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Evaluation of Microbial Profile in Patients with Polycystic Ovary Syndrome and Periodontal Disease: A Case-Control Study

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Abstract

Background: Polycystic ovary syndrome (PCOS) and oral health are found to share a reciprocal link. Previous substantiating evidences suggest that PCOS may have a confounding effect on periodontal health and may quantitatively modify the composition of the oral microbiome. To analyze the role of PCOS as a risk factor in causing periodontal disease, we compared and evaluated the levels of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in patients with polycystic ovary syndrome, polycystic ovary syndrome, chronic periodontitis, polycystic ovary syndrome, and gingivitis, and healthy controls.

Materials and Methods: In this case-control study, 40 female participants are enrolled and grouped into four groups which included healthy female individuals, patients with PCOS, patients with PCOS and gingivitis, and patients with PCOS and periodontitis. Periodontal examination is assessed primarily on all the participants using a UNC-15 probe. Dental plaque is then collected using a sterile curette in one stroke and transferred into an Eppendorf tube containing TE Buffer (Tris-EDTA buffer) solution. The level of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* was estimated by real-time polymerase chain reaction (PCR).

Results: The levels of *Fusobacterium nucleatum* were observed to be significantly higher in group with patients with PCOS and periodontitis.

Conclusion: PCOS may have an impact on the composition of oral microflora causing repercussions in periodontal health.

Keywords: Dental Plaque, Periodontitis, Polycystic Ovary Syndrome

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Introduction

Women of reproductive age may be affected by the complex, heterogeneous endocrine disorder known as polycystic ovary syndrome (PCOS) (1). The primary features of this condition encompass chronic anovulation, menstrual problems, and clinical or biochemical signs of hyperandrogenism (2). The Rotterdam criterion defines PCOS patients as those female patients with the presence of at least two of the following: polycystic ovaries, oligomenorrhea, and/or anovulation, hyperandrogenism (clinical and/or biochemical). According to the PCOS Consensus Workshop Group, the prevalence rate of PCOS has dispersed globally, ranging from 9.13 to 36% in India (3).

Periodontitis on the other hand is considered as a condition distinguished by inflammation of the supporting tissues of the teeth. The strong interplay between putative periodontal pathogens and host inflammatory response is attributed to the subsequent destruction of supporting tissue and tooth loss. The endocrine system has a significant impact on the homeostasis of the periodontium which is maintained through complex multifactorial relationships. The estrogen receptor (ER) and a progesterone receptor (PgR), present in the periodontium indicate that the periodontium is one of the target tissues for these hormones (4). The direct effects of estrogens were mediated through two distinct subtypes of receptors, ERα and Erβ (5). The ERα receptor is predominantly present in the different

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tissues such as the endometrium, ovaries, and mammary glands, whereas the ERβ receptor is present in the non-reproductive tissues, including periodontal ligament (6), gingival epithelium (7), and salivary glands (8). This explains the reason that changes in the amounts of circulating steroid hormones can affect periodontal tissues. Changes that take place in the level of sex hormones during different stages of females are observed to directly or indirectly affect oral health, subsequently influencing a person's susceptibility to periodontal disease. PCOS is defined by alteration in the level of sex hormone, where the progesterone levels remain low and the levels of estrogen and male androgenic hormones go high. The association of PCOS with an increase in the levels of inflammatory markers like C reactive protein, pro-inflammatory cytokines like interleukin-6, tumor necrosis factor-α (TNF-α), and chemokines, demonstrate this low-grade systemic inflammation (9).

On the other hand, periodontal diseases, like gingivitis and periodontitis, are common prevalent chronic infectious diseases. Such diseases are predominantly brought in by a bacterial plaque that grows in the subgingival area and are also found to increase systemic and local inflammatory markers such as TNF-α, C reactive protein, and interluekin-6 (IL-6) which is attributed to the destruction of periodontal tissue (10). Insulin resistance which is a prominent characteristic of PCOS is linked to low-grade inflammation through inflammatory cytokines like TNF-α, IL-1, and IL-6 and their signaling pathways (11). On the contrary, hormonal level alterations in PCOS are found to affect the salivary levels of potential periodontal pathogens and/or their systemic immune responses in the presence of gingival inflammation. This might be accounted to the deposition of active progesterone and oestrogen hormones, in the periodontal tissues' which supply the vital nutrients needed for the growth of the bacteria. Since insulin resistance is one of the main symptoms of the PCOS, periodontal organisms in subgingival plaque produce lipopolysaccharides that can cause considerable production of IL-1 and TNF-α. The insulin resistance can get exacerbated upon persistent elevation of cytokines which can ultimately in turn increase the condition of PCOS (12). Meanwhile, few studies showed an elevated oxidative stress level and alterations in the oral microbial flora of PCOS patients (12, 13). Evaluating the link between periodontal disease and PCOS, poor periodontal parameters were seen in patients with PCOS. While studies reported higher severity of periodontal disease in PCOS patients, hence it is mandatory to undergo periodontal examination and therapy periodically (14).

Hormonal disorders such as PCOS and oral health was shown to share a reciprocal link with each other. PCOS was found to bring about a repercussion in periodontal health by influencing the composition of the oral microflora. But there exists a paucity of literature evaluating change brought in by hormonal disorders, such as PCOS on the diversity of oral microbiota. The correlation between oral microbiota in dental plaque with the gingival inflammation in presence of PCOS is not been established to date. Therefore, this study aims to analyse the role of PCOS as a risk factor in causing periodontal disease.

Materials and Methods

Ethical guidelines

The present case-control study protocol was conducted in full accordance and approval of the Institutional Ethics Committee, SRIHER with the protocol number CSP/20/JAN/83/51. The nature of the study was discussed with patients orally and written informed consent was acquired from each patient prior to the initiation of the study.

Study population and study design

This case-control study was performed between December 2019 to March 2020 and included patients attending the Department of Gynaecology and Obstetrics and Department of Periodontology, Sri Ramachandra Institute of higher education and research. Forty female patients between the age of 18 to 40 years were recruited for the study. During screening, patients who fulfilled the inclusion criteria were provided with an informed consent form and were recruited into the study. The inclusion criteria for the PCOS group was based on the criteria by Rotterdam (2). The control group was healthy female individuals without PCOS and periodontitis. Patients in the periodontitis group were classified according to the definition of Eke et al. (15). Patients in the gingivitis group were classified according to the American Academy of Periodontology 1998. Patients with other medical conditions and those who had been under medications that could affect their periodontal status for the last 6 months were excluded from the study.

Using a one-way ANOVA test with a power of 0.95, alpha of 0.05 with an effect size of 19.25, a sample of 40 patients including the dropouts (10 members in each group) was calculated. Four groups were included in the study which include healthy female individuals, patients with PCOS, patients with PCOS and gingivitis and patients with PCOS and periodontitis.

Each patient's clinical periodontal evaluation, such as probing depth (PD), plaque index (PI), and bleeding on probing, was recorded after enrolment. Apart from third molars, with the help of Williams periodontal probe (Hu-Friedy, Chicago, IL) six sites of each tooth (mesiobuccal, mid-buccal, distobuccal, mesio-lingual, mid-lingual, and disto-lingual locations) were measured clinically. If bleeding on probing (BOP) happened within 15 seconds of applying the probe, it was considered positive. A single calibrated examiner conducted after the clinical periodontal measures. Subsequently, the periodontal inflammatory surface area for each patient was calculated.

Subgingival dental plaque sample collection

The tooth with the deepest PD was dried and isolated using cotton rolls. The subgingival plaque was collected by

inserting a sterile Gracey 5/6 curette 2 mm (Hue Friedy, Switzerland) into the gingival pocket followed by a pull stroke three subsequent times without evoking bleeding. The strokes had to sufficient force to remove plaque while no attempt was made to remove cementum from the root surface.

The Gracey 5/6 curette (Hue Friedy, Switzerland) was chosen to standardize as it permits the most effective method of placing the curette inside the transport tube. Immediately upon collection of the samples, the curettes were shaken into a transport tube with 200 µl of transport solution (Tris-Ethylenediaminetetraacetic acid buffer solution) (Himedia, Mumbai, India). The samples were then sealed and sent immediately to the testing facility for quantification of the periodontal pathogen *P. gingivalis* and *F. nucleatum*.

Sample processing

Following the transfer of the sample to the tube consisting of Tris-Ethylenediaminetetraacetic acid buffer (T.E.buffer) containing tris base powder and Ethylenediaminetetraacetic acid (Himedia, Mumbai, India). It was centrifuged (Model SLM-MCF-10K, Biobee tech, Bengaluru, India) at 5,000 rpm for 5 minutes at room temperature. After discarding the supernatant, 500 µl of fresh T.E. buffer was added again and 3 minutes of centrifugation at 5000 rpm was done. The same procedure was subsequently repeated 3 times with new T.E. buffer.

DNA extraction procedure

The sample processing was followed by DNA extraction by using modified proteinase-k method (16). After discarding the supernatant, 50 µl of lysis buffer I containing 1% Triton X-100, Tris-HCL pH=8.0, 10 mM, and EDTA 1 mM (Himedia, Mumbai, India) was added, vortexed, and allowed to stand for 5 minutes. Then, 10 µl of proteinase-K (100 µg/ml, Genei laboratories pvt. ltd, Bengaluru, India) and 50 microliters of Lysis buffer II contain Tris-HCl, pH=8.0, 50 mM, KCl, 50 mM, $MgCl_2$, 2.5 mM, Tween-20, 0.45%, and Nonidet P-40, 0.45% (Himedia, Mumbai, India) were added, and the mixture was vigorously vortexed. The samples are then held in a 60°C for two hours and then transferred to a boiling water bath for 10 minutes. Following this, the samples were centrifuged at 10,000 rpm for 3 minutes, and the DNA-containing supernatant is then collected in a new tube. The DNA was purified by using 3 M sodium acetate and absolute ethanol and reconstituted in 100 µl water. For PCR analysis, the DNA was kept at -200°C.

Bacterial quantification by quantitative real-time polymerase chain reaction

The qPCR reactions were used to determine the presence and levels of two different oral taxa in the subgingival plaque samples. Using an Eppendorf Real plex master cycler, real-time qPCR amplification and detection reactions were carried out in a 96-well format. Also, an 8 pm/µl of each P. gingivalis and F. nucleatum-specific primers were used in a total volume of 25 µl for the PCR reactions, along with 2 µl of template DNA and 12.5 µl of TB Green Premix Ex Taq (Tli RNaseH Plus) (Takara Bio inc., Kusatsu, Japan). All species-specific primers used in the qPCR reaction are given in Table 1 (17, 18). The reaction mixture was prepared in 0.2 ml qPCR tubes and kept in a thermal cycler (Realplex Master cycler, Eppendorf, Hamburg, Germany). qPCR reaction conditions were 95°C for 3 minutes, and 35 cycles of 95°C for 20 seconds and 60°C for 30 seconds, and 72°C for 30 seconds. Plotting the standard graph required running serial dilutions of DNA isolated (same procedure as described above) from the standard strains of P. gingivalis ATCC No. 33277 (Known quantity, (10^8 to 10^3 CFU/ml) and *F. nucleatum* ATCC No. 25886 (Known quantity, 10^8 to 10^3 CFU/ml). Deionised water served as a negative control. The amplification curves were obtained with fluorescent probes. Cycle threshold values (CT value) were obtained for DNA samples of known quantity (standards) and standard curve was plotted. Ct values of unknown samples were obtained from real time PCR runs. Then these CT values were plotted on the standard curve to get the corresponding quantity for unknown samples.

Statistical analysis

Statistical analysis was done using SPSS version 3.1.9.2 statistical software (International Business Machines Cooperation, Chicago). The data distribution was assessed by the Shapiro-Wilk normality test. After dividing the entire data below and above the median, Kruskal-Walli's test was used to compare all groups for non-normally distributed data (bacterial counts). For normally distributed variables (Periodontal Epithelial Surface Area & Periodontal Inflammatory Surface Area), the ordinary one-way ANOVA test was used. Statistical significance was set at $P \leq 0.05$ with a 95% level of confidence in the statistical analyses, which were carried out using the statistical program (SPSS version 3.1.9.2; International Business Machines Cooperation, Chicago).

Table 1: Species-specific primers

Organisms	Primer sequence (5´-3´)	Author (References)
Porphyromonas. gingivalis	F: AGGCAGCTTGCCATACTG CG R: ACTGTTAGCAACTACCGATGT	Kugaji et al. (17)
Fusobacterium nucleatum	F: GAAGAAACAAATGACGGT AACAAC R: GTCATCCCCACCTTCCTCCT	Yamaura et al .2005 (18)

Results

Demographic data

Full-mouth clinical assessments and demographic data were documented (Table 2). For all four groups, bleeding in probing (BOP), periodontal pocket depth (PPD), and clinical attachment loss (CAL) were determined. It was observed that the BOP, PPD, and CAL levels were significantly higher in the PCOS with periodontitis group.

Primary analysis results

Bacterial count analysis in plaque samples

The microbiological diversity of the dental plaque was assessed using qPCR. *Porphyromonas gingivalis* and *Fusobacterium nucleatum* bacteria were quantified. Although there was a difference in the level of *Porphyromonas gingivalis* among the four groups, the results were not statistically significant. Quantification of the level of *Fusobacterium nucleatum* showed an ascending growth pattern of the healthy group to group with PCOS and periodontitis (Table 2). On intergroup comparison in the levels of Fusobacterium nucleatum, a statistically significant difference was obtained between the healthy female individuals and patients with PCOS and periodontitis (P=0.04, Fig.1).

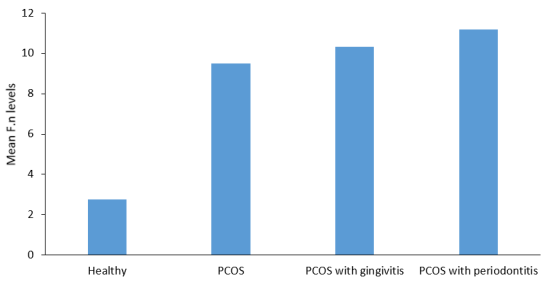

Fig.1: Subgingival *Fusobacterium nucleatum* levels. PCOS; Polycystic ovary syndrome.

Secondary results analysis

The periodontal epithelial surface area (PESA) accurately measures the surface area of the pocket epithelium, whereas periodontal inflammatory surface area (PISA) measures the amount of the inflamed periodontal tissue and as such assess the systemic inflammatory burden. A Microsoft Excel spreadsheet was developed to help with the estimation of Periodontal Inflammatory Surface Area and Periodontal Epithelial Surface Area using the formulas published by Hujoel et al. (20). PESA and PISA values were found to be statistically significant among the four groups (P=0.00). Among the intergroup comparison of periodontal inflammatory surface area scores, a significant difference was noted between healthy female individuals and patients with PCOS patients with gingivitis and periodontitis (P=0.00, Fig.2, Table 3).

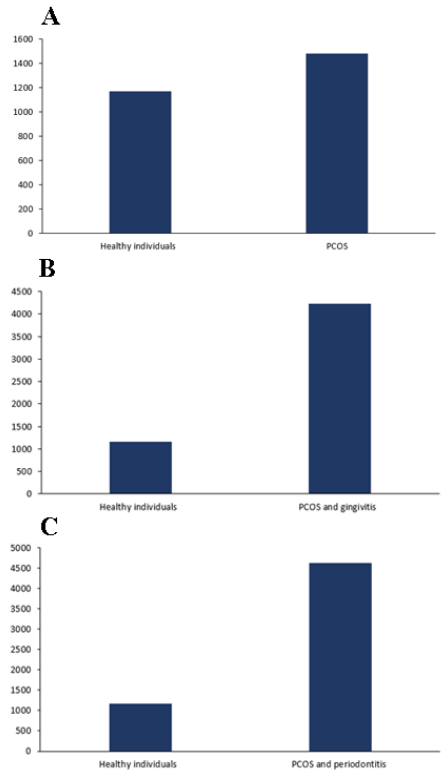

Fig.2: Intergroup PISA score comparison. **A.** PISA scores between healthy individuals and PCOS patients, **B.** PISA scores between healthy individuals and PCOS and gingivitis patients, and PISA scores between healthy individuals and PCOS and periodontitis patients. PISA; Periodontal inflammatory surface area and PCOS; Polycystic ovary syndrome.

Table 2: Demographic data and periodontal parameters

Demographic data	Healthy	PCOS	PCOS with gingivitis	PCOS with periodontitis
No of participants	10	10	10	10
Age (Y)	22.3 ± 3.3	22.9 ± 2.1	22.4 ± 3.1	27.6 ±3.4
Mean BOP	0.953 ± 0.33	1.055 ± 0.46	2.338 ± 1.65	2.647 ± 1.88
PPD (site specific)	0.59 ± 0.37	0.341 ± 0.53	3.861 ± 0.90	5.812 ± 0.91
CAL (site-specific)	1.211 ± 0.57	1.082 ± 0.57	3.431 ± 1.65	3.92 ± 1.75
MEAN PISA	116.590 ± 56.3	147.740 ± 60.49	423.320 ± 79.49	462.940 ± 84.5
MEAN PESA	818.010 ± 59.6	827.010 ± 80.1	941.180 ± 180.3	1090.840 ± 205.96

All data are based on mean ± SD. PCOS; Polycystic ovary syndrome, BOP; Bleeding on probing, PPD; Periodontal probing depth, CAL; Clinical attachment loss, PISA; Periodontal inflammatory surface area, and PESA; Periodontal Epithelial surface area calculation.

Table 3: Intergroup PISA score comparison

PESA and PISA scores	Sum of squares	df	Mean square	Sig.
PESA score (mm^2)				0.000
Between groups	485162.621	3	61720.874	
Within groups	132595.910	36	3683.220	
Total	617758.531	39		
PISA score (mm^2)				0.000
Between groups	979692.647	3	326564.216	
Within groups	17882.933	36	496.748	
Total	997575.580	39		

PISA; Periodontal inflammatory surface area and PESA; Periodontal epithelial surface area.

Discussion

The PCOS is an endocrine condition caused by a disruption in the hypothalamus pituitary axis, which results in abnormal gonadotropin secretion by the hypothalamus. This abnormal secretion of the gonadotropin hormone leads to an increased luteinizing hormone (LH) levels and normal or low follicle-stimulating hormone (FSH) levels (20). Bacterial plaque that causes periodontal diseases results in local and systemic elevations in levels of TNF-α, C reactive protein, and IL-6. These factors subsequently result in the destruction of periodontal tissue. Both, PCOS and periodontal disease, share chronic low-grade inflammation as a common risk factor. The existing studies reported that in comparison to healthy controls, patients with PCOS suffered from greater rates of BOP, periodontal probing depth (PPD), and CAL (21, 22).

Studies have also shown that periodontal disease may cause systemic inflammation and oxidative stress, both of which can aggravate insulin resistance. This suggests that there may be a bidirectional association between PCOS and PD (23). On the other hand, female sex hormones are found to affect the composition of oral microbiota in conditions like puberty, mensuration, and pregnancy. Also, studies confirm the increase in the levels of *Prevotella intermedia* in the subgingival plaque due to an increased plasma female sex hormones and alteration of the immune response (24, 25). There are limited data on PCOS effects on putative periodontal pathogens, which formed the principle objective of the current study.

Our findings suggest that PCOS has an amplifying effect on the levels of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* and their connection with gingival inflammation. Thus, the change in the microbiota in patients with PCOS could play a pivotal role in the propagation of gingival inflammation. Akcali et al. (12) observed an increase in the salivary levels of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* in PCOS patients when compared to healthy controls. Active progesterone and oestrogen was found to supply crucial nutrients needed for bacterial growth. In light of the accumulation of these hormones in periodontal tissues, the author concluded that hormonal alterations in PCOS may influence the salivary levels of putative periodontal pathogens and/or their systemic antibody responses. This is especially true in the presence of gingival inflammation (26).

Our results were in agreement with the study conducted by Porwal et al. (27) that observed an increase in the BOP in the newly diagnosed PCOS group when compared to the healthy group. These results indicate the impact of PCOS on the gingival inflammation.

Young female individuals between 18 to 35 years of age are generally not prone to periodontitis (28), but the results of our present study showed a comparatively high level of putative periodontal pathogens in PCOS patients which subsequently may lead to an increase in the inflammatory burden. This points us the fact that the presence of PCOS in young female individuals can predispose them to periodontal diseases. Hence, PCOS can be considered a viable risk factor in the initiation and progression of periodontal diseases in the young healthy female individuals. To our knowledge, this is the first study to assess the levels of suspected periodontal pathogens in dental plaque samples from non-obese women with PCOS. The limitations of the present study include a small sample size and site-specific based assessment of oral microbiota. Insulin resistance of patients wasn't considered in the present study and thereby could be a confounding factor. Future longitudinal studies with larger sample size, are needed to evaluate the influence of PCOS on the subgingival microbial environment and also, to investigate probable relationships between PCOS and periodontal disease.

Conclusion

The persistent polymicrobial challenge to the local host tissues often results in the progressive destruction of soft and hard tissues which is considered as the hallmark of periodontitis. Pathogenic consortium which causes these responses include the red complex bacteria such as *P. gingivalis*, *T. denticola* and *T. forsythia*. Additionally, higher concentration of possible periodontopathogens such as *Fusobacterium nucleatum*, *Prevotella species*, *Eikenella corrodens*, *Peptostreptococcus micros*, and *Campylobacter rectus* is found in deeper periodontal pockets. Oral health is often subjected to direct and indirect effects of the fluctuating levels of sex hormones during conditions like PCOS which can influence the

constituents of the oral microflora causing reverberation in periodontal health. Amongst the studied taxa in the present study, higher levels of *Fusobacterium. nucelatum* and *Porphyromonas. gingivalis* were observed in the subgingival plaque samples in patients with PCOS and periodontitis and patients with PCOS and gingivitis when compared to healthy individuals. Therefore, within the limitations of the study, it can be concluded that PCOS may quantitatively affect the constituents of oral microflora playing a pivotal role in subsequent gingival inflammation and periodontal health.

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Authors' Contributions

R.A.J.; Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Data curation, Writing Original draft, Review, and Editing. S.A., M.S.; Conceptualization, Methodology, Validation, Writing, Review, Editing, Validation, Supervision, and Project administration. S.K.B.; Writing, Review, Editing, Validation, Supervision, and Project administration. All authors read and approved the final manuscript.

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