BONE HEALTH IN CHILDHOOD: USEFULNESS OF BIOCHEMICAL BIOMARKERS

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Abstract

Development of the human skeleton begins in early embryonic life and continues through childhood into early adulthood. The acquisition of peak bone mass during these vulnerable periods may impact on skeletal fragility in later adult years. Once the skeleton has reached maturity, bone remodelling continues with periodic replacement of old bone with new at the same location. Bone biomarkers are specifically derived biomarkers that reflect both formation by osteoblasts and resorption by osteoclasts. Children have significantly higher concentrations of bone biomarkers than adults due to both skeletal growth and rapid bone turnover during childhood and adolescence. Biochemical assessment of markers of bone turnover may be important in the diagnosis, prognosis and management of metabolic bone disease. This review will discuss the various serum bone markers used for assessing bone health and the factors that influence their utility.

A. Introduction

A1. Bone Biology

Bone organizes itself to form a rigid skeleton and thus provides a framework for the body. It is a specialized, mineralized connective tissue that consists of a mainly organic collagen matrix and a mineral phase together with bone cells. (Vasikaran *et al*., 2008) The cellular composition of bone includes osteoblasts, osteocytes, bone lining cells and osteoclasts, and its matrix contains both organic and inorganic components (Downey *et al*., 2006). While bone cells or osteocytes and bone matrix proteins such as collagen are produced by actively synthesizing osteoblasts, bone resorption involves terminally-differentiated, multinucleated osteoclasts (Vasikaran *et al*., 2008)

A2. Peak Bone Mass and Bone Remodelling

Development of the human skeleton begins in early embryonic life by the differentiation of cells into chondrocytes (cartilage-forming cells). The velocity of skeletal growth is high immediately after birth, slows down for a short while and increases again later in infancy. At this

stage, longitudinal bone growth is more rapid in the appendicular (arms and legs) than axial skeleton (trunk) and remains so until puberty whereupon axial bone growth accelerates and long bones decelerate until the epiphyses fuse (Prentice *et al.,* 2006). The acquisition of peak bone mass also known as full adult bone mass is important for optimal skeletal health. It represents the ability to acquire the full genetic potential for bone strength, which can be considered as bone of a size and mass which has not been inhibited by nutrient insufficiencies and/or sub-optimal mechanical loading (Ho *et al*., 2005). It is achieved almost fully by late adolescence or early adulthood with more than one quarter of this being accrued during the 2 years around peak pubertal height velocity (Specker *et al.,* 2002). Chronic under-nutrition, as well as inadequate intakes of bone forming minerals (Ca, Zn) and micronutrients (vitamin D) can impair growth and hence bone production. Longitudinal studies helped determine that bone mass tracks through childhood and adolescence, and any interruptions in the normal trajectory of bone mass accretion during these vulnerable periods may impact on skeletal fragility in later adult years (Atkinson *et al.,* 2008).

Remodelling of bone starts in fetal life, and once, the skeleton is fully developed in young adulthood, accounts for most of the metabolic activity in bone. Bone remodelling or turnover is the continual process by which the skeleton is renewed through resorption (breakdown) of existing bone and formation of new bone. Since these processes occur in sequence, there is a temporary deficit of bone tissue called remodelling space (Prentice *et al*., 2006). There are several conditions such as, somatic growth, aging, metabolic bone disease, states of increased or decreased mobility, and therapeutic interventions, which can disturb the bone remodelling process producing a temporary phase lag while the system readjusts to the new remodelling rate often referred to as the “remodelling transient”. Children, with their rapid bone growth and reshaping, have larger remodelling spaces than adults and a shorter remodelling period (several weeks compared with 6-18 months for adults), therefore increasing the probability of detecting remodelling transients (Prentice *et al.,* 2006; Atkinson *et al.,* 2008; Seibel *et al.,* 2005) . The measurement of biochemical markers in both blood and urine is a means of assessing bone remodelling processes, whereas bone densitometry is an assessment of peak bone mass.

A3. Assessment of Bone Health

Bone mineral status in healthy individuals can be estimated using several densitometry techniques including the gold standard dual-energy x-ray absorptiometry (DXA) (Laskey *et al*., 2004; Ginty *et al.,* 2005). DXA quantifies fat, lean and bone mineral masses and is the only widely used technique than can measure regions in both axial and appendicular skeleton.

DXA is widely used for the measurement of bone status in children (Compston *et al.,* 1995). It has several advantages including: high accessibility; low ionizing radiation dose (equivalent to about less than 1 day naturally occurring radiation); precision; and more recently the availability of pediatric reference data for measures of whole body, lumbar spine and hip using Hologic DXA machines (Kalkwarf *et al.,* 2007) and a different reference data base using Lunar DXA machines (Van der Sluis *et al.,* 2002). In children, total body bone mineral content (BMC) is the preferred measure over bone mineral density (BMD), which is a two-dimensional measure (not volumetric) calculated as BMC divided by bone area. Consequently, BMD is not perceived to be a sensitive measure of bone acquisition in childhood due to the continuous change in bone geometry and the inability to differentiate change due to bone growth or mineralization (Prentice *et al.,* 2005; Goulding *et al*., 1996). Moreover, with increasing awareness of bone disorders , the interest in and the need for effective measures used in

screening, diagnosis and follow up of such pathologies have obviously grown. Thus, in contrast to static measures obtained from imaging techniques such as DXA, laboratory tests play an integral role in the assessment and differential diagnosis of metabolic bone disease, especially since they are non-invasive, comparatively inexpensive and when applied and interpreted correctly, helpful tools in the diagnostic and therapeutic assessment of metabolic bone disease (Seibel *et al*., 2006).

Biochemical markers of bone metabolism help to detect the dynamics of the metabolic imbalance itself (Seibel *et al*., 2005). Currently, bone markers include both enzymes and peptides derived from cellular and non-cellular compartments of bone and are classified according to the metabolic processes they reflect, namely bone formation markers and bone resorption markers (Seibel *et al.,* 2005 & 2006; Vasikaran *et al.,* 2008).

Circulating or excretion bone markers are high in the first 2 years of life, decrease in childhood, increase again during puberty and decrease thereafter to the lower levels seen in adulthood (Prentice *et al.,* 2006). Thus, the concentrations or outputs of these markers are significantly affected by both physiological (age, gender, growth velocity, nutritional status and pubertal status) and pathological conditions (prematurity, growth hormone deficiency, malnutrition, malabsorption, vitamin D deficiency, and metastatic bone disease) especially in children who, compared to adults have dramatically elevated bone marker concentrations due to high skeletal growth velocity and rapid bone turnover (Fares *et al.,* 2003; Yang &Grey*,* 2006; Atkinson, 2008). Biochemical assessment of markers of bone turnover may be important in the diagnosis, prognosis and management of bone metabolism disease (Vasikaran *et al.,* 2008). These markers can be assessed in both serum and urine samples. Compared to adults, in children, it is difficult to collect reliable serial samples especially from complete time urine collections. To adjust such data, they are often normalized and reported as analyte/creatinine ratio (Yang &Grey*,* 2006). This ratio itself is limited by considerable biological variation and change in creatinine levels with age as muscle mass increases (Szulc *et al*., 2000). For this reason it is preferable to measure bone biochemical markers in serum samples. This review will therefore discuss the various serum bone markers used for assessing bone health and the factors that may influence their utility.

B. Bone Formation Markers

Active osteoblasts produce bone formation markers. They are expressed during different phases of osteoblast development and are considered to reflect different aspects of osteoblast function and of bone formation. Serum markers of bone formation, include bone alkaline phosphatase, osteocalcin and procollagen I propeptides (Vasikaran *et al.,* 2008). Biological variation necessitates that particular attention be paid to the pre-analytical variables such as diurnal variation, stability of the marker and tissue specificity. Some of these are summarized in table 1A.

B1. Bone Alkaline Phosphatase

In serum, total alkaline phosphatase (AP) activity is a combination of four isoenzymes; liver/bone, placental, intestinal, and germ cell. During skeletal growth in children and adolescents the bone alkaline phosphatase (BAP) predominates and contributes up to 90% of the total alkaline phosphatase. (Van Hoof *et al.,* 1990; Magnusson *et al.,* 1999).

Older techniques to distinguish the different isoenzymes such as heat inactivation and electrophoresis are labor-intensive by nature and lack sensitivity and specificity. Measurement of AP activity both before and after lectin precipitation is inexpensive but lacks reproducibility due

to variation between batches of lectin. Immunoassays specific for BAP have been described and are now commercially available for clinical use (Alkphase-B kit, Metra Biosystems, USA; Ostase, Beckman Access, Beckman-Coulter Inc, USA) (Yang & Grey , 2006) . The ease of measurement, cost efficiency and higher specificity in detecting small changes in bone formation, makes BAP a good marker for bone formation (Vasikaran *et al.,* 2008). Additionally, BAP serum levels show no circadian variations, probably due to the relatively long half-live of 1 to 2 days (Yang & Grey , 2006). These assays represent an improvement on total alkaline phosphatase activity measurement and better reflect bone turnover both in postmenopausal osteoporosis and following antiresorptive therapy (Rauch *et al.,* 2002). However, one drawback of these assays is the residual low cross-reactivity (16%) with liver AP. Therefore, in subjects with high liver AP, results of BAP measurements may be artificially high, leading to false positive results (Langlois *et al.,* 1994; Woitge *et al.,* 1996; Martin *et al.,* 1997).

Age related changes in serum BAP levels correlate positively and significantly with height velocity in both genders. Age-related and pubertal staging reference values are available from several publications (table 1A). In growth hormone-deficient children serum BAP levels increase significantly after growth hormone therapy suggesting its use in monitoring therapy.

B2. Osteocalcin

Osteocalcin (OC) is a 5700 dalton protein synthesized by the osteoblast and the most abundant non-collagenous protein of bone matrix. Upon synthesis OC is released in circulation, where it has a short half-life and is rapidly (5 minutes) cleared by the kidneys. In addition to the intact molecule, various fragments that accumulate are also found in the circulation. Serum OC has been proposed as a specific and sensitive marker for bone formation because it is both present in bone tissue as well as increasing significantly during advanced and enhanced skeletal growth (Yang &Grey*,* 2006).

The hydroxyapatite-binding function of osteocalcin necessary for bone mineralisation requires the post-translational carboxylation of the 3 γ-glutamic acid residues (Glu) to γ--carboxyglutamic acid (Gla) by a vitamin K dependent carboxylase (Binkley & Suttie, 1995). Inadequate dietary vitamin K intake results in increased levels of undercarboxylated osteocalcin (Glu-OC) in healthy children (Van Summeren *et al.,* 2007). Methods have been developed to distinguish between carboxylated and undercarboxylated fractions of osteocalcin and % Glu-OC is often used as an indicator of vitamin K status (Knapen *et al.,* 1996; Gundberg *et al.,* 1998). Several studies have suggested that an increase in the percentage Glu-OC may be associated with an increased incidence of bone fractures in post-menopausal women. (Sokoll *et al.,* 1996; Vermeer *et al*., 2004; Booth *et al* 2003) . In girls, better vitamin K status (expressed as %Glu-OC) was associated with reduced bone turnover (Kalkwarf *et al.,* 2004) . Similarly, O'Connor *et al*, (2007) confirmed in peri-pubertal Danish girls that better vitamin K status, as indicated by lower Glu-OC, positively correlated with BMC of the total body and lumbar spine. Overall, it is reasonable to conclude that vitamin K status may play an important role in determining the bone quality in children.

Several immunoassays have been developed to measure serum OC concentrations and particular attention to pre-analytical parameters is important (Table 2). As there is no international standard for the measurement of osteocalcin and because both intact molecule and various accumulated fragments are present in circulation, the assay results can vary considerably. This reflects their differing abilities to detect these OC fragments. Since, a significant fraction of the intact osteocalcin molecule is rapidly converted to a large N-terminal mid-molecule (1-43

amino acids) fragment both in vivo and in vitro, assays that are specific to the intact molecule but also detect this large fragment are currently used because they are more robust as well as sensitive. These assays do not distinguish between Glu-OC and osteocalcin. Age-related and pubertal staging reference values for osteocalcin are available from several publications (Table 1A).

B3. Type I procollagen peptide

The procollagen type I peptides are derived from the most abundant form of collagen found in bone, collagen type I and considered quantitative measures of newly formed collagen type I because they are cleaved from the procollagen type 1 when it is secreted into the extracellular matrix (Liu *et al*., 1995). These precursor molecules are characterized by short terminal extension-peptides: the amino (N-) terminal propeptide (PINP) and the carboxy (C-) terminal propeptide (PICP) (Merry *et al.,* 1976). Type I collagen propeptides may also arise from other sources, such as skin, tendons and joints or cartilage. Both PICP and PINP can be measured in serum as markers of bone formation, and the factors affecting the assay are shown in Table 1A. Commercial immunoassays are available for both propeptides. PINP (Roche Elecsys, Mannheim, Germany) and PICP (sandwich ELISA, Metra Biosystems GmbH Osnabruck, Germany). In practice, PINP shows greater diagnostic value than PICP as a bone-formation marker (Robins *et al.,*1999; von der Mark *et al.,* 1999) .

C. Bone Resorption Markers

There are several bone resorption markers: hydroxyproline, hydroxylysine, and the various forms of pyridinium crosslinks. These are all products of collagen degradation released during degradation of bone-collagen. All are released into circulation and excreted into urine and thus are usually measured in urine (Seibel *et al.,* 2005). However, as mentioned above the difficulty of obtaining serial urine collections coupled with their relatively large intra-individual or biological variation limits their utility. The C- and N-telopeptides of type 1 collagen are commonly used serum bone resorption markers which can be measured in children (Table 1B).

C1. Cross-linked Telopeptides.

The crosslinked telopeptides are derived specifically from the amino terminal (NTX) and the carboxyterminal (CTX) regions of type I collagen during bone resorption. They are cross-linked through Pyr and DPD and are shown to be specific and sensitive markers of bone resorption (Yang & Grey, 2006; Siebel *et al*., 2006). There are four possible isomers of CTX the native α-L form and the β isomerised L (β-L) form. Originally, a radioimmunoassay (RIA) was developed against the C-terminal cross-linking telopeptide of type I collagen generated by matrix metalloproteinases (CTX-MMP, also called ICTP) in serum (Risteli *et al*., 1993). Since then, several assays against the C-terminal cross-linking telopeptide of type I collagen (CTX) have been developed and are grouped under the CrossLaps label. β-CTX in serum is measured using a sandwich ELISA, which utilizes a high-affinity polyclonal antibody against the β-isomerized C-terminal octapeptide attached to the ELISA plate (Bonde *et al*., 1997). Currently, a newer version of this assay uses two monoclonal antibodies against collagen type I C-telopeptide fragments containing two cross-linked EKAHD-β-GGR epitopes (Rosenquist *et al.,* 1998). An automated

CTX assay, the Beta-CrossLaps/serum assay on the Roche Elecsys (Roche Diagnostics, Mannheim, Germany) is now available (Okabe *et al.,* 2004). A serum NTX assay using a monoclonal antibody against an epitope on the alpha-2 chain of type I collagen (Gertz *et al*., 1994) is also available (Maeno *et al*., 2005). These assays appear to be stable at room temperature.

D. Biological Variation

There is significant within-subject variability in most bone turnover markers consequently limiting their practical and diagnostic utility. Knowledge of the sources of variability can reduce the "background noise" and provide more meaningful interpretation of bone markers. The ideal marker and assay is characterized by both excellent analytical performance (i.e. high precision and accuracy) and by minimal and predictable pre-analytical variability (Seibel *et al.,* 2005). The relevant pre-analytical factors affecting bone biomarker variability are summarized in Table 2.

There are two groups of pre-analytical variables: the first group includes the biological or uncontrollable factors, such as age, gender, ethnicity, pubertal or menopausal status, or recent fracture; second group are the controllable factors such as circadian rhythm, diet or menstrual cycle effects (Glover *et al.,* 2008). Many biological factors cannot be modified at all, while others are hard to control in clinical practice. Therefore, in establishing reference intervals factors such as age, gender and pubertal status must be taken into account. To minimise some of the limitations of the controllable factors linked to pre-analytical and analytical variability, standardised sampling (fasting, time of collection) and sample handling (analyte stability) are mandatory to obtain reliable and clinically relevant results. Even though most markers in current use are not significantly directly affected by food intake, it is possible to reduce the effects of diurnal variation by standardizing sample collection by ensuring the collection be done in the morning and in the fasting state. (Clowes *et al.,* 2002). The specific considerations for some of the biomarkers are presented in Table 1A &B. Overall, if bone turnover markers are to be used clinically, then appropriate interpretation of the biochemical markers must consider all sources of variability that include the pre-analytical variability and the analytical performance of the assay (Glover *et al.,* 2008).

When comparing measurements taken by DXA vs. laboratory testing using biochemical markers it is observed that although BMD measurement by DXA has low imprecision (around 1% at the lumbar spine), the magnitude of changes following treatment are also relatively small. On the other hand, bone-turnover markers may show more rapid changes soon after initiating treatment, which may be useful in management of bone disease. Standardizing pre-analytical procedures will limit inter-assay variability and therefore improve interpretation.

E. Pediatric Reference Range for Serum Bone Markers

Assessment of metabolic bone disorders and monitoring of antiresorptive therapy or disease progression requires the use of normal pediatric reference ranges for both serum bone formation and resorption markers (Rauchenzauner *et al.,* 2007). Usage of bone markers as risk of bone disease or monitoring therapy is rather different in adults vs. children. In adults, bone turnover markers primarily correspond to bone remodeling and are usually used as independent predictors of the risk of osteoporosis and fractures (Miller *et al*., 2005; Nishizawa *et al*., 2004), to monitor antiresorptive therapy (Hochberg *et al*., 2002; Bonnick *et al*., 2006) and also have a promising role in metastatic bone disease (Hannon *et al*., 2006). However, in children, these markers are released into the circulation during the processes of bone remodeling, modeling, and

growth in length. There are significant changes in levels of bone formation and resorption markers with age during skeletal growth and puberty and these also correlate with growth velocity (Crofton *et al*., 1998; de Ridder *et al.,* 1998). Therefore it is essential that longitudinal measurement in patients is compared to the age-related values of reference data.

For establishing paediatric reference ranges for bone markers and assessing their relation to sex, age, pubertal status and anthropometric data, a large population of healthy children is required. In the past, most such normative studies established reference ranges using low subject numbers. A recent study (Rauchenzauner *et al.,* 2007) conducted using a large cohort of 572 healthy children aged 2 months to 18 years, aimed to establish sex- and age specific reference equations for OC, BAP, ICTP and CTX in healthy children and adolescents, thus enabling calculation of SD-scores (SDS). Their results provided reference curves for OC, BALP, CTX and ICTP in healthy children. They found that taller and heavier individuals for age had greater bone marker concentrations, likely reflecting greater growth velocity. SDS for markers of bone formation, collagen degradation, and phosphatases were each independently correlated, suggesting they derive from the same biological processes. They suggested that with the possibility of calculating SDS, monitoring of antiresorptive therapy or disease progression in children with metabolic bone disease will be facilitated (Rauchenzauner *et al*., 2007).

Many studies have established reference ranges in healthy children. However, the status of the micronutrients that may influence bone health such as, vitamin D and vitamin K is not often taken into account. There is wide variation between nutrient-based dietary reference intakes (DRI) in various countries and there is some concern that even in a healthy child, intakes of some bone nutrients may not be optimal (Prentice *et al.,* 2006). One example is the recommendations for vitamin D. Recent studies have suggested that the optimal serum levels of 25OH vitamin D should be 75nmol/L. With this threshold, many healthy infants and children would have suboptimal levels (Gregory *et al.,* 2000; Gordon *et al*.,2008). There is also some concern that vitamin K intakes may be lower than desirable (Kalkwarf *et al*., 2004). In the establishment of normative reference values the relationship of bone biochemical markers to these nutritional parameters should be considered.

F. Conclusion

There is a systematic process of growth in children, starting with the synthesis of soft tissue, followed by epiphyseal bone growth and with extensive bone modeling and re-modeling. To assess changes in these processes, absolute bone mineral content, 2-D measurement of bone mass using DXA (BMD) or 3-D measurement of bone geometry, mass and density using quantitative computed tomography are used. However, the use of biochemical markers provides a dynamic picture of whole body bone turnover and thus complements the static measures of bone. These can be repeated at much shorter intervals; thus, the dynamic assessment helps to detect effects of disease or therapy much before changes in bone mass or progression in bone disease can be ascertained. The clinical usefulness of these tests is greatly enhanced if effort is made to minimize biological variation by using standardized sampling and sample handling. Since, bone marker concentrations can be similar in a child with high bone remodelling and low growth rate and in a normally growing child, it is essential to be aware of growth velocity and pubertal development to correctly interpret the results. Further attention should be given to the nutritional parameters of bone health in developing adequate reference values in healthy children.

Table 1A. Bone Formation Markers

Marker	Tissue	Analytical Method	Special Considerations	Reference Values
Bone specific alkaline phosphatase (BAP)	Bone	Eletrophoresis, Lectin precipitation, IRMA, EIA	Osteoblast product. Some immunoassays show 20% cross-reactivity with the liver isoenzyme. Does not show circadian rhythm (Yang & Grey, 2006), Half-life of 1-2 days, stable in serum.	Age, pubertal stage and gender in Thai population. (Chailurkit *et al.,* 2005), Age, pubertal stage and gender in Caucasian population (Ruachenzauner *et al., 2007)* Age, pubertal stage and gender in Caucasian population (Van der Sluis *et al., 2002*) Other information from review (Yang & Grey, 2006)
Osteocalcin (OC)	Bone, platelets	RIA, IRMA, ELISA	Osteoblast product Blood has several immunoreactive forms; Highest concentration found in morning samples. Some obtained from bone resorption. Lipemia, hemolysis falsely decreases result. Collect on ice and separate from cells within 1h to minimise degradation. Short half-life and is degraded into fragments that may be more stable in serum sample.	Age, pubertal stage and gender in Thai population. (Chailurkit *et al.,* 2005), Age, pubertal stage and gender in Caucasian population (Ruachenzauner *et al., 2007)* Age, pubertal stage and gender in Caucasian population (Van der Sluis *et al., 2002*) Other information from review (Yang & Grey, 2006)

Table 1B. Bone Resorption Markers

Marker	**Tissue**	**Analytical Method**	**Remarks**	**Reference Value**
Carboxy-terminal cross-linked telopeptide of type I collagen (ICTP, CTX-MMP)	Bone, skin	RIA, serum crosslaps, ELISA, and automated	Collagen type I, with most contribution from bone. Diurnal variation. Thermodegradation at room temperature. Fasting required	Age, pubertal stage and gender in Thai population. (Chailurkit *et al.*, 2005), Age, pubertal stage and gender in Caucasian population (Ruachenzauner *et al., 2007)* Age, pubertal stage and gender in Caucasian population (Van der Sluis *et al., 2002*) Other information from review (Yang & Grey, 2006)
Amino-terminal cross-linked telopeptide of type I collagen (NTX-I)	All tissues containing type I collagen	ELISA, CLIA, RIA	Collagen type I, with most contribution from bone. Diurnal variation. Fasting required. Decrease with exercise.	Age, pubertal stage and gender in Caucasian population (Van der Sluis *et al., 2002*) Other information from review (Yang & Grey, 2006

Table 2. Sources of pre-analytical variability

Sources of pre-analytical variability
Technical Sources
Type of specimen and mode of sample collection
Handling and storage of specimen
Thermodegradation
Time at which sample is collected
Variation between laboratories (Inter-laboratory variation)
Biological Sources
Age: pubertal status, somatic growth, ageing
Gender
Ethnicity
Fractures occurred recently
Drugs: Anti-resorptive agents, anticonvulsants, oral contraception
Non-skeletal diseases: Diabetes, liver disease, degenerative joint disease
Diet
Exercise
Temporal variability: Diurnal (circadian), menstrual, seasonal

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