating the oral microbiome is what happens when *S. mutans* attaches to the host tissue in the first place. It has been firmly established that *Streptococcus* adherence to host tissues is almost exclusively mediated by surface proteins (Nobbs et al., 2009). In the case of *S. mutans*, there are 3 surface proteins of major importance, two of which have been briefly mentioned, with regards to attaching to the tooth surface. These include glucosyltransferases (GTFs), glucan-binding proteins (GBPs), and I/II. All of these proteins have been vaccine targets for caries, vaccines that remain elusive. GBPs are surface-associated adhesins that play a substantial role in architectural development of the biofilm, GTFs are cell-associated secreted protein enzymes, and I/II is a cell-wall anchored adhesin.

In biofilm development, insoluble polysaccharides (these moieties are formed by GTF hydrolytic cleavage of sucrose into fructose and glucose, which are then polymerized) are essential, but they do not facilitate initial attachment to the salivary pellicle unless GBPs are present there. Thus, there are two processes of attachment, sucrose-dependent and sucrose-independent, respectively. I/II fits into the latter category and is thus very important since it can attach to the acquired pellicle without another factor needing to be present. I/II is firmly anchored to the cell wall by sortase A (SrtA) (Marraffini et al., 2006), which is

also ubiquitous in virtually every species of *Streptococcus* although there are 5 currently known sortases in the sortase family of transpeptidases. The acquired tooth pellicle is made up of an array of omnipresent salivary proteins such as acidic proline-rich proteins or salivary glycoproteins, such as glycoprotein-340 (gp340, otherwise known as salivary agglutinin [SAG] or Deleted in Malignant Brain Tumours 1 [DMBT1]). Human gp340 belongs to the family of innate immune proteins, which are mainly comprised of scavenger receptor cysteine-rich (SRCR) proteins. There are SRCR domains along the entire length of gp340, which serve as theoretical binding sites for the specific arrangement of particular regions within I/II. When in the planktonic form, SRCRs provide gp340 the ability to serve as a pattern recognition receptor (PRRs are discussed later) (Loimaranta et al., 2009). Thus, gp340 "promotes bacterial aggregation and clearance" when it encounters *S. mutans* planktonically, however, if the glycoprotein is embedded in the tooth pellicle (i.e., immobilized), it instead serves as the receptor in receptor-adhesin streptococcal attachment (Nobbs et al., 2009).

The Ingbritt strain of *S. mutans*, as well as other streptococcal species, has been shown to bind a very specific region of gp340, namely SRCR2 (there are 14 SRCR domains along the length of gp340) (Bikker et al., 2002). I/II is also extremely adept at adhering to an array of host and environmental receptors, as has been demonstrated in the cases of "collagen, [fibronectin], laminin, and other oral microorganisms," such as, "*Actinomyces naeslundii*, *S. oralis*, *Porphyromonas gingivalis*, and *Candida albicans*" (Nobbs et al., 2009). Thus, *S. mutans* can not only directly bind to the conditioned acquired pellicle, through gp340 interactions, it can also bind directly to other microbes populating the pellicle surface. Once attached, *S. mutans* has a biologically competitive advantage under a variety of circumstances as previously mentioned. Once I/II has facilitated the attachment

of *S. mutans* to the tooth pellicle, it also "influences biofilm formation, promotes collagen-dependent bacterial invasion of dentin, and mediates adherence to human epithelial cells" (Larson et al., 2010).

Host Immunogenetic Responses to a Caries Pathogen

The key thus far is that if a dental pathogen such as *S. mutans* begins to change the oral microflora, it is because it has first attached to the community (biofilm) and then displaced other commensals. This could be prevented if it was attacked early on during colonization or planktonically neutralized. There are perhaps different host immunogenetic profiles that allow such insertion and expansion of *S. mutans* into the microflora until the host is overwhelmed, and other profiles that respond immunologically early in the process, thus keeping *S. mutans* levels low. In the oral cavity, these profiles are functionally manifested at the level of mucosal immunity.

The mucosal immune system, which acts at mucosal surfaces, in contrast to the systemic immune system, is dominated in its secretions by one particular immunoglobulin (Ig) isotype, secretory IgA (SIgA). IgA is the most plentiful immunoglobulin in mammals (Macpherson et al., 2000) and makes up more than 70 (or perhaps more than 75) percent of all Ig produced (Macpherson and Slack, 2007; Macpherson et al., 2008). There is also evidence that SIgA not only plays a major role in adaptive immunity, but also in innate immunity (Macpherson et al., 2008). Considering that the IgA:IgG ratio in oral secretions from the parotid gland is 500 times greater than that of serum, and that IgA-positive (IgA^+) plasma cells (PCs), in comparison to the parotid gland, are double and triple the density in the submandibular and labial glands, it is clear how important this Ig is in the oral microbiome (Brandtzaeg, 2007). It is also very important in the instestinal microbiome, since the colonization of commensals serves as a necessary requirement and is modulated by SIgA induction at mucosal surfaces

(Macpherson et al., 2000). The entire mucosal immune system across the entire human microbiome revolves around SIgA, and without it the pathogenic threat all mammals have encountered since birth would be insuperable.

A majority of the evidence demonstrates an "inverse relationship" between caries and levels of salivary IgA in children and young adults (Brandtzaeg, 2007), meaning that someone highly susceptible to caries would have low levels of salivary IgA and someone more resistant would have high levels of salivary IgA. This summary statement must be understood in its context. There are many things to consider and understand if we are to ultimately know how host immunogenetics modifies the oral microbiome in the context of mucosal immunity. Innate and adapative immunity are involved, the nexus between them, and HLA genetics. Thus, these connections are essential (especially in terms of context) to understand how the proposed hypothesis purported in these pages may function in humans.

Innate Immunity

The difference between innate and adaptive immunity are the receptors that mediate their responses, pattern recognition receptors (PRRs) in innate functionality, and antigen receptors in the case of adaptive immunity (Medzhitov, 2007). Every immune cell in this system has a host receptor, a PRR, whose target ligand is typically a pathogen-associated molecular pattern(s) (PAMPs), even though pathogens, commensals and opportunitsts all express PAMPs. "Bacterial PAMPs are often components of the cell wall, such as lipopolysaccharide [LPS], peptidoglycan, lipoteichoic acids and cell-wall lipoproteins." It is important to note here that PRRs cannot discriminate between pathogens and other benign colonizers, yet they are essential in maintaining homeostatic symbiosis in a way that remains ambiguous.

There are two broad categories of PRRs, namely transmembrane receptors found on the cell surface (including endosomal receptors) and intracellular receptors (Medzhitov, 2007). One well known class of transmembrane receptors is the Toll-like receptors (TLRs) that recognize viruses, but more important for our focus, bacterial products such as LPS and lipoteichoic acids (Akira et al., 2006). We know that TLRs, once bound to their ligands, activate an array of antimicrobial responses, albeit indirectly in humans. Another class of PRRs, the Nod-like receptor (NLRs), which are intracellular receptors, recognize bacterial products within the cell and posses a nucleotide-binding oligomerization domain (NOD). NOD proteins as well as other subfamilies of NLRs, such as NALP (NACHT-LRR-PYD-containing protein; see later discussion) or NAIP (neuronal apoptosis inhibitor protein), have not been well characterized with regard to antimicrobial effect, but are nonetheless implicated in responding to bacterial infection. We shortly return to the connection between adaptive and innate immunity.

Adaptive Immunity

The acquired or adaptive immune system is mediated by two antigen receptors, the T cell and B cell receptors. There are also two types of lymphocyte, both of which carry these types of receptors, namely the conventional lymphocytes and the innate-like lymphocytes (some have even labeled them simply innate lymphocytes). The traditional lymphocytes are T cells and B cells. These T cells are mainly alpha, beta T cells ($\alpha\beta$ T cells) while the B cells are mainly B2 (bone marrow-derived) cells whose antigen receptors are essentially built randomly (Medzhitov, 2007). In contrast, the architectural scaffolding of innate-like lymphocytes is not random but restricted, yet these lymphocytes, consisting of some gamma, delta T cells ($\gamma\delta$ T cells), natural-killer T cells (NKT

cells), B1 cells (pleuroperitoneal cavities), and splenic marginal zone B cells, are the counterparts of the conventional lymphocytes.

Among the conventional $\alpha\beta$ T cells, there are two types, including T-helper cells (characterized by the co-receptor CD4) and cytotoxic T cells (characterized by CD8), which recognize HLA-II and I molecules respectively, positioning these cells to provide an almost limitless ability to recognize diverse antigens (Medzhitov, 2007). B2 cells can also recognize a multitude of antigenic epitopes. We will return to how conventional lymphocytes specifically function in the context of mucosal immunity in relation to the proposed hypothesis.

Although B1 cells cannot survey as wide a variety of antigens as their traditional counterparts, they are nonetheless significant in their own right. Though they are generally thought to secrete IgM in response to commensal bacteria (and self antigens), experiments conducted by Macpherson et al. and others (Fagarasan et al., 2001; He et al., 2007; Uematsu et al., 2008) confirm that these cells produce a prominent SIgA response in a T cell-independent (TI) and follicularly organized lymphoid tissue-independent manner, needing only intestinal lymphoid aggregates containing these B cells and antigen to activate (Macpherson et al., 2000) the mucosal response. TI pathways serve as the first line of defense during which the adaptive response can initiate and ultimately provide protective immunity by high-affinity SIgA (Dullaers et al., 2009). It is interesting to note that innate-like T cells identify with HLA-Ib proteins, and one protein in this category is MR1 (MHC-related protein I). In a specific case, a unique mucosal innate T cell called mucosal-associated invariant T cell or MAIT is speculated to serve as an intestinal regulator of SIgA production (Huang et al., 2008). Surprisingly, the non-classical HLA-I protein, MR1, is not dependent upon the HLA-I peptide loading complex, but instead relies greatly on an HLA-II antigen presentation pathway. Thus, it putatively allows this HLA-related

protein to sample endocytosed microbial antigens (the presumed APCs are B cells), thereby activating MAITs and the immune response. Finally, it is interesting to note that some non-classical HLA molecules

> might themselves be ligands for T-cell receptors, without presenting any other molecules. In this case, the expression of these molecules is thought to be inducible by the engagement of PRRs [e.g., TLRs] on specific cell types, such as mucosal epithelial cells (Medzhitov, 2007).

The Innate-Adaptive Nexus

Innate and adaptive immunity come together at the interface between PRRs recognizing PAMPs and antigen-lymphocyte interactions (Medzhitov, 2007). This nexus is represented by both T-independent and T-dependent antigens. For the latter, dendritic cells interpret PAMPs for T cells by initially sensing the pathogen via TLRs (or other PRRs such as NLRs) to upregulate the HLA-II pathway, and then presenting the processed antigen to TCRs via surface HLA-II molecules. For the former, this interface is directly represented by an antigen physically interacting with a PAMP (presumably by Fc receptor-antigen and PRR-PAMP co-engagement). In addition to conventional B lymphocytes, B1 cells may directly interact with PRRs thereby initiating SIgA secretion against bacterial antigens, in the same vein as the aforementioned TI pathway example. Additionally, innate-like T cells do recognize antigen presented by non-classical HLA molecules. In particular cases, these T cells do not need a peptide in the binding groove of these HLA molecules (PRR-induced) to initiate an immune response. As previously stated and as is widely accepted, T-helper cells are activated by dendritic cells or other antigen presenting cells even when antigens and PAMPs are not directly linked, thus initiating the adaptive response for long-term protective immunity. This immune response is the end-game of mucosal immunity.

Mucosal Immunity

The mucosal immune system is inextricably involved with the microbes it encounters (e.g., caries pathogens), SIgA (as explained earlier), and the host's HLA immunogenetics. Let us follow a caries pathogen, *S. mutans*, on its journey through the human microbiome, and the microbiome's regionalized mucosal immune system, in order to discuss how it relates to SIgA and HLA-II genetics.

S. mutans can be vertically transferred from mother to child or from adult to adult. Once *S. mutans* gains access to the human microbiome orally, its journey is complex and dynamic. Some bacterial siblings may localize to the tooth surface pellicle close to the gingival margin of the dentition or the variegated oral mucosa, while other *S. mutans* may localize to the lingual or palatine tonsils (oropharynx), or even the pharyngeal tonsil (nasopharynx), which together are sometimes referred to as Waldeyer's tonsillar ring, Waldeyer's pharyngeal ring, or simply Waldeyer's ring. In the context of mucosal immunity, however, this specialized region with its specialized lymph tissue is called the naso-oropharynx- or nasopharynx-associated lymphoid tissue (NALT). NALT is an important inductive site for SIgA in the salivary glands, but we will return to that theme later.

Although many an *S. mutans* bacterium will localize to the oral microbiome and NALT, others will be carried away planktonically during swallowing and inferiorly traverse the gastrointestinal (GI) tract by passing over the Laryngopharynx (laryngeal inlet closes during swallowing), moving through the Esophagus and esophageal sphincter into the acidic stomach where *S. mutans* can survive because of its aciduric nature. Moving through the pylorus into the duodenum, then into the jejunum, *S. mutans* finally moves into the distal ileum where it encounters a foci of mucosa-associated lymphoid tissue (MALT), a general term that refers to any principal inductive site for mucosal immunity in

any of the mucosal compartments of the body, which in this anatomical case is the gut-associated lymphoid tissue (GALT).

GALT consists of 3 types of inductive sites, including Peyer's Patch, the appendix, and isolated lymphoid follicles (ILFs) (Brandtzaeg et al., 2008). These inductive sites posses all the necessary cells (T-cells, B-cells, and antigen presenting cells) to generate a mucosal immune response (Kiyono and Fukuyama, 2004). Peyer's Patches (PPs) are quintessential immune inductive sites and are most dominant in the ileum. In early male adolescence, for example, approximately 61 percent of all PPs in the small intestine are located in the ileum, in contrast to only 37 percent in the jejunum, and 2 percent in the duodenum (Cornes, 1965). At birth, 78 percent of all PPs in the small intestine in males are located in the ileum, whereas only 22 percent reside in the jejunum, and are virtually non-existent in the duodenum. The main components of PPs are follicles whose residents include T and B cells (Makala et al., 2002). Between the follicles is the interfollicular area (or interfollicular region, IFR) that houses high endothelial venules (HEVs), which are made up of specialized endothelial cells that lymphocytes selectively bind to (these cells are taller and more cuboidal in appearance than their morphologically flat endothelial counterparts) (Johnson-Léger et al., 2000), and T lymphocytes. Above a follicle is a dome comprised of plasma cells and T and B cells, which is overlaid by what is termed the follicle-associated epithelium (FAE) that interfaces with the intestinal lumen. PP mucosal epithelium (i.e., FAE) is specialized in that it contains membrane or microfold (M) cells that play a major role in transferring antigen across (antigen transcytosis) the mucosal barrier. Thus, PPs in the small intestine and especially in the distal ileum serve as major surveyors of both symbiotic and pathogenic gut bacteria. If *S. mutans* has managed to escape immunological recognition before it arrives at the distal portion of the ileum, it is likely that the ileal patchwork of

inductive sites will recognize this dental pathogen and trigger the appropriate immune response, thus leading to SIgA in the compartment of the oral microbiome.

M cells have long been known to facilitate the internalization of antigen to PPs in GALT. Today, we know that they posses specialized receptors which facilitate glycoprotein 2 (GP2)-mediated transcytosis of bacterial antigens (Hase et al., 2009). One piece of evidence for this is that in ileal FAE GP2 receptors are highly expressed while their expression decreases exponentially in the villus epithelium. These results from the human (and murine) small intestine also hold for M cell GP2 receptors expressed in human NALT (Hase et al., 2009; Verbrugghe et al., 2008). M and intestinal epithelial cells intrinsically posses TLRs located on their apical surfaces, which help recognize PAMPs. TLRs are also coupled with intracellular adaptor proteins in most TLR-mediated signaling, and the protein almost always involved is called MyD88 (myeloid differentiation factor 88) (Brandl et al., 2007). TLR-mediated signaling initially induces epithelial secretion of a protein called RegIIIγ, which can directly kill Gram-positive bacteria (Pamer, 2007) like *S. mutans* in the short-term, but cannot ensure long-term protective immunity. Thus, the basic innate functions of the mucosal barrier, and especially PPs, involve M and mucosal epithelial cell TLRs (coupled with MyD88) recognizing PAMPs of *S. mutans*, and signaling not only the innate release of antimicrobial proteins, but also the activation of adaptive dendritic cells (DCs) making them immunogenic (Blander and Medzhitov, 2006). It is now known that DCs can extend their dendrites across tight junctions inter-epithelially to directly survey bacteria, in this case *S. mutans*, in the intestinal lumen, but this is also enhanced by TLR signaling (Maynard and Weaver, 2009). M cells appear to preferentially express TLR1, TLR2 and TLR4 as these TLRs are highly expressed relative to other M cell counterparts, namely the villus

epithelium and FAE (Cashman and Morgan, 2009). Interestingly, one study has suggested that *S. mutans* clearance is inhibited by the lack of TLR2 (Horst et al., 2009). In certain cases, TLR2 has been specifically linked to Th2-associated DC stimuli although there are certainly other mechanisms (MacDonald and Maizels, 2008). M cells recognize and transcytose pathogens such as *S. mutans* from the ileal lumen to an infolding of their basolateral membrane forming a pocket in which DCs (macrophages, and T and B cells also reside in close proximity) can endocytize *S. mutans* and present immunodominant antigens on its surface to T cells in the T-cell rich IFR.

Antigen Presentation

DCs are the most potent APCs in adaptive mucosal immunity. All subsets of DCs in the subepithelial dome (SED), and elsewhere in PPs, express HLA-II (Mercado-Lubo and McCormick, 2010), but one set (termed LysoDC) located in the SED and FAE expresses an extremely high level of HLA-II molecules (Lelouard et al., 2010), thus playing a crucial role in antigen presentation in the adaptive immune response across the mucosal barrier. It is interesting to note that ileal intestinal epithelial cells (which would include M cells) constitutively express HLA-II molecules (Buning et al., 2008) as well as lamina propria (LP) stromal cells, both of which may present antigen as non-professional APCs to T helper cells. In any case, antigen presentation occurs and initiates the adaptive immune response.

The immunodominant antigen of *S. mutans*, I/II, which is responsible for bacterial attachment to the tooth surface, is one of the major antigens that is processed and presented by DCs to T cells in the IFR in our scenario. Both the intracellular process and the actual presentation of I/II is modulated by particular biologically-related and HLA-II genes. After discussing these genes, I will describe the movement of I/II to intracellular locations of the APC to be

degraded, HLA-II ligation to I/II peptides, and the final trafficking of HLA-II-I/II peptide complexes to the APC surface.

HLA-II Transcriptional Regulation

It is very important here to recognize the nexus between innate and adaptive immunity as that interface is very important with regard to HLA-II expression in DCs (and other immune cells)—at the gene level. All structural genes have promoter or initiation sites, at which point along the DNA, RNA polymerase begins to synthesize messenger RNA (i.e., transcription). This ultimately leads to protein assembly (i.e., translation), and in this case, the α and β chains of HLA-II molecules that form the peptide binding groove. Because HLA-II molecules are constitutively expressed in DCs, and other immune cells already mentioned, there is a basal level of HLA-II transcription in these cells. Nonetheless, transcription factors greatly increase the production rate of mRNA and therefore the rate of HLA-II production, and enhancers also stimulate promoters. But transcription factors and enhancers can only be activated in response to a stimulus.

An important gene, *CIITA* (class II [meaning HLA class II] transactivator), is inextricably tied to regulating HLA-II transcription upregulation through its protein, which is a non-DNA-binding co-activator (Reith et al., 2005). I will briefly explain how this works. Upstream of the gene region encoding the HLA-II molecule is the promoter and also a regulatory module currently identified as composing 4 unique sequences or boxes, labeled in the 5′ to 3′ direction as S, X, X2, and Y. Each box in this regulatory module, initially known as the SXY module, can bind to ubiquitously available transcription factors. The currently known SXY binding factors include:

> the heterotrimeric X-box-binding factor regulatory factor X (RFX), which is composed of RFX5, RFX-associated protein (RFXAP) and

> RFX-associated ankyrin-containing protein (RFXANK); the X2-box-binding factor cyclic-AMP-responsive-element-binding protein (CREB); the Y-box-binding factor nuclear transcription factor Y (NFY); and an as-yet-unidentified S-box-binding factor (Reith et al., 2005).

Once RFX, CREB, and NF-Y (made up of 3 subunits NF-YA, NF-YB and NF-Y) (Leimgruber et al., 2009) have bound the SXY (now simply termed S-Y) module (Krawczyk and Reith, 2006), the entire protein complex is referred to as the HLA-II enhanceosome, and serves as a docking site for CIITA, which in turn, will recruit other necessary factors in activating HLA-II transcription.

As previously mentioned, stimuli must precede the binding of transcription factors to the S-Y module and the docking of CIITA. Of note here is the fact that CIITA has certain structural properties that fit into the context of PRRs, more specifically NLRs, which recognize PAMPs as previously discussed. As heretofore mentioned, there are two broad categories of PRRs, namely transmembrane receptors such as TLRs, and intracellular receptors such as NLRs. TLRs possess Toll–interleukin 1 receptor (TIR) domains (or alternatively called (IL)-1R homology domains), which extend into the cytoplasm of the cell and appear to be a link between TLRs and NLRs when they synergistically work in initiating an immune response to bacteria (Werts et al., 2006). For example, MyD88-dependent TLR signaling and Nod2 (an NLR) work together in initiating immune responses. NLRs involve four domains:

> an N-terminal effector domain, which can be a pyrin domain (PYD), a CARD [caspase activation and recruitment domain] or a Bir [baculovirus 'inhibitor of apoptosis' repeat] domain; a central NACHT domain (NACHT stands for domain present in Naip, CIITA, HET-E (plant *het* product involved in vegetative incompatibility) and TP-1 (telomerase-associated protein 1)); [a NOD domain] and a C-terminal LRR domain thought to constitute the microbe-sensing portion (Fritz et al., 2006).

Thus, NLRs are comprised of effector (PYD, CARD, or Bir), NACHT, NOD, and LRR domains. It seems that NLRs work in tandem or synergistically with TLRs thus bridging the extracellular and intracellular compartments and providing for DC activation in the face of antigens such as I/II. For example, TLRs have been reported to signal through NALPs (a subfamily of NLRs) for Gram-negative bacteria, and Nod2 (another NLR) has been linked to signaling immune upregulation against Gram-positive bacteria, such as *S. pneumonia* (Fritz et al., 2006), which possesses an antigenic region homologous to a region of the proline-rich area of I/II. CIITA, which is found in DCs, contains CARD, AD (activation domain), NACHT, NOD, and LRR domains. Similarly, Nod2 has the exact same structure minus AD. Thus, CIITA possesses the ability to recognize PAMPs and other signals in order to initiate transcription through a promoter (called pI) mainly active in DCs (Reith and Boss, 2008). Once *CIITA* has been transcribed and translated, CIITA can dock in the HLA-II enhanceosome and finish recruiting the final factors essential for HLA-II synthesis.

A defect in *any* of the HLA-II-biologically related genes, including *CIITA* leads to the severe consequences of bare lymphocyte syndrome, including the absence or virtual abolishment of antibodies to microbial antigens (100 percent prevalence) due to complete absence of HLA-II expression (Krawczyk and Reith, 2006). This means that if all factors are present, then HLA-II molecules will be synthesized appropriately. Defects in immune recognition can arise from HLA-II structural gene variations as well as transcriptional gene defects. DCs interpret PAMPs for T cells by internalizing *S. mutans* via TLR-signals (and/or other PRRs such as NLRs), to upregulate HLA transcription factors and CIITA in the HLA-II pathway, any alterations in class II display of I/II to TCRs may arise due to allelic variations in class II molecules. This notion is further bolstered because the promoter genes that control HLA-II transcription are not combinatorially

regulated as is the case in many other human genes, but are controlled by CIITA. Moreover, the exact same genetic transcription machinery is used in synthesizing HLA-DM and HLA-DO, which are important intracellular processors of HLA-II-peptide complexes (discussed shortly), as well as Invariant chain (Ii), which is also very important in regulating HLA-II-peptide loading (discussed shortly). Thus, differences in immunity against *S. mutans* will not manifest themselves in all-or-nothing ways but in more nuanced degrees of immunodeficiency or immunogenicity.

HLA-II Endocytic Pathway and Trafficking

The α and β proteins, which are encoded by separate HLA-II genes, are assembled in the endoplasmic reticulum (ER) thus creating the HLA-II molecule that will ultimately project from the cell surface and present I/II peptides in the binding groove to TCRs. However, a chaperone protein, Ii, is actually assembled concomitantly with both chains and not only stabilizes their conjoined structure, but also prevents immature and inappropriate antigen binding (Berger and Roche, 2009). The nascent $\alpha\beta$-Ii complex travels from the ER and traverses the trans-Golgi network (TGN) toward the endosomes.

Once the HLA-II-Ii complex has arrived in endocytic organelles, Ii is proteolytically processed and released from class II to permit the binding of antigenic peptides (Berger and Roche, 2009). In endosome and lysosome microenvirons, resident proteases such as cathepsins S and L, degrade Ii in a stepwise fashion until all that is left is a peptide fragment, CLIP (class-II-associated invariant chain peptide), which resides in the HLA-II binding groove. An HLA-II molecule, HLA-DM, which does not present antigen on the surface of the DC, but rather functions in endosomes, directly binds the HLA-II-CLIP complex and removes CLIP. It is thought that HLA-DM serves as a chaperone protein that stabilizes HLA-II as it samples antigenic fragments (during which

there are multiple bindings and disassociations) until it locks-in a high-affinity peptide. HLA-DO is the other protein thought to also act as an intracellular regulator of HLA-DM. Once the HLA-II-peptide complex has locked in, HLA-DM releases the HLA-II-peptide complex, and the resulting HLA-II-peptide complexes move to the cell surface.

Naïve T Cell Activation

When DC HLA-II complex display epitopes of I/II in their ligand groove, this can be recognized by CD4+ T cells in the IFR. As mentioned previously, TLRs express cytoplasmic Toll/interleukin (IL)-1R homology domains, and these regions stimulate DCs to secrete cytokines needed prior to and during HLA-II-peptide-TCR interactions (Wang et al., 2008). Activated DCs also increase expression of co-receptors on their surface, including class II, CD80 and CD86, the latter which bind CD28 on the T cell. Differentiation into T helper (Th) cells, such as Th1 or Th2, is dependent on the pathogen in question, and most Th1 responses come on the heels of viral or intracellular bacterial infection, which would exclude *S. mutans*. Thus, I/II epitopes with their costimulatory molecules and signals induce predominantly Th2 differentiation and stimulation. IL-4 secretion induces the expression of GATA-3, generally known as the Th2 master regulator (Fazilleau et al., 2009). This master regulator inhibits Th1 production and is essential for Th2 differentiation. Once the naïve T cells have committed to the Th2 path, they proliferate via Stat6 (activated by IL-4-IL-4R interactions) transduction pathways that are regulated by GATA-3. IL-2 also promotes naïve T cell activation through Stat5a. Once differentiated and activated, Th2 cells proliferate and respond to I/II epitopes by producing IL-4, IL-5, IL-13, and IL-25 in the PP follicle in order to activate B cells. One interesting point is that IL-10, either by itself or in combination with IL-4, inhibits Th1 activation, but IL-10 is also implicated in IgA switching.

B cell Induction, Proliferation, and Class Switch Recombination

Similar to DCs, resting B cells in PPs also uptake I/II and are able to present I/II epitopes to T cells, however, in this case they present HLA-II-I/II complexes to activated Th2 cells, which once bound to the TCR, induces CD154 expression on these T cells (Rodriguez-Pinto, 2005). This can generate cytokine signals directly secreted across the Th2-B cell synapse producing B cell migration into the germinal center of the follicle, proliferation, somatic hypermutation, and isotype switching or class switch recombination (CSR). All of these inductions occur because of T cell recognition of the HLA-II-I/II complex. The mechanism of somatic hypermutation remains unexplained, but mutations rapidly occur in heavy and light chains of the variable (V)-regions of Ig gene segments within the germinal center, ultimately leading to high affinity binding antibodies. The constant (C)-region genes of the B-cell Ig continue to change and mature during the adaptive immune response to I/II. This region is ultimately vital in recruiting help from other cells, and it confers functionally distinct properties to each isotype. Portions of the C-region of different isotypes are recognized by certain immune effector cells. The C-region also aids in transporting antibodies across barriers that would typically involve active transport, such as is the case with SIgA. During rapid B cell proliferation in the GC, the antibody C-region is modified, that while conserved in a general sense has now changed its functionality (i.e., CSR). CSR occurs in response to Th-produced cytokines (for CSR leading to IgA, the predominant cytokine is transforming growth factor (TGF)β with contributions from IL-2, IL-4, IL-5, IL-6, and IL-10) , which have been induced by HLA-II-I/II B cell-Th2 interactions in this case.

The gene segments responsible for the various isotypes are: Cμ (IgM), Cδ (IgD), Cγ (IgG), Cε (IgE), & Cα (IgA). Switching to other isotypes requires interacting with antigen, which in this case is controlled by HLA-II-I/II

recognition and induction. This mechanism is guided by switch regions in the DNA, stretches of intronic repetitive elements made up of approximately 150 repeats of the GAGCT and GGGGGT sequences. In the case of IgA, these introns lie between the V-region exon and the μ gene and, in this case, 5′ to the α gene. The switch regions are named: Sμ, Sγ, Sε, & Sα.

When the activated B-cell expressing IgM and IgD, reactive with I/II, receives a switch signal to express IgA, whose limiting player is that of the HLA-II-I/II complex, the Sα and Sμ regions undergo recombination and every coding region between these 2 regions is deleted, thus enabling the normal DNA machinery to transcribe the heavy-chain transcript of IgA. Since switch sequences lie within introns, they will not cause frameshift mutations and thus functional proteins will be encoded, which once ferried to the effector sites, can ultimately be secreted in order to neutralize *S. mutans* in the oral microbiome.

Homing to Effector Sites

GC IgA+ plasmablasts home to the salivary glands by traversing the afferent lymphatics where they course through the mesenteric lymph nodes (MLNs) (or the draining lymphatics), a location that also produces DC-Th2-B cell interactions and more effector cells, all of which find their way superiorally through the thoracic duct and enter the vasculature at the thoracic duct and subclavian vein junction. The B-cell homing system works by particular endothelial signals of HEVs in the various mucosal immune compartments, such as NALT, which includes the pharyngeal tonsil, the palatine tonsils, and the lingual tonsils. IgA plasmablasts programmed to play major roles in the oral microbiome express CCR10, which binds to the salivary gland ligand CCL28, as well as the $\alpha_4\beta_1$ integrin, which is attracted to VCAM-1 (vascular cell-adhesion molecule-1) (Macpherson et al., 2008). Thus, IgA-secreting cells find their way to the salivary glands and the oral cavity because of the mucosal immune response

to *S. mutans*, which fundamentally revolves around HLA-II-I/II complexes, and sets in motion long-term protective immunity. Although the traditional journey of adaptive mucosal immune cells, especially IgA+ B-cells (in response to I/II epitopes), has been described as induction in GALT (specifically PPs) and effector migration to the oral microbiome, there is substantial evidence that NALT "may be relatively more important than GALT as inductive sites for B cells destined to the salivary glands" (Brandtzaeg, 2007). This fact does not change any of the biologically essential mechanisms thus discussed, but instead provides perspective on other important inductive sites against immunodominant epitopes of cariogenic bacteria within the oral microbiome.

NALT is an Important Mucosal Inductive Site

It has become clearer that NALT functions in very similar ways as does GALT. One set of authors state the case clearly:

> NALT consists of follicle-associated epithelium (FAE), HEVs, and T-cell- and B-cell-enriched areas. Antigen-sampling M cells are present in the epithelium of NALT, which is specialized for antigen uptake . . . Antigen-presenting cells, including dendritic cells (DCs) and macrophages, are also found in NALT. So, NALT contains all of the lymphoid cells that are required for the induction and regulation of mucosal immune responses to antigens (Kiyono and Fukuyama, 2004).

Other authors point out that NALT is also very significant in generating memory B cells, thus arming the oral microbiome with high-affinity IgA (Jang et al., 2004) against I/II and other *S. mutans* antigens. It is also clear that NALT elicits regional or compartmental SIgA immunity (through cervical lymph nodes instead of mesenteric lymph nodes) (Brandtzaeg et al., 2008; Brandtzaeg, 2009). Finally, it is important to note that the mucosal immune system is much more compartmentalized or regionalized then has been traditionally accepted, and the

idea of a "common" system is now "obsolete," as explained by one set of authors:

> It is important to be aware of the extensive regionalization and compartmentalization that exist within the mucosal immune system. Thus, in contrast to extravasation events in the intestine, $\alpha_4\beta_7$ and MAdCAM-1 are not important homing molecules in the airways, where CD62L, $\alpha_4\beta_1$, CCR7, and CCR10 appear to play a more decisive role (Macpherson et al., 2008).

One of the foremost experts in SIgA, who has studied this antibody for more than 4 decades, observes "that intestinal immune induction is not so well reflected in the salivary IgA system" and that "the enteric–oral B cell homing axis" is probably overestimated (Brandtzaeg, 2007). A conservative estimate might anticipate that 60 percent of total SIgA activity in the oral microbiome is locally induced (by NALT) while 40 percent may be GALT-induced (one could even imagine a 70/30 split). Brandtzaeg has also been recognized for his work, which is now widely accepted as the model for SIgA epithelial transport (Macpherson et al., 2008), which we will now discuss.

Secretory Epithelial Transport in Oral Microbiome

Secretory epithelial transport of SIgA in the oral microbiome happens in both the intercalated regions of major salivary glands as well as across serous-type acini (Brandtzaeg, 2007). It is interesting to note that IgA-plasmablasts do not fully differentiate to IgA-PCs until they arrive in the LP or stroma of the oral glandular compartments. Taken together, the submandibular and labial glands contain two and three times more IgA+ PCs than the parotid gland. Moreover, we currently know that submandibular glands substantially exceed the parotid glands SIgA output. SIgA is transported with the help of a transmembrane epithelial receptor, pIgR (polymeric Ig receptor), previously known as membrane secretory component (mSC) (Brandtzaeg et al., 2008; Brandtzaeg, 2009). This

receptor facilitates pIgA (Polymeric IgA; refers mainly to dimers but also includes larger polymers of J-chain-containing IgA) transcytosis. PCs initiate pIgA formation via Joining (J)-chain ligation. The J-chain polymerizes IgA and creates a binding site for pIgR (Braathen et al., 2007). Once bound, SIgA is transported apically by cleavage of the pIgR. Thus, SIgA is released into the salivary glandular compartments that together comprise the oral microbiome biofilm where SIgA can neutralize *S. mutans* by high affinity I/II-SIgA binding.

Summary

The proposed hypothesis is therefore informed by the fact that the predominant effector antibody in mucosal immunity, SIgA, specifically targets immunodominant epitopes of *S. mutans*, and the predominant rate limiting step in this immune response fundamentally revolves around HLA-II structural genes and their molecules as has been discussed. Specifically, the central hypothesis is that there is an association between certain HLA-II allelic variants (which differ slightly at the structural gene level), specifically within *HLA-DRB1*04*, and inefficient HLA-DRB1*04-I/II peptide presentation to Th2 cells, thus eliminating in large measure, the SIgA response to *S. mutans*. In an HLA-I example, one study has demonstrated that structural gene variants do indeed yield variant binding grooves and a concomitant differential in affinity for particular epitopes. For example, HLA-B*4405, which presents an Epstein Barr Virus (EBV) epitope to its cognate TCR, exhibits a 10-fold increase in affinity when compared with HLA-B*4403 and HLA-B*4402, all of which differ by only one amino acid. Thus, 3 studies (1 laboratory and 2 clinical) were conducted to explore and test this hypothesis. While the two clinical studies sought to test the overall hypothesis in relation to HLA-II allelic variants and SIgA, the laboratory study served as a precursor to the latter two in that we sought to identify epitopes of I/II that had already been shown to induce adaptive effector responses in humans as opposed

to animal models. After identifying these immunodominant regions, I/II epitopes were synthesized and tested with human pooled saliva to explore which of these epitopes might play more predominant roles in the SIgA system. The materials and methods used to conduct these studies as well as their results will be discussed in the following two chapters.

Before that, it is important to discuss a link between a disease that produces salivary impairment and consistently creates an abnormally high nutrient-rich environment that *S. mutans* and other cariogenic pathogens can take advantage of. This disease is type 1 diabetes (T1D), and its connection to caries is partly explained by higher basal levels of cariogenic threat with concomitantly lower overall SIgA activity. At-risk T1D subjects are recruited in our third study, but the T1D-caries connection could not be tested in that study although this connection appears to be very important and future studies could be directed at testing its many potential hypotheses.

The Caries and Type 1 Diabetes Connection

The link between diabetes and a variety of oral diseases has received increasing attention (Hampton, 2008; Lamster et al., 2008). Some of the major oral diseases or disorders associated with diabetes include caries, periodontal disease, salivary gland dysfunction, fungal infections (e.g., oral candidiasis), lichen planus and other lichenoid reactions, and infection. Whether HLA-II gene inheritance plays a role in the increased caries increments associated with type 1 diabetes mellitus (T1D) has not been tested, but could be a contributing factor. Nonetheless, a link between caries and T1D exists.

Diabetes Mellitus has been associated with caries (Bakhshandeh et al., 2008). Although previous data between type 1 diabetes mellitus (T1D) and caries is uneven, the link has been supported and recently bolstered. Previous studies have suggested, for example, that T1D patients exhibit more "initial buccal

caries" compared to controls (Edblad et al., 2001). One reason diabetes may be linked to caries has to do with salivary dysfunction, a risk factor for caries and a condition caused by, among other systemic diseases, diabetes. In fact, xerostomia and salivary hypofunction are important risk factors in caries, and caries is very difficult to prevent under these conditions (Kidd et al., 2008). Thus, T1D patients' salivary secretion was greatly impaired (resting and stimulated) compared to controls in a matching study (Mata et al., 2004). Salivary flow is also important. Low resting salivary flow rate, a significant salivary parameter favoring caries because of impaired bacterial clearance, is observed in T1D subjects (Moreira et al., 2009). Thus, high caries experience in T1D patients is significantly associated with decreased resting salivary flow rate (Siudikiene et al., 2006).

Moreover, relatively high levels of salivary glucose coming from both the serum (via gingival crevicular fluid) and the saliva (via salivary glands), foster an environment in which cariogenic bacteria may flourish in creating the deleterious dental biofilm (interchangeably called the dental plaque) and producing caries. Thus, salivary glucose concentration (in either resting or stimulated saliva) has been found to be much higher in T1D patients than in control subjects (Jurysta et al., 2009), and high caries experience has been correlated with the cariogenicity of the dental plaque (Siudikiene et al., 2006). Decayed/missing/filled surface (DMFS), one measure of caries experience, was recently used in assessing the connection between diabetes and caries. The authors found that T1D patients' "DMFS increments" over a 2-year period "were associated with greater increments in salivary glucose concentrations" (Siudikiene et al., 2008). Moreover, T1D subjects exhibited increased increments of "dental plaque," a necessary prerequisite in caries, and concomitantly associated "higher increments in active caries lesions" compared to controls. Thus, the authors conclude that "diabetes-induced changes in salivary glucose" foster "caries

development" among T1D patients. The magnitude of this association may also depend on how well controlled T1D is. For example, blood glucose and glycosylated haemoglobin (HbA_{1c}) data demonstrate that poorly controlled T1D patients, compared to well controlled T1D counterparts, produce increased resting salivary glucose levels, and a significantly higher caries incidence (Twetman et al., 2002; Twetman et al., 2005). Thus, HLA gene expression, high salivary glucose and low salivary flow rates may contribute to caries development in T1D.

CHAPTER THREE

Materials and Methods

Study #1: Human Salivary IgA Reactivity for Selected I/II Peptides

General Description of Design

As previously mentioned, 3 studies (1 laboratory and 2 clinical) were conducted to explore and test the previously described hypothesis. The materials and methods used to conduct these studies as well as their results will be discussed in this and the next chapter.

We selected I/II peptides, obtained deidentified human saliva per Institutional Review Board (IRB) approval (#0304-58), and used a modified enzyme-linked immunosorbent assay (ELISA) (Gregory, 2001) to determine human salivary IgA reactivity for the peptides. We also determined which of the selected peptide(s) were more immunoreactive.

Selecting, Synthesizing, and Storing I/II Peptides

We selected I/II peptides that induce immune responses in humans, as opposed to animal models, by searching for articles between 1990 and 2008 that directly related to I/II (Web of Science search parameters: Topic=((I/II) AND (streptococcus mutans) AND (epitope)). With additional searches (Kelly et al., 1995; Senpuku et al., 2007), we selected the following peptides: NAKATYEAALKQYEADLAAVKKANAA (361-386), IETGKKPNIWYSLNGKIRAV (803-822), VPKVTKEKPTPPVKPTAPTK (824-843), PPVKPTAPTKPTYETEKPLK (834-853), ETEKPLEPAPVEPSYEAEPT (925-944), PTVHFHYFKLAVQPQVNKEI (985-1004), RNNNDINIDRTLVAKQSVVK (1005-1024), FQLKTADLPAGRDETTSFVL (1025-1044), TLATFNADLTKSVATIYPTV (1085-1104), KSVATIYPTVVGGVLNDGAT (1095-1114). Each peptide was synthesized (Peptide 2.0, Chantilly, Virginia) and lyophilized at the following purities (following the same order as above, in percent): 91.1, 92.2, 96.9, 98.0, 95.1,

96.6, 93.6, 80.6, 79.4, and 93.2. Each peptide was dissolved in distilled water to yield a 1mg mL^{-1} stock solution, which was pipetted into centrifuge tubes in 1.0 mL aliquots and stored at -20°C.

Human Saliva Collection and Storage

We obtained 3 separate unstimulated human saliva samples, which we could not trace back to any particular subject. After combining and vortexing the pooled sample, saliva was pipetted into 4 centrifuge tubes in 1.5 mL aliquots and stored at -20°C.

Enzyme-Linked Immunosorbent Assay

Using ELISA, we measured human salivary IgA binding to I/II peptides. Each peptide stock solution was assayed at two concentrations (10 μg mL^{-1} and 100 μg mL^{-1}). To prepare the appropriate peptide concentration, the appropriate volume of peptide stock solution was pipetted into the appropriate volume of carbonate buffer (CB) at a pH of 9.6, thus producing the I/II dilution. 100μL of the I/II dilution was pipetted into each predetermined experimental well in the ELISA plate (Fisher Scientific, Hanover Park, Illinois), incubated for 3 hours at 37°C, and refrigerated overnight at 4°C.

The next day, a 5 percent (weight/volume) skim milk CB dilution was prepared. Plates were washed 3 times (3X) in Tween Saline (TS), and each experimental well was treated for 1 hour at room temperature with 200 μL of the skim milk blocking agent (preventing unbound sites from later being bound by antibody). During the skim milk blocking treatment, serially diluted saliva samples at dilutions of 1:5, 1:10, and 1:20 were prepared in TS. Once the skim milk incubation terminated, plates were washed 3X in TS, and 100 μL of each saliva dilution was added to the corresponding predetermined wells. Saliva dilutions containing human salivary IgA were incubated at 37°C for 1 hour. In the meantime, we prepared a Horse Radish Peroxidase (HRP)-Anti-human IgA

conjugate from goat (HRP-Anti-IgA; Sigma-Aldrich, St. Louis, Missouri) TS dilution (Anti-IgA) of 1:500. Once the incubation period had ended, plates were washed in TS 3X, and incubated for 1 hour at 4°C after adding 100 μL of Anti-IgA to each well.

Salivary IgA antibodies which bound I/II peptides were determined by the combination of Anti-IgA incubation at 37°C for 1 hour and subsequent reaction with o-Phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St. Louis, Missouri) in CB (with 30% H_2O_2). Control wells (no saliva) were only subjected to TS, Anti-IgA, and OPD, respectively. After 35 minutes of color development, and quenching of the reaction by pipeting 100 μL of 2N H_2SO_4 into each well, the absorbance of each well in the plates were read at 490 nm with the SpectraMax 190 (Molecular Devices, Sunnyvale, California).

Data Analysis

All experiments were done in triplicate and are reported as means and standard errors of the mean.

Study #2: HLA-II Gene Association with SIgA in the Caries Process

Study Design

This clinical study was carried out per IUPUI/Clarian IRB approval (# 0811-50). We collected saliva from 15 subjects with known caries scores. These scores were previously determined using the International Caries Detection and Assessment System or ICDAS, and could be used for this study because subjects had had ICDAS exams done 1-2 years prior to this project by trained and calibrated examiners. Each subject provided two saliva samples, one for bacterial and antibody analysis (1 mL analyzed the same day, and 1-2 mL stored frozen at -20°C), and the other for genetic analysis (3 mL stored at room temperature). Chi square tests were used to determine the association between *HLA-DRB1*04* (an HLA-II allele), and levels of mutans streptococci, salivary IgA

activity, and caries. Salivary IgA was the primary outcome, while bacterial and caries outcomes were secondary (n=15).

Study Population

Subjects who had previously indicated interest in research at the Oral Health Research Institute, and had received an ICDAS exam within the last 1-2 years by a calibrated ICDAS examiner, were recruited. A Caucasian population was recruited as previous literature suggested an association between the biomarker being studied and caries in this population. Those who participated were remunerated $25.

Inclusion Criteria:

1. Willing to provide written informed consent and authorization for release of health information for research. For children 12-17, an assent was also obtained along with parent/legal guardian consent
2. Age must be between 12 and 35 years of age
3. Have on file records demonstrating either an ICDAS score of less than 3 (ICDAS = 0, 1, & 2) indicating a less severe caries state, or have 5 or more lesions with an ICDAS score greater than or equal to 3 (ICDAS = 3, 4, 5, & 6) indicating a more severe caries state.
4. Be White non-Hispanic.
5. Willing to attend one study visit and provide 2 saliva samples.

Exclusion Criteria:

1. Those that do not meet the inclusion criteria.

Clinical Study Procedures

Subjects fitting the inclusion criteria were contacted by phone and invited to participate. Those who participated were remunerated $25. At the study visit, after completing the informed consent process, subjects were asked to provide

their medical history, demographic information, and to answer questions regarding the inclusion or exclusion criteria.

Human Saliva Collection and Storage

For the first sample, unstimulated saliva was collected as subjects were instructed to provide at least 2 mL of saliva into a 50 mL sterile tube. After the samples had been transferred to the laboratory and vortexed, 1 mL was pipetted out into a separate, smaller centrifuge tube, stored on ice and processed the same day for bacterial analysis. The rest of the sample was divided into 1 mL aliquots and stored at -20°C to later use for salivary IgA analysis.

The second sample required approximately 3 mL of stimulated saliva (stimulated for 2 min by chewing on paraffin), which was expectorated into Oragene® DNA sample collection kits (DNA Genotek, Kanata, Ontario, Canada), and stored at room temperature for HLA DNA genotyping.

HLA Genotyping

Manual Purification of DNA from 0.5 mL of Oragene®•DNA/saliva

From the saliva samples that had been stored in Oragene® DNA sample collection kits (DNA Genotek, Kanata, Ontario, Canada) for HLA genotyping, the DNA was purified exactly as published in the above-named laboratory protocol (see http://www.dnagenotek.com/DNA_Genotek_Industry_AR_SCA_P.html) with the following exceptions. No glycogen was added in order to make the initial pellet more visible. We did, however, add an ethanol wash step in order to facilitate the abatement of any residual DNA inhibitors from the DNA pellet. After adding buffer to the DNA pellet, we added another step to ensure complete hydration of the DNA by vigorously pipetting and vortexing the sample. This was important since incomplete hydration causes inaccurate calculations of DNA concentration, and could interfere with our downstream

application. We quantified the DNA by absorbance according to the protocol. It is important to note that gloves were worn when pipetting, touching, or working in any way with the microcentrifuge tubes in order to prevent oils or other human DNA from contaminating the tubes or pipets.

We determined group-specific HLA-DRB1 alleles using HLA-DRB-specific fluorogenic probes, and employing polymerase chain reaction (PCR) using the LightCycler ® 480 Real-Time PCR System (Roche Applied Science, Indianapolis, Indiana). As controls, we used 3 cell lines (courtesy of Dr. Janice S. Blum) that were known to be homozygous for HLA-DRB1*0401 (Priess), heterozygous for HLA-DRB1*04 (Frev; also known for HLA-DRB1*01), or negative for HLA-DRB1*04 (Sweig; homozygous for HLA-DRB1*1101).

Accordingly, DRB1*04 exon primers were tailed with M13f–21 forward and M13 reverse sequences, respectively (Danzer et al., 2007). The amplification reactions were carried out in a final volume of 10 μL using the following master mixture: 5 μL of FastStart DNA Master Hybridization Probes mix (Roche Diagnostics); 1 μL of TaqMan probe; 1 μL of forward primer (M13f-21 Forward, 5′- GTA AAA CGA CGG CCA G -3′; Integrated DNA Technologies, Coralville, Iowa); 1 μL of the reverse primer (M13 Reverse, 5′- CAG GAA ACA GCT ATG AC -3′); and 1 μL of PCR Boost (Applied Biosystems), all of which had final concentrations of 200 nmol/L except for the PCR Boost, which we know minimizes water from the sample but whose formulation is proprietary. All primer oligonucleotides were synthesized by Integrated DNA Technologies. Quantification of how many ng/μL of DNA had been isolated for each sample was determined by pipetting 1 μL of sample onto the lower measurement pedestal of the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware), closing the sampling arm, and beginning measurement using the coupled operating software (after blanking the instrument by adding

the same concentration of carrier liquid). The DNA concentration of 10 ng/μL for each sample was achieved by adding the appropriate amount of water to each sample. Once the 9 μL of master mix was prepared and pipetted into a 1.5 mL centrifuge tubes, 1 μL of each DNA sample (at 10 ng/μL) was added. The following procedure was then used:

> The LightCycler program consisted of an initial denaturation for 10 minutes at 95°C followed by 10 cycles of 95°C for 5 seconds, 65°C for 20 seconds, and 25 cycles of 95°C for 5 seconds, 60°C for 20 seconds. In case of PCRs with intron primers, extension time was increased to 60 seconds. The PCR products were analyzed by Absolute Quantification selection in channel [465–510] nm (LightCycler 480). After evaluation for the presence or absence of a fluorescence signal from the group-specific PCR using the software readout, a 5 μL quantity of the PCR products was treated with 2 μL of ExoSAP-IT (Applied Biosystems) to remove primers and dNTPs, according to the manufacturer's instructions. Direct cycle sequencing was performed in a 9800 Fast Thermal Cycler (Applied Biosystems) in 10 μL of reaction volume containing 1 μL BigDye v 3.0 (Applied Biosystems), 1 μL sequencing buffer, and 400 nmol/l of the respective sequencing primer (M13rev; M12f–21)" (Danzer et al., 2007).

Bacterial Analysis

Whole saliva was diluted 1:10, 1:100 and 1: 1,000, double-plated on Mitis Salivarius agar supplemented with bacitracin and sucrose (MSSB) (Anaerobe Systems, Morgan Hill, California). The plates were incubated for 48 hours at 37°C, 5 percent CO_2, and subsequently the number of *S. mutans* colonies enumerated using Protocol (Synoptic LTD, North Cambridge, United Kingdom), thus yielding *S. mutans* colony-forming units mL^{-1} (CFU mL^{-1}).

Enzyme-Linked Immunosorbent Assay

Using ELISA, we measured human salivary IgA binding to I/II peptides. Each peptide stock solution was assayed at 10 μg mL^{-1}. To prepare the

appropriate peptide concentration, the appropriate volume of peptide stock solution was pipetted into the appropriate volume of CB at a pH of 9.6, thus producing the I/II dilution. 100μL of the I/II dilution was then pipetted into each predetermined experimental well in the ELISA plate (Fisher Scientific, Hanover Park, Illinois), incubated for 3 hours at 37°C, and refrigerated overnight at 4°C.

The next day, a 5 percent (weight/volume) skim milk CB dilution was prepared. Plates were then washed 3X in TS, and each experimental well was treated for 1 hour at room temperature with 200 μL of the skim milk blocking agent (preventing unbound sites from later being bound by antibody). During the skim milk blocking treatment, serially diluted saliva samples at dilutions of 1:5 and 1:10 were prepared. Once the skim milk incubation terminated, plates were washed 3X in TS, and 100 μL of each saliva dilution was added to the corresponding predetermined wells. Saliva dilutions containing human salivary IgA were incubated at 37°C for 1 hour. In the meantime, we prepared a HRP-Anti-IgA (Sigma-Aldrich, St. Louis, Missouri) TS dilution (Anti-IgA) of 1:500. Once the incubation period had ended, plates were washed in TS 3X, and incubated for 1 hour at 4°C after adding 100 μL of Anti-IgA to each well.

Salivary IgA antibodies which bound I/II peptides were determined by the combination of Anti-IgA incubation at 37°C for 1 hour and subsequent reaction with SIGMA*FAST*™ OPD (Sigma-Aldrich, St. Louis, Missouri). The ELISA plates were washed in TS 3X to discard unbound constituents. 100 μL of the OPD solution was pipetted into each well. After 15 minutes, the reaction was quenched by pipeting 100 μL in each well of 2N H_2S0_4, and the plates were read on the SpectraMax 190 (Molecular Devices, Sunnyvale, California) at 490 nm. Negative control wells (no saliva) were only subjected to TS, Anti-IgA, and OPD, respectively. These controls expressed any background noise, and this noise was subtracted after correcting raw data values. Interplate positive reference controls

(of human pooled saliva samples) were incubated with the I/II peptide, 1085-1104, and were subjected to the same procedures as all other sample wells. These controls allowed any interplate variability to be factored out of the analyses.

Data Analysis

All experiments were done in either duplicate or triplicate and are reported as means and standard errors of the mean. Differences (between HLA-DRB1*04 positive and negative groups as well as between high caries and low caries groups) in salivary IgA reactivity were determined using a chi square test or a student's t-test. ICDAS data were correlated with bacterial and salivary IgA data from those subjects for whom we had bacterial levels.

Study #3: HLA-II gene Influence in Caries Experience in an at-risk type 1 diabetes population on Salivary IgA Response.

Study Design

The study population was divided into two groups: an *04 NEGATIVE group (n=16) and an *04 POSITIVE group (n=16). After completing the informed consent process, subjects were asked to provide medical history and demographic information and to answer questions regarding the inclusion/exclusion criteria. Each subject received a clinical exam by a trained and calibrated examiner with regard to caries experience using ICDAS II, a novel yet validated and robust approach to detect and assess caries (Jablonski-Momeni et al., 2008; Rodrigues et al., 2008). Kappa values for intra-examiner reproducibility for the ICDAS-II examination were 0.68 (from a long term clinical trial), and 0.89 from a similar study during training with the gold standards of Drs. Ekstrand and Nyvaad. A recent 18 month follow-up study was reported by Prof. Eckert with a repeatability weighted kappa of 0.77. At the time of the subject's visit, 5-6 mL of unstimulated whole saliva was collected from both groups of subjects and stored at either -80°C or -20°C until laboratory analysis.

Bacterial numbers (mutans streptococci) were evaluated per mL of whole saliva. Levels of salivary IgA antibodies to I/II epitopes of *S. mutans* UA159, and whole cells of *S. mutans* (UA159, NG8 and PC3370) were detected using ELISA. Subjects did not brush their teeth at least a few hours before they participated in the study although this was not formally required, but verified post-hoc. Even if someone had done so, only bacterial data would have been compromised while SIgA data would remain unaffected. Differences between DRB1*04 positive and negative groups were examined using a two-sided, two-sample t-test.

Sample Size Calculation

A sample size of 17 subjects each from DRB1*04 positive and negative group (totaling 34 subjects) would have 80% power to detect a 1.0 standard deviation difference between the two groups, assuming a two-sided two-sample t-test and a 5% significance level. A literature review and preliminary data from the second clinical study demonstrated a mean (SD) for salivary IgA mg 100 mL^{-1} of 2.0 (0.5) for DRB1*04 positive subjects. The variability in the proposed study was assumed to be similar, thereby predicting an ability to detect a 25% difference in salivary IgA between the DRB1*04 positive and negative subjects.

Study Population

The predominant number of healthy subjects in this study were recruited from the TrialNet Natural History Study (TN NHS), where we recruited healthy yet at-risk for T1D subjects. Subjects who are DRB1*04 positive (as indicated from Phase 2 or 3 participation of TN NHS, or as indicated by a saliva test at the time of study visit) were invited to participate. Matched sex, race and age control subjects (see table 3) testing negative for DRB1*04 were also invited to participate. Those who participated were remunerated $50.

Inclusion Criteria:

1. Willing to provide written informed consent and authorization for release of health information for research. For children 6-17, an assent needed to be obtained along with parent/legal guardian consent.
2. Age must be matched in both the experimental and control groups.
3. Ethnicity must be matched (The IU TN NHS is an all Caucasian population and thus our sample was a Caucasian population; the literature suggests significant HLA differences among distinct populations, see (Ozawa et al., 2001)).
4. Sex must be matched.
5. Other pertinent demographic variables should be matched.
6. Willing to attend one study visit and provide 1 saliva sample.

Exclusion Criteria:

1. Those that do not meet the inclusion criteria.

Clinical Procedures

At the study visit, the subject or parent completed a subject's informed consent form and an authorization for the release of health information for research form. Children under 18 were given a similar letter of assent. The information presented to the subject and parent was explained during the Informed Consent Process and the subject and parent had an opportunity to ask questions and decide if they wished themselves or their child to participate in the study. Upon the subject's/parent's signing of the consent/assent forms, the subject's medical history and the inclusion/exclusion criteria were reviewed. The subjects accepted into the study received an oral soft tissue exam.

In some cases, 2 samples of saliva were collected. In the case where DNA had already been collected, and only one saliva sample was required, that sample was collected and later divided in the laboratory into 2 samples, one for bacterial analyses and the other for antibody analyses. Subjects were instructed to provide at least 5 mL of unstimulated saliva into a 50 mL sterile tube and timed in order to calculate a salivary flow rate. After the samples had been transferred to the laboratory and vortexed, 0.9 mL was pipetted out into a separate, smaller centrifuge tube and mixed with 0.2 mL of glycerin for each sample, vortexed again and then stored at -80°C until bacterial analysis. The rest of the sample was then divided into 1.5 mL aliquots and stored at -20°C so as to prevent unnecessary freeze/thaw cycles and allow repeat assays if needed.

In those cases requiring 2 saliva samples, the first sample included the one previously discussed, and the second sample was collected for DNA purposes (to determine if the participant was HLA-DRB1*04 positive or negative). This sample requires approximately 3 mL of stimulated saliva (stimulated for 2 min by chewing on paraffin). This DNA sample was only collected from participants for whom we did not have HLA typing data, and stored in Oragene® DNA sample collection kits (DNA Genotek, Kanata, Ontario, Canada) at room temperature. Following the saliva collection, subjects had their teeth brushed by study personnel. They then received an ICDAS exam by one calibrated examiner. Upon completion of the ICDAS exam, the subject's participation ended.

ICDAS-II Examination

The examiner performed a visual caries examination of all teeth in the dentition using the ICDAS-II criteria. Anterior teeth must have been fully erupted to be examined, while posterior teeth were examined if the full occlusal surface was visible. In the case of missing permanent teeth, the examiner attempted to establish the cause. This examination will be performed using

conventional dental equipment including dental chairs and lights, compressed air and plane surface mirrors. The criteria used in the ICDAS-II diagnostic system are as follows:

Tooth codes

S = sound

U = unerupted

X = extracted, presumed for the reasons of caries

Y = extracted for other reasons, e.g. orthodontic reasons

N = not gradable, e.g. for reasons of trauma

C = caries or caries related events such as fillings

0 = Sound tooth surface.

1 = First visual change in enamel.

2 = Distinct visual change in enamel.

3 = Localized enamel breakdown due to caries with no visible dentin.

4 = Underlying dark shadow from dentin +/- localized enamel breakdown.

5 = Distinct cavity with visible dentin.

6 = Extensive distinct cavity with visible dentin.

In addition, the activity of the caries lesions detected was determined, in accordance with the ICDAS-II recommendation:

1 = Not active

2 = Active lesion

Fillings:

1 = Sealant, partial

2 = Sealant, full

3 = Tooth colored restoration

4 = Amalgam restoration

5 = Stainless steel crown

6 = Crown, gold, porcelain or MCC

7 = Lost restoration

8 = Temporary restoration

9 = Other

Genetic Analyses (HLA typing)

Most HLA typing was already completed under the TrialNet protocols for phase 2 and 3 subjects in TH NHS. Any remaining procedures were accomplished exactly as specified on pages 51-53 of this chapter.

Bacterial Analyses

Aliquots of whole saliva (0.9 mL) with glycerin (0.2 mL) were stored at -80°C until they were thawed for analysis. Each sample was diluted 1:100 and 1:1,000 in sterile saline, double-plated on Mitis Salivarius agar supplemented with bacitracin and sucrose (MSSB) (Anaerobe Systems, Morgan Hill, California). The plates were incubated for 48 hours at 37°C, 5 percent CO_2, and subsequently the number of *S. mutans* colonies enumerated using Protocol (Synoptic LTD, North Cambridge, United Kingdom), thus yielding *S. mutans* colony-forming units/mL (CFU/mL).

Enzyme-Linked Immunosorbent Assay

In preparation of individual saliva samples being used to conduct ELISA SIgA antibody assays to the 10 selected epitopes, optimization assays were completed and repeated until refined (Figs. 6-8; Table 1). There were two important needs regarding these assays. Absolute absorbance numbers needed to be in the range of 0.40 to 1.20 in optimization samples in order to provide additional room for those individuals who would undoubtedly respond outside this range so as to not compromise the results in either direction. It was observed during the optimization assays that one unidentified subject was a low responder at a secondary antibody concentration of 1:15,000 (Fig. 7). Neither the

pooled nor other individual samples were too low at the 1:10,000 dilution of secondary antibody, nor did any of the samples provide too high an absorbance at this dilution (Figs. 6-8), whereas two out of three samples were too high at the 1:5,000 dilution. Additionally, it was considered important to make sure that both primary and secondary antibody concentrations produced TS negative control absorbance data that fell below 10 percent of each sample's reactivity (Table 1). Only an anti-IgA detection antibody concentration of 1:10,000 and a saliva dilution of 1:10 fulfilled both of these requirements.

Using ELISA, human salivary IgA binding to I/II peptides was measured. Each peptide stock solution was diluted to 1 μg mL^{-1}. To prepare the appropriate peptide concentration, the required volume of peptide stock solution was pipetted into the desired volume of CB, thus producing the 1 μg mL^{-1} I/II epitope dilution. For antigen coating, 100μL of the I/II dilution was pipetted into each predetermined experimental well in the ELISA plate (Fisher Scientific, Hanover Park, Illinois), incubated for 3 hours at 37°C, and refrigerated overnight at 4°C.

The next day, a 5 percent (weight/volume) skim milk CB dilution was prepared. Plates were washed 3X in TS, and each experimental well was blocked for 1 hour at room temperature with 200 μL of the skim milk blocking agent. During skim milk treatment, serially diluted saliva samples at dilutions of 1:5 and 1:10 in TS were prepared. Once the skim milk incubation terminated, plates were washed 3X in TS, and 100 μL of each saliva dilution was added to corresponding predetermined wells. Saliva dilutions containing human salivary IgA were incubated at 37°C for 1 hour. In the meantime, an HRP-anti-IgA (Sigma-Aldrich, St. Louis, Missouri) TS dilution (anti-IgA) of 1:10,000 was prepared. Once the incubation period ended, plates were washed in TS 3X, and incubated for 1 hour at 4°C after adding 100 μL of anti-IgA to each well.

The relative level of salivary IgA antibodies which bound I/II peptides were determined by the combination of anti-IgA incubation at 37°C for 1 hour and subsequent reaction with SIGMA*FAST*™ OPD (Sigma-Aldrich, St. Louis, Missouri). The ELISA plates were washed in TS 3X to discard unbound constituents and 100 μL of the OPD solution was pipetted into each well. After 30 minutes, the reaction was quenched by pipeting 100 μL in each well of 2N H_2S0_4, and the plates were read on the SpectraMax 190 (Molecular Devices, Sunnyvale, California) at 490nm. Negative control wells (no saliva) were only subjected to TS, anti-IgA, and OPD, respectively. These controls expressed any background noise, and this noise was subtracted out after correcting raw data values. Interplate positive reference controls were incubated with the I/II peptide, 1085-1104, and were subjected to the same procedures as all other sample wells. These controls allowed any interplate variability to be factored out of the analyses.

Using ELISA, human salivary IgA binding to 3 strains of *S. mutans* was also determined. Page Caufield at the University of Alabama, Birmingham, kindly provided the *S. mutans* UA159 strain to the Gregory Laboratory; it is also a part of the American Type Culture Collection (ATCC 700610). The UA159 genome can be electronically accessed (access # NC_004350) (Ajdić et al., 2002). The other two strains, NG8, and an NG8-derived I/II-mutant strain, PC3370 (Crowley et al., 1999), were kindly provided to the Gregory Laboratory by Dr. L. Jeannine Brady in the Department of Oral Biology, at the University of Florida, Gainesville, Florida. All three strains, UA159, NG8, and PC3370 had been stored at -80°C in glycerol. These cells were cultured separately in Todd-Hewitt broth (THB, Acumedia, Baltimore, MA) overnight in a 5% CO_2 air incubator at 37°C, centrifuged at 8500 RPM for 20 minutes the next day until the supernatant could be removed, leaving the initial bacterial cell pellet. The pellet was resuspended

by vortexing the solid pellet in 0.15 M saline, then washed by centrifugation again, which was repeated twice. The pellet was resuspended a third time in 100 mL of 0.15 M saline with 0.5 mL formaldehyde to give a 0.5% concentration. These cells were incubated with the formaldehyde mixture at room temperature for 48 hours to kill the cells. Then, the cells were washed 2X in saline in order to remove the formaldehyde. The cells were resuspended in sodium carbonate/bicarbonate buffer, and diluted until a 0.5 OD was achieved at 660 nm using the SpectraMax 190.

Data Analysis

All experiments were done in either duplicate or triplicate and are reported as means and standard errors of the mean. ICDAS-II data were correlated with lesion activity, bacterial levels, and salivary IgA data from all 43 subjects (n=18, DRB1*04 positive; n=25, DRB1*04 negative). However, to minimize exogenous variables in ultimately making any conclusions, a more conservative, matched sample of subjects was selected from the overall sample (n=16 in each group; see Table 3).

Paired t-tests were used to compare the matched pairs from the DRB1*04 positive and negative groups. When the analyses were not restricted to matched pairs, two-sample t-tests were used for the comparisons. Pearson correlation coefficients were calculated to assess the linear relationships between variables. A natural logarithm transformation of the variables was used for most analyses due to the non-normal distribution of the data.

CHAPTER FOUR

Results

From the outset, it is important to generally qualify data hereafter presented. Though certain groups may appear numerically distinct from other groups, comparisons are not statistically significant *unless explicitly stated*. Nonetheless, this research does provide some interesting trends, especially in the third study, as well as suggestions that provide added information and may provide researchers new directions in the pursuit of understanding caries at its fundamental level—in rational vaccine design and searching for biomarkers that predict caries susceptibility.

In the first study, at two separate dilutions, and across seven initially selected epitopes, one eptiope in the C-terminal domain (Fig. 1) located between the last proline-rich repeat in the P region (so named because of proline repeats) and the LPxTG anchor (1085-1104), was numerically but not statistically, more immunoreactive than the others (Figs. 2 and 3). These data came from a sample of pooled saliva (n=3), samples from subjects whose genetic makeup are unknown. Nonetheless, these data demonstrate that, at least in this particular sample, 1085-1104 is an important SIgA binding region on I/II in relation to the other regions studied despite the fact that these data cannot be directly compared. Another epitope straddling the P3 region in the P region, and the C-terminal region (985-1004), became equally immunoreactive when the peptide concentration was increased by 10 fold (Fig. 3). Additionally, there is a dichotomous relationship between the first three peptides, 803-822 (located in the variable, or V region, which is also a gp340 binding site), 824-843 (straddling the V region on the amino-terminus side and the P region, specifically P1, at the carboxy-terminus, which region crosses another gp340 binding site), and 834-853 (the same as 824-843 except that it is almost entirely located in P1 of the P region,

but also straddles the gp340 binding site), and the last four peptides, 925-944 (P3 of the P region), 985-1004 (straddling P4 of the P region and the C-terminal domain), 1085-1104 (C-terminal domain), and 1095-1114 (C-terminal domain); in that the combined mean immunoreactivity (OD 490 nm) of the former group is 0.11 ± 0.01 and 0.56 ± 0.14 for the latter ($p = 0.055$).

In the second and third studies, subjects having the HLA-DRB1*04 immunogenetic biomarker were identified using HLA-DRB-specific fluorogenic probes, by polymerase chain reaction (PCR) using the LightCycler ® 480 Real-Time PCR System (Fig. 4). In the second study, no correlations between higher caries and DRB1*04 positive subjects were observed. However, in seven out of 10 epitopes, DRB1*04 positive subjects had numerically, but not statistically, higher SIgA responses than their negative counterparts (Fig. 5). DRB1*04 positive subjects appeared to exhibit lower levels of *S. mutans* although these observations were not statistically significant (Fig. 9).

In the third study, reminiscent of the first laboratory study, all four downstream epitopes, 361-386 (straddles A2 and A3 in the A region, so named because of alanine repeats), 803-822, 824-843, and 834-853, located along I/II at the carboxy-terminus of the V region and the amino-terminus of the P region as previously described, induce less immunoreactivity (OD 490 nm) in both DRB1*04 positive and negative groups (0.76 ± 0.01) compared to the next 5 epitopes, 925-944, 985-1004, 1005-1025, 1025-1044, and 1085-1104, all located at the carboxy-terminus of the P region or in the C-terminal domain as previously described, which induce higher reactivity in both groups (0.98 ± 0.03). This difference is statistically significant ($p \leq 0.004$). The uppermost upstream epitope (1085-1104) was numerically, but not statistically, the least responsive of all 10 epitopes assayed (0.62 ± 0.06). DRB1*04 positive subjects (0.88 ± 0.09), compared

to their non-biomarker counterparts (0.79 ± 0.07), exhibited numerically, but not statistically, higher immunoreactivity to nine out of 10 epitopes.

Although the third study demonstrated that DRB1*04 positive subjects (n=18, unpaired; n=16 paired) had numerically, but not statistically, higher reactivity to these epitopes, the exception was 834-853 (Figs. 11, 14, 16). This epitope's amino-terminus straddles the carboxy-terminus of the V region and the epitope's carboxy-terminus encompasses most of the P1 region, which appears to be a very important interface between all three regions when biologically functional in three dimensions. Although not statistically significant, DRB1*04 positive subjects exhibited lower specific SIgA activity to 834-853 (Fig. 14) and also a lower reactivity ratio of 834-853/whole cell *S. mutans* UA159 (Fig. 16). Additionally, specific activity, and reactivity ratio figures exhibited the same dichotomous relationship (lower SIgA response versus higher SIgA response) between the lower downstream four epitopes, which account mainly for parts of the A and V regions, but also the first domain in the P region (361-386, 803-822, 824-843, and 834-853), and the subsequent upper 5 epitopes (925-944, 985-1004, 1005-1025, 1025-1044, and 1085-1104; accounting for the C-terminal end of the P region) in both the DRB1*04 positive and negative goups. DRB1*04 positive subjects also exhibited numerically, but not statistically significant, higher specific activity SIgA responses to all three *S. mutans* strains assayed (Fig. 17), and antibody reactivity to the UA159, NG8, and PC3370 strains (Fig. 15), except reactivity to NG8 in both groups is at a similar level. In an indirect measure of immunoreactivity against the entire I/II antigen (PC3370 subtracted from the NG8 strain), DRB1*04 positive subjects exhibited lower responses, though not statistically significant, to I/II in its entirety, suggesting that when encountering the entire antigen, in contrast to the selected peptides, these subjects were not able to respond as well as their non-biomarker counterparts (Fig. 18).

In contrast to exhibiting higher SIgA responses to most peptides, or to other substrates (whole cells of UA159, NG8 and PC3370), though statistically insignificant, DRB1*04 positive subjects demonstrated 25-30 μg mL^{-1} lower total IgA (TIgA) than negative subjects (Fig. 13). These determinations (Table 2) were accomplished by creating a standard curve fit to mean data points from standard concentrations of human colostral IgA, including 200, 100, 50, 25, 12.5, 3.1, and 0 μg mL^{-1}, respectively (Fig. 12). Absorbance values were used to predict actual concentrations of total salivary IgA by using the standard curve equation from a log-log function (R^2 = 0.991), and then plugging each subject's reactivity (1:15 saliva dilution in TS) to unlabeled, human IgA into the function, thus providing the correct concentration in μg mL^{-1}. The total salivary IgA concentrations in all 43 subjects ranged from 11.1-420.4 μg mL^{-1}, in agreement with a very recent report demonstrating total salivary IgA concentrations ranging from 13.7-483.0 μg mL^{-1} in 134 healthy individuals (Booth et al., 2009). DRB1*04 positive subjects (n=18, unpaired) had a numerically, but not statistically, lower salivary flow rate (0.55 ± 0.08 min^{-1} compared to 0.71 ± 0.10 min^{-1} in 25 negative subjects) (Fig. 10), and although this does not constitute a trend, these subjects may have a disadvantage of experiencing a 200 μg mL^{-1} salivary IgA deficit per day. When examining only the DRB1*04 positive subjects from the matched study (table 3; n=16), however, the differences in salivary flow rate disappeared making the groups indistinguishable with regard to this factor.

Tables 4 and 5 suggest interesting phenomena by themselves, although this phenomena does not hold up when taking into account other past caries experience in both the DRB1*04 positive and negative groups. If we were to rely solely on the data from Tables 4 and 5, we may conclude that there are more irreversible caries lesions in the DRB1*04 positive group compared to the negative group. However, by analyzing the full spectrum of data available, it is

clear that each group cannot be distinguished from each other when taking into account not only irreversible ICDAS-II lesions, but also past caries experience (Table 6).

Since ICDAS-II criteria represent the spectrum of caries progression, or the caries process, as it develops from its reversible stages to its irreversible development, it is thought that much can be gleaned by comparing differences between the reversible and irreversible processes of caries among groups. Unfortunately, it was impossible to control for a variety factors that may mask a putative negative effect the DRB1*04 biomarker may have.

In order to normalize the distribution among ICDAS-II scores, these data were transformed by taking the natural log of the scores. Then, ratios which reflected the irreversible predisposition of caries in each group, were calculated and refined by taking into account the breadth of the data available (table 6). The mean ratios of each group were statistically compared. A higher irreversible ratio number indicates that the caries process is more likely to cross the threshold from reversible to irreversible disease. Whereas a lower value indicates that the caries process is likely to abate before progressing to an irreversible level. The two groups were indistinguishable.

Table 1. Optimization of Tween Saline negative control*

Dilutions	Control % of Primary Antibody Reactivity			
Pooled Saliva, 1:10 Dilution	9.2%	5.6%	6.6%	6.6%
Pooled Saliva, 1:20 Dilution	11.6%	8.3%	9.7%	9.4%
Individual #1 Saliva, 1:10 Dilution	13.2%	7.9%	10.3%	10.7%
Individual #1 Saliva, 1:20 Dilution	18.0%	11.8%	15.6%	16.7%
Individual #2 Saliva, 1:10 Dilution	8.4%	5.4%	5.9%	5.6%
Individual #2 Saliva, 1:20 Dilution	10.8%	6.7%	8.2%	7.3%
Secondary Antibody Dilutions	1:5,000	1:10,000	1:15,000	1:20,000

*Optimization of Tween Saline (TS) negative control (control) as a percentage of salivary IgA immunoreactivity to one selected putative epitope of an *S. mutans* adhesin, I/II. Reactivity (1:10 and 1:20 saliva dilutions in TS) to the selected peptide (1085-1104; numbers reflect amino acid residues; 1 μg mL^{-1}) was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (at 4 separate dilutions: 1:5,000, 1:10,000, 1:15,000, and 1:20,000 in TS), and subsequent reaction with OPD. The TS negative control solution takes the place of saliva, and its reactivity values are presented in this table as percentages within the table itself, calculated by taking the control's reactivity, as measured by optical density absorbance (490 nm), and divding by the OD values observed for each of the saliva samples. Notice that in every case, a 1:10 dilution of saliva and a 1:10,000 secondary antibody dilution yields the lowest percentages within the table, including 5.6%, 7.9%, and 5.4%. These data are reported as contol OD percentages of each saliva sample's OD values.

Table 2. Unpaired subjects' raw data from the third clinical study*

Subject #	DRB1*04+ = 1	*S. mutans* (CFU mL^{-1})	Saliva Flow (mL min^{-1})	Total IgA (μg mL^{-1})
1001	0	2310000	0.23	136.37
1002	1	2560000	0.31	120.57
1003	1	1140000	0.22	30.63
1004	1	1020000	0.38	68.18
1005	1	80000	0.86	48.77
1006	0	1210000	0.35	420.36
1007	0	2730000	0.35	269.98
1008	0	2350000	0.70	78.21
1009	0	6420000	1.19	32.33
1010	0	1400000	0.25	125.66
1011	0	931831	0.12	106.29
1012	1	1370000	0.28	233.72
1013	1	1820000	0.63	233.72
1014	0	3510000	0.65	269.98
1015	1	88000	0.63	48.77
1016	1	831510	0.40	41.96
1017	0	426000	0.55	65.08
1018	0	2030000	0.98	56.45
1019	0	1090000	0.53	332.75
1020	1	2420000	1.00	39.87
1021	1	564000	0.70	34.09
1022	0	1580000	0.55	153.79
1023	0	1360000	0.70	19.50
1024	0	528000	0.20	32.33
1025	0	4640000	0.51	44.14
1026	0	6640000	0.63	24.56
1027	0	1530000	1.25	29.01
1028	0	1350000	0.52	27.46
1029	0	3160000	no data	166.38
1030	1	951860	1.79	136.37
1031	0	67000	0.57	15.32
1032	0	156000	1.57	23.21
1033	1	71000	0.48	11.14
1034	1	54000	0.54	19.50
1035	0	560000	0.84	23.21
1036	1	456000	0.33	74.75
1037	0	5700000	0.48	20.68
1038	0	2540000	0.91	23.21
1039	1	224000	0.18	51.23
1040	1	2260000	0.48	29.01
1041	1	72000	0.27	15.32
1042	0	85000	2.33	16.29
1043	1	3390000	0.50	34.09

*Unpaired subjects' raw data from the third clinical study (n=43, unpaired), including HLA-DRB1*04 biomarker information (0 = negative; 1 = positive),

mutans streptococci counts, unstimulated salivary flow rates, and total IgA concentration. Subject 1029 presents no data in the salivary flow column since the data was inadvertently not recorded.

Table 3. Matched DRB1*04 positive and negative subjects by age, sex within a Caucasian population*

Matched (Paired) Subjects (n = 32)			
DRB1*04 Positive		**DRB1*04 Negative**	
Age	Sex	Age	Sex
5.5	M	6	M
6	M	8	M
7	M	9	M
18	F	19	M
8	M	13	M
32	F	32	F
22	F	27	F
23	F	30	F
31	F	30	F
6	F	9	M
27	M	30	M
7	F	11	F
34	M	34	M
31	M	30	M
36	F	34	F
19	F	26	F
26	F	30	F
21	F	26	F

*Subjects were matched by age before any data analysis was performed. The algorithm used simply included matching sex perfectly, if at all possible, then matching age as close as possible within this Caucasian population. White Hispanics were not excluded, but were not seen.

Table 4. Raw ICDAS-II descriptive statistical data*

Untransformed ICDAS-II Data					Log Transformed ICDAS-II Data				
Descrip. Statistics	**# surfaces with ICDAS ≥ 1**	**# surfaces with ICDAS ≥ 3**	**# filled surfaces**	**# surfaces missing due to caries**	**Descrip. Statistics**	**# surfaces with ICDAS ≥ 1**	**# surfaces with ICDAS ≥ 3**	**# filled surfaces**	**# surfaces missing due to caries**
	DRB1*04+, Unpaired (n =18)					DRB1*04+, Unpaired (n =18)			
Mean	2.39	1.33	11.28	1.61	**Mean**	0.71	0.49	2.10	0.38
SD	4.38	2.54	8.35	3.96	**SD**	0.95	0.78	1.13	0.90
SE	1.03	0.60	1.97	0.93	**SE**	0.22	0.18	0.27	0.21
	Negative, Unpaired (n = 25)					Negative, Unpaired (n = 25)			
Mean	3.56	0.96	21.04	1.16	**Mean**	0.91	0.26	2.75	0.33
SD	6.14	3.45	15.44	2.94	**SD**	1.05	0.67	0.97	0.78
SE	1.23	0.69	3.09	0.59	**SE**	0.21	0.13	0.19	0.16
	DRB1*04+, Paired (n =16)					DRB1*04+, Paired (n =16)			
Mean	2.69	1.50	11.56	1.81	**Mean**	0.79	0.55	2.10	0.43
SD	4.57	2.66	8.57	4.17	**SD**	0.97	0.81	1.17	0.94
SE	1.14	0.66	2.14	1.04	**SE**	0.24	0.20	0.29	0.24
	Negative, Paired (n = 16)					Negative, Paired (n = 16)			
Mean	4.25	1.38	18.00	1.44	**Mean**	1.03	0.32	2.60	0.39
SD	7.01	4.29	12.98	3.39	**SD**	1.10	0.81	1.02	0.86
SE	1.75	1.07	3.24	0.85	**SE**	0.28	0.20	0.25	0.21

*Raw ICDAS-II descriptive statistical data (for both the unpaired, n=43 sample, and the paired, n=32 sample), describing the number of lesions fitting the criteria of ICDAS-II ≥ 1 or ICDAS-II ≥ 3, or the total number of filled or missing surfaces due to prior caries experience. In order to normalize the distribution among ICDAS-II scores, these data were transformed by taking the natural log (right side of the table).

Table 5. Numbers of ICDAS-II lesions in each ICDAS-II category for all subjects*

Descriptive Statistic Labels for DRB1*04 Positive and Negative	# surfaces with ICDAS=1	# surfaces with ICDAS=2	# surfaces with ICDAS=3	# surfaces with ICDAS=4	# surfaces with ICDAS=5	# surfaces with ICDAS=6
DRB1*04 positive-all	n = 18					
Mean	0.17	0.89	0.44	0.61	0.28	0.00
SD	0.38	2.05	1.04	1.69	0.67	0.00
SE	0.36	0.71	0.20	0.32	0.08	0.16
DRB1*04 negative-all	n = 25					
Mean	0.44	2.16	0.24	0.48	0.08	0.16
SD	1.80	3.57	1.01	1.61	0.40	0.80
SE	0.36	0.71	0.20	0.32	0.08	0.16
DRB1*04 positive-match	n = 16					
Mean	0.19	1.00	0.50	0.69	0.31	0.00
SD	0.40	2.16	1.10	1.78	0.70	0.00
SE	0.10	0.54	0.27	0.44	0.18	0.00
DRB1*04 negative-match	n = 16					
Mean	0.69	2.19	0.38	0.63	0.13	0.25
SD	2.24	3.43	1.26	2.00	0.50	1.00
SE	0.56	0.86	0.31	0.50	0.13	0.25

*Notice that the mean number of surfaces in the irreversible categories (ICDAS-II = 3, 4, and 5) are higher in the DRB1*04 positive group compared to its negative counterpart across all samples. The opposite is true with regard to reversible lesions (ICDAS-II = 1 or 2).

Table 6. Untransformed and transformed ICDAS-II data mimicking DMFS measures as well as irreversible to reversible ratios*

	ICDAS-II untransformed data			ICDAS-II log transformed data			Log transformed irreversible ratios		
Descriptive Statistic Labels for DRB1*04 Positive and Negative	# filled surfaces	# surfaces missing due to caries	# surfaces with ICDAS>=3 or filled	# filled surfaces	# surfaces missing due to caries	# surfaces with ICDAS>=3 or filled	ratio: # irreversible (ICDAS>=3) / # reversible (ICDAS=1 or 2)	ratio: # irreversible (ICDAS>=3 or filled) / # reversible (ICDAS=1 or 2)	ratio: # irreversible (ICDAS>=3 or filled or missing due to caries) / # reversible (ICDAS=1 or 2)
DRB1*04 positive-all	n = 18								
Mean	10.2	1.6	11.3	1.9	0.4	2.0	0.02	1.54	1.61
SD	8.6	4.0	9.6	1.2	0.9	1.2	0.42	1.20	1.23
SE	2.0	0.9	2.3	0.3	0.2	0.3	0.10	0.28	0.29
DRB1*04 negative-all	n = 25								
Mean	20.3	1.2	21.0	2.6	0.3	2.7	-0.55	1.85	1.90
SD	15.7	2.9	16.2	1.1	0.8	1.1	0.84	1.18	1.15
SE	3.1	0.6	3.2	0.2	0.2	0.2	0.17	0.24	0.23
DRB1*04 positive-matc	n = 16								
Mean	11.3	1.8	12.6	2.1	0.4	2.2	0.02	1.65	1.72
SD	8.5	4.2	9.4	1.2	0.9	1.2	0.44	1.21	1.23
SE	2.1	1.0	2.4	0.3	0.2	0.3	0.11	0.30	0.31
DRB1*04 negative-matc	n =16								
Mean	17.7	1.4	18.8	2.6	0.4	2.6	-0.61	1.71	1.78
SD	12.7	3.4	13.5	1.0	0.9	1.0	0.82	1.10	1.05
SE	3.2	0.8	3.4	0.3	0.2	0.3	0.21	0.28	0.26
Comparison	p-values								
2-sample t-test - all	0.01	0.69	0.02	0.06	0.84	0.08	0.01	0.40	0.43
2-sample t-test - match	0.11	0.78	0.14	0.20	0.90	0.24	0.01	0.88	0.88
paired t-test - match	0.12	0.80	0.16	0.21	0.91	0.24	0.02	0.88	0.88

*Filled surfaces, missing surfaces due to caries, as well as irreversible lesions are accounted for here. Moreover, the ratio of the number of irreversible lesions to the number of reversible lesions (# ICDAS>=3 / # ICDAS=1 or 2) has been calculated and presented. Furthermore, adding the number of filled surfaces to this measure and also the number of missing surfaces provides three separate ways to analyze the irreversible to reversible caries ratio. Although the ratio itself is statistically significant whether including all recruited subjects (n=43) or within the matched group (n=32), by adding the other irreversible measures (filled and missing surfaces), this ratio becomes statistically insignificant and the groups ultimately appear to be indistinguishable in caries outcomes. Notice that although some of the untransformed data is statistically significant when

analyzing the entire sample of subjects, these p-values are not valid since the distribution of scores is non-normal and therefore these numbers cannot be interpreted in any meaningful way.

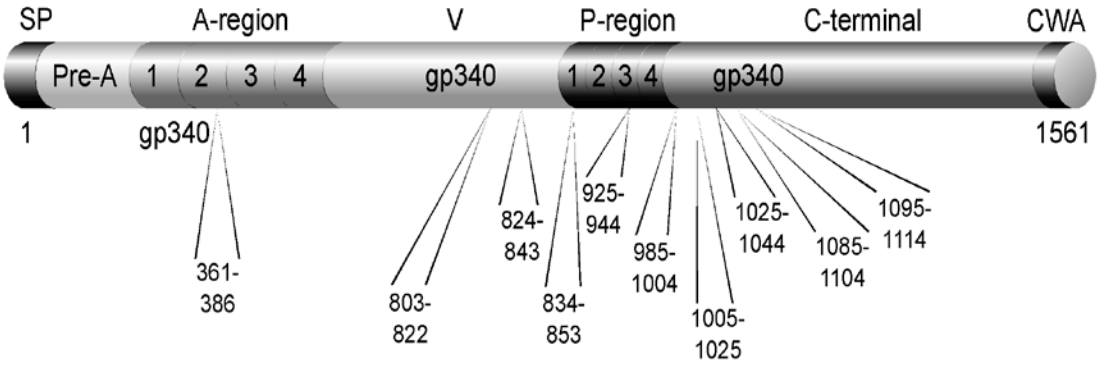

Figure 1. Schematic and diagramatic representations of the surface protein adhesin, I/II. I/II comprises the following regions (and residues): the signal sequence (1-38), a pre-A region (39-120), the A-region (121-447), V-region (aa 448-839), P-region (840-983), and the C-terminal region (984-1463), and regions for anchor proteins associated with the bacterial cell wall including the the LPxTG anchor region (CWA) (1464-1561). The A-region consists of three to four (represented here as four) alanine-rich tandem repeats, specifically A1 (121-201), A2 (202-283), A3 (284-365), and A4 (366-447). The P-region also consists of three to four repeated regions (represented here as four) with about 35 percent proline content, including P1 (840-878), P2 (879-917), P3 (918-956), and P4 (957-983) (Larson et al., 2010; van Dolleweerd et al., 2004).

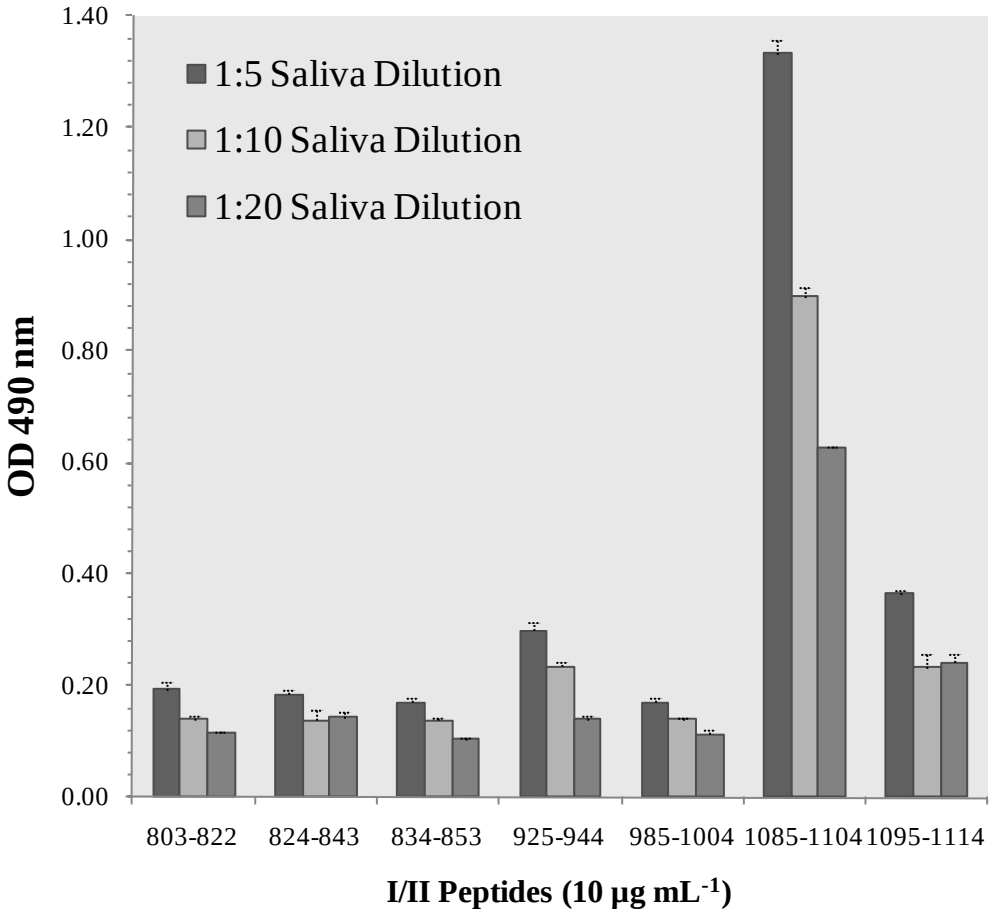

Figure 2. Human salivary IgA (from pooled samples, n=3) immunoreactivity to selected putative epitopes of an *S. mutans* adhesin, I/II. Reactivity for seven selected peptides (803-822, 824-843, 834-853, 925-944, 985-1004, 1085-1104, and 1095-1114; numbers reflect amino acid residues; 10 µg mL^{-1}) was determined in triplicate using ELISA, on low binding ELISA plates, and detected by a horse radish peroxidase (HRP)-labeled, anti-human IgA conjugate from goat (1:500 dilution in TS), and subsequent reaction with o-Phenylenediamine dihydrochloride (OPD). These data are reported in optical density absorbance (490 nm) as means and standard errors of the mean.

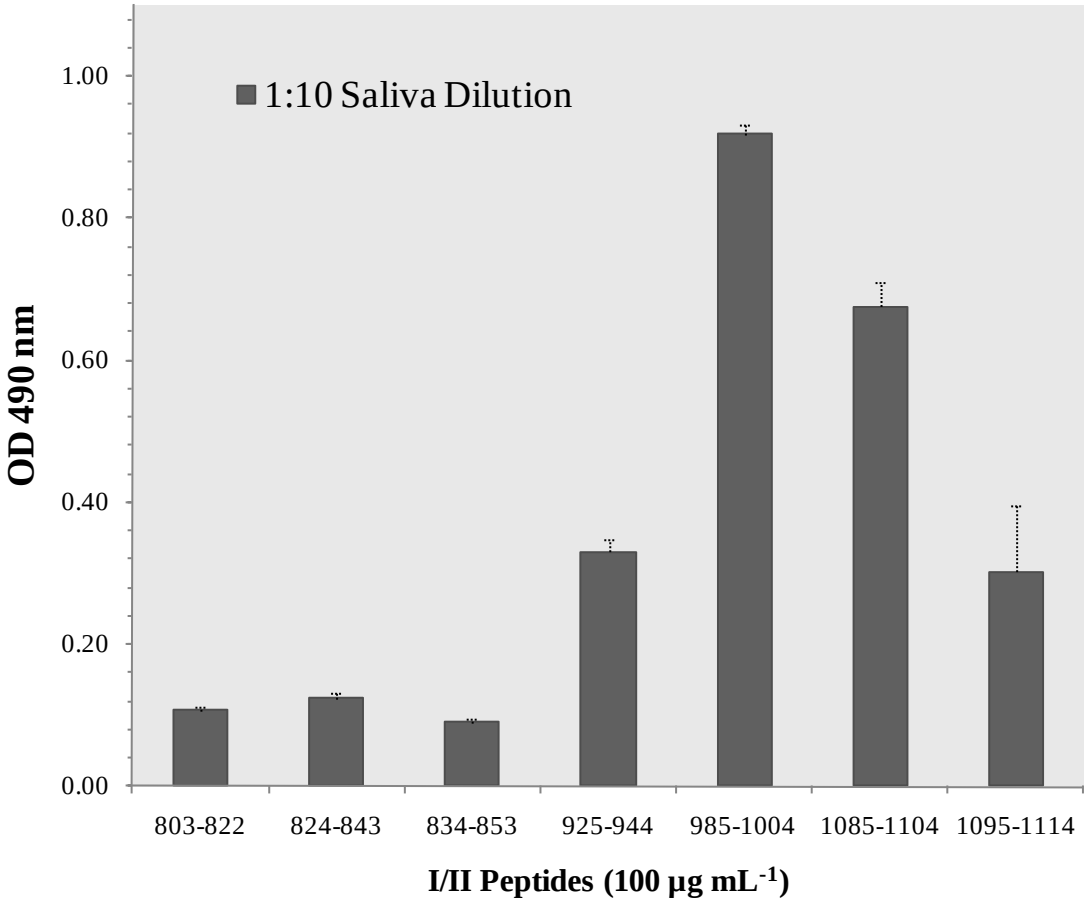

Figure 3. Human salivary IgA (from pooled samples, n=3) immunoreactivity to selected putative epitopes of an *S. mutans* adhesin, I/II. Reactivity for seven selected peptides (803-822, 824-843, 834-853, 925-944, 985-1004, 1085-1104, and 1095-1114; numbers reflect amino acid residues; 100 µg mL^{-1}) was determined in triplicate using ELISA, on low binding ELISA plates, and detected by a horse radish peroxidase (HRP)-labeled, anti-human IgA conjugate from goat (1:500 dilution in TS), and subsequent reaction with o-Phenylenediamine dihydrochloride (OPD). These data are reported in optical density absorbance (490 nm) as means and standard errors of the mean.

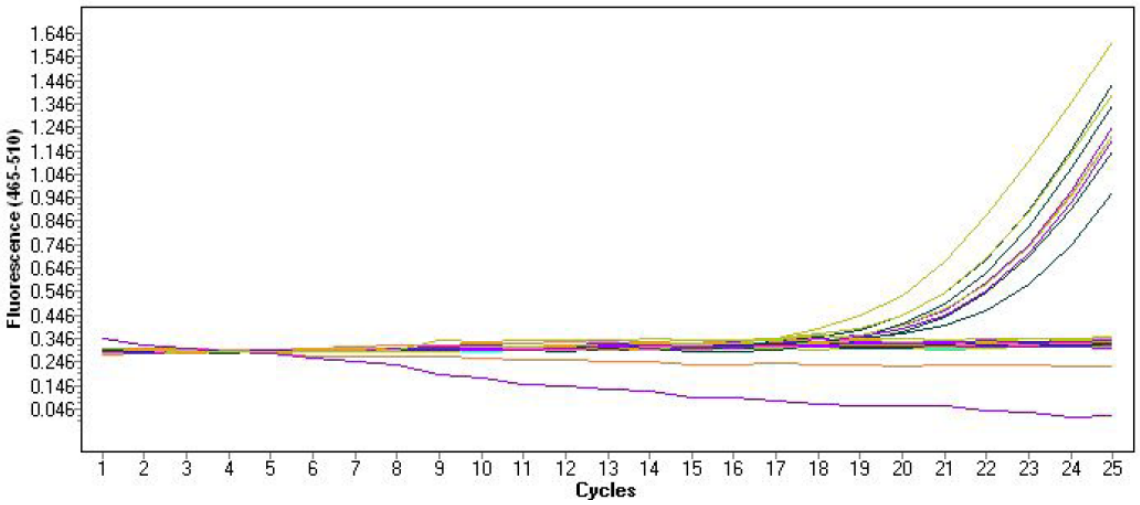

Figure 4. Selected amplification curves for Human HLA-DRB1*04 genotyping. Saliva samples were collected and stored in Oragene® DNA sample collection kits until DNA purification. DRB1*04 positive subjects (n=8) were determined by the presence of a fluorescence signal (exponential amplification curves) from HLA-DRB-specific fluorogenic probes, by polymerase chain reaction (PCR) for 25 cycles using the LightCycler ® 480 Real-Time PCR System. PCR products were analyzed at 465-510 nm. Three gold-standard cell lines were used as positive and negative controls, one being homozygous for the biomarker, the other heterozygous, and the third negative (see Materials and Methods). Negative subjects (n=17) are represented in the horizontal plane.

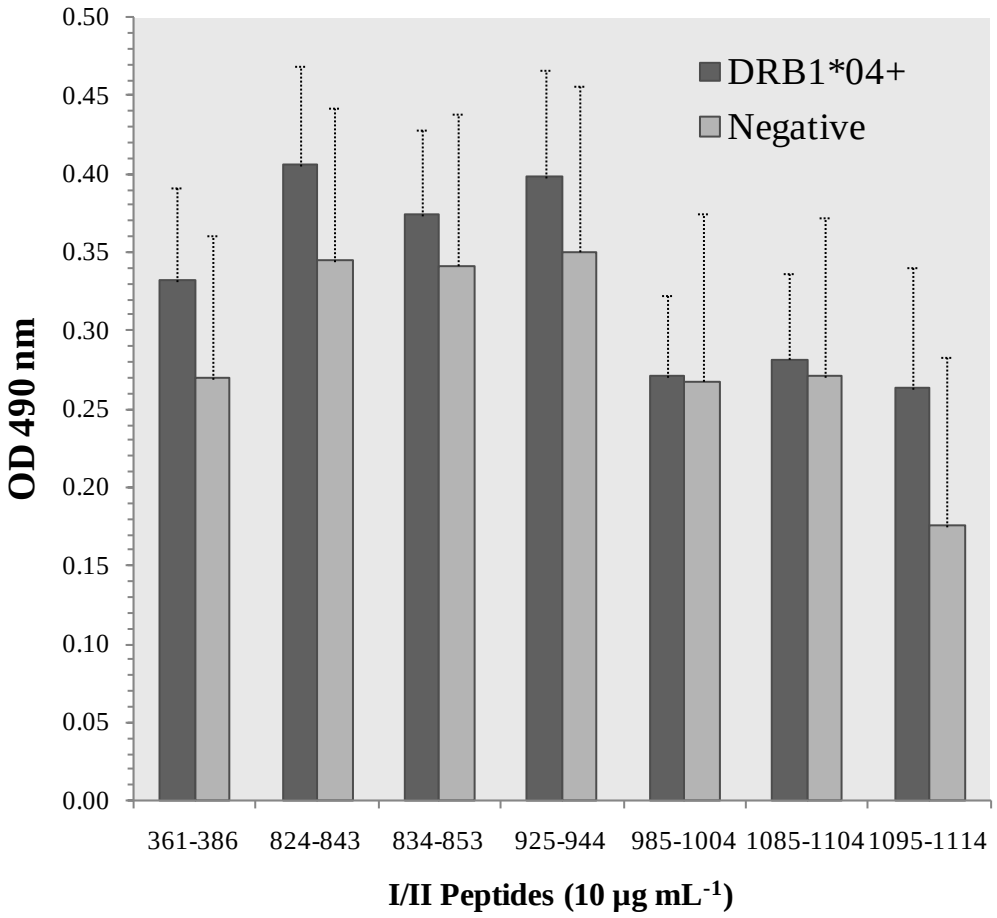

Figure 5. Human salivary IgA immunoreactivity of HLA-DRB1*04 positive (n=8) and negative subjects (n=7) to 7 of 10 selected putative epitopes of an *S. mutans* adhesin, I/II. Reactivity (1:10 saliva dilution in TS) to each selected peptide (361-386, 824-843, 834-853, 925-944, 985-1004, 1085-1104, and 1095-1114; numbers reflect amino acid residues; 10 µg mL^{-1}) was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (1:500 dilution in TS), and subsequent reaction with OPD. These data are reported in optical density absorbance (490 nm) as means and standard errors of the mean.

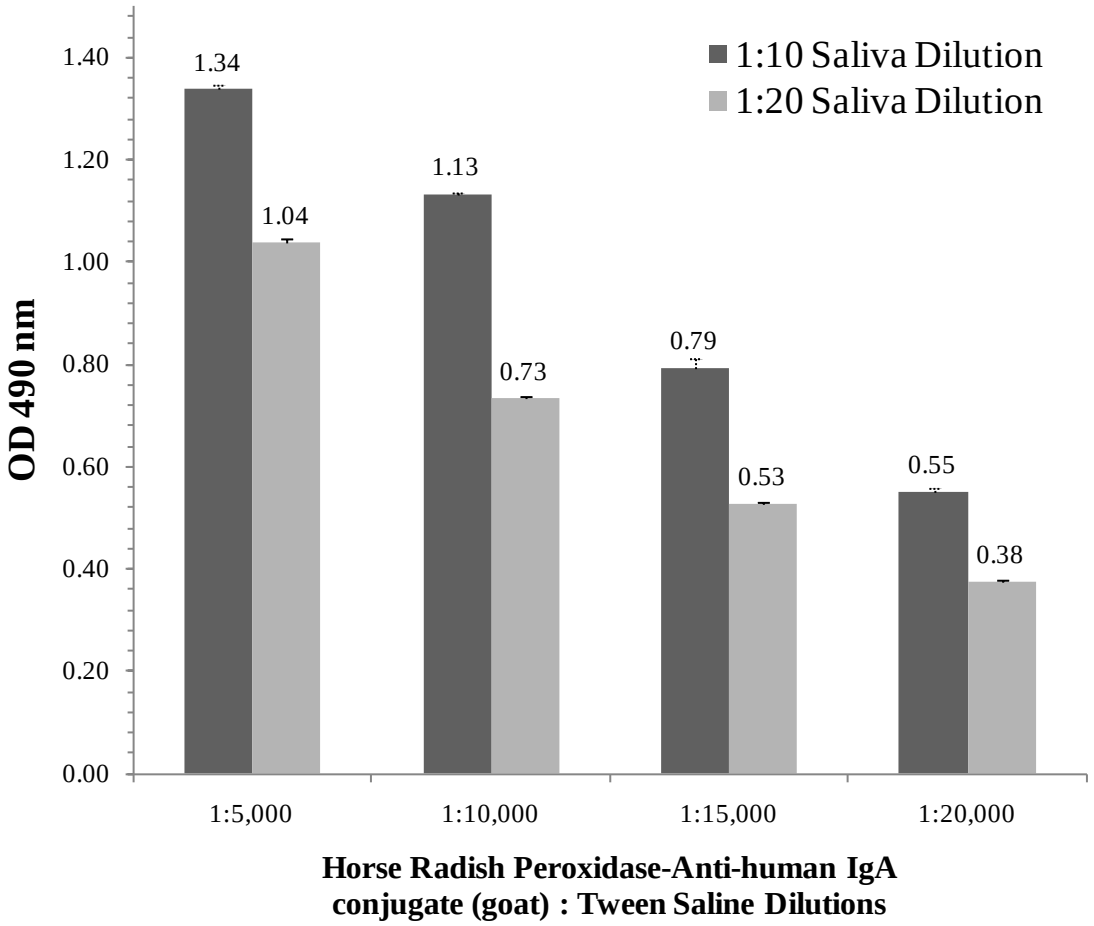

Figure 6. Optimization of human salivary IgA (pooled) immunoreactivity to one selected putative epitope of an *S. mutans* adhesin, I/II. Reactivity (1:10 and 1:20 saliva dilutions in TS) to the selected peptide (1085-1104; numbers reflect amino acid residues; 1 μg mL^{-1}) was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (at 4 separate dilutions: 1:5,000, 1:10,000, 1:15,000, and 1:20,000 in TS), and subsequent reaction with OPD. These data are reported in optical density absorbance (490 nm) as means and standard errors of the mean.

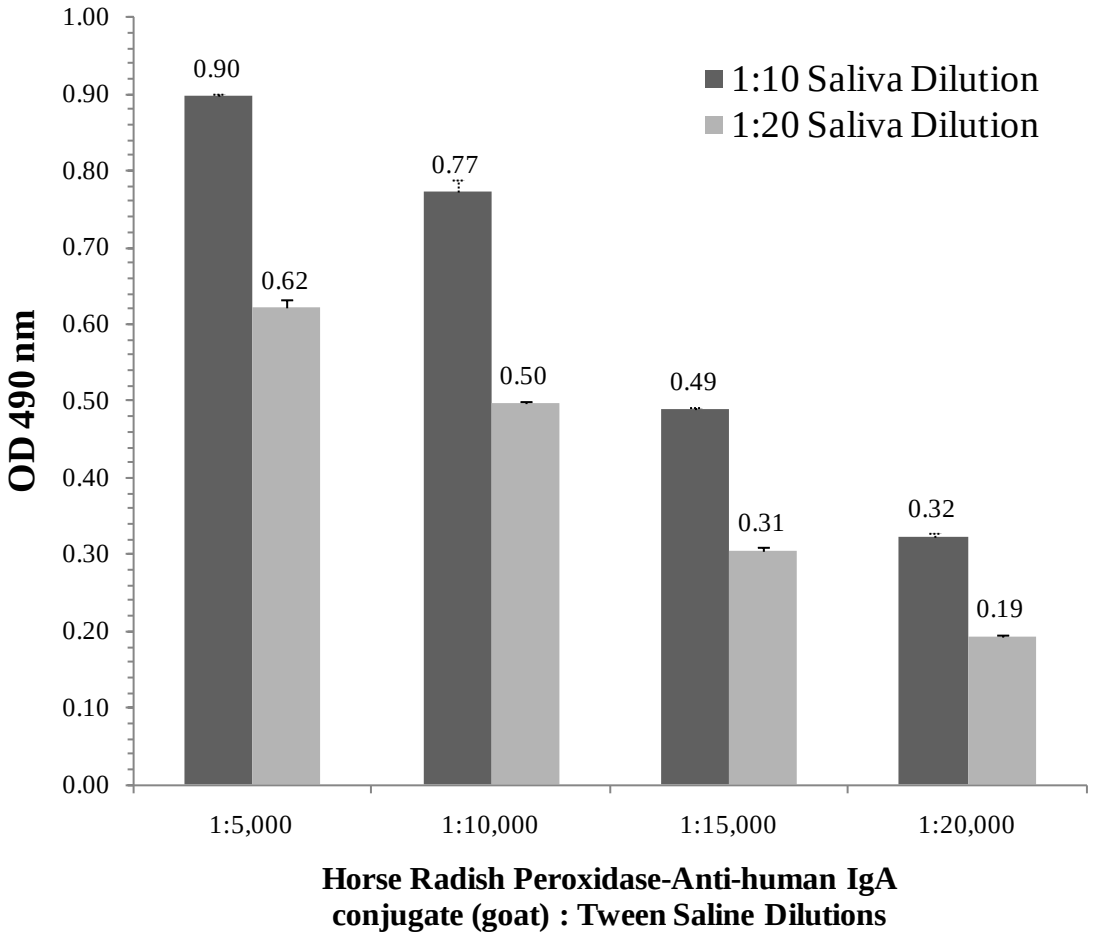

Figure 7. Optimization of human (unidentified subject #1) salivary IgA immunoreactivity to one selected putative epitope of an *S. mutans* adhesin, I/II. Reactivity (1:10 and 1:20 saliva dilutions in TS) to the selected peptide (1085-1104; numbers reflect amino acid residues; 1 μg mL^{-1}) was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (at 4 separate dilutions: 1:5,000, 1:10,000, 1:15,000, and 1:20,000 in TS), and subsequent reaction with OPD. These data are reported in optical density absorbance (490 nm) as means and standard errors of the mean.

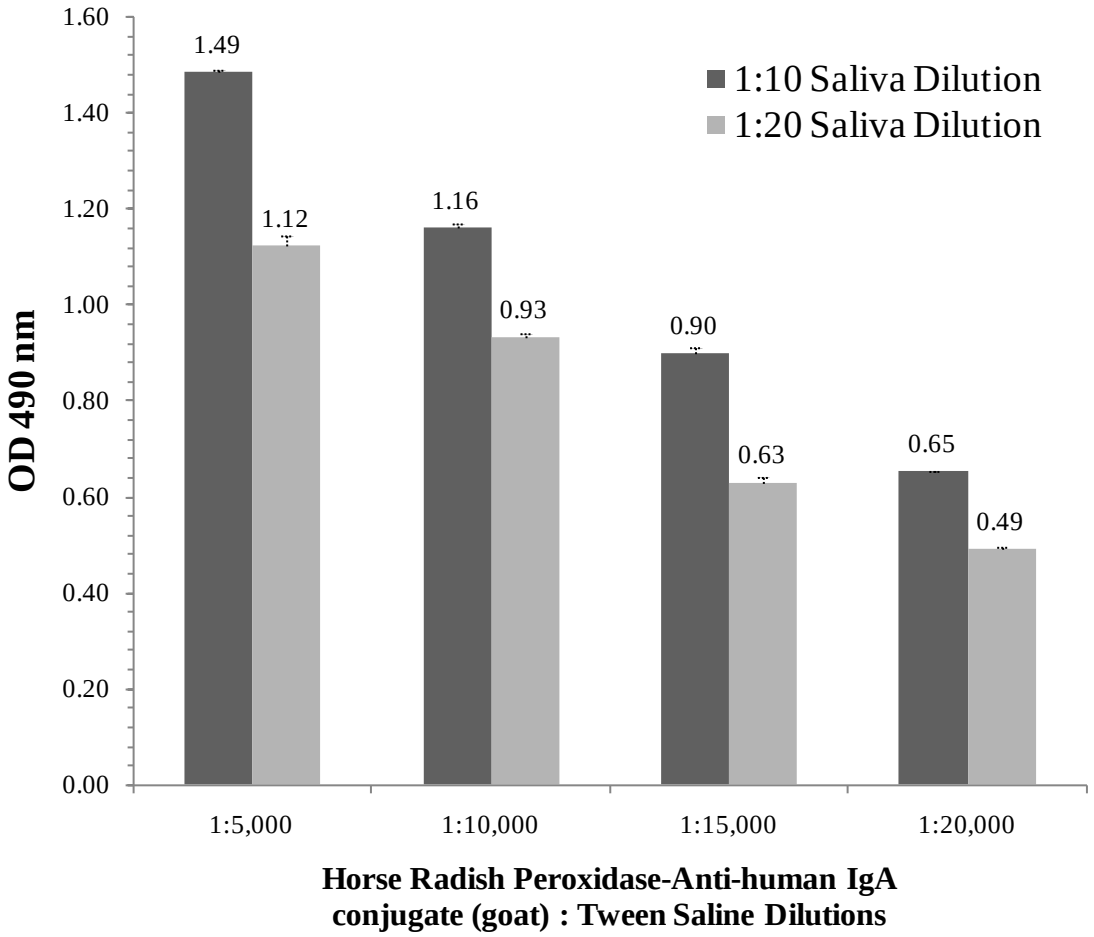

Figure 8. Optimization of human (unidentified subject #2) salivary IgA immunoreactivity to one selected putative epitope of an *S. mutans* adhesin, I/II. Reactivity (1:10 and 1:20 saliva dilutions in TS) to the selected peptide (1085-1104; numbers reflect amino acid residues; 1 μg mL^{-1}) was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (at 4 separate dilutions: 1:5,000, 1:10,000, 1:15,000, and 1:20,000 in TS), and subsequent reaction with OPD. These data are reported in optical density absorbance (490 nm) as means and standard errors of the mean.

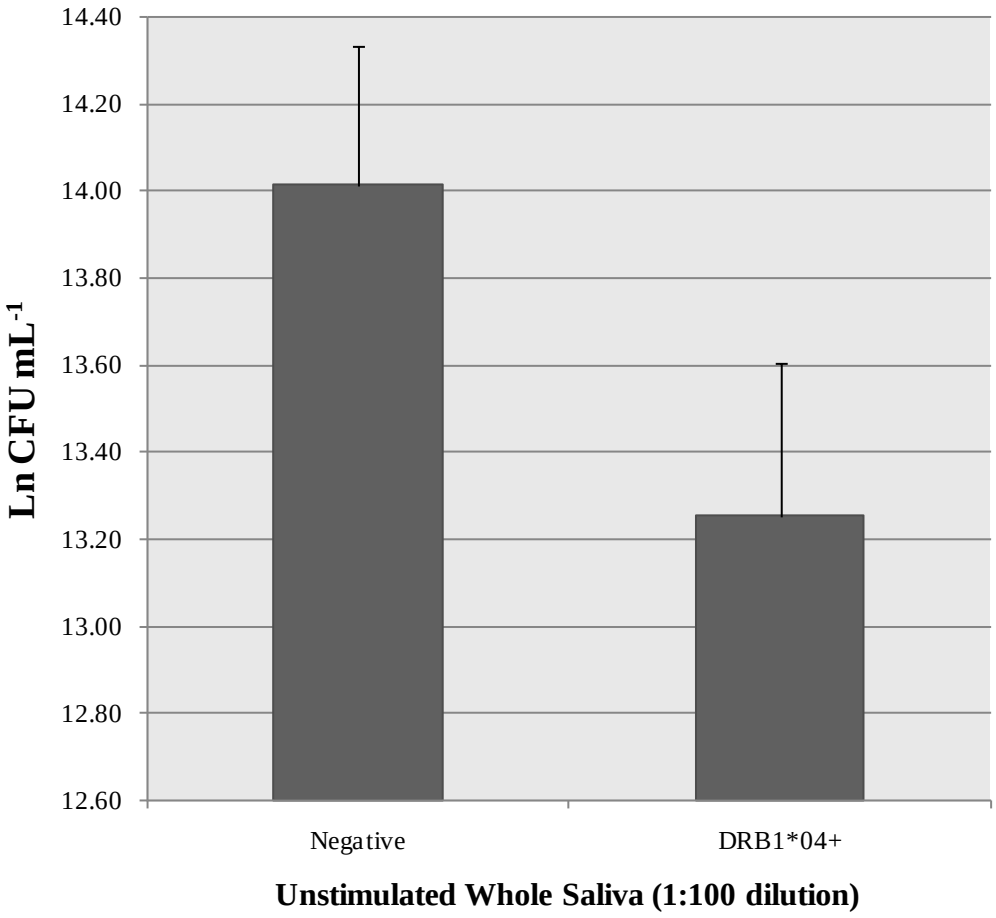

Figure 9. The log transformed number of *S. mutans* colony forming units mL^{-1} for DRB1*04 positive (n=16) and negative subjects (n=16). Aliquots of whole saliva (0.9 mL) were prepared with glycerin (0.2 mL), and were stored at -80°C until they were thawed for analysis. Each sample was diluted 1:100 in sterile saline, double-plated on Mitis Salivarius agar supplemented with bacitracin and sucrose (MSSB) (Anaerobe Systems, Morgan Hill, California). The plates were incubated for 48 hours at 37°C, 5 percent CO_2, and subsequently the number of *S. mutans* colonies enumerated using Protocol (Synoptic LTD, North Cambridge, UK), thus yielding *S. mutans* colony-forming units/mL (CFU mL^{-1}). These data are reported as natural log means and standard errors of the mean.

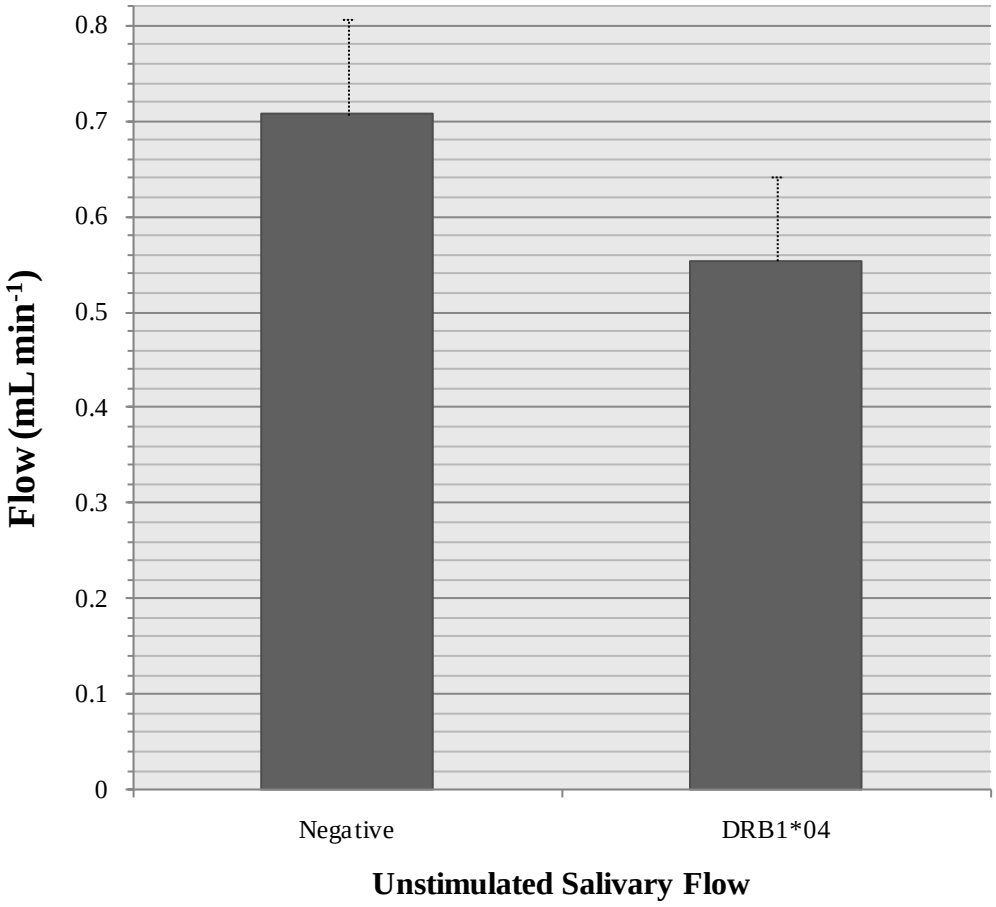

Figure 10. Salivary flow rate in mL min^{-1} for DRB1*04 positive (n=18) and negative subjects (n=25). Flow rate was calculated by taking the amount of saliva provided by each subject and dividing by the number of minutes it took them to yield that amount. These data are reported as means and standard errors of the mean.

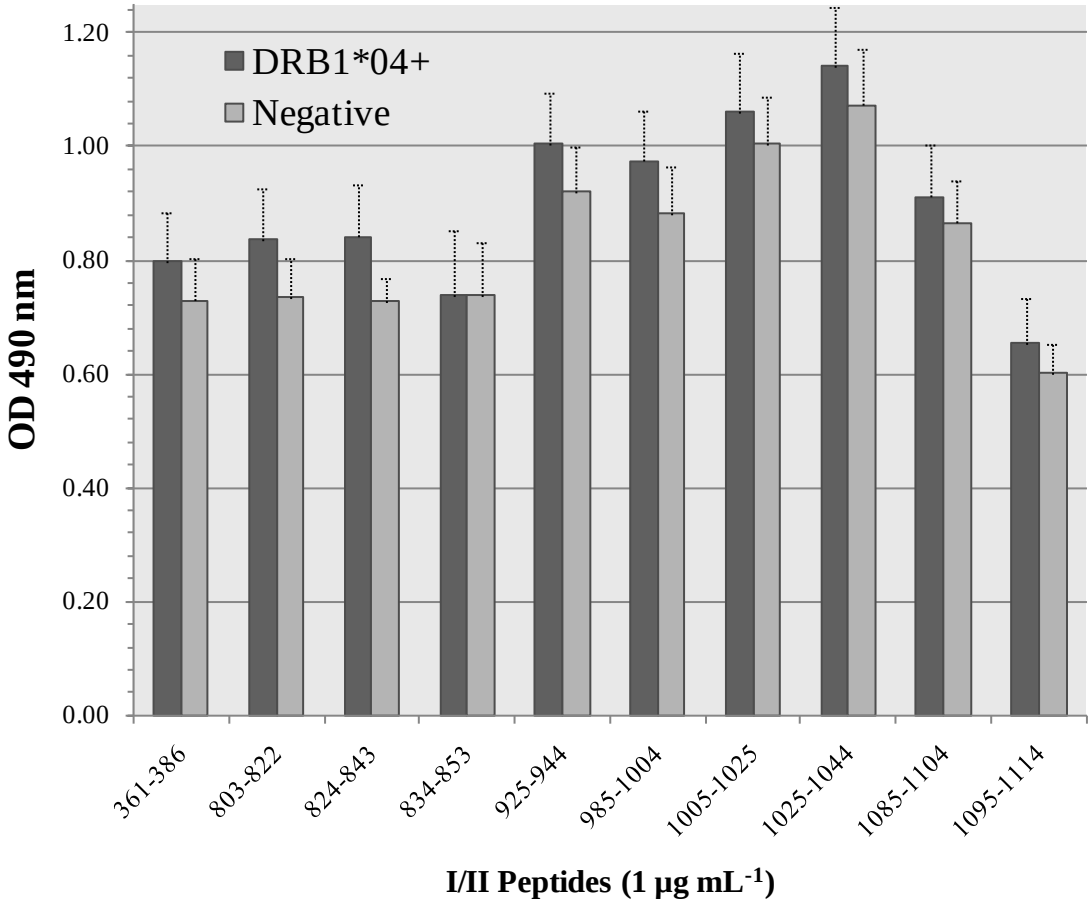

Figure 11. Human salivary IgA immunoreactivity of HLA-DRB1*04 positive (n=16) and negative subjects (n=16) to 10 selected putative epitopes of an *S. mutans* adhesin, I/II. Reactivity (1:10 saliva dilution in TS) to each selected peptide (361-386, 803-822, 824-843, 834-853, 925-944, 985-1004, 1005-1025, 1025-1044, 1085-1104, and 1095-1114; numbers reflect amino acid residues; 1 µg mL^{-1}) was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (1:10,000 dilution in TS), and subsequent reaction with OPD. These data are reported in optical density absorbance (490 nm) as means and standard errors of the mean.

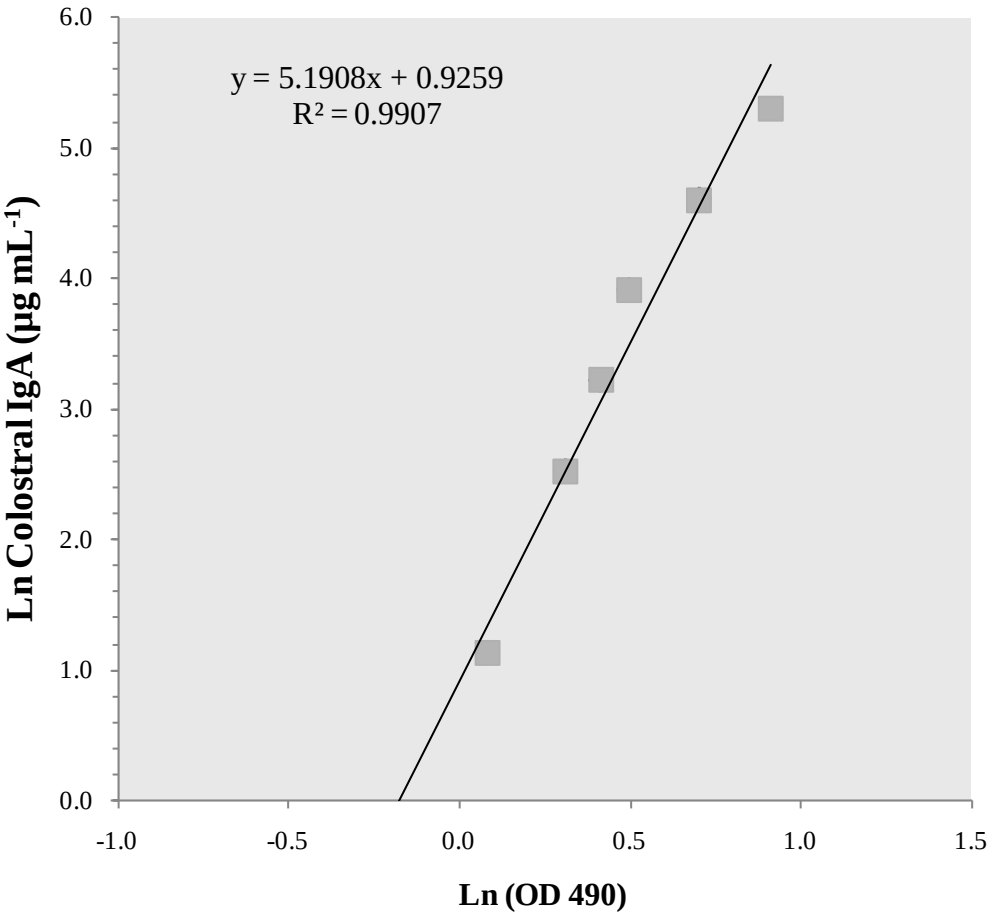

Figure 12. Standard curve fit to data points from standard concentrations of human colostral IgA, including 200, 100, 50, 25, 12.5, 3.1, and 0 µg mL^{-1}, respectively. Absorbance values are used to predict actual concentrations of total salivary IgA. Calculations were performed by using the standard curve equation from a log-log function (R^2 = 0.991), and then plugging each subject's reactivity (1:15 saliva dilution in TS) to unlabeled, human IgA into the function providing the correct concentration in µg mL^{-1}. The total salivary IgA concentrations in all 43 subjects ranged from 11.1-420.4 µg mL^{-1}, in agreement with recent data that demonstrated total salivary IgA concentrations range from 13.7-483.0 µg mL^{-1} in 134 healthy individuals (Booth et al., 2009).

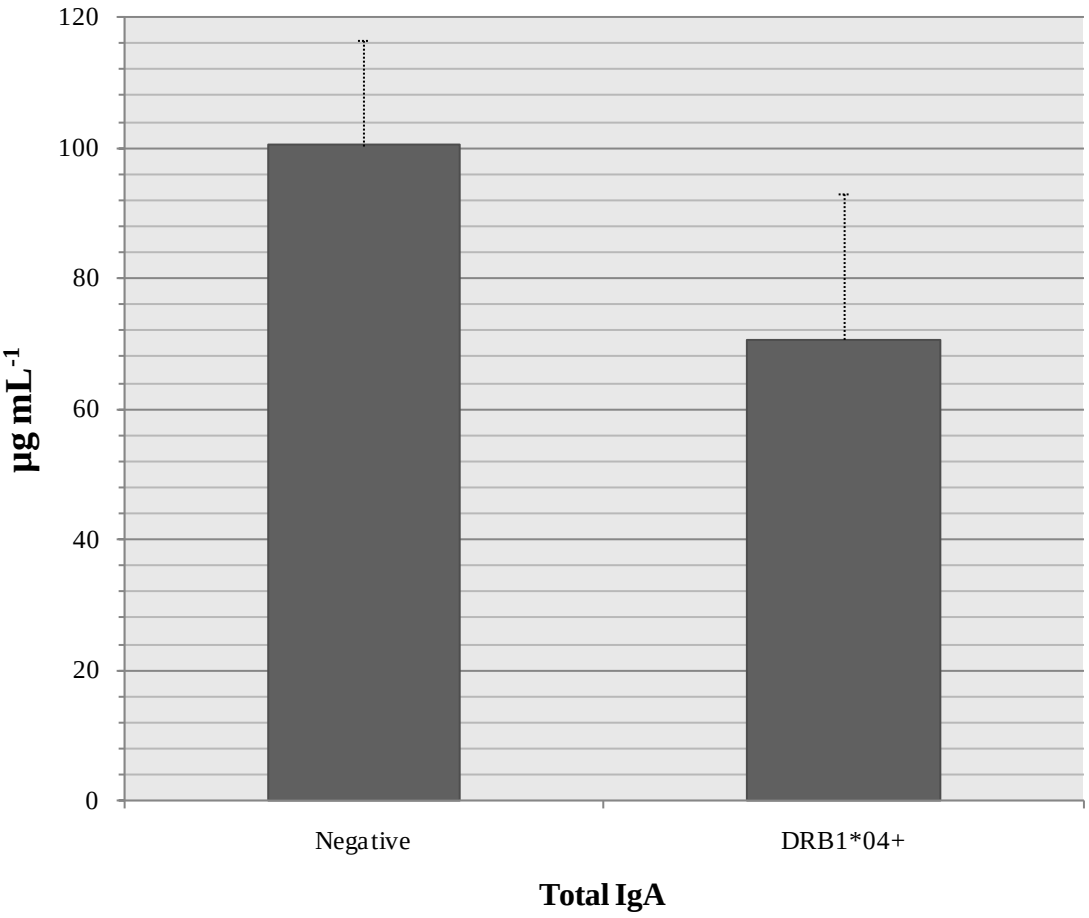

Figure 13. Human salivary total IgA among HLA-DRB1*04 positive (n=18) and negative subjects (n=25). Calculations were performed by creating a standard curve (see Fig. 12) by fitting data based on colostral IgA standard concentrations in a log-log function ($R^2 = 0.991$), and then plugging each subject's reactivity (1:15 saliva dilution in TS) to unlabeled, human IgA into the log-log function providing the value as illustrated in this figure. Reactivity was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (1:10,000 dilution in TS), and subsequent reaction with OPD. These data are reported as means and standard errors of the mean.

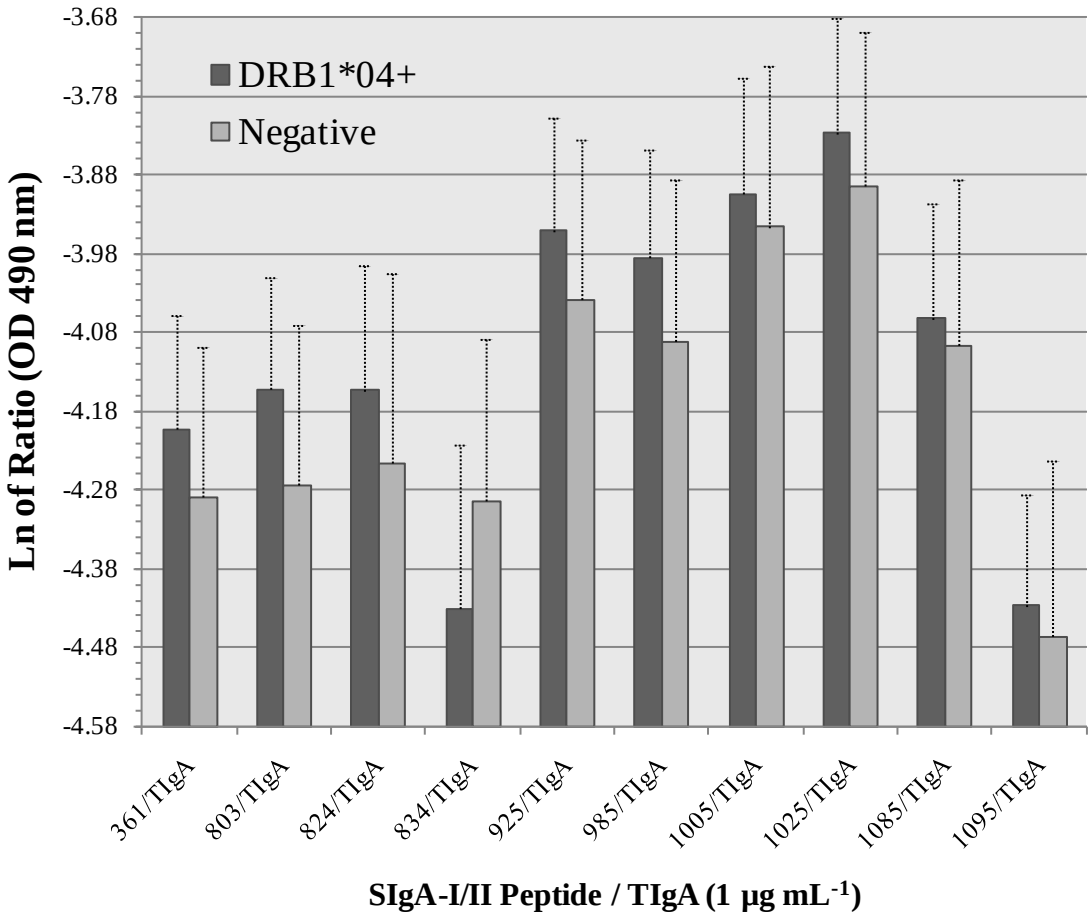

Figure 14. Human salivary IgA specific activity of HLA-DRB1*04 positive (n=16) and negative subjects (n=16) to 10 selected putative epitopes of an *S. mutans* adhesin, I/II. Specific activity was calculated using the natural log of the ratio of each subject's SIgA OD reading for each epitope to the total IgA OD for each subject. Reactivity (1:10 saliva dilution in TS) to each selected peptide was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (1:10,000 dilution in TS), and subsequent reaction with OPD. These data are reported in natural log values as means and standard errors of the mean.

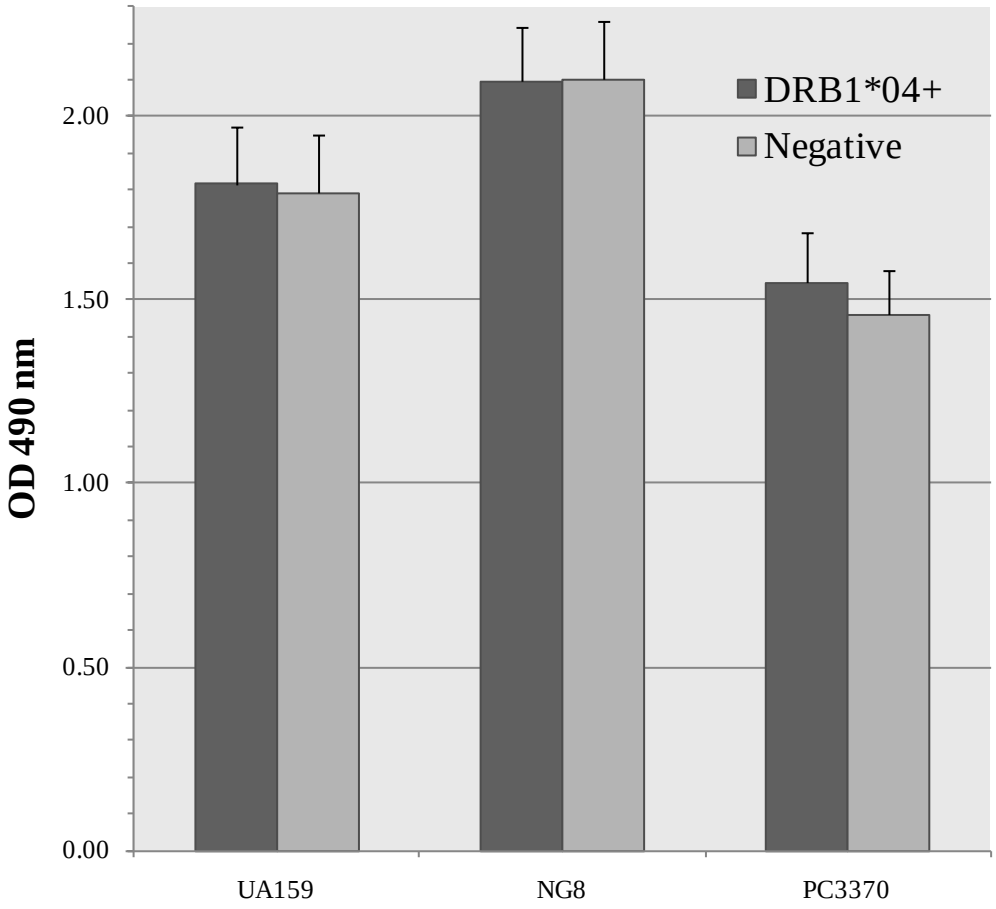

Figure 15. Human salivary IgA immunoreactivity of HLA-DRB1*04 positive (n=16) and negative subjects (n=16) to whole cells of *S. mutans* (UA159, NG8, and NG8-derived I/II-deficient, PC3370). Each strain was initially diluted in sodium carbonate/bicarbonate buffer until a 0.5 OD was achieved at 660 nm. Reactivity (1:4 saliva dilution in TS) to each strain was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (1:10,000 dilution in TS), and subsequent reaction with OPD. These data are reported in optical density absorbance (490 nm) as means and standard errors of the mean.

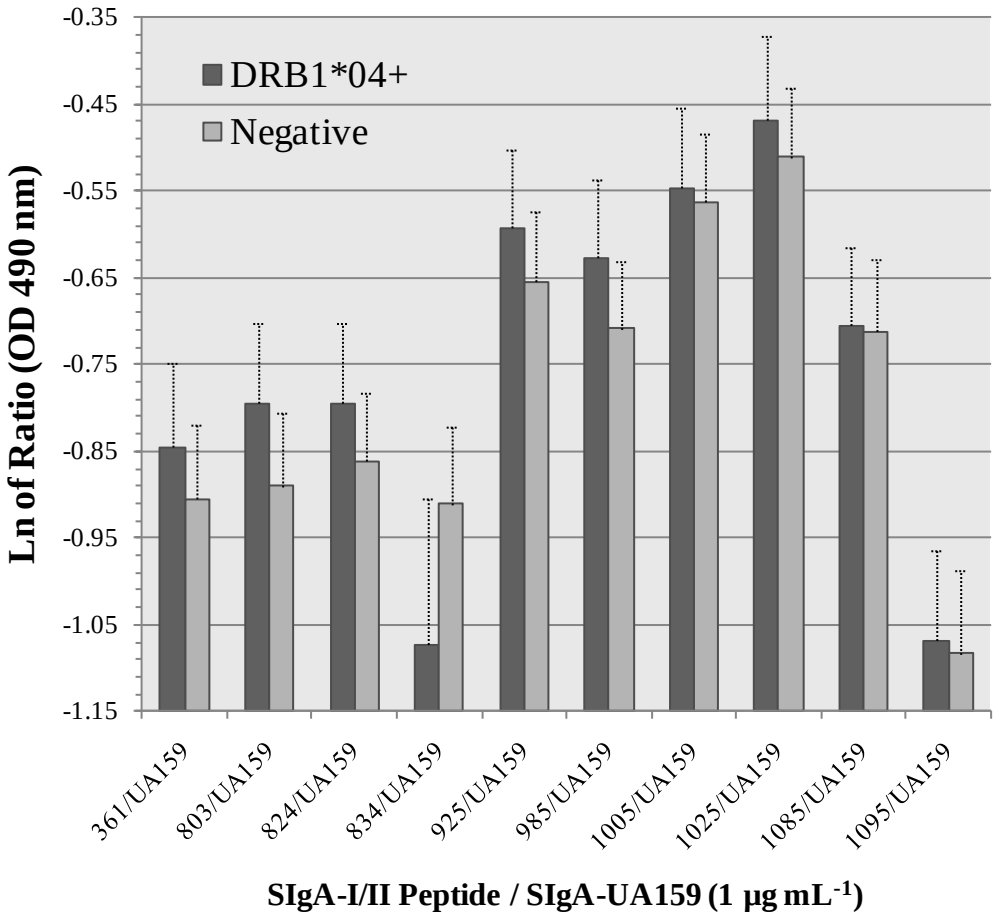

Figure 16. Human salivary IgA reactivity ratios of HLA-DRB1*04 positive (n=16) and negative subjects (n=16) to 10 selected putative epitopes of an *S. mutans* adhesin, I/II / whole cell *S. mutans* UA159 (UA159). Reactivity (1:10 saliva dilution in TS) to each selected peptide or to UA159 was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (1:10,000 dilution in TS), and subsequent reaction with OPD. These data are reported in natural log values as means and standard errors of the mean.

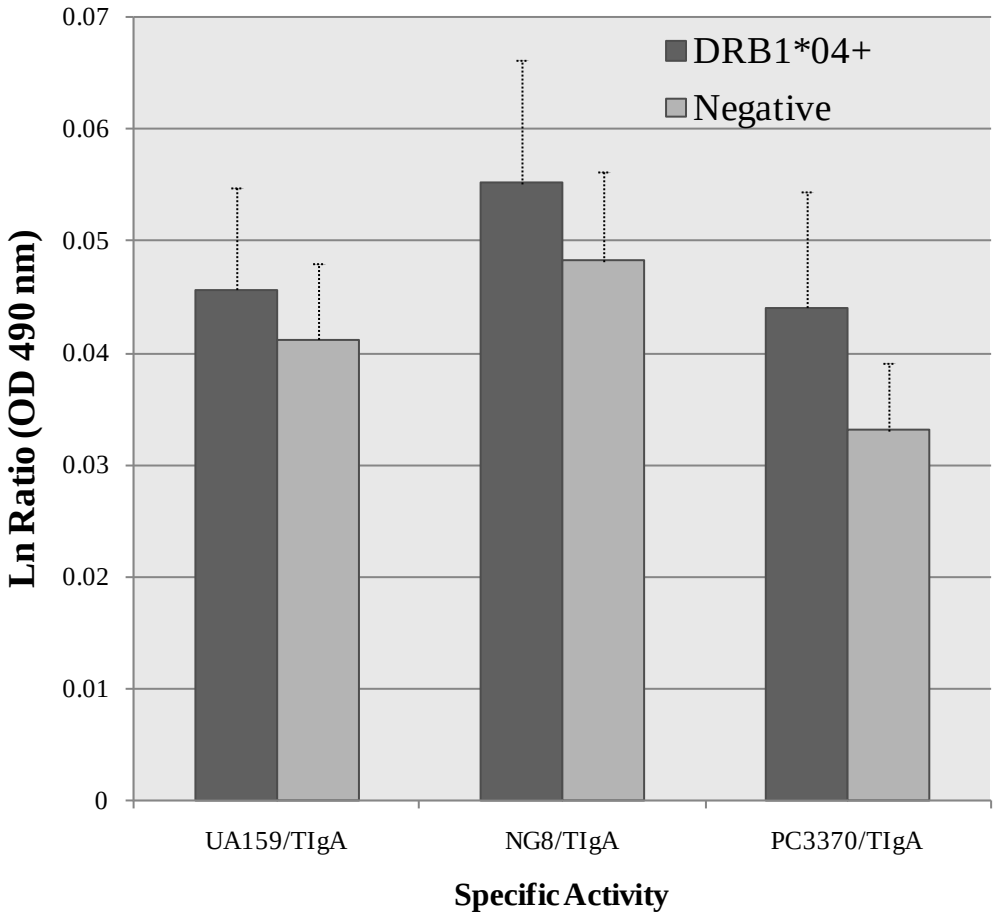

Figure 17. Human salivary IgA specific activity of HLA-DRB1*04 positive (n=18) and negative subjects (n=25) to three strains of whole cells of *S. mutans* (UA159, NG8, and NG8-derived I/II-deficient, PC3370). Specific activity was calculated using the natural log of the ratio of each subject's SIgA OD reading for each strain to the total IgA OD for each subject. Reactivity (1:10 saliva dilution in TS) to each strain was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (1:10,000 dilution in TS), and subsequent reaction with OPD. These data are reported in natural log values of optical density absorbance (490 nm) as means and standard errors of the mean.

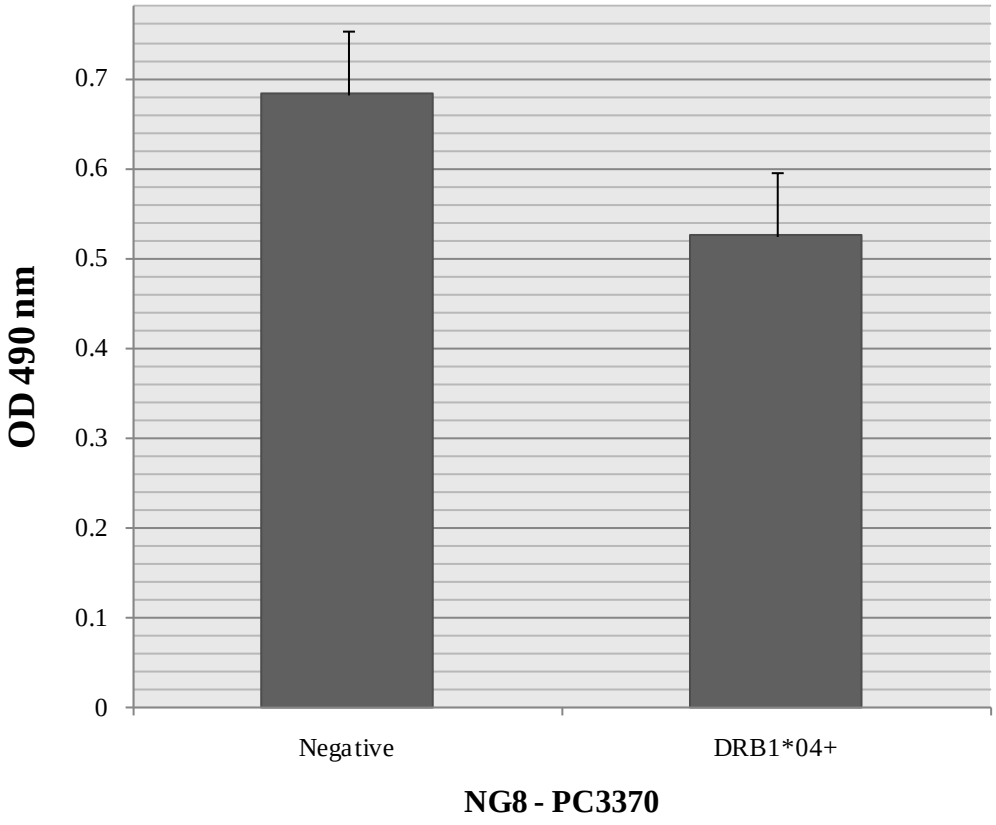

Figure 18. Human salivary IgA immunoreactivity to the entire I/II antigen on NG8 (indirect measure) in HLA-DRB1*04 positive (n=18) and negative (n=25) subjects. Reactivity (1:10 saliva dilution in TS) to NG8 and a I/II-deficient strain (PC3370) was determined in triplicate using ELISA, on high-binding ELISA plates, and detected by HRP-labeled, anti-human IgA (1:10,000 dilution in TS), and subsequent reaction with OPD. These data were calculated by subtracting OD 490 nm PC3370 values from NG8 values. Biologically, this means that in a simplified model the components of PC3370 (i.e., GTFs, and GBPs) are subtracted from all the components of NG8 (i.e., I/II, GTFs, and GBPs), thereby leaving only I/II to examine. These data are reported in optical density absorbance (490 nm) as means and standard errors of the mean.

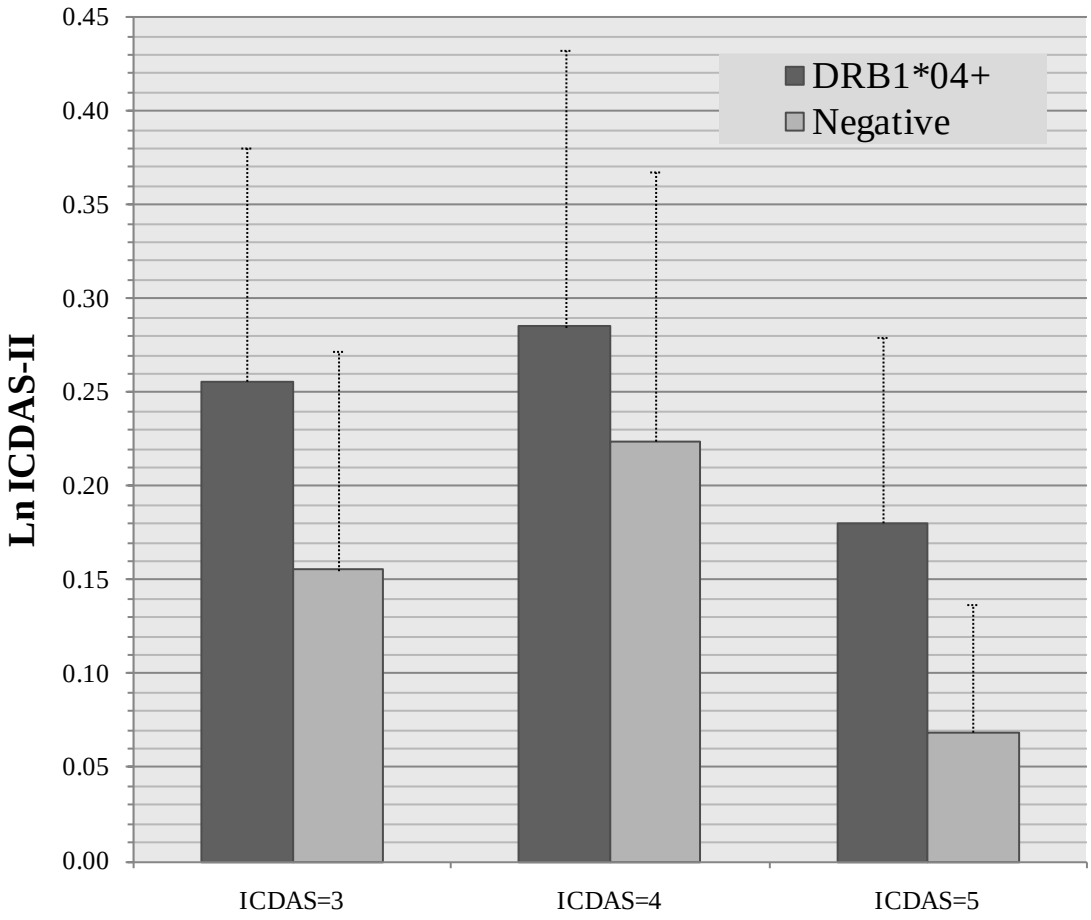

Figure 19. The HLA-DRB1*04 positive subjects (n=16) exhibit numerically, but not statistically significant, increased numbers of irreversible ICDAS-II scores compared to a negative group (n=16; see Materials and Methods for criteria details). Raw scores were natural log transformed. These data are reported in natural log values as means and standard errors of the mean.

CHAPTER FIVE

Discussion

Previous literature, as reviewed in chapter one, has suggested that HLA-DRB1*04 appears to be a caries susceptibility allele in Caucasian populations. These results do not disprove this notion, but argue for a more nuanced and complicated landscape regarding the immunogenetics of caries. Given the sample size in both of the current clinical studies (n=15) and (n=43, unpaired; n=32, paired), it was not anticipated to uncover any statistically significant results at the clinical level although trends were expected. Rather, power analyses were based on the primary outcome of SIgA reactivity to selected epitopes, which had not previously been studied in this context.

Clinically, the results confirmed that the sample size was too low for any major statistically significant clinical finding, however an emergent trend was also absent. Nonetheless, compared to their non-biomarker counterparts (n=25, unpaired; n=16, paired) DRB1*04 positive subjects (n=18, unpaired; n=16, paired) exhibited a statistically significant difference ($p < 0.01$, unpaired; $p < 0.01$, paired) with regard to the ratio of the number of irreversible lesions to the number of reversible lesions (# ICDAS>=3 / # ICDAS=1 or 2), where higher ratio values may suggest a greater progression of the irreversible caries process (table 6). Nonetheless, when adding other past caries experience measures to these ratios, the groups became indistinguishable from each other (table 6).

This is an interesting finding since DRB1*04 positive subjects exhibit a trend toward lower TIgA and lower *S. mutans* counts, both of which would lend themselves to an increase in the caries process. Instead, the DRB1*04 positive subjects in this study seemed to compensate (at least by staying even with their negative counterparts in terms of the number of lesions reported) somehow in

the face of these putative deficiencies, at least with regard to past experience—in comparison to their negative counterparts.

It is important at this juncture to briefly mention ICDAS-II, a novel approach to detect and assess caries. This system for detecting and assessing caries was developed by an ad hoc international committee that sought to create a unified visual system, with histological support, that researchers and practitioners alike could use, where data could be compared no matter the locale or point in time. The goal is that data will not only be comparable but also compatible and more easily shared the world over. The ICDAS-II "system is practical [and] has content validity, correlational validity, [as well as] discriminatory validity" (Ismail et al., 2007). Its reliability "has been found to be in the range of good to excellent (kappa coefficients range between 0.59 and 0.82)." Furthermore, researchers have demonstrated that the ICDAS-II non-cavitated "diagnostic threshold specificity was 0.74–0.91 and sensitivity was 0.59–0.73" (Jablonski-Momeni et al., 2008). The "ICDAS-II system demonstrates reproducibility and diagnostic accuracy for the detection of occlusal caries at varying stages of the disease process," and provides the added bonus of high validity and reliability compared to other caries measures. Disadvantages of this system involve its activity scale as it is not well validated. Additionally, interproximal lesions cannot be visualized. Nonetheless, it is as good or better than any other comprehensive caries lesion measurement criteria (Rodrigues et al., 2008).

With regard to I/II epitopes, the results shown here do not fully align with the hypothesis but are not inexplicable. In every case, except for one, DRB1*04 positive subjects exhibit a weak trend toward higher SIgA reactivity than their non-biomarker counterparts. At the SIgA level, this is contrary to the hypothesis, but appears to be the case though none of these data reach statistical significance.

Nonetheless, it is certainly possible that DRB1*04 positive subjects could nonetheless exhibit an increase in the irreversible stages of the caries process since there are many more epitopes of I/II than what was measured, and there are more adhesins involved in cariogenic colonization of *S. mutans* than simply I/II. It may be that the epitopes involved in this study have little to do with I/II attachment and virulence, or are only components of immunogenic discontinuous epitopes that must function together. In fact, data suggest that DRB1*04 positive subjects exhibit a lower SIgA response to I/II of *S. mutans* NG8 compared to their non-biomarker counterparts (Fig. 18). This further suggests that I/II is still important but that the selected epitopes do not tell the whole story. Moreover, both GTFs and GBPs are also important. Researchers have hypothesized that HLA-II alleles produce the differences in immunological responses observed to GbpB immunogenic epitopes (Nogueira et al., 2005). Nevertheless, the previous results demonstrate that DRB1*04 positive subjects are slightly higher responders to a I/II-deficient strain (PC3370) of *S. mutans*, as well as to the I/II replete NG8 and UA159 strains (Figs. 15 & 17), meaning that these subjects are immunologically competent to GBPs and other adhesins, compared to these negative counterparts. Additionally, by subtracting the I/II-deficient strain's reactivity from NG8's activity, an elegant indirect measure of reactivity to the entire I/II antigen is created, demonstrating that I/II is where DRB1*04 positive subjects may exhibit a lower immune response (Fig. 18). This is in agreement with a previous study where two DRB1*04 allelic variants demonstrated lower reactivity to SIgA than their negative counterparts to the entire I/II antigen (Wallengren et al., 2005). Unfortunately, a purified sample of the entire sequence of I/II was not available. This measure is especially helpful since I/II in its entirety is notoriously unstable and degrades rapidly in vitro; thus it it very difficult to study in isolation as a whole.

Continuing this theme, it is interesting to note that of all 10 epitopes assayed, one epitope (834-853; which straddles the carboxy-terminus of the V region and encompasses most of the P1 region) demonstrated the opposite trend across all measures (Figs. 11, 14, 16), a trend that nicely illustrates the original hypothesis. More interesting is that this epitope, along with one other (1085-1104; in the C-terminal domain), was originally identified in the first laboratory study, using pooled saliva, as being most important after normalizing those data (data not shown). These results regarding 834-853 support the initial hypothesis. Taking into account those data demonstrating lower reactivity to the entire I/II antigen, the evidence suggests that 834-853 may be one part of a discontinuous epitope involved in attachment to the acquired salivary pellicle on the tooth surface. There is new data which suggests that this may be the case.

Although the A and P regions have been known to be important as regions that interact and serve as docking sites for discontinuous epitopes, the variable region is now known to also be very important (Larson et al., 2010). Researchers have suggested that I/II presents a lectin-like (i.e., carbohydrate-binding) domain in the middle of the V region important in bacterial adhesion (Troffer-Charlier et al., 2002); for example, in binding fibronectin (Jakubovics et al., 2009) or "collagen, laminin, keratin, fibrinogen and other oral microorganisms" (McArthur et al., 2007). It is very interesting to note that 834-853 encompasses the very residues (interacting with 464-471, which were not examined in these studies) that make up the distal arm of the V region (the reactive part of the arm, which is made up of proximal and distal regions) of what has recently been called a hinge-like area of I/II, which interfaces with all three major regions (A, V, and P) of I/II and supports the flexure of what is now known to make up most of the length of I/II, the stalk (Larson et al., 2010). Not only does the distal arm provide flexing in unison by the A, V, and P regions, but

it is also involved in governing intermolecular interactions among the backbone of I/II such as solidifying the proximal arm's hydrophobicity, which is locked interiorly by hydrogen bonding and salt-bridges (Troffer-Charlier et al., 2002). The backbone of the V region is very important and features an unmistakable crevice, which is a calcium-dependent preformed binding site whose every feature suggests it is a carbohydrate-binding cleft that undergoes little conformational change in the face of ligand binding. Water and calcium appear to also function as "direct sugar ligands." Even more, the V region exhibits sequence homology with *S. mutans* GbpC, which of course can bind glucan that is synthesized by GTFs and aid the development of the biofilm. Nevertheless, it is clear that the V region does not act alone but rather functions in tandem via its distal arm (serving as a sort of center for functionality) with its sister domains.

In fact, it is the three-dimensional interaction of the A, V, and P regions that predominantly affects *S. mutans* attachment to a dental pellicle protein, gp340, alternatively called salivary agglutinin (SAG) or DMBT1 (deleted in malignant brain tumors 1). Human gp340 belongs to a family of innate immune proteins that are characterized by scavenger receptor cysteine-rich (SRCR) proteins. There are SRCR domains along the entire length of gp340, which serve as the binding sites for the specific arrangement of particular sections of the A, V, and P regions within I/II. When in the planktonic form, SRCRs provide gp340 the ability to serve as a pattern recognition receptor (Loimaranta et al., 2009). Thus, gp340 "promotes bacterial aggregation and clearance" (Nobbs et al., 2009) when it encounters *S. mutans* planktonically. However, when the glycoprotein is embedded in the tooth pellicle or on the epithelium or on microbes themselves (i.e., immobilized in dental biofilm), it instead serves as a receptor for streptococcal attachment (Jakubovics et al., 2005).

S. mutans I/II was recently demonstrated to be a singular protein, very different from what has been heretofore understood about other proteins in nature. Until 2 months ago, a hybrid structure of α-helices and polyproline type II (PPII) helices intimately associated with each other was unknown (Larson et al., 2010). Nonetheless, this is the case for I/II. Proline-rich areas are ubiquitous in functionally important proteins throughout nature (Jin et al., 2009). Many proline-rich regions, such as the P region within I/II, exhibit repeated motifs, which form "left-handed polyproline II helical conformations (PPII)." PPII helices are more flexible than other regular structures such as α-helices or β-sheets, but are nonetheless solid due to the "rigidity of the proline ring," echoing what was previously said about the functionality of the distal arm of the V region, which is also linked and a part of the amino terminus of the P region, whose existence provides a hinge-region allowing the I/II stalk to flex as a unit and yet maintain a V region hydrophobic core that is locked in place.

Larson et al. recently reported the entirely unique (the first of its kind) α-helical structure intimately conjoined with PPII helices:

> Here we report a high-resolution (1.8 Å) crystal structure of the A_3VP_1 fragment of *S. mutans* AgI/II that demonstrates a unique fibrillar structure (155 Å) formed by the intimate association of two widely separated segments within the primary sequence. The A_3 repeat of the alanine-rich domain was found to adopt a long α-helical structure that intertwines with the P_1 repeat polyproline type II (PPII) helix to form a highly extended stalk. . . . [There is] a high-affinity interaction between the α- and PPII helices. . . . together the three A/P repeat units account for the majority of the length of AgI/II. [We] identified two distinct binding sites on AgI/II for it's host receptor [gp340]. Finally, we propose a model for AgI/II's interaction with [gp340]. . . . It is now apparent that AgI/II is a highly extended fibrillar structure formed by a long and continuous A–P association. This architecture would position the globular V region at the tip of the stalk away from the cell surface,

with the C-terminal region positioned near the cell surface and the pre-A region in close proximity (Larson et al., 2010).

What insight does this new found structure provide? For one, it demonstrates that A3 and P1 (and V) interactions, of which 834-853 is crucially involved and center stage, serve as a primary gp340 binding domain. It also demonstrates that a secondary gp340 binding site is located in the C-terminal domain, meaning that after the globular V region (coupled with the amino terminus of the P region and the carboxy terminus of the A region) initially adheres to the salivary pellicle, the distal arm can flex to such a degree that the C-terminal domain, which abuts the anchor motif on *S. mutans* itself, can actually bind gp340 as well. Thus, if host SIgA sufficiently responds to this very particular region, I/II will not be able to take advantage of these binding sites and the process of caries will be abated or slowed. Alternatively, if there were low SIgA responses to this particular epitope located in the distal arm region, it would follow that I/II could take full advantage of its array of virulent mechanisms, which fits the hypothesis with regard to the DRB1*04 positive group and the epitope found at 834-853. That the negative group may exhibit a better ability to naturally fight the disease while still in the reversible stage is supported by the fact of higher reactivity to NG8 and a higher 834-853 epitope response.

Active oral immunization with antigen I/II in rats produces an increase in specific SIgA antibody and a concomitant decrease in *S. mutans* thus protecting against caries (no statistical difference in *S. mutans* was seen in the paired study), while systemic active immunization in monkeys induces specific serum IgG antibodies that also inhibit *S. mutans* colonization and inhibit the development of caries by expressing themselves on the tooth via the gingival crevicular fluid (Lehner et al., 1989; Ma et al., 1990). One reason why inducing these antibodies

inhibits caries is that SIgA antibody apparently binds at critical points along I/II, probably especially in the distal arm region, thus depriving *S. mutans* of its main adhesin, which normally facilitates attachment to the tooth surface. Since the goal of any type of immunization against caries is blocking bacterial adhesion, and thus preventing lesion formation (Fontana et al., 2000), pursuing this path is one way forward. Active immunization in these animal models clearly shows protection against caries.

But local passive immunization has been touted as less immunogenic and avoids the untoward consequences of a systemic immune response. One study showed that of two groups of rhesus monkeys exposed to a human-type diet, none that were immunized with IgG (i.e., the experimental group) developed caries over a one-year period (Lehner et al., 1985). Those who were not immunized (i.e., the control group), not only developed caries, but did so irrespective of whether the surface was smooth or fissured. The working hypothesis of how this happens is that antibodies interact with specific epitopes of I/II, perhaps the distal arm of the V region, priming *S. mutans* to become opsonized, phagocytosed, and destroyed by gingival neutrophils. An article in *Nature Medicine* used this approach in humans but synthesized SIgA antibodies from plant-derived material, which theoretically rendered the foreign antibodies even safer and more serologically compatible (Ma et al., 1998). In this work, four months after passive immunization with the plant antibodies the foreign salivary IgA significantly decreased microbial colonization.

Ultimately, however, how antibodies relate to their HLA gene encoding counterparts remains to be explored. As these fundamental mechanisms become better understood in the long-term, our ability to design vaccines will not only increase but also enable us to individually tailor immunizations.

What this means at the host level is that the DRB1*04 positive group may have some sort of decreased immune response with regard to clearing the infection by mounting an appropriate immune response, whereas the negative group is more immunocompetent. These findings diverge in some respects with previous findings, two of which are here mentioned, where a serologically determined DR4 positive group was related to high DMFS scores and a negative group was not (Lehner et al., 1981), and DRB1*04 positive subjects younger than 5 years of age were 10 times more likely to develop early childhood caries (ECC; defined as "one or more decayed (noncavitated or cavitated), missing (due to caries), or filled tooth surfaces in any primary tooth in a child 71 months of age or younger.") than their negative counterparts (Bagherian et al., 2008).

These studies have been interesting, but there is much more to uncover with regard to the biological aspects of caries. One could imagine that the genetic portion of this disease is something like 30 percent or higher, and that the environmental component is something like 70 percent. Yet, until recently little was known regarding the proportion of the caries disease process that is specifically affected by genetics, or what proportion environment contributes to the disease. In robust samples in twin populations, recent studies have demonstrated upwards of 50 percent of caries variability is explained by genetic factors (Bretz et al., 2005). Family studies, in contrast to twin studies, are better predictors of genetic contributions since they greatly minimize non-genetic confounding contributions to the disease. Very recently, the largest (n=2,600; 740 families), most well-designed caries heritability study ever completed was published (Wang et al., 2010). The authors found that "caries phenotypes in the primary dentition were highly heritable, with genes accounting for 54–70% of variation in caries scores. The heritability of caries scores in the permanent dentition was also substantial (35–55%, all $p < 0.01$)." It is important know why

exactly family studies are better at eliminating confounding environmental variables compared to twin studies. In the words of the authors:

> [The] strength of our family study design is that, compared to twin studies, it is less susceptible to inflated heritability estimates caused by non-genetic familial factors. This is because twins (including dizygotic twins) are likely to share unmeasured environmental and behavior exposures that track with kinship coefficient (degree of relatedness), whereas inclusion of more distant relatives, such as half-siblings raised separately, cousins, and parent-offspring pairs (reared at different times and in different environments), help insulate heritability estimates from the effects of familial non-genetic exposures.

This article demonstrated that in children genetic factors may account for upwards of 70 percent of the variation in caries scores, while in adults the contribution decreases to as low as 35 percent (but as high as 55 percent).

Nonetheless, it has long been known that caries is also affected in large part by exogenous variables, such as oral hygiene, diet, and fluoride exposure, as well as psycho-social factors, all of which may change over time in different ways. For example, a child's level of oral hygiene is more dependent on parents than the child, but could change dramatically as the child becomes older and cognizant of why it is important to brush. An example of how diet could change could be a college student who never has time or money to prepare nutrient dense foods that his or her parents prepared up until college began. Moreover, if this student had genes leading to an increased sucrose sweetness preference (Bretz et al., 2006; Fushan et al., 2009), then 4 years in college could completely alter caries experience. Fluoride exposure could change if someone moved from a fluoridated area to an area whose water supply was unfluoridated. Finally, depression and anxiety are two psychological factors that may lead people to value oral health much less leading to deleterious oral health outcomes. On the

other hand, cultural or social norms could lead some to become obsessive about oral health or others to disbelieve the education they receive from oral health care professionals. Interestingly, in children, sex and age explain very little of the variation in caries experience, yet a significant proportion in adults (Wang et al., 2010). The fact that many of these factors were not captured is a limitation to the studies thus presented. Moreover, these studies are not longitudinal. Much effort has gone into seeking to minimize, homogenize, and standardize these exogenous factors, primarily by carrying out a matched study where age, sex, and a Caucasian population were matched. In an ideal world, all factors could be accounted for. Then one could determine to a much better extent the role of both environment and genetics.

Conclusion

This research demonstrates both the biological and environmental complexity involved in caries. Taken alongside a growing body of literature regarding HLA alleles and caries, and its fundamental biological factors, it does not discount the notion that DRB1*04 is a biomarker that is involved in a lower mucosal immune response against particular discontinuous conformationally specific immunogenic epitopes that remain elusive. Although the tested epitopes reacted with SIgA to varying degrees, most did not appear to have a large impact in the caries process and may be relatively inconsequential with the significant exception of epitope 834-853. This epitope located in the distal arm of the V region (also connected to the P1 region) is probably very important, and provides a biologically sound connection between a lack of SIgA reactivity to its binding site, and therefore greater adherence to the tooth structure. All of this said, it is important to understand the limitations of this research in that we were unable to control for an array of environmental variables that can certainly affect things such as bacterial counts, and especially caries outcome data. Nevertheless, we

did design a paired study in order to minimize certain environmental factors that certainly affect the caries process.

Future Directions

Complex immunogenetic pathways involved in the caries disease process itself are not only crucial to understanding the fundamental nature of this infectious disease, but also essential in paving the way for new algorithms of diagnosis and detection (e.g., using biomarkers in lab-on-a-chip technologies to distinguish caries-susceptible and caries-resistant patients), vital for developing new therapeutic approaches (e.g., vaccines), and crucial in revealing common susceptibility alleles. Although HLA-II pathways account for only one piece of the caries genomic puzzle, they may prove even more important than suggested in this research. Although one HLA binding motif has been revealed within I/II, the newly revealed fibrillar structure of I/II "now provides a framework to design future studies and to re-examine historical data" (Brady et al., 2010).

Moreover, according to these authors, "one of the most promising strategies seems to be delivery of peptides, derived from glucan-binding protein B (GbpB) and antigen I/II (AgI/II) protein, via a mucosal (nasal) route." Another delivery method would involve something as commonplace as toothpaste especially to provide much more marketplace penetrance in underdeveloped countries. All of this will nonetheless require a definitive assessment of "the contribution of antibodies against complex discontinuous epitopes to caries protection, in either naturally-sensitized humans or in immunized animals" since this has yet to be done. In summary, Brady and colleagues are optimistic about the future:

> There is new potential for predicting and generating conformational epitopes eliciting protective antibodies. However, specificity of function in different *Streptococcus* species or strains is a major issue requiring more detailed and accurate analyses. This

may provide key information in understanding site-specificity of host colonization by different streptococci. In addition, this information is necessary if blocking of AgI/II-like protein functions with antibodies, mimetics or via small molecule inhibitors is to be developed as a therapeutic route.

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2008–2010	GRANT REVIEWER | Review Committee (4 members) for Graduate Student Educational Enhancement Grants, GSO
2008–2009	GRADUATE REPRESENTATIVE | Student Professional Conduct Committee (SPCC), IUSD
2009	ADVANCED GRADUATE LEADER | In Creation of Graduate Student Professional Conduct Committee, IUSD (working with the Associate Dean for Graduate Education)
2009–2010	CHAIR | Review Committee (4 members) for Graduate Student Educational Enhancement Grants, GSO
2009–2010	VICE-PRESIDENT | GSO, IUPUI (elected to serve over 8,000 graduate/professional students)
2009–2010	ADVANCED GRADUATE REPRESENTATIVE | Preparing Future Faculty Oversight Committee, IUPUI
2009–2010	ADVANCED GRADUATE REPRESENTATIVE | Hearing Meeting Committee (overseeing the entire campus of 30,000+ students) for reviewing misconduct, IUPUI
2010–2011	ORTHODONTIC RESIDENT REPRESENTATIVE | Graduate Student Professional Conduct Committee, IUSD

SELECTED HONORS AND AWARDS

2002–2003	National Dean's List
2003	Religious Studies Center Fellowship, BYU

2003	Institute for the Study and Preservation of Ancient Religious Texts Fellowship, BYU
2004–2005	Journalism Award: ASDA's Distinguished Chapter Newsletter Award for Design
2006	UNLV Teaching and Learning Center Grant for scenario based learning
2006	American Association for Dental Research (AADR) Bloc Travel Grant Recipient, awarded to the AADR by the National Institute of Dental and Craniofacial Research (NIDCR)
2005–2006	Journalism Award: ASDA's Distinguished Chapter Newsletter Award for Best of Competition
2007	Selected for *Who's Who in America*, 2007 (61st edition)
2007	Certificate in Recognition of valuable contributions to UNLV SDM Research Day
2007	United States Senate Certificate of Commendation
2007	The Pierre Fauchard Academy Undergraduate Certificate of Merit for Outstanding Academic Achievements in Dentistry
2007–2008	Fellowship in Oral Biology, IUSD
2008	Travel Fellowship Award, Indiana University
2008–2009	Indiana Clinical & Translational Sciences Institute (CTSI) Fellow Training Award
2008	Research Enterprise Spotlight, Office of the Vice Chancellor for Research, IUPUI
2009	Graduate Research Grant
2009–2010	Indiana Clinical & Translational Sciences Institute (CTSI) Fellow Training Award
2009	2 separate Graduate Travel Grants

PUBLICATIONS (2 articles, not shown; 1 submitted, 1 in preparation)

1. McCarlie VW. Shameful Plight: The Mentally Ill, Jails and Mental Health Care Facilities—A Quantitative Analysis. *Insight* 2000; **15**(2)**:** 22-31.

2. McArthur ED, Fairbanks DJ (eds). Respiratory and Physiological Characteristics in Subpopulations of Great Basin Cheatgrass. *Shrubland Ecosystem Genetics and Biodiversity*; Provo, Utah. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, 2000.

3. Fry EH, McCarlie VW (eds). Globalization and the Information Technology Revolution: Their Impact on North America's Federal Systems. *Globalization and the Information Technology Revolution*; Provo, Utah. David M. Kennedy Center for International Studies, 2001.

4. Smith BN, Harris LC, McCarlie VW, Stradling DL, Thygerson T, Walker J *et al.* Time, Plant Growth, Respiration, and Temperature. In: Pessarakli M (ed) *Handbook of plant and crop physiology*, 2nd , rev. and expanded edn. M. Dekker: New York, 2001, pp 1-12.

5. Fry EH, McCarlie VW. Mapping Globalization Along the Wasatch Front. In. Pacific Council on International Policy (division of the Council on Foreign Relations), 2002, pp 5-30.

6. Hansen LD, Church JN, Matheson S, McCarlie VW, Thygerson T, Criddle RS *et al.* Kinetics of plant growth and metabolism. *Thermochimica Acta* 2002; **388**(1-2)**:** 415-425.

7. McCarlie VW, Hansen LD, Smith BN, Monsen SB, Ellingson DJ. Anabolic rates measured by calorespirometry for eleven subpopulation of Bromus tectorum match temperature profiles of local microclimates. *Russian Journal of Plant Physiology* 2003; **50**(2)**:** 183-191.

8. Hansen LD, McCarlie VW. From foam rubber to volcanoes: The physical chemistry of foam formation. *Journal of Chemical Education* 2004; **81**(11)**:** 1581-1584.

9. Matheson S, Ellingson DJ, McCarlie VW, Smith BN, Criddle RS, Rodier L *et al.* Determination of growth and maintenance coefficients by calorespirometry. *Functional Plant Biology* 2004; **31**(9)**:** 929-939.

10. McCarlie VW. Dr. Robert Michael Sanders: Creative Pragmatist. *ASDA Impressions* 2005; **1**(1)**:** 1,6.

11. McCarlie VW. Remembering Dr. Nowlin. *ASDA Impressions* 2005; **1**(1)**:** 1,6.

12. McCarlie VW. Serving the 'Working Poor': A national insurance plan for dentistry? *ASDA Impressions* 2005; **1**(1)**:** 2.

13. McCarlie VW. UNLV Ready for Ortho Class, Pediatric Residency. *ASDA News* 2005; **35**(5)**:** 7.

14. McCarlie VW. Friday Legislative Session Inspires Members to Affect Change. *ASDA Leader* 2005.

15. McCarlie VW. Ideal Chapter is Minnesota. *ASDA Leader* 2005.

16. McCarlie VW. Timothy Levine: Pushing Forward When Life Pushes Back. *ASDA News* 2005; **35**(8)**:** 12.

17. McCarlie VW. Picture Secrets on Fundraising: Think outside the Box. *ASDA News* 2005; **35**(9)**:** 12.

18. McCarlie VW. Our Duty as Dentists: When we chose to become dentists, we also chose an inextricable obligation to serve unconditionally. *Mouth: Journal of the American Student Dental Association* 2006; **25**(4)**:** 5.

19. McCarlie VW. How We See Our Patients. *ASDA Impressions* 2006; **2**(1)**:** 1,6.

20. McCarlie VW. Be Careful How You Judge. *ASDA News* 2006; **36**(3)**:** 12.

21. McCarlie VW. The Road Less Traveled. *ASDA News* 2006; **36**(6)**:** 3.

22. McCarlie VW. What is a True Education. *ASDA News* 2006; **36**(9)**:** 2.

23. Orr DL, McCarlie VW. A Paradigm Shift for Peer Review? *ASDA News* 2006; **36**(10)**:** 2-3.

24. McCarlie VW. Lights in the Sky. *Mouth: Journal of the American Student Dental Association* 2007; **26**(4)**:** 3.

25. McCarlie VW. Reflections on Success. *ASDA News* 2007; **37**(3)**:** 3.

26. McCarlie VW. Achieving a Life Balance. *ASDA News* 2007; **37**(6)**:** 2.

27. Orr DL, McCarlie VW. Hospital Privileges for GPs. *Mouth: Journal of the American Student Dental Association* 2007; **27**(2)**:** 20-21.

28. McCarlie VW. Sidney B. Sperry: Father of Religious Education at BYU. *BYU Religious Education Review* 2009**:** 10-13.

29. McCarlie VW. The Oral-Systemic Connection. *Nevada Dental Association Journal* 2009; **11**(4)**:** 18-20.

30. McCarlie VW. Edward H. Angle and the Motives Behind His School of Orthodontia. *Journal of the History of Dentistry* 2010; **58**(1)**:** 48-55.

31. McCarlie VW. Viewing Craniofacial Diseases Through Nominalistic and Essentialistic Lenses. 2010**: Submitted**.

32. McCarlie VW, Orr DL, II. Health Science Education: Reviewing a Framework for Problem-Based Learning. *Journal of Dental Education* 2010; **74**(5)**:** 480-488.

ABSTRACTS (since 1999) AND PRESENTATIONS (since summer 2008)

1. 13th Annual Spring Research Conference 1999. Sponsored by Brigham Young University, College of Physical and Mathematical Sciences and the Central Utah Section of the American Chemical Society. Temperature dependence and respiration among cheatgrass (Bromus tectorum L.) populations. Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA, March 1999. [Abstract and Oral Presentation]
2. International Society for Biological Calorimetry XI. Respiration characteristics differ among cheatgrass populations. Department of Botany and Range Science, Brigham Young University, Provo, Utah 84602, USA, 6–10 June 1999. [Abstract and Poster Presentation]
3. XVI International Botanical Congress. Respiration characteristics differ among cheatgrass populations. Department of Botany and Range Science, Brigham Young University, Provo, Utah 84602, USA, 1–7 August 1999. [Abstract and Poster Presentation]
4. Religious Education Student Symposium. 25 February 2000. [Oral Presentation]
5. 11th Annual Wildland Shrub Symposium. Respiration and growth characteristics in subpopulations of Great Basin cheatgrass have adapted to local microclimates. Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA, 15 June 2000. [Abstract and Oral Presentation]

6. 85th annual meeting of the Ecological Society of America. Respiration and growth characteristics in subpopulations of Great Basin cheatgrass have adapted to local microclimates. Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA, 6 August 2000. [Abstract and Oral Presentation]
7. 14th Annual Spring Research Conference 2000. Sponsored by Brigham Young University, College of Physical and Mathematical Sciences and the Central Utah Section of the American Chemical Society. Respiration and growth characteristics in subpopulations of Great Basin cheatgrass which have adapted to local microclimates. Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA, March 2000. [Abstract and Oral Presentation]
8. 15th Annual Spring Research Conference 2001. Sponsored by Brigham Young University, College of Physical and Mathematical Sciences and the Central Utah Section of the American Chemical Society. Temperature dependence of respiration can distinguish among different populations of cheatgrass (Bromus tectorum L.). Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA, March 2001. [Abstract and Oral Presentation]
9. 16th Annual Spring Research Conference 2002. Sponsored by Brigham Young University, College of Physical and Mathematical Sciences and the Central Utah Section of the American Chemical Society. Popcorn Foaming on the Apricot Tree (physical chemistry of foam formation). Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA, March 2002. [Abstract and Oral Presentation]
10. 3rd Annual Clinic & Research Day 2005. Sponsored by the University of Nevada, Las Vegas, School of Dental Medicine and UNLV's chapter of the American Student Dental Association. Sex Appeal: Oral Piercing and Dentistry's Lack of Guidelines. UNLV School of Dental Medicine, Las Vegas, Nevada, USA, 20 May 2005. [Abstract and Poster Presentation]
11. 84th General Session and Exhibition of the International Association for Dental Research (IADR) 2006. Sponsored by the International Association for Dental Research. Oral Health and Smoking In Nevada Teens: Environmental Impact. UNLV School of Dental Medicine, Las Vegas, Nevada, USA. Brisbane Convention & Exhibition Centre, in Brisbane, Australia from June 28–July 1, 2006. [Abstract and Poster Presentation]
12. 85th General Session and Exhibition of the International Association for Dental Research IADR/AADR/CADR 2007. Second Hand Smoke, Sealants and Oral Health In Nevada Adolescents. UNLV School of Dental Medicine, Las Vegas, Nevada, USA. Ernest N. Morial Convention Center

in New Orleans, Louisiana from March 21–24, 2007. [Abstract and Poster Presentation]

13. Annual Meeting of the American Association for the Advancement of Curriculum Studies 2008. Indiana University, School of Dentistry, Indianapolis, Indiana, USA. Health Science Curriculum: The Scientific Mind. Teachers College, Columbia University, March 21–24, 2008. [Paper presentation]
14. Annual Meeting of the Indiana Clinical and Translational Sciences Institute (CTSI) 2009. Critical Antigen I/II Epitopes of *Streptococcus mutans*. Indiana University School of Dentistry, Indianapolis, Indiana, USA. Indiana University, School of Medicine, Health Information and Translational Sciences (HITS) Building, Collaboration Area, January 8, 2009. [Poster presentation]
15. Teaching, research, and service opportunities for up-and-coming academics, January 2009. Presented at the Preparing Future Faculty Orientation, Indiana University and Purdue University at Indianapolis, University Library, 1126, USA. [Oral presentation]
16. Dental Caries: Genetics, Immunology, & Microbiology, February 2009. Presented at Oral Examination for PhD Qualifying Exam, Indiana University, School of Dentistry, Indianapolis, Indiana, USA. [Oral presentation]
17. Research in Dental Medicine: Biological Connections, April 21, 2009. Presented at IUPUI Biology Club, Science Building, Indiana University and Purdue University, Indianapolis, Indiana, USA. [Oral Presentation]
18. 17th Annual Research Day, Indiana University School of Dentistry, 2009. Critical Antigen I/II Epitopes of *Streptococcus mutans* (an additional epitope highlighted from previous poster with same title). IUPUI Campus Center Room 409, Indianapolis, Indiana, USA, April 23–24, 2009. [Poster presentation]
19. Genetics, Human Leukocyte Antigen Complex Genes & Caries, November 10, 2009. Presented at the Graduate Student Research Seminar, Indiana University, School of Dentistry, Indianapolis, Indiana, USA. [Oral presentation]
20. Why Academic Dentistry? Thoughts on the Journey and Ethics in Dentistry, November 19, 2009. Presented at a Graduate Education Seminar for 1st year dental students. Indiana University, School of Dentistry, Indianapolis, Indiana, USA [Oral presentation]
21. Human Leukocyte Antigen Complex Genes & The Caries Process, December 4, 2009. Presented at the Indiana Association chapter of the

American Association of Dental Research, Indiana University, School of Dentistry, Indianapolis, Indiana, USA. [Oral presentation]

22. Antigen Processing & Presentation, March 11, 2010. Presented to Advanced Graduates in G959 Graduate Oral Microbiology. Indiana University, School of Dentistry, Indianapolis, Indiana, USA [Oral presentation].
23. 18th Annual Research Day, Indiana University School of Dentistry. Genetically Distinct Oral Immunity to Pathogenic Adhesin Epitopes. IUPUI Campus Center Room 409, Indianapolis, Indiana, USA, April 12, 2010. [Poster presentation].
24. Annual Meeting of the Indiana Clinical and Translational Sciences Institute (CTSI) 2010. Distinct Oral Immunity to Pathogenic Adhesin Epitopes. Indiana University, School of Medicine, Health Information and Translational Sciences (HITS) Building, Collaboration Area, April 19, 2010. [Poster presentation]
25. Annual National Meeting of Clinical and Translational Science Fellows 2010. Oral Immunity to Pathogenic Adhesin Epitopes. St. Louis, May 2-4, 2010. [Poster presentation]
26. Meeting of Second Year Indiana Clinical and Translational Science Fellows. The Immunogenetics of Dental Caries. Regenstrief Institute, 6th Floor Boardroom. May 6, 2010 [oral presentation].
27. Defense Seminar: The Immunogenetics of Dental Caries. Indiana University School of Dentistry, Indianapolis, Indiana, USA. June 4, 2010 [oral presentation].

PROFESSIONAL MEMBERSHIPS

American Academy of the History of Dentistry
American Association for the Advancement of Science
American/International Association for Dental Research
American Dental Association
American Dental Education Association
Fédération Dentaire Internationale (FDI) World Dental Federation
Society for Clinical and Translational Science