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Analytical

Age- and sex-matched reference curves for serum collagen type I C-telopeptides and bone alkaline phosphatase in children and adolescents: An alternative multivariate statistical analysis approach

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ABSTRACT

Introduction: The availability of pediatric age- and sex-matched reference curves for bone markers is essential for appropriate assessment of bone turnover and treatment follow-up of bone disorders. The aim of this work was to obtain updated reference equations for collagen type I C-telopeptides (CTX-1) and bone alkaline phosphatase (BAP) by using an alternative statistical approach.
The study included 1502 Italian pediatric subjects from 6 months to 16 years of age (686 females and 816 males) subjected to CTX-1 and BAP measurement during a six-year period.

Methods: The unselected population of patients was used for the calculation of age- and sex-matched reference curves by using a multivariate statistical analysis after an appropriate validation with a selected population of 184 healthy subjects (6 months-16 years; 88 females and 96 males). The effect of age, sex, puberty based on Tanner stage evaluation and anthropometrics on the variations of the two bone markers was then studied.

Results: Pediatric reference curves were obtained for CTX-1 and BAP from 3465 results retrieved by our Laboratory Information System. The equations for the calculation of reference values were obtained for boys and girls. The two bone markers markedly varied according to age, sex and pubertal stage with females displaying higher values during Tanner stages II and III and males during stages III and IV.

Conclusions: The application of a novel statistical approach provided reference curves for CTX-1 and BAP. This method, moreover, could be applied in pediatrics to obtain reference intervals for other biomarkers.
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1. Introduction

Serum collagen type I C-telopeptides (CTX-1) and bone alkaline phosphatase (BAP) are specific and sensitive markers of bone resorption and formation respectively [1]. Indeed, CTX-1 are short fragments of C-terminal domains of type I collagen, cleaved by osteoclasts during collagen degradation [2] while BAP is a glycoprotein involved in bone mineralization produced by osteoblasts and reflecting their degree of activity [3]. Both CTX-1 and BAP are released into circulation where their concentrations provide information regarding ongoing bone turnover [4]. The evaluation of biochemical bone markers is a useful tool for dynamic assessment of bone turnover, that, in association to the more "static" densitometry measures obtained by dual energy x-ray absorptiometry (DXA) or by the quantitative computed tomography (QCT) provides a more complete evaluation of bone mass status [5]. Moreover, measurement of biochemical bone markers can be repeated at short intervals allowing early detection of effects of disease or treatments, whereas DXA or QCT need longer intervals to reveal physical and densitometric changes in the bone.

Both CTX-1 and BAP concentrations can be easily determined by many commercial immunoassays in the routine laboratory [2]. Nevertheless no single test fulfills all the criteria as an ideal marker [6]. In particular in children no marker can be considered specific for any of the three different biological processes of modeling, remodeling and endochondral ossification at the growth plates in bone [7]; however, the combined use of at least one formation and one resorption marker could help providing a more complete picture of bone turnover [8].

Changes in bone markers are influenced by many physiological and pathological factors. In children they are expected to have higher values due to high skeletal growth velocity and rapid bone turnover, in particular in infancy and in mid-puberty when linear growth is maximized [9]. The availability of reference curves is thus an essential pre-requisite for evaluating children with bone diseases. To

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date, very few reports on pediatric reference intervals for CTX-1 and BAP are available in the literature [2] and they often suffer from heterogeneity of analytical methods or pre-analytical factors or are derived from low subject numbers [10–12]. In addition, very few reports use appropriate curve-fitting procedure which is an essential statistical approach for bone markers data [1]. International Federation of Clinical Chemistry (IFCC) and Clinical Laboratory and Standards Institute (CLSI) guidelines (C28-A3) recommend the use of local reference intervals related to the patient's population observed in a minimum of 120 healthy subjects preferably obtained using the nonparametric ranking method [13]. In addition, for pediatrics, various subpopulations (infant, pre-pubertal, pubertal and post-pubertal) should be considered as puberty is expected to play a critical role in determining variations of bone markers concentration [3]. Moreover, sex-related reference intervals should be obtained at least in the pubertal period taking into account the differential timing in the onset of puberty in the two sexes. As the collection of such a large number of samples from healthy individuals in children is difficult, time-consuming and expensive, to overcome this limitation an alternative approach consists of using the large number of laboratory results that are recorded by laboratory information systems (LIS) and to elaborate them with specific statistical methods [14,15].

The aim of this work was to obtain updated age and sex-matched reference equations for CTX-1 and BAP by reviewing the results obtained in our centre from an unselected population of pediatric patients from 6 months to 16 years of age. This work provided updated information of bone metabolism in support to appropriate clinical and densitometric evaluation of bone mass in the pediatric age. Furthermore the influences of pubertal stage and anthropometrics in the variation of these two biomarkers were investigated.

2. Material and methods

2.1. Subjects

The study included 1502 patients (age 6 months–16 years; 686 females and 816 males) diagnosed with various conditions potentially affecting bone health followed at the G. Gaslini Institute and subjected to routine CTX-1 and/or BAP measurement from April 2009 to May 2015. In 900 patients (448 females and 452 males) pubertal stage was evaluated according to Tanner criteria by the same endocrinologists (NDI and EB) [16]. A control group was selected from healthy subjects seen as outpatients for routine investigations requiring blood sampling; they all underwent height and weight evaluation and were accurately selected for BMI (between ± 2 SDs) and any absence of diseases or therapies that could interfere with bone resorption. Parents'/guardian's written informed consent and child's assent were given before any study-related procedure.

2.2. Laboratory analysis

Peripheral blood was drawn in a vacutainer with clot activator and silica gel between 8.00 and 10.00 a.m. to avoid any bias from diurnal variation. Leftover serum after routine analysis was used for the present study. Serum was obtained by centrifugation at $4000 \times g$ for 5 min at room temperature and stored at -20 °C until analyzed. CTX-1 and BAP serum levels were measured by using the Serum Crosslaps (Serum Crosslaps ELISA, Immunodiagnostic System Ltd., Boldon, UK) and the Microvue BAP ELISA (Quidel Corporation, San Diego CA, USA) kits respectively, following the manufacturer's instructions automated on a DSX system (Technogenetics-Bouty, Milano, Italy). Intra-assay imprecision in our laboratory was less than 3% and 4% for CTX-1 and BAP respectively, and inter-assay variation was less than 5% for both assays.

2.3. Statistics

Descriptive statistics were reported in terms of medians and percentiles for continuous variables. Normality of variable distribution was tested using the Kolmogorov–Smirnov test.

Cases and controls were compared by using the General Linear Model (GLM) univariate procedure.

In order to define the reference curves for both CTX-1 and BAP with age as a continuous variable, a multivariate statistical analysis approach was provided, using the SPSS Curve Estimation Regression Model. For each selected model the residuals (observed value of the dependent variable minus the model predicted value) of the regression model were plotted and analyzed. The variance was standardized in order to facilitate the interpretation of the plot.

The model was based on the relative goodness of fit where a single dependent variable is predicted by a time variable. Comparison of CTX-1 and BAP concentrations among the Tanner stage groups was made by the non-parametric analysis of variance (Kruskal–Wallis test). Reference intervals for CTX-1 and BAP for the different Tanner stage groups were calculated nonparametrically by obtaining the 10th and 90th percentiles.

Correlation between CTX-1 and BAP and anthropometric measurements was evaluated in the control group by the parametric Pearson's correlation coefficient (r) and interpreted according to Swinscow [17] as follows: $r < 0.20$: very weak; $r \geq 0.2$–0.39; weak, $r \geq 0.4$–0.59: moderate; $r \geq 0.6$–0.79: strong; $r \geq 0.8$ very strong correlation.

A multivariate statistical analysis for the evaluation of the effect of puberty on the variation of the two biomarkers was performed. All statistical tests were two-sided and a p value less than 0.05 was considered as statistically significant.

The software MedCalc (MedCalc Software bvba, Ostend, Belgium) and the Statistical package for Social Science (version 13.0; SPSS IBM) have been used for all the analyses.

3. Results

By retrieving the data of CTX-1 and BAP from our LIS, 3465 results were obtained for both analytes (1557 from females and 1908 from males). The results were not normally distributed. A control group was obtained from a population of 184 healthy subjects (age 6 months–16 years; 88 females and 96 males) equally distributed in the different age classes. Data obtained from the control group were normally distributed. To identify univariate outliers the model converted all of the scores for the dependent variable to standard scores (z-scores) and a single case was identified as an outlier if its standard score was ± 3.0 or beyond. After outlier exclusion the results obtained from the patients' group were compared to those obtained from the control group by using the GLM. After adjusting for age no significant differences were found between case and control groups in respect to CTX-1 or BAP. The patients' group was then used to obtain age and sex-matched reference curves.

The SPSS curve fit model showed that the cubic model followed the observed data points fairly well during the observed time period. This statistical approach confirmed that the cubic model was considered as the curve of best fit. The curves and the related equations for the median and the 10th and 90th percentiles obtained are shown in Fig. 1 and Table 1 respectively.

The variation of CTX-1 and BAP concentrations among the different Tanner stages in males and females has been then analyzed (Fig. 2). In males a significant difference could be observed between stage I and other Tanner stages ($p < 0.001$) for both BAP and CTX-1. The highest concentrations for both markers could be found in stage III. In females a significant difference could be observed between stage I and other Tanner stages ($p < 0.001$) for both BAP and CTX-1. In contrast to males, the highest concentrations for both

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