


10-12-2015

Characterization and Diurnal Measurement of Oral Inflammation in Association with Glycemic Control, Periodontal Status, & Glucose Stimulation

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Characterization and Diurnal Measurement of Oral Inflammation in Association with Glycemic
Control, Periodontal Status, & Glucose Stimulation

by

Melanie N. Kuehl

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cell Biology, Microbiology, and Molecular Biology
with a concentration in Cell and Molecular Biology
College of Arts and Sciences
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Date of Approval:
November 17, 2015

Keywords: diabetes, cytokines, hyperglycemia, saliva, circadian rhythms

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Dedication

I dedicate this work to my parents, Lauren and Dan Kuehl, whom taught me that I can achieve anything I desired as long as I believe in myself and keep my eye on the prize. I dedicate this work to my siblings, Danny, Kristie, and Arley for being there for me and providing me with continuous words of encouragement.

Acknowledgments

This work was funded by the Early Investigator Award to Dr. Amy Alman from the USF College of Public Health.

I would like to express a sincere appreciation to my mentor, Dr. Brant Burkhardt, for his exceptional support, understanding, and motivation throughout my education. Words cannot express how grateful I am for his expert guidance and encouragement.

I would like to thank my committee members, Dr. Amy Alman, Dr. Meera Nanjundan, and Dr. Stanley Stevens for the valuable scientific discussions and tremendous support and guidance.

A big thank you goes out to my family and friends for their caring, loving support and encouragement all throughout my life. I am blessed to have such a close family and great friends. Dr. Ann Ballesteros, having been through this journey, has provided me with great advice for all aspects of graduate school, along with words of encouragement.

I would like to thank my colleagues Whitney Ratliff, Katie MarElia, Tiffany Agius, and Mark (Bark) Athanason for providing immense support throughout my graduate education. Having you all to go through this journey with me has certainly been a fun experience.

Thank you to the employees of the University of South Florida's Diabetes Center for assisting me with collections and scheduling for my work. I would like to particularly thank Mrs. Danielle Henson for her efforts in the coordination of my T1D study population.

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Abbreviations

ANOVA: analysis of variance
ARNT: aryl hydrocarbon receptor nuclear translocator
BCA: Bicinchoninic Assay
BMI: body mass index
BSA: Bovine Serum Albumin
CI: confidence interval
CRP: C-reactive protein
DAISY: Diabetes and Autoimmunity Study in the Young
DMEM/F12: Dulbecco's Modified Eagle's medium and Ham's F-12 nutrient mixture
ECM: extracellular matrix
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
FADD: FAS associated death domain protein
FASL: FAS ligand
FBS: fetal bovine serum
GCF: gingival crevicular fluid
GDM: gestational diabetes mellitus
GWAS: genome-wide association studies
HbA1c: hemoglobin adult component A1c
HLA: human leukocyte antigen
HSG: human submandibular salivary gland
IFN- γ : interferon gamma
IgA: immunoglobulin A
IKK: I kappa B kinases
IL-1 β : interleukin 1 beta
IL-6: interleukin 6
IL-8: interleukin 8
IL-10: interleukin 10
iNOS: inducible nitric oxide synthase
JAK: Janus kinase
JNK: c-Jun N-terminal kinase
MinDC: minimum detectable concentration
MAPK: mitogen-activated protein kinase
MMP: matrix metalloproteinase
MPER: mammalian protein extraction reagent
NK: natural killer
NF- κ B: nuclear factor- kappa B
NO: nitric oxide
NOD: non-obese diabetic
OR: odds ratio
PBMC: peripheral blood mononuclear cells
PBS: phosphate buffered saline
PCA: principle components analysis

PVDF: polyvinylidene difluoride
QC: quality control
RIPK: receptor interacting serine/threonine protein kinase
SCN: suprachiasmatic nucleus
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SNP: single nucleotide polymorphism
SS: Primary Sjögren's Syndrome
T1D: Type 1 diabetes
T2D: Type 2 diabetes
TBST: Tris-buffered saline containing 0.1% Tween-20
TEDDY: The Environmental Determinants of Diabetes in the Young
TIMP: tissue inhibitors of metalloproteinases
TMB: tetramethylbenzidine buffer
TNF- α : tumor necrosis factor alpha
TLR: toll-like receptor

Abstract

Diabetes has afflicted 8.3%, approximately 25.8 million, of the United States population and is the seventh leading cause of death [1]. Type I diabetes (T1D) accounts for 5 to 10% of all diagnosed cases of diabetes in the United States [2]. If present trends continue, the rate of T1D incidence among children under the age of 14 will increase by 3% globally [3]. T1D is an autoimmune disorder in which the β -cells of the pancreatic islets are destroyed, leading to high blood sugar. Hyperglycemia and loss of immunological tolerance to self-antigens are common associations of T1D [4]. Periodontal disease impacts as much as 47% of the U.S. population and is a significant cause for tooth loss in adults [5]. Chronic infections from bacterial populations that colonize the tooth root surface result in the activation of immunological mediators and various metabolic byproducts, such as cytokines, chemokines, and tissue-destructive enzymes [6, 7]. Studies have demonstrated that the increased systemic inflammation associated with periodontal disease appears to contribute to several systemic diseases, particularly diabetes [8, 9], with a strong, bi-directional, relationship between diabetes and periodontal disease in which glycemic control is a major determinant. Improved biomarkers for T1D prediction are needed.

Cytokines serve an important part in the onset of T1D and strongly determine the ultimate fate of β cell destruction [10]. Such cytokines include interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interferon gamma (IFN- γ), and tumor necrosis factor alpha (TNF- α). Th1 cytokines such as IL-1 β , IFN- γ , and TNF- α have been demonstrated to induce pancreatic β -cell apoptosis. Matrix metalloproteinases (MMPs) have been demonstrated to participate in the pathogenesis of periodontitis. Hence, the measurement

of these inflammatory cytokines and MMPs may serve as a marker for the onset or progression of T1D and glycemic control. There have been no previous studies describing the distribution of salivary biomarkers of inflammation in T1D. Saliva represents a suitable bioreservoir for cytokines and can be collected noninvasively with very little to no stress upon the donor allowing for multiple collections if needed and it is easy to collect, store, and transport [11], however there are circadian patterns that must be accounted for. Therefore, we measured the levels of cytokines and MMPs in the saliva of T1D patients, characterized diurnal patterns of salivary cytokines in healthy subjects, and explored sources of inflammation to increase our understanding of salivary biomarkers of inflammation as a prediction of the progression of T1D and gum health and glycemic control.

This work demonstrated that specific salivary inflammatory markers - MMP-8, MMP-9, and TNF- α - in T1D subjects are associated with decreased glycemic control. Diurnal patterns of salivary proteins must be accounted for upon collection due to the unique rhythms of cytokine expression within each individual. Upon glucose stimulation, pro-inflammatory (Th2) cytokines, such as IFN- γ and IL-13, tend to decrease, whereas anti-inflammatory (Th1) cytokines, such as IL-10, tend to increase. Hyperglycemic conditions may promote an anti-inflammatory profile of human submandibular gland cells.

Chapter 1: Introduction & Background

Type 1 Diabetes

Diabetes mellitus, a Greek derived phrase from *diabainein* meaning “a siphon” and *mellitus* meaning “like honey”, is a metabolic disease characterized by defective insulin secretion and/or insulin action leading to hyperglycemia [12]. The word “diabetes” was derived from Aetius of Cappadocia (81-133 AD) and later “mellitus” was coined in 1675 by Thomas Willis after discovering the sweetness of urine and blood in diabetic patients [13]. Insulin is a hormone responsible for transporting glucose from the bloodstream to the body’s cells for energy. Inadequate insulin action results from a combination of impaired secretion of insulin from β cells of the pancreas and/or weakened tissue responses to insulin [12]. Diabetes is classified into three different types: Type 1 diabetes (T1D), Type 2 diabetes (T2D), and gestational diabetes. T1D is defined by a destruction of pancreatic β cells resulting in a complete failure of insulin production and is most common in younger individuals, but it can occur at any age. T2D originates with insulin resistance and an inadequate insulin secretory response, nevertheless the pancreas is still capable of producing little insulin. Gestational diabetes mellitus (GDM) is acquired by approximately 4% of all United States pregnancies and results from any form of glucose intolerance with onset of pregnancy, creating a risk for mothers developing T2D [14].

There are environmental influences, occurring early in life either in utero or infancy, that have an affect towards the onset of β cell autoimmunity [15]. Immune system development and the turnover of pancreatic cells could contribute towards pathogenic events. A precipitating event occurs, whether it be a genetic predisposition, infectious agents, or diet that triggers the

ultimate detrimental cascade of β cell decline. Islet autoantibodies (anti- β cell autoantibodies) arise forming a dysfunctional immune response followed by (depending on the individual, months or years later) glucose intolerance (hyperglycemia) and altered insulin secretion [15]. For unknown explanations, some individuals expressing these autoantibodies do not develop overt diabetes. Lastly, the C-peptide levels gradually decrease into absence, though some individuals produce low C-peptide levels long after onset [15]. This complete timeline of T1D is modeled in Figure 1.

Diabetes has afflicted 8.3%, approximately 25.8 million, of the United States population and is the seventh leading cause of death [1]. T1D accounts for 5 to 10% of all diagnosed cases of diabetes in the United States [2]. If present trends continue, the rate of T1D incidence among children under the age of 14 will increase by 3% globally [16]. Diabetics are at an increased risk of cardiovascular disease, renal failure, blindness and amputation, leading to a decreased quality of life and, quite often, a premature death. Given the importance of T1D management for overall improved clinical outcome, additional measures are needed to evaluate glycemic control and elucidate the contributing immunological mechanisms. With the rate of diagnosis of T1D globally increasing, the search for reliable biomarker and better understanding of the onset of the T1D is critical.

Potential Triggers of T1D

Genetics

There are many proposed factors that can contribute to the progression of T1D. Several loci are considered to increase the risk of T1D, with the most important genes being located in the human leukocyte antigen (HLA) class II locus on chromosome 6, a locus involved in the determination of specific $\alpha\beta$ T cells of the immune system [17, 18]. The HLA locus also contains genes that code for major histocompatibility complex (MHC) molecules that are

responsible for specific polymorphisms of the DQ and DR forms of MHC Class II molecules related to the genetic predisposition of T1D involving the HLA locus [19].

The theory that T1D is an immune mediated disease is supported by the fact that other genetic loci primarily expressed in cells modulating the immune system, such as gene coding regions for interleukin-2 receptor α , protein tyrosine phosphatase non-receptor type 22, and cytotoxic T lymphocyte antigen 4, have been found to be associated with increased risk of T1D [18]. Recently, there is increasing evidence that over 60% of T1D candidate genes, most of which are modulated by pro-inflammatory cytokines, are expressed in human pancreatic islets [20]. Genome-wide association studies (GWAS) screen single nucleotide polymorphisms (SNPs) to investigate the association of disease and genetic variants and a genetic variant located near the insulin gene, IRS1 (coding for the insulin receptor substrate-1), has been recognized to be associated with T2D, hyperglycemia, and insulin resistance [21, 22]. There are additional discoveries of an association of T1D with insulin (i.e. IDDM2 locus) and other β cell molecules (i.e. GLIS3) that may affect β cell function and antigen presentation [18, 19]. The monozygotic concordance rate of T1D is $\geq 50\%$ and there is a $\sim 6\%$ chance of developing the disease if a sibling is affected [18]. Hence, environmental factors must also play a role in genetic susceptibility of the disease.

Infectious Agents

Autoimmunity is thought to be triggered by some environmental factor(s) acting on an already genetically predisposed immune system [23, 24]. Some of these environmental factors of T1D include: infectious agents, such as viruses and enteroviruses, diet, psychosocial environment, and even perinatal factors. There are several theories of the mechanistic breakdown of viruses and their influence on islet autoimmunity and β cell decay. Many studies performed in animal models have demonstrated that infectious agents can trigger islet

autoimmunity through a variety of ways, either through exocrine or endocrine pancreatic tissue, molecular mimicry where antigens from infectious agents share epitopes with islet antigens, or activation of innate immunity [24]. It is known that innate nonspecific immune pathways, including inflammation and Toll-like receptor (TLR) signaling, contribute to specific adaptive responses, but these pathways may also assist in islet autoimmunity [25]. One study revealed that expression of certain TLRs in T1D patients were more elevated than in healthy individuals [26]. Components of viruses are considered to be robust activators of TLR signaling, yet the mechanisms involved remain elusive [26].

While it is difficult to determine the mechanisms involved with islet autoimmunity, recognizing relevant specific infections will elucidate the connections between infection and islet autoimmunity [24]. In one study, children whom contracted congenital rubella virus were found to have acquired T1D at a rate of 12% [24, 27]. Enteroviruses have been studied, most commonly Coxsackie B4, due to the fact that they infect the pancreas and tissues in contact with the gut-associated lymphoid. It is theorized that additional exposure events may accelerate the progressive downfall of β cell function once autoimmunity has been initiated and islet autoantibodies are present [24]. The relation between enteroviruses and T1D, however, is inconclusive despite the many years of research [24].

There are numerous studies conducted to examine the impact of viral infections on T1D. TEDDY (The Environmental Determinants of Diabetes in the Young), is an ongoing, international longitudinal study that enrolls children who are a first-degree relative of someone with T1D to investigate the infectious agents, dietary factors, or other environmental agents that lead to the development of T1D and is striving to address the incomprehensive etiology of the disease [28]. Current TEDDY data from Finland suggest a relationship between enterovirus infections and the development of islet autoantibodies [29]. Another study, The Diabetes and Autoimmunity Study in the Young (DAISY), investigates genetically predisposed children and their chances of developing T1D from environmental factors [30, 31]. Out of the children who

developed islet autoimmunity, approximately 36% progressed to T1D, due to viral RNA present in their blood [31]. In addition to viral-induced T1D, many studies have determined a link between diet and the progression of the disease [23, 32-34].

Diet

The first signs of β cell autoimmunity may occur during the first year of life, suggesting environmental risk factors are the cause. There are several controversial studies that suggest breastfeeding as well as early introduction of cow's milk proteins and cereal may or may not have an impact on the development of T1D [23]. Interestingly, too early of an introduction of fruits, berries, and roots may present an increased risk of β cell autoimmunity. Islet antibodies were found in blood samples from HLA-DQB1-conferred young children aged ≤ 4 months who were introduced to fruits, berries, and/or roots [34]. Other studies have shown an association with vitamin D supplementation [23] and a decreased diabetes risk, whereas cod liver oil (omega-3 polyunsaturated fatty acid) intake [32], and low levels of vitamin E [33] have been associated with an increased diabetes risk. Both TEDDY and DAISY studies are investigating the role diet plays on the progression of T1D through analysis of food introductions during early childhood years [32, 35, 36]. TEDDY is assessing infant and childhood diet through the collection of toenail clippings, plasma, and erythrocytes for potential dietary biomarker analysis and food diaries at 3-month intervals during the first year of life and biannually thereafter [36]. This will assist in uncovering the cause of T1D, yet the mechanisms behind the disease need further elucidation.

Immunological Mechanism

The immune system is divided into two components: the innate immune system (unspecified immune cells such as neutrophils, monocytes, natural killer (NK) cells) that is the first line of defense and attacks foreign antigens and the adaptive immune system (antigen-

specific immune cells such as lymphocytes T and B cells) that provides protection against additional exposure to the same pathogen [37]. T and B lymphocytes are responsible for providing protection to the adaptive immunity through utilization of clonal receptors. Activated immune cells destruct β cells through mechanisms involving perforins, granzymes, Fas/FasL, reactive oxygen and nitrogen species, and pro-inflammatory cytokines [19]. These cytokines will then bind to receptors on β cells activating MAP-kinases and transcription factors, STAT1 and NF κ -B, ultimately leading to apoptosis [19].

T1D is described as a T cell mediated autoimmune disease that is initiated by the processing of self-antigens by antigen presenting cells to auto-reactive CD4+ T cells in the pancreatic lymph nodes. Thereby, a pro-inflammatory detrimental cascade is initiated, resulting in the generation of autoantibody producing B cells, pro-inflammatory cytokine release, and activated cytotoxic CD8+ T cells [18, 38]. This immunological response of T1D is outlined in Figure 2.

There is supporting evidence that cellular immune responses contribute to the destruction of β cells: presence of T cells in insulinitis, detection of circulating autoreactive T cells, and disease progression is delayed by immunosuppressive drugs targeting T cells [17, 39]. CD8+ cell accumulation and MHC class I hyperexpression have been located in the pancreatic islets of donor patients with early onset and established T1D [40]. It is debated whether islet-autoreactive T lymphocytes are activated initially. MHC II molecules are believed to be involved in the activation of T cells through their presentation of autoantigenic determinants to self-reactive T cells [17, 18]. Nonetheless, there are many controversial theories on how the immune system provokes β cell death and whether or not it occurs through apoptosis or necrosis pathways. One method of β cell apoptosis is through the production of perforins and granzymes.

Perforins and Granzymes in β Cell Destruction

Both perforins and granzymes are located within lysosomal granules of natural killer (NK) cells and cytotoxic lymphocytes. Perforins are cytolytic proteins that function synergistically with granzymes, serine proteases that possess a strong preference for cleaving after specific aspartate sites [41]. Although the synergistic mechanism between the two is poorly understood, there is evidence that granzyme may enter cells solo, but requires perforin to mediate apoptosis [41]. One proposed theory is that activated CD8+ T cells release cytotoxic molecules, such as perforins and granzymes, which damage cell membranes. This destruction is particularly caused by perforin creating holes in the β cell membranes, allowing osmotic death and permitting protease granzyme B to enter, release cytochrome c, and activate caspases [41, 42]. This theory has been contested by a study that found non-obese diabetic (NOD) mice with perforin-deficient T cell receptors still developed diabetes more frequently than those with perforin-competence, however diabetes developed in a Fas-dependent manner [43]. This indicates that although perforins and granzymes play a minor role in β cell death, it is likely that cytokines are the major players.

Fas/FasL in β Cell Destruction

One of the more critical signaling routes of β -cell apoptosis is through the FAS ligand (FASL), which is located on activated CD4+ and CD8+ T cells [42, 43]. Several studies have shown that FASL is expressed in both human [44] and NOD mice [45] islets upon induction of cytokines. FASL will bind to the FAS receptor on β cells triggering the release of cytokines, the activation of caspase 8, followed by the activation of caspase 3, initiating permeabilization of mitochondrial membranes, ultimately leading to the fate of β cell apoptosis [42]. This process is outlined in Figure 3. Some studies have claimed FAS-deficient NOD mice were resistant to the progression of diabetes. Such studies were disproved by the event that diabetes is prevented via apoptosis of the adoptive transferred lymphocytes elicited by abnormal FASL expressing

lymphoid cells in FAS-deficient NOD mice [43]. Additionally, β cell destruction from Fas expression has been found to be nitric oxide-mediated in insulin-dependent diabetes [44].

Nitric Oxide in β Cell Destruction

Activated macrophages produce free radicals and oxidants such as superoxide, hydrogen peroxide, hydroxyl radicals, peroxynitrate, and nitric oxide (NO) [46]. Accordingly, free radicals, primarily NO, have been implicated as one of the key mediators of β -cell destruction [47, 48]. There is an extensive review of the role of NO in cytokine-mediated β cell destruction [41]. Cytokines, such as IL-1 β , activate inducible nitric oxide synthase (iNOS) in macrophages, generating high concentrations of NO via nuclear factors, such as nuclear factor κ B (NF- κ B) [41] as shown in Figure 3. NO will destroy β cells through a variety of methods, including 1) the inactivation of Krebs cycle enzymes preventing mitochondrial glucose oxidation and ATP formation, 2) the damage of DNA strands leading to activation of tumor suppressor proteins inducing apoptosis, or 3) the role of a redox mediator in cytokine-induced apoptosis [43].

T1D and Cytokine Involvement in Inflammatory Response

Cytokines are soluble molecules that are produced by many different cells in a highly regulated fashion that act at picomolar to nanomolar concentrations on cytokine receptors expressed by target cells [49]. Because cytokines are regulators as well as mediators of the immune response, it is evident that they may play a key role in the pathogenesis of T1D. It is believed that the initiation of the autoimmune response in T1D results from a disorder of immunoregulation [50].

Type 1 cytokines- IL-2, IFN- γ , TNF- α , and TNF- β - initiate a cascade of immune/inflammatory processes in the islet (insulinitis), culminating in β -cell destruction [46] (Figure 3). Type 2 cytokines- IL-4, IL-6, IL-10, and IL-13- are part of the humoral immune response and assist in downregulating the inflammatory actions of Th1 cells [51]. While T

helper 1 (Th1) cells secrete Th1 cytokines to play a pathogenic role, T helper 2 cells (Th2) secrete Th2 cytokines to mediate protection from inflammatory disease, such as T1D.

It has been shown in non-obese diabetic (NOD) mice and bio-breeding (BB) rats that cytokines are expressed in pancreatic insulinitis lesions [52]. The inflammatory infiltrate of the pancreatic islets consist mainly of CD4⁺ Th1 helper T cells, CD8⁺ T cells, dendritic cells, macrophages, and natural killer (NK) cells [53]. T1D is believed to develop when one or another immunoregulatory mechanism fails, allowing β -cell reactive T cells to become activated, reactive to β -cell antigens and islet cell antibodies produced by B cells, and expand clonally [46, 50, 54]. This arises a cascade of immune/inflammatory processes in the islet (insulinitis), leading to the ultimate fate of β -cell destruction [46].

Macrophages and dendritic cells migrate towards pancreatic islets, where they present β cell-specific antigens that initiate the differentiation of Th0 CD4⁺ T cells into T helper (Th) CD4⁺ cells [42]. T helper CD4⁺ cells may differentiate into subsets of effector cells that produce distinct sets of cytokines and perform different functions [55]. These subsets, Th1 and Th2 cells, may be distinguished not only by the cytokines they produce, but also by the cytokine receptors and adhesion molecules they express. For example, Th1 cells produce high amounts of IFN- γ and IL-12, which activate macrophage and CD8⁺ T cells, respectively, that interact specifically with β -cells and destroy them [55]. Additionally, cytokine release induces migration of activated CD8⁺ cytotoxic T cells to islets where β cells are stimulated to release even more cytokines and chemokines, which will lead to further immune activation [42].

Some or all of these mechanisms may be responsible for the end stage destruction of the insulin-producing pancreatic islet β -cells. Discovering the molecular mechanisms of β -cell destruction is crucial in understanding the immunopathogenesis of T1D and may provide novel insights into preventative and therapeutic interventions. Specific inflammatory markers, such as TNF- α , MMP-8, and MMP-9, of T1D subjects are associated with poor glycemic control [56]. Hyperglycemia is known to induce an increase in C-reactive protein (CRP) levels (a clinically

relevant marker of inflammation) and adiponectin levels, which in turn lead to increased inflammatory markers [57]. Yet, certain cytokines and chemokines have yet to be evaluated to determine their role in inflammation, glycemic control, and T1D.

IL-1 β and T1D

IL-1 β is a Th1 type cytokine that is produced by activated macrophages and neutrophils and its major function is to mediate and regulate innate immune responses [49]. In T1D, IL-1 β has been shown to be cytotoxic to pancreatic β -cells *in vitro* through the stimulation of increasing nitric oxide, prostaglandins, and ceramide in these cells [58]. In addition, electron microscopy exhibits that IL-1 β has a selective β -cell toxic effect in which islet integrity is destroyed and glucose-stimulated insulin release is almost completely inhibited [59]. It is believed that IL-1 β , as well as TNF- α , activates the transcription factor, nuclear factor- κ B (NF- κ B), which induces β -cell apoptosis [60].

TNF- α and T1D

TNF- α is a Th1 type cytokine that is produced mainly by activated macrophages, NK cells, mast cells, and Th1 cells and may induce macrophage production of IL-1 and IL-6, but its primary function is to recruit neutrophils and monocytes to infection sites and activate them upon their arrival [49]. Evidence that TNF- α plays a role in T1D development is supported by several studies. One study demonstrated a protection against diabetes in transgenic NOD mice expressing the TNF receptor to become neutralized of TNF [43]. Another study verified when the TNF- α receptor 1 is blocked in NOD mice, the onset and incidence of T1D is delayed [61]. Additionally, TNF- α (and IL-6) secretion from adipose tissue is associated with obesity and insulin resistance [62]. Altogether, these findings indicate that TNF- α is a strong candidate of initiating the pathogenesis of T1D.

IFN- γ and T1D

IFN- γ is a Th1 type cytokine that activates cell-mediated immunity, such as cytotoxic and inflammatory responses mediated by T-cells, natural killer (NK) cells and macrophages [49]. Evidence for IFN- γ being a β -cell cytotoxic cytokine in T1D derives from the findings that transgenic expression of IFN- γ by β -cells in normal mice leads to an autoimmune, lymphocyte-dependent infiltration of the islets by mononuclear cells (insulinitis), β -cell destruction, and T1D [63, 64]. Further supporting this role, diabetes is prevented by anti-IFN- γ antibodies in BB rats and by IFN- γ receptor α -chain mutations or IFN- γ deficiency in NOD mice [43].

IL-6 and T1D

IL-6 is produced by macrophages, endothelial cells, and activated T cells in response to IL-1 and TNF- α [49]. IL-6 activates Th2 cytokine production in CD4+ T lymphocytes and is also an important factor in the synthesis of C-reactive protein, which is increased in inflammatory diseases [65]. A previous study concluded that IL-6 impacts glucose oxidation by altering insulin secretion in rat pancreatic islets and discovered a possible role of B cell suppression and destruction during diabetes [66]. A recent study indicates that changes in IL-6 levels contribute to the change in insulin resistance over time in T1D patients [67].

IL-8 and T1D

IL-8 recruits neutrophils and is produced by macrophages, which amplify T cell responses and may cause direct β -cell dysfunction [38]. IL-8 has been thought to be a chemokine, which is produced either by cells in inflamed tissue or sites of antigen exposure, which induces adaptive immunity by recruiting leukocytes to inflammation sites [49]. IL-8 is considered to be one of the most potent chemokines present and previous studies have demonstrated that IL-8 levels are increased in both T1D and T2D through the promotion of a pro-inflammatory state in monocytes [68, 69].

IL-10 and T1D

IL-10 is a Th2 cytokine that inhibits cytokine synthesis (particularly Th1 cytokines and IL-12), activated macrophages, dendritic cells, and controls the innate immunity. The concept that expression of Th2 cytokines (IL-4 and IL-10) in islets protects against β -cell destructive insulinitis is supported by studies in which increased numbers of IL-4 and IL-10-producing cells were found in islets of NOD mice protected from diabetes development by various treatments, such as oral administration of insulin [46]. Further supporting this role, IL-10 has been shown to significantly reduce the onset of T1D in NOD mice via its inhibition of IFN- γ synthesis by Th1 cells [70]. Altogether, these data suggest a potential therapeutic role for T1D.

Stress Pathways and Cytokines

Inflammatory mediators released during disease activate several intracellular pathways, including the NF- κ B and MAPK pathways. NF- κ B is comprised of five members that are normally inactive in the cytoplasm through the binding of inhibitory I κ B proteins. Pro-inflammatory cytokines, such as IL-1 β , TNF- α , and IFN- γ , activate signaling pathways, including p38 MAPK and NF- κ B that direct pancreatic β -cell death and dysfunction [71]. IL-1 β activates the interleukin cell surface receptors to recruit receptor adaptor proteins, leading to signaling of I κ B kinases (IKKs) [71]. Subsequently, I κ B proteins are phosphorylated by IKKs, which stimulate the degradation of I κ B proteins, and release of NF- κ B proteins to translocate into the nucleus from the cytoplasm. Various NF- κ B subunits are now permitted to hetero- and homo-dimerize inside the nucleus to transactivate an assortment of genes involved in inflammatory responses that rise to tissue dysfunction and cell death [71]. One study suggested IL-1 β induces degradation of IKK β via the proteasome and TRAF6, TAK1, and JNK dependent signaling pathways in insulin-producing INS-1E cells [60]. TNF- α is believed to utilize a different strategy by activating the IKK complex (containing both IKK α and IKK β) via TRAF2 and receptor interacting serine/threonine protein kinase (RIPK) to induce β -cell death [42, 60]. IFN- γ may

induce phosphorylation of the Janus kinase (JAK) family, leading to the phosphorylation and recruitment of STAT1 [42]. Once STAT1 is phosphorylated, it will homodimerize and transport into the nucleus where it will activate apoptosis-inducing genes [42].

Several studies have indicated that p38 MAPK promotes pro-inflammatory cytokine production, yet the molecular mechanism is poorly understood [72, 73]. The mitogen-activated protein kinase (MAPK) signal transduction pathway plays a significant role in the recruitment of leukocytes to sites of inflammation. This stimulation of leukocytes by pro-inflammatory cytokines leads to the activation of p38 MAPK [74]. There are three members of the MAPK pathway: 1) ERK1 & 2, which is activated by chemoattractants and growth factors, 2) c-Jun N-terminal kinase (JNK), and 3) p38 MAPK, which are stress- and cytokine-activated [75]. MAPK members are regulated by phosphorylation of tyrosine and threonine residues [76]. Prior reports have indicated that IL-6 production is increased from salivary gland epithelial cells during Primary Sjögren's syndrome (SS) via activation of intracellular signaling cascades, including the mitogen-activated protein kinase (MAPK) and NF- κ B pathways. In a particular study, pharmacological inhibition of p38 MAPK or NF- κ B, but not JNK, significantly suppressed palmitate-induced IL-6 secretion in human parotid gland epithelial cells, suggesting that IL-6 secretion is induced through activation of said pathways [77].

Circadian Rhythms & Inflammation

Circadian, or diurnal, rhythms are biological processes over a 24 hour time period in organisms ranging from bacteria to humans, thus creating time of day-dependent variations [78]. There are several factors that influence the circadian rhythm of the body: light, melatonin/hormones, cognitive functions, mood, and core body temperature to name a few. In mammals, the master regulator of circadian rhythms is the suprachiasmatic nucleus (SCN) located in a tiny region of the hypothalamus, which regulates oscillations of clock genes in peripheral tissues [78, 79]. The circadian molecular clock consists of a network of

transcriptional-translational feedback loops that regulate the 24 hour expressions of clock proteins. Transcription factors, BMAL1 and CLOCK, heterodimerize and transcribe *Period* (murine *PER1*, *PER2*, and *PER3*) and *Cryptochrome* (*CRY1* and *CRY2*) genes, which in turn dimerize and translocate back into the nucleus, where they interact with the CLOCK:BMAL1 complex [79]. These molecular mechanisms have been examined primarily with mice. Little is known on the impact of altered circadian rhythms in oral inflammation in humans.

Local circadian clocks may be present in the liver, heart, skin, kidney, and cultured cell lines. It has been shown that in vitro culture conditions, cortisol-mediated cytokine secretion rhythms exist, suggesting a local macrophage-intrinsic clock [78]. Interestingly enough, one study examined human submandibular gland (HSG) cells and suggested internal circadian rhythms derived from the transcription factor, BMAL1, and its repressor, REV-ERB α [80]. This local circadian clock is utilized to regulate the type, amount, and content of saliva. Melatonin is well known to control phase shifts in circadian rhythms, is released into the saliva, and increases in response to periodontal inflammation [81, 82]. In addition to melatonin and cortisol being the primary regulators of circadian clocks and diurnal rhythms of cytokines, contradicting studies claim glucocorticoids may/may not play a role [83]. There is evidence of a connection to the progression of diabetes and the ablation of certain clock genes in mice that led to abnormal glucose tolerance, impaired insulin secretion, and altered islet gene expression [84]. Further research needs to be conducted to determine the impact of circadian rhythms and oral inflammation.

The recent discovery of clock genes in the gastrointestinal tract play a critical role in the progression of inflammation by impacting intestinal epithelial cell permeability [85]. Although it is well known that clock genes are one of the key regulators of cell functions, the mechanisms of how clock genes contribute to the regulation of cell proliferation during disease have yet to be elucidated [86]. Inflammatory mediators, such as TNF- α , can also suppress the expression of the period clock genes in murine fibroblasts *in vitro* and *in vivo* in TNF- α -infused mice [87].

Previous reports have supported the complex relationship of circadian rhythms and the regulation of the immune system, including CD4+ T cells, eosinophils, and macrophages by clock gene transcription [86]. Nonetheless, additional research is needed to gain a full comprehension of clock gene expression within the immune system. Inflammation can be triggered through a poor management of glycemic control and subsequent hyperglycemia.

Hyperglycemia and Glycemic Control

Hyperglycemia is a classic manifestation of T1D and if managed poorly, can lead to cardiovascular and periodontal disease [88]. Previous studies have demonstrated there is an association between biomarkers of inflammation and glycemic control in type 1 diabetics [89, 90]. Hyperglycemia leads to the non-enzymatic glycation of sugars that lead to the formation of Schiff bases and Amadori products that produce advanced glycation end products (AGEs) [91]. These AGEs interact with their receptors on monocytes and stimulate the inflammatory process [92]. This process is outlined in more detail in Figure 4. HbA_{1c} has been associated with increased levels of systemic inflammation [91], and inflammation in turn has also been associated with vascular complications of diabetes [92]. In addition, the pathogenic effect of hyperglycemia has been shown to activate stress-sensitive pathways, which secrete inflammatory cytokines [93]. There are no established noninvasive methods for glycemic control.

Human hemolysate contains several minor components designated HbA_{1a}, HbA_{1b}, HbA_{1c}, which are post-translational modifications of the major hemoglobin component A0 [94]. HbA_{1c} is the direct reaction of hemoglobin with glucose. HbA_{1c} is a test that measures glycosylated hemoglobin over a three month period, which is the approximate lifetime of an erythrocyte. The normal HbA_{1c} levels are between 4-5.6%, whereas diabetic levels are 6.5% or higher [95]. A high HbA_{1c} result has been associated with increased levels of inflammation and vascular complications of diabetes [95].

There is no real non-invasive measure of glycemic control. Based on the guidelines from the American Diabetes Association (ADA), T1D diabetics should monitor their glucose levels at least four times a day. Studies have demonstrated that continual self-monitoring of blood glucose (SMBG) results in better clinical outcomes and thereby reducing all-cause mortality such as those associated with myocardial infarction. In addition, SMBG appears to result in significant savings from a health economics standpoint. SMBG allows T1D patients to evaluate their individual response to therapy using a glucose meter and frequency is dictated by each patient's needs and goals. Effective clinical intervention to avoid the glycemic peaks and valleys typical of T1D management would greatly reduce the incidence of morbidity and mortality. Many patients with T1 or T2D associate SMBG with pain, frustration, and a continual demoralizing reminder that they have diabetes. Therefore, it is critical to develop alternative strategies for evaluation of patient compliance for management of T1D.

IL-13 and Hyperglycemia

IL-13 is a Th2 cytokine that mediates macrophage activation and inhibits inflammatory cytokine production. IL-13 is considered a critical mediator of mucus secretion in the lungs and in the regulation of cell-mediated immunity and tissue fibrosis [96]. In diabetes, IL-13 regulates hepatic glucose production via its receptor, IL-13 α 1, and the STAT3 signaling pathway in the liver [97]. It remains unclear what regulates the IL-13 (and IL-4) receptor expression and how that affects functional activity of IL-13.

IL-4 and Hyperglycemia

IL-4 is a Th2 cytokine and is functionally related to IL-13; it too inhibits inflammatory cytokine production. Studies have shown a prevention of diabetes in vivo when IL-4 is administered to NOD mice and in vitro when IL-4 is transgenically expressed in pancreatic β cells [51]. In another study analyzing plasma from 22 children with T1D with and without

hyperglycemia, it was concluded that cytokines IL-1 α , IL-6, and IL-4 were highly elevated during and remained elevated after correction of hyperglycemia [98].

IL-2 and Hyperglycemia

IL-2 is a Th1 cytokine produced by CD4+ T cells known to support regulatory T cell survival, whose function is to regulate pathogenic autoimmune responses in T1D [99]. In pokeweed mitogen-stimulated (activates T and B lymphocytes) human peripheral blood mononuclear cells, endogenous production of IL-2 (IL-6 and IL-10 as well) is inhibited by a suppressed immune response from high glucose-induced TGF- β production [100]. One study observed that as periodontal health worsened in type 1 diabetics, levels of IL-2 decreased, possibly due to periodontopathic bacteria metabolic products [101]. There is limited information on IL-2 secretion and its impact on hyperglycemia. Therefore, analysis of its role in hyperglycemic conditions in salivary gland cells may provide useful insight into understanding IL-2 expression in diabetes and/or periodontitis.

Saliva Production and Secretion

Saliva is a clear, slightly acidic mucoserous exocrine derived liquid containing a mixture of secretions from major and minor salivary glands and from gingival crevicular fluid (GCF) [102, 103]. Saliva is composed of 99% water, containing electrolytes (i.e. sodium, potassium, chloride, and phosphate), enzymes, immunoglobins, glycoproteins, and small amounts of albumin [101]. There are three major glands involved in saliva secretion: parotid, submandibular, and sublingual. The parotid gland is the largest and secretes serous saliva, while the others secrete saliva containing mucous [104]. The submandibular gland contains both serous and mucous cells. The sublingual gland consists mainly of mucous cells with very few serous cells. The localization of these glands can be depicted in Figure 5. Some biomolecules, such as steroid hormones and/or immunoglobulin A (IgA), enter the saliva from

blood by passive diffusion of lipophilic molecules, ultrafiltration, or through active transport of proteins via ligand-receptor binding [11]. These processes are modeled in Figure 6. Serum components can be identified in the saliva, however the components that are produced locally by the salivary glands may not be detected in plasma [105]. In addition, it has been shown that amines and peptides, such as melatonin and insulin, enter the saliva through these methods and exhibit an excellent correlation between saliva and blood concentrations [106]. Because saliva contains serum constituents and certain hormones that correlate to blood concentrations, it is a favorable diagnostic of systemic diseases.

Saliva as a Diagnostic Fluid

There has been an increase of interest in using saliva as a diagnostic medium over the past 10 years [11]. However, the use of saliva as a measurement for biomarkers in T1D has not yet been established. There are several reasons why saliva is advantageous in comparison to serum: it can be collected noninvasively with very little to no stress upon the donor allowing for multiple collections if needed, it does not require training or expertise for collection, and it is easy to collect, store, and transport [11]. Saliva provides a more accurate representation of the individual's health and well-being at the moment of collection. This is due to the fact that the salivary glands are exocrine glands that produce proteins indicative of one's health. Steroid hormones, proteins, and cytokines make their way into the saliva from the capillaries via active transport, ultrafiltration, or diffusion [107] as shown in Figure 6. Additionally, larger proteins can be transported into the saliva from the gum tissues and gingival fluid.

Studies have shown differential salivary protein expression in T1D adults and children in comparison to non-diabetics [108, 109]. More recently, a study described that peptidome data not only support a diabetes-related higher susceptibility of salivary proteins to proteolysis, but also evidenced an increased content of some specific protein fragments known to be related

with bacterial attachment and the accumulation of phosphopeptides involved in tooth protection [110].

Although it is quite difficult to quantify the inflammatory burden presented by diabetes and periodontitis, there have been several attempts collecting and analyzing GCF to assess the inflammatory response [111]. However, there are limitations of the collection of GCF since there is a need to collect multiple times throughout the dentition and this collection process only occurs at selected sites of the crevice of each tooth [111]. Collection of whole saliva includes GCF, sloughed epithelial cells, bacteria, and other constituents derived from the salivary glands rendering a wide assessment of the oral inflammatory burden [111].

The one disadvantage to using saliva as a diagnostic fluid is that the diurnal/circadian variations of cytokines and other biomolecules present in the saliva do not always reflect the concentrations of these molecules in serum [11]. High sensitive assays have been developed and are being improved to enhance sensitivity. Once the diurnal patterns of salivary components are identified, a better understanding towards salivary diagnostics can be achieved. Previous studies have shown that the saliva test for antibodies to HIV, which is the best known of the saliva-based diagnostic tools, approaches the sensitivity and specificity of a blood test [11].

Periodontal Disease and Diabetes

Periodontal disease is an inflammatory disease derived from pathogenic bacteria, such as *Porphyromonas gingivalis* (*P. gingivalis*), *Prevotella intermedia*, or *Aggregatibacter actinomycetemcomitans*, in the biofilm and may arise from genetic, developmental, traumatic, or inflammatory origins [112, 113]. Gingivitis is the mildest form of periodontal disease and results from the accumulation of bacterial biofilm on teeth flanking the gingiva, or gums [113]. Periodontitis destructs the underlying support structure of the teeth through the deterioration of connective tissue and reduction in alveolar bone [113].

Periodontal disease affects as much as 47% of the U.S. population and is a significant cause for tooth loss in adults [5]. Periodontal (gum) disease is more common in people with diabetes. Among young adults, those with diabetes have about twice the risk of those without diabetes [1]. Adults with poor glycemic control ($HbA_{1c} > 9\%$) were 2.9 times more likely to have severe periodontitis than those without diabetes [1]. Studies have demonstrated that the increased systemic inflammation associated with periodontal disease appears to contribute to several systemic diseases, particularly diabetes, with a strong, bi-directional, relationship between diabetes and periodontal disease in which glycemic control is a major determinant.

Periodontitis is driven by bacterial infections that colonize the tooth root surface [6]. Due to this pathogenic event, immunological mediators are activated and various metabolic byproducts such as cytokines, chemokines, and tissue-destructive enzymes are released [7]. Spillover of these immunological mediators into the general circulation is thought to play a role in the development and exacerbation of systemic diseases, particularly diabetes, where a bi-directional relationship between periodontal disease and glycemic control has been demonstrated [9, 114].

In the metabolic dysregulation of diabetes, persisting hyperglycemia causes non-enzymatic glycation and oxidation of proteins and lipids, and the subsequent formation of AGEs, which accumulate in the plasma and tissues [115]. Hyperglycemia and resultant AGE formation are considered to be a major causal factor in the pathogenesis of diabetes complications [115]. In addition, diabetes has been associated with altered collagen metabolism and increases the response of the periodontal tissue to pathogenic microorganisms, thereby increasing the severity of periodontal disease [116]. While the relationship between periodontal disease and glycemic control has been demonstrated in T1D, the association between oral immunological mediators and glycemic control in T1D is not well understood [117].

Matrix Metalloproteinases and Periodontitis

A complex network of cytokines is involved in the inflammatory and immune responses in the inflamed periodontal tissues during the pathogenesis of periodontitis [118]. Among these cytokines, matrix metalloproteinases (MMPs)—a family of zinc endopeptidases (break peptide bonds of nonterminal amino acids) that degrade the extracellular matrix (ECM) and regulate fibrosis formation—has been thought to play a central role in the regulation of periodontal tissue turnover in health and disease [118]. MMPs are proteolytic enzymes that regulate changes in the extracellular matrix (ECM) and maintain its normal physiology and pathology [119]. MMPs are divided into six major groups depending on their substrate specificity of extracellular matrix proteins: collagenase, stromelysin, gelatinase, elastase, matrilysin, and membrane-bound MMPs [120].

The expression and activity of MMPs in adult tissues is normally quite low, but increases significantly in various pathological conditions, including inflammatory diseases, that may lead into unwanted tissue destruction. MMP activity is increased due to an increased concentration of collagenase in periodontal pockets [121]. Several studies have shown that MMPs are useful as markers for people at risk for T1D [122, 123]. MMPs are produced by various cells (i.e. macrophages, lymphocytes and fibroblasts) and actively contribute to the destruction of periodontal tissues and eventual alveolar bone loss [124].

Specific MMP Involvement and Periodontitis

MMP-3 and Periodontitis

MMP-3, also known as stromelysin-1, breaks down collagen and degrades proteoglycans and fibronectin. MMP-3 levels have been shown to be elevated in GCF and from patients with periodontitis and in vitro in gingival fibroblasts [120]. Interestingly, extracellular levels were significantly elevated compared to intracellular levels. Furthermore, increased MMP-3 mRNA expression has also been demonstrated in gingival samples from periodontitis-

affected tissue. This production is shown to be enhanced by the cytokines IL-1 β and TNF α [120]. Human primary umbilical vein endothelial cells and monocyte-derived macrophages under high glucose conditions showed an increase in MMP-1 expression, while reducing MMP3 expression, suggesting a dysregulation of MMPs under hyperglycemic conditions [125].

MMP-8 and Periodontitis

MMP-8, also known as collagenase-2, has many roles, including breaking down collagenases, assisting with neutrophil migration, and inducing the expression of certain cytokines, such as IL-6 and IL-8 during inflammation [126, 127]. Several studies have shown that MMP-8 levels in the GCF and saliva are significantly elevated in periodontitis patients compared to healthy individuals [128, 129]. In one study investigating the various forms of MMP-8, it was discovered that less glycosylated forms are found to be derived from pro-inflammatory mediators, such as IL-1 β and TNF- α [130]. MMP-8 originates from mature neutrophils and degranulated neutrophils that have been attacked by bacteria. Plasma cells located within inflamed periodontal tissue express MMP-8, however their molecular form is still under investigation.

MMP-9 and Periodontitis

MMP-9, also known as gelatinase B, is involved tissue remodeling, wound healing, mobilization of matrix-bound growth factors and processing of cytokines [119]. MMP-9 is mainly secreted by polymorphonuclear leukocytes and degrades collagen (mainly type IV) present in gingival tissues and participates in basement membrane remodeling [131]. MMP-2 and MMP-9 serum levels are thought to be abnormal in obese patients and significantly higher in T1D patients in comparison to control groups [123]. In a particular study investigating MMP-9 levels in subjects with chronic periodontitis with and without diabetes, MMP-9 levels were increased three-fold in subjects with chronic periodontitis with diabetes compared to without [116]. In

chronic periodontitis, levels of TIMP (tissue inhibitors of metalloproteinases) are low and thus inadequate to inhibit elevated MMPs. Moreover, mobilization and activation of inflammatory cells, such as lymphocytes and neutrophils, alteration of immunomodulators and secretion of inflammatory proteases occur [131].

PCA Analysis

Principal components analysis (PCA) is a variable reduction technique used to account for redundancy between variables by producing uncorrelated components. These components account for a meaningful amount of the variance contained in the original set of variables but that can be used simultaneously in a regression analysis. The relationships between PCA components and HbA1c and gum health can be examined through multiple linear and logistic regression analyses.

Hypothesis and Aims

The goal of this work is to characterize and measure oral inflammation in association with glycemic control, periodontal status, and glucose stimulation. There have been no previous studies describing the distribution of salivary biomarkers of inflammation in T1D. Thus, our overall hypothesis is that **salivary biomarkers of inflammation will be associated with glycemic control and periodontal status in type 1 diabetes**. We will address the hypothesis via the following specific aims:

Specific Aim 1 (presented in Chapter 3): **Determine the association of salivary inflammatory burden, oral health, and glycemic control in T1D subjects.**

1.1 Recruit and collect saliva and metabolic parameters from 150 T1D patients.

1.2 Measure the salivary levels of TNF- α , IL-1 β , IFN- γ , IL-6, IL-8, and IL-10 as a determination of inflammatory burden.

1.3 Determine the salivary levels of MMP-3, MMP-8, and MMP-9 as a measure of periodontal status.

1.4 Utilize linear and logistic regression model analysis to evaluate the association of measured salivary inflammatory and periodontal markers with glycemic control and self-reported gum health.

Specific Aim 2 (presented in Chapter 4): Measure the salivary diurnal inflammatory rhythms within the saliva of healthy adult subjects.

2.1 Recruit and collect longitudinal intra-daily saliva samples from healthy adult subjects.

2.2 Measure the cytokine levels within salivary samples by multiplexing analysis.

2.3 Perform statistical analysis to determine if oral inflammatory diurnal rhythms are present and can be detected within the saliva.

Specific Aim 3 (presented in Chapter 5): Evaluate impact of hyperglycemia on the inflammatory profile from a HSG cell line.

3.1 Determine cytokine profile and measure cytokine production of the HSG cell line at basal and glucose stimulatory conditions.

3.2 Elucidate the cellular signaling mechanism of enhanced inflammatory production during glucose-stimulatory conditions.

Overall Impact and Significance

At the end of this work, our findings will allow us to better comprehend the impact of hyperglycemia on the inflammatory profile from salivary resources. Additionally, we will better comprehend the cytokine diurnal rhythms in the saliva. The objective of this research is to analyze the presence and distribution of salivary biomarkers of inflammation in T1D and periodontitis and investigate how these markers relate to glycemic control and self-reported gum

health. The long term goal of this proposed project is to investigate the causal mechanisms associating inflammation, periodontal disease, and diabetes using oral biomarkers. In addition, the results of this research could provide a new avenue into the investigation of using saliva instead of serum as a diagnostic fluid for evaluation of T1D management.

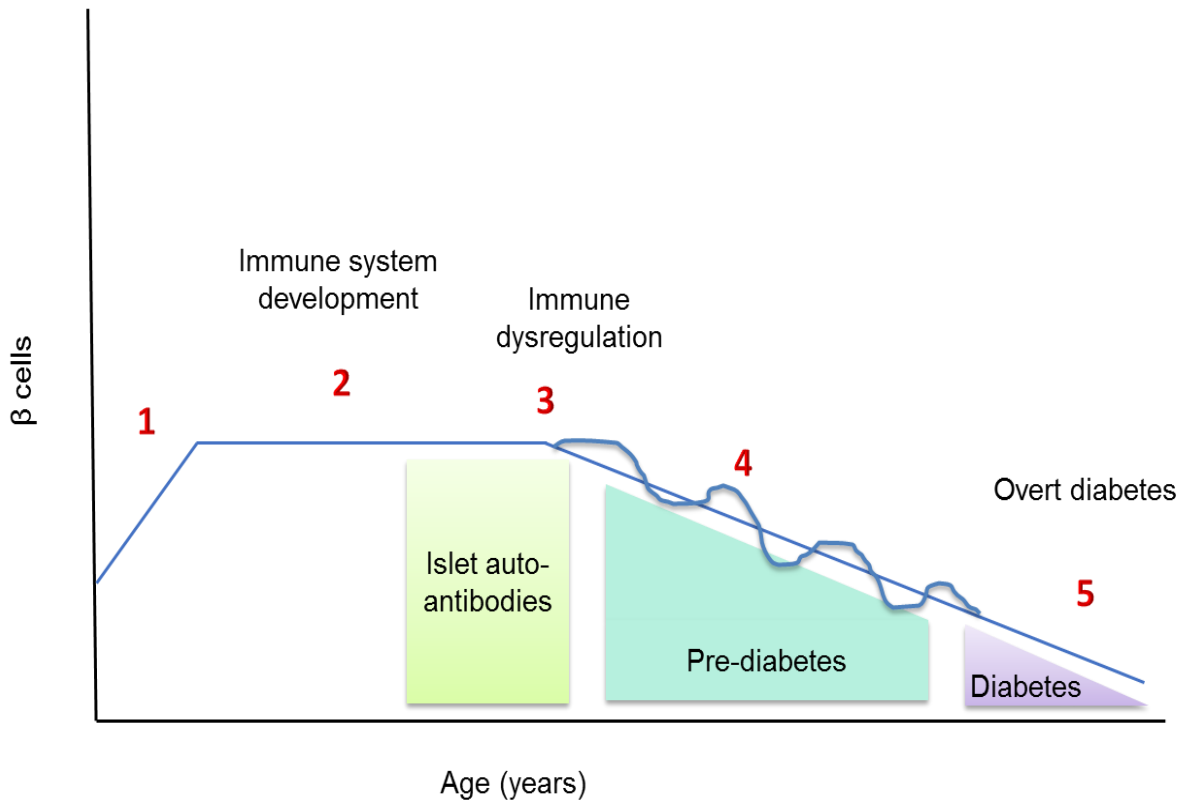


Figure 1: Timeline of Type 1 Diabetes

This graph depicts the overall timeline of events that occur in individuals whom develop T1D. **1)** A precipitating event or genetic predisposition occurs early in life. **2)** The immune system develops and turnover of islet cells begin. **3)** β cells start to destruct and autoantibodies form. **4)** Progressive loss of insulin release occurs and C-peptide levels decrease. Some individuals may experience β cell relapse creating wavering levels. **5)** Hyperglycemia, insulin resistance, and complete β cell loss results in overt diabetes (figure adapted from Atkinson et. al, 2014).

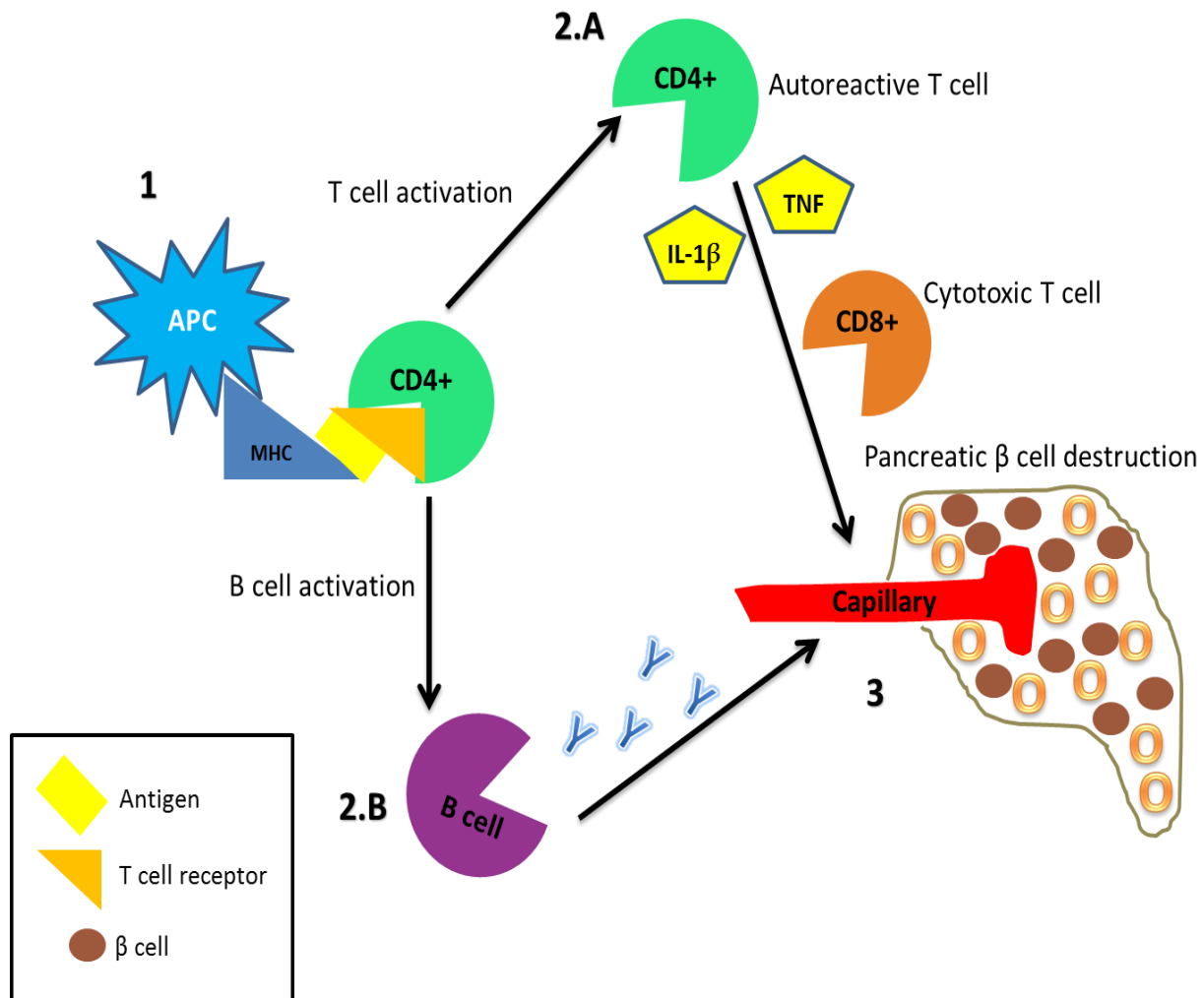


Figure 2: Immunological Mechanisms in Type 1 Diabetes

(1) An APC (antigen presenting cell) binds to T cell receptors to stimulate T cells and B cells to become either cytotoxic or helper CD4+ cells. **(2.A)** CD4+ cells will clonally expand and release cytokines, such as TNF and IL-1β, which recruit cytotoxic CD8+ T cells that release perforins and granzymes to damage pancreatic cell membranes or **(2.B)** B cells become activated and release autoantibodies that attack the pancreatic β cells. **(3)** Insulinitis of the pancreas occurs. MHC: major histocompatibility complex (Adapted from Waldron-Lynch, *et. al*, 2011).

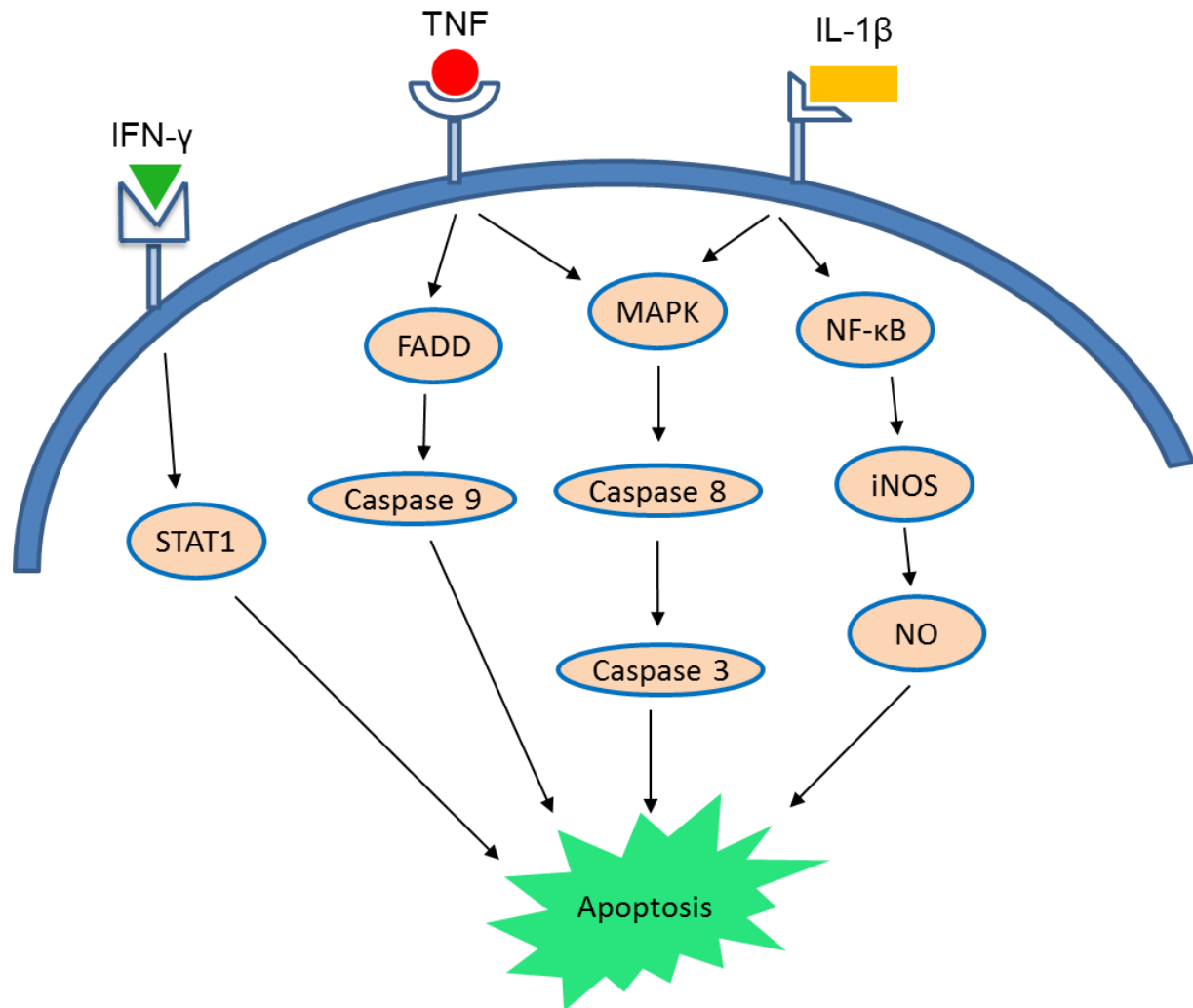


Figure 3: Overview of β Cell Apoptosis from an Inflammatory Standpoint

Cytokines bind to their respective receptors located on the β cell and each cytokine plays a unique role in triggering apoptosis. IFN- γ activates STAT1 via a Janus kinase (JAK)-signal transducer. TNF can utilize either the FAS associated death domain (FADD) protein or the mitogen activated protein kinase (MAPK) pathway, which activate caspases. Lastly, IL-1 β activates the MAPK or nuclear factor- κ B (NF- κ B) pathways, which then activates inducible nitric oxide synthase (iNOS) to increase the production of nitric oxide (NO) to initiate β cell death (Adapted from Vetere *et. al*, 2014).

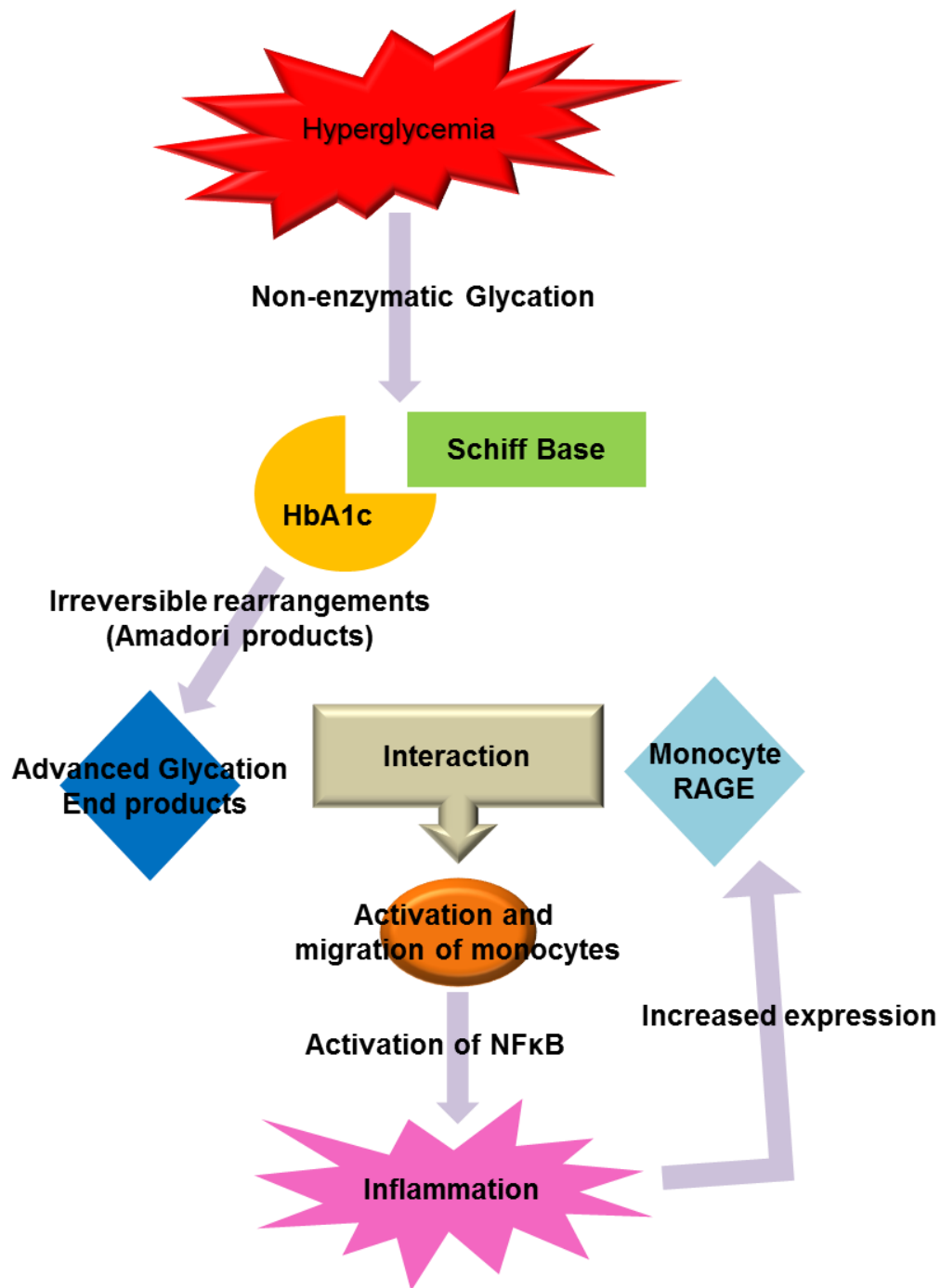


Figure 4: Hyperglycemic Events Leading to Inflammation

During hyperglycemia, sugars undergo non-enzymatic glycation, creating Schiff bases and Amadori products. These irreversible products are converted into advanced glycation end products (AGEs), which interact with receptors on monocytes and trigger inflammation via stress pathways, such as the NFκB pathway (Figure adapted from Dr. Alman).

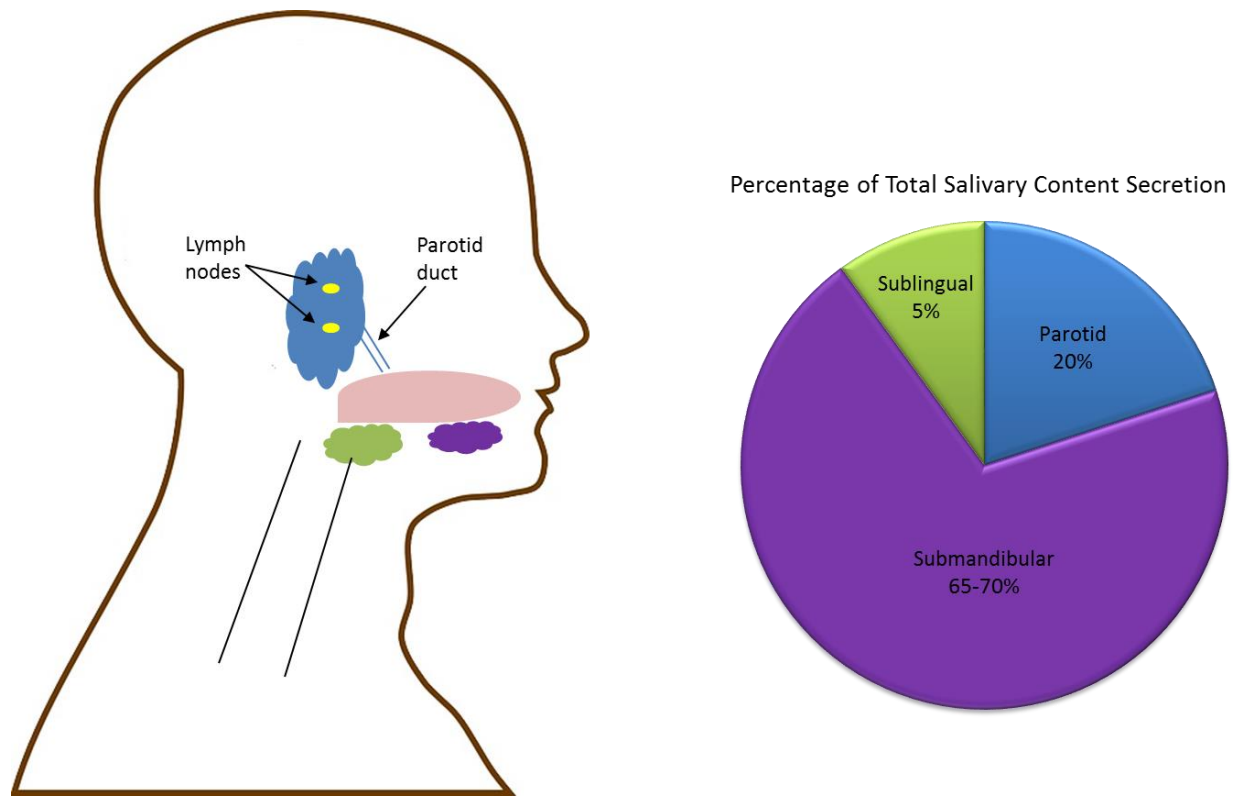


Figure 5: Summary of the 3 Main Salivary Glands

The parotid gland, located above the jaw and in front of the ear, secretes serous (water, electrolytes, enzymes) fluid through the parotid duct into the rear of the mouth. The submandibular and sublingual glands secrete mucin-containing fluids and are located at the back of the mouth and under the floor of the tongue, respectively. The submandibular gland secretes 65-75% of total salivary content, the sublingual gland secretes 5% of the total salivary content, and the parotid gland secretes 20% of the total salivary content (Image adapted by Pfaffe *et. al*, 2011).

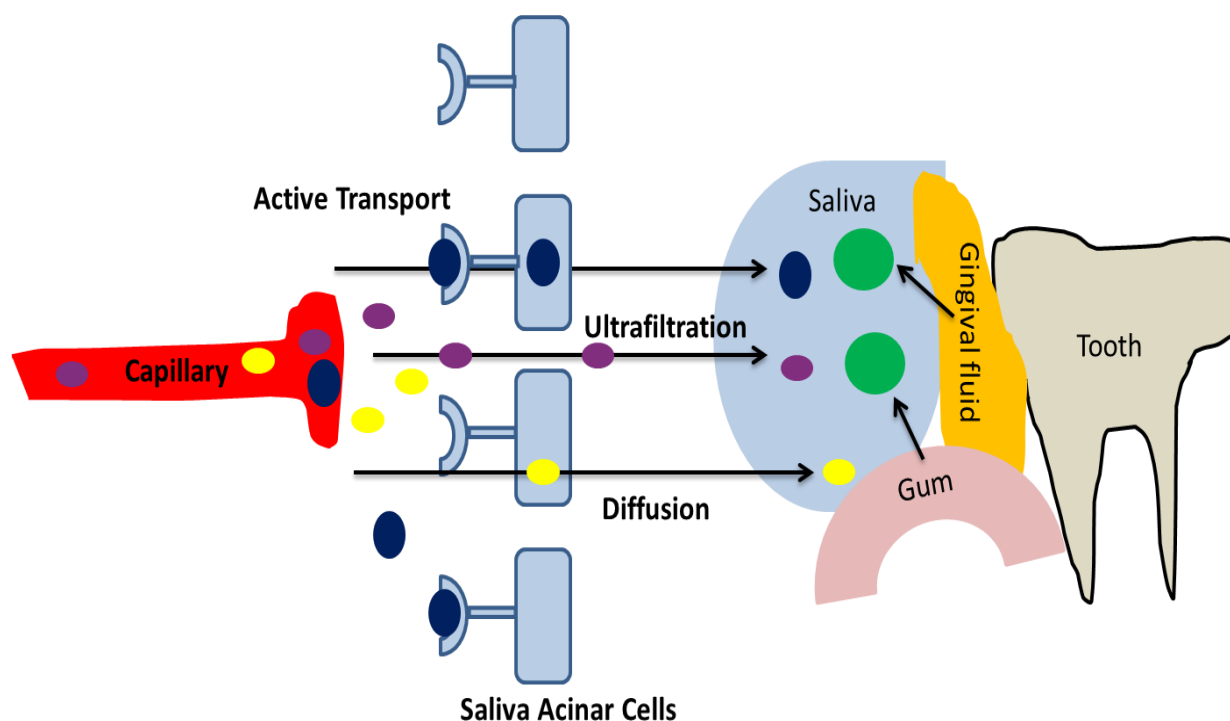


Figure 6: Overview of the Biomolecular Flow from Capillaries to Salivary Acinar Cells

From the capillaries, steroid hormones diffuse or actively transport into the saliva, whereas smaller molecules enter through the gap junctions. Large proteins can be transported through gingival fluid and gum tissue (Figure adapted from Punyadeera *et. al*, 2013).

Chapter 2: Materials & Methods

Saliva Collection and Enrollment of T1D Subjects

All human saliva collections were approved by the University of South Florida Institutional Review Board. 152 T1D patients of the Carol and Frank Morsani Diabetes Center at the University of South Florida, aged 18 or older, were enrolled. The study was explained to the subject and upon agreement, he/she was asked to passively drool in a 2 mL collection tube with a salivary collection aid attached to it. At least 0.3 mL of saliva was collected for triplicate analysis. Immediately upon receiving the saliva sample, a Thermo Scientific Halt™ protease and phosphatase inhibitor cocktail (EDTA-free) was added at 1x to prevent breakdown of proteins. The sample was centrifuged for 5 minutes at 5500 rpm in a refrigerated centrifuge set at -10°C. The saliva sample were distributed into 100 µL aliquots and stored at -80°C until assay. The metabolic parameters, such as BMI and HbA1c, in addition to duration of diabetes, race, sex, time of collection, and age were collected from each subject. Each subject was asked to answer two questions based on their oral health: 1) “Compared to others your age, how would you rate the present condition of your gums?” and 2) “Do you have a loose tooth?” The main steps of the enrollment process are summarized in Figure 7.

Quality Control Saliva Preparation

Commercially received pooled healthy human QC saliva (Innovative Research, Lot # 11656) was prepared in 100 µL aliquots with and without protease and phosphatase inhibitor. Thermo Scientific Halt™ protease and phosphatase inhibitor cocktail (EDTA-free) were added at 1x immediately when received. All samples were prepared in 100 µL aliquots and stored at -

80°C. QC saliva was diluted 1:2 in assay buffer prior to being tested to eliminate clogging of the probe in the Luminex Magpix® instrument.

Luminex Magpix® Assays

Measurement of Cytokine Levels

Cytokine levels were determined using a high sensitivity human cytokine multiplexed bead immunoassay (Millipore, Catalog number HSCYTMAG-60SK). T1D subject saliva, HSG lysate and media, and BNL lysate samples were performed in triplicate on each plate. Diurnal saliva samples were performed in duplicate for each plate. Prior to assay, further processing of the saliva samples included diluting them in a 1:2 ratio by adding 155 µL of saliva sample and 155 µL of Assay Buffer. Two quality control commercially received saliva samples (Innovative Research, Novi, MI) were also included in each run and they were diluted in a 1:2 ratio by adding 55 µL of sample and 55µL of Assay Buffer. Nine cytokines- IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, TNF-α, and IFN-γ- were measured using the high sensitivity human cytokine magnetic bead assay. The high sensitivity human cytokine magnetic bead kit provides a minimum detectable concentration of IL-1β (0.06 pg/mL), IL-2 (28.48 pg/mL), IL-4 (16.87 pg/mL), IL-6 (0.20 pg/mL), IL-8 (0.05 pg/mL), IL-10 (0.48 pg/mL), IL-13 (383.95 pg/mL), TNF-α (0.07 pg/mL), and IFN-γ (0.18 pg/mL). For every plate analyzed, concentrations were calculated by the StatLIA® Immunoassay Analysis software (Brendan Technologies), by measuring the true limits of detection for an assay by mathematically determining what the empirical Minimum Detectable Concentration (MinDC) would be if an infinite number of standard concentrations were run for the assay under the same conditions.

To start, 200 µL of Wash Buffer was added to each well and mixed on the plate shaker at 400 rpm for 10 minutes at room temperature. The Wash Buffer was decanted and residual amount was removed by inverting the plate and tapping it smartly onto absorbent towels several

times. 25 μ L of microsphere beads coated with monoclonal antibodies against the six different target analytes were added to the wells. Next, the plate was rested on a hand-held magnetic plate to allow complete settling of magnetic beads. The well contents were removed by gently decanting the plate and tapping on an absorbent towel to remove residual fluid. The plate was washed with 200 μ L of 1x Wash Buffer. The magnetic plate resting/wash process was repeated for a total of 2 times. The plate was then prepared by adding 50 μ L of each standard or control into appropriate wells. The 0 pg/mL standard (background wells) consisted of 50 μ L of Assay Buffer. 50 μ L of the diluted saliva samples was added in triplicate to the plate, resulting in a total of 24 samples to be analyzed per plate.

The plate was sealed, wrapped in foil, and incubated with agitation of 200-400 rpm on a plate shaker overnight at 4°C. The plate was warmed to room temperature, the contents removed, and washed twice. The detection bodies were warmed up to room temperature and 50 μ L was distributed to each well. The plate was sealed, protected from light, and incubated with agitation of 200-400 rpm on a plate shaker for 1 hour at room temperature. Then, 50 μ L of Streptavidin conjugated to the fluorescent protein, R-Phycoerythrin, was added to each well and incubated at room temperature, protected from light, on a plate shaker set to 200-400 rpm for 30 minutes. The well contents were removed and the plate washed twice. Lastly, 150 μ L of Wash Buffer was added to all wells and the beads were resuspended on a plate shaker set at 200-400rpm for 5 minutes. The plate was analyzed on the Luminex Magpix[®] using a 5-parameter logistic curve-fitting method for calculating cytokine concentrations in the saliva samples. This entire process is demonstrated in a brief summary in Figure 8.

Measurement of Salivary MMP Levels

MMP-3, MMP-8, and MMP-9 levels were determined using a multiplexed bead immunoassay (R&D systems Inc, Catalog number LMPM000, Minneapolis, MN). The high sensitivity human cytokine magnetic bead kits provide a minimum detectable concentration of

MMP-3 (35.3 pg/mL), (MMP-8 (195.47 pg/mL), MMP-9 (157.94 pg/mL). For every plate analyzed, concentrations were calculated by the StatLIA[®] Immunoassay Analysis software (Brendan Technologies), by measuring the true limits of detection for an assay by mathematically determining what the empirical Minimum Detectable Concentration (MinDC) would be if an infinite number of standard concentrations were run for the assay under the same conditions. Prior to assay, further processing of the saliva samples included diluting them in a 1:40 ratio by adding 10 μ L of saliva sample and 390 μ L of Calibrator Diluent.

Two quality control saliva samples were also included in each run and they were diluted in a 1:40 ratio by adding 10 μ L of sample and 390 μ L of Calibrator Diluent. MMP-3, MMP-8, and MMP-9 were measured using the high sensitivity human cytokine magnetic bead assay. 50 μ L of the microparticle cocktail, containing magnetic beads and MMP antibodies against the three target analytes, were added to every well of the microplate. The plate was then prepared by adding 50 μ L of each standard or control into appropriate wells. The 0 pg/mL standard (background wells) consisted of 50 μ L of Calibrator diluent. 50 μ L of the diluted saliva samples were added in triplicate to the plate, resulting in a total of 24 samples to be analyzed per plate.

The plate was sealed, wrapped in foil, and incubated with agitation of 200-400 rpm on a plate shaker for 2 hours at room temperature. Next, the plate was rested on a hand-held magnetic plate to allow complete settling of magnetic beads. The well contents were removed by gently decanting the plate and tapping on an absorbent towel to remove residual fluid. The plate was then washed with 100 μ L of 1x Wash Buffer. The magnetic plate resting/wash process was repeated for a total of 3 times. 50 μ L of the Biotin Antibody cocktail was distributed to each well. The plate was sealed, protected from light, and incubated with agitation of 200-400 rpm on a plate shaker for 1 hour at room temperature. Next, 50 μ L of Streptavidin conjugated to the fluorescent protein, R-Phycoerythrin, was added to each well and incubated at room temperature, protected from light, on a plate shaker set to 200-400 rpm for 30 minutes. The well contents were removed and the plate washed three times. Lastly, 100 μ L of Wash

Buffer was added to all wells and the beads were resuspended on a plate shaker set at 200-400 rpm for 5 minutes. The plate was analyzed on the Luminex Magpix[®] using a 5-parameter logistic curve-fitting method for calculating cytokine concentrations in the saliva samples.

Cytokine Array

We screened for 40 chemokines/cytokines for expression in 5.5 mM, 11 mM, and 22 mM-treated mouse liver BNL CL.2 (American Type Culture Collection, BNL CL.2, TIB-73) cells using a Mouse Cytokine Array Panel A (R&D Systems Inc, Catalog number ARY006, Minneapolis, MN). Cell lysate was collected from mouse liver BNL cells and used for this protocol. A nitrocellulose membrane coated with 40 different capture antibodies was added to a well of the 4-well multi-dish containing 2 mL of Array Buffer 6 and put on a rocking platform shaker for one hour. As recommended by R&D Systems Inc., 500 μ L of cell lysate was used and added to 0.5 mL of Array Buffer 4 and 1 mL of Array Buffer 6 was added to bring the final volume up to 1.5 mL. Next, 15 μ L of reconstituted Mouse Cytokine Array Panel A Detection Antibody Cocktail was added to each prepared sample, mixed, and incubated for 1.5 hours at room temperature. The well was aspirated and the sample/antibody mixtures were added. The well was incubated overnight at 2-8°C on a rocking platform shaker.

The membrane was then removed and placed into a plastic container with 20 mL of 1x wash buffer for 10 minutes on a rocking platform shaker. Two more washes were performed to make it a total of three washes. Streptavidin-HRP was diluted 1:2000 in Array Buffer 6, as recommended on the vial label, and added to the membrane for incubation of 30 minutes on a rocking platform shaker. Three washes were performed with 1x wash buffer and the membrane was placed on a plastic sheet protector and coated with 1 mL of Chemi Reagent Mix and incubated for one minute. The membrane was exposed for 10 minutes using standard chemiluminescence on a Fuji Image Reader LAS 3000.

Cotinine Measurement

A high sensitivity cotinine ELISA (Salimetrics, Catalog number 1-2002, State College, PA) was used to determine cotinine levels in all T1D and healthy saliva samples collected. Saliva samples were thawed on ice and centrifuged at 1500 g for 15 minutes to remove mucins and other particulate matter that may interfere with antibody binding. 20 μ L of standards, controls, and unknowns were pipetted into appropriate wells and were assayed in duplicate. Next, 100 μ L of conjugate solution was added to each well and then 100 μ L of antiserum was added to each well. Antiserum was not added to the nonspecific binding control wells. The plate was covered and incubated on a shaker for 1.5 hours at 37°C with constant mixing at 500-600 rpm. The plate was washed four times with 300 μ L of 1X wash buffer using the Multiwash III[®] microplate washer. Next, 200 μ L of TMB (tetramethylbenzidine) buffer was added to each well, mixed at 500 rpm for 5 minutes, and incubated in the dark for 25 minutes at room temperature. 50 μ L of stop solution was added and the plate mixed on a plate shaker at 500 rpm for 3 minutes. The plate was read using the VersaMaxPlus microplate reader within 10 minutes of adding stop solution at 450nm and correction at 630nm was calculated. Data was analyzed using the SoftMax Pro software version 5.4.1.

Cell Culture, Maintenance, and Propagation

The human submandibular gland (HSG) cell line was established from an irradiated human salivary submandibular gland [132] and was generously provided by Dr. Bruce Baum NIH/NIDCR. HSG cells were cultured using Dulbecco's Modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12) (catalog # 11330-032 GIBCO; Thermo Scientific, Rockford, IL) supplemented with 10% fetal bovine serum (FBS) (Hyclone; Thermo Scientific, Rockford, IL), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine in a CO₂ incubator maintained at 5% CO₂ and 37°C. BNL CL.2 cells (American Type Culture Collection, BNL CL.2, TIB-73) were cultured using Dulbecco's Modified Eagle's medium (DMEM) with 4.5 g/L glucose,

L-glutamine, and sodium pyruvate (catalog # 10-013-CV; Corning Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Hyclone; Thermo Scientific, Rockford, IL), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine in a CO₂ incubator maintained at 5% CO₂ and 37°C. When passaging the cells, the split ratio was performed at 1:10 and cells were detached from the tissue culture flask with 0.25% trypsin/1.0 mM ethylenediaminetetraacetic acid (EDTA) (Sigma–Aldrich Co., LLC St. Louis, MO). The DMEM/F12 and DMEM media were changed every two days, and cells were allowed to reach 80% confluence before exposure to glucose.

Glucose Treatment of Cell Lines

HSG and BNL cells were cultured as previously described and were seeded at 1 million cells per well in a 6-well culture dish. Following 24 hour overnight adherence of cells to the plate, the media in the wells were replaced with 3 mL of glucose-free media containing 1% FBS and starved for 5 hours. The glucose-free media containing 1% FBS was removed via pipetting. Cells were treated in duplicate with 1 mL of three different concentrations of glucose: 5.5mM, 11mM, and 22mM. These concentrations were determined from the calculations that reflect the high, middle, and low HbA1c recordings in the 152 T1D subject cohort previously mentioned. For example, the average HbA1c, 8.3%, is equivalent to 11mM using the formula for average blood glucose in mmol/L: $1.583 * \text{HbA1c}(\%) - 2.52$.

The BNL cell 6-well culture dish was incubated in a CO₂ incubator maintained at 5% CO₂ and 37°C for 24 hours to stimulate hyperglycemic conditions. The HSG cell 6-well culture dish was incubated in a CO₂ incubator maintained at 5% CO₂ and 37°C for 24 hours, 48 hours, and for 72 hours to stimulate hyperglycemic conditions. These differences in time to exposure were compared and analyzed. One experiment of 24 hour glucose stimulation was performed using BNL cells of similar passage number. Six repeat experiments of 24 hours, 48 hours, and 72

hours of glucose stimulation were performed using HSG cells of similar passage numbers. Cell media was collected and frozen at -80°C until assay.

Cell Lysate and Media Collection

HSG and BNL cells were washed with 1mL of sterile phosphate-buffered saline (PBS) and 300 µL of MPER (Mammalian Protein Extraction Reagent) was added to each well. For repeat experiments, 200 µL and 100 µL of MPER was utilized to maximize protein concentration. The solutions were collected and centrifuged at 10,000 rpm for 5 minutes. Supernatant was removed and stored in -80°C until assay. The total protein concentration was determined to normalize the amount of cytokines that were being released.

Bicinchoninic Protein Assay

The Microplate Procedure of the Bicinchoninic Assay (BCA) kit (Pierce, Thermo Scientific, Product number 23227, Rockford, IL) was performed to quantitate total protein content of all saliva collections, cell culture media, and cell lysate. Fresh standards (0, 25, 125, 250, 500, 750, 1000, 1500, and 2000 µg/mL) were prepared by diluting the contents of one Bovine Serum Albumin (BSA) Standard ampule (2 mg/mL albumin standard) into several tubes, using water or MPER as the diluent, for analysis of saliva or cell culture respectively. Each time the assay was performed, fresh standards were prepared. The determination of BCA Working Reagent volume was calculated utilizing the following formula: (# standards + # unknowns) x (# replicates) x (200 µL of Working Reagent) = total volume of Working Reagent required. The Working Reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part Reagent B (50:1, Reagent A:B). For a full microplate of 96 wells, 19.6 mL of Reagent A was added to 0.4 mL of Reagent B.

Once all reagents and standards were prepared, 25 µL of each standard and unknown sample (in duplicate or triplicate depending on available sample volume) were pipetted into a

microplate well. Next, 200 μ L of Working Reagent was added to each well and mixed on a plate shaker for 30 seconds, followed by an incubation at 37°C for 30 minutes. After cooling to room temperature, the plate was read at an absorbance of 562nm on the VersaMaxPlus microplate reader and data was analyzed using the SoftMax Pro software version 5.4.1.

Enzyme-Linked Immunosorbent Assay

To confirm trends discovered in the Luminex Magpix[®] kits, a multi-analyte ELISArray[™] kit (Catalog Number MEH-004A, Qiagen, Valencia, CA) was utilized to qualitatively measure levels of six cytokines: IL-1 β , IL-6, IL-8, IL-10, TNF- α , and IFN- γ . The protocol was followed per manufacturer's instructions. Sample Dilution Buffer 2 was utilized and saliva samples were not diluted as recommended by Qiagen. Data was analyzed using the SoftMax Pro software version 5.4.1.

An IL-8 ELISA (Enzyme-Linked Immunosorbent Assay) (R&D Systems Inc., Catalog number HS800, Minneapolis, MN) was performed to determine IL-8 levels in the HSG cell lysate and media to confirm the levels detected using the multi-plex Luminex Magpix[®] kit as previously mentioned. The protocol was followed as per manufacturer's instructions. The protocol calls for the subtraction of readings at 650 nm from readings at 450 nm. Analysis was performed on the results from this subtraction and data was analyzed using the SoftMax Pro software version 5.4.1.

An IL-13 ELISA (R&D Systems Inc., Catalog number D1300B, Minneapolis, MN) was performed to determine IL-8 levels in the HSG cell lysate and media to confirm IL-13 trends detected using the multi-plex Luminex Magpix[®] kit as previously mentioned. Data was analyzed using the SoftMax Pro software version 5.4.1.

Western Blotting Analysis

Human submandibular gland cells were lysed as previously described. Protein concentration was calculated using the BCA Protein Assay (Pierce, Fisher Scientific, Pittsburg, PA) as previously described. Protein concentration was normalized to the lowest sample. Premixed 4x Lammeli Sample Buffer (Bio-Rad Laboratories Inc., Catalog number 161-0737, Hercules, California) was diluted to 1x in samples. Samples were loaded in the appropriate volume (30 μ L or 50 μ L) to a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (4-15%, 10%, or 20%) gel. The gel was ran at 110 volts until adequate separation was achieved, typically 2 hours. The gel was transferred to a polyvinylidene difluoride (PVDF) membrane using a gel transfer stack (Novex, Life Technologies, Reference number IB401001) on the iBLOT™ (Invitrogen, iBlot®) apparatus.

The membrane was then washed for 10 minutes in TBST (Tris-buffered saline containing 0.1% Tween-20, Thermo Scientific, Rockford, IL) buffer on a plate rocker. The membrane was blocked for 1 hour in Startingblock™ T20 TBS Blocking Buffer (Thermo Scientific, Product number 37543). After blocking, the membrane was rinsed in TBST and the primary antibody, prepared with Startingblock™ buffer, was applied and incubated overnight at 4°C on a plate rocker. The next day, the primary antibody solution was collected and five 10 minute washes were performed. After all washes, an HRP-conjugated Goat Anti-Rabbit (Bio-Rad, Hercules, CA) secondary antibody was applied to the membrane and incubated at room temperature on a plate rocker for 1 hour. After incubation, five 10 minute washes were performed. Lastly, an enhanced chemiluminescence Western Blotting Substrate solution (Pierce, Thermo Scientific, Product number 32106) was applied to the membrane and exposed in a GE Amersham Imager 600 (GE Healthcare Life Sciences, Pittsburgh, PA) at the automated recommended time.

Statistical Analyses

Statistics were performed using the Tukey's Multiple Comparison, Two-Way Analysis of Variance (ANOVA), and One-way ANOVA tests using GraphPad Prism Version 5.04. Representation of p values is as follows: $p > 0.05$ (not significant), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

PCA and Prediction Model Analysis

PCA with orthogonal rotation was used to produce linear components of the cytokine and MMP variables with shared variance. Components may be thought of as independent constructs represented by the markers that load highly on that component. Factor loads to indicate correlations between individual markers were determined for each component. Prediction models, such as multiple linear (HbA1c) and logistic (gum health) regression analyses, were performed to examine the relationships between the PCA components and HbA1c and gum health. This analysis was adjusted for age, duration of diabetes, BMI, sex, and race. All data analyses were performed using SAS/STAT 9.3 software (SAS Institute Inc., Cary, NC).

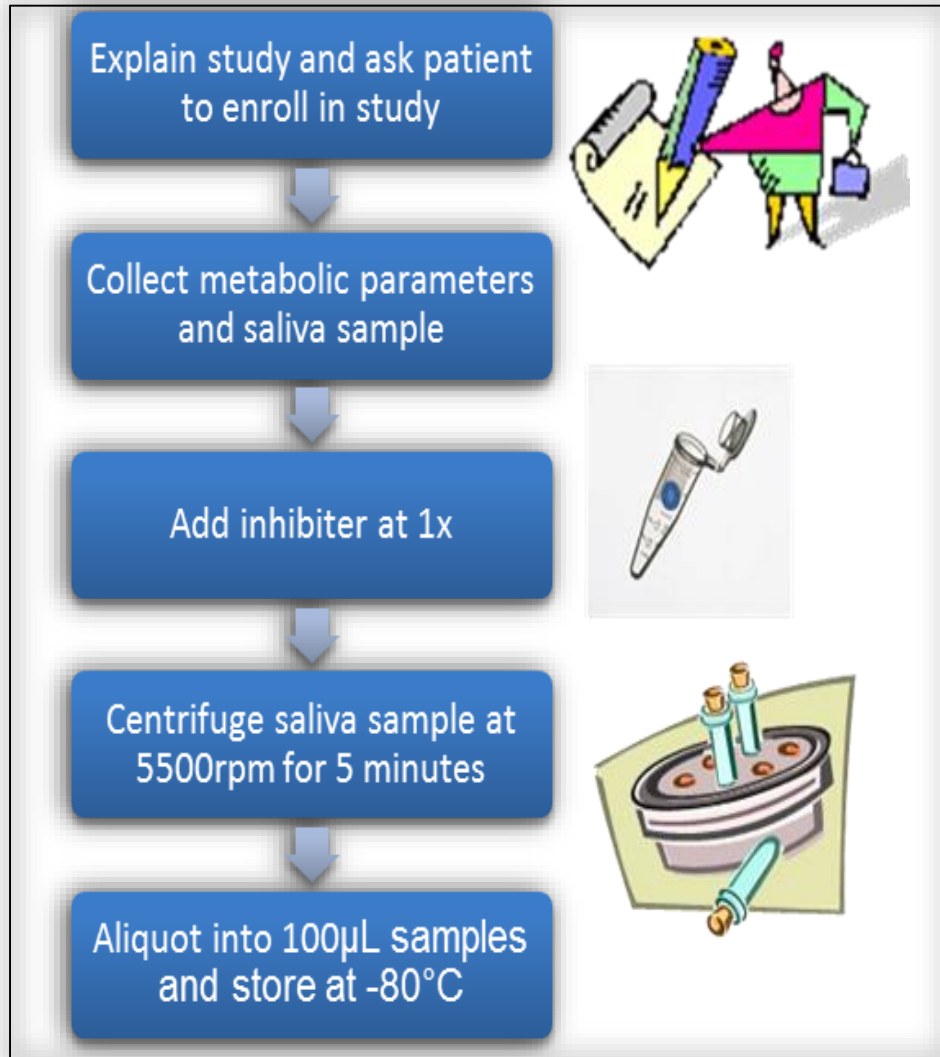


Figure 7: Overview of Enrollment and Collection Process

The main steps of the enrollment process and salivary collection for 150 T1D subjects are summarized here.

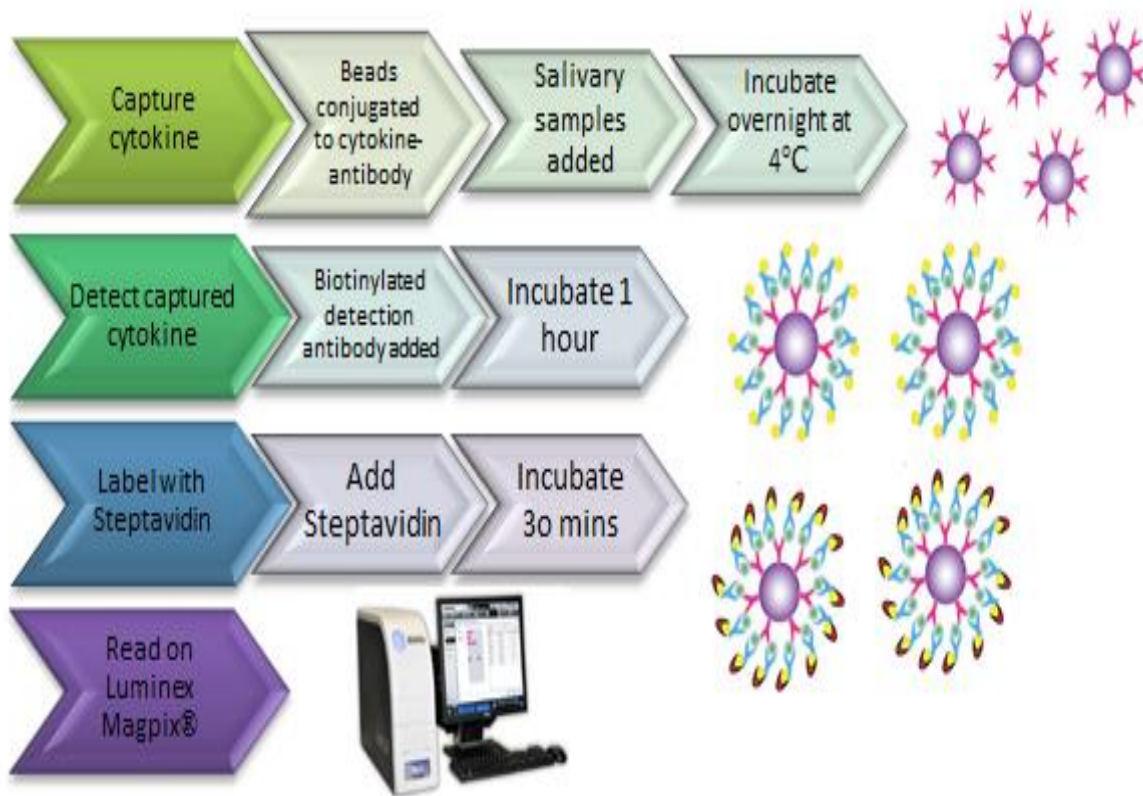


Figure 8: Detection of Cytokines Using Luminex Magpix® High Sensitivity Assay.

This scheme summarizes the important steps of detecting cytokines in salivary samples from T1D subjects (Picture of Luminex Magpix® instrument and computer adapted from www.millipore.com).

Chapter 3: Tumor Necrosis Factor-Alpha, Matrix-Metalloproteinases 8 and 9 Levels in the Saliva Are Associated With Increased Hemoglobin A_{1c} in Type 1 Diabetes Subjects

Note to Reader:

The majority of this chapter has been previously published in *Plos One* and have been reproduced with permission from *Plos One* Open Access. Dr. Burkhardt, Dr. Amy Alman, and Melanie Kuehl assisted with data analysis. Melanie Kuehl performed all data acquisition. Dr. Burkhardt, Melanie Kuehl, Dr. Amy Alman, and Dr. Henry Rodriguez assisted with study design.

Abstract

Background: Type 1 diabetes (T1D) is an autoimmune disease resulting in the targeted destruction of pancreatic β -cells and permanent loss of insulin production. Proper glucose management results in better clinical outcomes for T1D and provides a strong rationale to identify non-invasive biomarkers indicative or predictive of glycemic control. Therefore, we investigated the association of salivary inflammation with HbA_{1c} in a T1D cohort.

Methods: Unstimulated saliva was collected from 144 subjects with T1D at the USF Diabetes Center. BMI, duration of diabetes, and HbA_{1c} was recorded during clinical visit. Levels of interleukin (IL)-1 β , -6, -8, -10, IFN- γ , TNF- α , MMP-3, -8, and -9 were measured using multiplexing immunoassay analysis. To account for smoking status, salivary cotinine levels were also determined.

Results: Multiple linear (HbA_{1c}) and logistic (self-reported gingival condition) regression analyses were performed to examine the relationships between the Principal Component Analysis (PCA) components and HbA_{1c} and gingival condition (adjusted for age, duration of

diabetes, BMI, and sex; model for HbA1c also adjusted for gingival condition and model for gingival condition also adjusted for HbA1c). PCA components 1 (MMP-8 and MMP-9) and 3 (TNF- α) were significantly associated with HbA1c ($\beta=0.28 \pm 0.14$, $p=0.045$; $\beta=0.31 \pm 0.14$, $p=0.029$), while PCA component 2 (IL-6, IL-1 β , and IL-8) was significantly associated with gingival condition (OR 1.60 95% CI 1.09-2.34, $p=0.016$). In general, increased salivary inflammatory burden is associated with decreased glycemic control and self-reported gingival condition.

Conclusions: The saliva may represent a useful reservoir of novel noninvasive inflammatory biomarkers predictive of the progression and control of T1D.

Introduction

Periodontitis impacts as much as 47% of the U.S. population and is a significant cause for tooth loss in adults [5]. This destructive process is driven by bacterial infections that colonize the tooth root surface [6]. Due to this pathogenic event, immunological mediators are activated and various metabolic byproducts such as cytokines, chemokines and tissue-destructive enzymes such as matrix-metalloproteinases (MMPs) are released [7]. Spillover of these immunological mediators into the general circulation is thought to play a role in the development and exacerbation of systemic diseases, particularly poorly controlled diabetes, whereby a bi-directional relationship between periodontal disease and glycemic control has been suggested [9, 114]. Type 1 diabetes (T1D) is a highly complex polygenic autoimmune disease resulting in the loss of pancreatic β -cells and absence of insulin production [15]. While the relationship between periodontal disease and glycemic control has been demonstrated in T1D [117], the association between oral immunological mediators and glycemic control in T1D is not well understood and has not been precisely measured. The overall suspected relationship between periodontal disease and glycemic control provides a strong rationale for our central hypothesis that increased inflammatory burden and quantitative biomarkers of periodontal disease will be

associated with decreased glycemic control. To our knowledge, this has never been evaluated in a T1D cohort.

Saliva is a clear mucoserous exocrine derived liquid containing a mixture of secretions from the submandibular, parotid, sublingual and minor glands that provides a representation of overall health status and oral inflammatory burden [11, 102, 111]. Saliva can be obtained noninvasively, safely and economically with minimal processing and required training by personnel. Inflammatory molecules within the saliva are derived from the periodontium via influx of GCF and from the mucosa [133]. This bio-collection serves as a highly accessible and useful general measurement of oral inflammatory and periodontal burden. Despite the tremendous potential and utility of the saliva for the examination of biomarkers related to systemic disease, limited studies have been conducted in understanding and evaluating the salivary inflammatory burden specifically in T1D [108-110]. At present, numerous potential surrogate measures of existing periodontal disease and oral health have been identified and include cytokines and MMPs such as interleukin-1 β (IL-1 β), tumor necrosis factor (TNF)- α , and matrix metalloproteinase (MMP)-8 [111, 134-136]. The utility of these biomarkers has been demonstrated in terms of association with decreased oral health but there are currently no published reports to our knowledge that have examined the association between oral inflammation and levels of HbA1c within T1D.

To address this, we conducted an original study to examine the association between salivary inflammatory burden with glycemic control (HbA_{1c}) and self-reported gingival condition in adult T1D subjects recruited from the University of South Florida Diabetes Center.

Materials & Methods

Participants

A cross-sectional observation study of 152 T1D patients consecutively recruited from the Diabetes Center at the University of South Florida, aged 18 or older, was conducted to examine

the association between salivary inflammation and glycemic control. Subjects were recruited during regularly scheduled clinic visits. Of the 152 that were enrolled and that provided an unstimulated whole saliva sample (described below), 6 subjects were excluded from this analysis due to their saliva being very viscous and/or evidently contaminated with blood. Nine additional subjects were excluded from the MMP analysis due to inadequate quantity of saliva. Only 2 subjects approached refused to participate in the study on the basis of overall apprehension of salivary collection.

Since there are no published reports of the relationship between HbA1c and salivary cytokine levels in T1D, we have had to base sample size calculations on data from an independent cohort study of adult T1D that used serum measurements. The independent cohort we used did not have measurements for all of the inflammatory cytokines that we are proposing to measure, and due to the possibility that individual inflammatory markers may act on several different pathways to arrive at the same outcome, we based the sample size calculation on a measure of inflammatory burden. We would have a power of >80% to detect a difference in sample means of 0.29 in the inflammatory burden variable using a dichotomization of subjects on HbA1C of \geq mean vs. $<$ mean. The study was reviewed and approved by the University of South Florida Institutional Review Board. All participants provided informed written consent prior to participation in the study.

Clinical Data Collection

All enrolled patients in the study completed the oral health questionnaire. This questionnaire consisted of two questions regarding gingival condition: 1) Compared to others your age, how would you rate the current condition of your gums: poor, fair, good, excellent, and 2) Do you have a loose tooth? These questions were previously shown to be correlated with clinically determined periodontal health [137]. Additional information obtained from the patient

chart at the time of the clinic visit included body mass index (BMI), duration of diabetes, age, sex, race, and glycated hemoglobin (HbA_{1c}), a measure of glycemic control.

Saliva Collection

Each participant passively drooled in a 2 mL collection tube with an attached salivary collection aid (Saliva Biol LLC No. 61/524096, patent pending). Approximately 1 to 1.5 ml of saliva was collected from each subject. Immediately upon collection a protease and phosphatase inhibitor cocktail (EDTA-free Thermo Scientific Halt™, Thermo Fisher Scientific, Rockford, IL) was added at 1X to inhibit proteolysis. The sample was centrifuged for 5 minutes at 5500 rpm in a refrigerated centrifuge set at -10°C. The saliva sample was then distributed into 100 µL aliquots and stored immediately at -80°C until multiplexing analysis. Commercially received saliva (Innovative Research, Novi, MI) was also aliquoted and stored frozen to be later used within each multiplexing batch for the purpose of quality control.

Cytokine Analysis

Cytokine levels were determined using a multiplexed bead immunoassay and measured with a Luminex Magpix® instrument (Luminex, Austin, TX). Six cytokines: IL-1β, IL-6, IL-8, IL-10, TNF-α, and IFN-γ were measured using the high sensitivity human cytokine magnetic bead assay (EMD Millipore, Cat No. HSCYTMAG-60SK-06, Billerica, MA) following manufacturer instructions as described in Chapter 2. Quality control samples of non-T1D saliva were included in each assay run to account for any potential intra or inter-assay variability to ensure that % CV did not exceed 3%. Measurements were performed in triplicate.

MMP Analysis

Human matrix metalloproteinase (MMP) levels were determined using a multiplexed bead immunoassay as described in Chapter 2. Three MMPs: MMP-3, MMP-8, and MMP-9

were measured using the high sensitivity human MMP Base magnetic bead assay (R&D systems Inc, Catalog number LMPM000, Minneapolis, MN). The plate was analyzed on the Luminex Magpix[®] instrument as described in Chapter 2 for determination of cytokine concentration. Measurements were performed in triplicate.

Cotinine Analysis

Salivary cotinine levels were measured utilizing a commercial ELISA (Salimetrics, Catalog Number 1-2002, Carlsbad, CA) according to manufacturer's instructions as described in Chapter 2. Cotinine levels (ng/ml) were determined from 20 μ l of saliva and measured in duplicate. Due to saliva volume limitations of T1D subjects, cotinine levels were determined for 95 of the 144 subjects. Results are reported as means and standard deviations.

Statistical Analysis

Data are presented as means and standard deviations for continuous variables and as the number of subjects and percent for categorical data. Distributions of all of the cytokines and MMPs were found to be skewed and were log transformed for all analyses. For these variables, the data are presented as the geometric mean and interquartile range. All cytokines were adequately measured within the linear range of the generated standard curve with the exception of IFN- γ which was only measured in 2.1% of all salivary samples analyzed and therefore excluded from further analyses. Cotinine-derived smoking status was determined using a cutoff value of 15 ng/ml [138, 139].

Principal components analysis (PCA) with orthogonal rotation was used to produce linear components of the cytokine and MMP variables with shared variance. PCA is a variable reduction technique used to account for redundancy between variables by producing uncorrelated components that account for a meaningful amount of the variance contained in the original set of variables but that can be used simultaneously in a regression analysis.

Components may be thought of as independent constructs represented by the markers that load highly on that component. Interpretability of the final solution was a major determinant of the minimum number of components to be used in the analyses. Individual cytokines were considered to load highly on a given component with factor loads >0.6 . Factor loads indicate the correlation of individual markers on each component. Multiple linear (HbA_{1c}) and logistic (gingival condition) regression analyses were performed to examine the relationships between the PCA components and HbA_{1c} and gingival condition.

Both the linear (HbA_{1c}) and logistic (gingival condition) models were adjusted for age, sex, duration of diabetes, and BMI. The HbA_{1c} model was additionally adjusted for gingival condition and the gingival condition model was additionally adjusted for HbA_{1c}. Cotinine-derived smoking status, race, and time of saliva collection were evaluated as potential confounders and not found to alter the parameter estimates for the PCA components in either model by more than 10%, so these were not retained in the final models. All data analyses were performed using SAS/STAT 9.3 software (SAS Institute Inc., Cary, NC).

Results

Study Design and Concentrations of Cytokines and MMPs in Saliva

152 T1D subjects were enrolled in this study. Subjects 066 and 074 were not tested or analyzed due to missing information. Subject 031 was not analyzed for MMP levels due to lack of saliva sample quantity. The overall characteristics of the subject population are shown in Table 1 and Figure 9. The mean age of subjects in this study population was 35.8 (± 16.5) years. The study population had slightly more females than males (59% vs 41%) and was predominantly white (79.2%). The mean duration of diabetes was 18.4 (± 12.9) years, with a mean HbA_{1c} of 8.3 (± 1.7), and a mean BMI of 27.4 (± 6.3). Saliva was collected mostly in the afternoon (12:00 pm to 4:00 pm) and the majority of subjects (97%) reported no loose teeth.

The geometric means of the analytes ranged from 0.25 pg/ml for TNF- α to 305.0 ng/ml for MMP-9. Figures 10 and 11 portray the mean levels of MMP and cytokine concentrations, respectively, of commercially received quality control saliva ran on all six Luminex Magpix[®] plates. Figures 12 and 13 demonstrate the mean MMP and cytokine levels of the 144 T1D cohort, respectively.

Cotinine Analysis

Cotinine is a bi-product of nicotine and its levels can be detected up to 4 days for light smoking, 5 days for medium smokers, up to 10 days in heavy or addicted smokers, and for more than 10 days in people who chew tobacco [140]. Nicotine can only be detected up to 10 hours in the saliva [140], therefore we measured cotinine levels. Ninety-five subjects were analyzed for smoking status through detection of cotinine in the saliva. Cotinine values indicated that 18 (19.0%) were current smokers or exposed to second-hand smoke. Figure 14 shows the ELISA results of cotinine levels in the T1D participants' saliva.

Total Salivary Protein Levels

Total salivary protein was analyzed in each T1D subject (Figure #) following the protocol from the BCA protein kit as previously described in Chapter 2. Due to limited sample volume, only 47 T1D subjects could be analyzed. We did not observe any significant differences in protein content, as shown in Figure 15.

Principal Components Analysis

Table 2 presents the 5-component solution derived from the PCA. MMP-8 and MMP-9 were positively correlated with component 1 with factor loads of 0.89 and 0.88, respectively. IL-6, IL-1 β , and IL-8 (factor loads of 0.82, 0.70, and 0.63, respectively) were highly correlated with component 2. Components 3, 4, and 5 were correlated with single analytes, TNF- α (factor load

of 0.84), IL-10 (factor load of 0.87), and MMP-3 (factor load of 0.79), respectively. Subjects with high values on any or all of these components would also have high values for the respective markers that loaded highly on the component.

Multiple linear and logistic regression model of PCA components

Table 3 presents the results of the multiple linear regression model of the PCA components on HbA_{1c}, adjusted for age, sex, race, BMI, duration of diabetes, and gingival condition. In this model, a significant linear association was found between PCA component 1 (MMP-8 and MMP-9 loaded highly) and PCA component 3 (TNF- α loaded highly) with HbA_{1c} (β 0.28 \pm 0.14, $p=0.045$; β 0.31 \pm 0.14, $p=0.029$; respectively). The other PCA components were not found to be linearly associated with HbA_{1c} ($p>0.05$).

Table 4 summarizes the results of the multiple logistic regression model of the PCA components on the self-reported gingival condition. PCA component 2 (in which IL-6, IL-1 β , and IL-8 loaded highly) was significantly associated with poorer gingival condition (OR 1.60, 95% CI 1.09-2.34; p -value 0.016) after adjustment for age, duration of diabetes, HbA_{1c}, BMI, and sex. This result suggests that high values for these markers are associated with decreased gingival condition. None of the other PCA components were significantly associated with gingival condition ($p>0.05$).

Discussion

This study demonstrated that specific salivary inflammatory markers in T1D subjects are associated with decreased glycemic control. Two principal components were associated with decreased glycemic control. The inflammatory markers that loaded strongly on these components were MMP-8, MMP-9, and TNF- α . This is the first study that we are aware of to

examine the association of multiple salivary inflammatory biomarkers with glycemic control and self-reported gingival condition in T1D subjects.

Prior studies examining salivary inflammatory levels within general systemic diseases, T1D, and T2D have demonstrated that specific mediators of inflammation are elevated within the saliva of these respective cohorts. A large study examining the salivary concentrations of 1000 adults in southern Sweden for levels of IL-1 β , -6, -8, TNF- α , and MMP-8 (similar to inflammatory markers examined in this study) measured increased levels of: (1) IL-8 in subjects with tumor and bowel diseases, (2) MMP-8 in those following cardiac surgery or with diabetes and muscle diseases and (3) IL-1 β , -8, and MMP-8 in those having muscle or joint diseases [135]. With regard to salivary inflammatory burden in T1D, Dakovic *et al.* examined IL-8 salivary levels in T1D subjects with or without concomitant periodontitis versus the non-T1D cohort (n=20 per group) [141]. Measured levels of IL-8 were significantly elevated in T1D subjects as compared to the non-T1D group. Within the T1D cohort, IL-8 levels were not associated with either periodontitis or clinical parameters. In respect to T2D, Yoon *et al.* examined unstimulated saliva samples in 192 subjects with or without T2D and revealed that IL-1 β concentration in saliva was mainly associated with the degree of periodontal disease not diabetes [111].

Another investigation demonstrated that poor glycemic control (HbA_{1c} > 8) was significantly associated with increased IL-1 β levels in GCF in T2D [142]. In a later report, IL-8 levels did not associate with increased HbA_{1c} [143]. Taken together, these findings solidify the central hypothesis that salivary inflammatory burden can be associated with diseases of autoimmunity, metabolic control and periodontitis. However, the specific relationship between certain cytokines such as IL-1 β and either glycemic or periodontal status can be contradictory and requires further characterization.

Proteomic and peptidomic analysis has revealed significant differences in the saliva between those subjects with T1D and periodontitis versus those with T1D and without

periodontitis [108-110]. Concordantly, a recent study evaluating 153 subjects with T1D or T2D examined the proteomic profile of these individuals stratified by their HbA_{1c} levels ranging from <7 to >10 [144]. PCA and cluster analysis revealed that salivary proteomic changes were associated with HbA_{1c} sub-groupings and to some extent supported our findings in that systemic glycemic levels are reflected within the salivary milieu in an HbA_{1c} dependent manner. Their findings revealed that salivary proteomes are distinctly segregated when compared with low (HbA_{1c} <7), medium (8-9), and high (>10) HbA_{1c} levels. The proteomic changes based on HbA_{1c} were stronger in T1D rather than T2D subjects and the identified salivary proteins associated with HbA_{1c} changes in individual samples included albumin, hemoglobin, alpha-2-macroglobin, serum amyloid A, sereotransferrin, and numerous others. Interestingly, neither cytokines nor MMP's were identified within the salivary proteome as associated with HbA_{1c} within T1D. This may have been attributed to a sensitivity issue whereby these inflammatory mediators are potentially below the detection level for this proteomic approach. There was no mention of periodontal status within this study. Nonetheless, their findings in combination with our report clearly demonstrate that glycemic levels as reflected by HbA_{1c} can be associated and represented in the saliva as measured by various biomarkers that can originate from the salivary gland, serum, or host immune system.

A recent report by *Engbretson et al.* revealed that periodontal intervention (non-surgical) failed to promote glycemic control in T2D subjects displaying moderate to advanced chronic periodontitis [145]. These findings would be somewhat discordant with our conclusions indicating that increased inflammatory burden is association with decreased glycemic control. However, systematic reviews and meta-analysis of numerous studies examining this relationship have indicated that periodontal treatment can result in modest reduction of HbA_{1c} in combination with improvement of periodontal status in T2D subjects [146-149]. This type of comprehensive analysis has yet to be as extensively examined in T1D subjects with varied periodontal status as this was the primary cohort of our described study. Nonetheless, our

results are certainly consistent with the overall theme that oral inflammatory levels are associated with glycemic control and potentially autoimmunity in T1D.

A potential weakness of our study is the lack of a non-T1D control group. The primary focus of this study was to measure and determine the association of salivary inflammation with glycemic control within T1D. Since glycemic control is an important component of T1D clinical management, but not for those without diabetes, we felt that this population was particularly relevant for this initial study. Previous literature has examined the gross comparison of salivary inflammation between diabetic (T1D and T2D) and non-diabetic controls and therefore, we did not intend to repeat these examinations [111, 141]. While we are not able to perform a comparison with non-diabetic controls, we are able to demonstrate that salivary inflammatory markers are significantly associated with increasing HbA_{1c} in a linear model, after adjustment for potential confounders.

Another potential limitation of this study is absence of a clinical dental exam with recorded measurement of pocket depth and bleeding on probing. Unfortunately, we were unable to perform a clinical exam due to both economic and logistical reasons. However, the primary objective of this study was to measure and examine quantitative measures of salivary inflammation with glycemic status in a T1D cohort. Clinical measures of periodontitis and periodontal inflammation obtained from an examination is strictly a qualitative determination of the inflammatory response. For our analysis, clinical measurements would certainly have provided some utility but would not have provided the quantitative assessment needed for our objectives. Nonetheless, we still accounted for this via self-reported gingival condition. Measurement of inflammatory mediators in the saliva provides a more comprehensive analysis of oral inflammation [133]. In addition, the inflammatory mediators utilized within this study that had the greatest association with increased HbA_{1c} levels (TNF- α , MMP8 and MMP9) have all been previously documented to be increased and associated with decreased periodontal status [136, 150, 151]. Therefore, it is reasonable to speculate that those individuals with elevated

inflammatory burden also potentially had decreased periodontal health. Future studies are anticipated to include a comprehensive dental exam in combination with measurement of inflammatory burden and glycemic status.

Salivary diagnostics has tremendous translational potential for numerous biological and technical reasons. The outstanding utility of the saliva for serving as a clinical focal point during routine dental examinations or physician visits and potentially enabling large investigational studies certainly warrants this effort. As compared to blood collection, salivary evaluation is relatively easy to obtain with high patient compliance (in our study only 1% of subjects declined participation) and can be performed by minimally trained personnel with little post-collection processing. Our study has suggested that the salivary inflammatory burden may also be an indication of glycemic status or clinical management in T1D. These data can be further utilized to establish novel clinical diagnostic tools to promote patient compliance and enhance subsequent clinical application of interventional therapy. In addition, oral inflammatory burden with biomarkers described in this publication may also be combined with other biomarkers (i.e. autoantibodies in the case of T1D) either circulating or from the saliva that can be implemented to generate predictive models to identify subjects in the early stages of either developing autoimmunity or glucose intolerance.

Acknowledgements

Support for this study was provided by an Early Career Investigator Award to ACA from the USF College of Public Health. The authors would like to thank all participants and the supporting staff of the USF Diabetes Center. In particular, the authors would like to particularly thank Mrs. Danielle Henson for her efforts in the coordination of this study population. The authors report no conflict of interest.

Table 1: T1D Subject Characteristics (n=144)

Characteristics	
Age (years)*	35.8 ±16.5
Male (n)†	59 (41.0)
Race/ethnicity (n) †	
White	114 (79.2)
Black	13 (9.0)
Hispanic	12 (8.3)
Other	5 (3.5)
Duration of diabetes (years)*	18.4 ±12.9
HbA1c (%)*	8.3 ±1.7
BMI (kg/m²)*	27.4 ±6.3
BMI category (n)†	
Underweight (<18.5 kg/m²)	5 (3.5)
Normal (18.5-<25 kg/m²)	52 (36.1)
Overweight (25-<30 kg/m²)	51 (35.4)
Obese (≥30 kg/m²)	36 (25.0)
Cotinine (ng/ml)‡,§	1.1 (0.3–1.7)
Current smoker (n; cotinine > 25 ng/ml)†,§	18 (19.0)
Time of day of saliva collection (n)*	
Morning (8:50 am to 11:58 am)	54 (37.5)
Afternoon (12:00 pm to 5:07 pm)	90 (62.5)
Condition of gums (n)†	
Excellent	20 (13.9)
Very Good	43 (30.0)
Good	53 (36.8)
Fair	25 (17.4)
Poor	3 (2.1)
Has a loose tooth (n)†	5 (3.5)
IL-6 (pg/ml)‡	1.8 (0.61-6.8)
IL-8 (pg/ml)‡	54.6 (22.2-118.3)
IL-10 (pg/ml)‡	6.9 (2.6-18.6)
IL-1β (pg/ml)‡	1.1 (0.27-4.7)
TNF-α (pg/ml)‡	0.25 (0.13-0.50)
MMP-3 (pg/ml)‡, 	197.5 (77.3-377.0)
MMP-8 (ng/ml)‡, 	84.1 (35.5-183.6)
MMP-9 (ng/ml)‡, 	305.0 (140.4-680.7)

*Data presented as mean ±SD, †Data presented as number (%), ‡Data presented as geometric mean (25th-75th percentile), §N=95, ||N=135

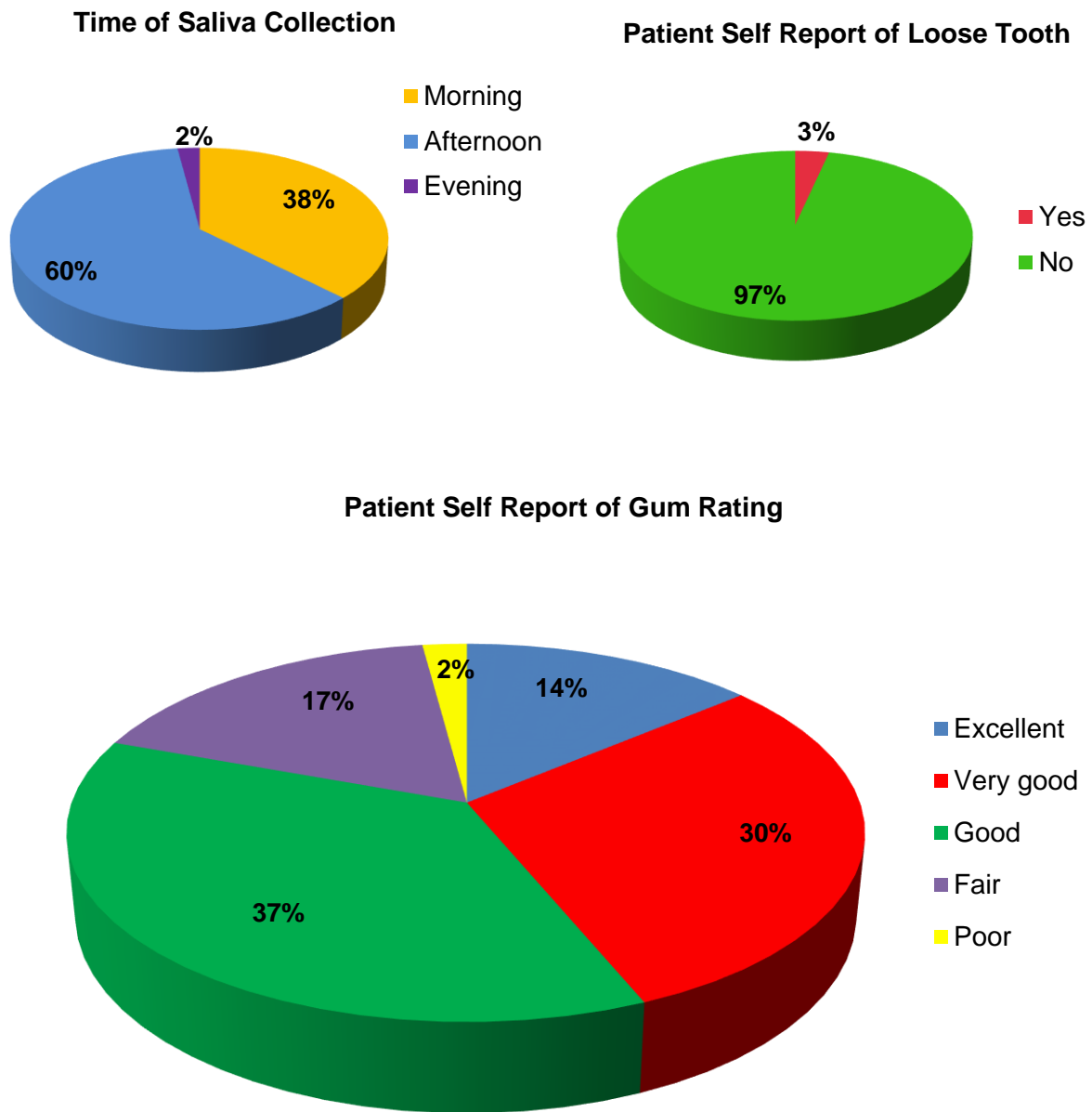


Figure 9: Statistical Collection Data of 144 T1D Subjects

(A) Patient saliva collection times. Morning is defined as 12:00am to 11:59am, afternoon is defined as 12:00pm to 4:00pm, and evening is defined as 4:59pm to 12:00am. **(B)** Percentage of answers to “Do you have a loose tooth?” **(C)** Percentages of answers to “Compared to others your age, how would you rate the present conditions of your gums?”

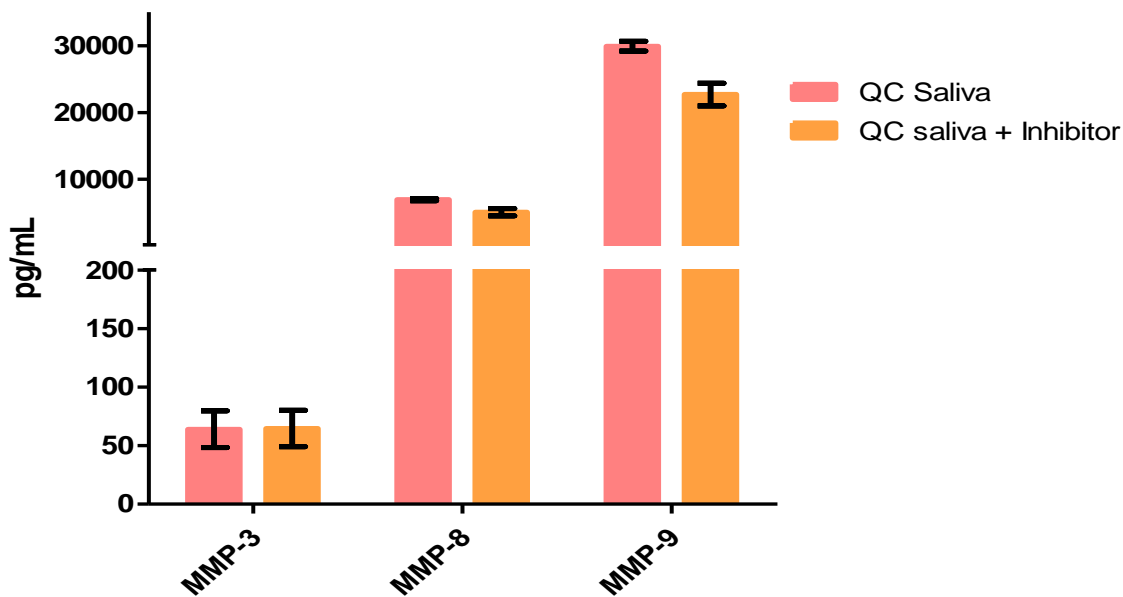


Figure 10: Mean MMP Levels of QC Saliva Samples

Commercially received Quality Control saliva was included on each Luminex Magpix® plate with the T1D saliva samples. Mean MMP levels were measured in these quality control samples with and without inhibitor to determine to demonstrate minimal inter-assay variability.

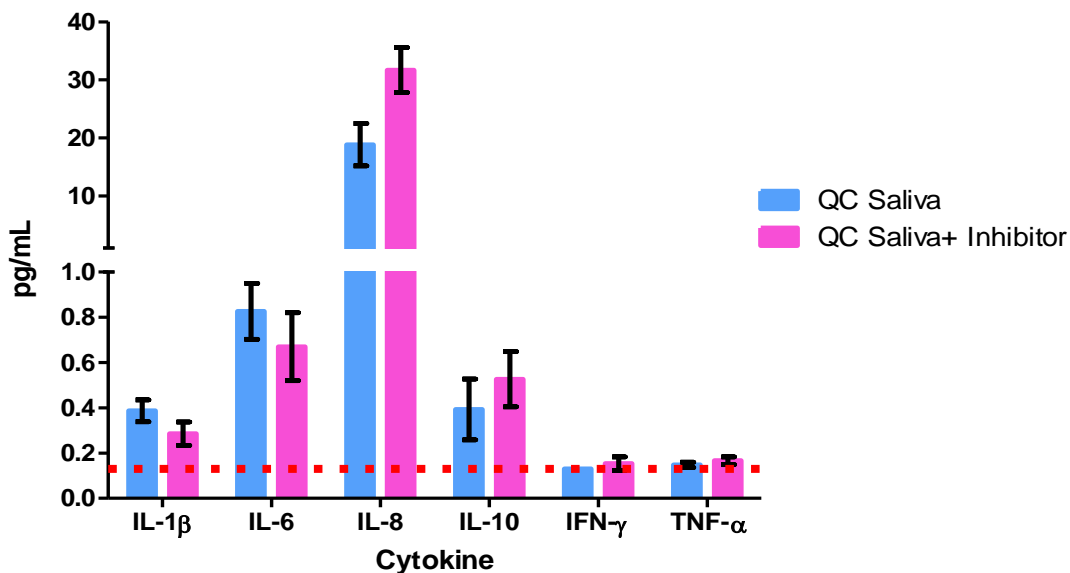


Figure 11: Mean Cytokine Levels of QC Saliva Samples

Commercially received Quality Control saliva was included on each Luminex Magpix® plate with the T1D saliva samples. Mean cytokine levels were measured in these quality control samples with and without inhibitor to determine to demonstrate minimal inter-assay variability. The red dotted line indicates the minimum detection concentration of the assay, which was 0.13 pg/mL. Error bars represent SEM.

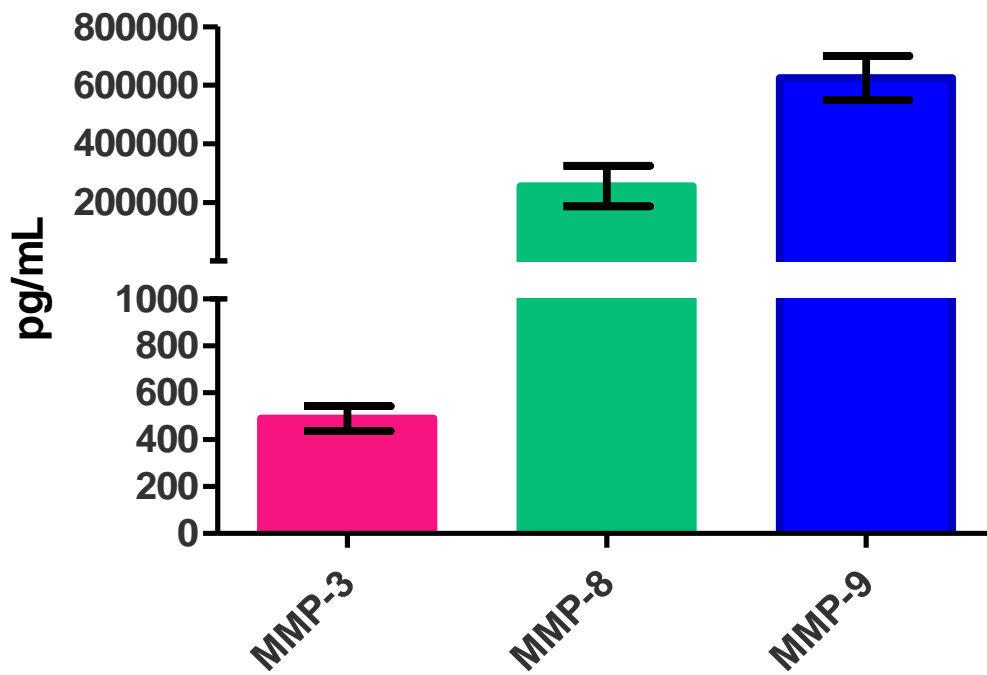


Figure 12: Mean MMP Levels of 144 T1D Subjects

Total combined salivary MMP levels of 144 T1D subjects across all 6 Luminex Magpix® plate runs. All results fell within the standard curve from mid- to low-range. Minimum detection levels were the following: MMP-3: 35.3 pg/mL, MMP-8: 81.9 pg/mL, and MMP-9: 100.3 pg/mL. Error bars represent SEM.

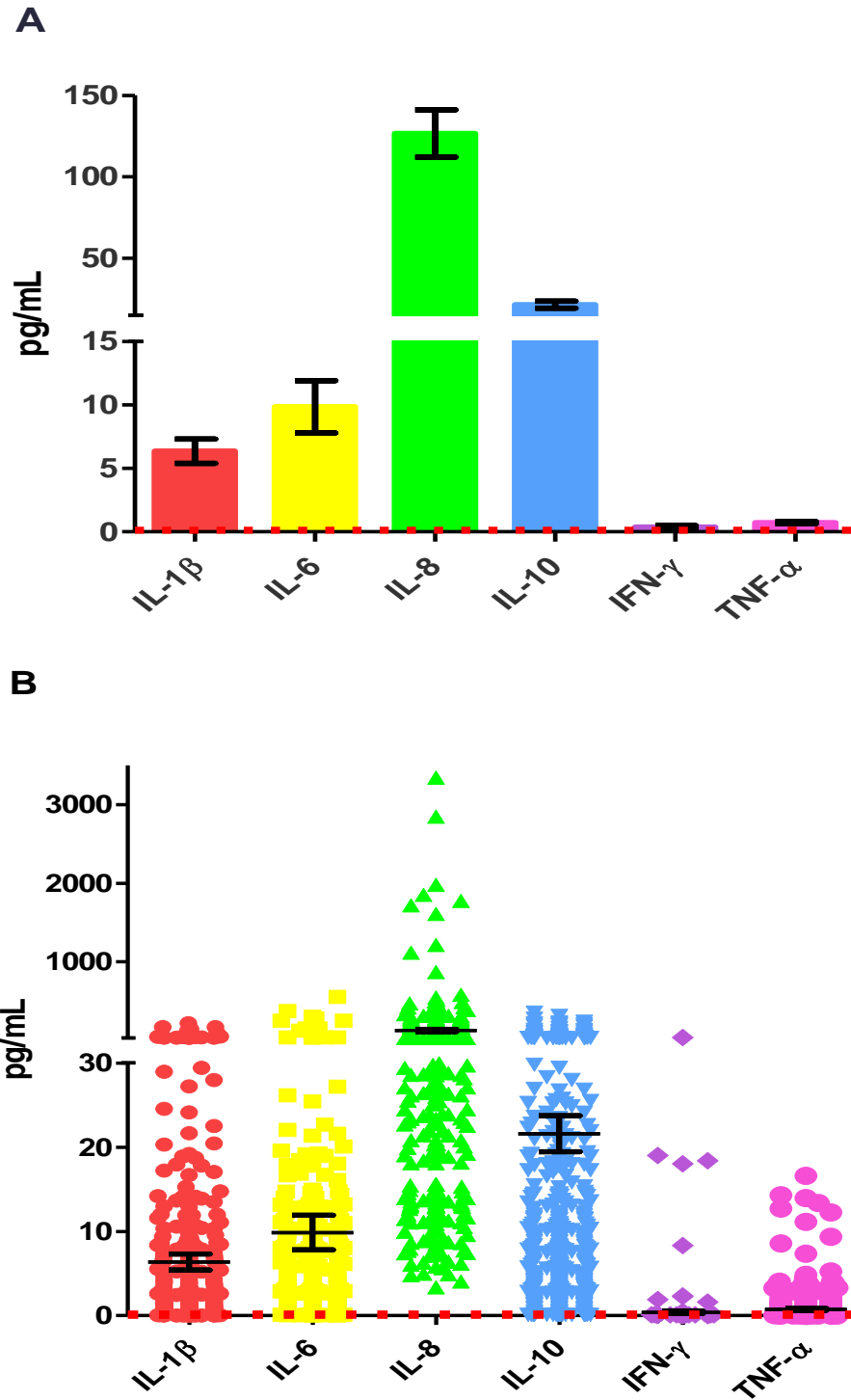


Figure 13: Mean Cytokine Levels of 144 T1D Subjects

Total combined salivary cytokine levels of 144 T1D subjects across all Luminex Magpix[®] plate runs. **A)** Shows the total mean in bar chart form. **B)** Demonstrates a scatter plot of all 144 T1D subjects. All results fell within the standard curve from mid- to low-range. The red dotted line indicates the minimum detection concentration of the assay, which was 0.13 pg/mL. Error bars represent SEM.

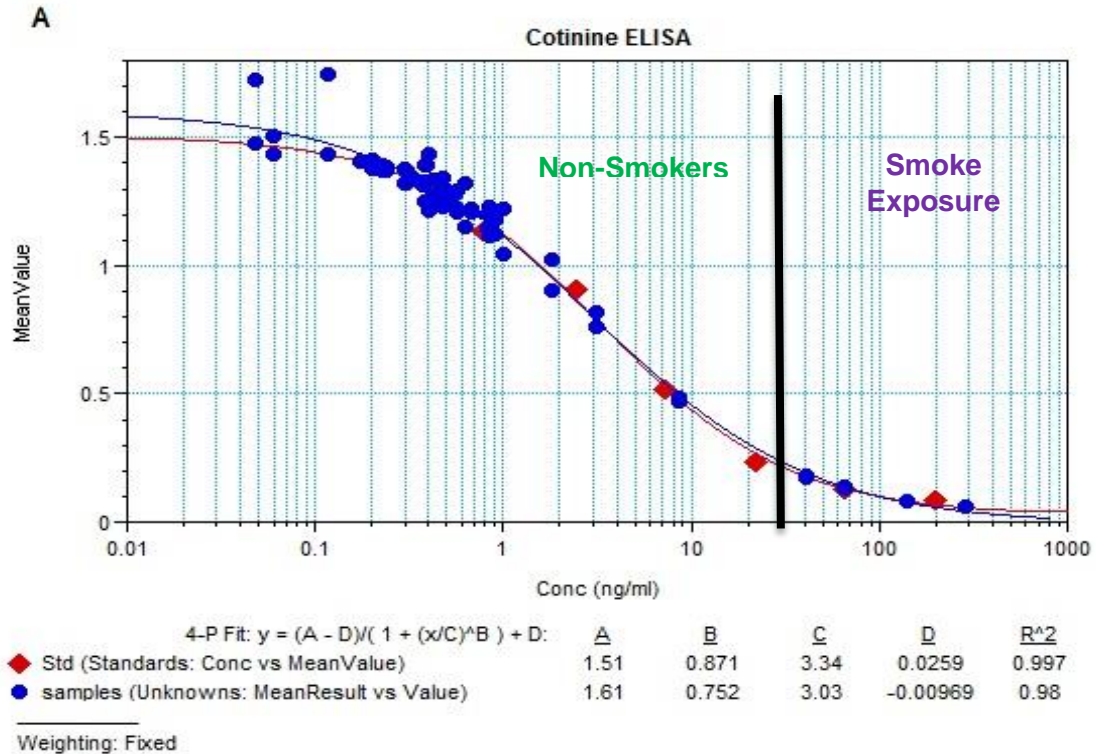


Figure 14: Cotinine Levels of 95 T1D Subjects

Unstimulated whole saliva was collected from 95 T1D subjects and their saliva was analyzed on 3 ELISA cotinine plates. **(A)** Mean result of 37 subjects (performed in duplicate) from the subject range 001 – 053, **(B)** Mean result of 39 subjects (performed in duplicate) from the subject range 054-130, **(C)** Mean result of 19 subjects (performed in duplicate) from the subject range 131-152. The standard curve is represented by red diamonds and the blue dots indicate each individual saliva sample. The standard curve ranged from 0.8 ng/mL to 200 ng/mL. Any result greater than 25 ng/mL indicates a smoker or an involvement of second-hand smoke.

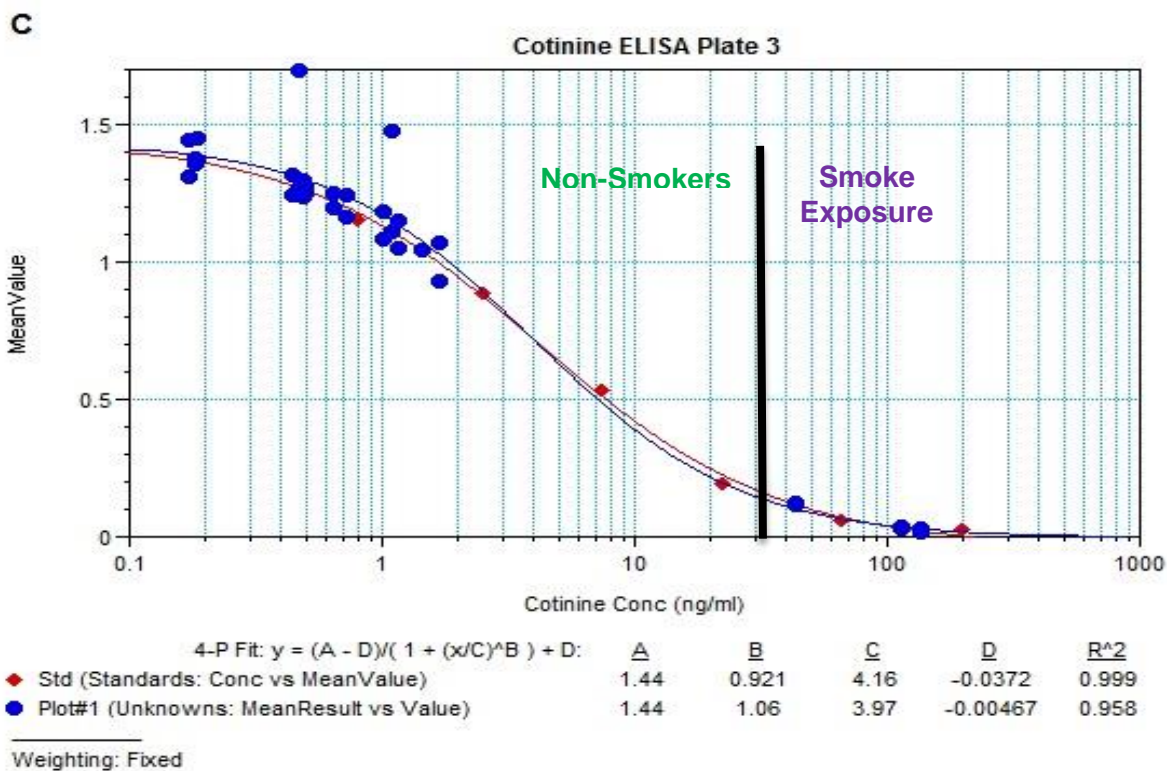
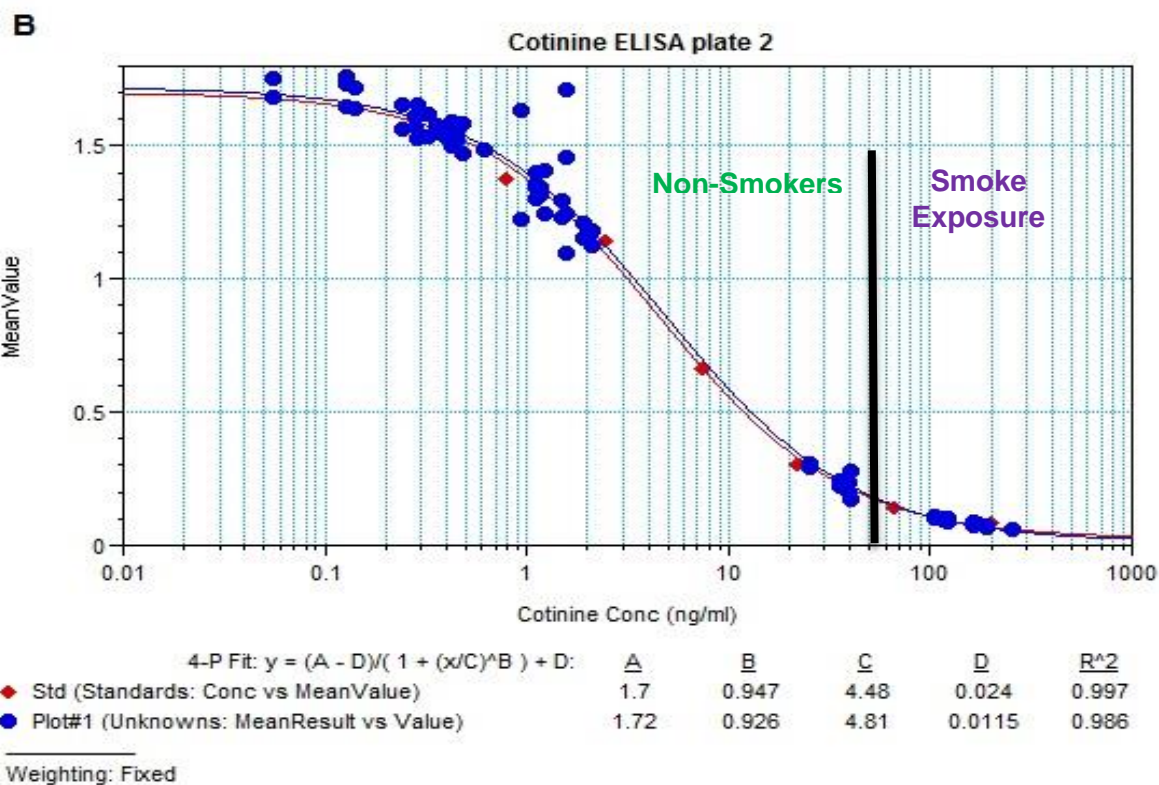


Figure 14 continued

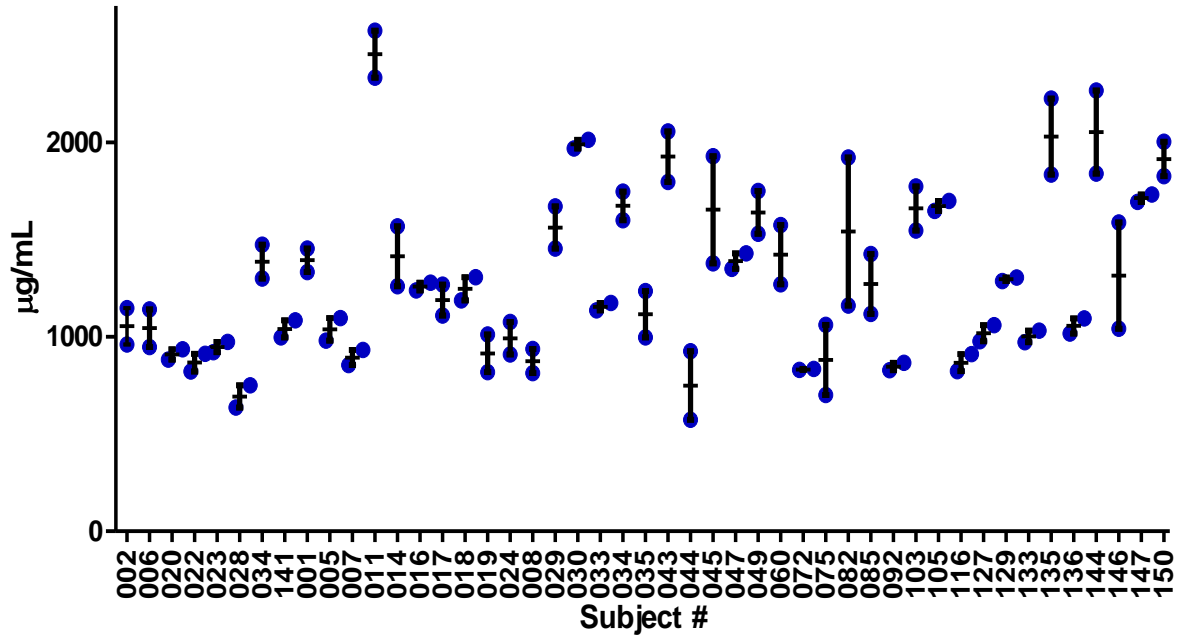


Figure 15: Salivary Protein Levels of 47 T1D Patients

Remaining saliva from 47 T1D subjects was tested using a BCA protein kit (Thermo Scientific, product number 23227, Rockford, IL). Due to limited sample, not all saliva could be analyzed. Standard curve ranged from 0 µg/mL to 2000 µg/mL. Bars represent the median result of the duplicate samples.

Table 2: Principal Components Analysis with Orthogonal Rotation of the Individual Cytokines and MMPs

Component 1		Component 2		Component 3		Component 4		Component 5	
Marker	Load*	Marker	Load*	Marker	Load*	Marker	Load*	Marker	Load*
MMP-8	0.89 [†]	IL-6	0.82 [†]	TNF- α	0.84 [†]	IL-10	0.87 [†]	MMP-3	0.79 [†]
MMP-9	0.88 [†]	IL-1 β	0.70 [†]	IL-1 β	0.40	IL-6	0.27	IL-6	0.34
IL-8	0.49	IL-8	0.63 [†]	IL-8	0.35	TNF- α	0.26	MMP-9	0.24
IL-1β	0.44	TNF- α	0.35	IL-10	0.24	IL-8	0.26	MMP-8	0.19
MMP-3	0.39	MMP-3	0.33	MMP-3	0.21	MMP-3	0.22	IL-10	0.19
IL-10	0.25	IL-10	0.28	IL-6	0.19	MMP-9	0.20	TNF- α	0.19
TNF-α	0.23	MMP-8	0.26	MMP-8	0.19	MMP-8	0.17	IL-8	0.15
IL-6	0.16	MMP-9	0.22	MMP-9	0.15	IL-1 β	0.15	IL-1 β	0.11

*Factor loads are determined by the Pearson correlation coefficient of the marker on the component, [†]Factor loads >0.6 were considered for the interpretation of the component

Table 3: Multiple* Linear Regression of PCA Components on HbA1c

Marker	$\beta \pm SE$	p-value
PCA Component 1 (MMP-8 & MMP-9)	0.28 \pm 0.14	0.045
PCA Component 2 (IL-6, IL-1β, & IL-8)	-0.02 \pm 0.14	0.901
PCA Component 3 (TNF-α)	0.31 \pm 0.14	0.029
PCA Component 4 (IL-10)	-0.11 \pm 0.14	0.421
PCA Component 5 (MMP-3)	0.21 \pm 0.14	0.123

*Adjusted for age, duration, BMI, sex, and gingival condition

Table 4: Multiple* Logistic Regression of PCA Components on Condition of Gums (Poor + Fair + Good vs. Very Good + Excellent)

Marker	OR (95% CI)	p-value
PCA Component 1 (MMP-8 & MMP-9)	1.16 (0.79, 1.70)	0.463
PCA Component 2 (IL-6, IL-1β, & IL-8)	1.60 (1.09, 2.34)	0.016
PCA Component 3 (TNF-α)	0.75 (0.52, 1.10)	0.141
PCA Component 4 (IL-10)	1.07 (0.74, 1.54)	0.717
PCA Component 5 (MMP-3)	1.05 (0.72, 1.53)	0.798

*Adjusted for age, duration of diabetes, HbA1c, BMI, sex

Chapter 4: Measurement of Diurnal Pattern of Oral Inflammation in Healthy Adults

Abstract

There has been an increased interest in utilizing the saliva as a diagnostic fluid, particularly with regard to assessing markers of inflammation for indicators of systemic diseases, periodontitis, or stress. However, future clinical utility of the saliva cannot be fully assessed without a detailed examination of standard circadian rhythm for these potential markers. In addition, numerous inflammatory diseases such as rheumatoid arthritis (RA) have revealed that onset of symptoms can correlate with diurnal fluctuations. This suggests that circadian rhythm may also regulate disease pathology. To examine the daily fluctuation of inflammation to provide necessary understanding into these potential diagnostic parameters, we measured levels of IL-1 β , IFN- γ , IL-6, IL-8, IL-10, and TNF- α simultaneously in the saliva of 12 healthy young adults. Saliva samples were collected 6 times a day according to individual's sleep schedule (wake, wake + 30 minutes, wake + 3 hours, wake + 6 hours, wake + 9 hours, and immediately preceding sleep). This study demonstrated that salivary inflammatory markers have unique diurnal patterns and may affect the overall inflammatory response. Additionally, diurnal patterns of IFN- γ and TNF- α remained elevated in the morning over three consecutive days. In summary, we have revealed that specific cytokines are regulated by circadian rhythm and indicate that utilizing these as biomarkers for systemic disease must be noted and observed in accordance with time of measurement with regard to sleep schedule.

Introduction

Saliva is a clear mucoserous exocrine derived liquid containing a mixture of secretions from the [102] three major glands involved in secretion: parotid, submandibular, and sublingual. The parotid gland is the largest and secretes serous saliva, while the others secrete saliva containing mucous [104]. Saliva has a multitude of biological functions including: lubrication, enamel maintenance, buffering action, food clearance, preservation of tooth integrity, inhibition of bacterial colonization and activity, and mastication and swallowing [103]. Typically, the average healthy adult produces 0.5 to 1.5 L of saliva daily, with an approximate rate of 0.5 ml/min [152].

Saliva contains serum constituents and certain hormones that correlate to blood concentrations and therefore has high potential to mirror systemic conditions [102, 103]. Saliva harbors a wide spectrum of components, including steroid hormones and IgA, that enter the saliva from blood capillaries by passive diffusion of lipophilic molecules, ultrafiltration through gingival crevices, or through active transport of proteins via ligand-receptor binding [11, 153]. It has been shown that amines and peptides, such as melatonin and insulin, enter the saliva through these methods and exhibit an excellent correlation between saliva and blood concentrations [106]. Several aspects of saliva and ease of collection make it an ideal diagnostic fluid for disease and overall health surveillance particularly in comparison to serum or plasma.

There has been an increase of interest in using saliva as a diagnostic medium over the past 10 years [11]. Saliva does provide a true representation of overall health as the salivary glands are exocrine glands that produce strongly detectable protein profiles of health status at the moment of collection [11]. Progress has been made in the development of salivary biomarkers for indication of several diseases such as cardiovascular disease [154], oral cancer [155], and breast cancer [156]. Saliva's use as a diagnostic fluid has been hindered due to a lack of understanding of biomolecules within saliva and their relevance to disease etiology, combined

with the diurnal/circadian variations of these biomolecules [11]. If saliva is to be utilized in the future as a non-invasive source of biomarkers indicating systemic disease, biological variation in association with diurnal rhythms must be demonstrated.

There are some limitations to salivary research. Salivary composition is not constant and varies with changes in flow. Saliva is composed of immunoglobulins, proteins, mucins, enzymes, and a variety of electrolytes all of which are multifunctional, redundant, and amphifunctional and contribute to the modulation of demineralization and remineralization of teeth, antimicrobial/antibacterial actions, oral microorganism activity of dental plaque metabolism, and oral pH [103]. In addition, the pH of saliva is not constant. The typical pH of saliva is slightly acidic being 6 to 7, however, salivary flows may alter the pH depending on flow rate from 5.3 (low flow) to 7.8 (high flow) [103]. Furthermore, salivary flow rates fluctuate depending on circadian rhythms, which also affect the concentrations of salivary components, such as inflammatory cytokines and matrix metalloproteinases (MMPs).

Inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis (RA), have shown a clear association with diurnal rhythms [85, 157]. Circadian clock genes can be turned off by inflammatory mediators (i.e. TNF- α) or these genes can impact the intestinal epithelial cell permeability leading to inflammation [85]. In RA patients, cytokine-mediated inflammation fluctuates on a diurnal pattern of the release of melatonin and cortisol and joint symptoms are most severe in the early morning [83, 158]. Furthermore, the circadian system in the human body has many influences on aging and disease progression. Significant disruption and aging in the circadian rhythms of the sleep-wake cycle can lead to the development of Alzheimer's, Parkinson's, and Huntington's disease [159, 160].

Increasing evidence supports a complex relationship between circadian rhythms and the regulation of both the innate and adaptive immune systems [86]. Circadian rhythm disruption has been shown to accelerate the progression of diabetes in mice [84]. A knockout of the circadian rhythm CLOCK genes, *Arntl1* and *Clock*, led to the activation of the NF- κ B pathway

and increased inflammation triggering the onset of diabetes, whereas the ablation of the clock gene, aryl hydrocarbon receptor nuclear translocator (ARNT), impaired insulin secretion, implemented abnormal glucose tolerance, and altered islet gene expression in mice [84]. In addition, there is evidence of the clock period circadian protein, PER3, length polymorphism in T2D patients [161]

Previous literature has indicated that plasma cortisol and possibly melatonin seem to regulate diurnal variations of cytokine production [83]. One study demonstrated that salivary IL-6 and C-reactive protein (CRP) levels peaked in the morning, but CRP remained constant while IL-6 peaked again at bedtime in 27 healthy individuals [162]. Circulating IL-6 and CRP levels showed similar trends indicating there could be a shared mechanism involving the regulation of diurnal patterns of cytokines in blood and saliva [162, 163]. Further research needs to be conducted to determine the impact of circadian rhythms on oral inflammation.

The human mouth is home to a diverse collection of bacteria, viruses, fungi, protozoa, and archaea, termed the oral microbiome, and consists of both pathogenic and commensal microbes [164]. For a variety of reasons, commensal microbes can become pathogenic or allow pathogens to colonize, leading to a disease state [164]. Little research has been done to investigate the variations in human responses to the oral microbiome and further research needs to be done to comprehend its role in the development of human diseases. Additionally, no studies to date have comprehensively examined the diurnal rhythms of the oral microbiome from saliva within healthy subjects.

To demonstrate biological variation within diurnal rhythms, we examined longitudinal saliva samples collected from healthy non-T1D donors and measured a panel of cytokines previously demonstrated to have potential diagnostic properties. The diurnal rhythms of saliva have been examined in twelve healthy adults. Samples were collected based on each individual's sleep schedule. In addition, we included commercial saliva (Innovative Research, Novi, MI) samples on each plate run to show consistency across plates.

To address this, we conducted an original study to examine the circadian rhythms of six inflammatory cytokine expressions within each individual. We chose these six cytokines- IL-1 β , IL-6, IL-8, IL-10, and TNF- α - since they play a role in the progression of diabetes, hyperglycemia, and/or glucose production or have been found to have high levels in periodontitis or T1D tissue [10, 58, 61, 63, 66, 68, 165]. It has been concluded that salivary cytokine concentration independently fluctuates based on diurnal rhythms. Inflammation is generally highest in the early morning and late at night.

Methods

Participants

Twelve healthy individuals aged 20-42 (mean age of 26.5) were recruited to participate in this study. The sample consisted of seven females and five Caucasian males. The mean body mass index of the participants was 23.08 kg/m² and all but one were non-smokers. None reported gross infections, immune disorders, or oral/dental disease, and none used any medications or dietary supplements that affect immune activity. The study was reviewed and approved by the University of South Florida Institutional Review Board. All participants provided informed written consent for participation in the study.

Saliva Collection

Saliva samples for IL-1 β , IL-6, IL-8, IL-10, and TNF- α measurements were obtained. The participants were asked to passively drool into a 2 mL collection vial with an attached salivary collection aid (Saliva Biol LLC No. 61/524096 patent pending) over six time-points throughout the day based on each subject's sleep schedule (Wake, Wake +30 minutes, Wake + 3 hours, Wake + 6 hours, Wake + 9 hours, and Prior to Sleep). Two of the subjects were asked to repeat this sampling procedure on 3 consecutive days to test the stability of the diurnal patterns. The passive drool collection method is recommended by previous studies as it

provides the least interference with the salivary immunoassay results for the cytokines measured [162]. Unstimulated whole saliva often correlates to systemic clinical conditions more accurately than stimulated saliva, since materials used to stimulate flow may change salivary composition [166]. The collection vials contained a protease and phosphatase inhibitor cocktail (EDTA-free Thermo Scientific Halt™, Thermo Fisher Scientific, Rockford, IL) at 1x to assist in the preservation of the saliva sample. In general, sampling was performed at the participants' homes with supplied and labeled tubes with instructions.

Following collection, saliva samples were frozen in the participants' home freezers and transported in insulated containers with ice-packs to laboratory following completion of the collected samples. The sample was centrifuged for 5 minutes at 4000 rpm in a refrigerated centrifuge set at 4°C. The saliva sample was then distributed into 100 µL aliquots and stored immediately at -80°C until multiplexing analysis. Commercially received saliva (Innovative Research, Novi, MI) was also aliquoted and stored frozen to be later used within each multiplexing batch for the purpose of quality control.

Procedures

The participants were instructed to provide saliva samples 6 times throughout the day (at wake, +30 minutes from wake, +3 hours from wake, +6 hours from wake, +9 hours from wake, and bedtime). The participants did not have to change their sleep/wake schedule for this study and each was encouraged to keep their normal sleep/wake routine. Two of the participants were asked to repeat this sampling procedure on 3 consecutive days to test the stability of the diurnal patterns. All participants were instructed to refrain from eating, drinking, heavy exercising, or brushing their teeth for 30 minutes after wakening and for 1 hour prior to each sampling time to eliminate effects of masticatory stimulation and reduce food debris. Written instructions about the saliva collection protocol were distributed. All participants completed an oral health questionnaire. This questionnaire consisted of two questions regarding gingival

condition: 1) Compared to others your age, how would you rate the current condition of your gums: poor, fair, good, excellent, and 2) Do you have a loose tooth? These questions were previously shown to be correlated with clinically determined periodontal health [56, 137].

Cytokine Analysis

Cytokine levels were determined using a multiplexed bead immunoassay and measured with a Luminex Magpix[®] instrument (Luminex, Austin, TX). Six cytokines: IL-1 β , IL-6, IL-8, IL-10, TNF- α , and IFN- γ were measured using the high sensitivity human cytokine magnetic bead assay (EMD Millipore, Cat. No. HSCYTMAG-28SK, Billerica, MA) following manufacturer instructions as described in Chapter 2. Quality control samples of non-T1D saliva were included in duplicate in each assay run to account for any potential intra or inter-assay variability.

Cotinine Analysis

Salivary cotinine levels were measured utilizing a commercial ELISA (Salimetrics, Catalog Number 1-2002, Carlsbad, CA) according to manufacturer's instructions as described in Chapter 2. Cotinine levels (ng/mL) were determined from 20 μ L of saliva and measured in duplicate. A measurement greater than 25 ng/mL indicated that the subject was exposed to second-hand smoke or was smoking up to 4 days prior to collection. Results are reported as means and standard deviations.

ELISA Analysis

Six cytokines- IL-1 β , IL-6, IL-8, IL-10, and TNF- α – were analyzed using a Qiagen Multi-Analyte ELISArray[™] kit (Catalog Number MEH-004A, Valencia, CA) qualitatively. The protocol was followed per manufacturer's instructions as described in Chapter 2. Sample Dilution Buffer 2 was utilized and saliva samples were not diluted as recommended by Qiagen.

Results

Participant Characteristics

Table 5 displays the characteristics of the 12 subjects who participated in this study. Table 6 provides a summary of the twelve healthy subjects enrolled, as well as their metabolic parameters including BMI and self-reported periodontal condition. We enrolled 5 Caucasian males, 2 Hispanic and 5 Caucasian females. The mean age was 26.5 and the mean BMI was 23.08 kg/m². None of the participants were considered obese or were taking any chronic medications for a documented medical condition. The average amount of time for the “Prior to sleep” collection time was 16.08 hours. The cotinine ELISA test showed that only one individual was considered to be exposed to second-hand smoke or was smoking on the day of collection.

Total Salivary Protein Levels

Total salivary protein was analyzed in each individual throughout the day at six different time-points (Figure 16) following the protocol from the BCA protein kit as previously described in Chapter 2. The Tukey’s Multiple Comparison and One-way ANOVA tests show that there is a significant difference ($p < 0.05$) in total protein content between “Wake” and all other time-points. The Two-way ANOVA test analyzed the total protein content of individual subject’s saliva throughout the day. Nine out of the twelve subjects showed a statistical difference between “Wake” vs. “Prior to sleep” total salivary protein content.

Diurnal Variations of Salivary Cytokines

Saliva was collected from twelve healthy subjects during six time-points throughout the day based on each individual’s sleep schedule. Additionally, commercially received quality control saliva was included on each plate and the mean levels are described in Figure 17. As shown in Figures 20-25 and Table 7, diurnal patterns were unique to each individual, yet for certain individuals, all cytokine levels peaked at the same time-point. Subject 01 was generally

inflamed in the evening, with the exception of IFN- γ , which was detectable in the middle of the day and IL-1 β , which peaked in the morning (there were significantly higher cytokine levels at “W + 6 hours” compared to the “Wake” and “Prior to Sleep” time-point, with the exception of IL-1 β , which was higher at “Wake” than at “W + 6 hours”). In Subject 02, there were significantly higher cytokine levels in the morning, whereas Subject 08 showed higher cytokine levels immediately before going to sleep. In Subject 03, cytokine levels were significantly higher at “Wake” than all other time-points and IFN- γ levels were undetectable. Subjects 04 and 11 demonstrated similar diurnal patterns in which all detectable cytokine levels were significantly higher at “Wake” with the exception of IL-10 and IL-6, respectively, which were most elevated at “Prior to Sleep”. In Subject 05, cytokine levels were significantly higher at “Prior to Sleep” than “Wake”, with the exception of IL-8, which was lowest at “Wake +3 hours” and highest at “Wake +6 hours”. There were mostly undetectable or no trend in cytokine levels for Subjects 06, 07, 09, and 12. In Subject 08, cytokine levels peaked at “Prior to Sleep”, with the exception of IFN- γ , which peaked at “Wake + 9 hours” time-point. In Subject 10, all cytokine levels were highest at “Wake + 9 hours” and/or “Prior to Sleep” time-points.

Figure 18 and Figure 19 portray the diurnal patterns of all six cytokines throughout the day in all subjects combined. IL-10 levels were significantly higher ($p < 0.01$) immediately before sleep than at wake for six subjects. Inversely, IL-1 β levels were significantly higher at wake than in the afternoon and evening for seven subjects. IL-10, TNF- α , and IFN- γ were mostly undetectable, however six of the subjects expressed higher levels of IFN- γ in the evening. Due to undetectable levels of certain time-points, ANOVA could not be performed for TNF- α and IFN- γ in all twelve subjects. Subjects 01 and 12 were the only subjects to have detectable levels of IL-10 in all time-points. There was a substantial error bar for IL-6 detection due to the fact that Subject 04 showed high levels of IL-6 (~400 pg/mL) at the “Wake” time-point compared to the rest of the subjects (~5 pg/mL). Nonetheless, when dropping out these values

in the combined graph, there is no trend for IL-6 production. To verify accurate results, an additional Luminex plate run was performed for Subjects 07-12.

Consecutive Diurnal Patterns

To test for diurnal pattern stability, two subjects, Subject 01 and Subject 02, provided saliva samples for six time-points throughout each day for three consecutive days. IL-1 β and TNF- α were analyzed for the three consecutive day analysis since they demonstrated the strongest diurnal trends. The trends were similar for each day: IL-1 β and TNF- α levels were mostly elevated in the morning in each subject as shown in Figure 26 and Figure 27, respectively.

Cotinine Analysis

Cotinine is a bi-product of nicotine and its levels can be detected up to 4 days for light smoking, 5 days for medium smokers, up to 10 days in heavy or addicted smokers, and for more than 10 days in people who chew tobacco [140]. Nicotine can only be detected up to 10 hours in the saliva [140]; therefore we measured cotinine levels. All twelve subjects were analyzed for smoking status through detection of cotinine in the saliva. Only one subject (Subject 02) was exposed to or had been smoking up to 4 days prior to collection. Figure 28 demonstrates these results.

ELISA and Luminex Magpix[®] Comparison

Figure 29 compares the ELISA and Luminex Magpix[®] results using two healthy adult female subjects: Subject A and B. These results have demonstrated the sensitivity of the Luminex assay and support our reasoning behind using the Luminex Magpix[®]. The trends of cytokine expression have been confirmed in the ELISA. Using an independent method shows

the same relative differences and relationships amongst cytokines, however as concentrations get lower, the ELISA method is unable to discriminate these relationships. Although the same relationships are shown with ELISA, the Luminex is much more sensitive.

Discussion

This study investigated the diurnal patterns of salivary IL-1 β , IL-6, IL-8, IL-10, IFN- γ , and TNF- α in healthy young adults. Although previous studies demonstrated the diurnal variations of salivary IL-6 [162, 167], to our knowledge, this is the first study to simultaneously examine diurnal levels of IL-1 β , IL-8, IL-10, and TNF- α throughout the day. Cytokines have their own specific diurnal rhythms and their concentrations are typically highest in either the early morning or late night. Generally speaking, there is a trend of an increase in expression of pro-inflammatory (Th1) cytokines, such as IL-1 β and TNF- α , in the morning and an increase in expression of anti-inflammatory (Th2) cytokines, such as IL-10, in the evening.

We found that the levels of TNF- α and IL-1 β remained elevated in the morning over three consecutive days. To our knowledge, this is the first study to analyze these salivary cytokines over three consecutive days. One study analyzed the diurnal patterns of salivary C-reactive protein and IL-6 in healthy adults over two consecutive days revealing CRP levels to be more stable than IL-6 [162]. Yet, there is much more information needed to examine the daily patterns and stability of salivary cytokines.

Studies have shown evidence of circadian rhythms having an impact on diabetes. A previous study demonstrated hyperglycemic conditions from an impairment in glucose tolerance and insulin secretion when the pancreatic clock gene, BMAL1, is knocked down in mice [168]. Knockdown of other clock genes, such as ARNT and CRY, have disclosed an abnormal glucose tolerance, increased inflammation, and altered islet gene expression in mice [84]. Another

study revealed that when diet-induced obesity is complimented with circadian disruption in male rats, β cells are impaired and subsequent type 2 diabetes occurs [169].

There is a possibility that melatonin levels could affect the inflammation of cytokines since it has an anti-inflammatory effect by reduced expressions of iNOS, IL-1 β , and IL-6 activities [170]. This study revealed that melatonin suppressed acrolein-induced IL-8 production in human pulmonary fibroblasts. Additionally, melatonin is suggested to play a role in restoring insulin resistance and impaired glucose intolerance derived from the disturbance of circadian rhythms [171]. This evidence further supports the importance of circadian rhythms in the development of diabetes.

To date, there are no studies that investigate the daily variations of cytokines and chemokines in human saliva. In a particular study, the daily variations of cytokines and chemokines in tears of healthy individuals were examined. Concentrations of 18 analytes were measured using multiplexing technology and IL-10 levels were undetectable in the afternoon yet detectable only in the evening, whereas IL-6 and IL-1 β levels were higher in the evening [172]. This is somewhat consistent with our results in that most of the IL-10 levels were undetectable in the twelve subject cohort. Additionally, six out the twelve subjects expressed higher IL-6 levels in the evening hours, five out the twelve subjects expressed higher IL-1 β levels in the evening. In general, although we were mostly unable to detect IL-10, IFN- γ , and TNF- α levels, they still may have critical diagnostic value.

It is known that the diurnal rhythm of plasma cortisol creates a higher expression of pro-inflammatory cytokines during the evening and a lower expression during the afternoon [173]. Izawa *et. al* found that IL-6 levels peaked at awakening, gradually declined throughout the day, and then peaked again back at night, whereas CRP levels peaked at awakening and lowered throughout the day in 27 healthy individuals [162]. We saw similar concentrations of IL-6, however the trends varied most likely due to sample size. This study analyzed salivary

cytokines from a sample size of individuals with the majority age being in the mid-20's and a small number of participants, hence these findings should be further analyzed in larger samples.

These findings indicate that each individual has a unique pro- and anti-inflammatory diurnal pattern. Circadian variability of inflammation is important for the approach of clinical relevance of inflammatory disease in addition to the optimization of clinical treatment times [174]. In previous reports, circadian rhythms of Th1 cytokines have been shown to peak during the morning and at night in rheumatoid arthritis patients, possibly due to changes in metabolism, and secretions of corticosteroids and plasma melatonin and cortisol [175].

The findings that salivary IL-8, IL-6, and IL-1 β , levels had strong diurnal rhythms could be useful for investigating the pathway linking sleeping patterns and disease. In addition, these results provide critical information of diurnal patterns to optimize salivary collection techniques. Environmental factors, such as cigarette/tobacco smoke have been found to disrupt circadian clock function causing an elevation in lung and peripheral blood mononuclear cell (PBMC) inflammation as well as lung destruction via the SIRT1-BMAL1 pathway [176, 177]. It was found that levels of IL-8, IL-6, and TNF- α in PBMCs were reduced in smokers compared to non-smokers [177]. Therefore, circadian rhythms could potentially lead to a dysregulation in inflammation with regard to particular cytokines.

The discovery and validity of salivary biomarkers relies on the important factor of the method of saliva collection: stimulated versus unstimulated. Stimulated saliva, primarily derived from the parotid glands, inserts bicarbonates that interfere with the composition of salivary components by diluting potential analytes and decreasing concentrations of salivary peroxidase, uric acid, sulfhydryls, and secretory IgA [105]. Thus, stimulated saliva, primarily derived from the submandibular and sublingual glands, is optimal since it offers the least variance of salivary biomolecules.

Table 5: Subject Characteristics (n=12)

Characteristics	
Age (years)*	26.5 (\pm 5.2)
Male (n)†	5 (41.7)
Prior to Sleep collection time (hours)	16.08
Race/ethnicity (n) †	
White	10 (83.3)
Hispanic	2 (16.7)
BMI (kg/m²)*	23.08 (\pm 3.5)
BMI category (n)†	
Underweight (<18.5 kg/m²) †	1 (8.3)
Normal (18.5-<25 kg/m²) †	6 (50)
Overweight (25-<30 kg/m²) †	5 (41.7)
Obese (\geq30 kg/m²) †	0 (0)
Cotinine (ng/ml)‡	5.12
Current smoker (n; cotinine > 25 ng/ml)†	1 (8.3)

*Data presented as mean \pm SD

†Data presented as number (%)

‡Data presented as geometric mean (25th-75th percentile)

Table 6: Healthy Subject Enrollment Log

Subject #	sample ID	Time	Race	Sex	Age	BMI	Gum Rating	Loose tooth?	Saliva Collected (mL)	Tubes (100µL each)	Amount of sleep on day of collection
001	001 A	7:02 AM	Caucasian	F	25	27.5	Good	no	1.5	4	8
	001 B	7:32 AM							1.5	5	
	001 C	10:33 AM							1.8	10	
	001 D	1:33 PM							1.8	8	
	001 E	4:28 PM							1.8	12	
	001 F	11:02 PM							1.8	14	
Day 2	001 A	7:29 AM							1.5	9	8
	001 B	8:00 AM							1.5	8	
	001 C	11:02 AM							1.5	9	
	001 D	2:02 PM							1.5	6	
	001 E	5:00 PM							1.5	0	
	001 F	12:21 AM							1.5	9	
Day 3	001 A	9:18 AM							1.8	8	8
	001 B	9:50 AM							1.8	9	
	001 C	12:50PM							1.6	9	
	001 D	3:50 PM							1.8	10	
	001 E	6:40 PM							1.5	10	
	001 F	12:00 AM							1.5	10	
002	002 A	6:35 AM	Caucasian	M	24	28.7	Good	no	1.8	9	7
	002 B	7:05 AM							2	12	
	002 C	9:34 AM							1.3	7	
	002 D	12:32 PM							1.5	11	
	002 E	3:37 PM							1.9	13	
	002 F	10:31 PM							1.8	14	
Day 2	002 A	6:20 AM							1.8	14	8
	002 B	6:50 AM							1.8	13	
	002 C	9:24 AM							1.8	13	
	002 D	12:36 PM							1.3	7	
	002 E	3:36 PM							1.8	14	
	002 F	10:52 PM							1.8	11	
Day 3	002 A	6:20 AM							1.8	13	7.5
	002 B	6:50 AM							1.8	12	

Subject #	sample ID	Time	Race	Sex	Age	BMI	Gum Rating	Loose tooth?	Saliva Collected (mL)	Tubes (100µL each)	Amount of sleep on day of collection
	002 C	9:36 AM							1.8	12	
	002 D	12:34 PM							1.3	7	
	002 E	3:31 PM							1.8	12	
	002 F	10:48 PM							1.8	13	
003	003 A	6:00 AM	Caucasian	M	42	23	Fair	no	1.8	7	6
	003 B	6:30 AM							1.25	5	
	003 C	9:00 AM							1.5	7	
	003 D	12:00 PM							1.8	8	
	003 E	3:00 PM							1.8	7	
	003 F	12:00 AM							1.8	10	
004	004 A	6:35 AM	Caucasian	F	29	18.8	Very Good	no	1.6	6	7.5
	004 B	7:10 AM							1.3	7	
	004 C	10:11 AM							1.25	6	
	004 D	1:13 PM							1.3	5	
	004 E	4:16 PM							1	5	
	004 F	11:42 PM							1.25	9	
005	005 A	9:30 AM	Caucasian	F	25	19.7	Very Good	no	1.8	12	8.5
	005 B	10:00 AM							1.8	13	
	005 C	1:07 PM							1.8	8	
	005 D	4:07 PM							1.5	6	
	005 E	7:05 PM							1.5	8	
	005 F	10:06 PM							1.5	8	
006	006 A	9:55 AM	Caucasian	F	24	27	Fair	no	1.5	9	6
	006 B	10:25 AM							1.5	12	
	006 C	12:55 PM							1.5	11	
	006 D	3:55 PM							1.5	11	
	006 E	6:55 PM							1.5	10	
	006 F	2:05 AM							1.5	11	
007	007A	9:00 AM	Caucasian	F	20	18.2	Excellent	no	1.25	6	7
	007 B	9:30 AM							1	6	
	007 C	12:00 PM							1.5	11	
	007 D	3:00 PM							1.4	9	
	007 E	6:00 PM							1.5	9	
	007 F	11:30 PM							1	5	

Subject #	sample ID	Time	Race	Sex	Age	BMI	Gum Rating	Loose tooth?	Saliva Collected (mL)	Tubes (100µL each)	Amount of sleep on day of collection
008	008 A	6:55 AM							1	3	
	008 B	7:30 AM							1.5	8	
	008 C	11:15 AM							1	5	
	008 D	2:30 PM							1	6	
	008 E	5:30 PM							1.5	7	
	008 F	1:00 AM							1	4	
009	009A	8:00 AM	Caucasian	M	22	25.77	Good	no	1	3	7
	009 B	8:30 AM							1.5	7	
	009 C	11:30 AM							1.5	7	
	009 D	2:30 PM							1	5	
	009 E	5:28 PM							1	6	
	009 F	1:00 AM							1	5	
010	010A	8:00 AM	Caucasian	M	26	21.1	Excellent	no	1	6	6.5
	010 B	8:31 AM							1.25	6	
	010 C	11:35 AM							1	6	
	010 D	2:35 PM							1	6	
	010 E	5:35 PM							1	6	
	010 F	1:07 AM							1.25	9	
011	011A	6:10 AM	Hispanic	F	26	22.3	Very Good	no	1.5	7	6.5
	011 B	6:40 AM							1	6	
	011 C	9:10 AM							1	5	
	011 D	12:10 PM							1	5	
	011 E	3:10 PM							1.5	5	
	011 F	11:00 PM							1	5	
012	011A	7:45 AM	Caucasian	F	27	19.7	Very Good	no	1.25	6	7.5
	011 B	8:15 AM							1.5	4	
	011 C	10:45 AM							1	5	
	011 D	1:45 PM							1	5	
	011 E	4:45 PM							1	7	
	011 F	11:00 PM							1	5	

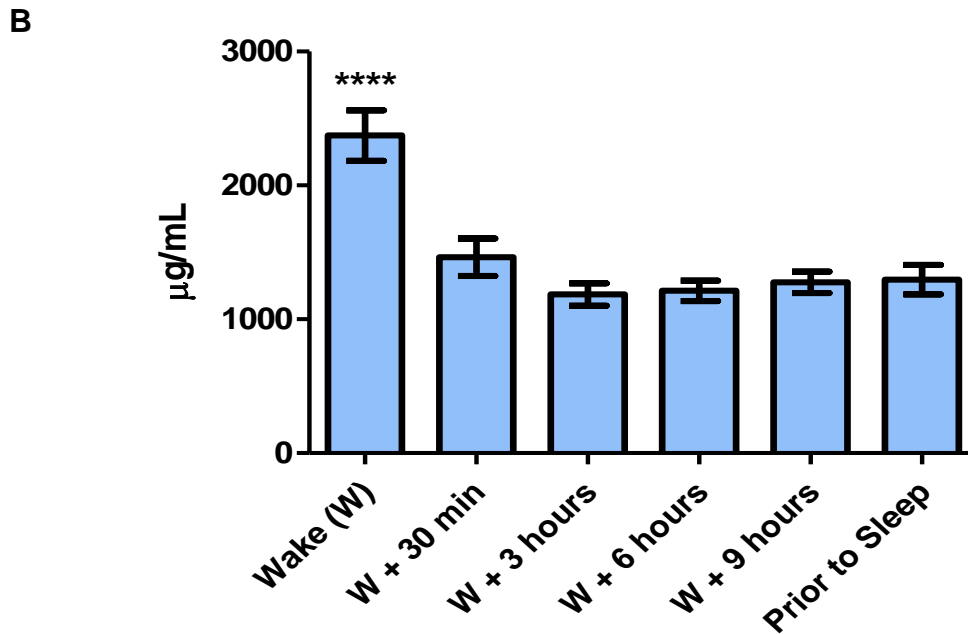
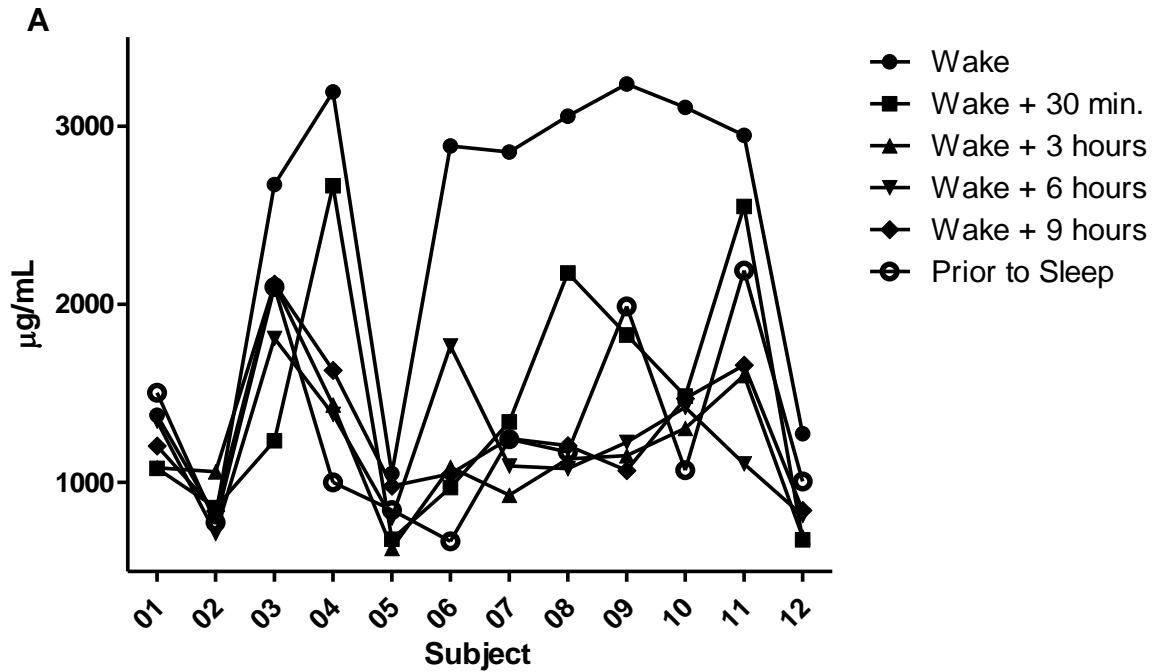


Figure 16: Total Protein Levels of Healthy Subjects' Diurnal Saliva Samples

Total protein content was determined using the BCA protein kit as previously described. **(A)** The diurnal patterns of total salivary protein content (ng/mL) in healthy subjects by time of day. Protein levels were highest in the majority of subjects at the “Wake” time-point. **(B)** Total salivary protein content (ng/mL) in all healthy subjects combined (mean and SEM). Error bars represent SEM. There is a statistical difference ($p \leq 0.0001$) between protein levels of Wake samples vs. all time-points.

Table 7: Cytokine Trends within Subjects Individually

This table depicts the amount of significant difference compared to “wake” time-point for each subject and each cytokine.

Subject #	01	02	03	04	05	06	07	08	09	10	11	12
IL-8	W+6 HIGHER (**)	All (***) LOWER	No trend	All (***) LOWER	W+3 LOWER (**), W+6 HIGHER (**)	All but W+30 min. LOWER	All (***) LOWER	W+30 min. (*), W+6, W+9 (***) LOWER, Prior to Sleep (***) HIGHER	W+30 min., W+6 (***) , W+3 (*) LOWER	Prior to Sleep (***) HIGHER	All LOWER (*)†	No trend
IL-6	W+6 HIGHER (***)	All undetectable or LOWER†	All undetectable or LOWER†	All (***) LOWER	W+3 (*) LOWER, W+9 (*), Prior to Sleep (***) HIGHER	No trend/ undetectable †	W+9 (*) HIGHER	Prior to Sleep HIGHER†	Prior to Sleep HIGHER†	W+9 (***) , Prior to Sleep (*) HIGHER	Prior to Sleep HIGHER†	W+3 (***) HIGHER
IL-1β	Prior to Sleep (*) LOWER	All (***) LOWER	All (***) LOWER	All (***) LOWER	W+30 min. (**), W+3 (***) , W+9 (***) LOWER, Prior to Sleep (***) HIGHER	All (***) LOWER	All (***) LOWER	Prior to Sleep HIGHER†	No trend/ undetectable †	W+9, Prior to Sleep HIGHER†	All LOWER †	No trend/ undetectable †
IL-10	W+6 (***) , W+9 (***) HIGHER	No trend/ undetectable †	All LOWER†	Prior to Sleep HIGHER†	Prior to Sleep HIGHER†	W+30 min., W+6 HIGHER†	W+6 HIGHER†	Prior to Sleep HIGHER†	W+9 HIGHER†	W+9 HIGHER†	All LOWER†	N/A
TNF-α	W+6, W+9 HIGHER†	All undetectable or LOWER†	All undetectable or LOWER†	All LOWER†	Prior to Sleep HIGHER†	No trend/ undetectable †	All undetectable or LOWER†	Prior to Sleep HIGHER†	N/A	Prior to Sleep HIGHER†	All undetectable or LOWER†	N/A
IFN-γ	W+3 HIGHER, rest undetectable †	N/A	N/A	All undetectable or LOWER†	Prior to Sleep HIGHER, rest undetectable†	N/A	W+6 HIGHER†	W+9 HIGHER†	W+9 HIGHER†	W+9 HIGHER†	No trend/ undetectable †	All higher, W undetectable †

Data in red (†): ANOVA could not be performed; trends can be seen though. N/A: all time-points undetectable. *: p<0.05, **: p<0.01, ***: p<0.0001.

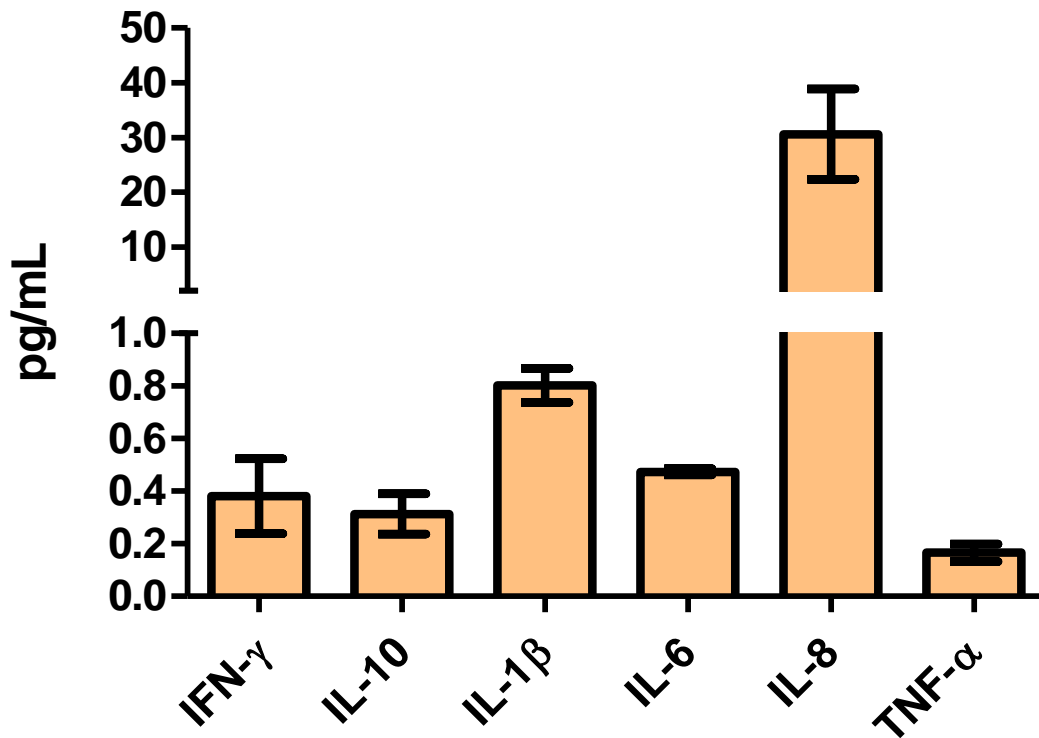


Figure 17: Mean Salivary Cytokine Levels in QC Saliva Samples

A commercially received quality control saliva sample (Innovative Research, Novi, MI) was included on each plate (n=4) for the diurnal salivary cytokine analysis. Mean cytokine levels were measured in these quality control samples with inhibitor to determine to demonstrate little inter-assay variability. Error bars represent SEM.

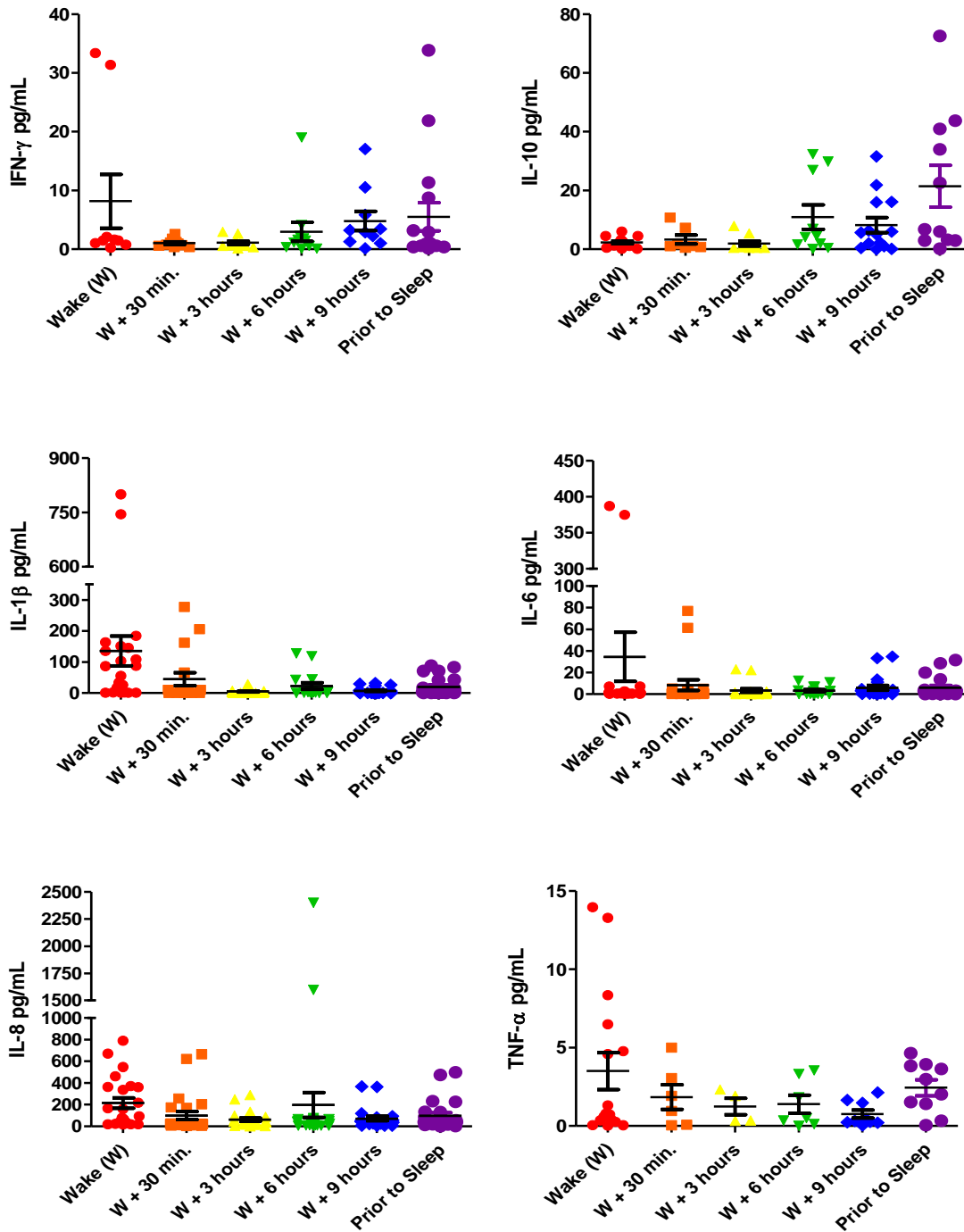


Figure 18: Cytokine Salivary Levels of IFN- γ , IL-10, IL-1 β , IL-6, IL-8, and TNF- α as Determined By Detected Levels

Saliva was collected at various time-points throughout the day based on each individual's sleep schedule. Each graph represents the mean undetectable and detectable levels of all 12 subjects combined (n=24). IL-6, IL-1 β , and TNF- α levels are generally highest in the morning, whereas IL-10 levels are generally higher in the evening. In general, IL-10, TNF- α , and IFN- γ levels were either low or undetectable. Error bars represent SEM.

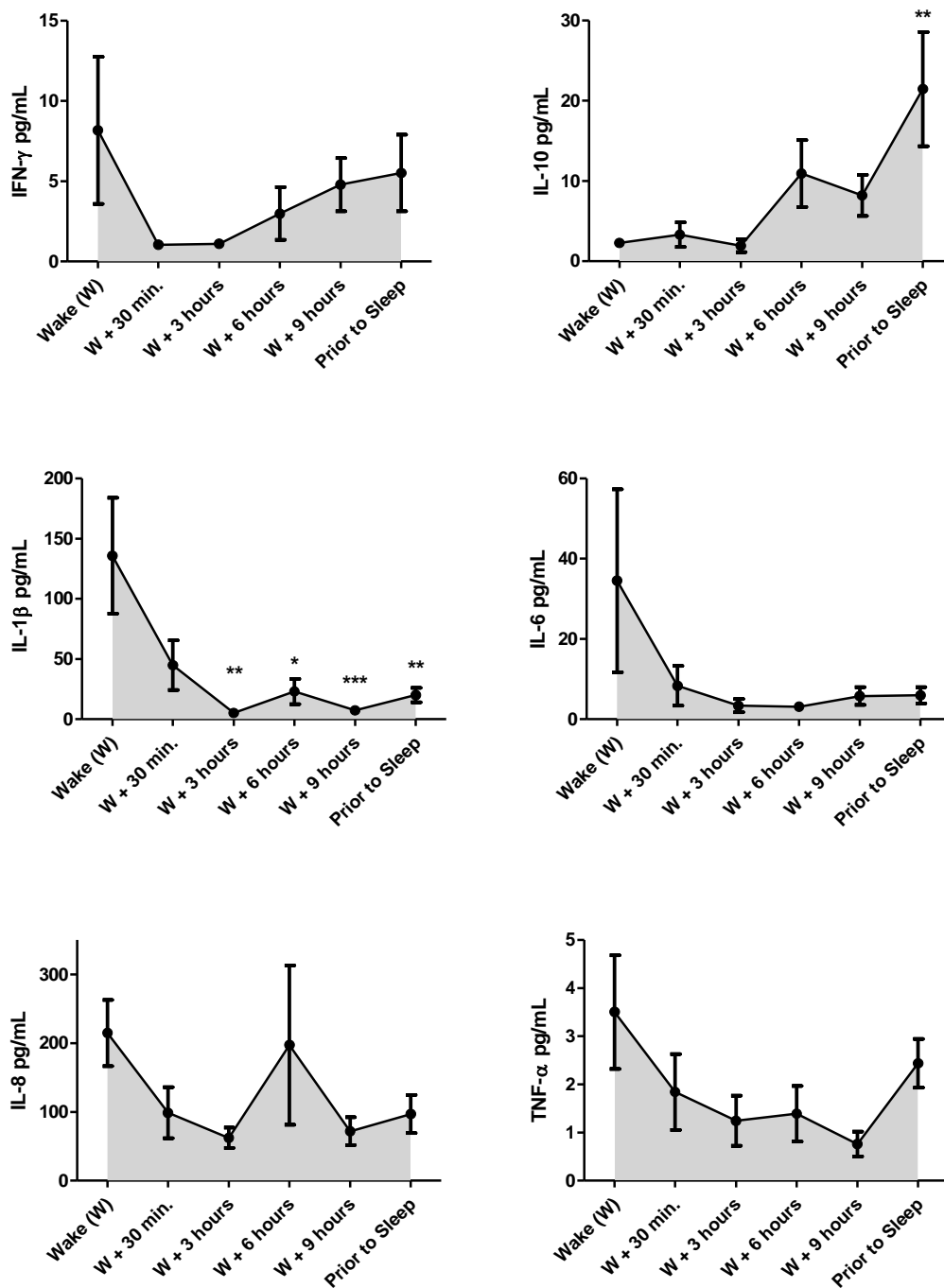


Figure 19: Cytokine Salivary Trends of IFN- γ , IL-10, IL-1 β , IL-6, IL-8, and TNF- α as Determined By Detected Levels in 12 Subjects Combined

Saliva was collected at various time-points throughout the day based on each individual's sleep schedule. Each graph represents the mean undetectable and detectable levels of all 12 subjects combined (n=24). In general, IL-10, TNF- α , and IFN- γ levels were either low or undetectable. Error bars represent SEM. IL-1 β levels are significantly higher in the morning, whereas IL-10 levels are significantly higher in the evening. All time-points are compared to Wake. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.0001$.

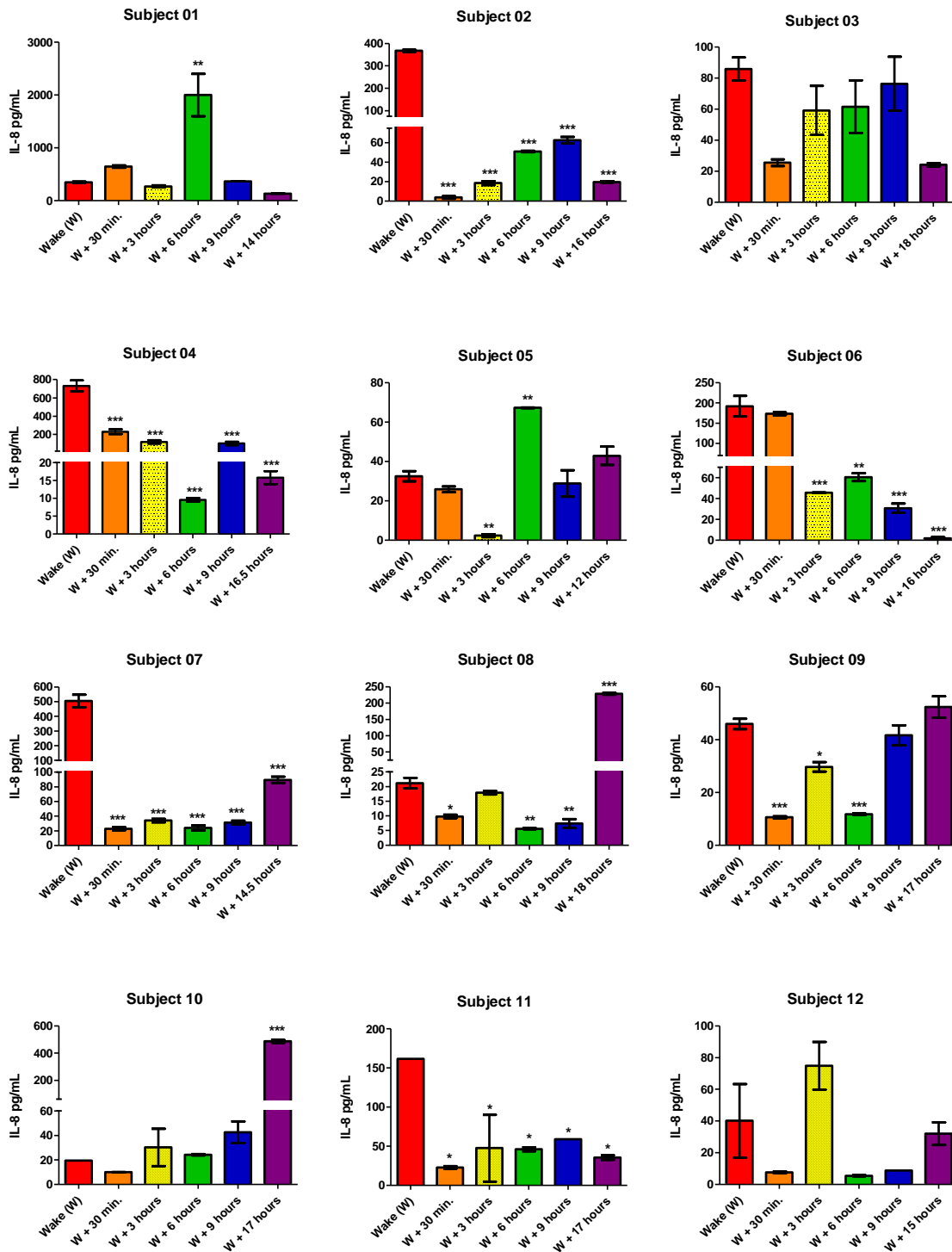


Figure 20: Cytokine Salivary Levels of IL-8 (Mean and SEM)

Saliva was collected at various time-points throughout the day based on each individual's sleep schedule. Subject 01 and Subject 02 graphs represent Day 3 saliva collection. Each graph represents the detectable levels of IL-8 of each time-point in each subject. Error bars represent SEM. All time-points are compared to Wake. *: p<0.05, **: p<0.01, ***: p<0.0001.

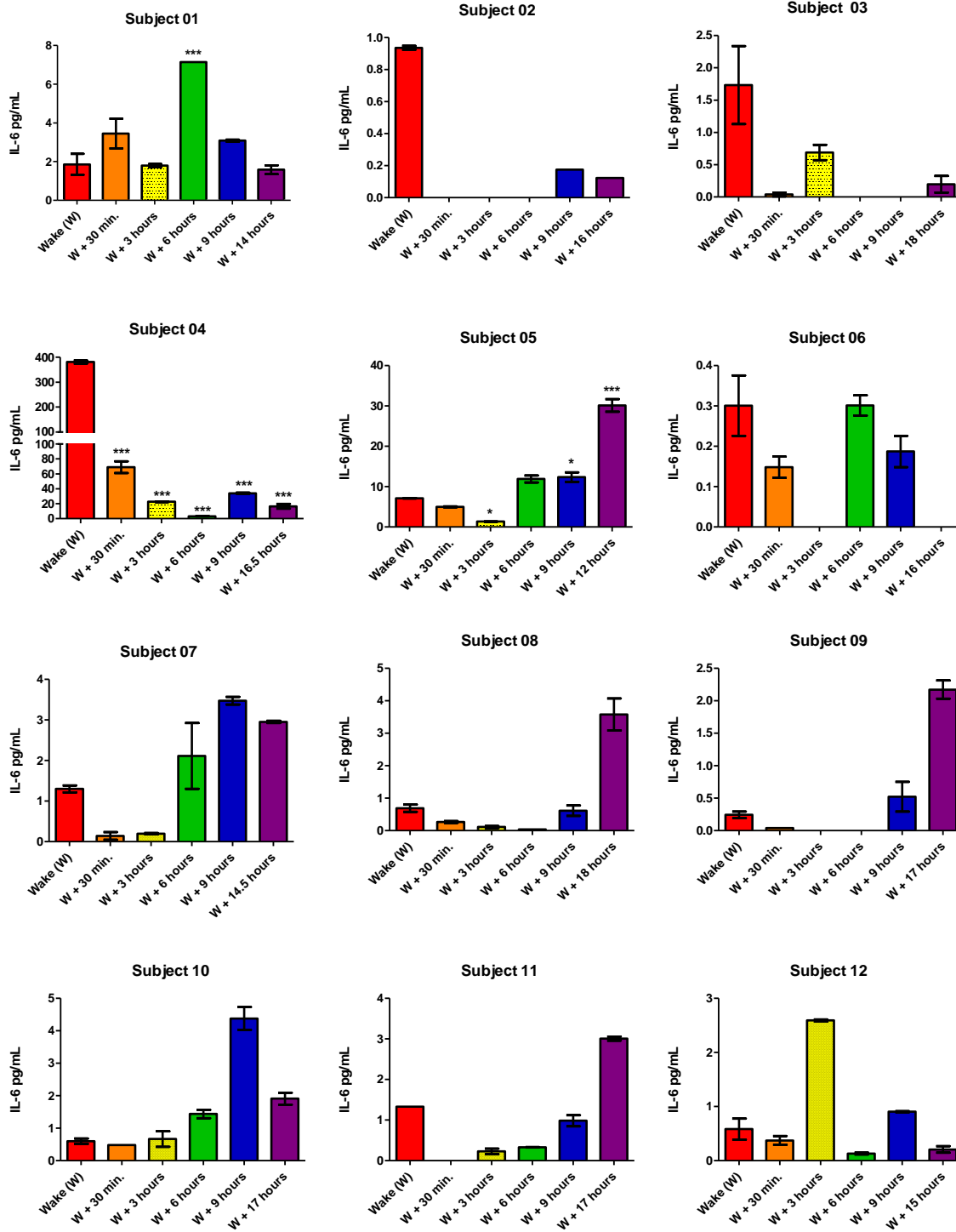


Figure 21: Cytokine Salivary Levels of IL-6 (Mean and SEM)

Saliva was collected at various time-points throughout the day based on each individual's sleep schedule. Subject 01 and Subject 02 graphs represent Day 3 saliva collection. Each graph represents the detectable levels of IL-6 of all time-points in each subject. Error bars represent SEM. All time-points are compared to Wake. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.0001$.

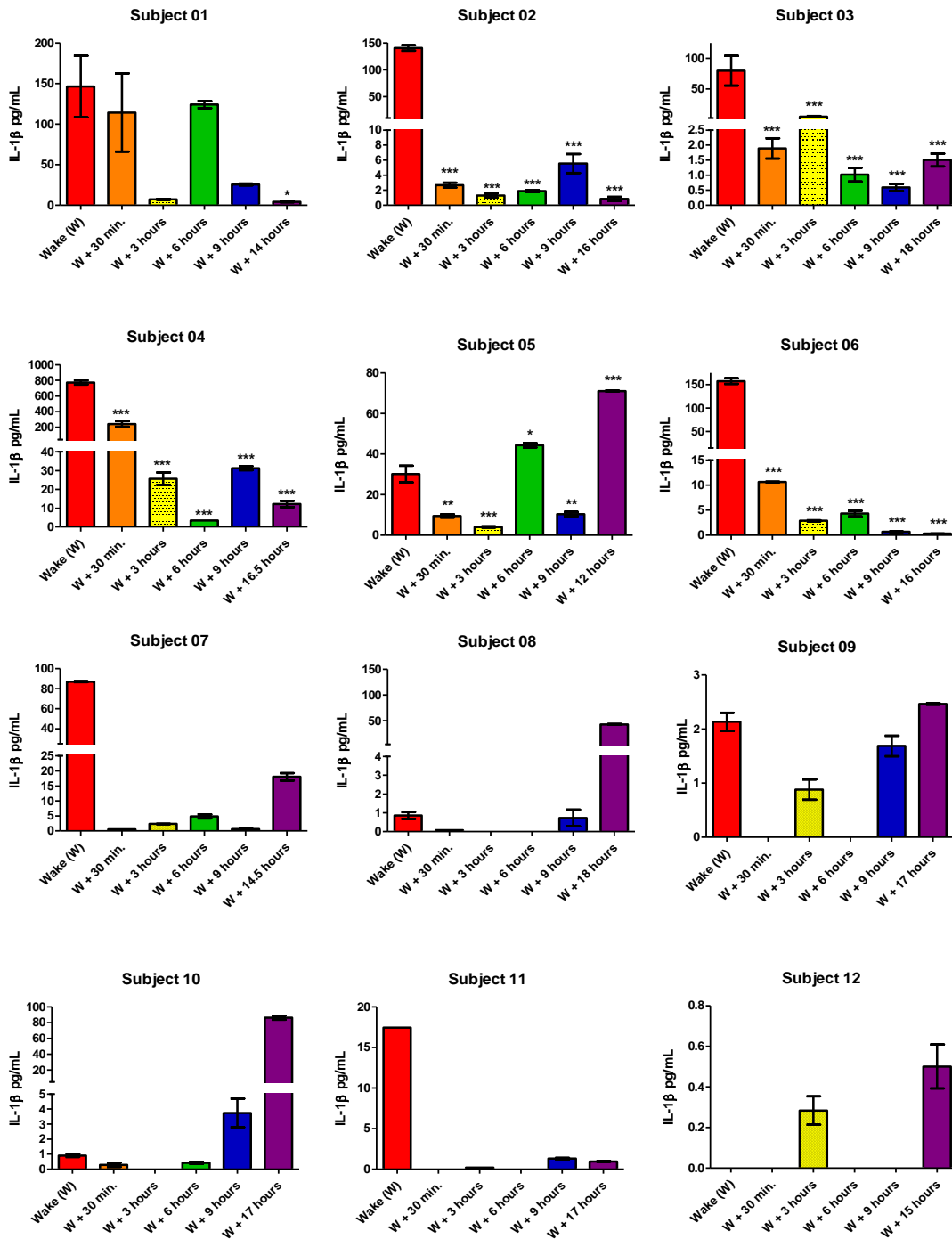


Figure 22: Cytokine Salivary Levels of IL-1β (Mean and SEM)

Saliva was collected at various time-points throughout the day based on each individual's sleep schedule. Subject 01 and Subject 02 graphs represent Day 3 saliva collection. Each graph represents the detectable levels of IL-1β of all time-points in each subject. Error bars represent SEM. All time-points are compared to Wake. *: p<0.05, **: p<0.01, ***: p<0.0001.

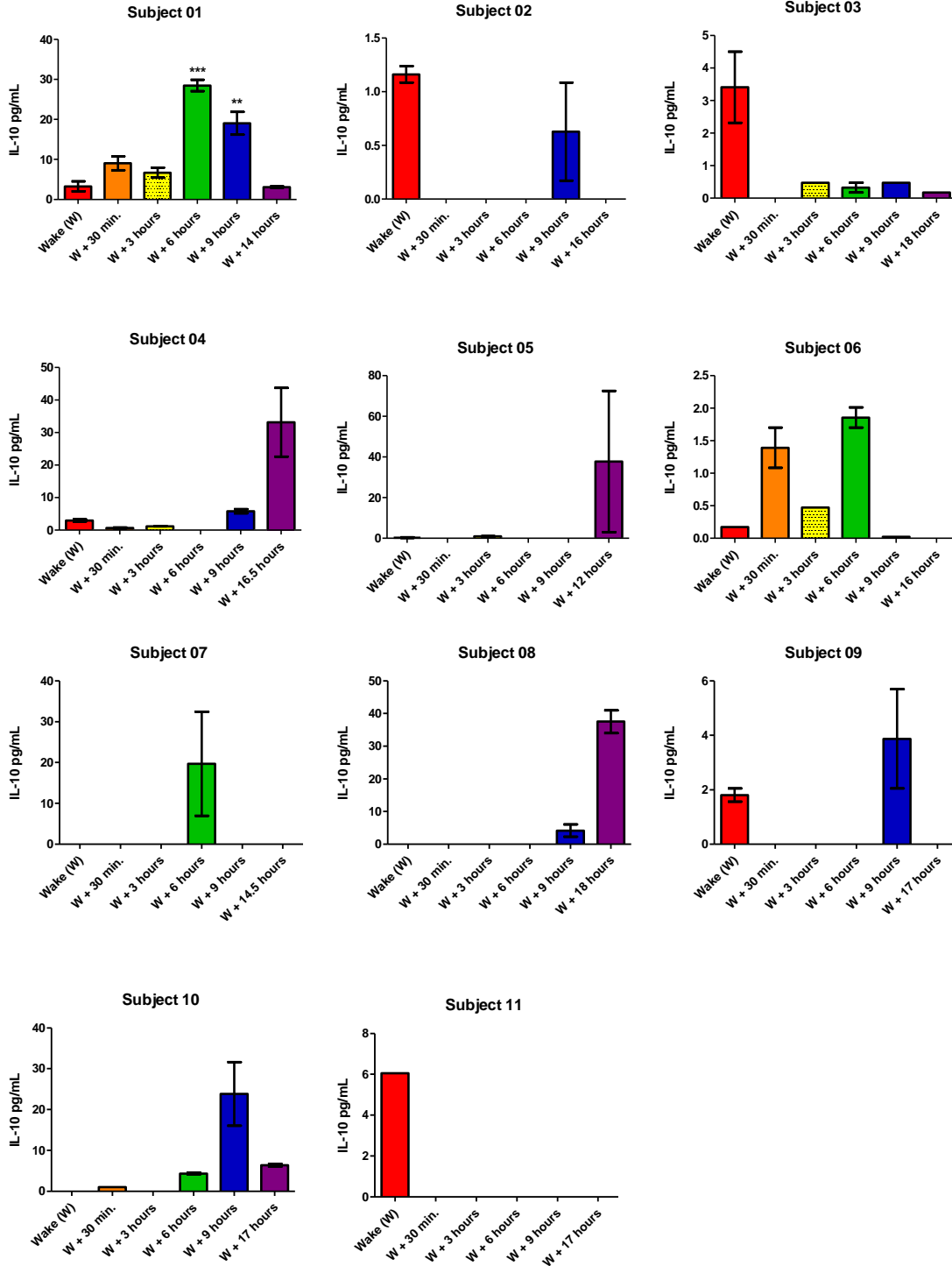


Figure 23: Cytokine Salivary Levels of IL-10 (Mean and SEM)

Saliva was collected at various time-points throughout the day based on each individual's sleep schedule. Subject 01 and Subject 02 graphs represent Day 3 saliva collection. Each graph represents the detectable levels of IL-10 of all time-points in each subject. Error bars represent SEM. All time-points are compared to Wake. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.0001$.

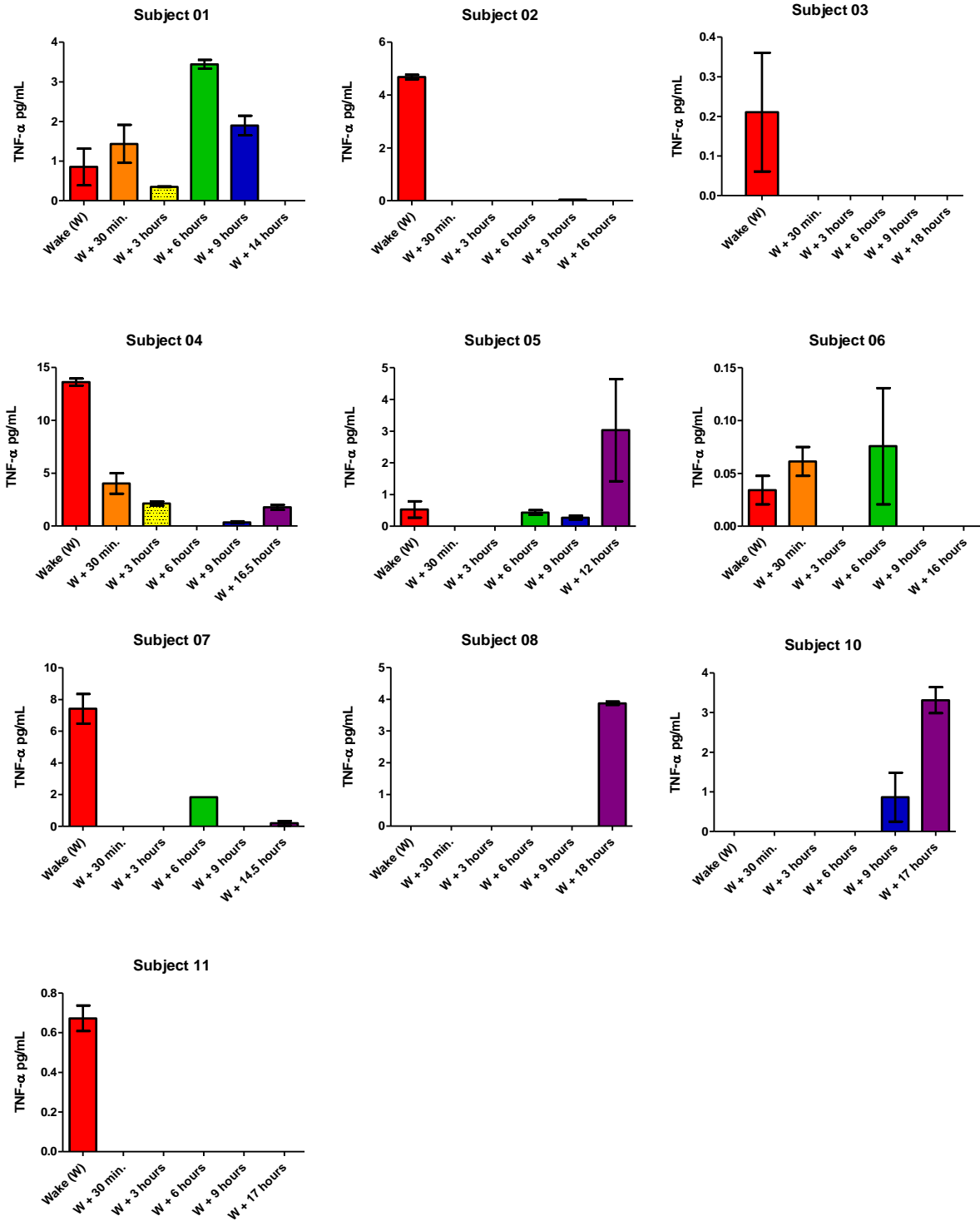


Figure 24: Cytokine Salivary Levels of TNF-α (Mean and SEM)

Saliva was collected at various time-points throughout the day based on each individual's sleep schedule. Subject 01 and Subject 02 graphs represent Day 3 saliva collection. Each graph represents the detectable levels of TNF-α of all time-points in each subject. Undetectable levels are not shown in the graphs. Error bars represent SEM. All time-points are compared to Wake.

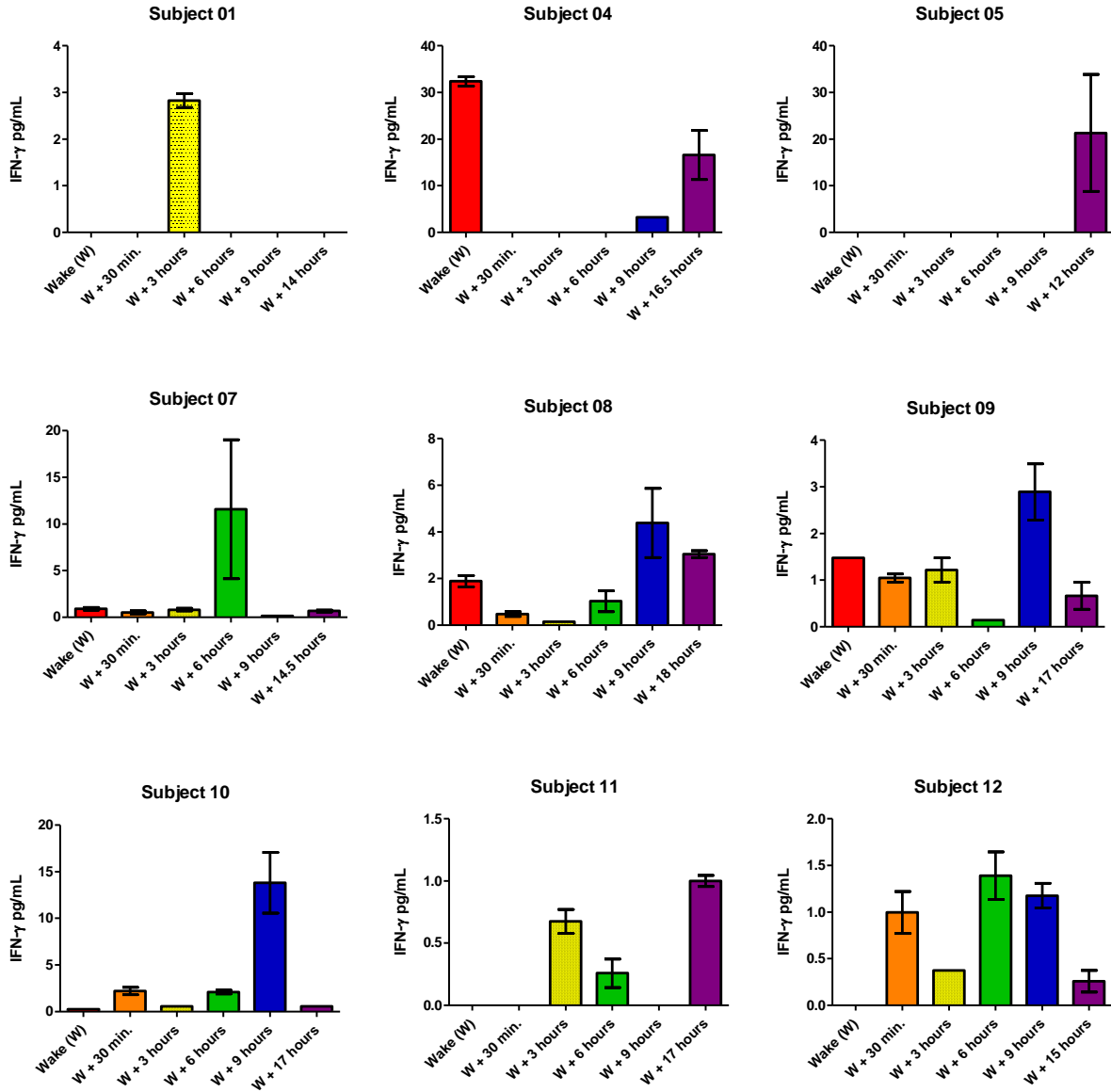


Figure 25: Cytokine Salivary Levels of IFN- γ (Mean and SEM)

Saliva was collected at various time-points throughout the day based on each individual's sleep schedule. Subject 01 and Subject 02 graphs represent Day 3 saliva collection. Each graph represents the detectable levels of IFN- γ of all time-points in each subject. Undetectable levels are not shown in these graphs. Error bars represent standard error of the mean. All time-points are compared to Wake.

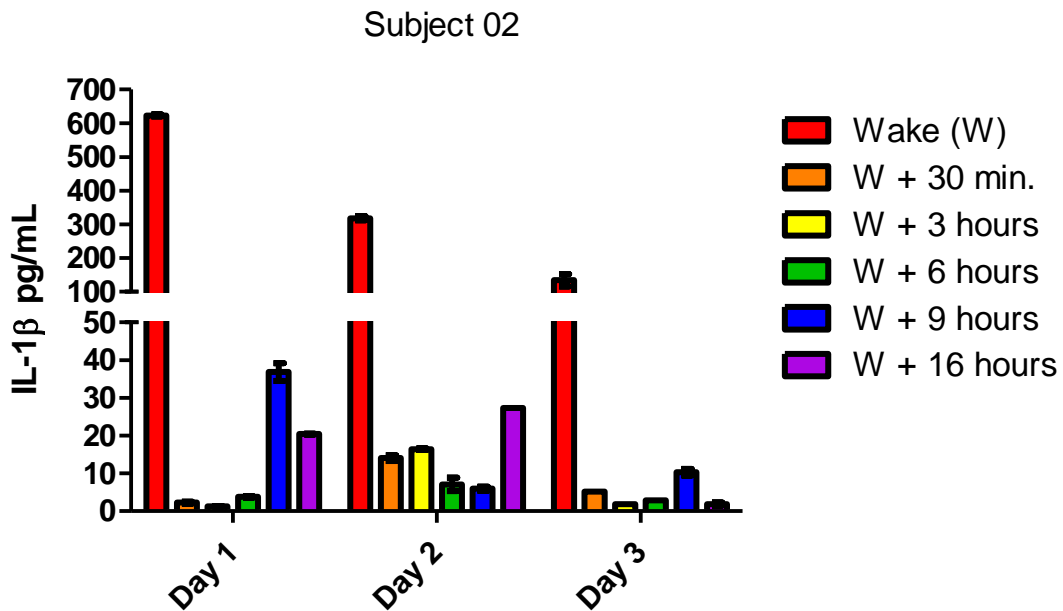
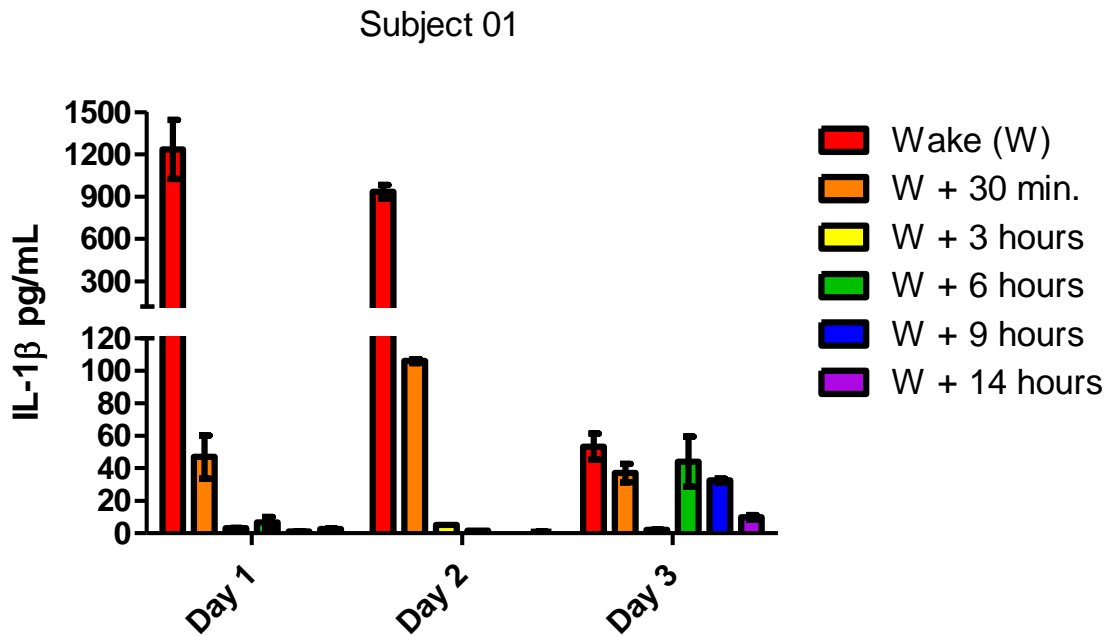
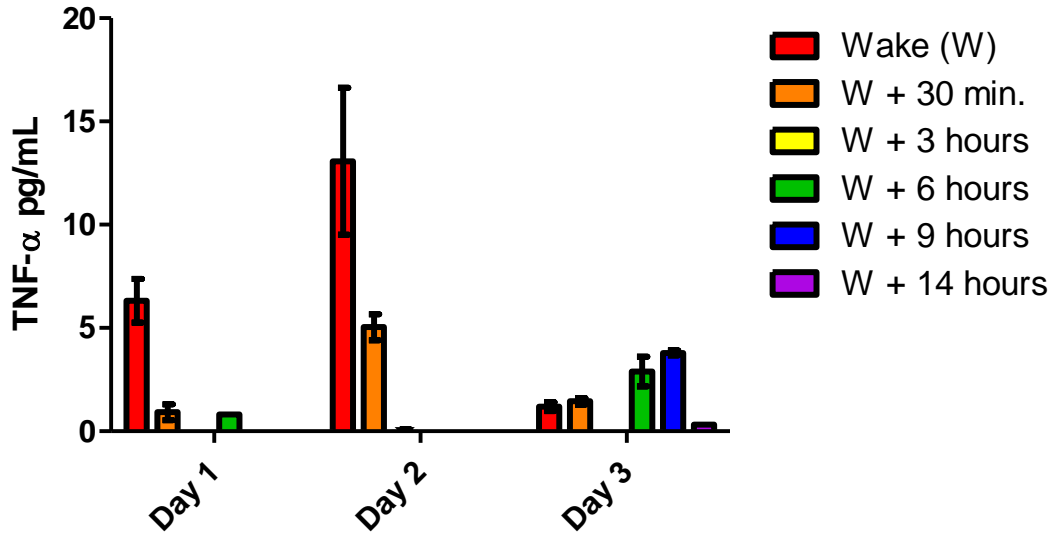


Figure 26: Cytokine Salivary Levels of IL-1 β Over Three Consecutive Days

Saliva was collected at various time-points throughout the day based on each individual's sleep schedule. Each graph represents the detectable levels of IL-1 β of all time-points in each subject. Undetectable levels are not shown. IL-1 β levels are generally elevated in the morning on each day. Error bars represent SEM.

Subject 01



Subject 02

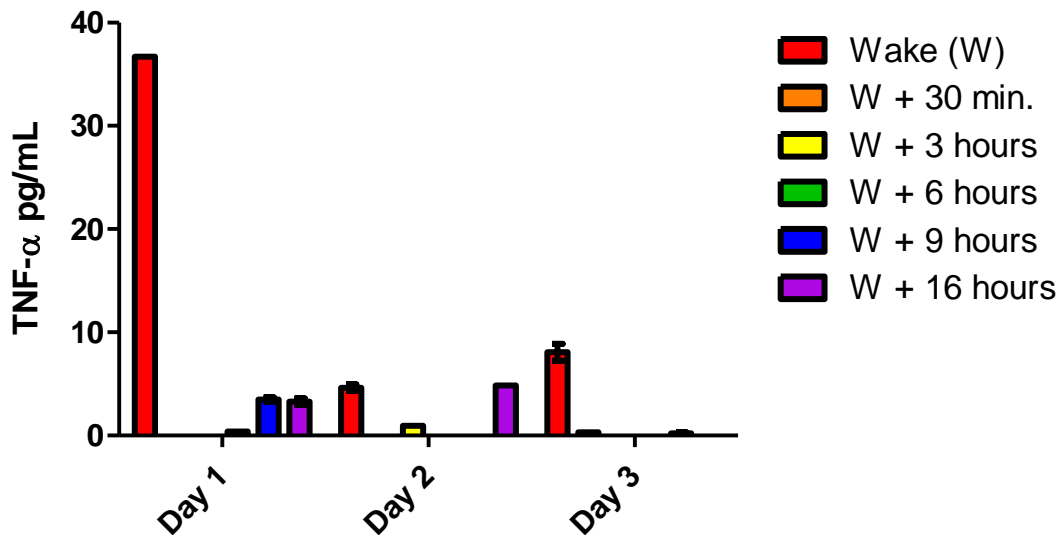


Figure 27: Cytokine Salivary Levels of TNF- α Over Three Consecutive Days

Saliva was collected at various time-points throughout the day based on each individual's sleep schedule for 3 consecutive days. Each graph represents the detectable levels of TNF- α of all time-points. Undetectable levels are not shown. TNF- α levels are generally elevated in the morning on each day. Error bars represent SEM.

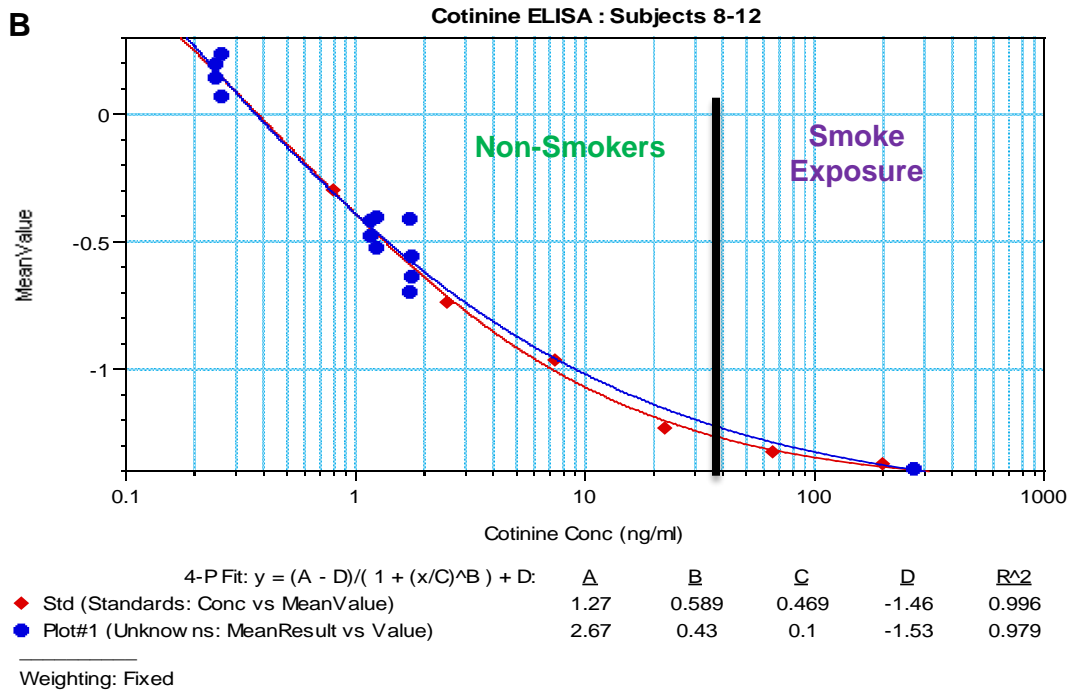
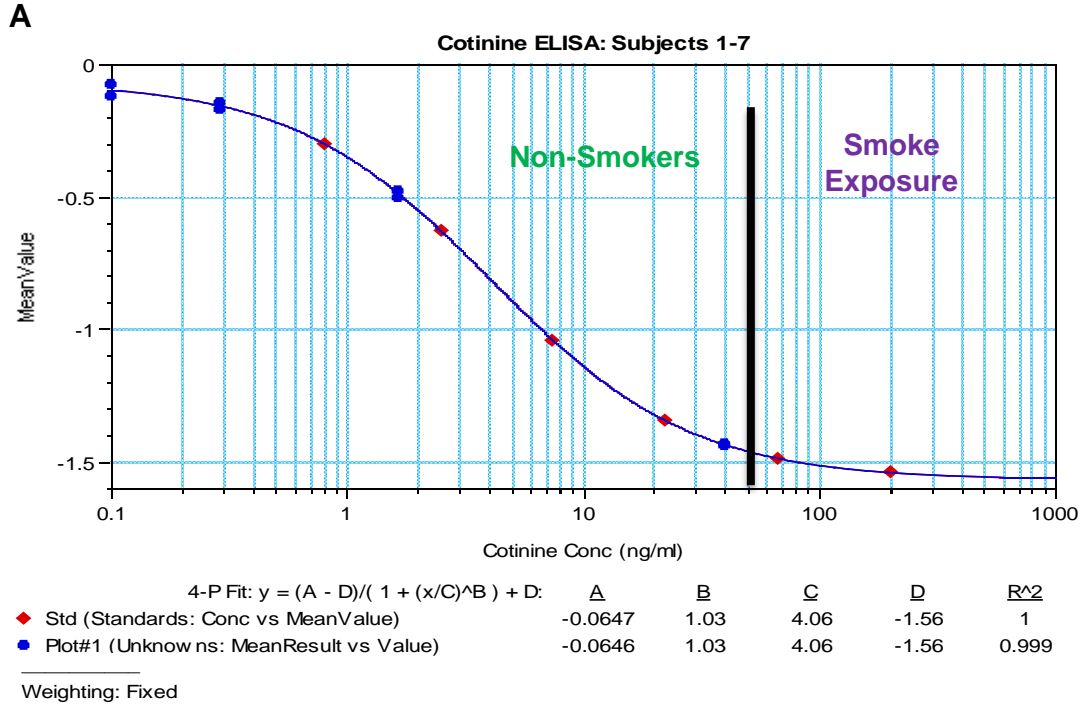


Figure 28: Cotinine Levels of 12 Healthy Subjects

Unstimulated whole saliva was collected from 12 healthy subjects and their saliva was analyzed on 2 ELISA cotinine plates. (A) Mean result of 7 subjects (performed in duplicate) from the subject range 01-07, (B) Mean result of 5 subjects (performed in duplicate) from the subject range 08-12. The standard curve is represented by red diamonds and the blue dots indicate each individual saliva sample. The standard curve ranged from 0.8 ng/mL to 200 ng/mL. Any result greater than 25 ng/mL indicates a smoker or an involvement of second-hand smoke.

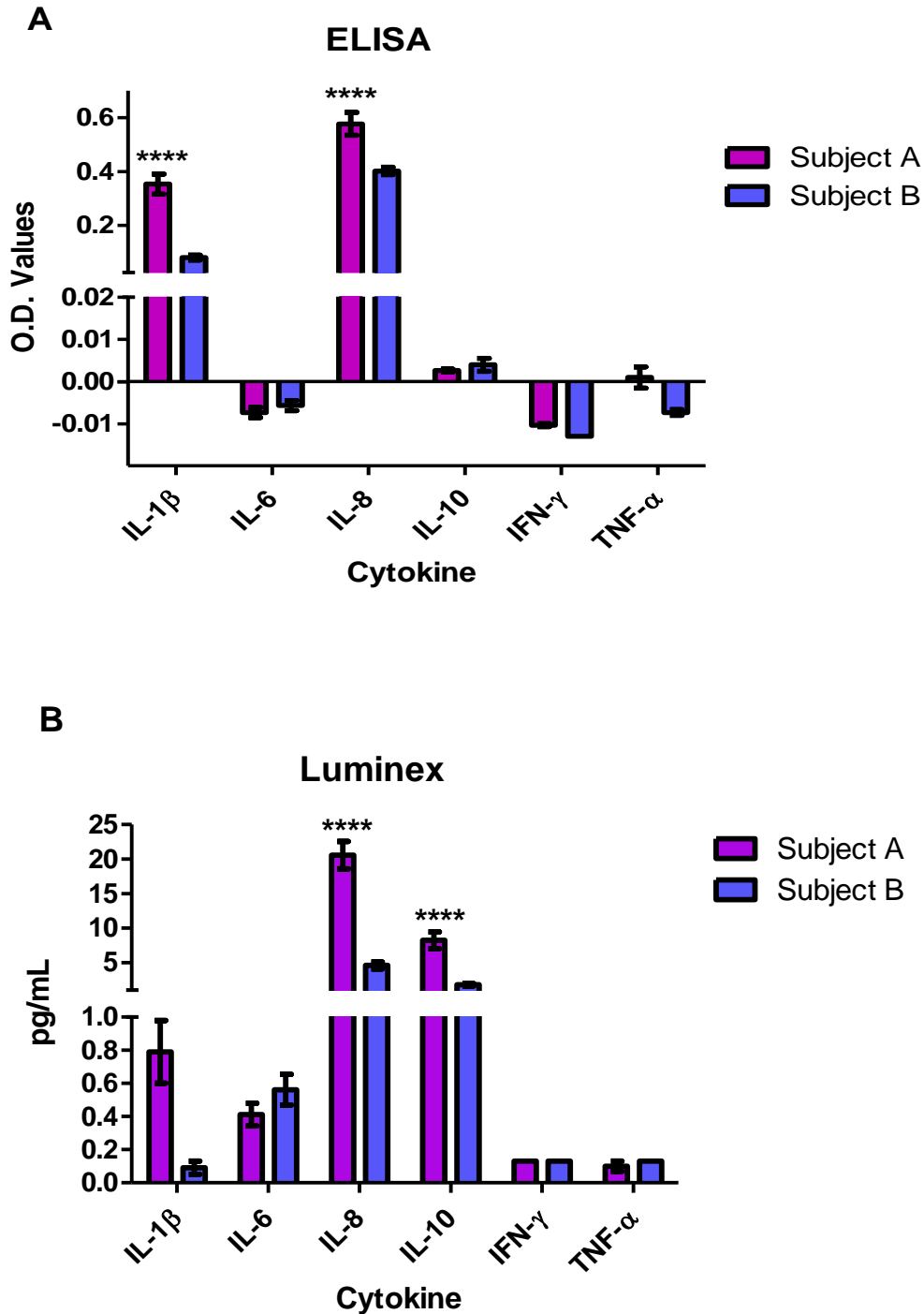


Figure 29: ELISA and Luminex Magpix[®] Cytokine Expression Comparison

Saliva was collected from two healthy adult subjects and the trends of cytokine expression were confirmed in a qualitative ELISA. **A)** ELISA results showed positive results IL-1 β , IL-8, and IL-10 for both subjects. Detection below 1 pg/mL were considered negative, with the exception of IL-1 β , in the ELISA experiment. **B)** Luminex results demonstrated quantitative cytokine expression in pictogram measurements and is clearly more sensitive. Significance was determined using Two-way ANOVA. Error bars represent standard error of the mean.

Chapter 5: Impact of Hyperglycemia on the Inflammatory Profile from a Human Submandibular Gland Cell Line

Abstract

The objective of this study is to address the gap in knowledge of salivary biomarkers of inflammation by measuring the distribution of select cytokines and MMPs previously demonstrated to have a putative causatory or protective role of T1D and periodontitis, respectively. The presence and variation of nine different salivary biomarkers- IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IFN- γ , and TNF- α – and two MMPs–, MMP-8, and MMP-9- from the human submandibular gland cell (HSG) line will be described. Biomarkers associated with distinct biological phases of periodontal disease (i.e. inflammation, collagen degradation, and bone remodeling) have been suggested to be useful for early recognition of patients with periodontitis [3]. The source of the increased salivary inflammatory burden is still being elucidated. Therefore, we examined the inflammatory profile of HSG cells, one of the primary glands of saliva secretion. Out of the eleven analytes analyzed, we found a significant increase of IL-13 ($p < 0.05$) and IL-10 ($p < 0.05$) in the HSG lysate from 5.5 mM to 22 mM glucose treatments. This suggests that glucose stimulates an anti-inflammatory response within the submandibular salivary gland in vitro. In addition, we discovered a significant decrease in IFN- γ ($p < 0.05$) in the HSG lysate from 5.5 mM to 22 mM glucose treatments. However, hyperglycemia does not grossly alter the inflammatory profile of the HSG cell line. Oral Th1 cytokines are potentially derived from other cell populations such as the liver.

Introduction

There is a bi-directional relationship between periodontal health and diabetes—periodontal infection creates an inflammatory response that contributes to hyperglycemia whereas increase blood glucose levels initiates the development and prolongation of periodontal disease [178]. It all begins with inflammation from the dental plaque and its oral microbiome, which triggers hyperinflammation that causes alveolar bone to break down. Both local and systemic inflammation occur via the periodontium and bacterial endotoxins, respectively. Added sugar from the diet, as well as poor glycemic control, initiates an inflammatory cascade generating a chronic overexpression of pro-inflammatory cytokines and MMPs. This, in turn, leads to inflammatory mediators acting as antagonists on insulin receptors generating insulin resistance [111] and stimulation of the liver to express CRP, serum amyloid A, and fibrogen, all of which contribute to hyperglycemia. This hyperglycemic condition stimulates oxidative and other cellular stress increases free radicals, ROS, and the irreversible production of AGEs to instigate further inflammatory dysfunction contributing to inflammation of the oral mucosal tissues and potentially exacerbating periodontal disease [111, 178].

The source of inflammation within the oral cavity occurs both locally and systemically [111], yet little is understood regarding the direct impact of glucose, either from the diet or chronic hyperglycemia, on the inflammatory profile of salivary cells. Secretions from the salivary glands contain many proteins and peptides responsible for the maintenance of the oral cavity, rendering the submandibular, parotid, and sublingual cells to have a vital role in the progression and development of periodontal disease [179]. Recent evidence has suggested that inflammatory cytokines may originate from the serum, host immune system, salivary glands, and oral mucosal tissues [144]. Other studies have demonstrated that free fatty acids such as palmitate can increase IL-6 production from human salivary gland epithelial cells and certainly can immunologically contribute to the local milieu of the saliva [77]. However, the impact of glucose on cytokine-mediated inflammation has yet to be investigated in salivary gland cells.

Our results presented in chapter 3 revealed that oral inflammation was associated with glycemic control and therefore glucose may be serving as a dominant secretagogue of locally induced inflammation. Thus, our goal is to even further identify and characterize the source of this inflammatory burden.

Ultimately, there is a critical need for the comprehension of the secretory profile of these salivary cells since they may provide a constant source of basal inflammation and prolong the detrimental effects of periodontitis and diabetes. To address this need, we characterized the immunological profile of the human submandibular gland (HSG) cell line, generously donated by Dr. Bruce Baum from the National Institute of Health/National Institute of Dental and Cranial Facial Research (NIH/NIDCR), under physiological and supraphysiological glucose and measured secreted cytokine levels. We investigated this particular cell line due to the fact that the human submandibular gland is the largest contributor to salivary total content secretion [103]. We screened for several cytokines and proteins including MMP-9, MMP-8, IL-4, IL-2, IL-13, IL-1 β , IL-6, IL-8, IL-10, TNF- α , and IFN- γ and found that IL-13, IFN- γ , and IL-10 were associated with supraphysiological glucose.

Materials and Methods

Cell Culture

We examined the two dominant cell types responsible for saliva production: submandibular gland and parotid PSY gland cell lines. The HSG cells were cultured as previously described in Chapter 2. Six independent experiments in duplicated were performed for 24 glucose stimulation analysis, whereas three independent experiments in duplicate were performed for 48 hour and 72 hour glucose stimulation analysis. We attempted to culture the human parotid PSY salivary gland cell line (American Type Culture Collection, CRL-7669 Hs 917.T, Lot 58078722), however it was unsuccessful. Despite several months of optimizing cell culture techniques and variations of media, the parotid cell line never reached confluency.

To explore the possible additional sources of inflammation, we cultured BNL murine liver cells using the same procedures of cell culture as described in Chapter 2. The BNL cells were stimulated with 5.5 mM, 11 mM, and 22 mM glucose concentrations over 24 hours.

Cytokine and MMP Analysis

Cytokine levels were determined using a multiplexed bead immunoassay and measured with a Luminex Magpix[®] instrument (Luminex, Austin, TX). Eleven cytokines/MMPs: MMP-9, MMP-8, IL-4, IL-2, IL-13, IL-1 β , IL-6, IL-8, IL-10, TNF- α , and IFN- γ were measured using the high sensitivity human cytokine magnetic bead assay (R&D Systems, Cat. No. LXSAHM, Minneapolis, MN, and EMD Millipore, Cat No. HSCYTMAG-60SK-06, Billerica, MA) following manufacturer instructions as discussed in Chapter 2. All eleven cytokines and MMPs were analyzed on the 24 hour glucose stimulated plates, whereas six cytokines- IL-6, IL-8, IL-10, IL-1 β , TNF- α , and IFN- γ - were analyzed on the 48 hour and 72 hour glucose stimulated plates.

Results

Total Lysate and Media Protein Levels

Figure 42 demonstrates that the total protein content of the BNL lysate remained consistent across all glucose treatments. The total protein content of the HSG lysate remained consistent across all glucose treatments. There was an increase in protein content of the HSG media samples as the concentration of glucose increased (Figure 30). Regardless, the variance of cytokine levels suggests the increase in IL-13 is not due to protein content. When using 200 μ L of MPER to lyse the cells, the protein content was too dilute as shown in Figure 30. Therefore, a repeat experiment of 24 hour, 48 hour, and 72 hour glucose treatment was performed and half the amount of MPER (100 μ L) was applied to the HSG cells and collected (Figure 31). The protein content increased significantly from 500 μ g/mL to 1500 μ g/mL.

Glucose Stimulation of HSG Cell Culture

To initially determine the impact of glucose on changes in the inflammatory profile (IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-1 β , TNF- α , IFN- γ , MMP-8, and MMP-9), we examined the HSG cell line. Glucose was administered over 24, 48, and 72 hour time-courses and the inflammatory profile was investigated in both the media and cell lysate. Th1 cytokine levels were unaffected by increased glucose concentration for 24 hours in both HSG cell lysate and media as demonstrated in Figure 32 and Figure 33, respectively. IFN- γ levels were undetectable in the media of 24 hour glucose stimulation. Th2 cytokine levels, with the exception of IL-13, were unaffected by increased glucose concentrations for 24 hours in both HSG cell lysate and media as shown in Figure 34 and Figure 35, respectively. IL-13 significantly increased ($p < 0.05$) from basal conditions to 22mM glucose stimulation. MMP-8, MMP-9, and IL-8 did not exhibit a significant trend as glucose levels increased in both HSG cell lysate and media as shown in Figure 36 and Figure 37, respectively.

We measured 6 cytokines in the 48-hour and 72-hour glucose treated HSG cell lysate and media. No significant trends were demonstrated in either the 48 hour glucose stimulated media or lysate (Figure 38 and Figure 39). IFN- γ levels were undetectable in the media of 48 hour and 72 hour glucose stimulation. There was a significant decrease in IFN- γ levels in 72 hour glucose stimulated lysate compared to basal conditions (Figure 40). There were no significant trends in the 72 hour glucose stimulated media (Figure 41). Multiplexing analysis revealed out of the eleven analytes, IL-13 and IL-10 levels were significantly elevated ($p < 0.05$) and IFN- γ levels significantly decreased ($p < 0.05$) as the concentration of glucose increased. Additionally, IL-8 levels decreased, but not significantly, at hyperglycemic conditions (22 mM) for 24 hours in HSG cell media and for 48 hours in HSG cell lysate.

IL-13 Analysis

We attempted to verify these results using an ELISA assay (Catalog number D1300B, R&D Systems Inc., Minneapolis, MN) per manufacturer's instructions as described in Chapter 2. Yet, the antibody to IL-13 was not sensitive enough for detection in our samples. Additionally, a Western Blot using a human IL-13 antibody (Catalog number AF-213-SP, R&D Systems Inc., Minneapolis, MN) was performed (as described in Chapter 2); however the antibody was not sensitive enough to detect cell lysate (53 µg/mL) despite protocol enhancements. This further validates the high sensitivity of the Luminex Magpix® method.

Glucose Stimulation of BNL Cell Culture

To determine the impact of glucose on changes in the inflammatory profile of other sources (IL-6, IL-8, IL-10, IL-1β, TNF-α, and IFN-γ), we examined the BNL cell line. Glucose was administered over a 24 hour time-course and the inflammatory profile was investigated in the cell lysate. There was a significant increase in TNF-α levels from 5.5 mM to 22 mM glucose stimulation, as shown in Figure 43. This suggests that the liver is a potential source of Th1 cytokine inflammation under hyperglycemic conditions.

Discussion

This is the first study to explore the inflammatory profile of hyperglycemic conditions of salivary submandibular gland cells. We identified that upon glucose stimulation, pro-inflammatory cytokines, such as IFN-γ and IL-13, decrease, whereas anti-inflammatory cytokines, such as IL-10, increase. Thus, hyperglycemic conditions may promote an anti-inflammatory profile of the HSG cell line. The majority of studies exploring the cytokine expressions of salivary gland cells often investigate inflammation associated with Sjögren's Syndrome (SS). One study compared IL-10 and IL-13 levels in biopsied salivary gland homogenates and peripheral blood mononuclear blood cells (PBMC) of SS and healthy subjects

and discovered expression of both cytokines in SS salivary gland samples and little to no expression in healthy controls, whereas only IL-13 gene expression was present in PBMC of SS subjects [180]. These results suggest a network of cytokines contributing to inflammation in a systemic and localized fashion.

Submandibular gland cells do not appear to be a significant source of glucose induced inflammatory cytokine production. The source of inflammation could potentially be derived from other sources, such as neutrophils. Hyperglycemia affects the cytokine expression of BNL cells. Our results suggest that oral Th1 cytokines are potentially derived from other cells populations, such as the liver.

The purpose of this study was to examine the direct impact of glucose on cells producing saliva. Added sugar from the diet, as well as poor glycemic control, initiates an inflammatory cascade generating a chronic overexpression of pro-inflammatory cytokines [178]. In one particular study, the effects of glucose-induced hyperglycemia on human monocytic cells was analyzed. They discovered a significant increase (2 to 5-fold) in the mRNA expression of TNF- α and IL-1 β [181]. The source of inflammation under glucose-induced hyperglycemia in the oral milieu has yet to be extensively studied. This study provides an increased understanding of the inflammatory profile within HSG cells under glucose-stimulated conditions.

Another study analyzed the immunohistochemical expressions of salivary cytokines in lymphocytic infiltrates and epithelial cells of NOD versus BALB/c submandibular glands and found that cytokine expression was greatest in NOD mice, suggesting that this expression is involved in the development of organ-localized autoimmunity in the salivary glands of NOD mice [182]. However, the cytokine expression levels of human submandibular glands have yet to be analyzed.

Limited studies have examined the effects of hyperglycemia on specific cellular populations within the mouth. In a particular study, pharmacological inhibition of p38 MAPK or NF- κ B, but not JNK, significantly suppressed palmitate-induced IL-6 secretion in human parotid

gland epithelial cells, suggesting that IL-6 secretion is induced through activation of said pathways [77]. Therefore, our next objective is to investigate the stress pathways activated from the increased expression of oral Th1 cytokines within HSG and BNL cells.

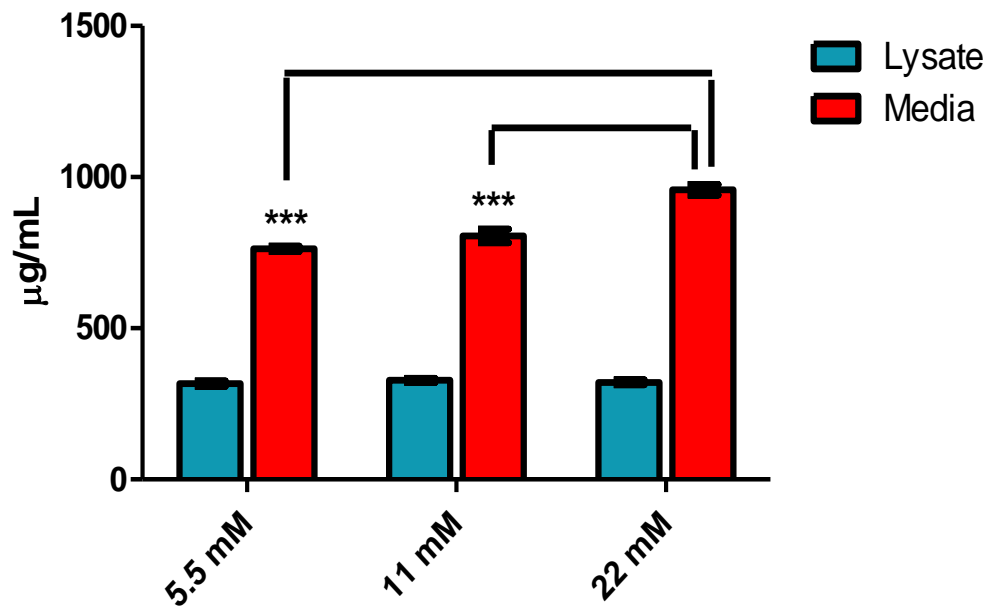


Figure 30: Protein Levels of 24 hour Glucose Stimulation of HSG Media and Cell Lysate Using 200 µL of MPER

This graph demonstrates the combined levels of 3 independent experiments of 24 hour glucose treatments in duplicate (n=6). Total protein content was determined using the BCA protein kit as previously described. There are no significant differences amongst glucose treatments for both cell lysate, however there is a statistical difference (p<0.001) between protein content of the media and glucose concentration. The error bars are represented by the standard error.

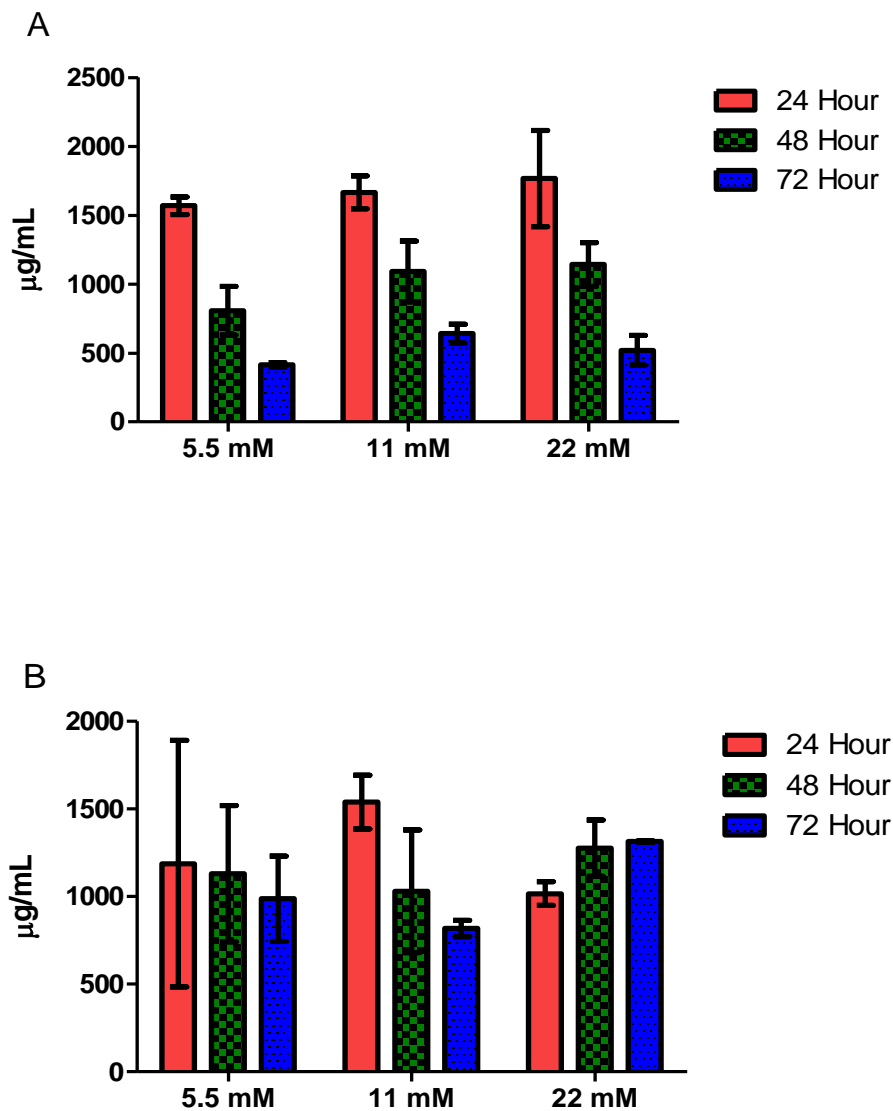


Figure 31: Protein Levels of Glucose Stimulation of HSG Media and Cell Lysate Using 100 µL of MPER

(A) This graph shows the HSG lysate levels of each glucose treatment of 24, 48, and 72 hours (n=3). **(B)** This graph shows the HSG media levels of each glucose treatment of 24, 48, and 72 hours (n=3). Three independent experiments of each glucose stimulation were performed in duplicate. The error bars are represented by the standard error. Total protein content was determined using the BCA protein kit as previously described in Chapter 2.

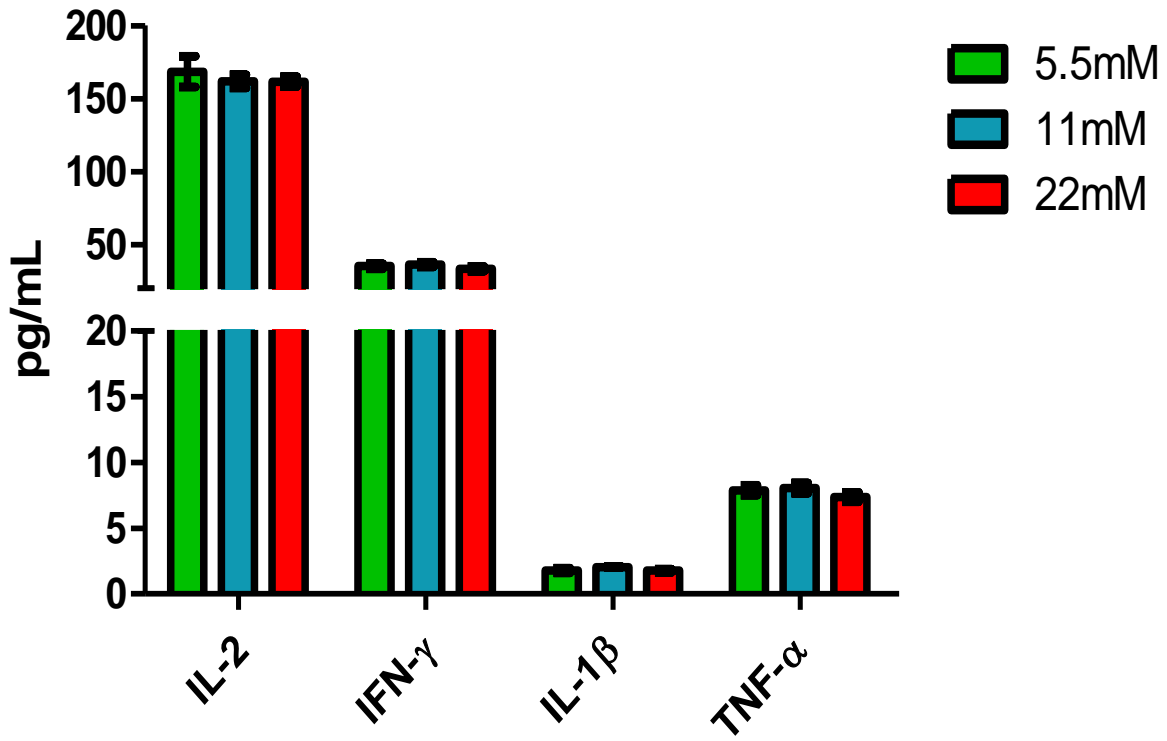


Figure 32: Th1 Cytokine Levels of 24 Hour Glucose Stimulation of HSG Cell Lysate

Human submandibular gland cells (HSGs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 24 hours, in six independent experiments in duplicate. Cell lysate was collected using 200 μ L of MPER and Th1 cytokine levels were measured via Luminex Magpix[®]. No significant trends were observed amongst various treatments. Error bars represent standard error of the mean.

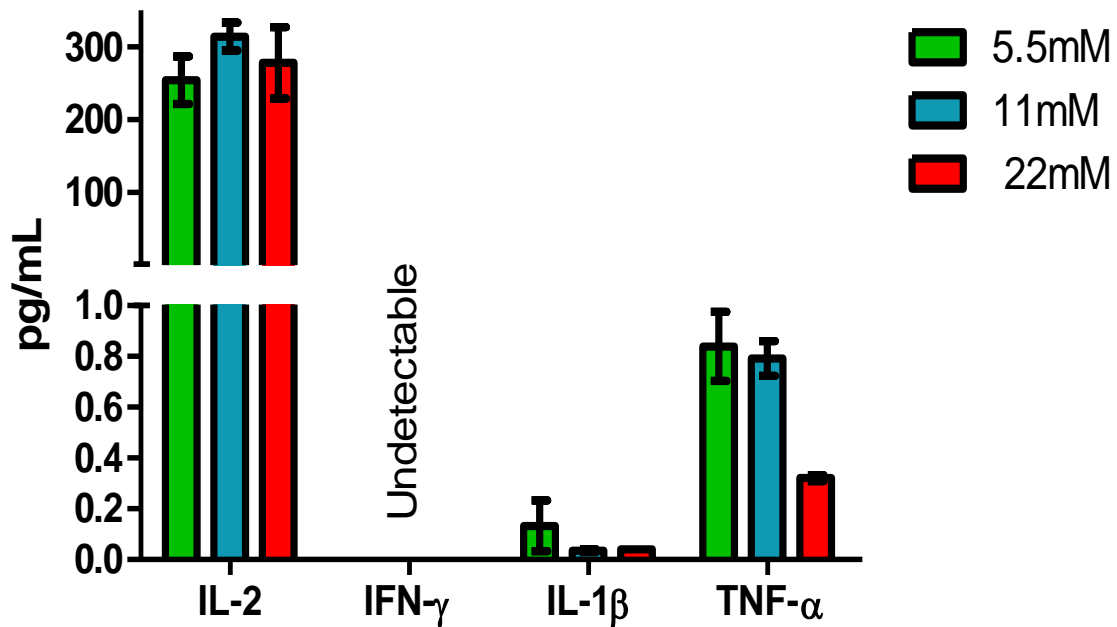


Figure 33: Th1 Cytokine Levels of 24 Hour Glucose Stimulation of HSG Cell Media

Human submandibular gland cells (HSGs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 24 hours, in six independent experiments in duplicate. MMP-8, MMP-9, and IL-8 levels in the media were measured via Luminex Magpix[®]. No significant trends were observed amongst various treatments. A Two-way ANOVA could not be performed due to undetectable levels of several data points. IFN- γ was completely undetectable in HSG media. Error bars represent standard error of the mean.

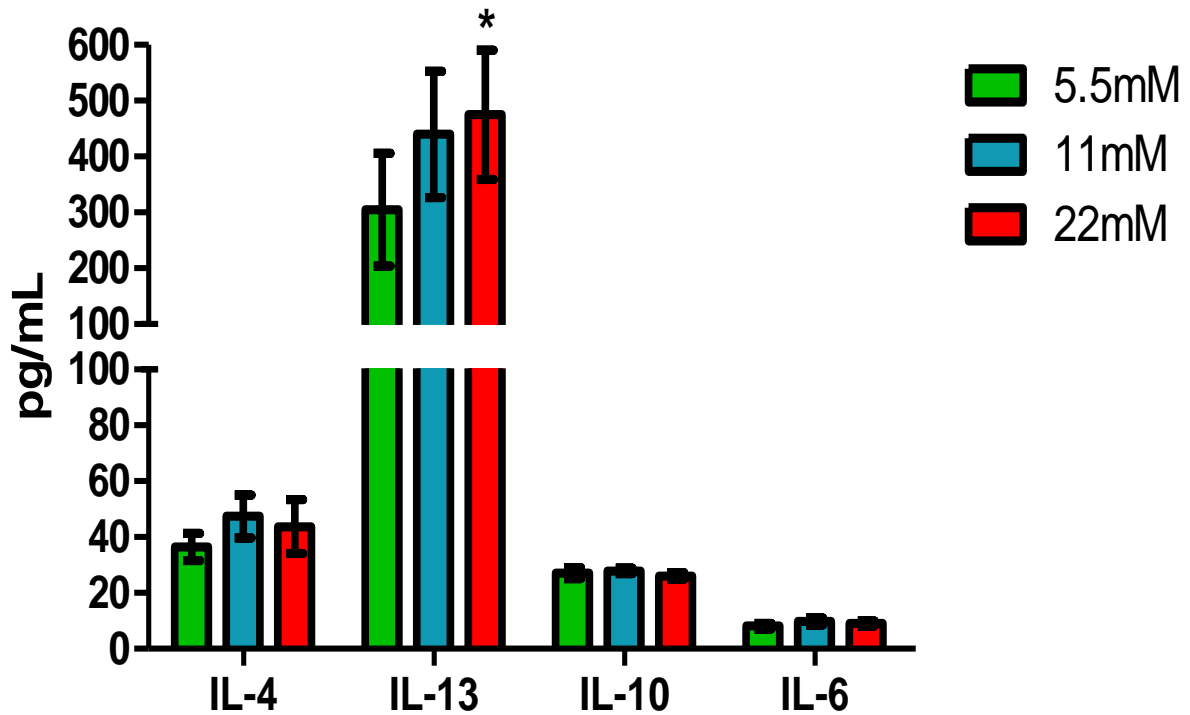


Figure 34: Th2 Cytokine Levels of 24 Hour Glucose Stimulation of HSG Cell Lysate

Human submandibular gland cells (HSGs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 24 hours, in six independent experiments in duplicate. Cell lysate was collected using 200 μ L of MPER and Th2 cytokine levels were measured via Luminex Magpix[®]. IL-13 showed a significant increase ($p < 0.05$) in expression from 5.5 mM to 22 mM glucose treatment. Error bars represent standard error of the mean.

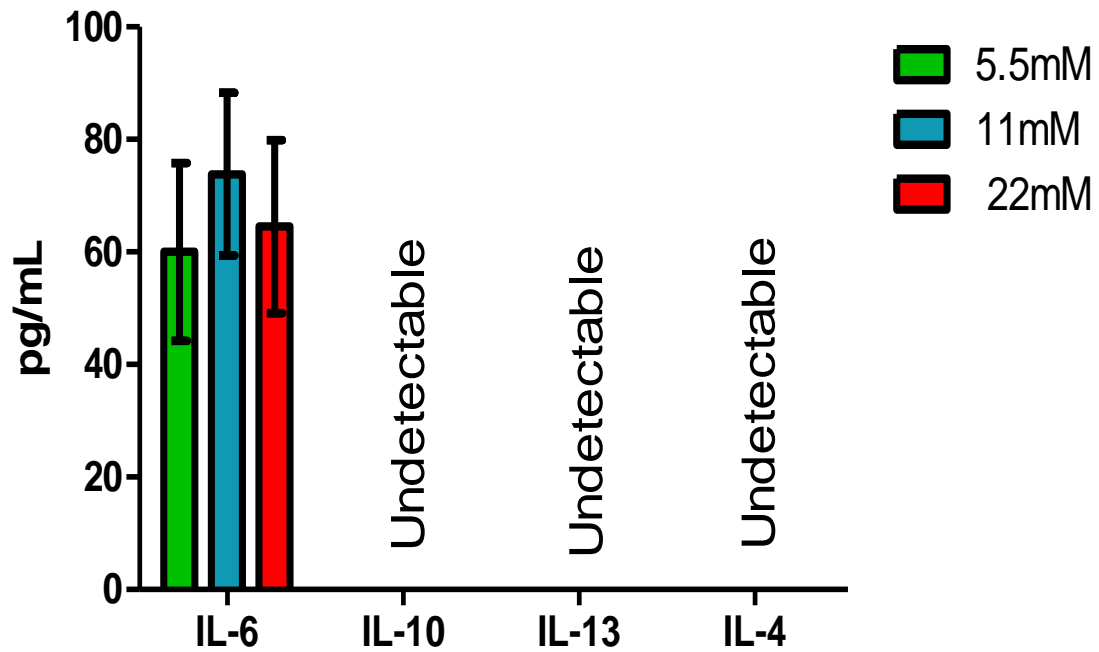


Figure 35: Th2 Cytokine Levels of 24 Hour Glucose Stimulation of HSG Cell Media

Human submandibular gland cells (HSGs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 24 hours, in six independent experiments in duplicate. Th2 cytokine levels were measured via Luminex Magpix[®]. No significant trends were observed amongst various treatments. IL-10, IL-13, and IL-4 levels were all undetectable amongst all glucose treatments. Error bars represent standard error of the mean.

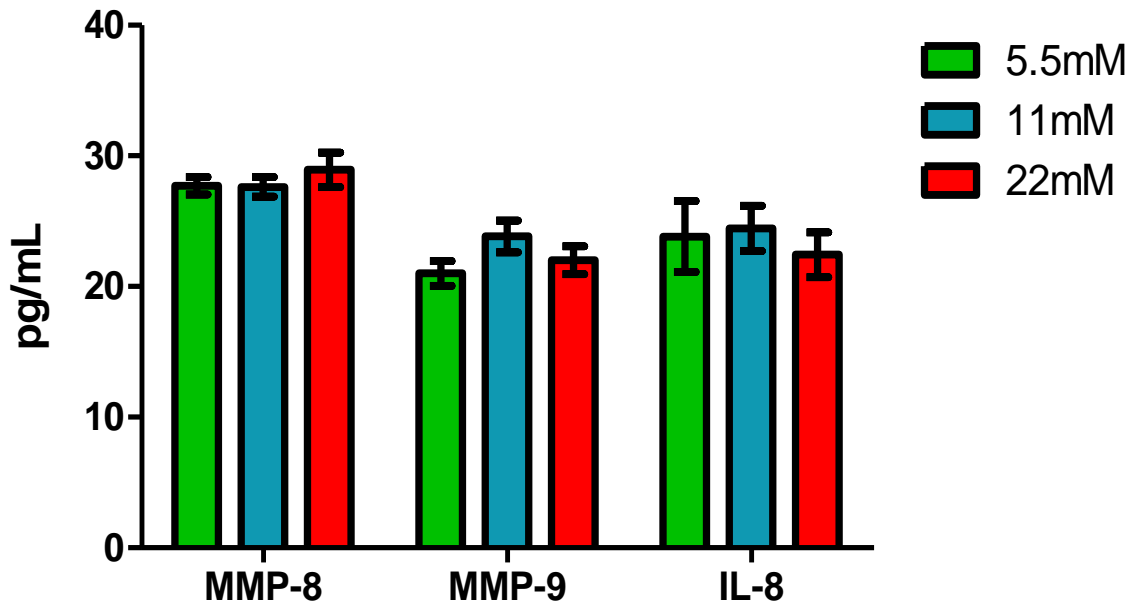


Figure 36: MMP and Chemokine Levels of 24 Hour Glucose Stimulation of HSG Cell Lysate

Human submandibular gland cells (HSGs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 24 hours, in six independent experiments in duplicate. Cell lysate was collected using 200 μ L of MPER and MMP-8, MMP-9, and IL-8 levels were measured via Luminex Magpix[®]. No significant trends were observed amongst various treatments. Error bars represent standard error of the mean.

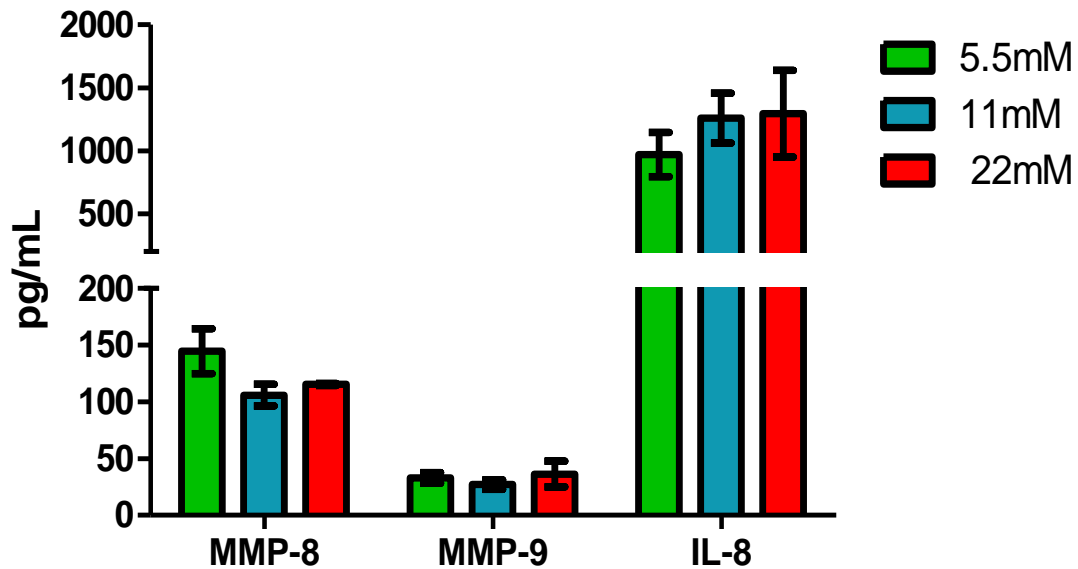


Figure 37: MMP and Chemokine Levels of 24 Hour Glucose Stimulation of HSG Cell Media

Human submandibular gland cells (HSGs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 24 hours, in six independent experiments in duplicate. MMP-8, MMP-9, and IL-8 levels in the media were measured via Luminex Magpix[®]. No significant trends were observed amongst treatments. Error bars represent standard error of the mean. IL-8 levels of 22 mM glucose treatment were mostly undetectable, hence a two-way ANOVA could not be performed.

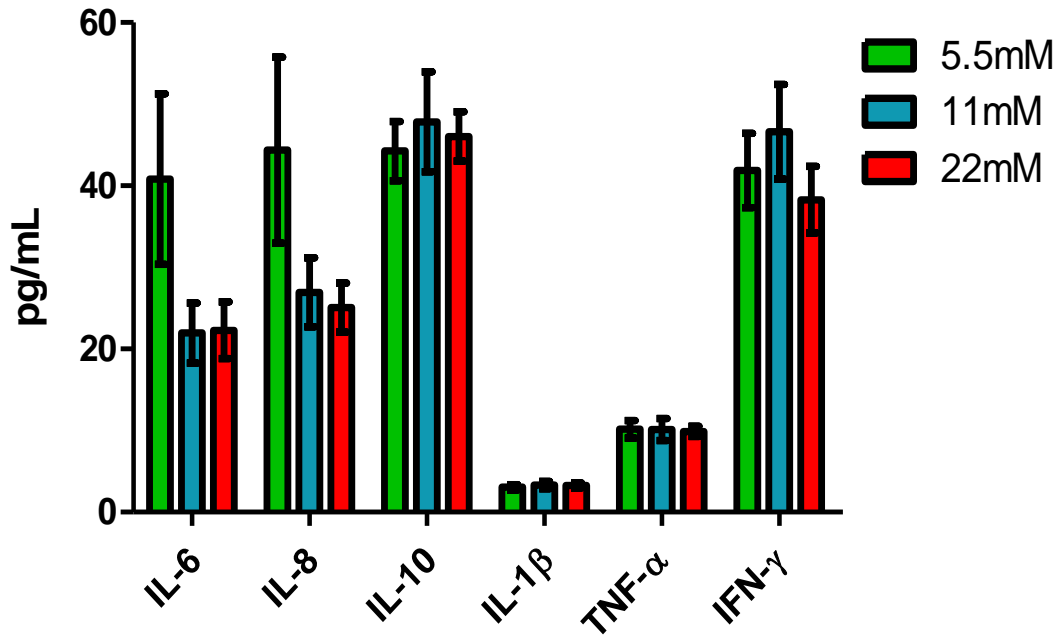


Figure 38: Cytokine Levels of 48 Hour Glucose Stimulation of HSG Cell Lysate

Human submandibular gland cells (HSGs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 48 hours, in three independent experiments in duplicate. Cell lysate was collected using 200 μ L of MPER and cytokine levels were measured via Luminex Magpix[®]. Although no significant trends were observed, IL-8 levels tend to decrease when comparing basal to hyperglycemic conditions. Error bars represent standard error of the mean.

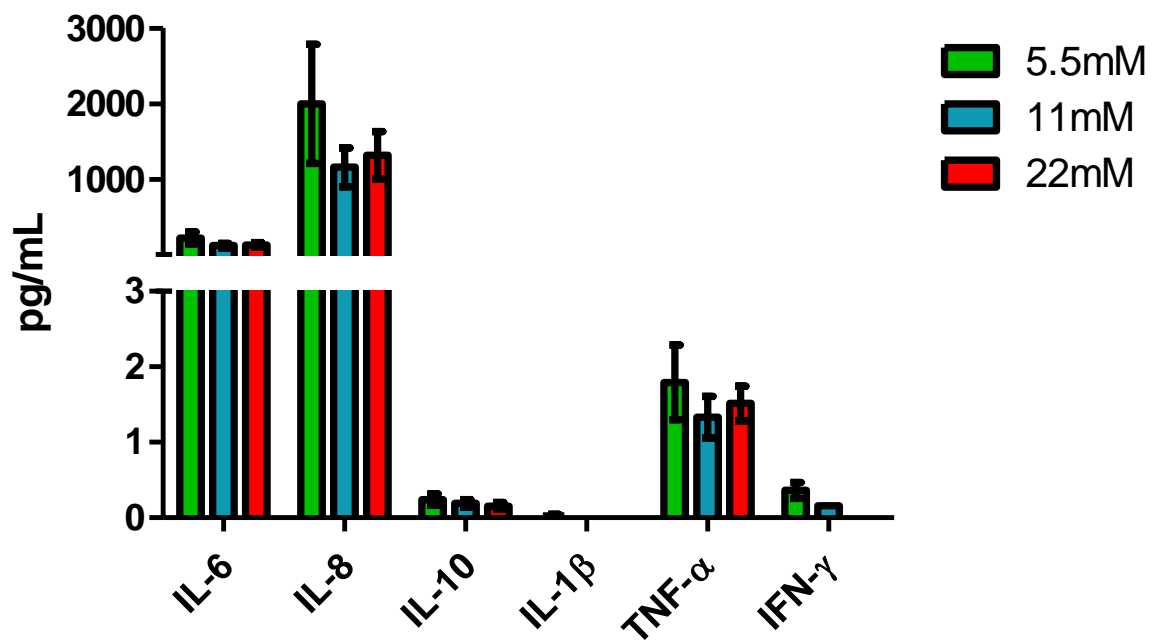


Figure 39: Cytokine Levels of 48 Hour Glucose Stimulation of HSG Cell Media

Human submandibular gland cells (HSGs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 48 hours, in three independent experiments in duplicate. IL-1 β and IFN- γ levels were mostly undetectable. No significant trends were observed amongst various treatments. Error bars represent standard error of the mean.

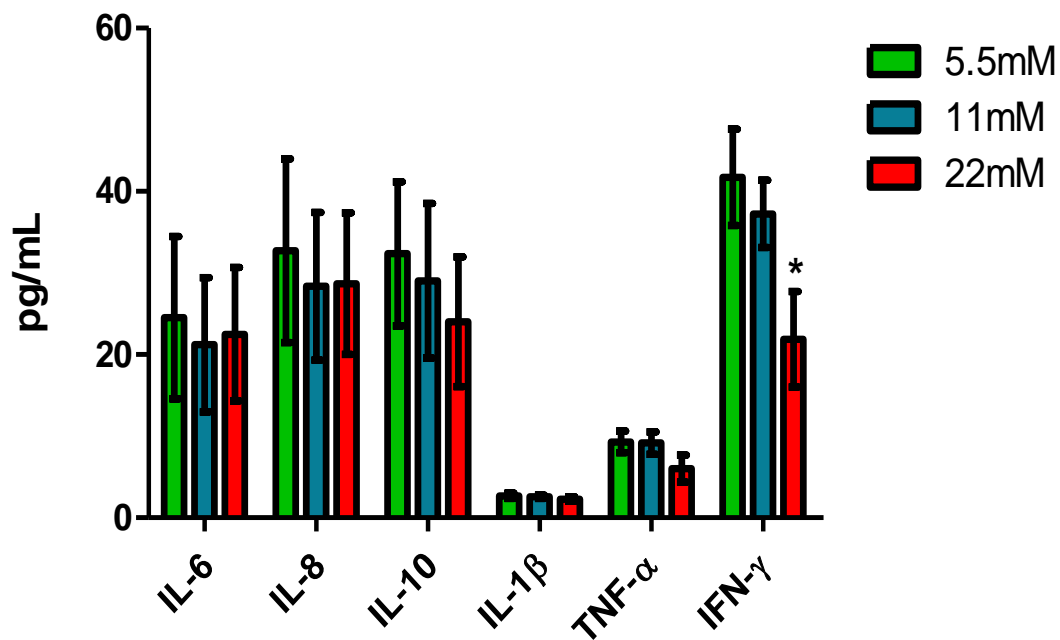


Figure 40: Cytokine Levels of 72 Hour Glucose Stimulation of HSG Cell Lysate

Human submandibular gland cells (HSGs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 72 hours, in three independent experiments in duplicate. Cell lysate was collected using 200 μ L of MPER and cytokine levels were measured via Luminex Magpix[®]. IFN- γ was significantly decreased ($p < 0.05$) from basal to hyperglycemic conditions. Error bars represent standard error of the mean.

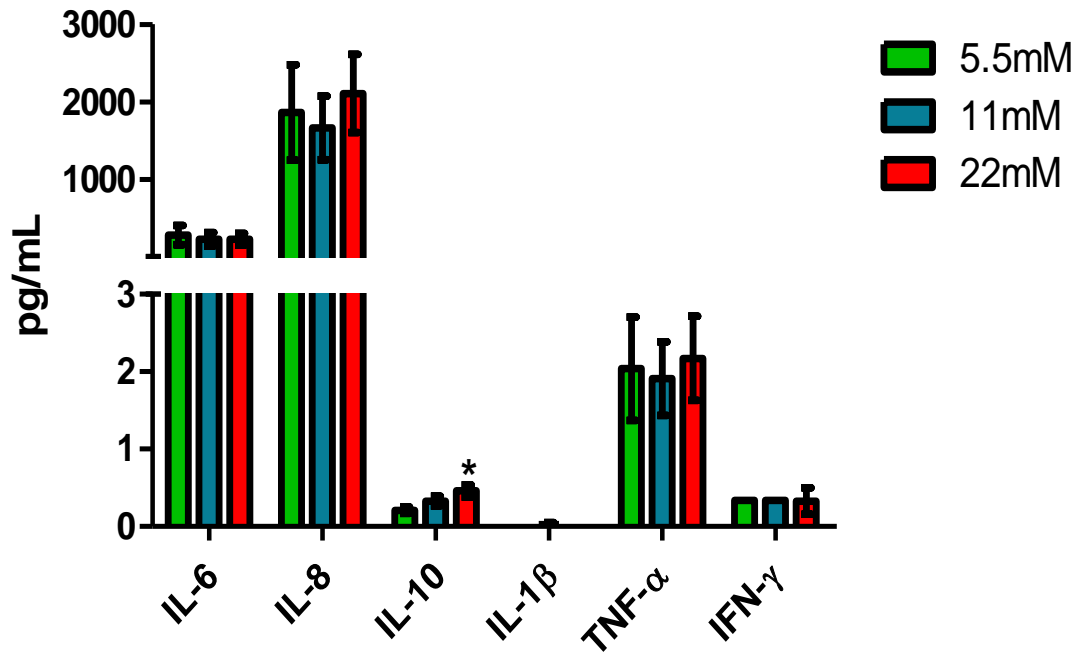


Figure 41: Cytokine Levels of 72 Hour Glucose Stimulation of HSG Cell Media

Human submandibular gland cells (HSGs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 72 hours, in three independent experiments in duplicate. IL-10 levels significantly increased ($p < 0.05$) from basal to hyperglycemic conditions. IL-1 β levels were mostly undetectable across all glucose treatments. Error bars represent standard error of the mean.

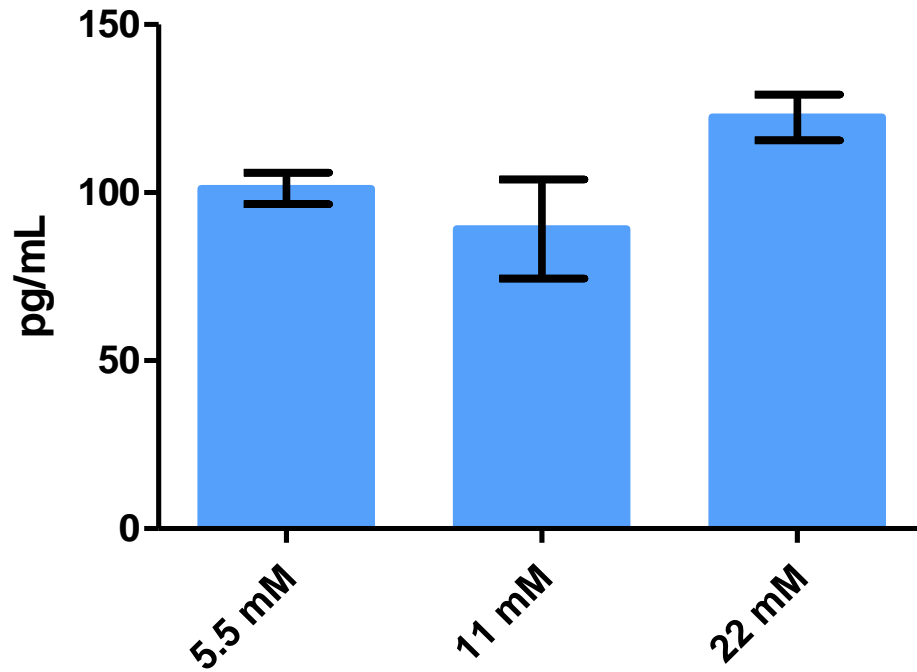


Figure 42: Protein Levels of 24 Hour Glucose Stimulation of BNL Cell Lysate Using 300 μ L of MPER

This graph represents a single experiment of 24 hour glucose treatments in duplicate. Total protein content was determined using the BCA protein kit as previously described. There are no significant differences amongst glucose treatments. The error bars are represented by the standard error.

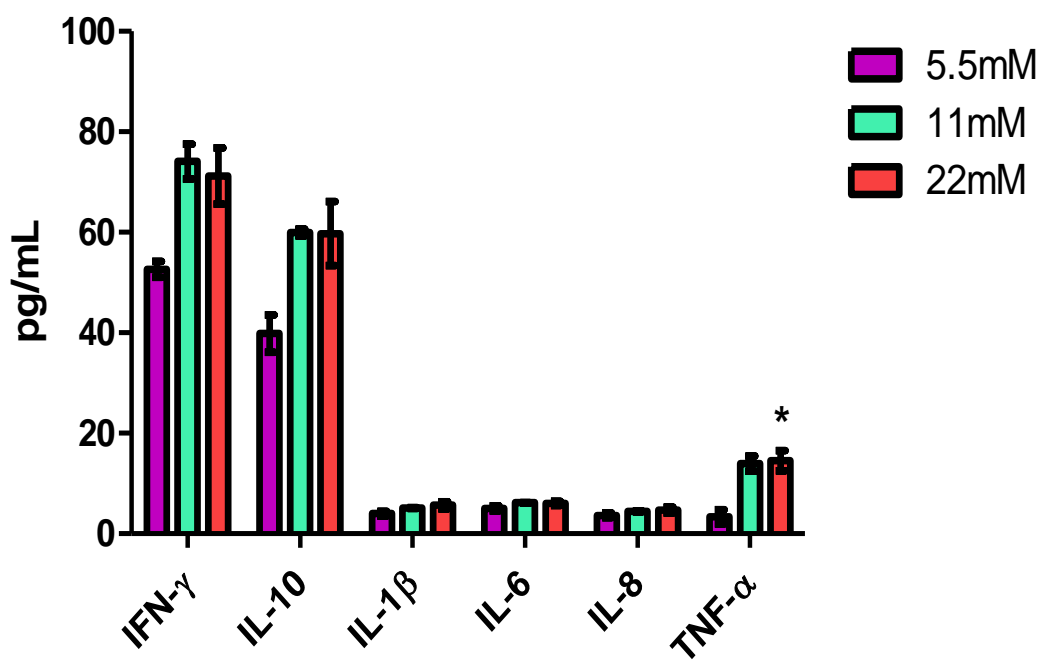


Figure 43: Cytokine Levels of 24 Hour Glucose Stimulation of BNL Cell Lysate

Mouse liver cells (BNLs) were starved for five hours in glucose-free medium and treated with 5.5 mM, 11 mM, and 22 mM concentrations of glucose over 24 hours, in an experiment in duplicate. Cytokine levels were measured via Luminex Magpix[®]. There was a significant increase in TNF- α ($p < 0.05$) as glucose levels increased. No significant trends were observed in the other cytokines using a T-test. Error bars represent standard error of the mean.

Conclusions and Future Directions

Conclusions

My dissertation is the first study to examine and describe the anticipated relationship between glycemic control and oral inflammation. Through use of multiplexing analysis, we revealed that specific salivary inflammatory markers, such as MMP-8, MMP-9, and TNF- α , in T1D subjects are associated with decreased glycemic control and self-reported gingival condition. We examined the saliva of 144 T1D subjects and found an association of oral inflammation with both HbA_{1c} and periodontal status.

Using high sensitivity multiplexing kits, we were able to analyze the specific circadian rhythms of six cytokines: IL-1 β , IL-6, IL-8, IL-10, TNF- α , and IFN- γ in twelve healthy subjects over six different time points throughout the day. Little is known on the impact of altered rhythms in oral inflammation and no studies to date have examined simultaneous measurements of numerous cytokines. We revealed that salivary inflammatory markers have unique diurnal patterns and may affect the overall inflammatory response. Cytokines have their own specific diurnal rhythms and their concentrations are typically highest in either the early morning or late night. Generally speaking, there is a trend of an increase in expression of pro-inflammatory (Th1) cytokines, such as IL-1 β and TNF- α , in the morning and an increase in expression of anti-inflammatory (Th2) cytokines, such as IL-10, in the evening.

We examined the inflammatory profile of eleven analytes in the human submandibular gland cell line to determine the source of oral inflammation. Glucose stimulation in concentrations of 5.5 mM, 11 mM, and 22 mM was applied over 24, 48, and 72 hours. This is the first study to explore the hyperglycemic conditions of human salivary glands. Submandibular gland cells do not appear to be a significant source of glucose-induced

inflammatory cytokine production. Although the source of inflammation is not from the human submandibular gland cells themselves, it could potentially be from other sources such as neutrophils. The oral Th1 cytokines are potentially derived from other cell populations, such as the liver. Overall, upon glucose stimulation, pro-inflammatory (Th2) cytokines, such as IFN- γ and IL-13, tend to decrease, whereas anti-inflammatory (Th1) cytokines, such as IL-10, tend to increase. Hyperglycemic conditions may promote an anti-inflammatory profile of human submandibular gland cells.

Diabetic complications and blood glucose levels are negatively affected by periodontal infection, however the evidence is scarce [178]. There are several risk factors for diabetes and periodontitis, including age, genetics, obesity, smoking, stress, diet, viruses, and smoking [178]. Figure 44 summarizes the overall relationship between diabetes periodontal disease, circadian rhythms and oral inflammation. There is a bi-directional relationship between periodontal health and diabetes— periodontal infection creates an inflammatory response that contributes to hyperglycemia whereas increase blood glucose levels initiates the development and prolongation of periodontal disease [178].

It all begins with inflammation from the dental plaque and its oral microbiome, which triggers hyperinflammation that causes alveolar bone to break down. Both local and systemic inflammation occurs via the periodontium and bacterial endotoxins, respectively. Added sugar from the diet, as well as poor glycemic control, initiates an inflammatory cascade generating a chronic overexpression of pro-inflammatory cytokines and MMPs. This, in turn, leads to inflammatory mediators acting as antagonists on insulin receptors generating insulin resistance [111] and stimulation of the liver to express CRP, serum amyloid A, and fibrogen, all of which contribute to hyperglycemia. This hyperglycemic condition stimulates oxidative and other cellular stress increases free radicals, ROS, and the irreversible production of AGEs to instigate further inflammatory dysfunction contributing to inflammation of the oral mucosal tissues and potentially exacerbating periodontal disease [111, 178].

Despite this bidirectional relationship, little is known on the association between glucose control and oral inflammation as well as diurnal variation and the source of inflammation. Therefore, we investigated generalized inflammation within the mouth over a 24 hour time course and discovered IL-13 increases as hyperglycemic conditions occur in human submandibular gland cells. Diurnal fluctuations of cytokine expression must be considered when analyzing salivary inflammation. Generally speaking, inflammation occurs primarily in the morning and night indicating hormonal influences on salivary inflammation. The alterations of inflammatory cytokines, MMPs, and chemokines might explain the variations of the oral environment during the day. The source of this inflammation is predominately systemic rather than localized within the salivary milieu. Overall, these results demonstrate the efficacy and potential utility of potential diagnostic salivary analysis of novel noninvasive biomarkers predictive of the progression and control of T1D.

Future Directions

The search for reliable biomarkers of type 1 diabetes continues. TEDDY has started actively collecting peripheral blood mononuclear cells (PBMC) in high risk children in May 2010 to study serum proteins as inflammatory or autoimmunity markers [35]. Advancements in this project will increase our understanding for discovering a potential breakthrough in the progression and diagnosis of diabetes.

Our findings that diurnal rhythms of cytokines do exist and have distinctive patterns pave the pathway in understanding the biological basis of these markers. Yet, the growth of salivary diagnostics has been impeded due to lack of sensitive detection methods, poor understanding of the biomolecular flow of proteins and hormones from the blood into the saliva, and circadian variations within saliva [183]. As newer, more sensitive assays are developed and further explorations of circadian rhythms of biomolecules within the saliva are completed, we could transition the discoveries of disease biomarkers to a clinical practice.

Our study has suggested that the salivary inflammatory burden may also be an indication of glycemic status or clinical management in T1D. These data can be further utilized to establish novel clinical diagnostic tools to promote patient compliance and enhance subsequent clinical application of interventional therapy. In addition, oral inflammatory burden with biomarkers described in this publication may also be combined with other biomarkers (i.e. autoantibodies in the case of T1D) either circulating or from the saliva that can be implemented to generate predictive models to identify subjects in the early stages of either developing autoimmunity or glucose intolerance.

Limited studies have examined the effects of hyperglycemia on specific cellular populations within the mouth. In a particular study, pharmacological inhibition of p38 MAPK or NF- κ B, but not JNK, significantly suppressed palmitate-induced IL-6 secretion in human parotid gland epithelial cells, suggesting that IL-6 secretion is induced through activation of said pathways [77]. Therefore, our next objective is to investigate the stress pathways activated from the increased expression of oral Th1 cytokines within HSG cells. Additionally, to broaden our comprehension of the source of inflammation, the investigation and analysis of activated stress pathways within additional cell populations, such as the liver, needs to be implemented.



Figure 44: Relationship of Diabetes, Periodontal Disease and Oral Inflammation

Oral inflammation contributes to periodontal disease and diabetes via numerous factors as listed. Time of collection is important due to the variation of cytokine and chemokine diurnal rhythms. Exogenous sugar from the diet contributes to oral inflammation through production of AGEs and increased cytokine expression creating an insulin resistance. Periodontal disease contributes to diabetes through the increased expression of MMPs leading to increased expression of cytokines. Lastly, one of the major causes of diabetes is hyperglycemia, which in turn, can lead to oral inflammation.

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Appendices

Appendix A: Copyright Permission

9/24/2015

University of South Florida Mail - Inquiry of Copyright



Melanie K <mkuehl@mail.usf.edu>

Inquiry of Copyright

2 messages

Melanie K <mkuehl@mail.usf.edu>
To: plos@plos.org

Fri, Sep 11, 2015 at 11:23 AM

To whom it may concern:

My name is Melanie Kuehl and I am a graduate student at the University of South Florida. I have published a first author paper in Plos One earlier this year entitled, "Tumor Necrosis Factor- α , Matrix-Metalloproteinases 8 and 9 Levels in the Saliva Are Associated with Increased Hemoglobin A1c in Type 1 Diabetes Subjects". May I have permission to use this article in my PhD dissertation for publication?

Thank you,

Melanie Kuehl

PLOS <PLOS@plos.org>
To: Melanie K <mkuehl@mail.usf.edu>

Fri, Sep 11, 2015 at 1:50 PM

Dear Melanie,

Thank you for your enquiry.

We are an open access publisher and as such everything we publish is freely available online throughout the world, for you to read, download, copy, distribute, and use (with attribution) any way you wish.

No permission required.

For information on the Creative Commons Attribution License please follow this link <http://www.plos.org/about/open-access/license/>

Hope this is helpful.

Thank you,

Tamaira Witherspoon

PLOS

11 September 2015

Appendix B: Consent Form for Salivary Biomarkers of Inflammation in Type 1 Diabetes

Study ID:Ame2_Pro00009518 Date Approved: 2/25/2013 Expiration Date: 10/15/2013



Informed Consent to Participate in Research Information to Consider Before Taking Part in this Research Study

IRB Study # Pro00009518

You are being asked to take part in a research study. Research studies include only people who choose to take part. This document is called an informed consent form. Please read this information carefully and take your time making your decision. Ask the researcher or study staff to discuss this consent form with you, please ask him/her to explain any words or information you do not clearly understand. We encourage you to talk with your family and friends before you decide to take part in this research study. The nature of the study, risks, inconveniences, discomforts, and other important information about the study are listed below.

Please tell the study doctor or study staff if you are taking part in another research study.

We are asking you to take part in a research study called:
Salivary Biomarkers of Inflammation in Type 1 Diabetes

The person who is in charge of this research study is Amy Alman, PhD. This person is called the Principal Investigator. However, other research staff may be involved and can act on behalf of the person in charge.

All research for this study will be conducted at the University of South Florida Morsani Center for Advanced Health Care, the USF College of Public Health, and the USF Department of Cell Biology, Microbiology and Molecular Biology.

Purpose of the study

The purpose of this study is to:

- Describe the presence of markers of inflammation in the saliva of adults with type 1 diabetes
- Determine if these markers are associated with glycemic control

Study Procedures

If you take part in this study, you will be asked to:

- Provide a saliva sample by passively drooling into a collection vial
- Answer two questions about your oral health
- Participation will take about 5-10 minutes of your time and can be completed at this clinic visit

- Your current BMI, hemoglobin A1c (a measure of glycemic control), duration of diabetes, race, gender, and age will be recorded from your medical record as a part of this study
- We will not record your name, address, phone number, or any other identifying information

Total Number of Participants

About 150 individuals will take part in this study at USF.

Alternatives

You do not have to participate in this research study.

Benefits

We are unsure if you will receive any benefits by taking part in this research study.

Risks or Discomfort

This research is considered to be minimal risk. That means that the risks associated with this study are the same as what you face every day. There are no known additional risks to those who take part in this study.

Compensation

You will receive no payment or other compensation for taking part in this study.

Cost

There will be no additional costs to you as a result of being in this study. However, routine medical care for your condition (care you would have received whether or not you were in this study) will be charged to you or your insurance company. You may wish to contact your insurance company to discuss this further.

Authorization to Use and Disclose Protected Health Information

Who will see your health information?

In this research study, we use and share your health information to the extent authorized (permitted) by you. We know that this information is private. The federal privacy regulations of the Health Insurance Portability & Accountability Act (HIPAA) protect your identifiable health information. If you authorize us to use your information we will protect it as required by the law.

This research is conducted at the University of South Florida (USF). By signing this form, you are permitting USF to use personal health information collected about you for research purposes within the USF health care system. You are also allowing USF to share your personal health information with individuals or organizations other than USF who are also involved in the research and listed below.

Who will disclose (share), receive, and/or use your information?

To conduct this research, USF and the people and organizations may use or share your information. They may only use and share your information:

- With the people and organizations on this list;
- With you or your personal representative; and
- As allowed by law.

In addition to the people and organizations listed below in the Privacy and Confidentiality section of this document, the following groups of people may also be able to see information about you and may use the information to conduct the research:

- The medical staff that takes care of you and those who are part of this research study;
- Each research site for this study. This includes the research and medical staff at each site and USF;
- Any laboratories, pharmacies, or other individuals and organizations that use your health information as part of the approved plan for this study;
- The designated peer review committees such as the Protocol Review and Monitoring Committee;

Who else can use and share this information?

Anyone listed above may use consultants in this research and for the purpose of this study, may share your information with them. If you have questions about who they are, you should ask the study team. Individuals who receive your health information for this research study may not be required by the HIPAA Privacy Rule to protect it and may share your information with others without your permission. They can only do so if permitted by the laws governing them. For example, the study sponsor may share your information with others. If the sponsor or others share your information, your information may no longer be protected under the HIPAA Privacy Rule.

How will my information be used?

By signing this form, you are giving your permission to use and/or share your health information as described in this document for any and all study/research related purposes. Your authorization to use your health information will not expire unless you revoke it in writing.

As part of this research, USF may collect, use, and share the following information:

- Your current BMI, hemoglobin A1c (a measure of glycemic control), duration of diabetes, race, gender, and age
- Levels of the inflammatory markers measured from your saliva
- Your collected saliva sample may be used for future studies

You can list any particular information that you do not want us to use or share in the space below. If you list nothing here, we can use and share all of the information listed above for this research but for nothing else.

For the Research Participant (you) to complete:

I am asking USF and the researchers not to include, use, or share the following health information in this research (if blank, then no information will be excluded):

Your Rights:

You can refuse to sign this form. If you do not sign this form you will not be able to take part in this research study and therefore not be able to receive the research related interventions. However, your health care outside of this study and benefits will not change.

How Do I Withdraw Permission to Use My Information?

You can revoke this form at any time by sending a letter clearly stating that you wish to withdraw your authorization to use of your health information in the research. If you revoke your permission:

- You will no longer be a participant in this research study;
- We will stop collecting new information about you;
- We will use the information collected prior to the revocation of your authorization. This information may already have been used or shared with other, or we may need it to complete and protect the validity of the research; and
- Staff may need to follow-up with you if there is a medical reason to do so.

To revoke this form, please write to:

Principal Investigator

For IRB Study # **Pro00009518**

University of South Florida

College of Public Health, Department of Epidemiology and Biostatistics

13201 Bruce B Downs Blvd, MDC 56

Tampa, FL 33612

While we are conducting the research study, we cannot let you see or copy the research information we have about you. After the research is completed, you have a right to see the information about you, as allowed by USF policies.

Privacy and Confidentiality

We will keep your study records private and confidential. Certain people may need to see your study records. By law, anyone who looks at your records must keep them completely confidential. The only people who will be allowed to see these records are:

- The research team, including the Principal Investigator, study coordinator, research nurses, and all other research staff.
- Certain government and university people who need to know more about the study. For example, individuals who provide oversight on this study may need to look at your records. This is done to make sure that we are doing the study in the right way. They also need to make sure that we are protecting your rights and your safety.
- Any agency of the federal, state, or local government that regulates this research. This includes the Florida Department of Health, and the Department of Health and Human Services (DHHS) and the Office for Human Research Protection (OHRP).

- The USF Institutional Review Board (IRB) and its related staff who have oversight responsibilities for this study, staff in the USF Office of Research and Innovation, USF Division of Research Integrity and Compliance, and other USF offices who oversee this research.

We may publish what we learn from this study. If we do, we will not include your name. We will not publish anything that would let people know who you are.

Voluntary Participation / Withdrawal

You should only take part in this study if you want to volunteer. You should not feel that there is any pressure to take part in the study. You are free to participate in this research or withdraw at any time. There will be no penalty or loss of benefits you are entitled to receive if you stop taking part in this study.

New information about the study

During the course of this study, we may find more information that could be important to you. This includes information that, once learned, might cause you to change your mind about being in the study. We will notify you as soon as possible if such information becomes available.

You can get the answers to your questions, concerns, or complaints

If you have any questions, concerns or complaints about this study, or experience an adverse event or unanticipated problem, call Amy Alman at 813-974-2235.

If you have questions about your rights as a participant in this study, general questions, or have complaints, concerns or issues you want to discuss with someone outside the research, call the USF IRB at (813) 974-5638.

Consent to Take Part in this Research Study

and Authorization to Collect, Use and Share Your Health Information

It is up to you to decide whether you want to take part in this study. If you want to take part, please sign the form, if the following statements are true.

I freely give my consent to take part in this study and authorize that my health information as agreed above, be collected/disclosed in this study. I understand that by signing this form I am agreeing to take part in research. I have received a copy of this form to take with me.

Signature of Person Taking Part in Study

Date

Printed Name of Person Taking Part in Study

Statement of Person Obtaining Informed Consent

I have carefully explained to the person taking part in the study what he or she can expect from their participation. I hereby certify that when this person signs this form, to the best of my knowledge, he/she understands:

- What the study is about;
- What procedures/interventions/investigational drugs or devices will be used;
- What the potential benefits might be; and
- What the known risks might be.

I can confirm that this research subject speaks the language that was used to explain this research and is receiving an informed consent form in the appropriate language. Additionally, this subject reads well enough to understand this document or, if not, this person is able to hear and understand when the form is read to him or her. This subject does not have a medical/psychological problem that would compromise comprehension and therefore makes it hard to understand what is being explained and can, therefore, give legally effective informed consent. This subject is not under any type of anesthesia or analgesic that may cloud their judgment or make it hard to understand what is being explained and, therefore, can be considered competent to give informed consent.

Signature of Person Obtaining Informed Consent / Research Authorization

Date

Printed Name of Person Obtaining Informed Consent / Research Authorization

Appendix C: Consent Form for Salivary Diurnal Inflammatory Study (SADI)

Study ID: Pro00018592 Date Approved: 9/22/2015 Expiration Date: 9/22/2016



Informed Consent to Participate in Research Involving Minimal Risk

Pro # Pro00018592

You are being asked to take part in a research study. Research studies include only people who choose to take part. This document is called an informed consent form. Please read this information carefully and take your time making your decision. Ask the researcher or study staff to discuss this consent form with you, please ask him/her to explain any words or information you do not clearly understand.

We are asking you to take part in a research study called:

Salivary Diurnal Inflammatory Study (SADI)

The person who is in charge of this research study is Brant R. Burkhardt, Ph.D.. This person is called the Principal Investigator. However, other research staff may be involved and can act on behalf of the person in charge.

The research will be conducted the University of South Florida USF Department of Cell Biology, Microbiology and Molecular Biology.

Purpose of the study

The purpose of this study is to:

- Characterize inflammation in the saliva of healthy adults
- Characterize the microbiome in the saliva of healthy adults

Why are you being asked to take part?

We are asking you to take part in this research study because you are in optimal health and have some degree of scientific training that enables you to understand and preserve self-collected saliva samples for subsequent analysis.

Study Procedures:

If you take part in this study, you will be asked to:

- Provide saliva samples over the course of a day by passively drooling into a collection vial (instructions will be give upon consent)
- Answer two questions about your oral health
- Participation can take place at home
- Your current BMI, race, gender, and age will be recorded for this study
- We will not record your name, address, phone number, or any other identifying information

Total Number of Participants

About 20 individuals will take part in this study at USF.

Alternatives / Voluntary Participation / Withdrawal

You do not have to participate in this research study.

You should only take part in this study if you want to volunteer. You should not feel that there is any pressure to take part in the study. You are free to participate in this research or withdraw at any time. There will be no penalty or loss of benefits you are entitled to receive if you stop taking part in this study. Decision to not participate will not affect your student status (course grade) or job status.

Benefits

We are unsure if you will receive any benefits by taking part in this research study.

Risks or Discomfort

This research is considered to be minimal risk. That means that the risks associated with this study are the same as what you face every day. There are no known additional risks to those who take part in this study.

Compensation

You will receive no payment or other compensation for taking part in this study.

Costs

There will be no additional costs to you as a result of being in this study.

Privacy and Confidentiality

We will keep your study records private and confidential. Certain people may need to see your study records. Anyone who looks at your records must keep them confidential. These individuals include:

- The research team, including the Principal Investigator, study coordinator, research nurses, and all other research staff.
- Certain government and university people who need to know more about the study, and individuals who provide oversight to ensure that we are doing the study in the right way.
- Any agency of the federal, state, or local government that regulates this research.
- The USF Institutional Review Board (IRB) and related staff who have oversight responsibilities for this study, including staff in USF Research Integrity and Compliance.

We may publish what we learn from this study. If we do, we will not include your name. We will not publish anything that would let people know who you are.

What if new information becomes available about the study?

During the course of this study, we may find more information that could be important to you. This includes information that, once learned, might cause you to change your mind about being in this study. We will notify you as soon as possible if such information becomes available.

You can get the answers to your questions, concerns, or complaints

If you have any questions, concerns or complaints about this study, or experience an adverse event or unanticipated problem, call Brant Burkhardt at 813-974-5968.

If you have questions about your rights as a participant in this study, or have complaints, concerns or issues you want to discuss with someone outside the research, call the USF IRB at (813) 974-5638.

As part of this research, USF may collect, use, and share the following information

- Your current BMI, race, gender, and age
- Levels of the inflammatory markers measured from your saliva
- Microbiome characterization
- Your collected saliva sample may be used for future studies

You can refuse to sign this form. If you do not sign this form you will not be able to take part in this research study. However, your care outside of this study and benefits will not change. Your authorization to use your health information will not expire unless you revoke (withdraw) it in writing. You can revoke this form at any time by sending a letter clearly stating that you wish to withdraw your authorization to use your health information in the research. If you revoke your permission:

- You will no longer be a participant in this research study;
- We will stop collecting new information about you;
- We will use the information collected prior to the revocation of your authorization. This information may already have been used or shared with others, or we may need it to complete and protect the validity of the research; and
- Staff may need to follow-up with you if there is a medical reason to do so.
- In addition to the procedures outlined above, we are asking you to allow us to obtain and store samples of your saliva for use in future research. The samples will be stored in a locked -80°C freezer in tubes labeled only with subject number and no identifying information.

To revoke this form, please write to:

Brant Burkhardt, Ph.D.
For IRB Study # Pro00018592
University of South Florida
4202 East Fowler Avenue
Tampa, FL 33620-5550

While we are conducting the research study, we cannot let you see or copy the research information we have about you. After the research is completed, you have a right to see the information about you, as allowed by USF policies.

Consent to Take Part in this Research Study

I freely give my consent to take part in this study. I understand that by signing this form I am agreeing to take part in research. I have received a copy of this form to take with me.

Signature of Person Taking Part in Study

Date

Printed Name of Person Taking Part in Study

Statement of Person Obtaining Informed Consent

I have carefully explained to the person taking part in the study what he or she can expect from their participation. I confirm that this research subject speaks the language that was used to explain this research and is receiving an informed consent form in their primary language. This research subject has provided legally effective informed consent.

Signature of Person obtaining Informed Consent

Date

Printed Name of Person Obtaining Informed Consent

Appendix D: IRB Approval for Salivary Biomarkers of Inflammation in Type 1 Diabetes



DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
Institutional Review Boards, FWA No. 00001669
12901 Bruce B. Downs Blvd., MDC035 • Tampa, FL 33612-4799
(813) 974-5638 • FAX (813) 974-5618

October 15, 2012

Amy Alman, PhD
Epidemiology and Biostatistics
13201 Bruce B Downs Blvd., MDC 56
Tampa, FL 33612

RE: **Expedited Approval** for Initial Review
IRB#: Pro00009518
Title: Salivary biomarkers of inflammation in type 1 diabetes

Dear Dr. Alman:

On 10/15/2012 the Institutional Review Board (IRB) reviewed and **APPROVED** the above referenced protocol. Please note that your approval for this study will expire on 10/15/2013.

Approved Items:

Protocol Document(s):

[Salivary Biomarkers of Inflammation Protocol](#)

[Salivary Biomarkers Abstraction Form](#)

Consent/Assent Documents:

[Revised Salivary inflammation Informed Consent.pdf](#)

Please note, the informed consent/assent documents are valid during the period indicated by the official, IRB-Approval stamp located on the form. Valid consent must be documented on a copy of the most recently IRB-approved consent form.

It was the determination of the IRB that your study qualified for expedited review which includes activities that (1) present no more than minimal risk to human subjects, and (2) involve only procedures listed in one or more of the categories outlined below. The IRB may review research through the expedited review procedure authorized by 45CFR46.110 and 21 CFR 56.110. The research proposed in this study is categorized under the following expedited review categories 3, 5 and 7:

(3) Prospective collection of biological specimens for research purposes by noninvasive means.

(5) Research involving materials (data, documents, records, or specimens) that have been collected, or will be collected solely for nonresearch purposes (such as medical treatment or diagnosis).

(7) Research on individual or group characteristics or behavior (including, but not limited to, research on perception, cognition, motivation, identity, language, communication, cultural beliefs or practices, and social behavior) or research employing survey, interview, oral history, focus group, program evaluation, human factors evaluation, or quality assurance methodologies.

As the principal investigator of this study, it is your responsibility to conduct this study in accordance with IRB policies and procedures and as approved by the IRB. Any changes to the approved research must be submitted to the IRB for review and approval by an amendment.

We appreciate your dedication to the ethical conduct of human subject research at the University of South Florida and your continued commitment to human research protections. If you have any questions regarding this matter, please call 813-974-5638.

Sincerely,

A handwritten signature in blue ink that reads "V. Jorgensen MD". The signature is written in a cursive style.

E. Verena Jorgensen, MD, Chairperson
USF Institutional Review Board

Appendix E: IRB Approval for Salivary Diurnal Inflammatory Analysis



RESEARCH INTEGRITY AND COMPLIANCE
Institutional Review Boards, FWA No. 00001669
12901 Bruce B. Downs Blvd., MDC035 • Tampa, FL 33612-4799
(813) 974-5638 • FAX(813)974-7091

9/22/2015

Brant Burkhardt, Ph.D.
Cellular, Molecular & Micro-Biology
Tampa, FL 33620

RE: **Expedited Approval for Initial Review**
IRB#: Pro00018592
Title: Salivary Diurnal Inflammatory Analysis (SADI)

Study Approval Period: 9/22/2015 to 9/22/2016

Dear Dr. Burkhardt:

On 9/22/2015, the Institutional Review Board (IRB) reviewed and **APPROVED** the above application and all documents contained within, including those outlined below.

Approved Item(s):

Protocol Document(s):

[Salivary Diurnal Inflammatory Analysis \(SADI\)](#)

[Salivary Inflammatory Manuscript](#)

Consent/Assent Document(s)*:

[SB Adult Minimal Risk v003_biomed_study_09222015.docx.pdf](#)

*Please use only the official IRB stamped informed consent/assent document(s) found under the "Attachments" tab. Please note, these consent/assent document(s) are only valid during the approval period indicated at the top of the form(s).

It was the determination of the IRB that your study qualified for expedited review which includes activities that (1) present no more than minimal risk to human subjects, and (2) involve only procedures listed in one or more of the categories outlined below. The IRB may review research through the expedited review procedure authorized by 45CFR46.110 and 21 CFR 56.110. The research proposed in this study is categorized under the following expedited review category:

(3) Prospective collection of biological specimens for research purposes by noninvasive means.

(7) Research on individual or group characteristics or behavior (including, but not limited to, research on perception, cognition, motivation, identity, language, communication, cultural beliefs or practices, and social behavior) or research employing survey, interview, oral history, focus group, program evaluation, human factors evaluation, or quality assurance methodologies.

As the principal investigator of this study, it is your responsibility to conduct this study in accordance with IRB policies and procedures and as approved by the IRB. Any changes to the approved research must be submitted to the IRB for review and approval via an amendment. Additionally, all unanticipated problems must be reported to the USF IRB within five (5) calendar days.

We appreciate your dedication to the ethical conduct of human subject research at the University of South Florida and your continued commitment to human research protections. If you have any questions regarding this matter, please call 813-974-5638.

Sincerely,

A handwritten signature in blue ink that reads "Janelle Perkins, Pharm.D.".

Janelle Perkins, Pharm.D., Chairperson
USF Institutional Review Board

Appendix F: Interview and Abstraction Forms

Salivary Biomarkers of Inflammation in Saliva Interview and Abstraction Form

Version 08/16/2012

Study ID: _____

Oral Health Interview Questions

1. Compared to others your age, how would you rate the condition of your gums? (Circle one)

Excellent Very Good Good Fair Poor

2. Do you have a loose tooth? YES NO

Clinical Variables

BMI _____

Race _____

Sex _____

Age _____

**Diurnal Rhythm of Salivary Biomarkers of Inflammation in Saliva
Interview and Abstraction Form
Version 05/20/2014**

Study ID: _____

Oral Health Interview Questions

1. Compared to others your age, how would you rate the condition of your gums? (Circle one)

Excellent Very Good Good Fair Poor

2. Do you have a loose tooth? YES NO

Clinical Variables

BMI _____

Race _____

Sex _____

Age _____