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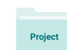
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ORIGINAL ARTICLE

Age-related trends and reference intervals of cross-linked C-telopeptide of type I collagen and procollagen type I N-propeptide from a reference population of Sri Lankan adult women

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Abstract

Summary Reference values of bone turnover markers (BTMs) are determined by factors that are country-specific. In Sri Lanka, unavailability of BTM reference data has led to their non-use in management of osteoporosis. The results of this study can be used as reference data for women in Sri Lanka.

Introduction This study was performed to establish age-related reference intervals for bone resorption marker; cross-linked C-telopeptide of type I collagen (CTX) and bone formation marker; procollagen type I N-propeptide (PINP) in a group of Sri Lankan adult women.

Methods Adult women ($n = 347$) aged 20–70 years were recruited using age-stratified random sampling technique and categorized into age groups by decades. Serum CTX and PINP concentration were measured using enzyme-linked immunosorbent assay (ELISA). The geometric mean (95% confidence interval) and 2.5th and 97.5th percentiles were calculated. ANOVA was used to compare the means between groups.

Results Mean CTX levels were relatively low and remained unchanged between 20 and 49 years. After the age of 49 years, mean CTXconcentration elevated significantly until the age of 70 years (43%, $p < 0.001$). Mean PINP concentrations were not significantly different between age categories ($p > 0.05$). Reference intervals of CTX and PINP were based on 2.5th and 97.5th percentile values. Reference intervals of CTX for the age groups of 20–29, 30–39, 40–49, 50–59, and 60–70 years were 0.19–0.97 ng/mL, 0.18–0.95 ng/mL, 0.20–1.29 ng/mL, 0.17–2.20 ng/mL, and 0.17–2.85 ng/mL respectively. Reference intervals of PINP for the same age groups were 118–810 pg/mL, 119–772 pg/mL, 116–645 pg/mL, 108–684 pg/mL, and 108–715 pg/mL respectively.

Conclusion In Sri Lanka, bone turnover markers are not used in evaluating patients mainly due to lack of normative data. These values can be used as reference data for women in this age group.

Keywords Adult women · Bone turnover markers · CTX · PINP · Reference intervals

Introduction

Bone mineral density (BMD) is the most used yardstick in diagnosing and treatment monitoring osteoporosis. Furthermore, BMD is used together with other clinical risk factors, in assessing the fracture risk [1]. Although widely used, there is no parallelism between BMD change and treatment efficacy and BMD does not detect early treatment response [2]. Cost and limited availability of dual X-ray absorptiometry (DXA) are additional constraints of using BMD [3].

Bone turnover markers (BTMs) provide information on bone metabolism beyond BMD. BTMs in serum and urine exhibit the metabolic activity of osteoblasts and osteoclasts in bone remodelling [4]. The availability of auto-analyzers in

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clinical chemistry laboratories is also increasing [5]. While BTMs of bone resorption are indicative of the activity of osteoclasts, those of bone formation reflect the activity of osteoblasts. Hence, the pattern of BTMs at a given time would depend on the state of bone metabolism determined by factors such as age, menopausal state, prevalent disease, and drug usage. Studies have shown that BTMs capture the efficacy of anti-resorptive treatment much earlier than DXA.

The behavior of BTMs is determined by country-specific factors such as ethnicity, geography, genetics, and epigenetics, hence, varies between countries [6]. Therefore, country-specific BTM reference values should be developed using representative study samples. Of the wide range of BTMs available, only a few are in current use [7]. Procollagen type I N-propeptide (PINP) and cross-linked C-telopeptide of type I collagen (CTX) have been recommended by the International Osteoporosis Foundation (IOF) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) as the standard bone formation and bone resorption markers, respectively [8]. While some countries have already developed country-specific reference values for CTX and PINP, several Asia–Pacific countries encourage reimbursement of PINP and CTX by national healthcare insurance programs to improve patient adherence and treatment outcomes [9].

BTM reference data would help in understanding age-related trends in bone metabolism in a given population. The pattern of bone mineral accrual in preadolescence and adolescence and loss in old age vary in different populations. Studies have shown delays in reaching peak bone mass in women in Sri Lanka, and BTMs would help in understanding such variations. Furthermore in Sri Lanka, BTMs are not used in clinical decision-making, partly due to non-availability of BTM reference values for the local population. Hence, this study was designed to establish age-specific reference values for serum PINP and CTX among Sri Lankan adult women. This information helps in understanding the age-related trends in BTMs in Sri Lankan adult women. When this information is available, clinicians would be able to identify patients with high fracture risk more accurately, offer patient targeted treatment, and recognize treatment failures early.

Materials and methods

Study population

This was a cross-sectional study conducted during the period of year 2017 to 2019. All study participants were long-term residents of Galle, a district located in the Southern province of Sri Lanka. The research protocol was approved by the Ethical Review Committee of the Faculty of Medicine, University of Ruhuna, Sri Lanka (Ref No 09.03.2016: 3.17). The latest electoral registers were used to identify women who were in the age range of 20 to 70 years. Participants were informed about the study purpose and written informed consent was obtained prior to data collection. A representative sample of adult women ($n = 347$) was recruited using an age-stratified random sampling technique in order to achieve a minimum of 60 subjects in five subgroups; 20–29 years, 30–39 years, 40–49 years, 50–59 years, and 60–70 years.

Data and sample collection

The data were collected using an interviewer-administered questionnaire and a focused clinical examination. Apart from clinical data, age, reproductive history (i.e., age at menarche, date of the last menstrual period, parity, and age at menopause), medical history (i.e., previous fracture, prevalent major diseases), and drug history were obtained. Participants were categorized as premenopausal and postmenopausal based on menstruation history of the last 12 months. Menopausal status was defined as the presence of amenorrhea for more than 12 consecutive months due to natural causes [10].

Women were excluded if they (1) had a history of endocrine disorders (disorders of thyroid, parathyroid, or adrenal glands, (2) had chronic kidney disease stage 4 or 5 (when eGFR is less than < 30 mL min^{-1} per 1.73 m^2), (3) had clinically evident chronic liver disease, (4) had metabolic or inherited bone diseases such as Paget's disease, osteomalacia, and arthritis, (5) had malignancies or cerebrovascular disease, (6) were immobilized or disabled, (7) had gastric surgery or major gastrointestinal disease that causes significant malabsorption, (8) were on any treatment which could alter the bone metabolism such as glucocorticoids, anti-osteoporotic drugs, anticonvulsants, thyroxine, calcium, vitamin D supplementation hormone replacement therapy, and androgen-stimulating therapy, (9) had experienced early menopause (before 45 years of age), (10) had fracture during the last 1 year, (11) were lactating or pregnant, (12) were on supervised dietary or exercise programs, and (13) were smoking.

Bone mineral density and anthropometry measurements

Bone mineral density and bone mineral content of the total body were measured using a DXA (dual X-ray absorptiometry) scanner (Hologic Discovery, Bedford, MA, USA) adhering to the manufacturer's protocols. Body weight was measured to the nearest 0.1 kg using a weighing scale, and height (cm) was measured to the nearest 0.1 cm using a stadiometer. Body mass index was calculated.

Blood sample collection and biochemical assays

Blood samples were collected between 0800 and 0900 h after an overnight fast. Serum samples were aliquot and stored at − 70 °C until being assayed.

Estimation of serum BTMs

Enzyme-linked immunosorbent assay (ELISA) was used to estimate serum CTX (Elabscience®, USA_Catalog No: E-EL-H0835) and intact PINP (Elabscience®, USA_Catalog No: E-EL-H0185) adhering to the manufacturer's protocol and laboratory quality control (QC) procedures. The manufacturer's QC serum (high and low) was used concurrently during the assays to maintain the accuracy of the test results. Sensitivity of the CTX and PINP test kits was 0.1 ng/mL and 9.38 pg/mL respectively. Respective detection ranges were 0.16–10 ng/mL and 15.63–1000 pg/mL. All the steps of the tests were completed without interruption, and each sample was assayed in duplicate. Percentage of intra- and inter-assay coefficient of variation (CV %) for CTX was 2.9% and 4.2% respectively ($n = 10$). Intra- and inter-assay CV % for PINP were 3.1% and 4.3% ($n = 10$).

Sandwich-ELISA principle was used in PINP assay. The micro-ELISA plate has been precoated with a mouse monoclonal antibody specific to human intact PINP. Standard (recombinant human PINP 23-161aa) was diluted to prepare the required series of concentrations. Standards, samples, and QC were added to the micro-ELISA plate wells and allowed to combine with the specific antibody. Then, a biotinylated detection antibody specific for human PINP and avidin-horseradish peroxidase (HRP) conjugate was added successively to each well and incubated. Free components were washed away and the substrate solution was added to each well. Wells that contain PINP appeared in blue in color. The enzyme–substrate reaction was terminated by the addition of stop solution and the color turned yellow. The optical density (OD) was measured at a wavelength of 450 nm ± 2 nm using an ELISA plate reader (Stat Fax® 4200, USA). The OD value was proportional to the concentration of PINP. Standard curve was automatically generated by the plate reader, and the concentrations were automatically estimated after comparing the OD of the samples to the standard curve. Assay procedure with similar principle was carried out for CTX using specific antibodies (mouse monoclonal antibody against CTX) and standards (recombinant human CTXI 1196-1218aa).

Serum 25-hydroxy vitamin D [25(OH)D] was measured using ELISA kits (DRG, Diagnostics GmbH, Germany), and intra- and inter-assay CV % were 2.6% and 3.5% ($n = 10$). Serum 25(OH)D cutoff levels were defined as deficient (< 20 ng/mL), insufficient (20–29 ng/mL), and sufficient (≥ 30 ng/mL) [11]. Serum creatinine (Biorex diagnostics, UK) was measured by a spectrophotometry-based assay kit and glomerular filtration rate was estimated (eGFR).

Statistical analysis

Mean (standard deviation) was used to interpret normally distributed data. Median (min–max) was used to interpret skewed data. The data on serum concentration of CTX and PINP were not normally distributed and they were log-transformed before statistical analysis. Therefore, CTX and PINP levels were presented as geometric means with a 95% confidence interval (95% CI). Reference intervals were defined as the central 95% range between 2.5th and 97.5th percentiles. Comparison of BTMs between age groups was done using one-way ANOVA with Bonferroni's multiple comparison test. Pearson's correlation and partial correlation (adjusted for possible confounders) were performed to elicit correlations between variables. Multiple linear regression was performed between BTMs and possible predictors to evaluate the associations.

Results

Descriptive data of the study sample is presented in Table 1. Mean CTX levels were relatively low and remained unchanged between 20 and 49 years. However, after the age of 49 years, mean CTXconcentration elevated significantly until the age of 70 years (43% difference, $p < 0.001$) (Fig. 1). The highest mean CTX concentration of 0.70 ng/mL was observed in the age group of 60–70 years, while the lowest mean concentration of 0.46 ng/mL was observed in the age group of 40–49 years (Fig. 1). Mean CTX concentrations were significantly different between age categories ($p < 0.05$). Notably, mean CTX concentration at the age of 60 s was significantly higher than that of the age of 20 s (46%, $p = 0.001$), 30 s (35%, $p = 0.027$), and 40 s (52%, $p < 0.001$) (Table 2). Moreover, mean CTX level of postmenopausal women was significantly higher than premenopausal women (38% difference, $p < 0.001$) (Table 1). The highest mean PINP concentration of 343 pg/mL was seen in the 20–29 age group while the lowest mean PINP concentration was seen in the age group of 40–49 years (Fig. 2). Mean PINP concentration was not significantly different between the age categories ($p > 0.05$). Mean PINP concentration of postmenopausal women was higher than premenopausal women, but this difference was not significant (3% difference, $p = 0.195$). Reference intervals of CTX and PINP were based on 2.5th–97.5th percentile values of 95% central distribution (Table 2).

CTX showed a significant positive correlation with age and negative correlation with body weight, BMI ($p < 0.03$), total body BMD and BMC ($p < 0.001$), but not with height

Table 1 Basic characteristics of the study population

Variable	All subjects ($n=347$)	Premenopause ($n=207$)	Postmenopause ($n=140$)
Age (years)	45 ± 14	35 ± 9	59 ± 5
Age at menarche (years)	14 ± 1	13 ± 1	14 ± 2
Years since menarche	31 ± 14	22 ± 9	45 ± 6
Age at menopause (years)	-	-	50 ± 3
Years since menopause	-	-	10 ± 6
Parity*	2 (1–6) ($n=251$)	2 (1–6) ($n=147$)	2 (1–6) ($n=104$)
Weight (kg)	54.9 ± 10.0	54.7 ± 10.4	55.3 ± 9.7
Height (cm)	151.6 ± 5.7	153.5 ± 4.9	148.7 ± 5.5
BMI (kgm^{-2})	23.95 ± 4.29	23.24 ± 4.27	25.01 ± 4.14
tBMC (g/cm^2)	1583.0 ± 267.9	1698.5 ± 221.7‡	1412.6 ± 248.2
tBMD (g/cm^2)	0.986 ± 0.102	1.029 ± 0.082‡	0.922 ± 0.095
CTX (ng/mL)†	0.55 (0.51–0.58)	0.48 (0.45–0.51)‡	0.66 (0.60–0.72)
PINP (pg/mL)†	302 (285–322)	312 (290–336)	288 (260–318)
25(OH)D** (ng/mL)	55.74 (13.9–96.6)	40.2 (13.0–90.4)	73.2 (15.6–100.8)
eGFR (mL/min/1.73 m^2)	92 ± 23	104 ± 21	75 ± 14

BMI body mass index, *tBMC* total body bone mineral content, *tBMD* total body bone mineral density, *CTX* cross-linked C-telopeptide of type I collagen, *PINP* procollagen type I N-propeptide, *25(OH)D* 25-hydroxy vitamin D, *eGFR* estimated glomerular filtration rate

Values are mean ± standard deviation if not otherwise specified

*Median (min–max), **median (interquartile range)

†Geometric mean (95% confidence interval)

‡Significantly different from postmenopausal group ($p < 0.001$)

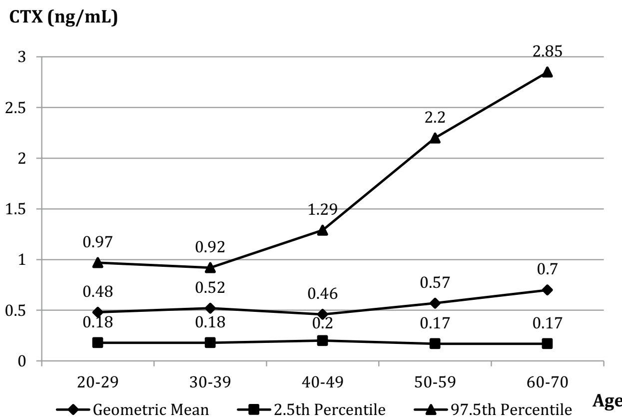

Fig. 1 Age trend of CTX in adult women

(Table 3). Regression analysis showed that 7% of variance in CTX explains by age and BMI while 16% explains by BMD. CTX (adjusted for age) showed a positive correlation with PINP ($r=0.19$, $p<0.001$). PINP showed a significant negative correlation with age ($p=0.046$) and total body BMD ($p=0.038$) but not with height, weight, or BMI (Table 3). Furthermore, CTX and PINP did not show significant associations with age of menarche and day of menstrual cycle in premenopausal women. Among women, 32% had vitamin D deficiency, 10% had insufficiency, and 58% had sufficient vitamin D levels.

Discussion

In this study, we report the first age-specific BTMs data for women aged 20–70 years in Sri Lanka. According to our findings, mean CTX concentrations were significantly different between age categories while mean PINP concentrations showed no such difference. The trough of the age trend of mean CTX and PINP was seen at the age of 40 s. Both CTX and PINP showed significant correlations with age in this study group. PINP and CTX reflect bone formation and resorption respectively during bone remodelling [12]. High mean PINP and CTX levels at the age of 20 s in this study group reflect high bone turnover in young age as previously seen [13]. Both mean CTX and PINP concentrations were higher in postmenopausal women than premenopausal women, but the change in CTX was more pronounced than PINP (CTX; 38% difference vs PINP; 3% difference) which reflect the rapid and unbalanced bone resorption following menopause.

Table 2 Age-specific reference intervals of CTX and PINP

Age group (years)	$N=347$	CTX (ng/mL)		PINP (pg/mL)		Menopause (%)
		Mean (95% CI)	Reference intervals	Mean (95% CI)	Reference intervals	
20–29	65	0.48 (0.43–0.55)*	0.19–0.97	343 (298–396)	118–810	-
30–39	66	0.53 (0.47–0.59)*	0.18–0.95	314 (275–358)	119–772	-
40–49	68	0.46 (0.41–0.52)*	0.20–1.29	282 (250–317)	116–645	6 (9%)
50–59	76	0.57 (0.50–0.66)	0.17–2.20	287 (250–329)	108–684	62 (82%)
60–70	72	0.70 (0.60–0.83)	0.17–2.85	297 (257–344)	108–715	72 (100%)

Values are geometric mean (95% confidence interval)

Reference intervals are 2.5th–97.5th percentile values

*Significantly lower than the mean value of 60–70 age group ($p<0.05$)

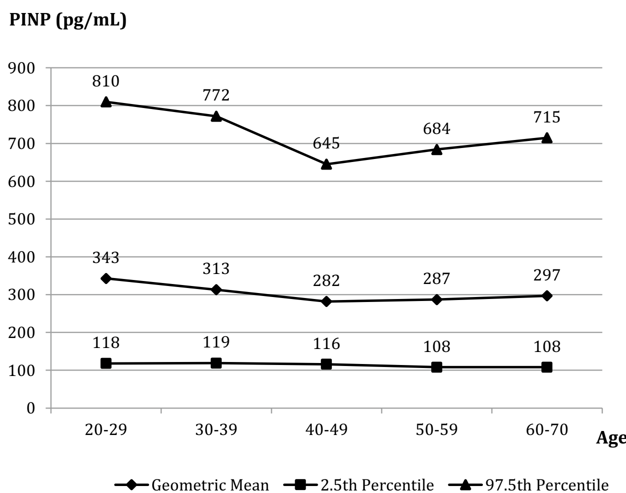

Fig. 2 Age trend of PINP in adult women

Table 3 Correlations of CTX and PINP with anthropometric measurements and BMD

Variable	CTX (ng/mL)		PINP (pg/mL)	
($n=347$)	r	p	r	p
Age (years)	0.24	<0.001	−0.11	0.046
Weight (kg)*	−0.13	0.015	−0.02‡	0.739
BMI (kg/m^2)*	−0.12	0.029	0.01‡	0.914
tBMC (g)†	−0.29	<0.001	−0.10‡	0.064
tBMD (g/cm^2)†	−0.32	<0.001	−0.11	0.038

BMI body mass index, *tBMC* total body bone mineral content, *tBMD* total body bone mineral density

*Adjusted for age

†Adjusted for age and BMI

‡Correlation is not significant at $p<0.05$

Similar to our findings, Ardawi et al. [14] reported that mean CTX concentrations increase significantly following menopause in Saudi Arabian women; Jenkins et al. [15] reported that there is no significant difference in PINP level between pre- and postmenopausal groups in Australian women; while Michelsen et al. [16] reported that mean PINP concentration is significantly higher in German postmenopausal women. Reduction of estrogen levels following menopause may contribute to increased levels of bone resorption markers and bone formation markers augments as coupling [17]. Studies conducted with Australian, Saudi Arabian, and Chinese women also show relatively similar age trend of CTX and PINP in line with our findings [14, 15, 18]. These populations show lowest mean CTX and PINP concentrations at the age of 40 s as our findings. This may be because the body may have reached the skeletal maturity at the age of 40 s in these populations [19, 20]. With reference to one of our previous publications, it is evident that peak bone mass of hip region of Sri Lankan adult women reaches at the age of 40 s [21]. These findings show substantial variation of mean CTX and PINP levels with aging and menopause, within and between populations.

Reference intervals for PINP and CTX have been established by many researchers. As recommended by other researchers, we considered 2.5th and 97.5th percentiles as reference intervals [22]. Age-specific reference intervals of CTX and PINP in our study are fairly concordant with those published in Australia [15] and Denmark [23]. However, reference intervals are broader after 40 years in our study when compared with the above studies probably due to small numbers in age categories. Although Jenkins et al. [15] and Jorgensen et al. [23] report that they used fasting serum samples, blood collection times were 0700–1300 h and 0730–1145 h, respectively. This may have influenced BTM levels due to diurnal variation in which BTMs show a nadir at afternoon [24]. Furthermore, participants in the study of Jenkins et al. [15] were probably not screened for bone metabolic diseases or use of drugs. When compared with the reference intervals reported from Japan [25],

Germany [16], and Spain [26], our reference intervals of CTX and PINP were broader in both pre- and postmenopausal age groups and the preanalytical variability may have partly contributed to these discrepancies. Martinez et al. [26] and Nomura et al. [25] have included a wider age range; 44–93 years and 44–83 years, respectively, for postmenopausal reference ranges. Furthermore, Michelsen et al. [16] report that they have collected non-fasting blood samples between 0800 and 2000 h and estimated intact PINP. While Jenkins et al. [15], Jorgensen et al. [23], Martinez et al. [26], and Michelsen et al. [16] have used an automated immunoassay system for CTX and PINP, Nomura et al. [25] have used ELISA for CTX and RIA (radioimmunoassay) for PINP. Among these studies, intra-assay and inter-assay ranges of CV % for CTX were 3.4–6.1% and 3.4–12.3%. Respective CV % ranges for PINP were 1.2–6.1% and 2.1–7.9%. The present study used the ELISA technique for PINP and CTX with intra- and inter-assay with CV % less than 5%. Differences present in immunoassay procedures may also have contributed to the observed variations in reference ranges among populations. These literature data show the presence of substantial variation of CTX and PINP concentrations within and between populations. We feel that preanalytical and analytical variability such as use of different epitopes for the same marker (i.e., intact PINP, fragmented PINP), different analytical methods, different age ranges, diurnal variation, fasting state, and blood collection time may have contributed to these differences [27].

We found negative correlations of CTX and PINP with BMD which probably indicate the occurrence of lower BMD at higher bone turnover levels. Two Chinese studies also reported negative correlations of CTX and PINP with BMD in women [17, 28]. We observed a significant positive correlation between CTXand PINP suggesting a synchronization of the bone formation and resorption during bone remodelling [29]. The respective positive and negative correlation of CTXand PINP with age may be due to the increased bone resorption and decreased bone formation with aging. Jorgensen et al. [23] report that age negatively correlated with PINP ($p < 0.001$) and CTX($p = 0.008$) levels in Danish women. Nevertheless, Zhao et al. [17] report neither CTXnor PINP correlated with age in Chinese women. BMIand weight were negatively but weakly correlated with CTXconcentration in our study. Similarly, a negative correlation was recorded between CTXand BMIin young UK women [30]. According to previous literature data, increased BMI and weight is associated with increased estrogen production in adipose tissue and subsequent inhibition of overall bone turnover [31]. CTX and PINP were not associated with age of onset of menarche and menstrual cycle in our study. Glover et al. [30] and Nomura et al. [25] also reported that menarche and menstrual cycle minimally affect the serum level of BTMs in line with our findings.

It was evident that the BTM reference ranges are highly variable between countries and within the same country [32]. This indicates the essentiality of establishing country-specific reference ranges and harmonization of them across and within countries [33]. Evidence shows that elevated levels of serum CTX and PINP are associated with osteoporosis, osteopenia, Paget's disease, hyperthyroidism, and metastasis of cancer [34]. Literature endorses that they could be used in the estimation of fracture risk at fewer DXA facilities [35]. Notably, clinical trial data confirms that there is a significant reduction in CTX and PINP at 3 months and 6 months following the anti-resorptive treatment [36]. Therefore, perhaps serum PINP and CTX may be useful in treatment monitoring and identification of bone diseases. Considering the above facts, certain countries have already included serum CTX and PINP in their health care policy guidelines and medical insurance [9]. The findings of our study will contribute to the appropriate assessment of bone health and monitoring treatment in Sri Lankan adult women for more than 20 years, because normative data are useful to predict bone turnover and suggest treatment targets.

This study has several strengths and weaknesses. The study sample was selected from community-dwelling women in a random manner to minimize the sample bias. The essential precautions were taken to minimize the preanalytical variability of BTMs. We collected fasting blood samples in the morning to minimize the effects of diet and diurnal variation [37] that are known to associate with BTM levels in serum. We did not recruit women who had eGFR less than < 30 mL min^{-1} per 1.73 m^2 to avoid advanced stages of chronic kidney disease since it significantly alter the serum CTX and PINP concentrations [38, 39]. We only excluded women who had diseases and on therapies which could affect bone metabolism, but women with other diseases were not excluded. The women in our study had never smoked and were not alcohol users. Among women, only 32% had vitamin D deficiency while 58% of them had sufficient vitamin D levels. These percentages were not significantly different from the values observed from the previous local studies [40]. Furthermore, all subjects were long-term residents of the same geographical location of the country where socioeconomic indices are comparable to the entire country according to the Department of Census and Statistics, Sri Lanka [41]. Crude mortality, infant mortality, life expectancy at birth, literacy, employment, poverty (proportion of people below the national poverty line), and ethnic composition of the area are comparable with national values, and hence our findings are generalizable to the entire country.

Assays were performed in two batches using test kits from the same manufacturer. QC sera (high and low levels) from the test kit manufacturer were used for each separate run and intra- and inter-assay CV % were measured and they

were less than 5%. All the tests were performed by the same technical officer using the same laboratory equipment and conditions using ELISA. A standard curve was developed for each assay to determine the concentrations. However, we detected a wide variation in PINP levels and we did not observe a significant increase in mean PINP levels after menopause. The data generated from this study would be useful as reference data until further data are generated in the country. There is a possible limitation in comparison of the reference data generated from this study with the reference data published by other countries due to variations in assay techniques and conditions. Relatively small sample size and lack of automated assay procedures are limitations of this study. Therefore, we encourage more studies with larger samples which could generate reference data from automated analyzers.

Conclusion

The data from this study indicate age-related trends in bone metabolism among Sri Lankan women. Study subjects were selected from one geographical area and more studies with larger samples and wider representation are needed to reconfirm our results. Our results can be used as a platform for future research in this area and as reference values until further data are generated.

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Declarations

Conflicts of interest None.

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