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ORIGINAL ARTICLE **OPEN ACCESS**

Estimation of Salivary Protein as A Potential Biomarker For Diagnosis of Type -II Diabetes Mellitus

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ABSTRACT
*Diabetes mellitus (DM) is fast gaining the status of potential epidemic disease in India with more than 62 million individuals currently diagnosed with this disease. Commonly we are using blood as gold standard body fluid for diagnose of DM even it is invasive and pain full process of sample collection. Recently saliva compromises an alternative to blood as a biological fluid; many salivary parameters have been used to characterize disease states because of non-invasive method of sampling. Protein is a biomarker for the identification of different diseases like diabetes, cancer and coronary syndrome. Diabetes is known to influence salivary composition and functions. In the present study focussed to investigate the salivary protein profile in Type –II DM patient and compared with healthy control. It is important to note that several proteins have at least one biological pathway related to diabetes and measuring the changes as biomarkers may allow to understand the clinical situations of individual patients and may in the future to provide greater personalized health care for patients. Hence in the present study to find non-invasive diagnostic tool using 200 healthy Control and 200 patients (Type –II DM), were selected for the study purpose. Fasting blood and salivary investigations were performed using unstimulated whole Saliva. A significant correlation(p-Value 0.0001 ***) was found between salivary(104.781 ± 10.983mg/dl) and blood (Protein 7.676 ±0.451 g/dl) concentrations in the diabetic patient and healthy control people(Salivary protein 100.273 ± 14.653mg/dl , blood 6.481 ± 0.348gm/dl).This finding suggests that salivary protein measurement can be used reliably for reflecting, monitoring and best biomarker for prognosis of Type –II DM.*
Key words: *Diabetes mellitus, Proteins, Blood biomarkers, saliva, Protein profile.*

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INTRODUCTION

Diabetes Mellitus (DM) is a clinically and genetically heterogeneous metabolic disorder characterized by abnormalities in carbohydrate, lipid and protein metabolism that results in occurrence of various complications such as retinopathy, nephropathy, neuropathy, cardiovascular symptoms etc[1]. It is immense, growing, silent epidemic disease that has the potential to affect the health services in all parts of the world[2].Worldwide prevalence rate of DM is rapidly increasing as number was expected to rise to 300 million by year 2025, 366 million by the year 2030 [3]. According to WHO estimate, 70% diabetics reside in developing countries and India has world's largest diabetes population with 50.8 million people followed by China[4].In India approximately 57.2 million people will be affected with DM in the year 2025 [5].DM is classified, according to its aetiology as Type-I DM and Type -II DM. The Type –I DM account for only 5 - 10% of these diabetic people, the remaining 90% percentage of people having Type -II DM [6-8]. Among the elderly people Type II DM is the fifth most common condition and the sixth leading cause of mortality. The quality of life is diminished with shortening of the patient's lifespan [9].

Currently, a diagnosis of DM is accomplished by estimating blood glucose levels. In diabetic people blood sugar level is closely monitored. It involves repeated estimation of plasma glucose either by finger pricks or by intravenous blood sampling that causes unnecessary discomfort, mental trauma to patients and also it required technically trained person to collect blood sample. Thus, it would be optimal to use another biological fluid [10]. Hence a much simpler and non-invasive tool for the diagnosis and monitoring of diabetes is very much needed in the present situation.

Saliva offers distinct advantages, when compared with other commonly used biological fluids can be collected non-invasively by individuals with limited training; costs towards the procedure are

dramatically reduced as no special equipment for the collection of saliva. In addition to that saliva is "a real time" fluid because it is secreted from the exocrine glands that produce protein profile indication of an individual's health and wellbeing status at the time of collection of sample. Because of these characteristics, it is possible to monitor several biomarkers in infants, children's, elderly persons, uncooperative patients in some circumstance in which blood and urine sampling not able to collect [11,12]. Protein is best biomarker for the identification of different diseases like diabetes, cancer[13].Salivary proteins have been shown to be increased in medically compromised patients. Human saliva contains a large number of proteins and peptides that are easily accessible and may serve as a potential source of biomarkers to monitor changes that occur under pathological conditions [14].
Hence the present investigation was undertaken to study the significance of saliva proteomics as a precise, reliable and non-invasive diagnostic tool instead of blood to diagnose Type -II DM, among a specific study population but for the present investigation both saliva and blood sample were collected to comparatively diagnosis to know their level of significance.

MATERIAL AND MATHODS

The present study area, Thiruporur is situated in Kanchipuram Dist of Tamil Nadu. Diabetic camp was conducted in different villages like Amma pet, Venbedu, Kottamedu and Nellikuppam village to collect the sample from 200 control groups and 200 Type - II diabetic people with different age group from the selected study area of Thiruporur. The study was approved by the members of the Institutional Ethical Committee. Exclusion criteria for the study were pregnancy, alcohol dependency, smoking, any chronic diseases and history of diabetes within the previous years. The inclusion criteria for the study voluntary participation • Age inclusion: 21years to 70 Years • Sex included both the genders.All patients were explained in detail about this study and informed consent was obtained in their native languages to prevent language bias and later was subjected to the collection of saliva and blood for examination. All subjects were asked not to eat, drink (except water) for an overnight period prior to collection of unstimulated whole saliva samples. In fasting condition the blood and saliva samples were collected. The patient was instructed to spit in a sterile plastic container over a period of 5 minutes. The blood sample was collected by vein puncture . The blood was drawn from the patients in sitting position with help of technically well trained person. Collected samples were centrifuged for 15 minutes at 2,000 rpm, stored at - 20^0 C for further investigations.

Estimation of Serum Total Protein (Biuret Method)

Serum total protein estimation was done based on the Biuret method. Cupric ions in an alkaline solution react with the peptide bonds of proteins and polypeptides containing at least two peptide bonds to produce a violet colored complex. The absorbance of the complex at 546 nm is directly proportional to the concentration of protein in the sample.

Calculation

$$\frac{A\ Sample\ X\ C\ Standard}{A\ Standard}\ C\ sample$$

A = Absorbance, C = Concentration

Estimation of protein in saliva (Pyrogallol Red Method)

Salivary protein was estimated by using pyrogallol red method. Proteins in an acidic medium, combines with Pyrogallol red and Molybdate to form a blue purple coloured complex. Intensity of the colour formed is directly proportional to the amount of proteins present in the sample.

High Linearity Assay

$$Micro\ Protein\ in\ mg\ /l\ = \frac{Abs.T}{Abs.s} X\ 1000$$

Estimation of Albumin (BCG method)

Albumin act as cation and binds with anionic dye bromocresol Green in a buffered medium to form a green coloured complex. The absorbance of final colour is measured at 630 nm. The intensity of the colour formed is directly proportional to the amount of albumin present in the sample.

Calculation

$$Albumin\ in\ gm\ /dl = \frac{Abs.T}{Abs.s} X\ 4$$

Estimation of Serum Globulin

The serum globulin concentration was estimated by subtracting albumin concentration from Total protein concentration. The globulin level was calculated by using following formula

Globulin in gm/ dl= $(Total\ Protein\ in\ gm\ /dl) - (Albumin\ in\ gm/dl)$

Estimation of Salivary Globulin

The salivary globulin concentration was estimated by subtracting albumin concentration from salivary protein concentration. The globulin level was calculated by using formula:

Globulin in mg / L = $(Protein\ in\ mg\ /L) - (Albumin\ in\ mg/L)$

Estimation of serum and salivary Albumin / Globulin ratio (A/G Ratio)

The serum and salivary Albumin / globulin ratio was estimated by using the following formula.

A/G ration= $Albumin\ /Globulin$

STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad software. Descriptive statistics on each study variable including mean and standard deviations were analysed. The results obtained from the analysis were expressed by using Mean ± SD with their level of significance at p <0.0001.

RESULT AND DISCUSSION

There are some proteins which may be up- and down regulated in the serum /plasma of diabetes mellitus especially in Type- II diabetes which will reflect in saliva of the patients.The protein biomarkers are very helpful for predicting long-term mortality in patients with diabetes, cancer, and coronary syndromes. Searching for novel biomarkers can be done using tissues and/or bio fluids (blood, serum, plasma, and urine). The present study emphasised to identify these markers in diabetics using saliva as a diagnostic fluid as well as the above parameters was investigated in blood samples forcomparison. The results of the study were presented in table -1 & 2.

TABLE -1: COMPARISON OF PROTEIN PROFILE IN BLOOD SAMPLE OF CONTROL AND DIABETIC PATIENTS (Mean / SD)

S.no	Parameters	BLOOD (in serum)		p- value (Between control and diabetic)
		Control People (n-200)	Diabetic patients (n-200)	
1	Total Protein (g/dl)	6.481 ± 0.348	7.676 ±0.451	0.0001 ***
2	Albumin (g/dl)	4.25 ± 0.267	4.494 ± 0.533	0.0001 ***
3	Globulin (g/dl)	2.231± 0.266	3.181 ± 0.567	0.0001***
4	A /G Ratio	1.933 ± 0.276	1.537 ± 1.099	0.0001 ***

* - Not Significant, ** - Significant, *** - Highly statistically significant

The present study results showed significantly increased levels of total Protein, Albumin, Globulin and A/G ratio in blood of diabetic people as compared with control subjects. In the blood of diabetic people a significantly increased mean total protein (7.676 ±0.451 g /dl), Albumin level (4.494 ± 0.533 g/dl), Globulin level (3.181 ± 0.567g /dl) and A/G ratio (1.537 ± 1.099) were observed as compared to control group.

TABLE -2: COMPARISON OF PROTEIN PROFILE IN SALIVA SAMPLE OF CONTROL AND DIABETIC PATIENTS (Mean / SD)

S.no	Parameters	SALIVA		p- value (Between control and diabetic)
		Control People (n-200)	Diabetic patients (n-200)	
1	Total Protein (mg/dl)	100.273 ± 14.653	104.781±10.983	0.0006 ***
2	Albumin (mg/dl)	68.529 ± 14.383	76.488 ± 14.318	0.0001 ***
3	Globulin (mg/dl)	31.744 ± 11.192	28.292 ± 11.110	0.0021 **
4	A /G Ratio	2.581 ± 1.465	3.335 ± 1.981	0.0001 ***

* - Not Significant, ** - Significant, *** - Highly statistically significant

Similarly in saliva of diabetic patient significantly increased level of Protein levels 104.781 ± 10.983mg/dl, Albumin level is76.488 ± 14.318mg/dl and A/G ratio level is 3.335 ± 1.98 was recorded. Slightly less amount globulins was observed in diabetic patients than control. The increased level of salivary protein profile is due to increased basement membrane permeability which is often associated with diabetes, is one of the possibilities for the increased passage of proteins from the exocrine glands into their secretions [15]. Whereas the reverse condition were observed in blood and saliva of control subjects. Results were observed to be highly significant at p< 0.0001. The present study results were found to be similar with studies of Ben- Aryeh[16] and Dodd's *et al,*[17], Lopez et *al,*[18], also reported the increased salivary total protein level in diabetic group as compared with the control. In accordance to our study Mata *et al.,* also reported that increased salivary protein concentration in diabetic Patients, could be attributed to reduce salivary fluid secretion in both Type - I and Type -II Diabetics

[19].Significantly increased levels of protein profile were observed in following their studies of Carda*et al.,* [20], Al-Maroof *et al.,*[21]and Sathyapriya *et al.,* [22].

Gheena *et al.,* in their study reported that higher concentration of blood protein, albumin, was detected in saliva of diabetic children as compared to saliva of healthy children, but without statistical significance [23]. But in another study reported by Rao *et al*., and Al Kawas *et al.,* indicated that analysis and identification of proteome is important not only for understanding of oral pathophysiology, but also as a potential biomarker for systemic disease [24,25]. They further confirmed that thousand salivary proteins with different role in metabolic processes, immune regulation, cell adhesion and communication, etc. In saliva of patients with Type -II diabetes, the proteome out of 487 proteins, 65 proteins were significant for differentiation of diseased patients from the control group.

The present study showed slightly higher values of serum albumin in DM patients (4.494 ± 0.553 mg/dl) than controls (4.25 ± 0.267 mg/dl). The present results were in accordance with the study of Carda*et al.,* showed higher mean serum albumin and globulin levels in diabetics than non-diabetics [20]. The present study also recorded significantly higher salivary A/G ratio in diabetics as compared to control [26].

In another study observed contradictory to our results, Dodds and Dodds found no significant relationship between salivary total protein level in diabetic and non-diabetic individuals [27]. Rao*et al.,* and Border *et al*., revealed that salivary proteomics offer an interesting option for those who prefer a less invasive approach for screening diabetes and also observed 52 differently expressed proteins in saliva as diabetes-related inflammatory biomarkers in diabetics as compared to control [24, 28]. Therefore, protein profiling in saliva could be an interesting avenue to diagnose and monitor diabetes in the future.

CONCLUSION

Saliva is regularly used in clinical laboratories for finding of IgA antibodies, determination of salivary cortisol, hormones and for other genetic studies. This study result highlights the possibility of using saliva as a diagnostic tool to assess the protein concentration in diabetic people. The highly significant correlation was found between serum and salivary levels of protein, albumin, A/G ratio in DM patients in comparison with non-diabetic healthy control. A significant positive correlation was established between blood and salivary protein profile levels. Hence, it can be concluded that fasting salivary protein profile levels can be used as a non-invasive diagnostic, as well as a monitoring tool to assess the glycaemic status of diabetes. Taken collectively along with this research work, the most recent data in the literature also indicates that salivary proteomics can offer many new perspectives into checking a considerable number of human diseases and conditions especially to the ones who requiring frequent and long-term monitoring diseases.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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