2022

Relationship Between Diabetic Status and Levels of Salivary Statherin

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"Relationship Between Diabetic Status and Levels of Salivary Statherin"

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A Thesis Submitted to the Graduate Committee
of the Department of Endodontics
University of the Pacific
Arthur A. Dugoni School of Dentistry

In Partial Fulfillment of the Requirements for the Degree
Master of Science in Dentistry (Endodontics)

San Francisco
2022

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Determining the Relationship between Diabetes and level of Salivary Statherin Production via Western Blot analysis.

Abstract:

Aim: The aim of this study is to determine the varying levels of salivary statherin production in patients with varying levels of risk for diabetes. The goal is to identify a causal relationship and thus, statherin could be used as a preliminary biomarker for identifying patients with diabetes.

Materials and Methods: Saliva from 47 participants were collected in order to quantify the levels of statherin production via western blot analysis. Participants were also asked to fill out self-reported questionnaires regarding risk factors for type 2 diabetes. The questionnaire consisted of 7 questions regarding age, sex, history of diabetes, hypertension, level of physical activity, and weight class. Each individual factor as well as total risk for type two diabetes was compared to levels of salivary statherin levels via the unpaired t-test and one way ANOVA testing.

Results: Risk factors for type two diabetes such as; age, sex, history of diabetes, hypertension, level of physical activity, and weight class showed no correlation to levels of salivary statherin secretion. All risk factors combined as a total risk level for type two diabetes also did not show a correlation to levels of salivary statherin secretion.

Conclusions: It can be concluded in this study that salivary statherin protein does not show a correlation for risk or status of type two diabetes. Salivary statherin does not act as a useful biomarker for detection of type two diabetes.

Introduction:

Statherin is a protein encoded by the STATH gene, produced in the human salivary glands, and secreted into the oral cavity which serves many functions for the health of the oral environment (1). It is a calcium binding protein that prevents the precipitation of calcium and phosphate, thus the prevention of calculus build-up on dental hard tissues. This results in the maintenance of higher levels of free calcium levels in saliva for remineralization of our enamel, also resulting in higher free phosphate levels for buffering (2). Further research has also shown that during post-eruptive enamel maturation (when most of the enamel mineral content is acquired), statherin contributes to the acellular layer of protective hydroxyapatite-binding salivary proteins which covers the newly exposed enamel, referred to as the acquired enamel pellicle (AEP)(3). The statherin content of AEP directly contributes to the post-eruptive maturation of enamel via the transport of calcium and phosphate from the salivary glands, into the oral cavity, and directly to the enamel surface (4).

Saliva, which is supersaturated with respect to calcium and phosphate ions as well as its conjugate calcium phosphate, reserves the natural driving force for plaque mineralization and formation of calculus (5). One of the natural factors which act to counteract the propensity of calculus formation is statherin. It was shown through a cohort study by Pateel et al. that patients with naturally lower statherin levels had higher calculus scores (5). More than just calculus formation, the level of statherin in whole saliva has been reported to be higher in caries-free patients than in caries-susceptible patients and those with elevated decayed, missing, and filled teeth (DMFT) indices (8). Thus based on all observations mentioned, it can be

concluded that statherin plays a significant role in a system which provides a protective, reparative, and stable environment for the teeth.

There have been reports that demonstrate a relationship between systemic disease and levels of salivary statherin secretion, such as diabetes. Diabetes, affecting nearly 10 percent of the American population (6), has been shown to have an inverse positive effect on salivary statherin levels (7). It is still unknown whether naturally lower statherin levels contribute as a risk factor for development of diabetes, or whether diabetes is the direct cause and effect for lower statherin levels. Regardless, the direct relationship between diabetes and statherin has been established (7).

We have immediate testing modalities for diabetes such as fasting blood glucose and HbA1c tests, however these tests are invasive, and would normally not be utilized without some sort of suspicion of glucose intolerance. Collection of saliva is a non-invasive and inexpensive sample collection method that has be used in medicine to test for analytes and specific biomarkers of interest such as cortisol, growth factors, immunoglobulins, and interleukins have been used to diagnose diseases such as oral leukoplakia and squamous cell carcinoma. If a quantitative relationship between salivary statherin and diabetes could be established, a collection of patients' saliva could be used as a non-invasive diagnostic measure for diabetes.

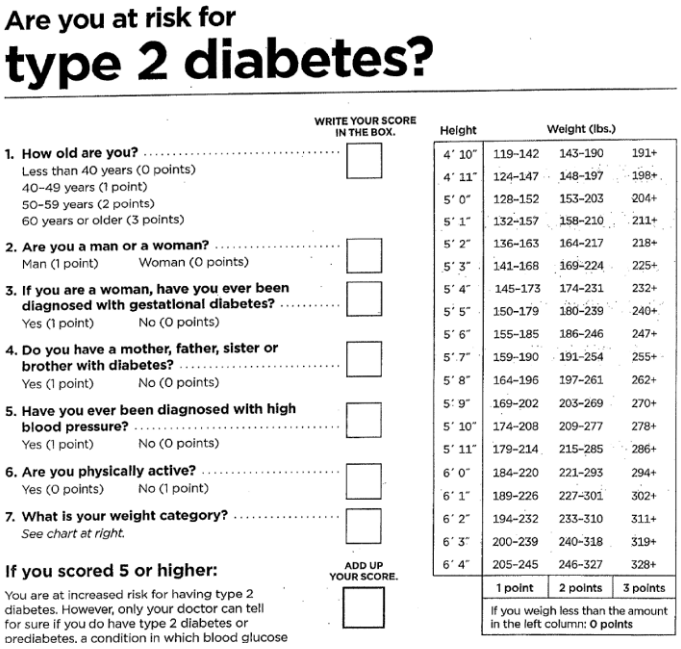
The aim of this project is to identify a relationship between one's diabetic status, and its effect on salivary statherin levels. The detection of statherin levels could be used as a non-invasive biomarker for preliminary screening and detection of patient with diabetes.

Materials and Methods:

Sample Collection:

This research was approved by the institutional review board (IRB #: IRB2022-133). 47 randomized participants from the University of the Pacific in San Francisco were included in this study. The participants ranged from staff, faculty, students, and patients of all ages and gender. After consent was obtained, participants were asked to fill out a self-reported questionnaire pertaining to lifestyle and risk factors for type two diabetes mellitus. The questionnaire consisted of 7 questions regarding the patients age, gender, personal and family history of diabetes, history of hypertension, level of physical activity, and weight category (Image 1). For each question, multiple choice answers were provided with different numerical values. Values were added which equated to a numerical score, indicating one's risk for having type two diabetes. After completion of the questionnaire, subjects were asked to provide 2-3 mL of saliva into a 10 mL collection tube which was kept at -20 °C until further processing.

Figure 1.



Are you at risk for
type 2 diabetes?

WRITE YOUR SCORE IN THE BOX.

1. **How old are you?**
   Less than 40 years (0 points)
   40–49 years (1 point)
   50–59 years (2 points)
   60 years or older (3 points)
2. **Are you a man or a woman?**
   Man (1 point) Woman (0 points)
3. **If you are a woman, have you ever been diagnosed with gestational diabetes?**
   Yes (1 point) No (0 points)
4. **Do you have a mother, father, sister or brother with diabetes?**
   Yes (1 point) No (0 points)
5. **Have you ever been diagnosed with high blood pressure?**
   Yes (1 point) No (0 points)
6. **Are you physically active?**
   Yes (0 points) No (1 point)
7. **What is your weight category?**
   See chart at right.

If you scored 5 or higher:
You are at increased risk for having type 2 diabetes. However, only your doctor can tell for sure if you do have type 2 diabetes or prediabetes, a condition in which blood glucose

ADD UP YOUR SCORE.

Height	Weight (lbs.)		
4′ 10″	119–142	143–190	191+
4′ 11″	124–147	148–197	198+
5′ 0″	128–152	153–203	204+
5′ 1″	132–157	158–210	211+
5′ 2″	136–163	164–217	218+
5′ 3″	141–168	169–224	225+
5′ 4″	145–173	174–231	232+
5′ 5″	150–179	180–239	240+
5′ 6″	155–185	186–246	247+
5′ 7″	159–190	191–254	255+
5′ 8″	164–196	197–261	262+
5′ 9″	169–202	203–269	270+
5′ 10″	174–208	209–277	278+
5′ 11″	179–214	215–285	286+
6′ 0″	184–220	221–293	294+
6′ 1″	189–226	227–301	302+
6′ 2″	194–232	233–310	311+
6′ 3″	200–239	240–318	319+
6′ 4″	205–245	246–327	328+
	1 point	2 points	3 points

If you weigh less than the amount in the left column: **0 points**

Sample Preparation:

From each saliva sample, 1 mL of saliva was transferred to a 1.5 mL Eppendorf tube, centrifuged for 10 min at 13,500 rcf to pellet and remove debris. Samples which could not extract a full mL from were discarded, leaving only 47 samples.

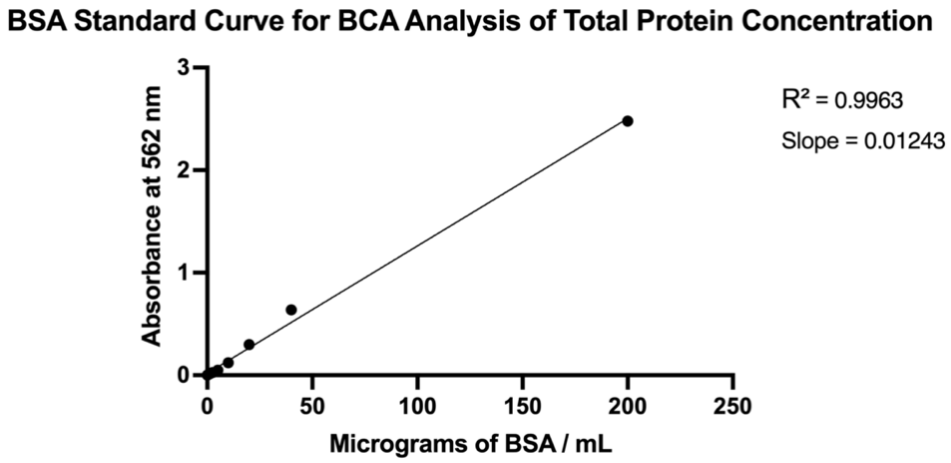
From those tubes, 500 µL of supernatant was transferred into a clean microcentrifuge tube labeled 1-47 to match the assigned number for the self-reported questionnaire data.

Protein Analysis:

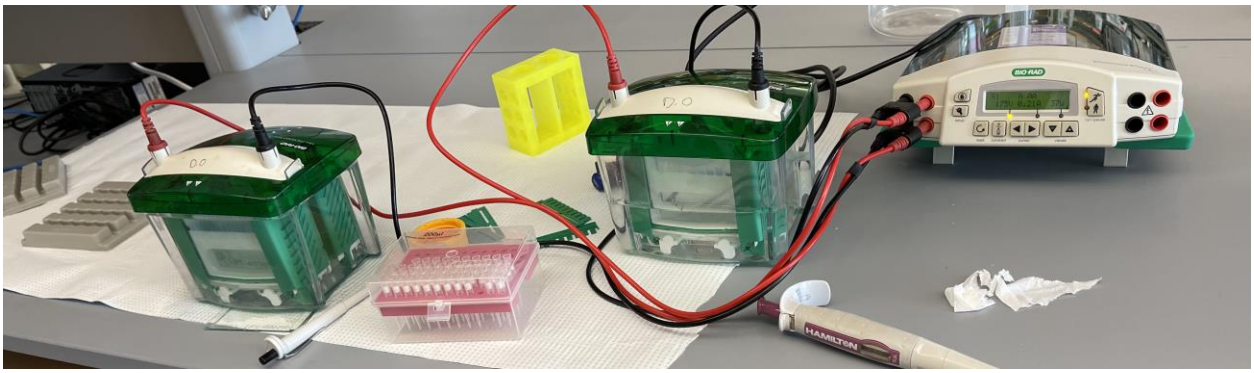
To quantify the total protein content in each sample, a BCA assay (Thermo Scientific) was ran as per manufacturer's instructions using a BSA standard curve. Assay was read on a plate reader (SpectraMax iD3, Molecular Devices). Then the electrophoresis gel was prepared and loaded with 30 µg of protein per well for each sample. A standard curve of commercially prepared statherin (Sino Biological Inc.) was prepared **(Table 1)** and ran on the electrophoresis

gel for Western Blot analysis after proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE)**(Figure 2)**.

(Table 1)



(Image 2)

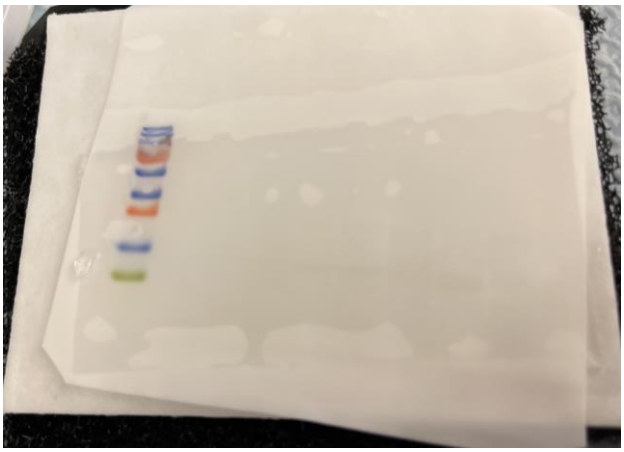


Western Blot Analysis:

Once separated via electrophoresis, the total protein content from each gel was transferred onto a PVDF Membrane (Amersham Hybond GE 10600023) **(Figure 3)**. Membranes were then blocked with BSA (5% in TBST) for 1 hour and then incubated with primary antibody

(Biorbyt orb638385) overnight at room temperature on a shaker. Membranes were then incubated with an HRP conjugated secondary antibody (Sino Biological Inc.) for one hour at room temperature on a shaker.

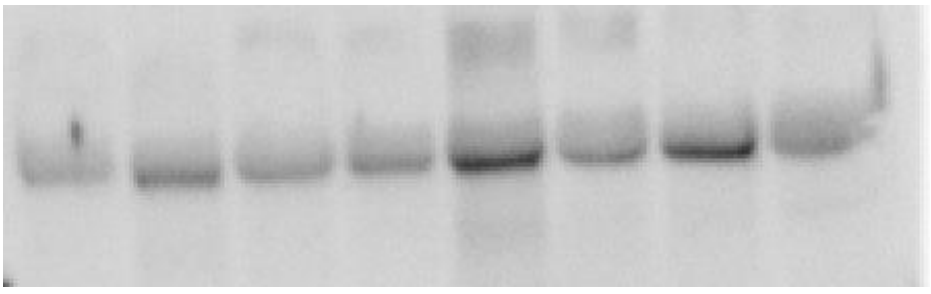
(Figure 3)



Data Analysis:

Images were captured using a Bio-rad Chemidoc MP imaging system and images were analyzed using Image lab (Bio-Rad) and ImageJ for band quantification (**Figure 4)**. An unpaired t-test was used to test for statistical significance between each category on the questionnaire. For groups with more than two categories for answers, a series of one-way ANOVA tests were performed in order to compare between groups.

Figure 4: Image of membrane incubated with substrate to visualize bands of proteins



Results:

Following statistical analysis, forest plots were constructed for each question as well as the total score for the questionnaire. Although most participants were female and under the age of 40 years old, there was no statistical significance between age or sex and levels of salivary statherin levels **(Tables 1 and 2)**

Table 2:

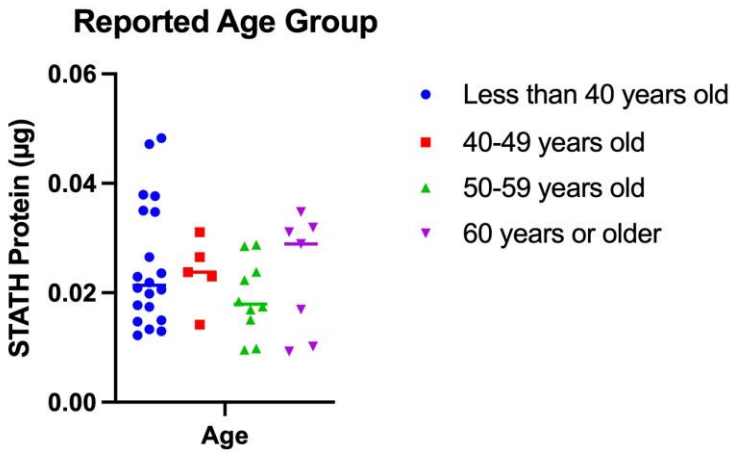
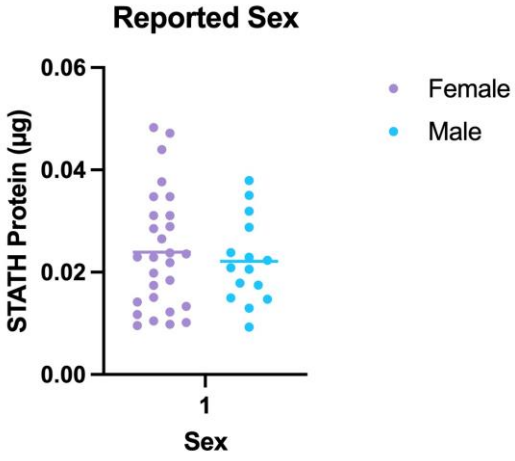


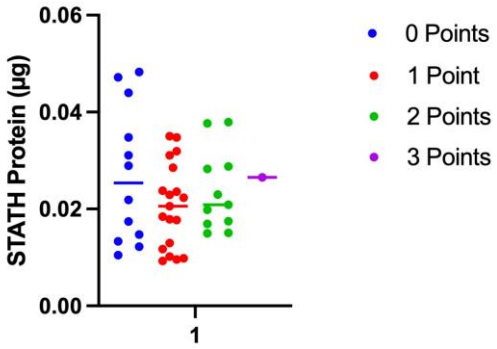
Table 3:



Under questionnaire number 3, The weight class most closely approximated BMI. With most values falling within scores 0-2, there was no statistical significance between weight class and levels of statherin secretion **(Table 4).**

Table 4:

Weight Catagory per Model on Survey



Under the questions regarding familial history of diabetes and personal history of gestational diabetes, 66% of participants answered no. However, there was still no statistical significance associated with either category or levels of statherin secretion.

Table 5:

Parent or Sibling Diagnosed With Diabetes?

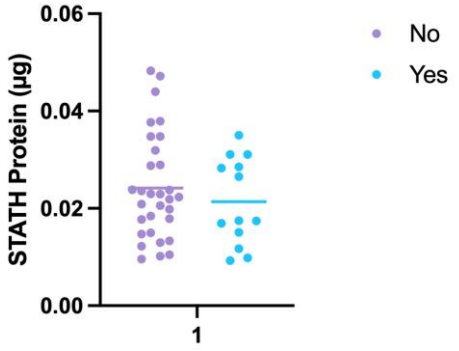
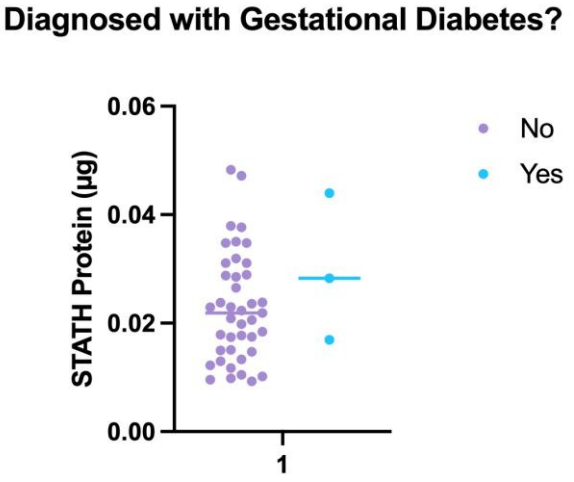


Table 6:



There also appears to be no statistical significance between hypertensive status or physical activity and levels of statherin secretion **(Table 7 and 8).**

Table 7:

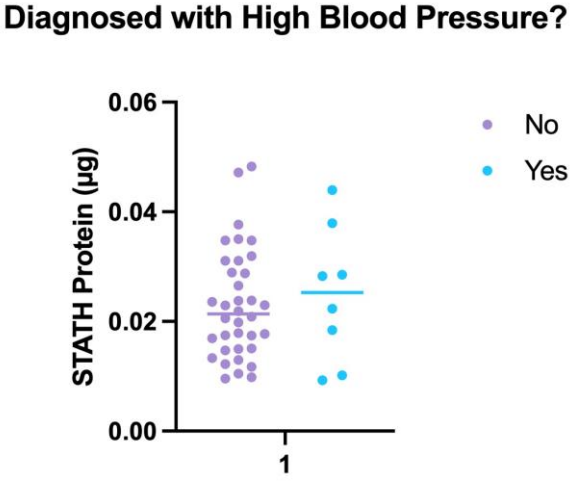
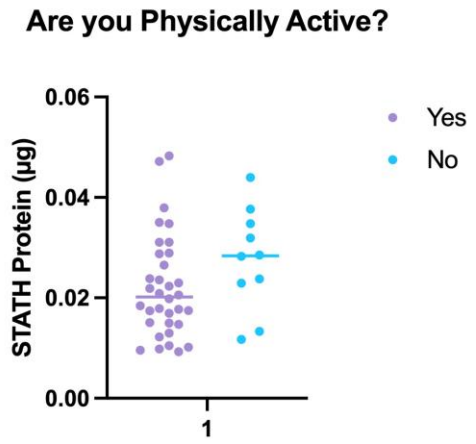
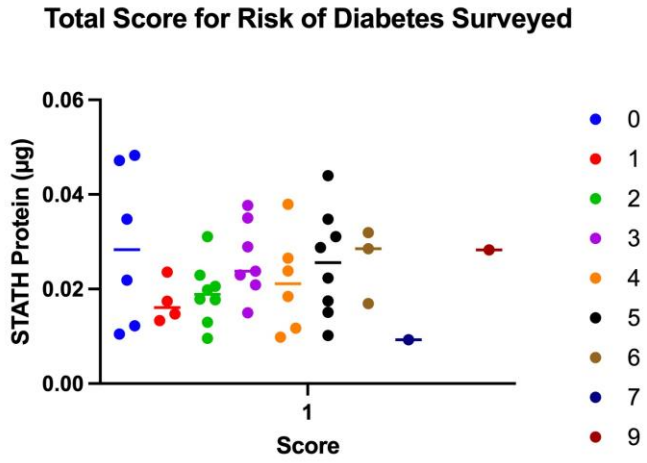


Table 8:



The total scores achievable on the questionnaire was anywhere from 0 to 11. The higher the score, the higher the risk for type two diabetes. The values obtained from the participants ranged from 0 to 9. Again, there was no statistical significance found between total risk for type two diabetes and levels of statherin secretion **(Table 9).**

Table 9:



Discussion:

It was expected to detect around 0.02 g/L of statherin from each participant's sample, which is the expected average concentration of human statherin based on current studies (5). We, on the other hand, detected levels consistently below that, in the microgram/L range. Upon deeper evaluation of our experimental methods, speculation was made on why greater values of statherin were not detected. During sample collection, the tubes of participants' saliva were not immediately kept on ice. There was a significant amount of time lapse (2-3 hours) between complete collection of samples and placement into the freezer, especially with the initial samples. Saliva is abundant with proteases which assist in the initial chemical breakdown of our foods. Salivary statherin is not immune to this protease activity. It is possible that if a buffer or protease inhibitor was placed in the collection tube with the saliva samples, that greater levels of statherin could be detected. A higher quality lab centrifuge could have also helped pellet more debris and yield greater results as well. Our n value was greater than previous studies, however if we collected from more participants and from a more diverse population, such as a larger collection from a population with confirmed or known diabetes, different protein values may have resulted.

A self-reported questionnaire and survey present with many weaknesses. With questions pertaining to one's health, lifestyle, and weight, participants may be inclined to not answer accurately. A blood sample in conjugation with the questionnaire could have been useful to obtain an objective fasting blood glucose level, and/or HbA1c, rather than subjective answering to the questionnaire.

Oral diseases, such as periodontitis, dental caries, fungal infections, xerostomia, and salivary gland dysfunction, are all major complications associated with diabetes (9). Thus, the early detection of diabetes would not only be beneficial for a person's systemic health, but also their oral health. Further, oral biomarkers can help achieve just that. Biomarkers are utilized for many applications in modern medicine, including screening and diagnosis of various diseases. With more established associations made between oral biomarkers and oral/systemic diseases, a multiplex assay could be employed as a one-stop-shop analysis. A multiplex assay is a type of immunoassay that can simultaneously measure multiple analytes In a single sample, such as saliva, and help detect the risk or establishment of multiple diseases at once.

Although the results in this study did not show any correlation between salivary statherin as an oral biomarker and diabetic status, other studies with altered methodologies may. If ultimately statherin is not found to be linked to diabetes, It is possible that other salivary biomarkers may still be. Areas of future research include exploration of other salivary proteins and biomarkers, and their potential correlation to other oral and systemic diseases.

Conclusion:

From the samples collected from this specific population and under these condicitons, there was no statistical significance between any of the categories in the questionnaire and the levels of statherin measured by western blot analysis. When adding the total score from the questionnaire, there was still no statistical significance between relative risk for type 2 diabetes

and levels of salivary statherin secretion. Further refinements to the sample collection method and expansion of the sampled population to include a more diverse distribution of age and diabetic risk could yield a stronger correlation.

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