Soluble CD163 as a biomarker of periodontal disease – A biochemical study using enzyme-linked immunosorbent assay

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INTRODUCTION

Periodontitis is a chronic inflammatory condition of the periodontium that affects the connective tissue attachment and alveolar bone around the teeth.\[11\] It is characterized by microbially associated, host-mediated inflammation that results in loss of periodontal attachment. The net result is apical migration of the junctional epithelium and pathological deepening of the gingival sulcus, allowing the pathogenic bacteria to enter the host tissue.\[2\] All the key pathways of the pathophysiology are mediated by the immune system of the host. The PMNs constitute to the innate immunity, whereas the lymphocytes constitute the adaptive immunity.\[3\] The bridging between the innate and the adaptive immunities is played by the mononuclear cells, i.e., the monocyte/macrophage lineage of cells.\[4\]

Macrophages release an array of cytokines that activate T cytotoxic lymphocytes for pathogen killing and B-lymphocytes to secrete immunoglobulins.\[5\] The categorization or polarization of macrophages into the M1 (classical activation) and M2 (alternative activation) subtypes is a simplified operation-based classification depending on the specific signals from the microenvironment, location, and disease state.\[6-8\] CD163 is a glycosylated membrane protein that is expressed almost exclusively on M2 macrophages and to a lesser extent on M1 macrophages.\[9,10\] CD163 belongs to the scavenger receptor cysteine-rich (SRCR) family and comprises nine extracellular SRCR protein domains that are linked to a short transmembrane segment and a short cytoplasmic tail. The locus for synthesis of CD163 lies on chromosome 12p13 and is composed of 17 exons.\[12\]

Abstract:
Background: The aim of the study was to evaluate the levels of soluble CD163 (sCD163) in gingival crevicular fluid (GCF) and blood serum of individuals having periodontitis, gingivitis, and healthy periodontium. Further, the role of sCD163 as a biomarker of periodontal disease was also assessed. Materials and Methods: A minimum of 5-µl GCF and 10 ml of venous blood was collected using a micropipette and 10-ml syringe, respectively, from the study population which was divided into three groups as healthy (Group I, n = 10), gingivitis (Group II, n = 10), and periodontitis (Group III, n = 10). sCD163 samples were assessed using a commercially available sCD163 enzyme-linked immunosorbent assay kit. Clinical parameters such as oral hygiene index simplified, gingival index (GI), percentage of sites with bleeding on probing, probing depth, and clinical attachment loss were recorded. Results: The mean serum sCD13 levels were 743.45 ± 51.17 ng/ml, 563.25 ± 103.74 ng/ml, and 431.0 ± 31.08 ng/ml when compared to the mean GCF sCD163 levels which were 59.81 ± 7.61 ng/ml, 38.93 ± 12.42 ng/ml, and 30.49 ± 12.60 ng/ml for periodontitis, gingivitis, and healthy individuals, respectively. The sCD163 levels were higher in patients with periodontitis when compared to the periodontally healthy individuals. Conclusion: Within the limitations of the present study, it can be concluded that sCD163 levels can be used as a diagnostic marker of disease as its levels are remarkably increased in GCFs of patients having periodontitis.

Key words:
Enzyme-linked immunosorbent assay, gingival crevicular fluid, macrophages, periodontal diseases, serum

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Soluble CD163 (sCD163) is shed in large quantities as a response to increased inflammatory condition to reduce the damage caused to the host by the host-derived enzymes and in reduced quantities during health by the anti-inflammatory cytokines to maintain tissue homeostasis.[12,13] Several studies have proven the increased presence of sCD163 in other body fluids during chronic inflammatory conditions such as rheumatoid arthritis, atherosclerosis, and metabolic syndrome-related conditions such as diabetes, obesity, and chronic kidney disease.[13-15] Although increased levels of sCD163 were found in serum and saliva of periodontitis patients,[15] the exact variation of the same in the gingival crevicular fluid (GCF) of periodontal health and disease is not clear till date. sCD163 is one such marker of macrophages that can be measured consistently even in smaller amounts with low intra-individual variability. Due to high specificity of sCD163 on monocyte-macrophage lineage and well-documented evidences of its (sCD163) presence in other chronic inflammatory conditions, sCD163 was chosen to be assessed in this present study. Hence, the aim of this study was to assess the sCD163 levels in GCF and serum from periodontal health to disease.

MATERIALS AND METHODS

Study population and clinical examination
The present cross-sectional study was conducted by selecting 30 individuals from the outpatient department of periodontology and implantology. The study sample included 17 males and 13 females, with age group ranging from 22 to 42 years. The study protocol was approved by the institutional ethics committee and was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. Return informed consent was obtained from the individuals, and then, they were included in the study. The individuals were included in the study if they showed clinical signs of health and disease[16,17] and minimum age of at least 21 years and not extending more than 65 years and had at least 20 teeth. Individuals who underwent periodontal therapy in the past 3 months, those taking any sort of medication that could alter the course of disease or influence inflammatory state, those suffering from any sort of systemic disease that could contribute to disease occurrence and progression, pregnant and lactating females, current smokers, and alcoholics were excluded from the study. The study was conducted from June 2019 to September 2019, and the individuals were stratified into healthy, gingivitis, and periodontitis groups, respectively, as Groups I, II, and III. Periodontal health was considered when an individual did not show any signs of bleeding on probing (BoP) and not having clinical attachment loss (CAL). Gingivitis was defined when an individual showed BoP and inflammation without any periodontal pocket or CAL. An individual was considered to be having periodontitis if at least two nonadjacent teeth showed interproximal CAL of ≥3 mm with radiographic evidence of bone loss. Oral hygiene status and inflammatory status were recorded using oral hygiene index simplified (OHS)[18] and gingival index (GI)[19] at the time of clinical examination. BoP was recorded as a dichotomous index[13] (present or absent) and was expressed in percentage of bleeding. To maintain standardization and intra-examiner reproducibility probing depth (PD), CAL was recorded by a single examiner using a UNC-15 probe.

Gingival crevicular fluid collection
All the clinical parameters were recorded on the 1st day, whereas the sample collection was done on the subsequent day. This was done to prevent bias that would have been caused due to stimulated secretion of GCF due to mechanical handling of tissues while recording clinical measurements, and the sample collection was done according to the procedure described by Pradeep et al.[20] Briefly, the site chosen for sample collection was gently dried and isolated using cotton rolls to prevent saliva contamination. Supragingival plaque was removed, and a 10-µl micropipette (Nipchip Ex-Plus II pipette, MERCK, © 2020 Merck KGaA, Darmstadt, Germany) was introduced into the gingival sulcus for collecting at least 5-µl GCF. One site per individual was selected for collection of GCF in gingivitis and periodontitis patients, whereas at least 3 sites per individual were used in the healthy group to ensure adequate collection of GCF. Samples contaminated with blood or saliva were discarded, and new sites were chosen for sample collection. Sites having the highest amount of inflammation and sites having the highest CAL were chosen for the collection of GCF sample in gingivitis and periodontitis patients, respectively. Air-protected plastic vials that were maintained at −70°C were used for storing GCF samples till enzyme-linked immunosorbent assay (ELISA) was performed.

Serum collection
Required quantity (10 ml) of blood was drawn using a 20G syringe from the antecubital vein. The collected blood sample was allowed to clot and was subjected to low-speed centrifugation at 2800 rpm for 5 min. Due to centrifugation, the serum got separated from the blood. The separated serum was stored separately in air protected plastic vials at-70 C.

Soluble CD163 analysis
sCD163 levels in both GCF and serum were analyzed using a commercially available aCD163 ELISA kit (Human CD163 ELISA Kit, MERCK, © 2020 Merck KGaA, Darmstadt, Germany). The sandwich method was performed according to the procedural instructions provided along with the kit. Briefly, all the samples and the reagents of the kit were brought to room temperature (18°C–25°C) and were diluted to required proportions. Five hundred microliters of standard and sample solution (GCF and serum) was coated to the wells of ELISA kit and allowed to incubate for 2.5 h at room temperature. After incubation, the wells were washed 4 times with 300-µl phosphate-buffered saline. Later, 500 µl of detection antibody was coated on to the wells and were allowed to incubate for 1 h at room temperature after which the wells were again washed 4 times with 300-µl phosphate-buffered saline. One hundred microliters of streptavidin solution was added to all the wells, incubated for 45 min at room temperature, and was again washed 4 times with 300-µl phosphate-buffered saline. One hundred microliters of tetramethylbenzidine was added which imparts color to the wells and was incubated for 30 min at room temperature after which 50 µl of stop solution was added to stop the coloring reaction. Then, the ELISA plate was fed into an optical plate reader, and the absorbance of standard was read at 450 nm. The absorbance of the samples was compared with the absorbance of standard, and the concentrations of sCD163 were charted accordingly.
Statistical analysis
The data were entered into a Microsoft Excel spreadsheet and imported to the SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) for statistical analysis. The result was presented in the form of mean and standard deviation. One-way ANOVA test was performed for intergroup comparison of sCD163 levels and clinical parameters. Student’s unpaired t-test was used for pairwise comparison of OHIS and GI, whereas Chi-square test was used to find association in bleeding index in between different groups. \( P < 0.05 \) was considered statistically significant.

RESULTS
Table 1 shows the demographic data of the study population in mean ± standard deviation. Unpaired t-test for intergroup comparison for OHIS showed a significant difference (\( P < 0.001 \)) in all the three groups. The mean OHIS score in periodontitis (4.80 ± 0.68) was higher than that of the gingivitis (2.38 ± 0.58) and healthy (0.87 ± 0.41) groups, as shown in the table. The inflammatory index scores were also significant among the three groups. The mean scores for inflammatory index in the periodontitis group (2.69 ± 0.29) were higher than those in the gingivitis (1.54 ± 0.20) and healthy groups (0.57 ± 0.42).

One-way ANOVA of sCD163 levels [Table 1] showed statistically significant differences among all the three groups. The mean serum sCD163 levels in the periodontitis group (743.45 ± 51.17 ng/ml) were more than the mean serum sCD163 levels in the gingivitis group (563.25 ± 103.74 ng/ml) and healthy group (431.03 ± 31.08 ng/ml). Similarly, the mean GCF sCD163 levels in the periodontitis group (59.81 ± 7.61 ng/ml) were more when compared to the GCF samples in the gingivitis group (38.93 ± 12.42 ng/ml) and healthy group (30.49 ± 12.60 ng/ml) which were almost near to the lower sensitivity range of the ELISA kit (30 ng/ml).

Chi-square test [Table 2] for bleeding index was done for intergroup comparison as the bleeding index was taken as dichotomous variables, i.e., bleeding present or absent. The intergroup comparison for bleeding index showed a significant difference (<0.001) among all the three groups.

Multiple comparisons were done using unpaired t-test [Table 3], which was performed to confirm which group or groups differed statistically at the 5% level of significance. All the comparisons showed statistically significant differences for both GCF and serum sCD163 samples.

DISCUSSION
sCD163 is a hemoglobin-scavenging protein which is increased during inflammatory conditions to counteract the destruction caused by classically activated macrophages (M1 macrophages). Several studies have been performed in which sCD163 was observed in various other body fluids,[21-23] but there is little evidence demonstrating the presence of sCD163 in GCF.

The results of the present study showed that the patients with periodontitis showed higher levels of sCD163 levels both in serum and GCF when compared to healthier individuals. The results also correlated with the increase in PDs along with the increase in GCF levels of sCD163 levels. This study reported a mean serum sCD163 level of 743.45 ± 51.17 ng/ml in periodontitis patients, 563.25 ± 103.74 ng/ml in gingivitis patients, and 431.03 ± 31.08 ng/ml in healthy individuals. A study done by Detzen et al.[15] in saliva and serum of periodontitis patients demonstrated elevated serum levels of sCD163 (720.0 ± 330.6 ng/ml) which are comparable to the serum sCD163 levels of the present study. The subdue decrease in the GCF sCD163 levels from the serum sCD163 levels may be due to the molecular weight of sCD163 (130 kDa) which is higher when compared to the most commonly found proteins in GCF which are <79 kDa.[24] The tissue barriers and cellular transport of CD163 are some other factors which play a role in expression of sCD163 in GCF.[25] The two-way interrelationship between periodontal disease and systemic health might be the reason for increase in the serum sCD163 levels even in the systemically healthy individuals.[26,27]

Free hemoglobin that is released into the blood forms hemoglobin/haptoglobin complexes which is one of the strong causes of oxidative stress and pro-inflammatory changes that occur in the tissues.[12,28,29] The best-known function of CD163 is the clearance of these hemoglobin/haptoglobin complexes in its cell-free form and participate as an anti-inflammatory soluble factor, exhibiting cytokine-driven functions. Furthermore, transforming growth factor (TGF)-beta has a role in downregulating CD163 secretion.[30] It downregulates the expression of CD163 secretion. However, due to biologic synergism of interleukin-6 (IL-6) and macrophage colony-stimulating factor (M-CSF) which are abundantly found in periodontal inflammation, the downregulation caused by TGF-beta might be overtaken by the upregulation caused by IL-6 and M-CSF.[31] In vitro studies of cultured monocytes/macrophages have shown that the shedding of sCD163 can be induced by toll-like receptor (TLR) activation by lipopolysaccharide (LPS) or phorbol

Table 1: Descriptive data (mean±standard deviation) of the study population and the soluble CD163 levels with one-way ANOVA test

| Parameters            | Group I (n=10) | Group II (n=10) | Group III (n=10) | One-way ANOVA (F) |
|-----------------------|---------------|-----------------|-----------------|------------------|
| Age                   | 22.2±3.46     | 35.7±8.12       | 42.4±6.84       | -                |
| OHIS                  | 0.87±0.41     | 2.38±0.58       | 4.80±0.68       | 121.73*          |
| GI                    | 0.57±0.42     | 1.54±0.20       | 2.69±0.29       | 111.25*          |
| PD                    | 1.91±0.74     | 2.46±0.31       | 5.16±0.60       | 90.10*           |
| CAL                   | 0             | 0               | 5.40±0.82       | -                |
| GCF sCD163 levels     | 30.49±12.60   | 38.93±12.42     | 59.81±7.61      | 14.73*           |
| Serum sCD163 levels   | 431.03±31.08  | 563.25±103.74   | 743.45±51.17    | 38.72*           |

*Statistically significant at \( P<0.001 \). OHIS – Oral hygiene index simplified; GI – Gingival index; PD – Probing depth; CAL – Clinical attachment loss; sCD163 – Soluble CD163; GCF – Gingival crevicular fluid; n – No of subjects; F – F static value for ANOVA test.
This is the first study to use GCF for assessing the levels of sCD163 in quantifying the health and disease states during the epidemiologic studies and scientific research directed toward developing a chairside test for sCD163 in periodontitis. Pre operative, post operative and follow up interval levels of sCD163 could also be measured for arriving at a stronger evidences of considering sCD163 as a marker for periodontal disease.

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**Conflicts of interest**
There are no conflicts of interest.

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