Pediatric reference Raman data for material characteristics of iliac trabecular bone


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 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].

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].
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 ν3PO4 (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 ν3PO4 (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/CH3 (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 (−1288 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>
<thead>
<tr>
<th>Parameter</th>
<th>% COV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral/matrix</td>
<td>1.582</td>
</tr>
<tr>
<td>Relative proteoglycan content</td>
<td>2.606</td>
</tr>
<tr>
<td>Relative lipids content</td>
<td>5.202</td>
</tr>
<tr>
<td>Mineral maturity/crystallinity (ν3PO4 FWHH)</td>
<td>1.458</td>
</tr>
<tr>
<td>Relative pyridinoline content</td>
<td>2.021</td>
</tr>
</tbody>
</table>

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.
Statistical analysis

For each individual, the nine values/microanatomical location, representative of every tissue age specific region, were averaged and the resultant value was treated as a single statistical unit for the appropriate anatomical location and tissue age. The data (with the exception of FWHH) were not normally distributed (as per Kolmogorov–Smirnov test), thus comparisons between male and female groups at the four distinct tissue ages considered were made using Mann–Whitney U-test. Comparisons as a function of tissue age were made using one-way ANOVA without assumption of Gaussian distribution (Kruskal–Wallis), followed by Dunn's post-hoc comparison. Statistical significance was assigned to p < 0.05. Correlations amongst the parameters monitored and subject age were analyzed by Spearman’s nonparametric test.

Results

Information on the technical variance for each parameter reported is provided in Table 1, as % COV.

Raman spectra were obtained at tissue areas of four different ages (Fig. 1). Fig. 1a shows the fluorescent labels and the three positions close to the fluorescence labels (tissue that is 1–3, 4–20, and >20 days old) where Raman spectra were acquired as visualized by the light microscope (fluorescence mode) of the Raman instrument. Additional Raman spectra were obtained in the geometrical center of these trabeculae (Fig. 1b), representative of older tissue age (CENTER) compared to that of the first 3 measurements. Fig. 1c is a stack of typical Raman spectra at the 4 different tissue ages in the same trabeculum, with the peaks of interest appropriately labeled. In Fig. 1d the area from 1200 to 1500 cm$^{-1}$ of the 1–3 day-old tissue Raman spectra is enlarged to mark the proteoglycan Raman band (1376 cm$^{-1}$; CH$_3$) and the Raman vibration (CH$_2$) from the lipids.

A summary of all Raman spectroscopic outcomes pooled for both sexes and all subject ages (median, 25th and 75th percentiles) at the different tissue ages is provided in Table 2. Sex differences as tested by Mann–Whitney comparison (data not shown) were not significant for any of the Raman outcomes. Subject age as tested by Spearman Rank order correlation (Figs. 2a to 6a) was not a confounder for Raman outcomes with one exception. For the less well-defined oldest analyzed tissue age (CENTER) mineral maturity/crystallinity index revealed a weak significant positive regression (Spearman $r = 0.360$, p = 0.005) (Fig. 3a). As a consequence, the data were pooled for subsequent analyses.

The mineral/matrix ratio significantly increased continuously at early tissue ages from day 3 to day 20 by about 485%. A further increase (83%) occurred with respect to age of the central bone region (Fig. 2b).

Mineral maturity/crystallinity also significantly increased as a function of early tissue age from days 3 to 20 by about 20%. However, there was no difference between day 20 and beyond (center region of the trabeculae) (Fig. 3b).

Relative Pyd content was significantly increased as a function of early tissue ages (~14% from days 3 to 20) (Fig. 4b), but there was no further increase between older (day 20) and beyond (center region of the trabeculae).

Fig. 1. (a) Fluorescence light photomicrograph (objective lens 50×) of a forming trabecular surface with the fluorescent double label evident showing the positions of the 3 first measurements (red crosses), (b) a picture through the 20× lens showing where the position where the 4th measurement was obtained (red cross; although the picture was obtained with the 20× lens for presentation purposes, the actual measurements were obtained through the 50× lens so as to ensure identical volume of analysis with the 3 first measurements). Typical Raman spectra are shown in (c) with the spectral regions of interest appropriately labeled, while the spectral region of ~1200–1500 cm$^{-1}$ is isolated and zoomed for better visualization of the peaks that were utilized for proteoglycan and lipids considerations (d).
Relative proteoglycan content expressed as PG/Amide III ratio was similar between the 3 early tissue ages, but was significantly higher at the oldest tissue (CENTER) compared to the younger tissues (~121%) (Fig. 5b).

Relative lipid content significantly decreased as a function of tissue age from 1–3 to ~20 days (~−22%) and from >20 days old to CENTER further ~−20% (Fig. 6b). Evidence for the specificity for lipids of the Raman peak used, and its independence from tissue organization/orientation is provided in Appendix A.

Discussion

The purpose of the present study was to establish reference values of bone material characteristics for Caucasian children and young adults as can be accessed by Raman microspectroscopy from transiliac bone biopsy sample blocks as a function of sex, subject, and tissue age. The tissue age normalization is expected to minimize any effects of divergent bone turnover rates amongst the individuals investigated.

The fluorescence markers of routine tetracycline labeling were used to define age and the early maturation stages of the bone tissue (1–3 to 20 days). We did not observe any dependency of the Raman outcomes at these defined tissue ages on subject age or gender. Therefore, a singular Raman data set is necessary to characterize the material characteristics of the reference cohort containing 54 subjects (aged 1.5 to 23 years).

In the present work, five different material parameters of bone tissue were considered:

1) The mineral/matrix ratio provides information on the amount of mineral normalized for the amount of organic matrix within the volume of bone material analyzed. The results of the present study indicate that this ratio is very sensitive to tissue age, in agreement with previously published reports on mineral mass fractions or ratios at different bone tissue ages in humans and animal models [11,44–47]. The mineral/matrix ratio increased from days 1–3 to day 20 and was the highest at the oldest tissue at

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Table 2
Summary of intrinsic material characteristics (median, and 25th and 75th percentile values) of children (age range 1.5–23 years, sample size N = 54) monitored at each specific tissue site. Since the tissue age of the trabecular geometrical centers is not precisely known, it is denoted by anatomical/geometrical location.

<table>
<thead>
<tr>
<th>Tissue age (days)</th>
<th>Mineral/matrix</th>
<th>Mineral maturity/crystallinity (1/FWHH v2PO4)</th>
<th>Relative pyridinoline content</th>
<th>PG/matrix</th>
<th>Lipids/matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–3 Median</td>
<td>0.085</td>
<td>0.038</td>
<td>0.014</td>
<td>0.057</td>
<td>0.091</td>
</tr>
<tr>
<td>25th percentile</td>
<td>0.064</td>
<td>0.036</td>
<td>0.012</td>
<td>0.045</td>
<td>0.081</td>
</tr>
<tr>
<td>75th percentile</td>
<td>0.153</td>
<td>0.040</td>
<td>0.016</td>
<td>0.069</td>
<td>0.099</td>
</tr>
<tr>
<td>4–20 Median</td>
<td>0.176</td>
<td>0.043</td>
<td>0.015</td>
<td>0.041</td>
<td>0.086</td>
</tr>
<tr>
<td>25th percentile</td>
<td>0.10</td>
<td>0.042</td>
<td>0.014</td>
<td>0.030</td>
<td>0.075</td>
</tr>
<tr>
<td>75th percentile</td>
<td>0.285</td>
<td>0.046</td>
<td>0.017</td>
<td>0.058</td>
<td>0.098</td>
</tr>
<tr>
<td>&gt;20 Median</td>
<td>0.497</td>
<td>0.047</td>
<td>0.016</td>
<td>0.059</td>
<td>0.071</td>
</tr>
<tr>
<td>25th percentile</td>
<td>0.391</td>
<td>0.046</td>
<td>0.016</td>
<td>0.045</td>
<td>0.062</td>
</tr>
<tr>
<td>75th percentile</td>
<td>0.622</td>
<td>0.047</td>
<td>0.017</td>
<td>0.082</td>
<td>0.077</td>
</tr>
<tr>
<td>Center Median</td>
<td>0.911</td>
<td>0.047</td>
<td>0.016</td>
<td>0.126</td>
<td>0.057</td>
</tr>
<tr>
<td>25th percentile</td>
<td>0.702</td>
<td>0.046</td>
<td>0.016</td>
<td>0.101</td>
<td>0.047</td>
</tr>
<tr>
<td>75th percentile</td>
<td>1.025</td>
<td>0.047</td>
<td>0.016</td>
<td>0.151</td>
<td>0.063</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01, *** p < 0.0001, vs 1–3 day-old tissue.
# p < 0.05, ## p < 0.01, ### p < 0.0001, vs 4–20 day-old tissue.
§ p < 0.05, §§ p < 0.01, §§§ p < 0.0001, vs >20 day-old tissue.
central trabecular area in agreement with the well-known time course of bone matrix mineralization, which is rapid in its initial phase (primary mineralization) and slows down with prolonged duration of mineralization (secondary mineralization) [10,11,48]. Interestingly, in recent reports comparing groups of adult patients with similar BMD but divergent fracture history, this mineral/matrix ratio measured between double fluorescent labels was significantly different between these groups (fracture vs. non-fracture) [9,49,50] emphasizing its relevance in predicting bone strength.

2) Bone mineral maturity/crystallinity is a measure reflecting the chemical makeup of the apatite crystallites and by extrapolation their size [3,16,51]. Theoretical models predict that larger crystallites in general result in compromised mechanical behavior [3,52–54].

Fig. 3. (a) Scatter plots indicate that the mineral maturity/crystallinity, estimated from the inverse of the Full Width at Half Height (FWHH) of the $v_1$PO$_4$ band was independent of subject age at the 3 younger tissue ages analyzed. It was however dependent on subject age only at the oldest of the ages examined, namely at the trabecular center (Spearman $\tau = 0.360$, $p = 0.005$). It was also significantly dependent on tissue age. The linear regression line is also shown for the different 4 tissue ages considered. (b). As can be seen, this parameter significantly increases as a function of tissue age (b) ($***p < 0.0001$).

Fig. 4. (a) Scatter plots show that the relative pyridinoline collagen cross-link content was independent of subject age at all 4 tissue ages analyzed. The linear regression line is also shown for the different 4 tissue ages considered. On the other hand, it significantly increased as a function of tissue age (b) (*$p < 0.05$; ***$p < 0.0001$).
Additionally, its considerable contribution in the determination of bone strength may be inferred from the changes described in mineral maturity/crystallinity index in disease and in response to therapies [16,50,55–59]. Moreover, in cases where divergence between BMD and fracture incidence is noted, this parameter correlated with the latter [50]. In the present study, mineral maturity/crystallinity exhibited a distinct increase with tissue maturation (early tissue ages). There was no further increase from tissue age day 20 on to the oldest analyzed tissue at the geometrical center of trabeculae. The values of these oldest analyzed tissue regions displayed a weak subject age-dependency. The reason for this is unclear, and no definite argument may be put forth due to the uncertainty in the exact tissue age of the specific anatomical location considered (there may be differences ranging from months to years depending on the individual).

3) The relative pyridinoline content (normalized to organic matrix content) is part of one of the most distinct feature of type I collagen in mineralized tissues, namely its cross-linking chemistry and molecular packing structure [15]. The intermolecular crosslinking provides the fibrillar matrices with various mechanical properties such as tensile strength and viscoelasticity. The importance of collagen intermolecular crosslinks to the mechanical performance of bone is also...
very apparent in the pyridoxine deficient chick animal model [60–62], as well as in lathyrisin [38,63,64]. Changes in these crosslinks, even at confined anatomical locations, are sufficient to influence bone strength in the absence of alterations in either mineral quantity or quality [38]. Finally, as is the case with mineral maturity/crystallinity, in cases where deviation between BMD values and fracture incidence is encountered, collagen crosslinks correlate with the latter in animal models and in humans [9,50,65,66]. Pyridinoline is a mature, non-reducible, trivalent collagen crosslink abundant in mineralizing type I collagen [15]. In the present study, relative pyridinoline content was found to increase within early tissue ages, but from day 20 on to old tissue (central area) no further increase was observed.

4) The relative proteoglycan content (normalized to organic matrix); in vitro, in situ, and in vivo experiments have shown proteoglycans to be negative modulators of mineralization [67–69]. Proteoglycans have also been identified in perilacunar matrix around the osteocyte lacunae, and around the canaliculi [70] in compact lamellar rat and human bone. Recently, it was proposed that a plausible role of these osteocyte-related proteoglycans (and in particular perlecan/Hspg2 (PLN)) was to prevent mineralization in the pericellular space of the lacunocanalicular network so as to ensure uninhibited interstitial fluid movement [71]. In the present study, significant differences were observed between the oldest tissue age and the 3 younger ones examined. This increase may be due to several possibilities. Different proteoglycans may be present at the formation surfaces and in the older tissue, having different numbers of glycosaminoglycan chains/molecule. Moreover, proteoglycans are known to undergo extensive post-translational modifications including addition of glycosaminoglycan chains as well as N- and O-linked oligosaccharides along other changes as a function of age [13]. Unfortunately, Raman microspectroscopic analysis methods to date are not discriminant enough to identify whether this increase in glycosaminoglycans is due to different proteoglycans, or due to the same proteoglycans that have undergone different post-translational modifications. Another possible explanation for the difference in relative proteoglycan content as determined by the glycosaminoglycan concentration normalized to the total amount of organic matrix between the oldest and the 3 younger tissue ages may be due to differences in canalicular network density between actively bone forming surfaces and densely mineralized bone.

5) Relative lipids content (normalized to organic matrix); lipids have been implicated in the literature as mineral nucleators, responsible for mineralization of collagen [12,13]. Lipid complexes have been shown to increase in concentration during bone formation, and calcium-acidic phospholipid-phosphate dyads have been shown to mediate collagen calcification in compact bone formation, and calcium-acid phospholipid-phosphate complexes have been shown to increase in concentration during cartilage calcification and early bone formation [72–75]. In the present study, the relative lipid content was significantly dependent on tissue age, with the highest values encountered in youngest bone formed, in agreement with what has been reported in the literature concerning their role.

In general the variability in the parameters monitored in the present study (as seen in the interquartile range) was greater at the sites of younger tissue age versus those of older tissue age (center site). This is most likely due to the fact that in zone of young tissue changes of tissue properties are rapidly occurring due to maturation processes. For example, close behind the mineralization front the mineral concentration in the bone matrix is rapidly increasing within few days from 0% up to 70% of fully mineralized bone tissue (primary mineralization) [11,45]. In contrast in the center zone only secondary mineralization occurs with only a relatively slow increase in mineral concentrations towards full mineralization (in scales of months to years) [10,11,44,45]. Thus the zone with relatively old tissue age is less heterogeneous. On the other hand, establishing values at the trabecular centers may prove useful in the future, in situations where unlabeled specimens are available and need to be compared to a normative database.

An important finding of the present study is that when similar tissue ages are considered, the monitored parameters are statistically similar regardless of the subject age (in the present study the subject age range was 1.5–23 years). This fact suggests that the osteoblasts of the subjects having different ages are producing a bone matrix, which is of similar quality with respect to identical tissue/matrix ages. Further, it highlights the need for tissue age normalization when microscopic techniques such as Raman and Fourier transform infrared microspectroscopy or Imaging are used to investigate bone material within single bone structural units (BSUs, bone packets and osteons), irrespective of subject age. Additionally, the information obtained through such techniques, while complementary, may not be in concert with the information obtained through techniques utilizing the whole bone tissue, because it is a composition of numerous BSUs exhibiting a certain age distribution strongly dependent on the bone formation/turnover rate of the subject. Consequently it is important to consider and compare either the whole tissue, or tissue-age normalized anatomical areas. Finally, there was no dependence of the intrinsic bone material properties on sex.

A limitation of the present work is that, although Raman spectroscopic analysis is capable of providing information on proteoglycan and lipid concentrations, it is not discriminant enough to date to readily distinguish between different proteoglycans and different lipids. On the other hand, since Raman analysis of PGs is based on GAGs, it should be kept in mind that in bone, chondroitin 4-sulfate constitutes ~90% of the total GAG content and is found predominantly in biglycan and decorin [76]. Another limitation of the present study is that the measurements were performed in iliac crest biopsies from Caucasian children only (as they were the only ones available to us at the present time), and further studies are needed to decipher whether these observations hold for other ethnicities as well.

In conclusion, the results of the present study indicate that bone material characteristics like mineral/matrix ratio, mineral maturity/crystallinity, relative pyridinoline collagen cross-link content, relative proteoglycan, and relative lipids content in trabecular bone of children and young adults (1.5–23 years old) are highly dependent on tissue age, while at any given tissue age, sex or subject age is not a confounding factor. As it is state of the art to perform bone biopsies with prior double tetracycline fluorescence labeling for differential diagnosis, the presented method of Raman microspectroscopic examination can be applied additionally to such biopsy samples. The established reference database allows now the detection of disturbances in material characteristics of pediatric bone biopsy samples.

Acknowledgments

This study was supported by the AUVA (Research funds of the Austrian workers compensation board), by the WGKK (Viennese sickness insurance funds) and the Shriners of North America.

Appendix A

The relative lipid content was expressed as the ratio of the integrated area of the lipid band – 1298 cm$^{-1}$/amide III [43]. To further demonstrate the selectivity of the band – 1298 cm$^{-1}$ for lipids, Raman spectra of fatty tissue from porcine skin (obtained at a local slaughterhouse), purified type I bovine bone collagen (kindly provided by Dr. S. Robins, Rowett Institute, UK), and commercially available biglycan (Sigma, Aldrich) are shown in the figure below.
We have also shown previously that certain Raman bands are sensitive to tissue organization/orientation [47]. To examine whether the ratio used in the present paper to determine the relative lipids content is dependent on tissue orientation, we calculated the lipids/amide III ratio in spectra previously obtained in the mineralizing turkey leg tendon (a model system for highly oriented collagen and apatite crystals) animal model as a function of Raman laser polarization and the results are presented in the graph below:

As may be seen, this ratio is independent of Raman laser polarization, thus independent of tissue organization/orientation. The instrument set-up was the same as in the published study and for each polarization, 5 Raman spectra were obtained and calculated.

References


