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Proinflammatory (CD14+CD16++) monocytes in type 2 diabetes mellitus patients with/without chronic periodontitis

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ABSTRACT

Background: Until date, the proportion of nonclassic monocytes in type 2 diabetic mellitus patients with and without chronic periodontitis has not been evaluated based on glycemic control. The objective of this study was to compare the proportion of CD14+CD16++ monocytes in type 2 diabetic patients with and without chronic periodontitis.

Materials and Methods: In this cross sectional study A total of sixty individuals with type 2 diabetes mellitus (n = 15/group) were recruited. Individuals were grouped based on glycosylated hemoglobin A (HbA 1c) values and the presence of chronic periodontitis; Group 1 (diabetes mellitus with good glycemic control), Group 2 (diabetes mellitus with poor glycemic control), Group 3 (diabetic mellitus with chronic periodontitis and good glycemic control), Group 4 (diabetic mellitus with chronic periodontitis and poor glycemic control). Fluorochrome-conjugated monoclonal antibodies against CD14, CD16, and human leukocyte antigen–antigen D related was used to analyze the proportion of nonclassic monocytes by flow cytometry. One-way ANOVA with Tukey's *post-hoc* test was used to assess the significant differences in monocyte subpopulations. The Pearson's correlation test was used to assess the relationship between hemoglobin A1c values and percentage of nonclassical monocytes. In both the above statistical tools, the value of $P < 0.05$ is considered as significant level.

Results: Group 4 had the highest percentage of CD14+CD16++ monocytes 14.67% + 4.70%, followed by Group 3-9.73% + 0.60%, Group 2-9.32% + 2.03% and Group 1-5.92% + 0.63% ($P < 0.001$). Further, a statistically significant positive correlation between HbA (1c) levels and the proportion of CD14+CD16++ monocytes was observed.

Conclusion: In the present study, we observed type 2 diabetes mellitus patients with poor glycemic control and chronic periodontitis showed more than two-fold increase in the proportion of nonclassic monocytes compared to type 2 diabetes mellitus patients with good glycemic control.

Key Words: Chronic periodontitis, diabetes mellitus, monocytes

INTRODUCTION

Diabetes mellitus, a common chronic inflammatory disease characterized by altered glucose metabolism resulting in various micro–macrovascular complication[1] has a high prevalence globally with 366 million individuals suffering from it as on 2011 which is likely to increase to around 566 million by 2030.[2] India is regarded to be the diabetes capital of the world.[3] Indian Council of Medical

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Research-INdia DIABetes study conducted on 13,055 individuals in 2011 projected that 77.2 million individuals living in India were pre-diabetes state and 62.4 million individuals had diabetes mellitus.[4] Glycemic control plays a pivotal role in the prevention of diabetes complications.[5,6] The prevalence of diabetes complication and its burden in the south Indian population were studied in Chennai Urban Population Study and Chennai Urban Rural Epidemiology Study and reported a high prevalence of cardiovascular complications, neuropathy, microalbuminuria, and retinopathy.[7-10] Mohan *et al*.[11] conducted a study involving 20,554 Indian type 2 diabetes mellitus patients and concluded that glycemic control was very poor among Indian diabetes mellitus patients with a mean hemoglobin A1c (HbA1c) of 9.2% (77 mmol/mol). Chronic periodontitis, an inflammatory disease of microbial etiology affects the supporting structures of the teeth causing resorption of alveolar bone and eventually leading to tooth mobility and tooth loss.[12] The two-way relationship between diabetes mellitus and chronic periodontitis has been studied extensively over decades.[13] Diabetes mellitus is also considered to be a risk factor for chronic periodontitis.[12] Chronic periodontitis is considered to be the 6th complication of diabetes mellitus.[14] Maintaining a healthy periodontium is essential for achieving good long-term control of diabetes mellitus.[15] Inflammation has been suggested to be a link between diabetes mellitus and chronic periodontitis and immune response seems to modulate the pathogenesis of the diseases. Monocytes are one of the potent immune cells which are known to exist in three subsets in humans.[16] The monocytes are categorized into classical, intermediate, and nonclassical monocyte subsets based on the expression of CD14 and CD16 receptors. Classical monocytes account for 90% of circulatory blood monocytes and express only CD14 and do not express CD16 (CD14++ CD16-), the nonclassical monocytes express both CD14 and CD16 (CD14+CD16++) and account for about 10% of the monocytes.[16] The nonclassical subsets of monocytes are known to be elevated in infection and inflammation.[17] The nonclassical monocytes are also referred to as pro-inflammatory monocytes owing to its increased production of pro-inflammatory cytokines in response to bacterial challenge.[18] Yang *et al*.[19] suggested that the disturbance of pro-inflammatory monocytes occurs in type 2 diabetes mellitus patients. Nagasawa *et al*.[20] revealed an increase in pro-inflammatory (CD16+) monocytes in chronic periodontitis patients compared to healthy controls and concluded that monitoring the surface expression of CD14 and CD16 receptors in chronic periodontitis patients could help in studying the alteration of monocytes in these patients. Furthermore, we have recently shown that in chronic periodontitis patients the nonclassical subset of the CD16-positive monocytes is increased compared to healthy controls.[21] Until date, the proportion of nonclassical (CD14+CD16++) monocytes in type 2 diabetes mellitus with and without chronic periodontitis have not been evaluated based on glycemic control. The primary objective of the present study was to compare the proportion of CD14+CD16++ monocytes in peripheral blood of type 2 diabetes mellitus patients with and without chronic periodontitis. The secondary objective was to determine whether a correlation exists between the HbA1c levels in type 2 diabetes mellitus patients and the proportion of CD14+CD16++monocyte subset.

MATERIALS AND METHODS

In this cross sectional study a total of 60 individuals with type 2 diabetes mellitus in the age range of 45–55 years were recruited from January 2014 to May 2014. The study was approved by the Institutional Ethics Committee (REF: IECNI/10/MAY/16/14). The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. Individuals were recruited after obtaining a written informed consent.

Sample size

Studies evaluating the monocyte subpopulation in diabetes patients with or without chronic periodontitis have not been performed earlier. Hence, the sample size for the present study was calculated based on the studies by Nagasawa *et al*.[20] and Bae *et al*.[22] The mean percentage of nonclassical monocytes in type 2 diabetes mellitus patients without cardiovascular diseases[22] was found to be 10.1% ± 1.4% and in chronic periodontitis patients[20] it was found to be 13% ± 1.3%. Based on this mean ± standard deviation (SD); to achieve a power of 90% and α = 5%, the sample size of 15 per group was obtained.

Patient selection

Patients were diagnosed to have type 2 diabetes mellitus based on the guidelines of American Diabetic Association standards of medical care in diabetes.[23] In brief, the diagnosis of diabetes was

made if HbA1c ≥6.5% (48 mmol/mol) or fasting plasma glucose of ≥126 mg/dl; fasting is defined as no caloric intake for at least 8 h or 2 h plasma glucose of ≥200 mg/dl or a patient with classical symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose of ≥200 mg/dl. The case definition for chronic periodontitis was based on the guidelines given by International Workshop for a Classification of Periodontal Diseases and Conditions 1999[24] (i.e.,) presence of at least 10 natural teeth; ≥30% of periodontal sites examined with ≥1 mm of attachment loss. HbA1c value of <7% (<53 mmol/mol) was considered good glycemic control and HbA1c value of >7% (>53 mmol/mol) was considered poor glycemic control.[25] A total of 60 patients were recruited into four groups (n = 15) based on the HbA1c values and the presence of chronic periodontitis. Patients were sampled separately for each group to obtain the desired sample size of 15 per group. Group 1 included type 2 diabetes mellitus patients with good glycemic control without chronic periodontitis, Group 2 included type 2 diabetes mellitus patients with poor glycemic control without chronic periodontitis, Group 3 included type 2 diabetes mellitus patients with good glycemic control and chronic periodontitis and Group 4 included type 2 diabetes mellitus with poor glycemic control and chronic periodontitis. Exclusion criteria for all the groups were as follows: pregnant and lactating mothers, smokers both former and present, any periodontal or antibiotic therapy in the past 6 months, any systemic disease or condition other than diabetes mellitus, and patients with diabetes-related complication. All the study participants underwent medical examination to rule out diabetes-related complications. The absence of retinopathy was confirmed by fundus examination to rule out microaneurysms. The study participants were considered to have the macrovascular disease if they had reported a history of abnormal investigation or prior macrovascular event. All patients underwent a complete full mouth periodontal examination on all teeth excluding 3rd molars using a manual periodontal probe (UNC15, Hu-Friedy, Chicago, IL). Pocket probing depth (distance between gingival margin to base of gingival sulcus, in millimeters), clinical attachment loss, (distance between cement-enamel junction and base of gingival sulcus in millimeters), were recorded.

Peripheral blood collection

Three milliliters of peripheral blood samples were taken collected in two separate vacutainers from each patient. One sample was analyzed for plasma glucose levels and HbA1c level. HbA1c was measured by high-performance liquid chromatography. The blood from the second vacutainer was subjected to flow cytometric analysis.

Flow cytometric analysis

Monocyte subpopulation was analyzed based on the specific fluorochrome-conjugated monoclonal anti-human antibodies (BD Biosciences, San Jose, CA) CD14 phycoerythrin (PE) and CD16 fluorescein isothiocyanate (FITC). The CD14 clone is derived from hybridization of mouse Sp2/0 myeloma cells with spleen cells from BALB/c mice immunized with peripheral blood monocytes from a patient with rheumatoid arthritis. The CD16 clone is 3G8 FITC mouse anti-human antibody. Human leukocyte antigen–antigen D-related (HLA DR) Clone is G46-6 allophycocyanin (APC) mouse anti-human antibody. In addition, the following reagents (BD Biosciences, San Jose, CA) were used; fluorescent-activated cell sorting (FACS) lysis solution, phosphate-buffered saline (PBS) (pH 7.4), PBS with 10% fetal bovine serum (FBS), and 1% paraformaldehyde. The sample preparation and analysis of monocyte subpopulations was done as described previously.[21] In brief, 200 μL of the whole-blood was taken in a FACS tube, and the fluorochrome-conjugated monoclonal antihuman antibodies of 7 μL CD14 (PE), 10 μL CD16 (FITC), and 5 μL HLA-DR (APC) were added and gently mixed. It was incubated for 30 min in the dark at room temperature (20°C–25°C); then 2 mL ×1 FACS lysing solution was added and incubated for 10 min in the dark at room temperature. The mixture was centrifuged at 500 × g for 5 min, and the supernatant was discarded. Three milliliers wash buffer (PBS with 10% FBS) was added, the centrifugation step was repeated, and the supernatant was discarded. Finally, 0.5 mL 1% paraformaldehyde solution was added and mixed thoroughly. The cells were acquired in a flow cytometer (FACS Calibur, BD Biosciences), and the results were analyzed using software (Flowjo software, Tree Star, Ashland, OR.).

Statistical analysis

The collected data were analyzed with IBM. SPSS statistics software 23.0 Version (IBM, Armonk, NY, USA). To describe about the data descriptive statistics mean and SD were used. The Shapiro Wilk's test for normality shows the data was normally distributed, hence to find the significant difference in the multivariate analysis the one-way ANOVA

with Tukey's *post-hoc* test was used. The Pearson's correlation test was used to assess the relationship between HbA1c values and percentage of nonclassical monocytes. Data were analyzed using statistical software package (SPSS for Windows v. 23, IBM, Armonk, NY, USA). In both the above statistical tools, the $P < 0.05$ is considered as statistically significant level.

RESULTS

The demographic data, clinical indices, mean HbA1c values of the study population are presented in Table 1. The processed blood samples were acquired in the flow cytometer. Unstained sample of peripheral blood was analyzed based on forward and sideward scatter [Figure 1a] and monocytes are gated as R2 [Figure 1b]. The numbers next to R2 represent the percentage of gated cells out of the total acquired cells. In the forward and side scatter (SSC) plot among the three subsets (lymphocytes, monocytes, neutrophils) monocytes (R2) alone were gated [Figure 1c]. Further cells in R2 gate were plotted with SSC in Y-axis and HLA-DR in X-axis [Figure 1d]. Only the HLA-DR+ cells were gated and monocyte subpopulations were analyzed based on CD 14 and CD16 expression.[26] Three subpopulations of monocytes could be identified; CD14++CD16-(classical monocytes), CD14++CD16+ (intermediate monocytes), CD14+CD16++ [nonclassical monocytes; Figure 1e]. Figure 2 shows monocyte subpopulations of one sample from each group.

Monocyte subpopulations in peripheral blood

The percentage of subset of monocyte in each group is presented in Table 2. Group 4 had the highest percentage of CD14+CD16++ monocytes 14.54% ± 3.7%, followed by Group 3-9.93% ± 1.6%, Group 2-9.42% ± 1.93% and Group 1-5.82% ± 1.3% ($P < 0.001$, one-way ANOVA). *Post hoc* analysis using Tukey HSD test revealed statistically significant difference in the mean proportion of classical and intermediate monocytes among all the four groups $P < 0.001$. Were as in the nonclassical monocytes Group 2 vs. Group 3 alone did not show statistical significance.

All three monocyte subpopulations differed significantly in HLA-DR expression in all the four groups as measured by the median fluorescent intensity (MFI). Intermediate monocytes had the highest MFI for HLA-DR (861.7 ± 24.4 – Group 1, 883.1 ± 26.2 – Group 2, 885.8 ± 60.7 – Group 3, 882.1 ± 54.2 – Group 4), followed by nonclassical monocytes (270.3 ± 41.4 – Group 1, 272.5 ± 38.7 – Group 2, 261.1 ± 56.4 – Group 3, 312.3 ± 36.7 – Group 4) and classical monocytes (96.3 ± 9.2 – Group 1, 96.5 ± 6.8 – Group 2, 95.1 ± 8.0 – Group 3, 93.6 ± 13.3 – Group 4), respectively. This finding is in accordance with previously published studies.[27,28]

Glycosylated hemoglobin and CD14+CD16++ monocytes

The correlation between HbA1c values and CD14+CD16++ monocytes was analyzed using Pearson's correlation. HbA1c value showed a significant positive correlation with the percentage of CD14+CD16++ monocytes in Group 1, Group 2, and Group 4 [Figure 3].

DISCUSSION

Several evidence-based studies have shown increased periodontal destruction in diabetes mellitus patients.[29-35] Diabetes mellitus is regarded as one of the risk factors for chronic periodontitis.[36,37] Diabetes patients in general have higher prevalence of periodontitis, more severe periodontal destruction compared to non-diabetes controls.[38,39] Taylor[40] suggested a bi-directional relationship between

Table 1: Demographic data of study population

Variable	Group 1 (*n*=15)	Group 2 (*n*=15)	Group 3 (*n*=15)	Group 4 (*n*=15)
Diabetes with HbA1c	<7%	>7%	<7% periodontitis	>7% periodontitis
Age (years)	51.2±3.2*	51.8±2.9*	50.6±3.9*	51.9±2.9*
Gender (females/males)	7/8	8/7	7/8	7/8
Clinical attachment loss (mm)	0±0	0±0	4.1±0.5*	4.5±0.7*
Probing depth (mm)	1.4±0.3*	1.8±0.3*	5.1±0.3*	5.3±0.2*
Glycosylated hemoglobin (%)	5.91±0.43* (41 mmol/mol)	8.34±0.65* (68 mmol/mol)	6.13±0.40* (43 mmol/mol)	8.76±0.70* (72 mmol/mol)

*Values expressed as mean±SD. Group 1: Diabetes mellitus type 2 good glycemic control; Group 2: Diabetes mellitus type 2 poor glycemic control; Group 3: Diabetes mellitus type 2 good glycemic control with chronic periodontitis; Group 4: Diabetes mellitus type 2 poor glycemic control with chronic periodontitis; SD: Standard deviation; HbA1c: Hemoglobin A1c

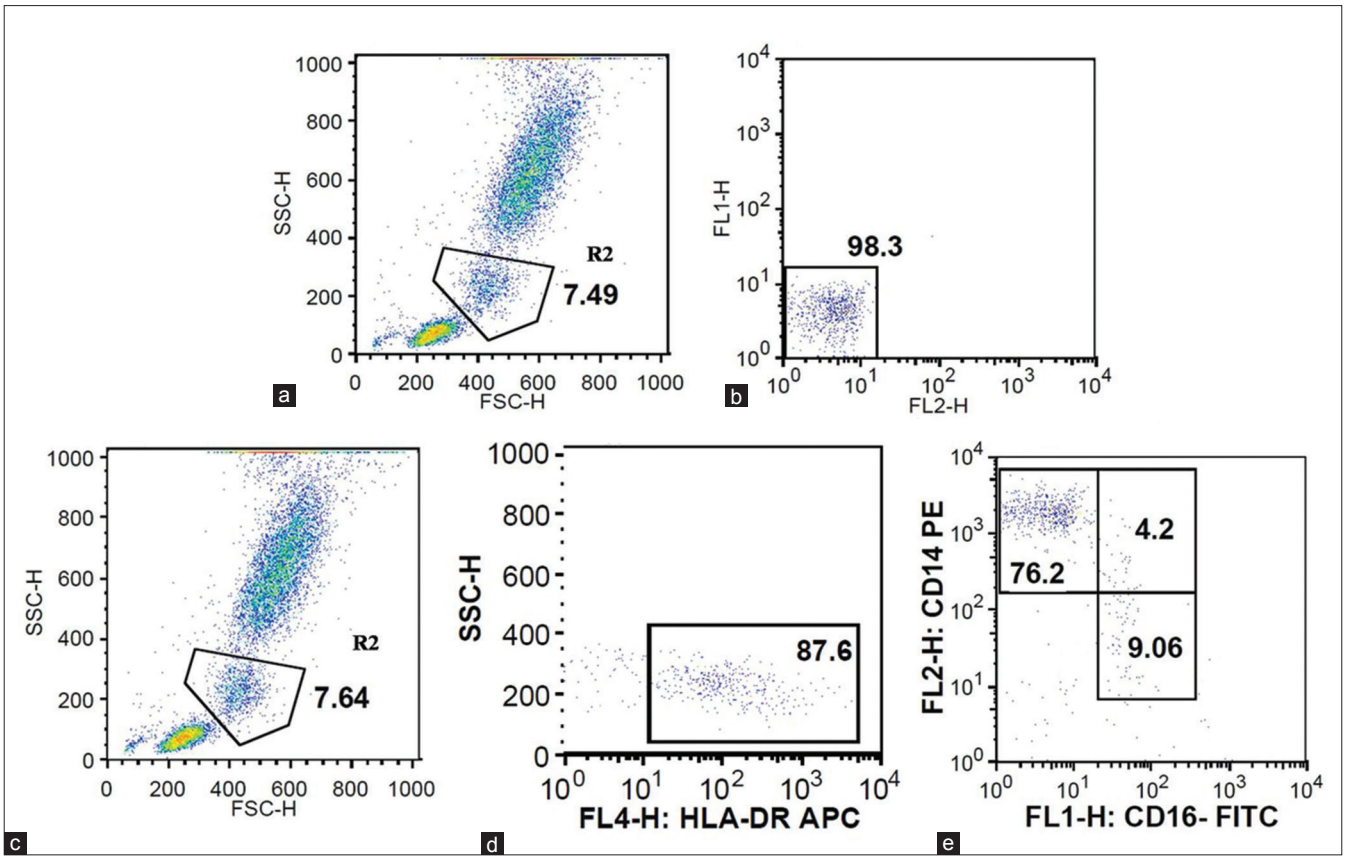

Figure 1: Flow cytometric analysis and gating of monocytes in peripheral blood. (a) Unstained sample of peripheral blood analyzed based on forward and sideward scatter and monocytes are gated as R2. The numbers next to R2 represent the percentage of gated cells out of the total acquired cells. (b) Unstained sample of peripheral blood; the cells in gate R2 were analyzed. (c) Peripheral blood sample was stained with phycoerythrin-conjugated anti-CD14; fluorescein isothiocyanate-conjugated anti-CD16 and allophycocyanin-conjugated anti-human leukocyte antigen–antigen D-related. Based on forward and sideward scatter among the three subsets (lymphocytes, monocytes, neutrophils) monocytes alone were gated as R2. (d) The cells in gate R2 were analyzed based on side scatter and human leukocyte antigen–antigen D-related expression. (e) Human leukocyte antigen–antigen D-related + cells were gated and monocyte subpopulations were analysed based on the expression of phycoerythrin-conjugated anti-CD14, fluorescein isothiocyanate-conjugated anti-CD16. Upper left gate (CD14++CD16-classical monocytes), Upper right gate (CD14++CD16+ Intermediate monocytes), Lower right gate (CD14+CD16++ Non classical monocytes).

diabetes mellitus and chronic periodontitis. The author hypothesized that diabetes mellitus could increase the susceptibility to develop chronic periodontitis and chronic periodontitis could contribute to increase hyperglycemia in diabetes patients and cause elevation of HbA1c in nondiabetes individuals.[40] Chen *et al*.[39] reported full mouth mean probing depth to be a predictor variable for increased HbA1c; after adjusting for age, gender, and Body Mass Index. Bandyopadhyay *et al*.[41] stated that individuals with poor glycemic control had significantly higher odds of progression of pocket probing depth compared to those with good glycemic control. Nesse *et al*.[38] assessed a dose-response relationship between periodontal inflamed surface area and HbA1c levels in type 2 diabetes mellitus and concluded that periodontal inflammation could contribute to poor glycemic control which in turn could increase the severity of periodontitis.[38] Inflammation has shown to be a negative influence on glycemic control.[42] Pro-inflammatory mediators such as tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-1 on entering systemic circulation could induce insulin resistance.[43] The human periodontium acts as a reservoir of cytokines and poses an inflammatory burden; whereby pro-inflammatory cytokines could enter systemic circulation and cause insulin resistance.[44] Increased periodontal destruction seen in type 2 diabetes mellitus patients has been attributed to the hyperinflammatory monocyte trait in these individuals.[45] CD14+CD16++ subpopulation of circulating monocytes has been

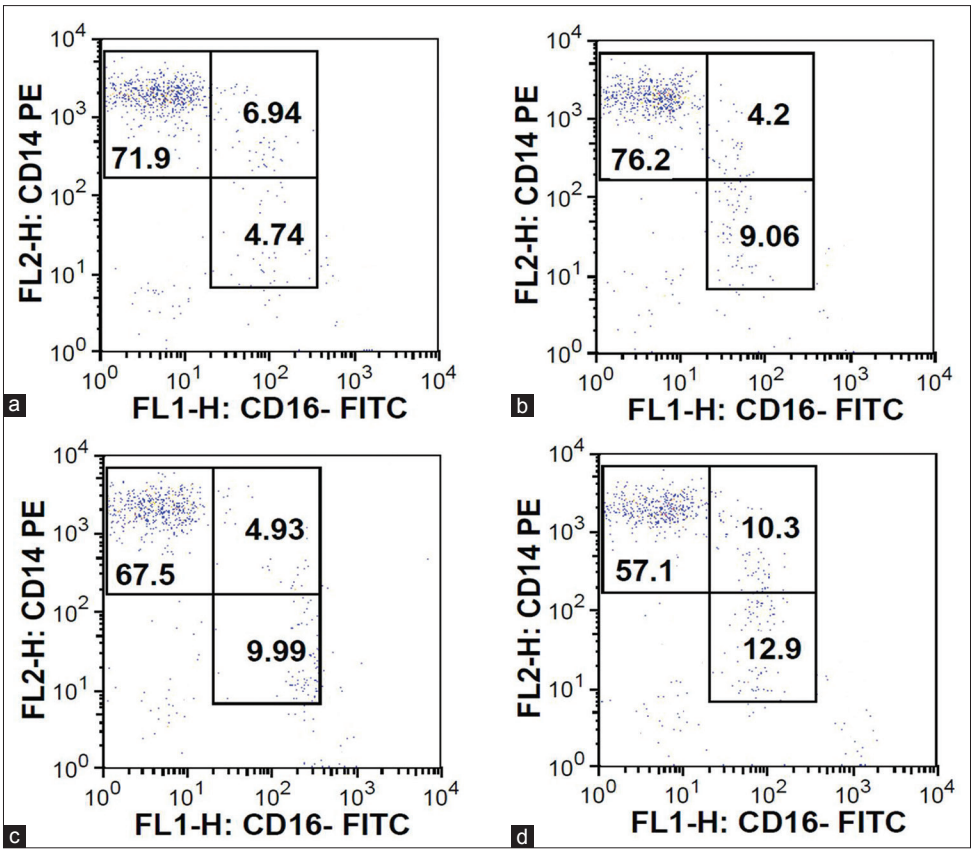

Figure 2: Representative sample from four groups (a) Group 1 (diabetes mellitus with good glycemic control). (b) Group 2 (diabetes mellitus with poor glycemic control). (c) Group 3 (diabetes mellitus with chronic periodontitis and good glycemic control). (d) Group 4 (diabetes mellitus with chronic periodontitis and poor glycemic control).

Table 2: Percentage of monocyte subpopulation in each group

Monocyte subpopulation	Group	Mean±SD	*F*	*P*
Classical monocytes (%)	1	70.9±2.6	467.313	0.000
	2	75.2±2.1		
	3	66.5±2.4		
	4	56.1±2.6		
Intermediate monocytes (%)	1	6.84±1.4	282.381	0.000
	2	4.1±1.6		
	3	4.83±1.3		
	4	10.1±1.3		
Nonclassical monocytes (%)	1	5.82±1.3	26.914	0.000
	2	9.42±1.93		
	3	9.93±1.6		
	4	14.54±3.7		

Group 1: Diabetes mellitus Type 2 good glycemic control; Group 2: Diabetes mellitus Type 2 poor glycemic control; Group 3: Diabetes mellitus Type 2 good glycemic control with chronic periodontitis; Group 4: Diabetes mellitus Type 2 poor glycemic control with chronic periodontitis. Statistically significant *P*<0.001, One-way ANOVA. SD: Standard deviation

shown to be elevated in type 2 diabetes mellitus patients as compared to normal controls.[22] Similarly, this subpopulation of monocytes was expanded in chronic periodontitis patients compared to healthy controls[20,21] In the present study, we observed that type 2 diabetes mellitus patients with poor glycemic control and chronic periodontitis showed more than two-fold increase in the proportion of nonclassical monocytes compared to type 2 diabetes mellitus patients with good glycemic control. Further a positive correlation between HbA1c levels and the proportion of CD14+CD16++ monocytes was observed. Yang *et al.*[19] stated that disturbance of nonclassical monocytes observed in type 2 diabetes mellitus patients could be related to the activation of TLR-4/NFκB signaling pathways underlying the immune abnormalities of CD14+CD16++ monocytes. Diabetes-related complication status has been reported to have an impact on the circulating monocyte phenotype.[46] Min *et al.*[46] analyzed the alterations of monocyte CD16 in diabetes patients with and without complications. The authors reported that compared to controls, the percentage of circulating nonclassical monocytes were higher in diabetes patients without complications. The authors concluded by stating that diabetes did not affect the white blood cells but increased the monocytes. Further, they reported a significant reduction in the

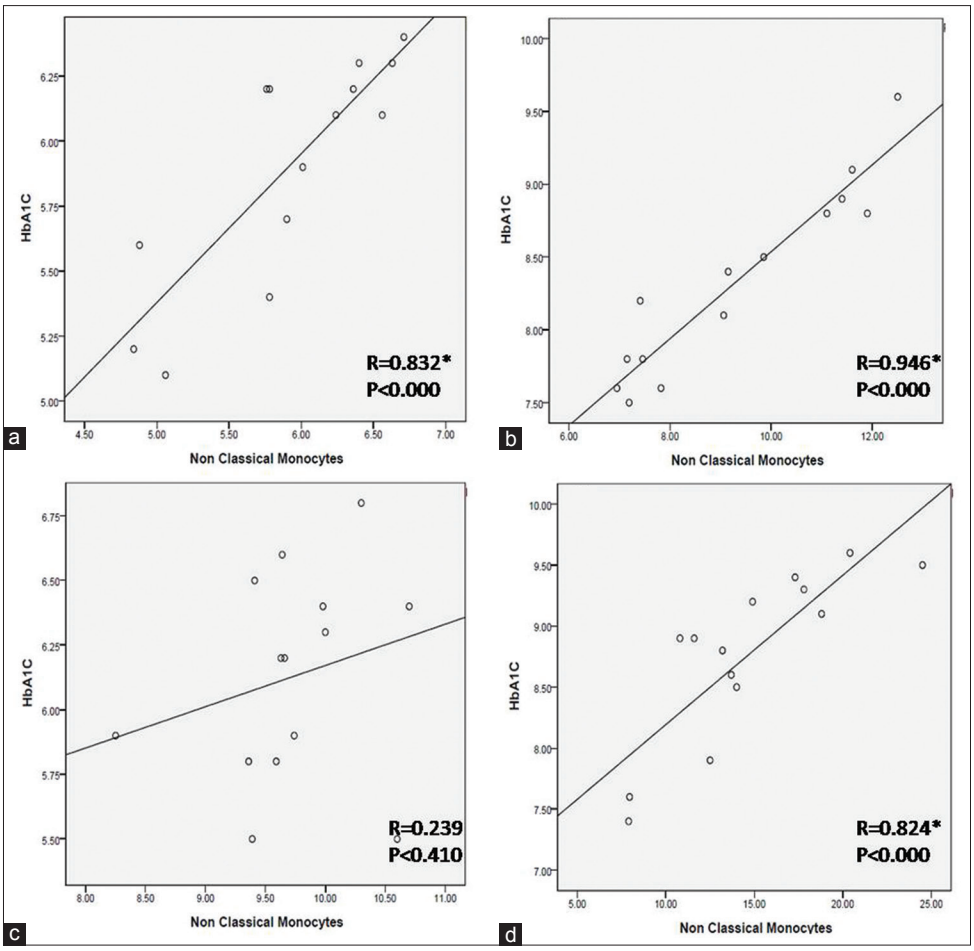

Figure 3: Pearson's Correlation test to assess the relationship between hemoglobin A1c values and percentage of non classical monocytes. (a) Diabetes Mellitus Type 2 good glycemic control, (b) Diabetes Mellitus Type 2 poor glycemic control, (c) diabetes Mellitus Type 2 good glycemic control with chronic periodontitis, (d) Diabetes Mellitus Type 2 poor glycemic control with chronic periodontitis. *Statistically significant $P < 0.001$.

number of non-classical monocytes in patients with diabetes-related complications. In the present study, none of the recruited patients had diabetes-related complications. CD14, CD16, HLA-DR fluorochrome antibody combination is considered to be reliable for the analysis of monocyte subsets[47] and has been used in several studies.[48-50]

CONCLUSION

One of the limitations of the present study is that the association between fasting blood glucose (FBG) and nonclassic monocytes was not assessed. FBG levels are influenced by diet, physical exercise and compliance to medication. HbA1c serves as a marker for average blood glucose level over the previous 2–3 months period. We hypothesize that the cytokine milieu or the underlying inflammatory process in periodontitis and hyperglycemic status in type 2 diabetes mellitus could trigger the classic monocytes to get converted to nonclassic monocytes, thereby contributing to increased periodontal destruction. Although this needs further investigation the results need to be interpreted with caution. Further studies/clinical trials based on the treatment of periodontitis, resolution of inflammation and alteration of nonclassical monocyte subpopulation could provide conclusive evidence on the role of these cells in the pathogenesis of chronic periodontitis and type 2 diabetes mellitus.

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Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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