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Pediatric reference Raman data for material characteristics of iliac trabecular bone

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ABSTRACT

Bone material characteristics are important contributors in the determination of bone strength. Raman spectroscopic analysis provides information on mineral/matrix ratio, mineral maturity/crystallinity, relative pyridinoline (Pyd) collagen cross-link content, relative proteoglycan content and relative lipid content. However, published reference data are available only for adults. The purpose of the present study was to establish reference data of Raman outcomes pertaining to bone quality in trabecular bone for children and young adults. To this end, tissue age defined Raman microspectroscopic analysis was performed on bone samples from 54 individuals between 1.5 and 23 years with no metabolic bone disease, which have been previously used to establish histomorphometric and bone mineralization density distribution reference values. Four distinct tissue ages, three well defined by the fluorescent double labels representing early stages of bone formation and tissue maturation (days 3, 12, 20 of tissue mineralization) and a fourth representing old mature tissue at the geometrical center of the trabeculae, were analyzed. In general, significant dependencies of the measured parameters on tissue age were found, while at any given tissue age, sex and subject age were not confounders. Specifically, mineral/matrix ratio, mineral maturity/crystallinity index and relative pyridinoline collagen cross-link content index increased by 485%, 20% and 14%, respectively between days 3 and 20. The relative proteoglycan content index was unchanged between days 3 and 20 but was elevated in the old tissue compared to young tissue by 121%. The relative lipid content decreased within days 3 to 20 by −22%. Thus, the method allows not only the monitoring of material characteristics at a specific tissue age but also the kinetics of tissue maturation as well. The established reference Raman database will serve as sensitive tool to diagnose disturbances in material characteristics of pediatric bone biopsy samples.

Introduction

In addition to bone mass and architecture, bone material quality is an important determinant of bone strength, and there is an increasing interest in assessing properties of the extracellular matrix for the diagnosis of bone diseases [1–3]. Connective tissue disorders like Osteogenesis Imperfecta or Ehlers–Danlos syndrome are characterized by altered composition and structure of the bone tissue [4]. Also metabolic disorders of mineral homeostasis like hyperparathyroidism [5], chronic kidney diseases [6] or diabetes [7] affect bone strength and lead to fragility fractures that are often not associated with changes in bone mineral density, and the same holds for idiopathic osteoporosis [8,9].

Due to continuous remodeling activity by the bone cells, material characteristics of the organic and inorganic bone matrix are highly dynamic at the microscopic level. The mineralization process occurs in a biphasic way: a rapid phase where the organic matrix becomes mineralized to about 70% of its final value within a few days (primary mineralization), followed by a second phase where the rate of mineralization is much lower taking months or years to achieve plateau level (secondary mineralization) [10–12]. Similarly, organic matrix components such as type I collagen and proteoglycans undergo significant post-translational modifications after having been synthesized by the relevant bone cells [13–15]. As a result, both mineral and organic matrix properties at the microscopic level are expected to be different depending on the relative tissue age or remodeling rates [11,16].

Children and adolescents have high bone turnover rates. Indeed, during growth, the skeleton is not only modeled to increase external size and shape of the bones but is also highly remodeled [17].

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However, in sharp contrast to the situation in high turnover osteoporosis, during growth there is a positive imbalance and bone formation prevails over bone resorption leading to a net increase in trabecular thickness after each remodeling cycle [17]. In children undergoing a "growth spurt", a situation that is inherently linked to markedly increased bone turnover, a transient "weakening" of the bones has been observed [18]. All together these observations underline the necessity to gain further insights in bone material characteristics of the developing skeleton.

For establishing a diagnosis in an individual patient or for investigating treatment follow-up, a comparison with normal values from healthy subjects is of crucial importance. A precise tool to allow the study of bone metabolism in individual patient disorders is bone biopsies. Transiliac bone biopsies are traditionally used to perform bone histomorphometry that allows the quantification of the size and amount of the mineralized and unmineralized bone tissue and evaluation of bone cell function [19,20]. Dynamic evaluation of cell function requires that the biopsy is fluorescent labeled *i.e.*, the patient has received at least two courses of tetracycline or other fluorescent drugs prior to biopsy. Further information in particular about bone material quality can be extracted from the available biopsy bone tissue applying different techniques. The tetracycline labels allow specific tissue ages in cancellous bone to be distinguished. Glorieux et al. presented reference data for pediatric iliac bone histomorphometry data based on the evaluation of 58 bone biopsies from a healthy population from 1.5 to 23 years [21]. These biopsies were further used to assess the bone mineral density distribution (BMDD) by quantitative backscattered electron imaging (qBEI) [21,22]. The combination of the histomorphometric and BMDD reference variables has raised the possibility of obtaining new insights within bone structural and material characteristics in the developing skeleton, as recently demonstrated in pediatric patients with genetic disorders like osteogenesis imperfecta or in dialyzed patients to evaluate the skeletal effects of growth hormone therapy [23–27]. However until now, such pediatric reference data assessing the organic component of the bone matrix is still lacking yet necessary as changes in the organic bone composition have already been observed in fracture prone children [28,29].

Raman microspectroscopy is a vibrational spectroscopy technique that allows the determination of bone material characteristics (of both mineral and organic matrix bone components) in bone biopsy blocks with a spatial resolution of ~1 μm, by measuring the wavelength and intensity of inelastically scattered light from molecules [30,31]. In combination with fluorescence labeling, this offers the capability to establish these properties as a function of tissue age [30–34].

The purpose of the present study was to establish a reference database of trabecular bone with the Raman derived bone quality indices for growing children, to complement the already existing ones based on histomorphometry and BMDD data [21,22], in the same iliac crest biopsy cohort, as a function of sex-, subject-, and tissue-age. The tissue age normalization is of particular interest as it minimizes the effect of bone turnover on the monitored bone quality indices and allows the description of the kinetics of maturation of the material characteristics investigated in this normal subject cohort [30–33].

Material & methods

Subjects

The study population comprised 54 Caucasian subjects aged 1.5 to 23 years (32 female, 22 male) without any known metabolic bone disorder, as previously described [21,22]. Transiliac bone biopsy samples were acquired during surgery for various conditions, such as lower limb deformities, scoliosis, clubfeet and other conditions requiring corrective surgery. The bone specimens were collected on day 4 or 5 after dual labeling with demeclocycline (15–20 mg per kg body weight per day) taken orally during two two-day periods separated by a 10-day-free interval. All subjects were ambulatory, had normal renal function (assessed by serum creatinine measurements) and had no evidence of any metabolic bone disease. None of the included subjects was either immobilized prior to surgery or received medications known to affect bone metabolism.

Raman analysis

Raman microspectroscopic analyses employed a Senterra (Bruker Optik GmbH) instrument. A continuous laser beam was focused onto the sample through a Raman fluorescence microscope (Olympus BX51, objective 50×) with an excitation of 785 nm (100 mW) and a lateral resolution of ~0.6 μm [32,33]. The Raman spectra were acquired from the biopsy block polished surface, using a thermo-electric–cooled charge-coupled device (CCD) (Bruker Optik GmbH). All data analyses were done with the Opus Ident software package (OPUS 6.5, Bruker Optik GmbH). Once acquired, the Raman spectra were baseline corrected (rubber band, 5 iterations) to account for fluorescence, and the following Raman parameters were calculated as published elsewhere [32,33]. The mineral/matrix ratio was expressed as the ratio of the integrated areas of the ν_2PO_4 (410–460 cm^{-1}) to the amide III (1215–1300 cm^{-1}) bands. The maturity/crystallinity of the bone mineral apatite crystallites was approximated from the inverse of the full width at half height (FWHH) of the ν_1PO_4 (930–980 cm^{-1}) band [31,35]. The relative pyridinoline (Pyd) content (a major trivalent collagen cross-link) was calculated as the absorbance height at 1660 cm^{-1}/area of the Amide I band (1620–1700 cm^{-1}) [31,36–39]. The relative proteoglycan (PG) content was defined as the PG/matrix ratio, which was calculated from the ratio of the integrated areas of the proteoglycan/CH_3 (1365–1390 cm^{-1}) band representative of glycosaminoglycans (GAGs) [40–42], to the amide III (1215–1300 cm^{-1}) bands, respectively. Finally, the relative lipid content was expressed as lipids (~1298 cm^{-1})/amide III [43].

The technical variance for each parameter monitored in the present study was calculated through the acquisition of 20 repeated measurements of the same anatomical location, and expressed as % coefficient of variation (% COV) (Table 1).

Anatomical area selection criteria

For each biopsy, three trabeculae with clearly discernible double tetracycline labels were analyzed in the following regions (see Fig. 1): Between the 2nd label and mineralizing front (2 μm before 2nd label) corresponding to a mineralized tissue age of 1–3 days, between the two labels corresponding to a tissue age of about 4–20 days and right behind the 1st label (2 μm behind 1st label) corresponding to a tissue age of slightly greater than 20 days, related to the tissue that was formed just before the first dose of demeclocycline was taken. In each of these regions three measurements were obtained, for a total of nine measurements per individual per anatomical area. Additionally, three measurements in the geometrical center of each trabecula utilized were also acquired, representative of old tissue (tissue age much greater than 20 days) with prolonged secondary mineralization designated as "CENTER".

Table 1
Summary of the technical variance for each parameter reported in the present study, expressed as percentage of coefficient of variation (% COV), calculated from 25 repeated measurements of the exact same anatomical location.

Parameter	% COV
Mineral/matrix	1.582
Relative proteoglycan content	2.606
Relative lipids content	5.202
Mineral maturity/crystallinity (ν_1PO_4 FWHH)	1.508
Relative pyridinoline content	2.021