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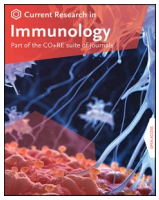

Cytokine levels in gingival tissues as an indicator to understand periodontal disease severity

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ABSTRACT

Cytokines regulate periodontal pathogenesis and are relevant estimates of current disease activity. There is sparse information on status of cytokine protein levels in periodontal pocket (gingival) tissues. The current study analysed proteins and transcripts of selected cytokines in varying severity of periodontal disease and elucidated cytokine/cytokine ratios that best indicated periodontal disease severity, in gingival tissues. A total of 92 participants comprising of generalised moderate periodontitis (GMP, n = 18), generalised severe periodontitis (GSP, n = 46) and periodontally healthy controls (PHC, n = 25) were recruited for the study. Interproximal gingival tissue samples were utilised for cytokine protein estimation and mRNA quantification by qRT-PCR and ELISA respectively. Selected key pro and anti-inflammatory cytokines, also representative of various Th subsets were analysed. ROC curve analysis was performed and Youden index was calculated for individual cytokines and pro/anti-inflammatory cytokine ratio to estimate the best indicator of periodontal severity/progression in tissues. IL-1β, TGF-β and IFN-γ cytokine protein levels varied significantly ($p \leq 0.05$) with severity of periodontal disease between groups. On comparison between deep and shallow sites within same participant, deep sites showed significant elevation of TGF-β ($p \leq 0.01$) and IFN-γ ($p \leq 0.05$) and IL-17 cytokines and shallow sites showed elevation of IL-4($p \leq 0.01$) and IL-1β ($p \leq 0.05$) cytokines. Analysis of transcripts showed IFN-γ and IL-1β transcript predominance in GSP ($p = 0.01$) compared to PHC. ROC analysis illustrated 97% sensitivity, 93% specificity with Youden index of 90% for IL-1β cytokine and 81%sensitivity, 79% specificity with a Youden index of 60% for IL-1β/TGF-β ratio In periodontal pocket tissue, a lack of distinct predominance of specific cytokines between study groups or between shallow and deep sites affected by periodontal disease was observed. However, ROC analysis of cytokines revealed IL-1β cytokine and IL-1β/TGF-β ratio as promising indicators of periodontal disease severity in gingival tissues.

1. Introduction

Periodontitis is a chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms and characterized by progressive destruction of the tooth-supporting apparatus(Papapanou et al., 2018). Though multiple factors are implicated in initiation and progression of periodontitis, the current understanding of periodontal pathogenesis is that the host-immune response plays a prominent role in enhancing dysbiosis and resultant tissue destruction in periodontal pocket tissue (Van Dyke et al., 2020). In line with this, various cytokines has been shown to play a crucial role both in tissue homeostasis as well as in destruction in severe periodontal disease(Pan et al., 2019). For instance, early studies have estimated the levels of pro/anti-inflammatory cytokines (IL-1β, IL-6, IL-17, IL-10, TGF-β, TNF-α), other inflammatory mediators (matrix metalloproteinases, chemokines, growth factors) and their association with periodontal pathogenesis and bone resorption

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(Becerik et al., 2012; Nicolas Dutzan et al., 2009a; Figueredo et al., 2019a; Gaffen and Hajishengallis, 2008; Konopka et al., 2012; Teles et al., 2010; Toker et al., 2008). The balance or imbalance of such pro and anti-inflammatory cytokines determines progression of periodontitis and the framework of pathogenesis of periodontal disease (Gaffen and Hajishengallis, 2008).

IL-1β and IL-17 have strong pro-inflammatory activity and promote tissue destruction by inducement of matrix metalloproteinases (MMPs) and RANKL and stimulate osteoclastogenesis(Cheng et al., 2020; Gürkan et al., 2006a; Vikram et al., 2015). TGF-β has both pro-inflammatory and immunosuppressive properties and is responsible for regulation of immune response in periodontitis(Gürkan et al., 2006b; Vikram et al., 2015). IL-4 and TGF-β cytokines downregulate IL-1β, IL-6 as well as TNF-α cytokines. They also inhibit MMPs and induce tissue inhibitors of MMPs in tissues(Donnelly et al., 1990; Essner et al., 1989). Further, TGF-β also downregulates transcription of pro-inflammatory factors such as IL-1β (Nicolás Dutzan et al., 2009; Vernal et al., 2005). In addition, IFN-γ has been demonstrated to be associated with progressive and severe forms of periodontitis (Nicolas Dutzan et al., 2009a; Garlet, 2010; Honda et al., 2006).

Cytokines or their ratios inform us about current disease activity in periodontitis affected sites unlike clinical parameters that represent past disease activity. Therefore these cell mediators have diagnostic or predictive potential to assess progression and severity of periodontal disease(Ghallab, 2018). Majority of studies have used saliva and gingival crevicular fluid (GCF) as sample for cytokine analysis(Haririan et al., 2021; Stadler et al., 2016). Most of the hypotheses of periodontal disease initiation and progression as well as biomarkers of disease activity identified in periodontitis is not derived from analysis of gingival/periodontal pocket tissue which is the key anatomo-pathologic lesion in periodontitis directly indicative of inflammatory events, but from analysis of GCF or saliva(Górska et al., 2003). GCF/saliva is assumed to be only a representative fluid reflecting inflammatory events of underlying pathological processes in the periodontal pocket tissue(Wassall and Preshaw, 2016).

Despite benefits such as non-invasive sample collection and site specificity, disadvantages such as inconsistencies in the methodological aspect of sampling, storage and processing of GCF samples exists, which could impact results and interpretation as is reported in literature (Wassall and Preshaw, 2016; Zhou et al., 2010). Very few reports of analysis of cytokine protein levels in gingival tissues are present in literature as tissue collection is invasive (Ejeil et al., 2003; Johnson et al., 2004; McGee et al., 1998; Stashenko et al., 1991). Moreover correlation of protein levels of cytokines in gingival tissue and other biologic sources/matrices such as GCF, saliva or serum is not yet well established in literature. Studies on cytokines in gingival tissue, states that GCF may not express all the markers in the same proportion at the corresponding tissue(Duarte et al., 2019; Lomba et al., 2015).

The aim of the study is therefore to quantify selected cytokine proteins and transcripts (IL-1β, IL-4 (Th2), IL-17 (Th17), IFN- γ (Th1) and TGF-β (Treg) cytokines) in periodontal pocket/gingival tissue in varying severity of periodontal disease in a section of Indian population and to determine the diagnostic/predictive potential of cytokine/cytokine ratio in the cohort. Cytokine protein levels have also been estimated between shallow and deep sites within same individual in the study. The study analysed pro/anti-inflammatory cytokine balance and cytokine ratios in gingival pocket tissues. ROC analysis was done, in order to determine the selected cytokine's diagnostic ability and Youden index calculation (sensitivity + specificity −1) was done to assess the accuracy of periodontal disease predictive potential of cytokine or cytokine ratio (any value > 50% is a reasonable predictor) in gingival tissues.

2. Materials and methods

2.1. Study participants recruitment

The study was approved by institutional human ethics committee (Project No: Faculty project/2/2019/2) and was conducted in accordance to the Helsinki declaration. The study was explained and informed consent was obtained from all participants of the study. Sample size was calculated based on mean difference of IL-1β levels between study groups based on a previous study (α = 0.05 and β = 0.2) With 80% power of study, the sample size was calculated as n = 32 per group (Górska et al., 2003)

A total of 92 study participants were recruited into control and test groups. Participants who reported to the outpatient section of Department of Periodontology of Indira Gandhi Institute of Dental Sciences (IGIDS) or Mahatma Gandhi Post Graduate Institute of Dental Sciences (MGPGIDS), Pondicherry and diagnosed with periodontitis were recruited to the study based on inclusion and exclusion criteria. Only participants who were clinically healthy, non-smokers without any underlying systemic conditions were selected. Subjects who reported antibiotic intake within a period of six months, those with underlying systemic conditions and pregnant or lactating women were excluded from the study. The test groups consisted of generalised periodontitis (GP) subjects (interproximal, CAL of ≥2 mm or ≥3 mm at ≥2 non-adjacent teeth) diagnosed as per analytical criteria of European federation of periodontology (EFP) 2017(Papapanou et al., 2018). The generalised moderate and severe periodontitis groups comprised of participants who met criteria of Stage 2 with Grade B and stage 3 or 4 with grade C periodontitis respectively as per EFP 2017 classification. The controls consisted of periodontally healthy individuals (PHC) (n = 25) who reported for orthodontic extractions or crown lengthening procedures, who presented with clinically healthy gingiva without bleeding on probing (BOP) (Gingival index of <2) and no site in the oral cavity with periodontal disease. The test group participants with generalised moderate periodontitis (GMP) (n = 18, generalised probing pocket depth (PPD) & clinical attachment loss (CAL) 4–5 mm, Gingival index (GI) score of ≥2) and those with generalised severe periodontitis (GSP) (n = 46, PPD >6 mm and CAL > 6 mm, GI score of ≥2) with BOP were included in the study. Further to elicit site specific differences within same individual, from generalised severe periodontitis (GSP) participants n = 18, samples were collected from shallow (PPD & CAL of 4 to ≤6 mm, GI score of ≥2) and deep sites (PPD >6 mm & CAL >6 mm, GI score of ≥2). The participant data is presented in Table 1.

2.2. Clinical procedure

All participants underwent a complete periodontal examination. PPD and clinical attachment loss CAL was measured using UNC-15 probe in six sites per tooth. Bleeding on probing was marked as present or absent on probing of buccal/labial, palatal/lingual, mesial and distal sites. GI

Table 1
Primer sequence for selected cytokine genes.

S.No	Primer Name	Primer sequence
1.	IL-1β F	ATGCTGGTTCCCTGCCCACA
	IL-1β R	CGGAGCGTGCAGTTCAGTGA
2.	IL-4 F	TCACCTCCCAACTGCTTCCC
	IL-4 R	CGGTCAACTCGGTGCACAGA
3.	IFN-ɣ F	TGGCTTTTCAGCTCTGCATC
	IFN-ɣ R	GGAGACAATTTGGCTCTGCA
4.	IL-17 F	TCCCACGAAATCCAGGATGCC
	IL-17 R	TGCGGTGGAGATTCCAAGGTG
5.	TGF-β F	GCGGGACTATCCACCTGCAA
	TGF-β R	GCTGTTGTACAGGGCGAGCA

scores were recorded and participants with GI (score of ≥2) were included for study in test groups. Recordings were performed by a calibrated examiner with reproducible assessments as determined in 10% of weekly registrations (substantial intra-examiner reliability with kappa score of k̤ = 0.84). After a thorough full mouth clinical and radiographic examination, two periodontists arrived at a diagnosis by consensus of generalised moderate/severe periodontitis.

2.3. Sample collection

After full mouth assessment, supra gingival scaling was done and in the subsequent appointment, gingival tissue sample collection was done. Interproximal gingival tissue harvested consisted of gingival epithelium, connective tissue and granulation tissue from periodontitis participants. The tissue samples were obtained using sterile curettes after incisions placed. The gingival tissue in test group participants were collected from deepest sites in molar and premolar sites. Tissues were weighed to find the mean tissue size. The tissue samples collected were divided into two, one half used for protein extraction and analysis and the other half used for mRNA estimation. The sample for ELISA was placed in PBS buffer and that for qRT-PCR was placed in trizol and immediately stored at −80 °C to be processed later. Periodontal therapy was completed in subsequent appointments after sample collection. For healthy controls, gingival samples were collected from participants who reported for crown lengthening or orthodontic extractions.

2.4. Estimation of cytokine levels by ELISA

Protein was extracted from the samples using RIPA lysis buffer (Thermo Scientific™). 1 ml of ice cold RIPA buffer was added to tissue samples sonicated (LABMAN probe sonicator PRO650) on ice at 50% pulse and tissue completely homogenised. This procedure caused cell lysis and liberated cytokines from both inter and intracellular compartments. After incubation on ice for 15 min with periodic pipetting, the sample was centrifuged at 14000×*g* for 15 min, at 4 °C supernatant was transferred to fresh tube for downstream applications. The total protein was quantified using Bradford assay. The protein extract was normalised to have equal concentration of proteins in all samples and subjected to estimate the levels of IL-1β, IL-4, IFN-γ, TGF-β and IL-17 using human specific ELISA kits (Bioassay Technology Laboratory) as per manufacturer's instructions.

2.5. Estimation of mRNA by qRT-PCR

RNA was isolated from gingival tissue of healthy controls and periodontitis (test group) samples using RNeasy kit (Qiagen, USA) according to manufacturer's instructions with mRNA concentration ranging between 45 and 50 μg/mL Universal thermal cycling conditions were used as follows: 2 min at 95 °C, 50 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s each respectively. Melt ramp from 60 °C to 95 °C, hold for 90 s on the 1st step, hold for 5sec on next steps, Melt A (Green). Only samples with adequate mRNA concentration was used for the analysis which amounted to n = 22 samples in test and control groups respectively, for qRT-PCR analysis Quantitative Real Time PCR (qPCR) analysis was carried out using SYBR Green mix (Qiagen, USA cat. nos. 208,052, 208,054, 208,056) on Rotor Gene 5 Plex (Qiagen, USA) for measuring the mRNA expression of five key cytokines: IFN-γ, IL-1, IL-4, IL-17 & TGF-β. The comparative threshold cycle (Ct) method was used to calculate fold change. Beta actin was used as the reference control to normalize the expression values(Ghaderi et al., 2014; Silver et al., 2008). Duplicate reactions were performed for each gene and the relative gene expression level was calculated using 2-ddct method. The primers for the genes expression studied is represented in Table 1.

2.6. Statistical analysis

Shapiro Wilk test was used to analyse normal distribution of data. Participant demographic data is presented as mean and standard deviation. mRNA expression and ELISA values are described as either as Mean (standard deviation)/Median (IQR) based on data obtained. ANOVA was used for comparison between three groups (PHC, GMP & GSP) and when significant, Post-hoc Bonferroni analysis was done to assess significance between groups. Correlation between mRNA and or protein values with clinical parameters (PPD, CAL) was done using Pearson's/Spearman's correlation. ROC curve analysis was performed to study the ratio of cytokines for their predictive potential for periodontal disease severity. Youden index (sensitivity + specificity −1) was used as measure of accuracy of predictability potential of cytokine ratios analysed.

3. Results

3.1. Participant characteristics

(Table 2):Baseline periodontal parameters significantly varied between groups ($p \leq 0.01$). Age in the periodontally healthy group was significantly lesser ($p \leq 0.05$) than test groups as gingival tissue samples in the group could be obtained from young participants undergoing therapeutic extractions for orthodontic management. Gingival index scores were 0.8 ± 0.14, 2.46 ± 0.54 and 2.63 ± 0.73 in healthy controls, moderate and severe periodontitis respectively.

3.2. Estimation and comparison of cytokine protein concentrations in periodontally healthy (PHC), generalised moderate periodontitis (GMP) and generalised severe periodontitis (GSP)

(Table 3):An overall increase in concentrations of IL-1β, IL-17, TGF-β and IFN-γ in periodontitis participants was observed in gingival tissues of test groups (All periodontitis participants (GP = GMP + GSP) when compared with PHC group and the difference was statistically significant ($p \leq 0.05$) except IL-4. IL-4 concentration in tissues did not show any significant difference between periodontally healthy controls and periodontitis participants (Fig. 1). When the cytokine proteins were compared between study groups (PHC, GMP and GSP), IL-1β, IFN-γ and TGF-β showed significant differences in concentrations between the three groups (p = 0.001, 0.02 and 0.001 respectively) by ANOVA showing increase in cytokine levels with severity of disease. Bonferroni corrections for comparison of cytokines between groups reveal that IL-1β, IFN-γ and TGF-β concentrations varied significantly between PHC and GMP or PHC and GSP groups ($p \leq 0.001$). However, the IFN-γ and TGF-β levels, when compared between moderate and severe

Table 2
Participant parameters at baseline examination.

Parameters	Periodontally healthy controls PHC) (n = 25) Male = 10, Female-15	Generalised Periodontitis (GP) (test group) (n = 64)	
		Generalised moderate Periodontitis (GMP) (n- = 18; M = 5,F = 13)	Generalised severe periodontitis (GSP) (n = 46; M = 18,F = 28)
Age(yrs)	20 ± 5.9	36.15 ± 5.05	39.92 ± 5.94
Mean PPD (mm)	2.27 ± 0.28	4.5 ± 0.71**	6.41 ± 1.20**
Mean CAL (mm)	0	4.74 ± 1.23	6.54 ± 1.29**
Bleeding	Absent	Present in all sites	Present in all sites
Gingival index	0.8 ± 0.14	2.46 ± 0.54**	2.63 ± 0.73**

** Significant at p = 0.001 on comparison with HC & between moderate and severe generalised periodontitis subgroups.

Table 3
Comparison of concentrations of cytokine proteins in periodontally healthy, Generalised moderate and generalised severe periodontitis.

Cytokine concentrations	Periodontally Healthy controls (PHC) (n = 25)	Generalised moderate periodontitis (GMP, n = 18)	Generalised severe periodontitis (GSP, n = 46)	F value/chi-Square	Sig
IL-4[#] (ng/mg)	2.28 (1.01–4.14)	2.35(2.08–2.62)	2.35(1.07–3.78)	2.028	0.363
IL-1β (ng/mg)	59.4 ± 5.373	135.07 ± 63.347[a]	170.14 ± 34.078[b,c]	29.712	0.000
IL-17 (ng/mg)	5.8 ± 4.507	10.83 ± 7.165	13.23 ± 13.187	2.486	0.094
TGF- β (ng/mg)	0.281 ± 0.061	0.397 ± 0.066[a]	0.401 ± 0.080[b]	14.073	0.000
INF-γ (ng/mg)	9.83 ± 4.564	20.28 ± 14.96[a]	19.81 ± 11.596[b]	4.011	0.025

[#] IL-4 values given as Median(IQR) due to non-parametric data;
[a] Significant Post-hoc Bonferroni correction between PHC and GMP is indicated by ;
[b] Indicates significance between PHC and GSP;
[c] Indicates significance between GMP and GSP.

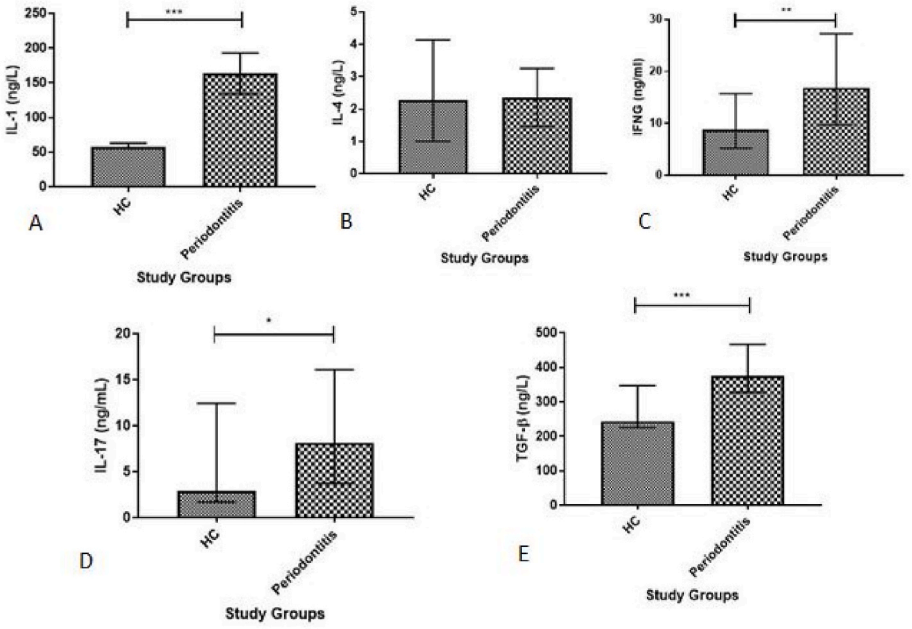

Fig. 1. Comparison of concentrations of cytokines in periodontally healthy controls Vs generalised periodontitis.
Expressed as median &IQR. * indicates p ≤ 0.05; ** indicates p ≤ 0.01; *** indicates p ≤ 0.001 respectively when compared in study groups.

periodontitis did not show statistical significance. IL-1β levels on the other hand significantly varied between GMP and GSP also and approached statistical significance indicating that the cytokine is a promising marker of periodontal disease severity in gingival tissues (p = 0.001). IL-4 levels did not show significant changes between the three groups (p = 0.36). IL-17 concentrations were elevated in GMP and GSP when compared with PHC but the difference did not approach statistical significance.

3.3. Comparison of cytokine concentrations between deep and shallow sites within same participant (GSP participant)

(Table 4, Fig. 2):Higher levels of IL-4 was present in shallow sites compared to deep sites within the same GSP subject (p = 0.01) even though IL-4 levels did not vary much when compared between test groups. Interestingly IL-1β was also elevated in shallow sites (p = 0.01) compared to deep sites. However, TGF-β (p = 0.01), IFN-γ (p = 0.01) and IL-17 levels (p = 0.08) were elevated in deep sites compared to shallow sites within the same GSP participant in periodontal pocket tissues.

3.4. Comparison of transcripts of selected cytokines in study groups

(Table 5) Comparison of transcript levels in gingival tissues was done only between PHC and GP groups and not between subgroups (GMP and GSP). We observed a statistically significant increase in gene expression

Table 4
Comparison of concentration of cytokine proteins in deep vs shallow sites in generalised severe periodontitis (GSP) participants.

	Deep/Shallow sites in same patient	
	Deep (n = 18)	Shallow (n = 18)
IL-4(ng/mg)	3.26(2.201–4.75)	5.21(4.319–8.6)**
IL-1β(ng/mg)	142.9(136.9–168.6)	157.7(142.5–202.3)*
IL-17(ng/mg)	16.12(10.27–23.2)	13.2(9.049–16.85)
TGF- β(ng/mg)	0.431(0.36–0.50)**	0.301(0.27–0.31)
INF-γ(ng/mg)	19.86 (8.696–25.22)*	10.14 (3.623–14.78)

Cytokine concentration values given as Median(IQR) due to non-parametric data.
[*] indicates p ≤ 0.05;
[**] indicates p ≤ 0.01 when shallow sites are compared to deep sites.

of IL-1β (p = 0.01) and IFN-γ (Th1 cytokine) (p = 0.04) in periodontitis samples. Though there was an increase in expression of IL-4 and IL-17 in periodontitis as compared to controls, the increase was statistically not significant. A decrease in expression of mRNA levels of TGF-β in periodontitis samples compared to healthy controls was observed.

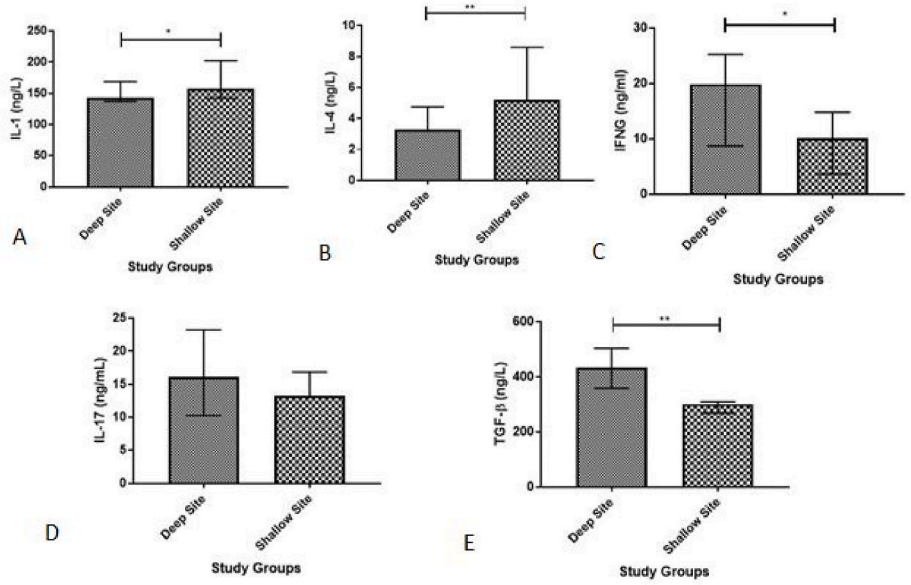

Fig. 2. Comparison of concentrations of cytokines in shallow Vs deep sites within same individual with generalised severe periodontitis. Expressed as median (IQR). * indicates $p \leq 0.05$; ** indicates $p \leq 0.01$ respectively when compared to study group.

Table 5
Comparison of mRNA expression of selected cytokines in test and control groups.

Cytokines	Cytokine expression in healthy controls (n = 22) (Mean ± SD)	Cytokine expression Periodontitis (n = 22) (Mean ± SD)	P value
Th1 IL-1	1.02 ± 0.511	1.89 ± 0.672	0.01*
INF-γ	0.83 ± 1.35	162.67 ± 174.90	0.04*
Th2 IL-4	1.207 ± 0.906	1.522 ± 1.103	0.51
Th17 IL-17	1.151 ± 0.784	2.087 ± 2.591	0.34
Treg TGF-β	1.325 ± 1.165	1.178 ± 0.678	0.74

The cytokine expression is described as Mean ± SD and.
* $p \leq 0.05$ is considered as significant.

3.5. Correlation of protein and transcript levels of cytokines with periodontal parameters

A weak insignificant correlation was observed between transcript levels of all cytokines investigated and periodontal disease parameters (PPD, CAL). With relation to cytokine protein levels, a moderate positive correlation was present between IL-17 and PPD & CAL. A weak negative correlation of TGF-β and IL-1β levels with PPD and CAL was also observed. IL-4 and IFN-γ showed no correlation between the protein levels and periodontal parameters (Table 6).

3.6. ROC analysis of ratio of cytokines to estimate disease predictive potential

(Table 7, Fig. 3, 4**):** ROC analysis was done to obtain the best cut-off value for individual cytokines as well as for the following ratios of cytokines: IFN- γ/IL-4, IL-17/TGF-β, IL-1/TGF-β and IFN-γ/TGF-β in gingival tissues. ROC analysis of all selected cytokines revealed cytokine IL-1β as the best predictor of periodontal disease in the present study with $p = 0.001$; sensitivity 97.3% and specificity of 93%, cut-off value of 68.33 and Youden index (sensitivity + specificity-1) of 90%. Among the ratios of pro and anti-inflammatory cytokine assessed, IL-1β/TGF-β cytokine ratio had a Youden index of 60% with a cut-off value of 0.25, 81% sensitivity & 79% sensitivity and best predicts periodontal disease in gingival tissue samples in this study (see Fig. 4).

4. Discussion

While majority of studies analysing cytokine protein concentrations have been done in GCF or saliva, the current study estimated cytokine protein transcript levels of key cytokines in gingival tissue (periodontal pocket tissue) and their predictive capacity using ROC analysis. Among the cytokines investigated in literature, IL-1β, IL-4 (Th2), IL-17 (Th17), IFN- γ (Th1) and TGF-β (Treg) cytokines are the key molecules of various Th subsets as well as for the pro/anti-inflammatory response in periodontitis (Figueredo et al., 2019b) Since these cytokines are secreted in gingival connective tissues by T cells, B cells, fibroblasts, macrophages, mast cells, osteoblasts and keratinocytes(Garlet, 2010), they have been selected in this present study to understand their dynamics in gingival tissue.

The current study reports cytokine protein and transcript profile in gingival tissues of a section of Indian population. Even though individual variations accounts for differences in gene expression, it is reported that population differences account for about10–15% of variations in gene expressions(Storey et al., 2007; Townsend et al., 2003). Despite the high prevalence of periodontitis in Indian population, there is paucity of information analysing cytokine protein and transcript profile in gingival

Table 6
Correlation of selected cytokines with periodontal parameters in periodontitis participants.

Correlation (n = 64)	INF-γ		IL-4		IL-17		TGF-β		IL-1	
	r	p	r	p	r	p	r	p	r	p
PPD	0.16	0.42	0.05	0.80	**0.67**	**0.000***	−0.17	0.41	−0.2	0.32
CAL	0.21	0.29	0.05	0.80	**0.52**	**0.01***	−0.18	0.39	−0.12	0.55

* Pearson's correlation is significant at $p \leq 0.05$ level.

Table 7
Sensitivity/specificity and cut off values of individual cytokines and cytokine ratios in varying severity of periodontal disease.

Cytokines/cytokine ratios in PHC/GMP/GSP	Cut-off value	Sensitivity	Specificity	Youd-ens index	AUC± SEM	Confidence Interval	p-value
INF-γ	9.93	70	57	0.27	0.749 ± 0.07	0.604–0.875	0.009*
IL-17	5.00	68	71	0.39	0.67 ± 0.01	0.547–0.845	0.32
IL-1β	68.33	97.3	93	0.90	0.994 ± 0.01	0.981–1.007	0.000*
TGF-β	0.34	78	86	0.64	0.898 ± 0.51	0.798–0.997	0.000*
IL-4	1.99	73	43	0.16	0.555 ± 0.09	0.374–0.736	0.547
INF-γ/IL-4	4.15	59	57	0.17	0.569 ± 0.94	0.385–0.754	0.45
IL-17/TGF-β	0.15	59	57	0.17	0.598 ± 0.08	0.431–0.746	0.33
IL-1β/TGF-β	0.25	81	79	0.60	0.855 ± 0.53	0.750–0.960	0.000*
INF-γ/TGF-β	0.032	59	57	0.17	0.625 ± 0.08	0.470–0.781	0.17

ROC curve analysis.
* Denotes significance $p \leq 0.05$; IL-1 cytokine & IL-1/TGF-β ratio best predicts periodontal disease.

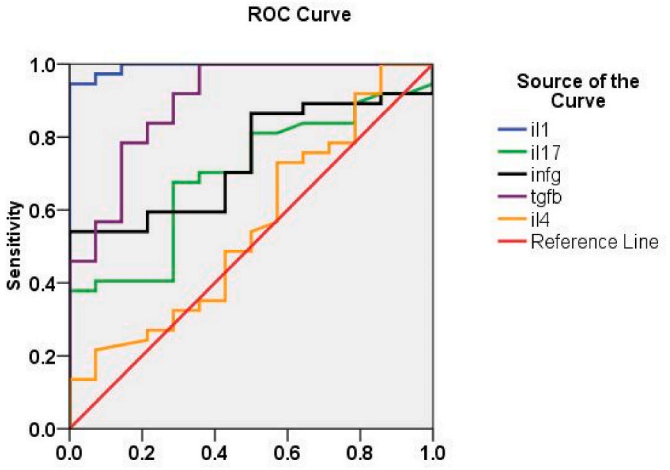

Fig. 3. Sensitivity/specificity and cut off values of individual cytokines in varying severity of periodontal disease.
Receiver Operating Curve analysis : IL-1 is significant with p = 0.001 with sensitivity 97.3% and specificity of 93% and cut-off value of 68.33 and Youden index of 90%.

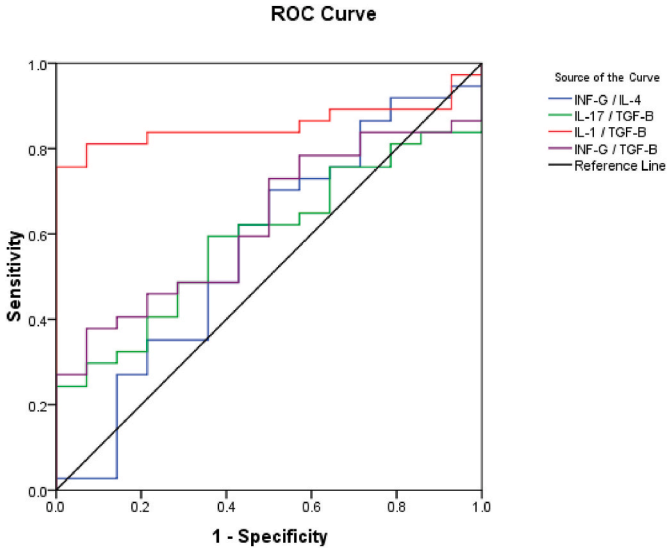

Fig. 4. Sensitivity/specificity and cut off values of cytokine ratios in varying severity of periodontal disease.
Receiver Operating Curve analysis : IL-1/TGF-β ratio is significant with p = 0.001 with sensitivity 81% and specificity of 79% and cut-off value of 0.25 and Youden index of 60%.

tissues (Shaju et al., 2011). Therefore the study was undertaken to report cytokine profile in this population.

The results indicated a simultaneous predominance of inflammatory cytokines (IL-1β, TGF-β, IL-17 and IFN-γ) in gingival tissues of periodontitis participants (GP) (p = 0.01) except IL-4 compared to non-periodontitis healthy controls (PHC) (Fig. 1). A similar observation was noted in previous studies, which demonstrated a higher concentrations of IL-1β, IL-4, IFN-γ and IL-17 in periodontitis compared to periodontal health in various sample matrices (Behfarnia et al., 2010; Górska et al., 2003; Steinsvoll et al., 1999; Takahashi et al., 2005). In GCF sample analysis multiple reports undisputedly are in concurrence with the results of this study where an overall elevation of IL-1β, TGF-β, IL-17 and IFN-γ in periodontitis is present indicating that these cytokines, whether analysed from GCF or from pocket tissue samples, discriminate well between periodontally healthy individuals with complete absence of periodontal disease and affected sites in generalised periodontitis patient(Stadler et al., 2016).

However, in the current study, when cytokines were analysed in increasing severity of periodontitis (between GMP and GSP subjects), only IL-1β significantly increased with severity of periodontal disease in gingiva. The other cytokines TGF-β, IL-17 and IFN-γ did not discriminate well between GMP and GSP groups which indicates that IL-1β is involved in all stages of initiation and progression of periodontitis. IL-1β is currently reported to be a biomarker with diagnostic potential for periodontal disease in saliva as well an accurate predictor of periodontal disease in GCF (Nazar Majeed et al., 2016; Sexton et al., 2011; Teles et al., 2010). Further, ROC analysis revealed that IL-1β levels could be the best indicator of periodontal disease (Table 7). On the other hand, IL-1β levels were decreased in deep sites when compared to shallow sites, which may be due to the presence of IL-4 cytokine that has been reported to antagonise IL-1 and IFN-γ production(Donnelly et al., 1990). Another reason possibly is that shallow sites in periodontitis individuals is said to potentially harbour more pathogenic microbiota compared to healthy sites in healthy individuals which could result in severe inflammation leading to increase in IL-1β levels prior to actual destruction of tissue (Miranda et al., 2020).

Gorska et al. reported a lack of vigorous production of IL-4 in gingival tissues of periodontitis patients and that the cytokine protein was equally observed in healthy biopsies (Górska et al., 2003). Also, reported either no change in levels of IL-4 between controls and periodontitis participants or a decrease in IL-4 levels in periodontitis compared to controls in GCF samples(Pradeep et al., 2008; Zhao et al., 2011). A similar result were obtained in our study, which indicates that IL-4 levels in the present study is not consistent with periodontal destruction(Papathanasiou et al., 2014; Pradeep et al., 2008). In fact, improvement of IL-4 levels in reports after non-surgical periodontal therapy further indicates that IL-4 cytokine is a better indicator of remission of periodontal disease (Giannopoulou et al., 2003; Pradeep et al., 2008). Further, a significant increase (p = 0.01) of IL-4 in shallow sites compared to deep sites within the same GSP participant was observed. This may be because of higher reparative activity at shallow

site than in deep sites where a more severe inflammatory destruction of tissues is underway.

Moreover, the levels of IFN-γ, TGF-β and IL-17 are significantly elevated in deep sites of GSP participants compared to shallow sites. In this regard, previous studies have reported that IFN-γ levels are associated with progressive lesions which explains increased levels of INF-γ in present study in deep sites (Nicolas Dutzan et al., 2009b; Gemmell and Seymour, 2004; Honda et al., 2006). Simultaneous elevation of IL-17 in deep sites is indicative of bone resorption activity of IL-17 as reported in few studies (Dutzan and Abusleme, 2019; Kotake et al., 2012; Takahashi et al., 2005; Zenobia and Hajishengallis, 2015). However, increased levels of IL-17 in gingival tissues could also mean higher anti-microbial activity of IL-17 against periodontal pathogens as reported in literature (Khader et al., 2009; Liang et al., 2006). Moreover high TGF-β concentrations in deep sites explains the nature of activity in deep sites in the present study. TGF-β is reported to have a dual role *ie.* both pro and anti-inflammatory properties. Skaleric reported that TGF-β induces mononuclear cells to secrete pro-inflammatory cytokines like IL-6 and IL-1β and that it is also a chemoattractant for neutrophils, monocytes and lymphocytes, the degranulation of which creates a highly inflammatory milieu(Skalerič et al., 1997). Further, it has been suggested that a fraction of TGF-β cells to lose its immunosuppressive actions in an inflammatory enriched environment (Nicolás Dutzan et al., 2009; Ernst et al., 2007).

Tomas et al. demonstrated that IL-1α, IL-1β and IL-17A levels in GCF could be potential biomarkers for distinguishing patients with chronic periodontitis from periodontally healthy individuals using mathematical models (Tomás et al., 2017). The current study, utilised ratios (Pro/anti-inflammatory) of selected cytokines in gingival tissues considering the different role of the cytokines involved in the inflammatory process based on literature evidence. Our results revealed that IL-1β cytokine and IL-1β/TGF-β ratio as best indicators of periodontal disease progression in gingival tissues with a Youden index of 90% and 60% respectively (Figs. 2 and 3 & Table 7).

Transcript levels of cytokines reveal a significant increase in the expression of IFN-γ ($p = 0.04$) and IL-1β ($p = 0.01$) expression in severe periodontitis lesions, which has been consistent with previous reports (Berglundh et al., 2002; Rajesh et al., 2015). It has been suggested that chronic elevation of IFN-γ expression is due to constant antigen stimuli and activation of inflammatory pathways leading to MMP release and bone destruction which correlates with IFN-γ levels in progressive lesions (Nicolas Dutzan et al., 2009b; Franco-Topete et al., 2018). A marginal increase of IL-4 and IL-17 transcripts is observed in the present study. IL-1 cytokine is reported to influence development of Th2 cell differentiation and enhance production of both INF-γ and IL-4 and is also is reported to have a synergistic role along with TNF-α in Th17 differentiation.(Santarlasci et al., 2013) which explains the increase of IL-17 and IL-4 transcripts though marginal without statistical significance.

In the current study, IL-17 levels correlated well ($r = 0.67$ & 0.52) with clinical periodontal parameters of PPD and CAL. Absence of robust production of IL-4 in test groups explains the lack of correlation of the cytokine with clinical parameters. Despite the increase in concentration of IL-1β, TGF-β and IFN-γ, we failed to observe correlation of these cytokines with clinical parameters which is in contrast to few reports (Górska et al., 2003; McGee et al., 1998). Also a high variability in cytokine concentrations between individuals may explain discrepancy between our results from previous publications. However one report points out lack of correlation of IL-1β with periodontal parameters which supports results in our study (Hou et al., 2003).

We attribute the uneven number of samples in GMP and GSP to lack of correlation of few cytokines which needs to be further clarified with further research in the population. We also admit the statistically significant difference ($p = 0.01$) in age between controls and test groups as an inherent limitation of the study due to ethical reasons in obtaining samples from healthy sites. A post hoc power calculation was done due to uneven sample size in study groups and it ranged between 70 and 95%. Assessment of correlation between tissue cytokine levels with GCF levels of cytokines would have given a definitive picture of both tissue levels and GCF levels which was not done in the present study.

5. Conclusion

Within limitations, we report a skewing towards IFN-γ (Th1 subset) at transcript level. In gingival tissue cytokine protein analysis in this population, IL-1β cytokine and IL-1β/TGF-β ratio have emerged as best indicators of periodontal disease severity with high Youden index. Simultaneous presence of high concentrations of IL-17, TGF-β and IFN-γ in deep sites signifies that there is no single cytokine predominance either between individuals or within the same individual, rather, the cytokines synergistically act in an inflammatory milieu and regulate outcomes in terms of tissue destruction or repair.

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Disclosure statement

The authors report no competing interests to declare.

Ethics approval statement

Approval Number: Project No: Faculty project/2/2019/2)

Consent to participate

Written informed consent was obtained from all individual participants included in the study.

Consent to publish

Patients signed informed consent regarding publishing their data.

CRediT authorship contribution statement

Pratebha Balu: Conceptualization, Visualization, Methodology, Investigation, Formal analysis, Writing – review & editing. **Agiesh Kumar Balakrishna Pillai:** Methodology, Supervision, Data curation, Formal analysis, Writing – review & editing. **Vignesh Mariappan:** Methodology, Statistics, Writing – review & editing. **Sudhakar Ramalingam:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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