POTENTIAL ROLE OF OSTEOCALCIN AND OSTEOPROTEGRIN IN DETERMINING THE CONNECTION BETWEEN RHEUMATOID ARTHRITIS AND PERIODONTITIS: PERSPECTIVE CASE STUDY FROM LOCAL POPULATION OF PUNJAB, PAKISTAN

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ABSTRACT

Objective: *The aim of this study was to determine the association between Rheumatoid Arthritis and Periodontitis with the help of biomarkers Osteocalcin and Osteoprotegerin.*

Materials and Methods: *One hundred and twenty subjects were enrolled using consecutive non-probability sampling for four groups, i.e., controls, clinically diagnosed cases of Rheumatoid Arthritis and Periodontitis, and both after informed consent (attached in the end). Serum Osteocalcin and Osteoprotegerin were measured along with Anti-CCP antibodies and clinical measures of severity of Periodontitis with the help of bleeding on probing (BOP), clinical attachment loss (CAL) and periodontal pocket depth (PPD). ANOVA and post hoc analysis were used to determine the difference of mean in different groups using SPSS 21.*

Results: *Mean Osteocalcin and Osteoprotegerin were equally distributed among all four groups, although showing trends of increase and decrease in mean plots, respectively (using ANOVA, p value= 0.711, 0.15, respectively). Anti-CCP antibodies, mean CAL, mean BOP, mean PPD, and age were significantly different in four groups (p-value < 0.05 for all). Gender and RA factor were homogenous in all groups (p-value > 0.05 for both), i.e., there was the same number of males and females in each group, and the Rheumatoid factor was equally distributed in patients of all four groups. This proves the fact that the RA factor is just a sensitive test and not a specific test for the diagnosis of Rheumatoid Arthritis.*

Conclusion: *It is concluded that at the current sample size of 120 subjects, i.e., 30 controls and 30 patients each in with either Rheumatoid Arthritis or Periodontitis or both, there is no difference in mean Osteocalcin and Osteoprotegerin levels.*

Keywords: *Osteocalcin, Osteoprotegerin, Rheumatoid Arthritis, Periodontitis*

INTRODUCTION

A chronic destructive inflammatory disease of unknown etiology is Rheumatoid arthritis (RA) that primarily involves joints. Arthritis may be remitting and symmetrical and is characterized by the buildup

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and persistence of an inflammatory infiltrate within the synovial membrane.[1] RA is a systemic disease that has extra-articular manifestations in various systems of the body, such as pulmonary, ocular, oral, vascular, and other organs or systems.[2] In addition to environmental factors, hormonal, genetic, infectious, and other variables also contribute to RA in some manner.[3] Chronic adult periodontitis, which is characterized by gingival inflammation with accompanying loss of supportive connective tissues including alveolar bone, results in loss of attachment of the periodontal ligament to the cementum. Periodontitis has also been proposed as having a role in various diseases of the body involving cardiovascular, cerebrovascular, diabetes, and respiratory diseases. It also plays a modulating role in pregnancy.[4] In recent previous years, studies have been done on pathological relationships between RA and PD. In past studies, some findings support the fact that oral infections play a role in the pathogenesis of RA.[5]

Evaluation of rheumatoid arthritis includes RA factor, C-reactive protein, erythrocyte sedimentation rate, and acute-phase proteins.[6] Oral dryness and salivary gland swelling can be seen in such patients at later stages. Patients with RA come across more periodontal problems than other 2.Periodontal disease has been associated with RA in a number of studies 6; a relationship with disease severity has also been suggested.[7] It was also found that the patients having PD had a higher prevalence of RA as compared to the general population. Previously an animal model was used as additional evidence to show a significant relationship between PD and RA.[8] In this study, they induced experimental arthritis in the rat (known as adjuvant arthritis), which resulted in periodontal breakdown with alveolar bone loss and increased activity of matrix metalloproteinase in the adjacent gingival tissue.

MATERIALS AND METHODS

This comparative cross-sectional study was conducted at the Centre for Research in Molecular Medicine, The University of Lahore. One hundred twenty patients were selected for four groups. Rheumatoid Arthritis was labeled if patients had RA diagnosed by a consultant physician and clinical record of taking DMARD for at least a year. Periodontitis was defined as the clinical involvement of at least ten teeth in patients with at least 20 teeth present. Patients were equally divided into four groups according to the disease presence i.e.Group I: Patients having Rheumatoid Arthritis (RA) and Periodontitis (PD) Group II: Patients having RA only Group III: Patients having Periodontitis only Group IV: Control group

Data Collection:

Basic demographic data, along with detailed history and examination, was recorded using a pretested questionnaire. Blood samples were drawn, stored, and analyzed and recorded for statistical analysis. A complete dental checkup was performed by the researcher herself. Under aseptic conditions, a phlebotomist drew 5ml blood from an anti-cubital vein from each patient. 2ml blood was shifted immediately to anti-coagulant EDTA vacutainers and transported in iceboxes to Biochemistry and Molecular Biology Laboratory of CRIMM for further analysis.

Serum Sampling For Bioassay By Human Bone Magnetic Bead Panel

Blood was allowed to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Serum and assay were immediately removed and stored at-20°C. Before using frozen samples, the samples were thawed completely, mixed well by vortexing and centrifuged before use in the assay to remove particulates. Serum samples were diluted 1:2 in the assay buffer provided by the manufacturer.

60 µL from each antibody bead was added to the mixing Bottle and made the final volume to 3 mL with bead diluent. Mixed beads were vortexed well. Before use, Quality Control 1 and Quality Control 2 with 250 µL deionized water was reconstituted by inverting the vial several times to mix and vortex. The vial was allowed to sit for 5-10 minutes. 10X Wash Buffer was brought to room temperature and mixed to convert all salts into solution. 60 mL of 10X Wash Buffer (two bottles) was diluted with 540 mL deionized water.1.0 mL of Deionized Water and 1.0 mL of Assay Buffer was added to the Bottle containing lyophilized Serum Matrix and mixed for complete reconstitution. Before use, the Human Bone Standard was reconstituted with 250 µL deionized water to obtain the required concentration. The vial was inverted several times to mix and vortexed for 10 seconds. This was used and labeled as the Standard

Six polypropylene microfuge tubes were Label as Standard 6, Standard 5, Standard 4, Standard 3,

Standard 2, and Standard 1. 150 µL of Assay Buffer was added to each of the six tubes. 1:4 serial dilutions were prepared by adding 50 µL of the reconstituted Standard 7 to the Standard 6 .mixed well and 50 µL transferred of the Standard 6 to the Standard 5 tube, mixed well and 50 µL transferred of the Standard 5 to the Standard 4 tube, mixed well and 50 µL transferred of the Standard 4 to the Standard 3 tube, mixed well and 50 µL transferred of the Standard 3 to the Standard 2 tube, mixed well and 50 µL transferred of the Standard 2 to the Standard 1 tube, The 0 Standard (Background) was the Assay Buffer

Immunoassay Procedure

200 µL of Assay Buffer was added into each well of the Plate. Sealed and mixed on a plate shaker for 10 minutes at room temperature (20-25°C). Assay Buffer was decanted and removed the residual amount from all wells by inverting the Plate and tapping it smartly onto absorbent towels several times. Mixing Bottle was vortexed, and 25 µL of the Mixed or Premixed Beads were added to each well. (Note: During the addition of Beads, the bead bottle was shaken intermittently to avoid settling. The Plate was sealed with a plate sealer. The Plate was wrapped with foil and incubated with agitation on a plate shaker overnight (16–18 hours) at 4°C or incubated with agitation on a plate shaker 2 hours at RT (20-25°C). Well contents were gently removed, and Plate was washed three times. 50 µL of Detection Antibodies were added into each well. The tube was sealed, covered with foil and incubated with agitation on a plate shaker for 30 minutes at room temperature. 50 µL Streptavidin-Phycoerythrin was added to each well containing the 50 µL of Detection Antibodies. The tube was sealed again, covered with foil and incubated with agitation on a plate shaker for 30 minutes at room temperature. Well, the contents were gently removed, and the Plate was washed three times. 100 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) was added to all wells. The beads were resuspended on a plate shaker for 5 minutes. The Plate was run on Luminex 200TM, HTS, FLEXMAP 3DTM, or MAGPIX® with exponent software. The Median Fluorescent Intensity (MFI) data was saved and analyzed using a 5-parameter logistic or spline curve-fitting method for calculating analytic concentrations in samples.

ACCP Kit Protocol

The sample material was Human serum or EDTA, heparin, or citrate plasma. Ten µl of diluted patient sample was added to 1.0 ml sample buffer and assorted well by vortexing (sample pipettes are not suitable for mixing). 100 µl of the calibrators, positive and negative controls, or diluted patient samples were added into the individual microplate wells according to the pipetting protocol. The pipetting should not take longer than 15 minutes. Then hatched it for 60 minutes at room temperature (+18°C to 25°C). Then wells were made empty and afterward washed three times using 300 µl of working strength wash buffer for each wash. 100µl of enzyme conjugate was transferred (peroxidase-labeled anti-human IgG) into each of the microplate wells through a pipette and then incubated for 30 minutes at room temperature (+18°C to 25°C). Wells were drained and washed. Using a Pipette, 100 µl of chromogen/substrate solution was added into each of the microplate wells. Then wells were incubated for 30 minutes at room temperature (+18°C to 25°C). Also protected from direct sunlight. 100 µl of stop solution was then added into each of the microplate wells using a pipette. Measurement of the color intensity was taken by using a photometer. Duplicate determinations further improved the dependability of the ELISA test for each sample. Both positive and negative controls served as internal controls for the reliability of the test procedure. They were assayed with each test run. 5 - point calibrated analysis.

ELISA KIT

The ELISA test kit provides a semi-quantitative or quantitative in vitro assay for human lgG Rheumatoid factors (antibody of lgG class against human lgG) in serum or plasma. The test kit was contained with strips, each with eight break-off reagent well encrusted with lgG. In the first reaction step, diluted perfect samples were incubated in the wells. In the case of positive samples, specific lgG antibodies (also lgA and lgM) will bind to antigens. A second incubation was carried out using an enzyme-labeled and human lgG (enzyme conjugate), catalyzing a color reaction to direct bond antibodies.100 ml of chromogen substrate solution was added into each of the microplate wells through the pipette. Then incubated for 15 minutes at room temperature.100 ml stop solution was transferred into each of microplate

wells in the same order, and the same speed as the chromogen/ substrate was introduced. Photometric measurement of the same intensity was made at a wavelength of 450 nm and the reference wavelength between 620nm and 650nm within 30 minutes of adding the stop solution. 100 ml of calibrators, positive or negative, controlled, or diluted patient samples were transferred into individual micro platting wells according to the pipetting protocol and then incubated at room temperature (for 30 minutes 18-25°C). The wells were made empty and subsequently washed three times using 300 ml of working strength wash buffer for each wash.

Reagents wells were washed three times, with 450 ml washing strength wash buffer. After washing thoroughly, all liquids from the micro Plate were disposed of by tapping on the absorbent paper, opening face downward to remove all residual buffer.100 ml of enzyme conjugate (peroxidase-labeled anti-human IgG) was added into each of the microplate wells and then incubated for 30 minutes at room temperature.

ELISA Incubation: There were eight beakers taken for conducting the test of ELISA incubation coated with antigen wells. 100 ml of calibrators, controls, samples were added into each of the beakers by using a pipette. Then all the beakers were left to rest for 30 minutes at room temperature i-e 18-25°C. After 30 minutes, each of the beakers was washed with 300 ml (man)/450 ml (aut.) per well with the estimated reaction time of 30-60 seconds. The beakers were refilled with samples of 100 ml per well of enzyme conjugate and rested for 30 minutes at room temperature followed by 2nd washing with 300ml (man.)/450 ml (aut.) per well with a reaction time of 30-60seconds. The optimal temperature of enzyme activity was room temperature (i.e., 18 °C-25°C). The third time, each well was filled by 100 ml of chromogen/substrate using a pipette and was left to settle at room temperature for 15 minutes. Then 100 ml stop solution was added into well, and a photometer was used to calculate it (450nm).

100 ml of calibrators containing positive or negative control or diluted patient samples were added into each of individual microplates according to the pipetting protocol and then incubated for 30 minutes at room temperature (18-25°C). The wells were then made empty and washed subsequently using three times 300 ml of working strength wash buffer for each wash.100 ml of enzyme conjugate (peroxidase-labeled anti-human lgG) was transferred into each of microplate wells using a pipette and incubated for 30 minutes at room temperature. (+18°C-25°C). All wells were made empty and washed. 100 ml of chromogen substrate solution was added by using pipette into each of the microplates wells and incubated for 15 minutes at room temperature. Also protected from direct sunlight. 10 ml of stop solution was added through pipette into each microplate wells in the same order and at the same speed as the chromogen/ substrate solution was introduced. A photometric Measurement of the color intensity at the wavelength of 450 nm and the reference wavelength of between 620 nm and 550 nm was made within 30 minutes of adding the stop solution. Before measuring, microplate wells were carefully shacked to ensure the homogenous distribution of the solution.

RESULTS

the p-value for RA factor lesser than 0.358 makes our result Non-significant (Table I). The p-value for anti-CCP lesser than 0.001 makes our result highly significant (Table: II). For osteoprotegerin p-value lesser than 0.003 makes our result highly significant (Table: III). Osteocalcin p-value lesser than 0.003 makes our result highly significant (Table: IV).

Table 1: Descriptive Statistics for RA Factor

	Mean	SD	SEM	Min	Max
Control	2.74	4.044	.738	0	15
Periodontitis	6.11	10.746	1.962	0	52
Rheumatoid Arthritis	3.78	7.163	1.308	0	24
Rheumatoid Arthritis & Periodontitis	3.73	6.474	1.182	0	27
Total	4.09	7.509	.685	0	52

Using ANOVA, p value= 0.358 (Non-significant)

Table 2: Descriptive Statistics for Anti-CCP

	Mean	SD	SEM	Min	Max
Control	1.44	1.242	.227	0	6
Periodontitis	2.74	3.359	.613	0	16
Rheumatoid Arthritis	34.42	64.674	11.808	0	265
RA & P	42.21	70.732	12.914	1	253
Total	20.20	50.796	4.637	0	265

Using ANOVA, p value < 0.001 (Highly Significant)

Table 3: Descriptive Statistics for Osteoprotegeri

	Mean	SD	SEM	Min	Max
Control	1188.73	1210.7	221.1	156	4776
P	1572.45	1270.9	232.0	215	3552
Rh Ar	1931.6	1689.6	308.4	178	5354
R A & P	2128.91	2061.858	376.4	183	7646
Total	1705.45	1616.332	147.5	156	7646

Using ANOVA, p value= 0.115 (Non-significant)

Table 4: Descriptive Statistics for Osteocalcin

	Mean	SD	SEM	Min	Max
Control	49974.83	44350.03	8097.171	9441	185958
Periodontitis	38194.41	54003.24	9859.598	6455	269488
Rheumatoid Arthritis	39446.91	51429.48	9389.696	3654	282827
Rheumatoid Arthritis & Periodontitis	37566.80	37038.31	6762.240	7637	155875
Total	41295.74	46847.78	4276.598	3654	282827

Using ANOVA, p value=0.711 (Non-significant)

Table 5: Post Hoc Multiple Comparisons using Games-Howell

Dependent Variable: Osteoprotegerin				
Grouping Variable	**Grouping Variable**	**Difference**	**Std. Error**	**Sig.**
Control	Periodintitis	-383.721	320.486	.631
	Rheumatid Arthiritis	-742.968	379.511	.217
	Rheumatid Arthiritis and Periodointitis	-940.188	436.551	.151
Periodontitis	Control	383.721	320.486	.631
	Rheumatid Arthiritis	-359.247	386.010	.789
	Rheumatid Arthiritis and Periodointitis	-556.466	442.213	.593
Rheumatoid Arthritis	Control	742.968	379.511	.217
	Periodintitis	359.247	386.010	.789
	Rheumatid Arthiritis and Periodointitis	-197.220	486.692	.977
Rheumatoid Arthritis and Periodontitis	Control	940.188	436.551	.151
	Periodintitis	556.466	442.213	.593
	Rheumatid Arthiritis	197.220	486.692	.977

DISCUSSION

Rheumatoid Arthritis is a multisystem syndrome with a variable spectrum of presentation ranging from small joint involvement to multisystem involvement the hallmark of Rheumatoid Arthritis is persistent inflammatory synovitis involving the small joints symmetrically.[9] The life of a patient with Rheumatoid Arthritis is quite morbid and costly. Similarly, Periodontitis is an inflammatory disorder of hard and soft tissues around teeth. It is also a destructive process ignited by bacteria, especially gram-negative facultative anaerobe present on tooth surface.[10] Periodontitis has its spectrum ranging from the benign form of gingivitis to aggressive Periodontitis. There is a reported association of diabetes, myocardial infarction, and stroke with Periodontitis showing increased risk in these patients of developing chronic diseases.[11]

Few studies narrate the association between Rheumatoid Arthritis and Periodontitis, and only a few studies have quantitatively tried to explain the common underlying mechanism between Rheumatoid Arthritis and Periodontitis.[6,12] The results of these quantitative studies had been conflicting. Still, the mechanism of progression of both diseases shows that some unidentified disablement or erratic physiological mechanism is operating in both diseases.[6,13] In search of the underline mechanism, the current study was undertaken, and two important markers, i.e., Osteocalcin and Osteoprotegerin, were hypothesized. The expression of Osteoprotegerin was found to be increased as the disease progressed while Osteocalcin level decreases with the progression of both diseases. In our study, we measured Osteoprotegerin level in four groups, in control, Periodontitis, Rheumatoid Arthritis, Rheumatoid Arthritis, and Periodontitis and there was a rising trend in the level of Osteoprotegerin as we move from control to Periodontitis to combined disease. Statistical analysis, although it showed a non-significant association between the Osteoprotegerin level and disease group, we may observe a rising trend when we plotted a graph of mean OPG level with disease group ($p = 0.115$, using ANOVA). We cannot comment on the status of Osteoprotegerin as an underlying mechanism in both Rheumatoid Arthritis and Periodontitis. The reasons for not finding statistical significance between mean Osteoprotegerin level in controls and diseased may be different age distribution and disease status.

The second biomarker, which we hypothesized to be a common underlying mechanism in Rheumatoid Arthritis and Periodontitis, was Osteocalcin. There should be a significant difference between controls and diseased individuals regarding Osteocalcin. We found a downward trend in mean Osteocalcin level as we go from control to Periodontitis to Rheumatoid Arthritis. Still, here too analysis of variances (ANOVA) showed non-significant differences in mean Osteocalcin level in all groups. Post hoc. Analysis using Games-Howell showed a non-significant difference between the two groups.

This result disproved Osteocalcin as the common underlying mechanism in RA and PD.[14,15] The reason may be different age distribution and not taking into account the disease classification and duration of disease. Both Rheumatoid Arthritis and Periodontitis are dynamic processes of bone and associated tissue formation and destruction; Osteocalcin may indicate increased destruction and low formation in the diseased individual. Still, currently in our study, we are unable to reject the null hypothesis to label lower levels of Osteocalcin as a marker of disease severity. Osteocalcin concentration varies with age and sex.[16,17]

The most specific test for Rheumatoid Arthritis is the presence of anti-CCP antibodies. Those are more than 90% specific and sensitive in diagnosing Rheumatoid Arthritis. Our results matched the known fact and Anti- CCP antibody titer was high and significantly different in Rheumatoid Arthritis patients.[18]

CONCLUSION

It was concluded that with a sample size of 120 subjects, i.e., 30 controls, 30 patients each in with either Rheumatoid Arthritis or Periodontitis or both, there was no difference in mean Osteocalcin and Osteoprotegerin levels. The strength of our study is that there is no published data to the best of our knowledge, which quantified the biomarkers to find a common pathway between two diseases. Limitation of our study is a small sample size, skewed age distribution, and disease severity confounding. A new study taking into account the above limitations may open new perspectives and may add new interventional modalities in treating these chronic diseases.

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