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THE IMPLICATION OF SERUM CALCIUM-PHOSPHORUS RATIO TO ASSESS RHEUMATOID ARTHRITIS (RA) IN WOMEN OF TRIPURA, A NORTH-EASTERN STATE OF INDIA

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ABSTRACT

Background: Systemic inflammation during rheumatoid arthritis (RA) affects multiple organs and metabolic pathways. Interleukin-18 (IL-18) and tumour necrosis factor-alpha (TNF-α) are the novel cytokines in RA, which mediate synovial inflammation and articular manifestations through the modulation of osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa β Ligand (RANKL) expression. This might be reflected into serum calcium to phosphorus ratio (Ca/P) of RA patients. In this context, our present study investigates the role of serum Ca/P in the assessment of systemic inflammation in treatment-naive RA patients. **Methods:** This cross-sectional study (2017-2019), conducted at Tripura, India, included 146 RA women (duration ≤15 months) and 40 age and gender-matched healthy control (HC). All participants were divided into pre-menopausal (PreM) and post-menopausal (PostM) group. We measured serum Ca, P, IL-18, TNF-α, OPG, and RANKL in all patients and HC. Pearson's bivariate correlation(r) and partial correlation (pR) analysis were performed to correlate Ca/P with IL-18, TNF-α and OPG to RANKL ratio (OPG/RANKL). **Results:** Serum Ca/P was found to be negatively correlated with IL-18 (PreM, r=-0.68, p=0.01; pR=-0.54, p=0.01 and PostM, r=-0.53, p=0.01; pR=-0.66, p=0.01) and TNF-α (PreM, r=-0.56, p=0.01; pR=-0.45, p=0.01 and PostM, r=-0.66, p=0.01; pR=-0.51, p=0.01). On contrary, significant positive correlation was observed between Ca/P and OPG/RANKL (PreM, r=-0.81, p=0.01; pR=-0.73, p=0.01 and PostM, r=-0.77, p=0.01; pR=-0.68, p=0.01). **Conclusions:** The study highlights a strong association between Ca/P and systemic inflammation in RA. This implicates that Ca/P could be used as a marker for the real-time evaluation of RA.

KEYWORDS: Ca/P; cytokines; OPG/RANKL; Osteoprotegerin; Rheumatoid Arthritis.

INTRODUCTION

Rheumatoid Arthritis (RA) is a systemic autoimmune disease associated with progressive disability, premature death, and socioeconomic burdens.[1] The autoimmune response evoked during the pathogenesis of RA primarily affects the lining of the synovial tissue, thus causing swollen and painful joints in patients.[1,2] It is reported that the exaggerated immune response mediated by the inflammatory cytokines in RA may affect multiple metabolic pathways, including those involved in bone mineral homeostasis.[3] A range of cytokines, including IL-1, IL-4, IL-10, TNF-α, IL-17, IL-18, are reported to participate in the modulation of the inflammatory response during the pathogenesis of RA.[4,5]

Several studies have revealed that interleukin-18 (IL-18) is spontaneously released in RA synovial tissue, and it seems to participate in the development of inflammatory and destructive alterations of joints via induction of tumour necrosis factor-alpha (TNF-α). TNF- α, in turn activates T cells and promotes the recruitment of osteoclasts at the site of tissue destruction.[6-8] The activated T cells secrete receptor activator of nuclear factor-kappa β Ligand (RANKL) which binds to (receptor activator of nuclear factor-kappa β) RANK. The RANK is expressed on the surface of osteoclasts that convey the signal for osteoclastogenesis through binding to RANKL. This RANK/RANKL signalling pathway is hindered when osteoprotegerin (OPG), a decoy receptor for RANKL, competes with RANKL for binding to RANK. However, this binding is dependent of the relative concentration of OPG and RANKL, suggesting the importance of OPG to RANKL ratio (OPG/RANKL). The binding of OPG to RANK prevents osteoclastogenesis, thereby regulating bone resorption.[9,10] Therefore, IL-18 and TNF-α plays significant role in bone

resorption during the pathogenesis of RA by modulating the OPG/RANKL/RANK system.

Bone resorption in RA can be well monitored by the evaluation of serum calcium to phosphorus ratio (Ca/P) since our bone is the reservoir of Ca and P in the form of crystalline hydroxyapatite [$Ca_{10}(PO4)_6(OH)_2$].[11] It is reported that oxidative stress in RA causes lipid peroxidation in the synovial joints, which leads to synovial tissue damage and an increase in serum P level.[12-14] On the other hand, increased rate of Ca metabolism and increased Ca excretion in RA patients leads to a reduced serum Ca level.[15,16] Overall, this results in reduced serum Ca/P in RA patients. Therefore, correlating the Ca/P with the inflammatory markers may explore the possible link between systemic inflammation and modulation of calcium-phosphate metabolism in RA. This approach might help real-time evaluation of RA patients by developing early diagnostic procedures, therefore better prognosis of the disease. In the present study, we have investigated serum Ca/P in a representative population of RA women and explored its association with IL-18, TNF-α, and OPG/RANKL in them. We have included only women in our study because of three specific reasons. Firstly, RA is three to six times more prevalent in women than in males.[17,18] Secondly, the woman exhibits more powerful cellular and humoral immune responses than men. The heightened immune responses in women are partly attributable to the X chromosome bearing genes for sex hormones, particularly estrogen that contributes to the development and activity of the immune system. The price of having two X chromosomes in women is a higher risk of autoimmune disease.[19] The third one, women, undergo massive physiological changes throughout their life due to a shift in their reproductive and hormonal life events, suggesting a role for sex hormones.[20] The predominant sex hormone estrogen level varies with reproductive and hormonal life events in women, and estrogen stimulates autoimmunity. The difference in hormone level of pre and post-menopausal women plays a significant role in disease progression.[21] Therefore, we have classified the enrolled patients in pre and post-menopausal subgroups of RA to explore the relationship between immune modulations and Ca-P metabolism.

MATERIALS AND METHODS

Study participants

Our present study included 146 RA women with disease duration of less than 15 months and 40 age and gender-matched healthy control (HC). The study was performed during 2017–2019 and a total of 472 RA patients were screened during this period from where only treatment-naive 146 RA patients were enrolled in the study. The selected patients were divided into pre (n=70) and post-menopausal (n=76) group with 20 respective HC in each group. All the patients registered at the outpatients' department (OPD) of orthopedics of Tripura Medical College & Dr. B.R. Ambedkar Memorial Teaching Hospital, Tripura India, and compatible with the diagnosis of RA as per the criteria of American College of Rheumatology/European League against Rheumatism (ACR/EULAR).[22] All patients gave their informed consent to participate in the study in accordance with the Helsinki Declaration. The study was approved by the ethics committee of the Tripura Medical College & Dr. B.R. Ambedkar Memorial Teaching Hospital, Tripura, India (Ethical clearance approval No.- F.3 (PO-75)/Inst. Ethical Com./SFTMC/2010-11/123284-123301, Date-18/01/2017).

The inclusion criteria were: women with age over 18 years, signs and symptoms compatible with the diagnosis of RA without any other health problems such as diabetes, hypertension, chronic obstructive pulmonary disease (COPD), coronary artery disease (CAD) and functional limitations. The exclusion criteria were: RA patients who had been treated with disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and corticosteroids. We have excluded the patients taking any Ca supplements (CS) and hormone replacement therapy (HRT). The inclusion and exclusion process of the study participants is presented in Fig. 1. Peripheral blood was drawn from all patients and HC after overnight fasting. Blood samples were collected aseptically by trained health professionals of concern OPD.

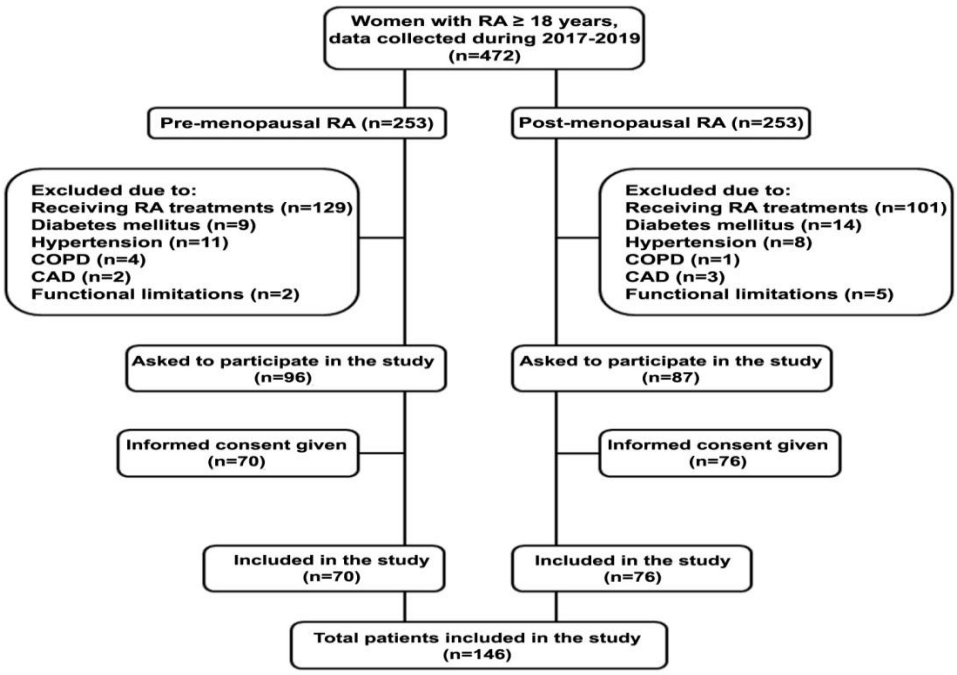

Figure-1: Flowchart of the inclusion and exclusion process in the study.

Figure abbreviations: *RA- rheumatoid arthritis, COPD- chronic obstructive pulmonary disease, CAD- coronary artery disease.*

Basic characteristics of the patients and healthy subjects

The demographic and clinical characteristics of the patients were obtained through standard self-assessment questionnaire. The demographic and clinical variables included in the study are age, body mass index (BMI), age at onset of disease, disease duration, percentage of rheumatoid factor (RF) positive patient, number of joints affected, pain score and disease activity score of 28 joints (DAS-28). The pain score was measured on a visual analogue scale (VAS; 0–10 mm), a numerical rating scale of pain (ranged from 0 to 10 as 0 means there is no pain, 10 means it is the worst possible pain patient had felt). An erythrocyte sedimentation rate (ESR) based DAS-28 was measured using an online DAS-28 calculator (http://www.4s-dawn.com/DAS28/). A DAS-28 score of greater than 5.1 implies high disease activity, 3.2 to 5.1 moderate disease activity, 2.6 to 3.2 low disease activity, and less than 2.6 remissions.[23]

Detection and measurement of inflammatory markers

RF in the patient's serum was qualitatively determined by the available commercial kits (AGAPPE, India).The serum concentration of IL-18, TNF-α, OPG, and RANKL were measured by using commercially available ELISA kits (ABCAM, USA) according to the manufacturer's instructions. The test principle is based on the standard enzyme-linked immune-sorbent assay (ELISA). Human IL-18, TNF-α, OPG, and RANKL specific capture antibody was pre-coated onto 96-well plates. Appropriately diluted serum samples and standards were added to the wells and followed by washing with 1X phosphate-buffered saline (PBS). Biotin conjugated detection antibody was added to each well and washed with 1X PBS. Followed by washing, Horseradish Peroxidase (HRP) conjugated streptavidin was added to each well and washed as earlier. HRP substrate 3,3′,5,5′-Tetramethylbenzidine (TMB) was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue coloured product that changed into yellow after adding the acidic stop solution. The density of yellowish product is proportional to the concentration of cytokine of interest (human IL-18, TNF-α, OPG, and RANKL) in the serum sample. [Optical density (O.D.)- 450nm, BioTek, Synergy H1 Hybrid Reader, USA**].** (Kit Sensitivity: IL-18- < 20 pg/ml, TNF-α - <4.32 pg/ml, OPG- <11 pg/mL, RANKL- < 10 pg/ml).

Measurement of serum calcium and phosphorus

Serum Ca and P levels were determined by the colorimetric method using a commercially available kit (AGAPPE, India). Briefly, the serum samples and standards were mixed separately with the test reagent. The O.D. of the resulting coloured reactions was measured against the blank (test reagent) using a spectrophotometer (EPPENDORF, Bio Spectrometer basic, Germany). (O.D., Ca-570nm, P-340 nm)

Statistical analysis

Statistical analyses were performed using the Statistics Package for Social Sciences (SPSS for Windows, version 24.0; SPSS Inc, Chicago, IL, USA). We have tested the normality of data distribution using SPSS software. The

unpaired t-test was done using the GraphPad t-test calculator online (https://www.graphpad.com/quickcalcs/ttest1.cfm) to examine the significant difference of a parameter between the two independent groups of RA. We performed Pearson's bivariate correlation to find the correlation between serum Ca/P and pro-inflammatory cytokines that include IL-18 and TNF-α, the correlation between Ca/P and OPG/RANKL in both pre and post-menopausal subjects. The partial correlation was sought to re-evaluate the correlation between two variables by adjusting or controlling the effect of other co-variables.[24] All the experimental data were expressed as Mean ± SD. A p-value of less than 0.05 was considered significant, less than 0.01 was considered highly significant, and less than 0.0001 considered extremely significant.

RESULTS

Demography of pre and post-menopausal RA and HC subjects and their health status

The demographical and clinical data of the patients enrolled in this study are shown in Table-1. The mean age of the pre-menopausal RA group was 36.82 ± 5.41 years, and the post-menopausal RA group was 56.5 ± 7.54 years. The BMI of the post-menopausal RA subjects was found to be lesser (20.69 ± 2.81 kg/m^2) as compared to pre-menopausal RA subjects (22.78 ± 3.2 kg/m^2). BMI was also found to be lower in pre and post-menopausal RA as compared to their respective HC. The mean age at onset of disease was lower in pre-menopausal RA (34.68 ± 5.83 years) compared to post-menopausal RA (51.33 ± 6.9 years). For disease duration, there is no significant difference observed between the pre-menopausal RA (1.24 ± 0.26 years) and post-menopausal RA (1.32 ± 0.31years) (p=0.094). 66 % of the pre-menopausal RA women were found RF positive, and 59.24 % of post-menopausal RA women were found RF positive. The post-menopausal RA women were found to exhibit higher disease activity than pre-menopausal women. The mean pain score of pre-menopausal women was 2.24 ± 1.19, and post-menopausal women was 3.73 ± 1.47. The post-menopausal women showed significantly higher DAS-28 (4.9 ± 1.51) than pre-menopausal subjects (3.39 ± 1.03) (p=0.0001).

Table-1: Basic characteristics of pre and post-menopausal rheumatoid arthritis (RA) patients with respective healthy control (HC).

Basic characteristics (Demographic and Clinical variables)	**Pre-menopausal RA (n=70)**	**Pre-menopausal HC (n=20)**	**Post-menopausal RA (n=76)**	**Post-menopausal HC (n=20)**
Age, years	36.82 ± 5.41	37.4 ± 7.44	56.5 ± 7.54	57.55 ± 8.01
BMI (Kg/m^2)	22.78 ± 3.2	23.45 ± 2.28	20.69 ± 2.81	21.69 ± 2.7
Age at disease onset, years	34.68 ± 5.8	-	51.33 ± 6.9	-
Disease duration, years	1.24 ± 0.26	-	1.32 ± 0.31	-
RF %	66%	-	59.2 %	-
No of joints affected	11.65 ± 4.05	-	15.98 ± 4.83	-
Pain score, VAS (1-10)	2.24 ± 1.19	-	3.73 ± 1.47	-
DAS-28	3.39 ± 1.03	-	4.9 ± 1.51	-

Values are expressed in mean ± SD, except where otherwise indicated. BMI = body mass index, VAS = Visual Analogue Scale; DAS-28 = Disease Activity Score of 28 Joints.

Evaluation of inflammation in pre and post-menopausal RA

The serum concentration of IL-18 and TNF-α in pre and postmenopausal RA and their age and sex-matched HC were determined and given in Table-2 and Table-3. IL-18 concentration was found to be higher in pre-menopausal RA women (239.13 ± 43.27 pg/ml) as compared to pre-menopausal HC (81.75 ± 16.33 pg/ml) (p=0.0001). Serum IL-18 was also higher in post-menopausal RA women (253.75 ± 32.67 pg/ml) in comparison to the post-menopausal HC group (104.63 ± 17.79 pg/ml) (p=0.0001). IL-18 concentration was found to be significantly higher in post-menopausal RA women (253.75 ± 32.67 pg/ml) as compared to pre-menopausal RA women (239.13 ± 43.27 pg/ml) (p=0.022) (Table-4). The level of TNF-α was found to be higher in pre-menopausal RA (27.93 ± 11.68 pg/ml) than pre-menopausal HC (4.09 ± 2.15 pg/ml) (p=0.0001). In case of post-menopausal RA, TNF-α concentration (28.92 ± 7.28 pg/ml) showed a similar trend when compared with post-menopausal HC (5.58 ± 3.02pg/ml) (p=0.0001). But the serum concentration of TNF-α was not significantly differed in postmenopausal RA women (28.9 ± 7.28 pg/ml) as compared to pre-menopausal RA women (27.93 ± 11.68 pg/ml) (p=0.544) (Table-4). Serum OPG level was significantly reduced in RA subjects as compared to HC (p=0.0001). The serum concentrations of OPG in pre and postmenopausal RA are 329 ± 41.11 pg/ml and 254.59 ± 38.12 pg/ml. These are less than the serum concentration observed in pre and postmenopausal HC (i.e., 464.65 ± 96.33 pg/ml and 460.74 ± 129.83 pg/ml, respectively). Serum OPG was significantly reduced in post-menopausal RA women (254.59 ± 38.12 pg/ml) in comparison to pre-menopausal RA women (329 ± 41.11 pg/ml) (p=0.0001) (Table-4). On the contrary, the level of Serum RANKL was found to be

higher in RA in comparison to HC (p=0.0001). The serum concentrations of RANKL in pre and postmenopausal RA are 5.32 ± 0.60 pg/ml and 6.37 ± 1.36 pg/ml, respectively; in pre and postmenopausal healthy women are 2.91 ± 1.32 pg/ml and 3.14 ± 0.83 pg/ml, respectively. RANKL was also increased significantly in post-menopausal RA women (6.3 ± 1.36 pg/ml) than pre-menopausal RA women (5.32 ± 0.60 pg/ml) (p=0.0001) (Table-4). Overall, this leads to a reduced OPG/RANKL in postmenopausal RA women (42.13 ± 10.14) as compared to pre-menopausal RA women (62.65 ± 11.09) (p<=0.0001) (Table-4).

Change in serum Ca/P in pre and post-menopausal RA

The serum concentration of Ca, P, and Ca/P in pre and postmenopausal RA women and their age and sex-matched HC were determined and given in Table-2 and Table-3. The level of serum Ca was found to be lesser in pre-menopausal RA women (8.95 ± 0.68 mg/dL) as compared to pre-menopausal HC (11.5 ± 1.25 mg/dL) (p=0.0001). Serum Ca in post–menopausal RA women (7.40 ± 0.99 mg/dL) is also lesser in comparison to HC (8.51 ± 0.77 mg/dL) (p=0.0001). On the contrary, serum P level was found to be higher in both pre-menopausal (3.32 ± 0.58 mg/dL) and post-menopausal (4.63 ± 0.85 mg/dL) RA women as compared to pre-menopausal (2.61 ± 0.46 mg/dL) and post-menopausal (3.17 ± 0.31 mg/dL) HC (p=0.0001).This results in decreased Ca/P in RA women as compared to HC. The serum Ca/P was also found to be significantly reduced in postmenopausal RA women (1.70 ± 0.61) as compared to pre-menopausal RA women (2.43 ± 0.66) (p=0.0001) (Table 4).

Table-2: Measures of inflammatory and biochemical markers of pre-menopausal healthy controls (HC) and rheumatoid arthritis (RA) patients.

Inflammatory and biochemical markers	PreM HC (n=20)	PreM RA (n=70)	P-value
IL-18 (pg/ml)	81.75 ± 16.33	239.13 ± 43.27	0.0001
TNF-α (pg/ml)	4.09 ± 2.15	27.93 ± 11.68	0.0001
OPG (pg/ml)	464.65 ± 96.33	329 ± 41.11	0.0001
RANKL (pg/ml)	2.91 ± 1.32	5.32 ± 0.60	0.0001
OPG/RANKL	199.02 ± 109.54	62.65 ± 11.09	0.0001
Ca (mg/dL)	11.15 ± 1.25	8.95 ± 0.68	0.0001
P (mg/dL)	2.61 ± 0.46	3.32 ± 0.58	0.0001
Ca/P	4.46 ± 1.15	2.43 ± 0.66	0.0001

Values are expressed in mean + SD; PreM= Pre-menopausal, PostM= Post-menopausal, IL-18= Interleukin-18; TNF-α= Tumour Necrosis Factor-alpha; OPG= Osteoprotegerin; RANKL= Receptor Activator of Nuclear Factor kappa β Ligand, Ca/P= Calcium to Phosphorous ratio, OPG/RANKL= OPG to RANKL ratio

Table-3: Measures of inflammatory and biochemical markers of post-menopausal healthy controls (HC) and rheumatoid arthritis (RA) patients.

Inflammatory and biochemical markers	PostM HC (n=20)	PostM RA (n=76)	P-value
IL-18 (pg/ml)	104.63 ± 17.79	253.75 ± 32.67	0.0001
TNF-α (pg/ml)	5.58 ± 3.02	28.92 ± 7.28	0.0001
OPG (pg/ml)	460.74 ± 129.83	254.59 ± 38.12	0.0001
RANKL (pg/ml)	3.14 ± 0.83	6.37 ± 1.36	0.0001
OPG/RANKL	157.92 ± 70.45	42.13 ± 10.14	0.0001
Ca (mg/dL)	8.51± 0.77	7.40 ± 0.99	0.0001
P (mg/dL)	3.17 ± 0.31	4.63 ± 0.85	0.0001
Ca/P	2.72 ± 0.48	1.70 ± 0.61	0.0001

Values are expressed in mean ± SD; PreM= Pre-menopausal, PostM= Post-menopausal, IL-18= Interleukin-18; TNF-α= Tumour Necrosis Factor-alpha; OPG= Osteoprotegerin; RANKL= Receptor Activator of Nuclear Factor kappa β Ligand, Ca/P= Calcium to Phosphorous ratio, OPG/RANKL= OPG to RANKL ratio

Table-4: Measures of inflammatory and biochemical markers of pre and post-menopausal rheumatoid arthritis patients (RA).

Inflammatory and biochemical markers	PreM RA (n=70)	PostM RA (n=76)	P-value
IL-18 (pg/ml)	239.13 ± 43.27	253.75 ± 32.67	0.022

TNF-α (pg/ml)	27.93 ± 11.68	28.9 ± 7.28	0.544
OPG (pg/ml)	329 ± 41.11	254.59 ± 38.12	0.0001
RANKL (pg/ml)	5.32 ± 0.60	6.3 ± 1.36	0.0001
OPG/RANKL	62.65 ± 11.09	42.13 ± 10.14	0.0001
Ca (mg/dL)	8.95 ± 0.68	7.40 ± 0.99	0.0001
P (mg/dL)	3.32 ± 0.58	4.63 ± 0.85	0.0001
Ca/P	2.43 ± 0.66	1.70 ± 0.61	0.0001

Values are expressed in mean ± SD; PreM= Pre-menopausal, PostM= Post-menopausal, IL-18= Interleukin-18; TNF-α= Tumour Necrosis Factor-alpha; OPG= Osteoprotegerin; RANKL= Receptor Activator of Nuclear Factor kappa β Ligand, Ca/P= Calcium to Phosphorous ratio, OPG/RANKL= OPG to RANKL ratio

Correlation between serum Ca/P and inflammatory markers in pre and post-menopausal RA

The result of Pearson's bivariate correlation was given in Table-5. Significant correlations were observed between serum Ca/P and markers of inflammation in RA women. In pre-menopausal RA women the ratio of Ca to P was found to be negatively correlated with IL-18 (r= -0.68, p=0.01) and TNF- α (r= -0.56, p=0.01), whereas a positive correlation was observed between Ca/P and OPG/RANKL ratio (r= 0.81, p=0.01). In the post-menopausal RA women the Ca/P was also found to be negatively correlated with IL-18 (r= -0.53, p=0.01) and TNF- α (r= -0.66, p=0.01), and positively with OPG/RANKL (r= 0.77, p=0.01).

The result of partial correlation remains same as Pearson's bivariate correlation (Table-5). A significant correlation was observed between the Ca/P ratio and the pro-inflammatory cytokines. The partial correlation (pR) between Ca/P and IL-18 was found to be pR= -0.54, p=0.01 in pre-menopausal RA women and pR= -0.66, p=0.01 in post-menopausal RA women while adjusted for TNF- α, the correlation between Ca/P and TNF- α was found to be pR = - 0.45, p=0.01 and pR= -0.51, p=0.01 in pre and post-menopausal RA women respectively while eliminating the effect of IL-18. The correlation between Ca/P and OPG/RANKL was pR = 0.73, p=0.01 in pre-menopausal RA women, and pR= 0.68, p=0.01 in post-menopausal RA women on adjustment of both IL-18 and TNF- α.

Table-5: Bivariate and Partial correlation among Ca/P, pro-inflammatory markers (IL-18 and TNF-α) and OPG/RANKL in pre and post-menopausal rheumatoid arthritis (RA) patients.

	IL-18				TNF-α				OPG/RANKL			
Ca/P	**r**	PreM RA	-0.68	0.01*	**r**	PreM RA	-0.56	0.01*	**r**	PreM RA	0.81	0.01*
		PostM RA	-0.53	0.01*		PostM RA	-0.66	0.01*		PostM RA	0.77	0.01*
	pR[1]	PreM RA	-0.54	0.01*	**pR[2]**	PreM RA	-0.45	0.01*	**pR[3]**	PreM RA	0.73	0.01*
		PostM RA	-0.66	0.01*		PostM RA	-0.51	0.01*		PostM RA	0.68	0.01*

*PreM= Pre-menopausal, PostM= Post-menopausal, RA= Rheumatoid Arthritis, IL-18= Interleukin-18, TNF-α= Tumour Necrosis Factor-alpha, OPG= Osteoprotegerin, RANKL= Receptor Activator of Nuclear Factor kappa β Ligand, Ca/P= Calcium to Phosphorous ratio, OPG/RANKL= OPG to RANKL ratio, r= Pearson's bivariate correlation, pR^1= partial correlation between Ca/P and IL-18 (adjusted for TNF- α), pR^2= partial correlation between Ca/P and TNF- α (adjusted for IL-18), pR^3= partial correlation between Ca/P and OPG/RANKL (adjusted for IL-18 and TNF- α), *= p value.*

DISCUSSION

The present study is based on the postulation that autoimmune response mediated by pro-inflammatory cytokines in RA may alter the serum Ca/P in patients. The demographic data of our study shows reduced BMI in post-menopausal women compared to pre-menopausal women as a consequence of higher age. However, the significantly reduced BMI in both pre and post-menopausal RA women compared to their respective control groups might be associated with RA manifestations. The increased disability and gradual deterioration in body mass of RA subjects reflected in their BMI value.[25] A higher percentage of RF positive cases was observed in both pre and post-menopausal RA. Earlier studies have also reported a linear relationship between RF positivity and disease progression.[26] The mean age at onset of the disease is significantly higher in post-menopausal RA women since the hormonal imbalance during menopause increases susceptibility to RA.[21,27] The higher disease duration in the post-menopausal group may be due to heightened and persistent inflammation. This might be stemmed from scarcity in hormones involved in the prevention of inflammatory response in women with RA in the post-menopausal group. Studies have reported that post-menopausal hormone therapy can improve disease conditions in subjects with RA.[28] We have found a higher pain score and disease activity score in post-menopausal RA women compared to pre-menopausal women with RA. This might result from sustained and more robust inflammation in post-menopausal RA, leading to swellings and pain in multiple joints.

It has long been known that IL-18 and TNF-α are associated with an inflammatory response in several autoimmune diseases.[29,30] These two pro-inflammatory cytokines play a pivotal role in the development of RA, especially in post-menopausal women, since they are

more prone to RA.[28,31] Although TNF-α in our study is not significantly increased in postmenopausal RA women as compared to pre-menopausal RA. But our present study showed that IL-18 and TNF-α were significantly higher in pre and postmenopausal RA women than age and sex-matched respective HC groups.

We have observed decreased OPG/RANKL in both pre and post-menopausal RA women in comparison to HC. These data are corroborated with the findings that IL-18 induces TNF-α secretion, which raises RANKL production by activated T cells, thereby reducing OPG/RANKL.[10] The higher levels of IL-18 observed in our study subjects might be associated with an increased level of TNF-α and thus lower the value of OPG/RANKL. This might lead to increased osteoclastogenesis by binding RANKL to its receptor RANK on osteoclasts of bone and leading to bone resorption. In this process, the bone mineral constituents are passed into the sera of RA patients, which might reflect through serum Ca/P.

Our correlation analysis showed that serum Ca/P is negatively correlated with IL-18 and TNF-α in pre- and post-menopausal RA groups. In RA patients, activated T cells infiltration releases pro-inflammatory cytokines into synovial joints, leading to hypertrophy and hyperplasia of synovial tissue and bone. This creates a hypoxic environment and generation of reactive oxygen species. This, in turn, causes degradation of membrane phospholipids and an increase in the serum level of P and lipid peroxide.[12,32] Garrett et al., 1990 have shown that the inflammatory response in RA is associated with a negative correlation between Ca/P and lipid peroxide.[14] However, the decrease in serum Ca also is a major reason for decreased Ca/P in RA. It was reported earlier that RA patients exhibit increased Ca metabolism and increased Ca excretion, thus leads to reduced serum Ca.[15,16]

CONCLUSION

Increased expression of IL-18 and TNF-α in RA causes a decrease in serum Ca/P, which establishes a negative correlation between Ca/P and pro-inflammatory cytokines of our study (IL-18 and TNF-α). On the contrary, the positive correlation between Ca/P and OPG/RANKL justifies that raised IL-18 and TNF-α in RA causes an increase in RANKL production and hence reduction in OPG/RANKL. This finding of our present study highlights the importance of serum Ca/P in the assessment of systemic inflammation in both pre and post-menopausal women with RA.

In the current study, we have mainly focused on circulating cytokines (IL-18 and TNF-α), but we cannot collect the data for the synovial cytokines. Although it is worth mentioning that cytokine expression may show distinct values while evaluating in the serum and synovial fluid.[33] Besides, all patients in the current study are treatment naïve. So, we cannot follow-up patients for change in the Ca/P with amelioration of disease. Moreover, it is unknown in this study whether treatment restores the Ca/P back to normal in those RA patients. Due to a small group of patients in this study, authors think that cumulative data from many studies or more extensive population-based studies could reveal the implication of the Ca/P in the field to assess the progression and efficacy of the treatment of RA. However, our current study establishes that alteration in serum Ca/P is closely associated with inflammatory manifestations in RA. Therefore, the Ca/P in RA could be implied to identify the disease progression, manage therapeutic efficacy, and provide useful prognostic information about the patient's long-term clinical condition.

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Ethical Approval and Informed Consent

All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional research committee of Tripura Medical College & Dr. B.R. Ambedkar Memorial Teaching Hospital, Tripura India (Ethical clearance approval No.- F.3 (PO-75)/Inst. Ethical Com./SFTMC/2010-11/123284-123301, Date- 18.01. 2017) and with the 2013 Helsinki declaration. All patients gave their informed consent prior to their inclusion in the study.

CONFLICT OF INTEREST

Each author certifies that they do not have any commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangements) that might pose a conflict of interest in connection with the submitted article. There is no financial or nonfinancial conflict of interest to be reported for any of the authors.

REFERENCES

1. Guo Q, Wang Y, Xu D, Nossent J, Pavlos NJ, Xu J. Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. Bone Res, 2011; 6: 15.
2. Heidari B. Rheumatoid Arthritis: Early diagnosis and treatment outcomes. Caspian J Intern Med, 2011; 2: 161–70.
3. da Cunha VR, Brenol CV, Brenol JC, Fuchs SC, Arlindo EM, Melo IM, et al. Metabolic syndrome prevalence is increased in rheumatoid arthritis patients and is associated with disease activity. Scand J Rheumatol, 2012; 41: 186-91.
4. Brennan FM, McInnes IB. Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest, 2008; 118: 3537–45.
5. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol, 2007; 7:429-42.
6. Gracie JA, Forsey RJ, Chan WL, Gilmour A, Leung BP, Greer MR, et al. A proinflammatory role for IL-18 in rheumatoid arthritis. J Clin Invest, 1999; 104: 1393-1401.
7. Dai SM, Matsuno H, Nakamura H, Nishioka K, Yudoh K. Interleukin-18 enhances monocyte tumor necrosis factor α and interleukin-1β production induced by direct contact with T lymphocytes: Implications in rheumatoid arthritis. Arthritis Rheum, 2004; 50: 432-43.
8. Yao Z, Li P, Zhang Q, Schwarz EM, Keng P, Arbini A, et al. Tumor necrosis factor-alpha increases circulating osteoclast precursor numbers by promoting their proliferation and differentiation in the bone marrow through up-regulation of c-Fms expression. J Biol Chem, 2006; 281: 11846–55.
9. Geusens P. The role of RANK ligand/osteoprotegerin in rheumatoid arthritis. Ther adv Musculoskel, 2012; 4: 225-33.
10. Papadaki M, Rinotas V, Violitzi F, Thireou T, Panayotou G, Samiotaki M, et al. New insights for RANKL as a proinflammatory modulator in modeled inflammatory arthritis. Front Immunol, 2019; 10: 97.
11. Von Euw S, Wang Y, Laurent G, Drouet C, Babonneau F, Nassif N, et al. Bone mineral: new insights into its chemical composition. Sci Rep, 2019; 9: 8456.
12. Walwadkar SD, Suryakar AN, Katkam RV, Kumbar KM, Ankush RD. Oxidative stress and calcium-phosphorus levels in rheumatoid arthritis. Indian J Clin Biochem, 2006; 21: 134-7.
13. Mateen S, Moin S, Khan AQ, Zafar A, Fatima N. Increased reactive oxygen species formation and oxidative stress in rheumatoid arthritis. PLoS One, 2016; 11: e0152925.
14. Fonseca LJ, Nunes-Souza V, Goulart MO, Rabelo LA. Oxidative Stress in Rheumatoid Arthritis: What the Future Might Hold regarding Novel Biomarkers and Add-On Therapies. Oxidative med cell longev, 2019; 7536805.
15. Ropes MW, Rossmeisl EC, Bauer W. Calcium and phosphorus metabolism in rheumatoid arthritis and degenerative joint disease. J Clin Invest, 1943; 22: 785-90.
16. Annamalai R, Kumar AN. Study of biochemical profile and 25-hydroxy Vitamin D association with disease activity in rheumatoid arthritis patients. J Orthop Allied Sci, 2018; 6: 17.
17. Van Vollenhoven RF. Sex differences in rheumatoid arthritis: more than meets the eye. BMC Med, 2008; 7: 12.
18. Barragán-Martínez C, Amaya-Amaya J, Pineda-Tamayo R, Mantilla RD, Castellanos-De La Hoz J, Bernal-Macías S, et al. Gender differences in Latin-American patients with rheumatoid arthritis. Gend Med, 2012; 9: 490-510.
19. Rubtsova K, Marrack P, Rubtsov AV. Sexual dimorphism in autoimmunity. J Clin Invest, 2015; 125: 2187-93.
20. Intriago M, Maldonado G, Cárdenas J, et al. Clinical characteristics in patients with rheumatoid arthritis: differences between genders. Scientific World Journal, 2019; 2019: 8103812.
21. Pikwer M, Nilsson JÅ, Bergström U, et. al. Early menopause and severity of rheumatoid arthritis in women older than 45 years. Arthritis Res Ther, 2012; 14: R190.
22. Aydın SZ, Castillo-Gallego C, Nam J, Freeston J, Horton S, Wakefield RJ, et al. The new ACR/EULAR criteria for rheumatoid arthritis can identify patients with same disease activity but less damage by ultrasound. E.ur J Rheumatol, 2017; 4: 118-21.
23. Uslu AU, Küçük A, Şahin A, Ugan Y, Yılmaz R, Güngör T, et al. Two new inflammatory markers associated with Disease Activity Score-28 in patients with rheumatoid arthritis: neutrophil-lymphocyte ratio and platelet-lymphocyte ratio. Int J Rheum Dis, 2015; 7: 731-35.
24. Gonzalez-Gay MA, Llorca J, Garcia-Unzueta MT, Gonzalez-Juanatey C, Matias JD, Martin J et al. High-grade inflammation, circulating adiponectin concentrations and cardiovascular risk factors in severe rheumatoid arthritis. Clin Exp Rheumatol, 2008; 26: 596.
25. Munro R, Capell H. Prevalence of low body mass in rheumatoid arthritis: association with the acute phase response. Ann Rheum Dis, 1997; 56: 326-29.
26. Wilson D. Rheumatoid factors in patients with rheumatoid arthritis. Can Fam Physician, 2006, 52: 180-1.
27. Goemaere S, Ackerman C, Goethals K, et al. Onset of symptoms of rheumatoid arthritis in relation to age, sex and menopausal transition. J Rheumatology, 1990; 17: 1620-1622.
28. Orellana C, Saevarsdottir S, Klareskog L, Karlson EW, Alfredsson L, Bengtsson C. Postmenopausal hormone therapy and the risk of rheumatoid arthritis: results from the Swedish EIRA population-

based case-control study. Eur J Epidemiol, 2015; 30: 449-57.
29. Volin MV, Koch AE. Interleukin-18: a mediator of inflammation and angiogenesis in rheumatoid arthritis. J Interferon Cytokine Res, 2011; 31: 745–51.
30. Fragoso JM, Vargas GA, Jiménez SM, Reyes OH, Ramírez JB. Tumor necrosis factor alpha (TNF-α) in autoimmune diseases (AIDs): molecular biology and genetics. Gac Med Mex, 2014; 150: 334-44.
31. Alunno A, Carubbi F, Giacomelli R, Gerli R. Cytokines in the pathogenesis of rheumatoid arthritis: new players and therapeutic targets. BMC Rheumatol, 2017; 1: 3.
32. Reddy P. Study of oxidative stress, serum calcium, phosphorus and ratio in rheumatoid arthritis patients. Int J Sci Res, 2019; 8: 33-4.
33. van den Ham HJ, de Jager W, Bijlsma JWJ, Prakken BJ, de Boer RJ. Differential cytokine profiles in juvenile idiopathic arthritis subtypes revealed by cluster analysis. Rheumatology (Oxford), 2009; 48: 899–905.