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ORIGINAL ARTICLE

Reference intervals for CTX and P1NP in a multi-ethnic Malaysian cohort

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Abstract

Background: Well defined reference intervals are central to the utility of serum C-terminal telopeptide of type 1 collagen (CTX) and N-terminal propeptide of type I procollagen (P1NP), designated as reference markers in osteoporosis, and useful for monitoring therapeutic response in that condition. This study reports the reference intervals for plasma CTX and serum P1NP in a multi-ethnic Malaysian population. Methods: Ethnic Malay, Chinese or Indian subjects aged 45-90 years old were recruited from Selangor, Malaysia from June 2016 to August 2018. Subjects with known medical conditions (e.g., bone disorders, malnutrition, immobilisation, renal impairment, hormonal disorders) and medications (including regular calcium or vitamin D supplements) that may affect CTX and P1NP were excluded. Additionally, subjects with osteoporosis or fracture on imaging studies were excluded. The blood samples were collected between 8 a.m. and 9 a.m. in fasting state. The CTX and P1NP were measured on Roche e411 platform in batches. Results: The 2.5th-97.5th percentiles reference intervals (and bootstrapped 90%CI) for plasma CTX in men (n = 91) were 132 (94-175) – 775 (667-990) ng/L; in post-menopausal women (n = 132) 152 (134-177) – 1025 (834-1293) ng/L. The serum P1NP reference intervals in men were 23.7 (19.1-26.4) – 83.9 (74.0-105.0) µg/L, and in post-menopausal women, 25.9 (19.5-29.3) – 142.1 (104.7-229.7) µg/L. Conclusion: The reference intervals for plasma CTX and serum PINP for older Malaysian men and post-menopausal women are somewhat different to other published studies from the region, emphasising the importance of establishing specific reference intervals for each population.

Keywords: bone turnover markers, CTX, P1NP, C-terminal telopeptide of type 1 collagen, N-terminal propeptide of type I procollagen, reference intervals

INTRODUCTION

Bone turnover markers (BTM) are a family of biomarkers that are released during bone remodelling and are classified as markers of bone formation or resorption depending on the phase of the remodelling cycle during which they are formed and released.[1,2] Since BTM measured in blood or urine reflect bone formation and resorption rates, they may aid in the diagnosis and or monitoring of conditions affecting bone turnover such as Paget's disease (of the bone), osteoporosis, metabolic bone disease of chronic kidney disease (CKD-MBD) and osteomalacia.[3-5] Serum C-terminal telopeptide of type 1 collagen (CTX) and N-terminal propeptide of type I procollagen (P1NP) are designated as reference markers in osteoporosis, where they may be useful in monitoring therapeutic response.[6,7] CTX is released during bone resorption and is a specific marker of the degradation of mature type-1 collagen of the bone organic matrix. By contrast, P1NP reflects the rate of synthesis of type-1 collagen, being the terminal extension peptide cleaved during collagen molecule formation.

Central to the clinical utility of these markers is a requirement for well-defined reference intervals to allow optimal clinical interpretation of the markers, and several studies have examined reference intervals mostly in Caucasian populations.[8-16] However, there is currently

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paucity in reference interval data for CTX and P1NP in the multi-ethnic country of Malaysia that is made up mainly of the Malays, Chinese and Indians. This study is a secondary analysis of a previous study,[17] which investigated the prevalence of osteoporosis and osteopenia in an urban adult Malaysian population, in order to derive reference intervals for plasma CTX and serum P1NP.

MATERIALS AND METHODS

Details of the recruitment of study subjects have been published elsewhere.[17] Briefly, subjects aged 45-90 years old and above who were (self-reported) ethnic Malay, Chinese or Indian were recruited from Selangor, Malaysia from June 2016 to August 2018. Using standardised questionnaire, subjects were preliminarily excluded if they reported existing diagnosis of osteoporosis, were taking/ had taken medication for osteoporosis (including calcitriol or alfacalcidol), had a known secondary cause of osteoporosis, subjects with renal impairment (estimated glomerular filtration rate of <60 ml/min/1.73 m^2), known to have or had metabolic bone disorders (including primary hyperparathyroidism), malabsorption, thyroid disease, immobilisation or taking other medication that affected bone homeostasis (e.g. corticosteroids, phenytoin, methotrexate, cyclosporine, oral contraceptive pill) or had had a computerised tomography scan in the past one year. Subjects were also excluded if they took regular calcium or vitamin D supplements as P1NP and CTX levels may be affected. Vitamin D repletion, particularly in the setting of vitamin D deficiency will increase calcium absorption leading to decreased PTH, thus decreasing BTM.

The eligible subjects were then invited for a face-to-face clinical assessment and bone mineral density measurement by dual-energy x-ray absorptiometry. Osteopenia and osteoporosis were assessed using T-scores of the bone mineral density measurements. Additionally, the subjects were screened for vertebral fracture using plain radiograph examination in the antero-posterior and lateral views of lumbar spine, using the Genant classification.[18] Subjects with osteoporosis and vertebral fracture on the imaging studies were excluded.

The CTX in K_2EDTA plasma samples and P1NP in serum samples collected between 8 a.m. and 9 a.m. from fasting subjects were measured in a single run on the Roche e411 platform (Roche Diagnostics, Hamburg, Germany) using the electrochemiluminescence immunoassay. Once collected, the samples were processed and archived at -80°C until batch analysis. For the automated CTX immunoassay, the sample and the biotinylated monoclonal anti-β-CrossLaps antibody are incubated together. Addition of streptavidin-coated microparticles and monoclonal anti-β-CrossLaps antibody labelled with a ruthenium complex result in a sandwich complex bound to the solid phase via the interaction between biotin and streptavidin. These complexes are aspirated into the measuring cell where they are measured by a photomultiplier. P1NP measurement uses similar principle except that a biotinylated monoclonal P1NP-specific antibody is used. The within-run/ between-run analytical imprecision of P1NP and CTX were 2.6%/4.1%, and 2.1%/3.8%, respectively.

The 2.5^{th} and 97.5^{th} percentile values, representing the lower and upper reference limits, along with the 90% confidence intervals were calculated using bootstrap sampling with replacement method. To obtain point estimates and 90^{th} confidence interval for the 2.5^{th} and 97.5^{th} percentile values for each stratified population, 1000 bootstrapped samples of the same size as the original one was generated by sampling with replacement. The 2.5^{th} and 97.5^{th} percentile values from each bootstrapped sample was obtained. The mean of the 2.5^{th}/97.5^{th} percentile was the average of the 2.5^{th}/97.5^{th} percentile values from the 1000 samples. To obtain the 90% confidence interval, the 2.5^{th}/97.5^{th} percentile values from the bootstrapped samples were sorted in ascending order. The 5^{th} and 95^{th} values after sorting were reported. Bootstrapping is done using the choice function in the random module within the Numpy package applied in Python. The choice function allows drawing of the same number of samples from the original dataset with replacement.

Ethics

The study protocol received approval from the Ethics Committee of Universiti Putra Malaysia (reference number: FPSK(FR16)P002). Informed consent was obtained from all subjects in the study.

RESULTS

In total, 411 subjects were interviewed, of whom 21 did not meet the screening eligibility criteria. Four subjects who did not have radiography examination, and three subjects who did not

have bone mineral density measurement were excluded. A further 65 subjects who had fractures (38 self-reported, 27 diagnosed by radiography) and 62 subjects who were diagnosed with osteoporosis/osteopenia by bone mineral density were excluded. The final analysis excluded 27 pre-menopausal women (Figure 1).

After application of the exclusion criteria, 95 men (median age, interquartile range: 58 years, 12.5 years; median body mass index, interquartile range: 25.3, 3.5) and 134 post-menopausal women (median age, interquartile range: 59 years, 11 years; median body mass index, interquartile range: 25.3, 5.4) were included in the derivation of the reference intervals. There were 54 Malays (26 males; 28 females), 104 Chinese (41 males; 63 females) and 71 Indians (28 males; 43 females).

The reference intervals for CTX in EDTA plasma and P1NP in serum are summarised in Table 1. A preliminary statistical exploration (ANOVA) of ethnic differences in the bone turnover markers showed no significant differences between the three ethnic groups except for P1NP in post-menopausal Malay and Chinese women. However, this study was under-powered to make firm conclusion regarding inter-ethnic differences.

DISCUSSION

This study reported the reference intervals for males >45 years and post-menopausal females of a multi-ethnic Malaysian population. The main strength of the study is the detailed clinico-radiological examination of the subjects, which included radiological assessment of the bone mineral density and radiograph of the lumbar spine in excluding subjects with osteoporosis/osteopenia as well as other metabolic bone disorders. Combined with the detailed and structured questionnaire, the subjects included in the reference intervals study are representative of a reference population.

We were able to estimate reference intervals for post-menopausal women and older men, but unable to examine reference intervals for pre-menopausal women due to the small number of subjects in the latter group. There is a need to establish reference intervals in pre-menopausal women also, as pre-menopausal median values are used as treatment targets for anti-resorptive treatment in osteoporosis.[19] In addition, the relatively small number of subjects limited the ability to statistically interrogate ethnic differences in CTX and P1NP with confidence. Due to inter-assay differences,[7] these results should be considered specific to the automated Roche assay.

The plasma CTX reference limits derived from this study were significantly higher for males and for menopausal females, respectively, when compared to the harmonised reference intervals suggested by the Australasian Association for Clinical Biochemistry and Laboratory Medicine for the same assay used in our study (AACB, males <70 years: 100-600 ng/L; post-menopausal females: 150-800 ng/L; Table 2).[14,15] Similarly, the reference limits for serum P1NP in our study were higher for males and menopausal females when compared to the AACB harmonised reference intervals using the same assay as in our study (males 15-80 μg/L and post-menopausal females: 15-90 μg/L).

A recent study reported the reference values (95% confidence intervals of geometric mean)

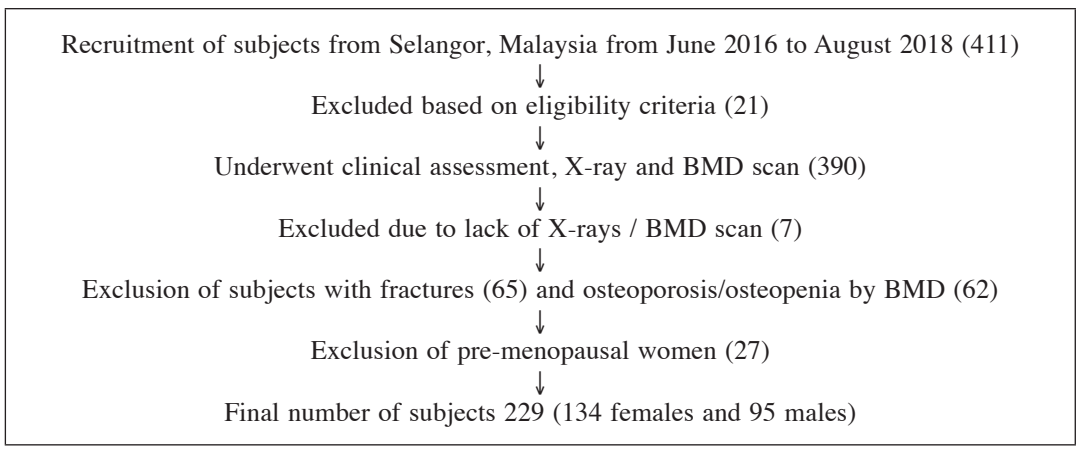

FIG. 1: Recruitment Flow Chart.

TABLE 1: Upper and lower reference limits, representing the mean of the bootstrapped 2.5th and 97.5th percentile values and their 90% confidence intervals of plasma C-terminal telopeptide of type 1 collagen (CTX) and serum N-terminal propeptide of type I procollagen (P1NP) of the reference population.

Percentile values		**2.5th**		**97.5th**	
CTX (ng/L)	No. of subjects	mean	90% confidence	mean	90% confidence
Men	91	132	94-175	775	667-990
Women (post-menopause)	132	152	134-177	1025	834-1293
P1NP (µg/L)					
Men	86	23.7	19.1-26.4	83.9	74.0-105.0
Women (post-menopause)	132	25.9	19.5-29.3	142.1	104.7-229.7

of serum CTX in a young (35-45 years old) Shanghainese (Chinese) population of 112-497 ng/L for pre-menopausal females and 100-612 ng/L for young males.[20] These values were lower than those observed in our study of menopausal females and older males, respectively. P1NP in the Shanghainese population were 13.7-58.7 µg/L for females and 16.9-65.5 µg/L for males, which were also lower than those observed in our study.

The difference in our results in women is likely explained by the difference in age and menopausal status in the two studies. BTM are known to increase around menopause and remain raised compared to pre-menopause.[21,22] The differences in BTM in men are less easily explained by age difference as studies have shown that BTM peak in young adult males and decrease with age till 60 years after which resorption markers may increase somewhat whilst formation markers remain stable.[22,23]

There are other possible causes for the apparent differences in reference intervals among the studies. These include the use of different analytical methods that are not well standardised,[7] but the above two studies used the same method as that used in our study. Ethnic differences, the stringency of the selection criteria of the study subjects, statistical analysis, the demographics of the subjects - with age-related changes observed for these markers may explain these differences.[14,20]

Ideally, subjects used for establishing BTM reference intervals should have normal vitamin D status, a criterion not used for selecting subjects in these studies including ours. Lower vitamin D status may be associated with higher BTM concentrations.[24] Diurnal variation and fasting status are particularly important for CTX measurement.[25] Ideally, samples should be collected in the morning in the fasting state, as was the case in our study. When patient's results are compared with the reported reference intervals, interpretation is enhanced if sample

TABLE 2: Recommended harmonised Australian reference intervals by AACB[15] and population-based reference intervals in a Chinese population by Hu *et al*.[20]. Reference intervals are calculated using mean ± 1.96 SD in Hu *et al*.

Measurand	**Source**	**Subject**	**Age (years)**	**Reference intervals**
CTX (ng/L)	AACB	Men	25-70	100-600
			>70	100-750
		Menopausal women	50-70	150-800
	Hu *et al*.	Men	35-45	100-612
		Women	35-45	112-497
P1NP (µg/L)	AACB	Men	25-70	15-80
			>70	15-115
		Menopausal women	50-70	15-90
	Hu *et al*.	Men	35-45	16.9-65.5
		Women	35-45	13.7-58.7

handling was similar for both studies. The above reported adult reference intervals pave the pathway towards the harmonisation of reporting of serum CTX and PINP results by laboratories within Malaysia using the Roche method.

CONCLUSION

We have reported reference intervals for serum CTX and PINP for older men and post-menopausal women in a multi-ethnic Malaysian population. These intervals are somewhat different to other published studies from the region, emphasising the importance of establishing specific intervals for each population. These results would be useful for interpretation of BTM results in patients in Malaysia.

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