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ABSTRACT

Aim. The study aims to investigate the presence of a specific gene, cytotoxic associated gene-A, as a potential salivary biomarker in individuals with periodontitis.

Materials and Methods. 45 salivary samples (15 from healthy patients and 15 from periodontitis patients with diabetes mellitus, 15 from periodontitis patients without diabetes mellitus) are appropriately collected, labelled, and stored. Pre-coated anti-human cytotoxic associated gene-A antibody is used to detect biotinylated polyclonal antibodies. The measurement of the colour intensity, which is positively correlated with the quantity of the target in the sample.

Outcomes. The mean concentration of Cytokine associated gene-A in saliva was increased consistently as the disease progressed from healthy state to periodontitis. It was highest in group 2 and lowest in group 1. Mean concentration of Cytokine associated gene-A in group 1 was 118.42ng/ml, 584.32ng/ml in group 2 and 465.72ng/ml in group 3 respectively. The results state the presence of cytotoxic associated gene-A salivary biomarker in elevated levels in the salivary samples of the periodontitis patients when compared with samples of the healthy patients.

Conclusion. The cytotoxin associated gene-A salivary biomarker level is elevated in the periodontitis patients with diabetes mellitus and it can be used for the prior detection of periodontitis.

INTRODUCTION

Periodontal disease is a chronic inflammatory condition that affects the supporting structures of the teeth, collectively known as the periodontium. The periodontium includes gingiva, periodontal ligament, cementum and alveolar bone [1].Periodontitis is initiated by the presence of dental plaque and their interaction with the host's immune response. The immune system's response can inadvertently damage the surrounding tissues, contributing to the progression of the disease. The shift in the balance of the oral microbiome, often triggered by factors like poor oral hygiene, can lead to an overgrowth of pathogenic bacteria [2]. The intricate interplay between these factors should be understood to prevent and manage periodontal disease. Periodontitis is a widespread health issue affecting a large number of people. Research studies and surveys may be subject to various biases, including selection bias, recall bias, or reporting bias, which can influence the recorded prevalence and incidence rates. The accuracy of diagnosis and classification of periodontal disease cases can also affect the reported statistics [3].

Diabetes mellitus is consistently identified in the literature as an important systemic risk factor for periodontitis. Diabetes can contribute to the initiation and progression of this oral condition [4].There is a well-established bidirectional relationship between diabetes mellitus and periodontal disease which includes periodontal ligament destruction, a critical component of periodontal disease. This destruction can ultimately lead to tooth loss in individuals with diabetes. In individuals with diabetes and periodontitis, the gingival crevicular fluid(GCF) often contains higher levels of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α) [5]. This suggests that the inflammatory response is more pronounced in diabetic individuals with periodontitis [6]. The joint workshops between the organisations identified a dose-response relationship that implies that as the severity of periodontal disease increases, the adverse consequences for individuals with diabetes become more pronounced [7]. As periodontal disease worsens, the negative impact on diabetes becomes more significant. This underscores the importance of addressing periodontitis in individuals with diabetes for their overall health management [8].

Traditionally, there has been a prevalent belief that bacteria are the primary factors responsible for the occurrence of periodontal disease. Recent studies have challenged the idea that bacteria are solely responsible for periodontal disease. Emerging evidence implies that the body's immune system, including the production of inflammatory molecules, contributes to the progression of periodontal disease[9]. Cytokines, specifically those belonging to the cytokine superfamily which are involved in diverse biological processes, including influencing growth and

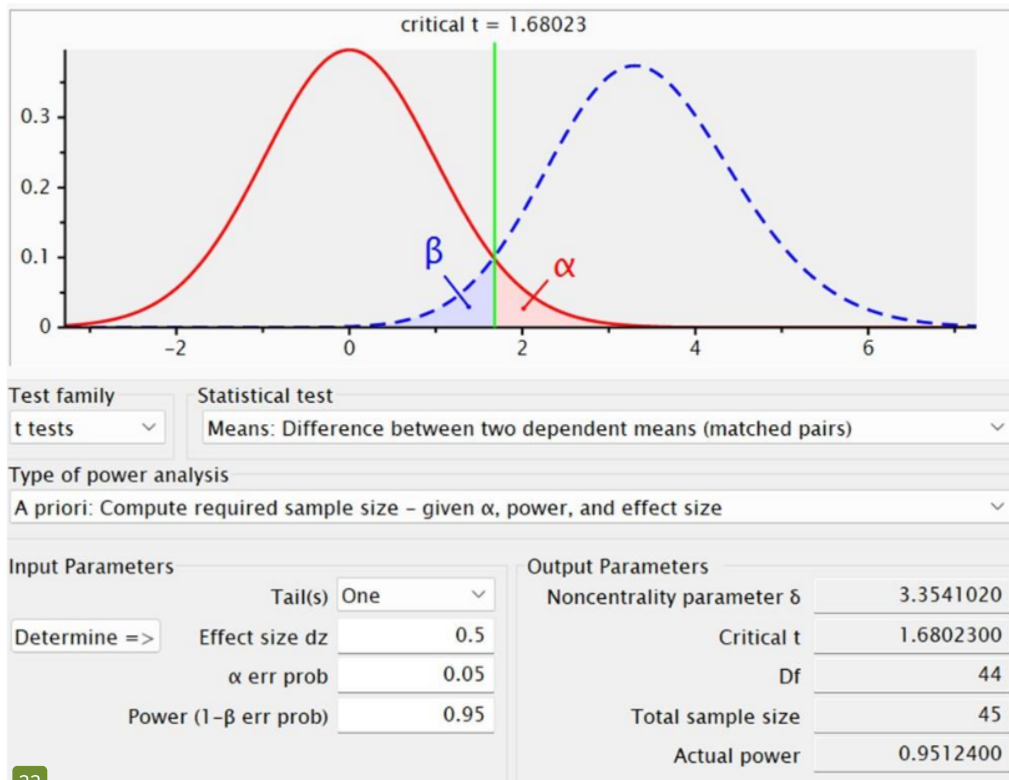
development, hematopoiesis (formation of blood cells), recruitment of lymphocytes (a type of white blood cell), differentiation of T cell subsets, and modulation of inflammation [10-11].

Cytokine was given by Cohen in 1974 as a replacement for the term "lymphokine." "Lymphokine" had been coined in the late 1960s to describe soluble proteins derived from lymphocytes that had immunological effects [12]. In 1979, at Second International Lymphokine Workshop, the IL (interleukin) system of nomenclature was proposed to simplify the list of cytokines. The nomenclature for cytokines today is a mix of accepted interleukins [13]. Cytokine Associated gene A gene was being considered as a new marker of systemic inflammation due to its association with cytokine production or regulation along with its connection to periodontitis and diabetes mellitus. This study aims to understand the presence of salivary biomarker cytokine associated gene-A in periodontitis patients with and without diabetes mellitus thus to explore its role in the inflammatory responses and to evaluate its potency as biomarker.

MATERIALS AND METHODS

Study population:

Total 45 individuals who have visited Saveetha Dental College and Hospitals and diagnosed with periodontitis were selected based on their visits to the clinic within a time frame from January to February 2021.The study has received approval from the Institutional Human Ethics Committee at Saveetha Dental College, Chennai (ethical clearance number: IHEC/SDC/PERIO2301/23/274). Each patient was explained about the study in detail and informed consent was taken. Randomisation was done using G*power software.



Inclusion Criteria:

(a) Patients with a history of periodontitis with pocket depth of greater than 5mm.
(b) With an age of more than 35 years.

Exclusion Criteria:

a) <18 years old.
b) Systemic diseases such as hypertension, hypothyroidism.

Patients were divided into three distinct Groups(n=15). Group-A: Periodontitis patients with diabetes mellitus, Group-B: Periodontitis patients without diabetes mellitus, Group-C: Systematically healthy individuals. The study began with the assessment of overall periodontal status followed by dental examination of the participants. This is followed by supragingival scaling, patients were instructed for oral rinsing and a wait of 15 minutes was done for the pooling of saliva which is free of debris and other oral fluids for saliva collection. 5 ml of saliva was collected from 9am and 12pm to minimise diurnal (daily) fluctuations in salivary composition. The collected samples underwent a centrifugation process with a centrifugal force of 10,000rpm for 5 minutes. The processed saliva samples were transferred to 1.5ml tubes and stored at -80°C after which biochemical investigations of the samples were conducted.

Analysis of cytokines:

The materials required to perform this procedure are

1. Microplate Reader (wavelength 450 nm): To measure the absorbance or fluorescence of substances in microplate wells.
2. 37°C Incubator : For cell culture, bacterial growth.
3. Automated Plate Washer: As it helps remove unbound substances, reducing background interference in assays.
4. Precision Pipettes: To accurately measure and transfer small volumes of liquid.
5. Clean Tubes and Eppendorf Tubes with Samples: To store and aliquot samples.
6. Deionized Water.

Specimen processing:

The specimens (saliva samples) were labelled and treated by lysis buffer as it inactivates the viruses in the samples, then ELISA(enzyme-linked immunosorbent assay) test was performed where the Cag-A biomarker level was assessed in both the control group and experimental group.

The Double Antibody Sandwich ELISA technique was implemented in a 96 well-plate format where the pre-coated anti-Human CagA monoclonal antibody was pre-adsorbed onto the wells and it captures the specific biotinylated polyclonal antibody by forming a sandwich with the target protein. The plates were incubated with standards and samples at 37°C for 90 minutes during which any CagA present in the samples will bind to the pre-coated capture antibody after which 100 µl of the biotin detection antibody working solution to each well and incubate for 60 minutes at 37°C. Followed by addition of 100 µl of the SABC (streptavidin-biotin complex) working solution to each well as this amplifies the signal and enhances detection. The Optical Density (O.D) reading at 450 nm within 15 minutes after colour development(from blue to yellow) was done as it reflects the presence and concentration of CagA in the samples.

RESULTS

SPSS version 23 was used for data analysis where the normality of data was assessed by the Shapiro-Wilk test. Intergroup comparison was done with paired t-test followed by the Mann-Whitney U test. The results of the data analysis were presented in Table 1.

Serial No	Different groups	Mean value	P- value
1.	Group- I Group- II	118.42 584.32	*0.004
2.	Group- II Group- III	584.32 465.72	*0.004
3.	Group- III Group- I	465.72 118.42	*0.004

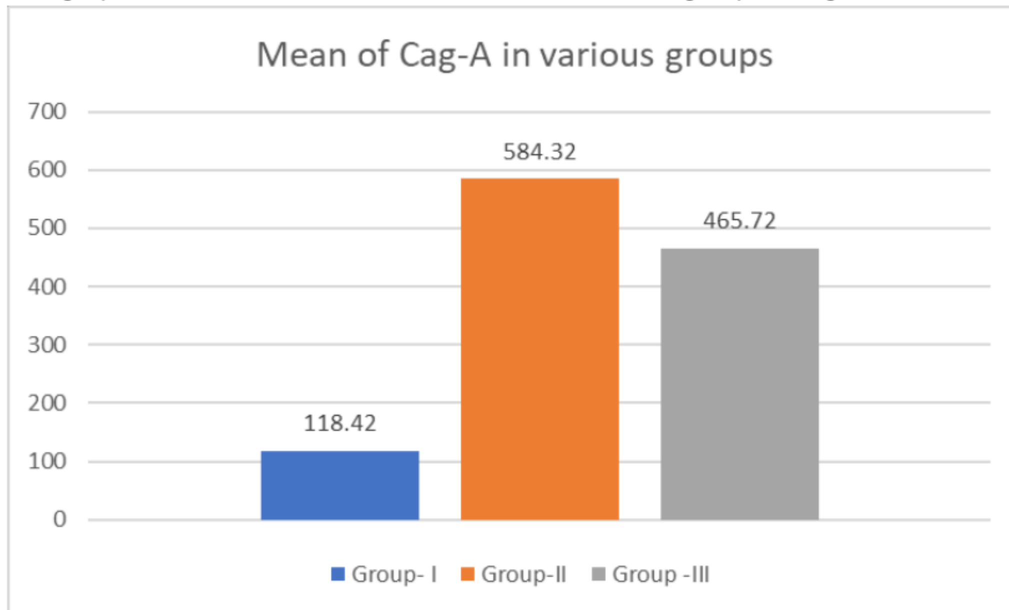
P-Value is less than 0.005, significant difference in mean values between the groups

Group- I = Healthy Individuals

Group- II = Periodontitis with Diabetes mellitus

Group-III = Periodontitis

The graph shows the mean difference between different groups in Fig.1.



The p-value <0.05, indicating that the difference between the groups is highly significant.

The results of the current study reveal that mean cytokine associated gene-A concentration in saliva was highest in group 2. The study population consisted of 45 participants with an age range of 35- 50 years, out of which 18 were males and 22 were females. The p-value for mann whitney test was less than 0.05, revealing a significant statistical difference in expression among the groups, that is healthy individuals and periodontitis with diabetes mellitus (p=0.001), periodontitis with diabetes mellitus and periodontitis (p=0.001), periodontitis alone vs healthy individuals (p=0.001).

DISCUSSION

Periodontitis initially originates from the gingival tissue and if left untreated can progress to affect deeper tissues leading to tooth loss. This progression underscores the importance of early diagnosis and intervention in managing periodontal disease [14]. Periodontal disease requires comprehensive understanding of the disease's causes and risk factors as it is multifactorial. The primary factor for periodontitis is dental plaque which contains a diverse community of bacteria and helps in initiation and progression of periodontal disease [15,16]. The interaction between the immune system and biofilm can influence the severity of the disease. Several other factors contribute to the development and progression of periodontitis, including local factors such as plaque and calculus, genetic makeup of the person, environmental factors, systemic health and social determinants [17].

Periodontopathogens can have negative effects on the periodontium and the systemic health of patients. This highlights the potential link between periodontal disease and broader health concerns [18]. The immune response in periodontal tissue involves the production of proinflammatory cytokines, chemokines and matrix metalloproteinases(MMPs) which promotes periodontal disease progression [19,20]. There are variations in immune response to bacterial infections that are attributed to genetic and risk factors. Genetic factors affect the activation of immune cells, synthesis of pro-inflammatory mediators [21]. Studies suggested that cytokine polymorphisms and variations in specific genes impact the immune system of the individual to infections [22].

Cytokines are the first responders to pathogens and stimuli at barrier sites in the body and facilitate the communication between different cell types such as lymphocytes, these molecules play a crucial role in maintaining homeostasis and regulating inflammatory processes [23]. Recent studies identified single nucleotide polymorphisms (SNPs) in cytokines suggesting the genetic variations in cytokine-related genes can lead to dysregulated cytokine responses, which may initiate and accelerate the development of periodontitis [24]. Various therapies for target cytokines showed promising preclinical trials suggesting that targeting specific cytokines could be a potential approach to treating periodontal disease [25]. Understanding the regulation of cytokines is crucial for developing effective therapies to treat periodontitis.

This study found that salivary cytokine associated gene A levels were significantly higher in two groups: the control group(periodontitis alone) and experimental group (periodontitis with diabetes mellitus) which suggests that cytokine associated gene A levels in saliva may be associated with the presence of periodontitis and are even

higher in individuals with both periodontitis and diabetes. This research is the first to investigate salivary cytokine associated gene A levels in three distinct groups: systemically healthy individuals, those with periodontitis, and those with both periodontitis and diabetes mellitus. Previous research showed variations in the cytokines of periodontal disease cells when compared to healthy cells. The study used a non-human primate model to study periodontitis induced by ligature placement allowing to examine the changes in cytokine profiles during the development of periodontal disease [26].Earlier studies reported changes in pro-inflammatory mediators such as IL-1β. The overexpression of IL-1β (crucial for Th17 type of immune response) during the acute phase of periodontitis explains its significant role in the tissue destruction associated with periodontitis [27]. These findings align with previous research and contribute to our understanding of the role of these cytokines in periodontal disease pathology.

Several studies examined the associations between IL-1α and IL-1β gene polymorphisms and microbiota in HIV-infected individuals where they did not find association between the gene polymorphisms and the studied parameters [28]. Another study analysed the association of IL-1α and IL-1β gene polymorphisms in periodontal tissue which suggested a relationship between IL-10, IL-1, and TNF-α gene polymorphisms in chronic periodontitis patients [29]. The complex interplay between genetic factors(cytokine gene polymorphisms) and bacteria in individuals with different conditions such as HIV infection and chronic periodontitis [30]. While some associations were observed in certain studies, the relationships between genetic polymorphisms and periodontal health and disease are multifaceted and may vary depending on the specific cytokines and patient populations studied. The limitations of the study are small sample size. There is the need for more research to better understand the role of Cytokine associated gene-A in the aetiology of periodontal disease.

CONCLUSION

The study shows that the cytokine associated gene-A(Cag-A) levels are higher in the of the patients suffering from periodontitis along with diabetes mellitus when compared with the patient with periodontitis(without any systemic disease) and healthy individuals. Hence, this Cag-A serves as a potential biomarker in diagnosing periodontal disease when screened in large populations along with its lead in the development of new therapeutic techniques.

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