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RESEARCH ARTICLE

ESTABLISHMENT OF REFERENCE INTERVAL FOR SOME BONE TURNOVER MARKERS IN HEALTHY, YOUNG, PREMENOPAUSAL EGYPTIAN FEMALES.

*Rania Mohamed El Sharkawy¹, Noha S. Kandil¹ and Moataza M. Abdel Wahab².

1. Medical Research Institute, Alexandria University, 165 El-Horreya Avenue, El-Hadara, Alexandria, Egypt.
2. Department of Biostatistics, High Institute of Public Health, Alexandria University & FAMCO at UoD.

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Abstract

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Introduction:-

The appropriateness of Laboratory tests selection and reliability of reference interval is crucial for healthcare delivery and clinical diagnosis. This is one of the main functions of clinical laboratory and test manufacturers.¹

With the increasing life expectancy in Middle East countries, osteoporosis should be set as one of the health priorities; addressed by the International Osteoporosis Foundation (IOF). According to the Egyptian national nutrition institute, osteoporosis is a major health problem in Egypt. Among adult females, 12.2% to 15.3% have osteoporosis. This percent reaches 28.4% to third of postmenopausal women.^{2,3,4,5} Moreover, it has been calculated that more than one-quarter of females aged 10-19 years and up to 53.9% of postmenopausal were relatively osteopenic. In the 40 to 50 years age group, 42% of females have low bone mineral density.^{3,5} Besides, almost 50% suffer vitamin D deficiency.⁶

Bone remodeling is a physiological process that occurs continuously in healthy adult bone tissue with coupling of both resorption and formation. Every year around 20% of total bone tissue is replaced.⁸ This process begins in the intra uterine life and continues all through life.⁸ Bone turnover is monitored either by dynamic histomorphometry or bone turnover markers.⁹ The dynamic histomorphometry had some impracticability and imprecision,⁹ Thus the need for development of highly specific, accurate and fully automated noninvasive bone turnover markers was essentially needed.¹⁰

Bone turnover markers (BTMs) are classified as either bone formation, including peptides and enzymes secreted by osteoblasts during bone formation, or resorption markers, which are the degradation products of bone collagen or enzymes secreted by osteoclasts.¹¹ Their level in blood or urine reflect bone formation and resorption rates, respectively, this is affected by several variables either physiological or pathological.^{12,13} These changes in BTM proves the coupling of formation and resorption.

Biochemical markers of bone turnover are used in the initial assessment of osteoporosis, including the suspicion of secondary causes and the identification of patients with rapid bone loss, but the most important function is to monitor the response to treatment.^{14,15,16}

Corresponding Author:- Rania Mohamed El Sharkawy.

Address:- Medical Research Institute, Alexandria University, 165 El-Horreya Avenue, El-Hadara, Alexandria, Egypt.

The biochemical markers levels varies rapidly and correspondingly to bone turnover, thus could be used to assess response and efficacy of pharmacological agents used in treatment of bone diseases such as osteoporosis.¹⁷ There are so many causes of high BTM levels other than osteoporosis, such as hyperparathyroidism, myeloma or thyrotoxicosis.^{18,19} Vitamin D insufficiency as defined by 25(OH)D levels between 30 and 50 nmol/L is associated with an increase in parathyroid hormone (PTH) levels that is attributed to decreased calcium absorption. This may indicate secondary hyperparathyroidism in postmenopausal women.¹⁵

The aim of antiresorptive treatments is to reduce BTMs to the lower part of the healthy premenopausal reference interval.²⁰ Thus the lower limits of BTMs became more important than previously, as not only has it been questioned if decreases of BTMs below the reference interval could harm microdamage repair of the bone, but they also may be helpful in taking decisions in clinical practice, such as whether or not to give the next dose of a potent antiresorptive drug.^{21,22}

Therefore accurate and robust reference intervals for bone turn over markers become mandatory.²³ Unfortunately the available reference ranges are released through commercial laboratory kits, which lack standardization and don't take in consideration preanalytical variables.²⁴ As a result establishment of population based reference intervals that takes in consideration the preanalytical variables including age is considered a priority.

Several BTM reference intervals for healthy premenopausal women have previously been established.^{17, 25,26, 27} In the last few years, reference intervals in premenopausal women from different countries in Europe (UK, France, Belgium and Denmark)^{27,17} and the US²⁸, have been reported. In addition, data from Germany on men and women have been recently published.²⁹ In Spain, there are data on Procollagen I intact N-Terminal (PINP) and Carboxy-terminal collagen cross links (CTX-I) in older men and in postmenopausal women^{30,31,32} as well as on CTX-I in a subset of 50 premenopausal women.³³

To our knowledge up till this moment there is no published data regarding reference values in premenopausal Egyptian females. Thus establishment of a valid and robust reference interval will allow comparing BTMs among different studied groups, differentiate between normal and osteoporotic patients³⁴ also predict the likelihood of fracture in osteoporotic patients and monitoring the suggested effective treatment response which cannot be done by bone mineral density as the latter can't detect minor and acute changes caused by therapeutic agents.^{17,35}

It is reported that nearly 70-80% of adult BMD is attained by the age of 18 years. One study indicated that there was no significant bone loss in 30- 45 year-old women who were calcium balanced and of optimal bone health; we chose women aged 30-45 yrs for reference interval calculations because these women have achieved peak bone mass and are not yet perimenopausal.³⁶ This study aims to establish robust reference intervals for some bone turnover markers in healthy premenopausal Egyptian females between the age of 30-45 yrs.

Material and method:-

Study population:-

This study protocol was approved by the Medical Research Institute, Alexandria University Ethics committee in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

This cross sectional study was conducted in Alexandria governorate. The specimens used in this study were obtained from an age stratified random sample. All subjects provided written consent.

The clinical and Laboratory Standards Institute (CLSI) released the EP28-A3c document that recommends 120 reference values as an adequate sample size for each reference population or subclass. This study included one hundred and fifty one healthy, non pregnant, regularly menstruating (10 or more periods per year), premenopausal females who were recruited as volunteers from the outpatient clinic of the MRI, Alexandria university, family planning clinics as well as volunteers from the medical, nursing, or secretarial staff of the units or their relatives from April 2015 till November 2015. The sample of this study was age-stratified random sample, the participants where 30-45 years of age; as women in this age have their bone turnover markers stabilized, making them suitable for calculation of clinically useful reference ranges for BTMs.²¹ (mean age 36.6 ± 4.5), with a body mass index (ranging from 17-27 Kg/m²) (mean 22.8), each having regular cyclic menses (12/year) , Weighed between (55-85Kg)

Women under this range were excluded as they might have elevated BTMs due to skeletal immaturity, while those above 45 were excluded as they tend to have increased markers in the perimenausal period with normal estrogen levels.²⁵

Exclusion criteria:-

None of the subjects taking part in the study had cancer, chronic disease, or any medical condition known to affect bone metabolism. All subjects were not receiving, within a year of starting the study, any bone metabolism affecting medications such as : selective estrogen receptor modulators , phytoestrogens , anticonvulsants , calcitonin , anabolic agents , steroids , vitamin D supplements , hormonal treatment for endocrinopathies , NSAID and depo-provera as well as calcium supplements, serotonin uptake inhibitors, L-troxin , antihypertensive drug and antiresorptive treatments . Subjects with history of alcoholism, smoking, recent clinical fracture, pregnancy, lactation, renal and endocrinal disorders were excluded. All participants were subjected to a detailed questionnaire concerning demographic data, medical history, lifestyle, etc.

Sample collection:-

Venous blood were taken from all subjects on a gel separator tube, as well as urine sample after overnight fasting from 8am till 10 am in the second week after menstruation . Venous samples were kept for 10 minutes at room temperature and centrifuged under standard guidelines (2500 g) for 10 min and serum was separated and stored at - 80 C , no freezing and thawing is allowed only once upon analysis.

CTX has significant diurnal rhythm with a peak in the morning and a nadir in the afternoon. Moreover the circulating levels are influenced by food intake. In non fasting conditions, the peak is reached at night, followed by a significant decrease and nadir from 1100 to 1500 h. Therefore all the samples were collected after overnight fast in the morning within a standardized time period ideally from 8 and 10 a.m. thus reducing the day to day variation^{12,37}

Analytical standardization and biochemical analysis:-

Standard Operating Procedures were followed during pre-analytical and analytical phases of the study with two levels of internal quality control included in each run. Corrective actions were done based on Westgard rules . The following biochemical markers were measured at the same time point using a single lot of reagents and the same batch.

Alkaline phosphatase , 25 (OH) vitamin D , PTH and CTX were measured in serum by immunoenzymatic assay on Cobas 411e (Roche diagnostics) with an intra-assay CV of 0.6-0.9 % , 1.7-7.8% , 1.4-7.4% and 1.0-4.6% the interassay CV was 0.9- 2.4 % , 2.2- 10.7% , 3.1-9.4% and 1.6-4.7% while the limit of detection (LOD) was 5 U/L , 3.00 ng/ml (7.5 nmol/L), 5.5-2300 pg/ml (0.583-244 pmol/L) , 0.01 ng/ml (10 pg/ml) respectively.

While Calcium and Phosphorus were carried out on Bekman coulter Olympus with an intra-assay CV of 0.46- 0.55% and 0.6-1.9%, the interassay CV of 0.68-1.34% and 0.9-2.1% respectively. The limit of detection (LOD) was 0.13mg/dl and 1mg/dl respectively.

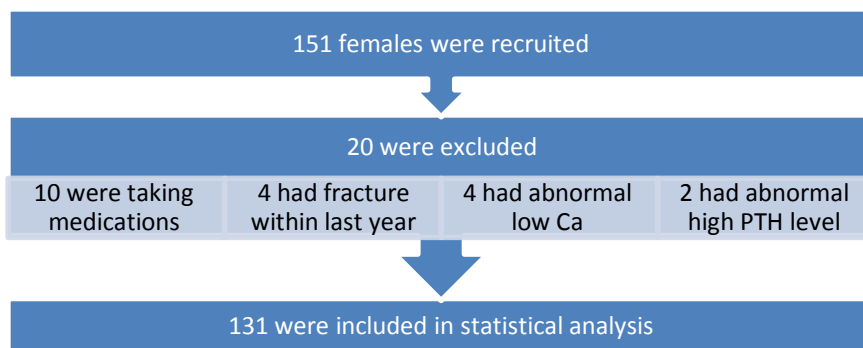
Statistical analysis:-

Statistical analyses were performed using SPSS version 22.0. The data were analyzed using nonparametric statistical techniques.

The initially collected 151 values were tested for outliers (outliers were considered for data point below $Q1 - 1.5 \times IQR$ or above $Q3 + 1.5 \times IQR$), outliers were investigated and overall, 20 cases were excluded from all the parameters for the reasons mentioned above.

Results:-

In this study one hundred fifty one females participated, of these women; four were excluded for having a fracture within one year, ten were excluded for medication intake (vitamin D, calcium and oral contraceptive pills), 4 were excluded for having abnormally low calcium levels and 2 were excluded for having abnormally high PTH level. 131 women completed all lab tests and the questionnaire fig(1)

**Fig (1)**

The age selected was between 30-45, the sample was restricted to this age group and our results showed no statistically significant correlation between age and any of the studied parameters except phosphorus (weak indirect correlation $r_s = -0.189$) and Vit D (intermediate correlation $r_s = -0.58$); thus the norms were established from the whole participants for each of the 6 parameters, furthermore VitD values were presented for the age categories 30-<35yrs ($n=45$), 35-<40yrs ($n=42$) and 40-45 yrs ($n=44$) as the values of VitD varied between the 3 age categories using Kruskal Wallis test. And phosphorous was presented by the age groups <40 ($n=87$) and 40+ years ($n=44$) as values differed using Mannwhitney U test.

Guided by the Clinical and Laboratory Standards Institute (CLSI) analysis of reference values¹, and Adult Reference Intervals on the Basis of the Canadian Health Measures³⁸, values were presented as median (90%CL) and the Survey reference values were constructed using 2.5th and 97.5th percentiles as lower and upper reference limits. The 90% confidence intervals around the estimates of the limits of the reference intervals were constructed by bootstrapping of 500 random samples of the same size.³⁵

Table (1) displays the range and Median (90%CI) values of the 6 parameters (Ca, Phosphorous, Alkaline phosphatase, PTH, CTX and Vit D) for the studied sample ($n=131$). The Coefficient of Variation (CV) of all the studied parameters lay in the accepted values of the Canadian quality standards except CTX which exceeded 33.3%. In the present study the premenopausal women had a median value for calcium 9.4 mg/dl (9.3-9.4), phosphorous 3.5mg/dl (3.4- 3.6), alkaline phosphatase 66 U/L (64-68), PTH 44 pg/ml (39.1- 45.6), CTX of 188 pg/ml (178 – 191pg/ml) and vitamin D 16 (15-16) ng/ml.

Table (1):- Reference intervals for 6 biochemical BTM

Parameter	Range	CV	Median (90%CI)	Bootstrap no. of samples	Lower value 90% CI	Higher value 90% CI	Mean \pm SD	Normal values set by manufacturer
Calcium (mg/dl)	8.5-10.5	2.7%	9.4 (9.3-9.4)	500	8.5-8.7	9.9-10.41	9.34 \pm 0.36	(min-max)8.6-10
Phosphorous(mg/dl)	2.5-4.7	10.0%	3.5 (3.4-3.6)	500	2.5-2.73	4.37-4.64	3.54 \pm 0.49	2.5-4.8
Age(yrs)								
30-<40(n=87)	2.5-4.7	8.3%	3.6 (3.5-3.7)	500	2.5-2.82	4.38-4.7		
40+(n=44)	2.5-4.4	10.6%	3.3 (3.2-3.5)	500	2.5-2.8	4.1-4.4		
Alkaline phosphatase (U/L)	41-99	13.6%	66 (64-68)	500	41.0-44.3	95.0-99.0	67.89 \pm 14.6	35-104
PTH (pg/ml)	21-77	20.5%	44 (39.1-45.6)	500	21.78-24.2	71.35-75.5	45.00 \pm 14.3	(mean) 31.3
CTX(pg/ml)	91-430	42.8%	188 (178-191)	500	91.6-97.95	396.1-428.8	213.9 \pm 95.34	299
Vitamin D (ng/ml)	10-24	15.6%	16 (15-16)	500	10.0-10.0	21-23.7	15.70 \pm 3.40	21.6
Age (yrs)								
30-<35(n=45)	10-24	9.7%	18 (17-19)	500	10.0-13.34	22.8-24		
35-<40(n=42)	10-23	15.4%	17 (16-17.5)	500	10.0-11.0	20.9-23		
40-45(n=44)	10-16	14.4%	13 (12-14)	500	10.0-10.0	15-16		

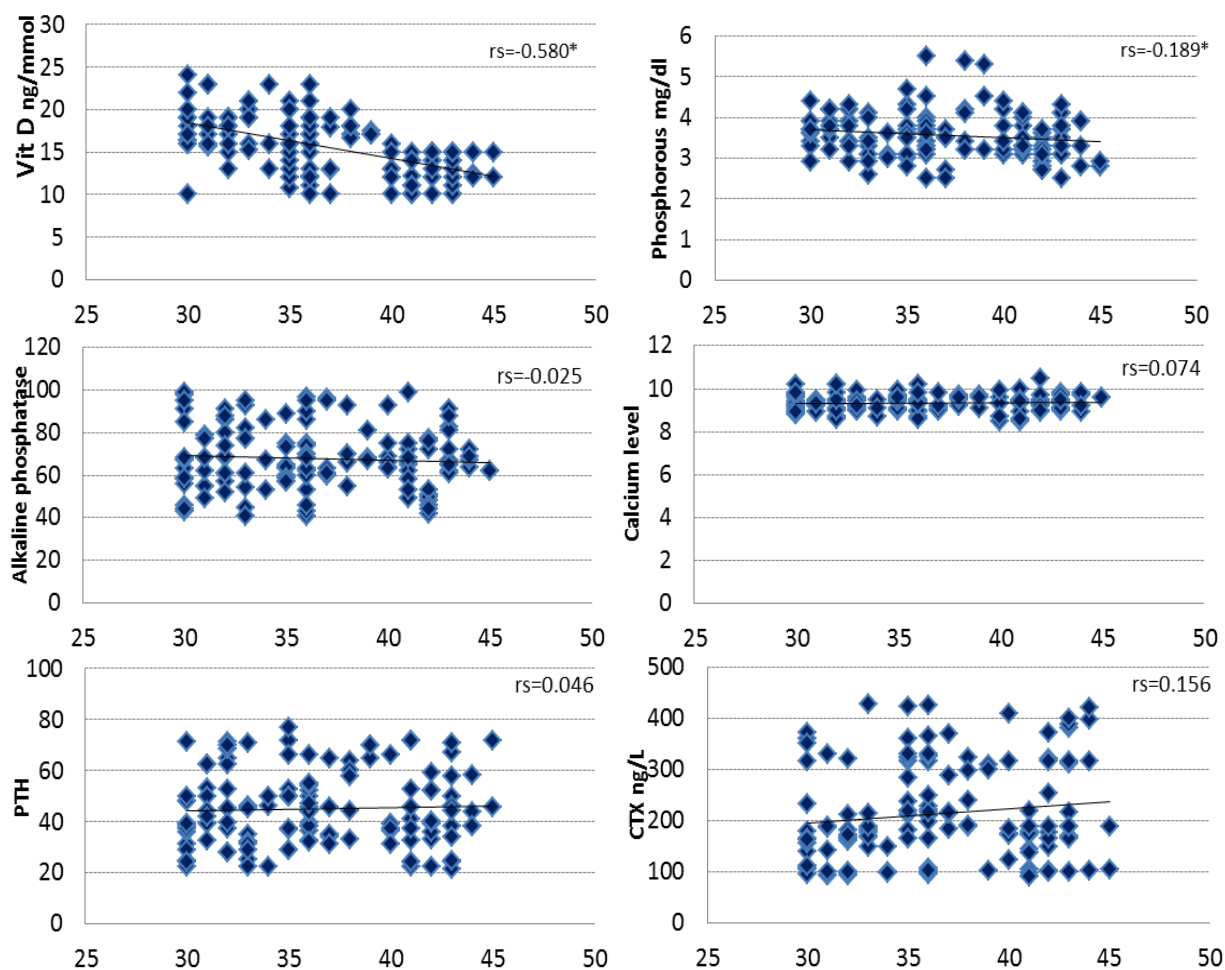


Figure 2:- Correlation between age and each of the 6 biochemical BTM

The new values for Ca and phosphorous did not statistically differ from the manufacturer though accepted higher upper limit (10.4) for Ca and a lower upper limit for phosphorous (4.64).

The new values for ALP as well did not statistically differ from the manufacturer. Concerning CTX and Vit D the new averages were lower than that set by the manufacturer, the differences were statistically significant ($t=-10.2$, $p=0.0001$ for CTX, and $t=-19.7$, $p=0.0001$ for Vit D). While, the new average for PTH was higher than that of the manufacturer, the difference was statistically significant ($t=10.9$, $p=0.0001$).

Discussion:-

The importance of establishment of a reliable reference interval is because most medically important decisions are based on comparing the laboratory tests with their reference intervals.

In fact most reference intervals used in many laboratories are those of the manufacturer. These reference intervals don't represent the actual population and thus needs to be verified or established for the relevant population.

Bone turnover markers are convenient tool used to assess systemic bone turnover rates, both in clinical practice and in clinical trials.

It has been recommended that normal reference ranges for BTMs shall be established in large samples of healthy premenopausal women.¹⁷ However, the reference ranges currently reported by commercial laboratories are often not rigorously established and fail to account for relevant preanalytical variables.¹² Some of these variables including fracture, metabolic bone disease, chronic disease (including; malignancies, rheumatoid arthritis, diabetes), abnormal laboratory results of serum calcium, phosphorous.¹⁷

In the last few years many reference intervals in premenopausal women in different countries like UK, France, Belgium, Denmark, US and Germany have been established and to our knowledge this is the first for Egyptian population.^{17,27,28,29}

The present study aimed to establish robust reference interval of some bone turn over markers in healthy premenopausal females aged from 30 to 45 years with mean age 36.6 ± 4.5 years. This age range was taken because BTM levels were considered to be stable by Glover et al. in their study assessing the reference interval in premenopausal British women which is beneficial in the assessment of response to antiresorptive therapy in postmenopausal osteoporosis.⁽¹⁷⁾

One of the main limiting factors was to include larger number of healthy individuals based on the literature exclusion criteria.¹⁷ The CLSI released EP28-A3c document to help researchers by trying to unify reference interval studies. However bone turn over markers required detailed history due to many factors influencing their level.

The women who participated in the current study fulfilled the detailed inclusion criteria and provided additional information that was used to characterize the population as a whole.

The exclusion criteria to the studied group included: vitamin D supplements, history of fracture, smoking, consumption of alcohol, metabolic bone disease and chronic diseases.¹⁷

All these preanalytical variables contribute to the BTM markers in premenopausal females.

This study includes 151 premenopausal females who reached 131 after application of the above exclusion criteria. Calcium, phosphorous, Alkaline phosphatase, 25 (OH) vitamin D, PTH and CTX were done to all the candidates of this study.

As shown in table (1) the Coefficient of Variation (CV) of all the studied parameters lay in the accepted values of the Canadian quality standards except CTX which exceeded 33.3%. In the present study the premenopausal women had a median CTX value of 188 ng/L (178 - 191 ng/L). This is in accordance with Gossielet al 2014²⁰ who reported a median CTX value of 190 ng/L.

Although these values were lower than those reported by Jenkins et al^{xxxix}, De Papp et al¹⁰, Glover et al²⁵, Martinez et al³⁰ and Lenora et al^{xi} where the median CTx values were 289, 280, 300, 387, 312 ng/L respectively. These discrepancies could be attributed to inter-assay variability of BTM, which represents a significant problem in their clinical application. Variability of collection as well as diurnal variability and intra individual variability have been described and lead to wider reference intervals and less diagnostic certainty. It is known that circadian variation is greater for bone resorption than bone formation. For example serum CTX exhibits a circadian rhythm with an amplitude of 80% of the 24 hour mean.^{xli}

Also Figure (2) shows scatter plots for the age range 30-45 years, showing that VitD values decrease by age (intermediate inverse correlation) and phosphorous values as well decrease by age but with a weak inverse correlation. Otherwise the correlation between age and the other 4 parameters was not statistically significant.

Other studies shows negative correlation between BTM and age, but these studies included women aged from 21-54, so they included women with skeletal immaturity.^{35,xlii} Other study included the same age group as ours and reported higher CTX between the age of 30-34 suggesting they haven't yet reached skeletal maturity.²⁵ This highlights the need for determination of the age at which each bone mass is reached. In our study this problem was unlikely and therefore the selected age group was the optimum for bone turnover markers reference interval establishment.

Comparing the norms resulted from the current research with those set by the manufacturer it was found that:

The new values for Ca and phosphorous did not statistically differ from the manufacturer though accepted higher upper limit (10.4) for Ca and a lower upper limit for phosphorous (4.64).

The new values for ALP as well did not statistically differ from the manufacturer. Concerning CTX and Vit D the new averages were lower than that set by the manufacturer, the differences were statistically significant ($t=-10.2$, $p=0.0001$ for CTX, and $t=-19.7$, $p=0.0001$ for Vit D).

While, the new average for PTH was higher than that of the manufacturer, the difference was statistically significant ($t=10.9$, $p=0.0001$).

The low level of vit D in the Egyptian population may be due to lack of direct sun exposure and reduced intake of vit D supplements as well as reduced calcium, this decreased vit D stimulates the parathyroid gland to secrete more parathyroid hormone and this explains the increased level of PTH which exceeds the manufacturer reference range.

Conclusion:-

BTM are widely used in therapeutic monitoring of osteoporosis or other bone diseases as well as having a significant impact in research. Our trial for establishment of reference interval in Egyptian population may help in harmonization of reporting by Egyptian laboratories. The strength points of this study are the standardization of the preanalytical and analytical conditions with adherence to the CLSI guidelines recommendations together with considering seasonal variation. The small sample size and the absence of the bone mineral density data with relatively low level of 25OHD were the limitations of this study. For a new or improved assay for BTM it is important to include a larger sample size with for a clinical reliable reference range to be used in assessment of the efficacy of the antiresorptive therapy.

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