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TOTAL SALIVARY PROTEIN CONCENTRATION AND ITS CORRELATION TO DENTAL CARIES

by

EMYLI PERALTA

A thesis submitted in fulfillment of the requirements
for the Honors in the Major Program in Biomedical Sciences
in the College of Medicine
and in the Burnett Honors College
at the University of Central Florida
Orlando, Florida

Fall Term
2019

Thesis Chair: Robert Borgon, PhD

ABSTRACT

Introduction: According to the World Health Organization, dental cavities are the number one chronic disease in children. Saliva coats the teeth all day and can serve many functions to maintain and protect teeth. Saliva has many proteins that can be both detrimental and essential to the preservation of tooth enamel. The purpose of this study is to determine if a correlation exists between the total protein concentration in saliva and the prevalence of cavities in the mouth. We hypothesized that there would be a positive correlation with total salivary protein concentration and the prevalence of cavities in the participant. **Methods:** Saliva samples were taken from the patient during their comprehensive exam at the University of Central Florida (UCF) Dental Center and were analyzed using the DC assay to determine the protein concentration in the saliva. These results were compared to the number of cavities found in the mouth of each patient to determine if a correlation exists between protein concentration and cavity number. **Results:** The correlation between the variables was fairly weak, indicating that the data from this study does not support a correlation between salivary protein concentration and cavity number. **Discussion:** Future research should look at specific salivary proteins, control the time of day of collection, and take into account more variables in order to get a more precise study.

AWCKNOWLEDGEMENTS

Thank you to my parents and sisters for cheering me on during my research process; even if you didn't fully understand what it was all about. Every time I had any success in my research, you guys were always proud of me, which means the world to me. I love you guys!

A very special thank you to my fantastic research partner, Jonathan Joseph. You were supporting me in every step of this process; I could not have made this project happen without your constant help! I love you!

Thank you to the wonderful dentists that I work with, Dr. Gary Lease and Dr. Aisha Manon. Dr. Lease, thank you for being so helpful with setting the office up for days that we needed to collect samples and allowing me to work in the office. Dr. Manon, thank you for pushing me to do research three years ago, I thought it wasn't an option for me and had given up on it, but every semester you kept bringing it up until I made it happen.

I would also like to thank Dr. Mary Schmidt-Owens for assisting me with the sample collection protocol writing and guiding me through the IRB process.

I would like to thank Dr. Zhu for your patience and guidance with the statistical analysis of this data.

I am grateful for Ms. Nicole Verity for accepting me into the PILOT program and helping us develop the protein quantification protocol.

Last, but certainly not least, I would like to thank Dr. Robert Borgon for dealing with me throughout this entire process. From when I first came to you with three different project ideas to finally deciding on one project and then completing it; you have always been happy to help me and answer my questions, no matter how many there were. I cannot thank you enough for all of your help throughout this journey and for providing me with the skills I needed to complete my very own research project.

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CHAPTER 1. INTRODUCTION

In a 1983 issue of the New York Times, the headline read "End of Most Tooth Decay Predicted for the Near Future" (Lyons, 1983). Fast forward 17 years, and in 2000, the US Surgeon General, David Satcher, stated: "we must recognize that oral health and general health are inseparable." In this report, Satcher warned of a "silent epidemic" regarding dental and oral health.

According to the World Health Organization, dental cavities are the number one chronic disease in children. Worldwide, nearly 60-90% of school children have dental cavities, which can lead to pain, discomfort, infection, and even systemic issues (World Health Organization, 2012). Tooth decay is mostly preventable through fluoride treatments, professional cleanings, sealants, and following proper hygiene techniques at home. Keeping up with oral health routines by visiting a dentist twice a year is essential to prevent oral diseases.

Oral diseases do not stop at the mouth. The oral cavity is the main entryway to the rest of the body, and microbes can travel through the oral cavity and affect systemic health (Gray & Lewis, 2000; Li, Kolltveit, Tronstad, & Olsen, 2000). Oral infection has been linked to endocarditis, myocarditis, and orbital cellulitis (Li, Kolltveit, Tronstad, & Olsen, 2000). In 2007, an uninsured twelve-year-old boy died because the bacteria from a dental abscess spread to his brain (Gallagher, 2018).

The American Dental Association recommends brushing twice a day, flossing once a day, and seeing the dentist regularly in order to prevent dental disease (American Dental Association, 2001). More frequent visits to the doctor allows for earlier preventative measures and earlier diagnoses, which can keep the cost of their treatment low and help to maintain a patient's health.

In dentistry, there are no set diagnostic measures for predicting the prevalence of cavities an individual may have. Working towards the goal of one day having a salivary diagnostic test for examining the risk of an individual developing cavities and being able to use this information to provide a patient more preventative care, this thesis sought to explore the relationship between total salivary protein concentration and the prevalence of cavities.

The mouth encounters many different substances and microbes. Accordingly, the oral cavity has many different defense mechanisms including the anatomy, oral microbiome, immune system, and saliva. Enamel is the hardest substance in the human body, and it is made of hydroxyapatite (Harris, Garcia-Godoy, & Nathe, 2014). Enamel is constantly being remineralized and demineralized, which means that calcium, phosphate, and other ions are being removed and added, similar to the mechanism of osteoclast and osteoblast activity in long bone. Acidity leads to the demineralization of the enamel, as seen in **Figure 1**, and enamel kept in an acidic environment for too long without enough time to remineralize can lead to caries formation.

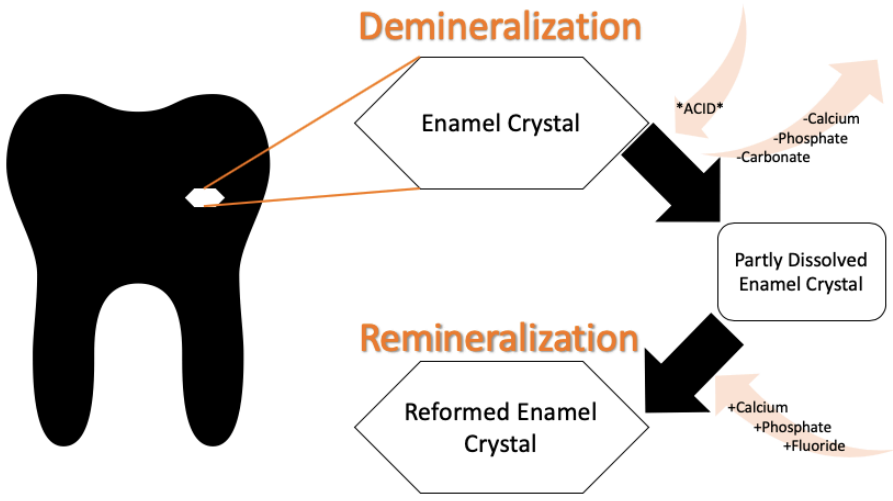

Figure 1. Enamel Demineralization and Remineralization

Parathyroid hormone is a key regulator of calcium in the blood; it is released by the parathyroid gland in reaction to low blood calcium levels. In 1994, a study found that parathyroid hormone did not seem to affect enamel formation and mineralization in rats the same way that it does for bone (Ranggard, 1994). Calcitonin works to oppose parathyroid hormone by decreasing blood calcium and phosphorus levels. It does so by inhibiting osteoclast activity in bones and inhibiting the kidneys from reabsorbing calcium and phosphate into the bloodstream. No research has been completed to show if calcitonin affects enamel remineralization. Bisphosphonates are a class of drug that have a high affinity to hydroxyapatite crystals and works to inhibit osteoclast activity (Drake, Clarke, & Khosla, 2008). A 2008 study found that a single five-minute treatment with bisphosphonate effectively increased the remineralization of outer enamel but has no effect on the inner enamel (Cate, 2008).

Plaque is a substance that adheres onto the surfaces of teeth and provides a scaffold for bacteria. Plaque's potential to harm the enamel of the tooth depends on the microbes that are living inside of it (Kolenbrander, et al., 2000). Plaque formation starts with an acquired pellicle, which is made from mostly glycoproteins from the saliva as well as carbohydrates (Harris, Garcia-Godoy, & Nathe, 2014). Bacteria then begin to colonize the acquired pellicle, forming a biofilm, which then becomes plaque. Plaque can house over 150 different species of microbes that can come from the environment randomly (Harris, Garcia-Godoy, & Nathe, 2014). Microorganisms living in the biofilm can produce a slime layer to protect themselves from being dislodged from the tooth as well as fibrils or appendages to aid in attachment to the enamel. It only takes 2 hours to make a plaque, two days for it to double in size, and 21 days for it to become so large that the microorganisms inside can no longer have access to oxygen, which results in metabolism switching to anaerobic glycolysis (Baier & Glantz, 289-30; Tanzer &

Johnson, 1976; Marsh, 1999). Anaerobic glycolysis occurs because oxygen cannot cross more than 0.1 mm into the plaque (Van der Hoeven, de Jong, & Kolenbrander, 1985; Globerman & Kleinberg, 1979). Bacterial anaerobic glycolysis has been shown to produce acidic byproducts capable of dropping the pH in its environment from 7.5 to 4.6, which can be detrimental since enamel demineralizes at a pH of 5.5 (Harris, Garcia-Godoy, & Nathe, 2014).

Veillonella is a species of bacteria that has lactate fermenting abilities; thus it can utilize the lactic acid produced by the anaerobic metabolism of *Streptococcus mutans* and *Lactobacillus* (Harris, Garcia-Godoy, & Nathe, 2014). Having *Veillonella* present in plaque helps decrease the risk of caries; therefore, it is a beneficial microbe to have. *Streptococcus mutans* and *Lactobacillus* are considered to be cariogenic microbes, meaning that they are related to caries formation (Harris, Garcia-Godoy, & Nathe, 2014). *S. mutans* uses sucrose as its main form of energy and then can quickly start making acid, intracellular polysaccharides as a stored energy source, as well as extracellular polysaccharides like dextrans and levans that help form the intracellular plaque matrix (Takahashi & Nyvad, 2008). *S. mutans* is outcompeted by *Lactobacillus* after the pH of the pellicle becomes more acidic due to *Lactobacillus* being more able to withstand acid (Harris, Garcia-Godoy, & Nathe, 2014).

Saliva is a mucosal secretion that is released in the mouth by salivary glands. There are three major salivary glands, the parotid gland that secretes by the upper second molars, the sublingual gland that secretes under the tongue, and the submandibular gland that also secretes under the tongue. There are also many minor salivary glands found throughout the mouth that aid with mastication (Harris, Garcia-Godoy, & Nathe, 2014). The secretions of these different glands can be serous or mucous and their concentrations of proteins secreted can vary, but studying whole saliva is the most relevant because it coats the teeth (Rudney, Krig, Neuvar, Soberay, &

Iverson, 1991). Saliva has multiple functions, which include lubrication, flushing of microbes and plaque, holding chemicals, aiding with antimicrobial processes, maintaining calcium and phosphorus concentrations that help with the remineralization of enamel, and buffering of acidogenesis (Dowd, 1995; Lageroff, 1998).

Salivary proteins can have different purposes. The four main salivary protein interactions studied are aggregation, adherence, cell killing, and nutrition (Scannapieco, 1994). The limit of many studies on these four protein interactions is that most are performed *in vitro*, and it is difficult to confirm these interactions *in vivo*. Aggregation *in vitro* has been shown to help form pellicles that are thought to possibly clear bacteria out of suspension, but it has not been confirmed *in vivo* (Scannapieco, 1994). Adherence could help the bacteria bind to the pellicle and allow for colonization. The cell killing property of proteins can assist with fighting microbes in the pellicle and inhibit their metabolic activity. Lastly, nutritionally, salivary proteins can help break down complex macromolecules for usage by bacteria when the host is in a fasting state.

Secretory IgA is a well-studied salivary protein that does not activate complement, but helps to promote aggregation (Liljemark, Bloomquist, & Ofstehage, 1979). Lysozyme is a muramidase that can assist with cell killing of bacteria by lysing bacterial cell walls; it has also been shown to aid with aggregation and adherence (Golub, Cheruka, Boosz, Davis, & Malamud, 1985; Tellefson & Germaine, 1986). Lactoferrin is a protein that can sequester iron which may be used to induce bacteriostasis or as a source of iron by bacteria (Arnold, Russell, Champion, Brewer, & Gauthier, 1982; Herrington & Sparling, 1985). This depends on whether the iron is stored to sequester or supply the bacteria with iron for metabolism. Lactoferrin can also be seen to aid with aggregation and adherence (Soukka, Tenovuo, & Rundegren, 1993). Glycoproteins can assist with aggregation in solution or adherence to pellicles (Rudney J. D., 1995). Acidic

proline-rich proteins can undergo a conformational change when they are absorbed by hydroxyapatite and expose other epitopes that can lead to adherence of oral bacteria to the pellicle and allow for colonization (Gibbons, Hay, & Schlesinger, 1991). Amylase can bind to oral *Streptococci* and aid with adherence to the pellicle, as well as digestion of starch from the host for the microbes to use as a source of energy (Scannapieco, Torres, & Levine, 1993).

In a study completed by Vibhakar, Patankar, Yadav, & Vibbhakar, thirty-nine patients were selected, had saliva samples collected, and the samples were analyzed for protein concentration. The total salivary protein levels showed a positive correlation with the Decayed, Missing, Filled Total (DMFT) teeth index (Vibhakar, Patankar, Yadav, & Vibbhakar, 2013). A poor positive relationship was found between the total salivary protein concentration and the number of patient's dental caries. For future studies, they suggested a larger sample size to confirm the data and then further analysis into specific salivary proteins and the roles they play, whether it be protective or detrimental. This study aims to explore the relationship between total salivary protein and the prevalence of cavities.

CHAPTER 2. RESEARCH DESIGN AND METHODS

The Decayed, Missing, Filled Surface (DMFS) index encodes how many surfaces of the participants' teeth have had or presently have decay, are missing, or are filled due to decay. If there is a filling present that was done for a cosmetic purpose, it is not counted towards the DMFS index (Lo, 2019). When excluding wisdom teeth, which we did for our study, the maximum value an individual could have for DMFS is 140. A higher score on the DMFS index means that the participant has had more cavities that have been addressed and/or presently has cavities that need to be addressed. In this study, the DMFS index was used to correlate the protein concentration to the prevalence of caries in the participant.

The DC Protein Assay uses alkaline copper tartrate solution to bind copper to the peptide bonds of a protein's polypeptide chain. Then Folin reagent is added, which will bind to the peptide-copper complex in order to form a blue product that has a maximum absorbance at 750nm. We measured the absorbance of our samples using this method by comparing the values we get to a standard curve.

UCF Institutional Review Board (IRB) approval was obtained on April 5, 2019 for this study (STUDY00000275).

Participants were UCF students and faculty between the ages of 18-29, who had presented to Student Health Services for a dental appointment and had a comprehensive or periodic exam completed in 2018 or 2019. Patients who were pregnant, frequent smokers, or had oral pathologies, such as cancer or periodontitis, which could affect their salivary protein concentrations were excluded from the study. During their dental visit, participants were asked to rinse their mouth with water to remove food residue and then waited 10 minutes after rinsing to avoid sample dilution before saliva collection. Sterile containers were used to obtain a 0.5-1 mL

sample of saliva and the DMFSI number was diagnosed by the doctor. At the end of the collection period, samples were stored in a freezer maintained at -20°C until the DC Protein Assay was performed.

Samples were thawed out on ice, resuspended by vortexing, and then centrifuged at 17,000 x g for 25 minutes to pellet out any blood, bacteria, or food debris that may have been collected. The DC protein assay was then performed using a serial dilution of bovine serum albumin standard curve at concentrations 4, 8, 12, 16, and 20 mg/mL. Then the absorbance of the saliva samples was measured and plugged into the standard curve equation to determine the protein concentration.

CHAPTER 3. RESULTS

Forty-three samples were collected and the Decayed, Missing, Filled Surface (DMFS) index and total protein concentration was determined. The result of linear regression analysis showed that the relationship between total protein concentration and DMFSI was not significant (R^2=0.0036, Figure 2). We split the patients into 3 groups according to their total protein concentration: those with protein concentration ranging 0-1 µg/mL defined as low, >1-2 µg/mL as medium, and >2 µg/mL as high respectively. We then performed ANOVA. The result showed that the average DMSI was not significantly different among the 3 groups (F=0.106, p=0.899, Table 1, Figure 3).

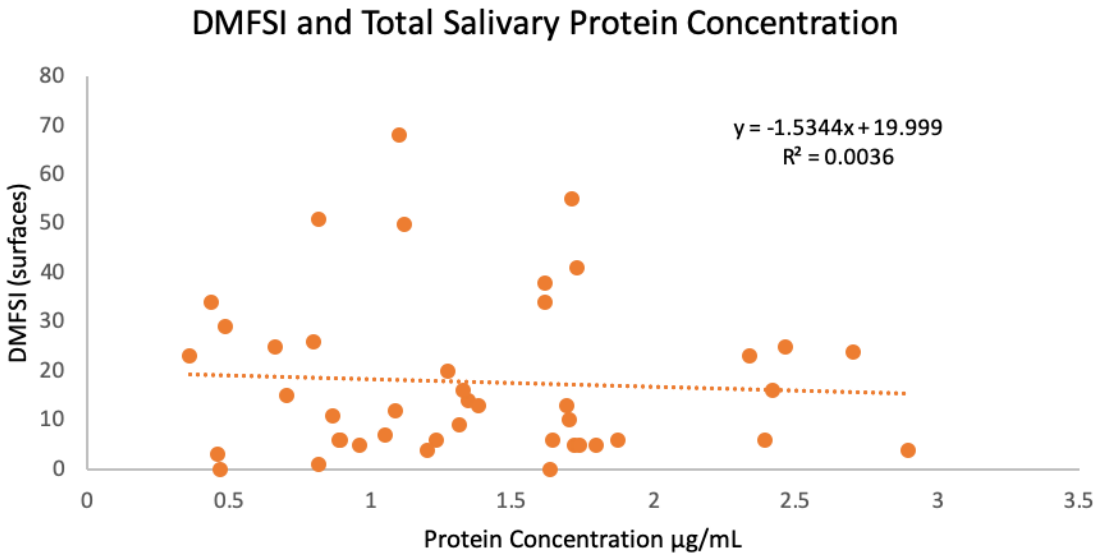

Figure 2. DMFSI vs Total Protein with linear regression model.

Anova: Single Factor					
SUMMARY					
Groups	# of Samples	Sum	Average	Variance	Standard Error
0-1 µg/mL	14	235	16.7857143	225.873626	4.016693971
>1-2 µg/mL	23	437	19	358.636364	3.948782771
>2-3 µg/mL	6	98	16.3333333	87.4666667	3.818085617
ANOVA					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	59.937430	2	29.968715	0.106425	0.8992953	3.231727
Within Groups	11263.690	40	281.59226			
Total	11323.627	42				

Table 1 Results of Anova Test showing Data Analysis

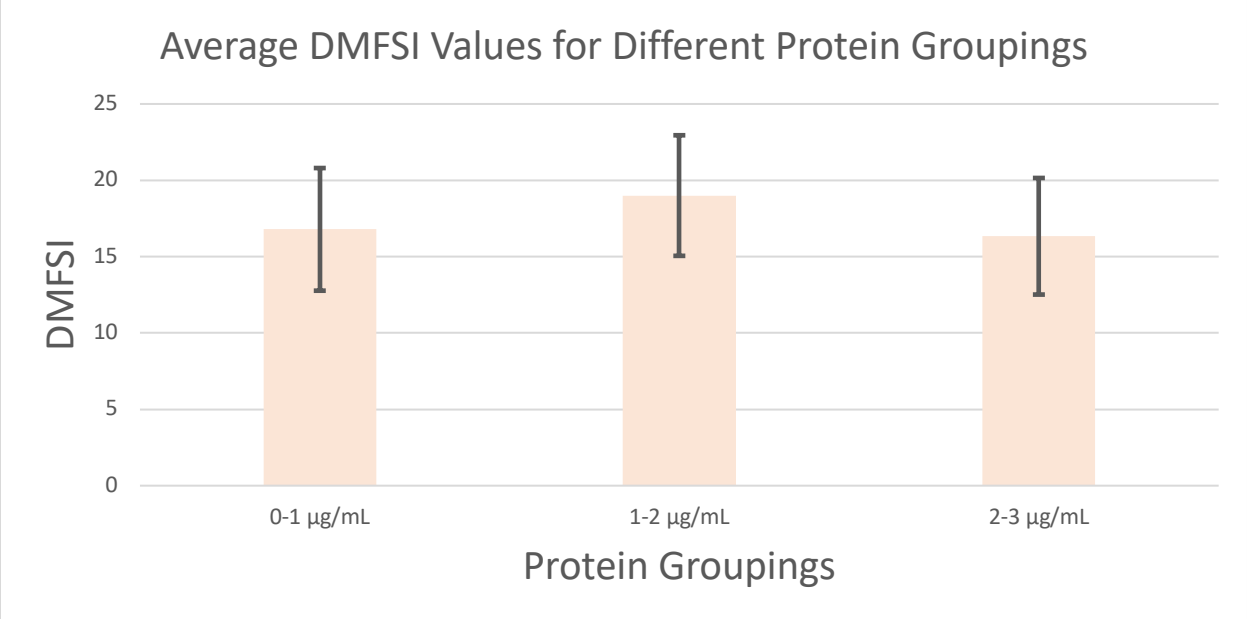

Figure 3 Protein Concentration Groupings and DMFSI Values with Standard Error

The results of the Anova test showed that our P-value was 0.899, which means that there is an 89.9% chance that our null hypothesis is correct. Additionally, the F statistic, which measures the ratio of between-group to within-group MS, was 0.106, indicating that the observed

variance in DMFSI was primarily due to random error that we did not control. This analysis demonstrates that there is more variation in DMFSI within each group than between groups. Given our current data, we cannot determine any significant association between total salivary protein concentration and DMFSI.

CHAPTER 4. LIMITATIONS

Caries formation has many different factors that lead to the disease state, and some are more controllable than others. The factors that influence caries, as seen in **Figure 4**, all need to be present at the same time for the disease state to occur (Harris, Garcia-Godoy, & Nathe, 2014). The susceptibility of the tooth, as in how strong the enamel matrix is to begin with, is one of the factors. Having cariogenic microbes in oral flora and plaque is also necessary for there to be acid accumulation on the enamel surface. Additionally, having a diet that consists of sugars that can be used by those cariogenic bacteria can aid in the production of acid. Lastly, time is needed for the disease state to progress from a slightly acidic plaque to a full formed cavity.

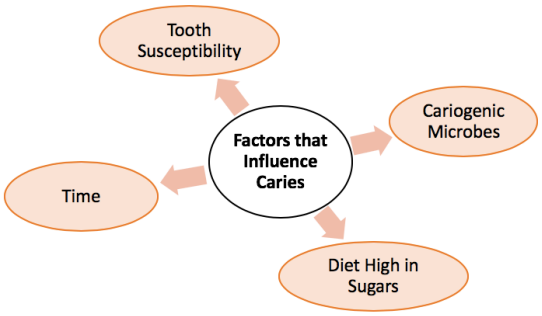

Figure 4. Factors that Influence Caries

In this study we focused on protein concentration which can be related to making an environment where cariogenic microbes can thrive or die. This is only one of the factors that influence caries development as we could not fully control the rest of these variables.

Circadian rhythm can affect concentration of salivary proteins during different times of the day (Dawes & Chebib, 1972). The highest protein concentration is typically found in the late afternoon, which can lead to some limitations of our study since all samples were collected between 8:30 am-4:30 pm. It was difficult to control the exact time of day all samples were collected because it was based on the office's work hours, the researchers' availability to collect samples, and if the patients were eligible to participate in the study. Other factors that could have

led to variations in protein concentration were gingivitis, menstrual cycles, and stress (Rudney J. D., 1995).

CHAPTER 5. DISCUSSION

This thesis served to determine if a correlation could be observed between total salivary protein concentration and the prevalence of cavities in an individual. Based on the sample size and experimental limitations, there was no significant trend in our data. The correlation between the variables under investigation was not as strong as the prior research that this study was based off of, completed by Vibhakar, Patankar, Yadav, and Vibbhakar (2013). After meeting with a biostatistician, we realize that this may have been due to an insufficient amount of information having been collected from participants and the small sample size.

Even though our study was focusing on the linear relationship of total salivary protein concentration and the prevalence of cavities the patients had, other factors outside of our interests should have been collected as confounding variables. Variables such as age, gender, and time of collection could have been used as controls in a multiple linear regression model for other variables that we could not control at the sampling stage. Our current data failed to show a significant linear relationship because: (1) we failed to control for confounding variables; (2) the R^2 value was too small to detect with such a small sample size; or (3) there is not a linear relationship between DMFS and total protein concentration.

In order to eventually develop a diagnostic salivary test for patient's caries risk, future studies would need to look at specific proteins in the saliva, as well as include more demographic information from participants. This was a broad study looking at total protein concentration rather than a specific protein due to limited time and resources for the study. Future studies could focus on more specific proteins and analysis of the specific mechanisms of action that can lead to caries development.

The results of this experiment revealed the need for more factors to be taken into account when comparing total protein to DMFSI. Future studies should include the age of participants as well as the time of day that samples are collected. Narrowing the scope of the study to investigate a specific protein and its relationship to DMFSI may also produce better results.

This was a general study to look at one of the possible factors that could contribute to dental caries formation. Once enough research has been completed and we have a better understanding of these mechanisms, diagnostic measures for those specific proteins as a marker for risk of caries prevalence can be developed. These tools could allow for improved preventative dentistry, reducing the need for restorative dentistry and allowing individuals to live healthier lives overall.

APPENDIX: IRB APPROVAL LETTER

UNIVERSITY OF CENTRAL FLORIDA

Institutional Review Board
FWA00000351
IRB00001138Office of Research
12201 Research Parkway
Orlando, FL 32826-3246

APPROVAL

April 5, 2019

Dear Robert Borgon:

On 4/5/2019, the IRB reviewed the following submission:

Type of Review:	Initial Study
Title:	Total Salivary Protein Concentration in UCF Population and it's Correlation to Dental Caries
Investigator:	Robert Borgon
IRB ID:	STUDY00000275
Funding:	None
Grant ID:	None
IND, IDE, or HDE:	None
Documents Reviewed:	• Consent Script.docx, Category: Recruitment Materials; • Protocol- Password is "Science", Category: IRB Protocol; • HRP-502 - Consent Document.pdf, Category: Consent Form;

In conducting this protocol, you are required to follow the requirements listed in the Investigator Manual (HRP-103), which can be found by navigating to the IRB Library within the IRB system.

If you have any questions, please contact the UCF IRB at 407-823-2901 or irb@ucf.edu. Please include your project title and IRB number in all correspondence with this office.

Sincerely,

Adrienne Showman
Designated Reviewer

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