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The role of bone intrinsic properties measured by infrared spectroscopy in whole lumbar vertebra mechanics: Organic rather than inorganic bone matrix?

Julien Wegrzyn ^{a,b,*}, Jean-Paul Roux ^{a,1}, Delphine Farlay ^{a,1}, Hlne Follet ^a, Roland Chapurlat ^a

^a INSERM, UMR 1033, Université de Lyon, Lyon, France

^b Department of Orthopedic Surgery – Pavillon T, Hôpital Edouard Herriot, Lyon, France

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ABSTRACT

Whole bone strength is determined by bone mass, microarchitecture and intrinsic properties of the bone matrix. However, few studies have directly investigated the contribution of bone tissue material properties to whole bone strength in humans. This study assessed the role of bone matrix composition on whole lumbar vertebra mechanics. We obtained 17 fresh frozen human lumbar spines (8 W, 9 M, aged 76 ± 11 years). L3 bone mass was measured by DXA and microarchitecture by μ -CT with a 35 μ m-isotropic resolution. Microarchitectural parameters were directly measured: Tb.BV/TV, SMI, Tb.Th, DA, Ct.Th, Ct.Po and radius of anterior cortical curvature. Failure load (N), stiffness (N/mm) and work to failure (N.mm) were extracted from quasi-static uniaxial compressive testing performed on L3 vertebral bodies. FTIRM analysis was performed on 2 μ m-thick sections from L2 trabecular cores, with a Perkin-Elmer GXII Auto-image Microscope equipped with a wide band detector. Twenty measurements per sample were performed at 30×100 μ m of spatial resolution. Each spectrum was collected at 4 cm^{-1} resolution and 50 scans in transmission mode. Mineral and collagen maturity, and mineralization and crystallinity index were measured. There was no association between the bone matrix characteristics and bone mass or microarchitecture. Mineral maturity, mineralization and crystallinity index were not related to whole vertebra mechanics. However, collagen maturity was positively correlated with whole vertebra failure load and stiffness ($r = 0.64$, $p = 0.005$ and $r = 0.54$, $p = 0.025$, respectively). The collagen maturity (3rd step) in combination with bone mass (i.e. BMC, 1st step) and microarchitecture (i.e. Tb.Th, 2nd step) improved the prediction of whole vertebra mechanical properties in forward stepwise multiple regression models, together explaining 71% of the variability in whole vertebra stiffness ($p = 0.001$). In conclusion, we demonstrated a substantial contribution of collagen maturity, but not mineralization parameters, to whole bone strength of human lumbar vertebrae that was independent of bone mass and microarchitecture.

Introduction

Mechanical principles dictate that whole bone strength is determined by bone mass, bone microarchitecture and intrinsic properties of the bone matrix [1,2]. Beside bone mass, the contribution of microarchitecture and its spatial distribution (i.e.; microarchitecture heterogeneity) has been extensively explored biomechanically and clinically. It is probably the best understood among the different levels of analysis [3–7]. Specifically, impairment in trabecular and cortical microarchitecture impacts dramatically on whole bone strength and the risk of fragility fracture, independently of areal bone mineral density (aBMD) [3,4]. In addition, the post-fracture mechanical behavior of vertebrae after initial mild fracture was demonstrated ex-vivo to be related to bone microarchitecture but

not bone mass [5]. Abnormalities in age-related enzymatic and non-enzymatic collagen cross-links affect the mineralization process, and can lead to microdamage accumulation and impaired bone mechanical behavior therefore contributing to fracture risk prediction. However, the direct contribution of bone matrix properties to bone strength is more difficult to assess and therefore, remains poorly understood at the whole bone level in humans [2,8–11]. Micro- or nano-indentation techniques in human iliac bone samples demonstrated a strong relationship between the bone matrix and local tissue mechanical behavior [12,13]. Along with mineralized matrix, the organic matrix accounts for one third of the variance in bone microhardness at the bone structural unit level [12]. Particularly, collagen maturity explained plastic mechanical properties whereas elastic mechanical properties were explained by mineralization [13]. In addition, at the whole-bone level in rat humerus, bone tissue material composition was a strong predictor of mechanical behavior, accounting for up to 83% of the variability in bone mechanics [14]. Therefore, the mechanical properties of the bone matrix are important parameters to explore to enhance the understanding of

* Corresponding author at: Department of Orthopedic Surgery – Pavillon T, Hôpital Edouard Herriot, 5, Place d'Arsonval, 69437 Lyon, France. Fax: +33 4 72 11 76 37.

E-mail address: julien.wegrzyn@chu-lyon.fr (J. Wegrzyn).

¹ These authors contributed equally to this work.

mechanisms involved in bone fragility. For example, in cohort studies, bisphosphonates impacted on bone matrix formation, in addition to their well-established antiresorptive effect. They contribute, therefore, to fragility fracture prevention and highlight the necessity for assessment of the bone matrix contribution to whole bone strength [15,16].

This study aimed to investigate the direct contribution of the organic and inorganic bone matrix properties to the mechanical behavior of whole human lumbar vertebrae. We hypothesized that bone matrix directly impacts mechanical behavior at the whole bone level, independently of bone mass and microarchitecture.

Material and methods

Bone specimens

Lumbar spines (L1–L5) were harvested fresh from 17 Caucasian elderly human donors (8 women and 9 men) aged 76 ± 11 years-old. Source of the donors was anatomical donation and their available medical history was limited to the cause of the death. The absence of prevalent fractures or significant bone diseases involving the lumbar spine (i.e., bone metastasis, Paget's disease, or Kellgren–Lawrence grades 3 and 4 lumbar spine osteoarthritis) was assessed using high-resolution lateral radiographs of the whole lumbar spine (Faxitron X-ray Corp., Lincolnshire, IL, USA). Then, the L2 and L3 vertebrae were separated from the lumbar spines and frozen at -20°C wrapped in saline-soaked gauze. Bone mass, trabecular and cortical microarchitecture and bone mechanics were measured on the L3 vertebrae [3–5]. The organic and inorganic trabecular bone matrix properties were assessed on the L2 vertebrae using Fourier transform infrared microspectroscopy (FTIRM) [17].

Bone mass and microarchitecture assessment

After thawing at room temperature, bone mineral content (BMC, g) and areal lateral bone mineral density (aBMD, g/cm^2) of the L3 vertebrae was measured using dual-energy X-ray absorptiometry (DXA; Delphi W, Hologic, Waltham, MA, USA). Then, the posterior arches and surrounding soft tissues including the intervertebral disks were carefully removed. Microarchitecture was measured using a μ -CT device (Skyscan 1076, Aartselaar, Belgium) on L3 vertebral bodies immersed in Ashman's solution. A nominal isotropic voxel size of $35\ \mu\text{m}$ was used (field of view $70\ \text{mm}$, 2000×2000 pixels, X-ray source: $100\ \text{kV}$ – $100\ \mu\text{A}$). Two- to three-dimension processing, analysis and visualization were performed using Skyscan Ant@ software. The following microarchitectural parameters were directly measured: trabecular bone volume per tissue volume (Tb.BV/TV, %), trabecular thickness (Tb.Th, mm), structure model index (SMI, #), degree of anisotropy (DA, #), anterior cortical thickness (Ct.Th, mm) and porosity (Ct.Po, %), and anterior cortical radius of curvature (Ct.Curv, mm).

Mechanical testing

After μ -CT acquisition, L3 vertebral bodies were kept moist at $+4^\circ\text{C}$ with Ashman's solution until mechanical testing. A polyester resin interface (Soloplast V11, Vosschemie, Saint-Egrève, France) with a quick-setting polymerization at low temperature (maximum exothermic peak $< +40^\circ\text{C}$) was applied to each endplate of the L3 vertebral body to achieve parallel surfaces for load application. Then, quasi-static uniaxial compressive testing was performed on the whole vertebral body submerged in $+37^\circ\text{C}$ -controlled Ashman's solution using a screw-driven testing machine (Schenck RSA-250, Darmstadt, Germany) under displacement control at $0.5\ \text{mm}/\text{mm}$ until failure. The compressive load and displacement were measured, respectively, using a $5000\ \text{N}$ load cell (F 501 TC, TME, Signes, France) and a displacement transducer mounted directly on the vertebral resin endplates (Mecanum mechanical engineering, Lyon, France). Preconditioning was performed prior to testing (10 cycles with

loading at $100\ \text{N}$ and unloading at $50\ \text{N}$). The following parameters were determined from the load-displacement data: failure load (N), defined by the force at the maximum on the load-displacement curve, stiffness (N/mm), defined by the linear part of load-displacement curve slope between 25% and 75% of the failure load and, work to failure (N.mm), defined by the area under the load-displacement curve to the failure load.

FTIRM (Fourier Transform InfraRed Microspectroscopy) analysis of bone matrix

L2 vertebrae were sectioned in half using an Isomet Buehler 4000 micro-saw (Buehler GmbH, Düsseldorf, Germany). A cylindrical core sample of trabecular bone was removed in the cranio-caudal direction from the anterior quadrant of the right half of vertebrae using an $8.25\ \text{mm}$ -diameter diamond tipped coring tool. The end plate of each core was removed with the micro-saw. Trabecular cores were fixed in 70% ethanol for 2 weeks, dehydrated for 48 h in absolute alcohol, substituted in methylcyclohexane for 48 h and then embedded in polymethylmethacrylate (PMMA). FTIRM was performed in transmission mode on $2\ \mu\text{m}$ -thick sections with a Perkin-Elmer GXII Auto-image Microscope (Norwalk, CT, USA) equipped with a wideband detector (mercury-cadmium-telluride) (7800 – $400\ \text{cm}^{-1}$). A Cassegrain objective with numerical aperture of 0.6 was used with a spatial resolution of $10\ \mu\text{m}$ at typical mid-infrared wavelengths. Twenty measurements per sample were done at $30 \times 100\ \mu\text{m}$ of spatial resolution to cover the whole surface of the vertebral trabecular core. Each spectrum was collected at $4\ \text{cm}^{-1}$ resolution and 50 scans by spectrum in the transmission mode. Contribution of air and PMMA were subtracted from the original spectrum. After automatic baseline correction (Spectrum Software) and curve fitting of every individual spectrum, GRAMS/AI software (Thermo Galactic, Salem, NH, USA) was used to quantify the characteristics of the spectra (Fig. 1). The following parameters were determined: the mineral crystallinity index which is inversely proportional to the full width at half-maximum of the $604\ \text{cm}^{-1}$ peak (apatitic phosphate environment) and corresponds to both crystal size and perfection [18], the mineralization index which is the area ratio of the bands of mineral matrix over organic matrix (1184 – $910\ \text{cm}^{-1}/1712$ – $1592\ \text{cm}^{-1}$) [17], the mineral maturity which is calculated as the area ratio of the apatitic phosphate over nonapatitic phosphate ($1030/1110\ \text{cm}^{-1}$ area ratio) and reflects the age of mineral [18], and the collagen maturity which is calculated as the ratio of organic matrix bands ($1660/1690\ \text{cm}^{-1}$ area ratio) [18] and reflects the change in secondary structure of collagen in relation to the mineralization process [19] (Fig. 1).

Statistical analyses

Shapiro–Wilk tests were used to assess the normality of the distributions. For Ct.Th, Ct.Po, Ct.Curv and work to failure, distributions

Fig. 1. Typical FTIRM spectra characteristics of a L2 core biopsy showing the peaks of amides (1600 – $1700\ \text{cm}^{-1}$) of the $\nu_4\text{PO}_4$ domain (500 – $650\ \text{cm}^{-1}$) and of the $\nu_1\nu_3\text{PO}_4$ domain (900 – $1200\ \text{cm}^{-1}$).

were normalized using logarithmic transformation. Data were presented as the mean $\pm$ standard deviation. The following tests were used: 1/paired t-tests for the comparison of two continuous variables, 2/Pearson coefficients of correlation for the analysis of the relationships between two continuous variables between gender and 3/forward stepwise multiple regression models for the selection of variables explaining the mechanical behavior. Results were considered as significant if p-value < 0.05. All statistical analyses were performed using SPSS® 16.0 (SPSS Inc., Chicago, IL).

Results

Descriptive statistics for bone mass, microarchitecture, matrix characteristics and whole vertebra mechanics parameters are shown in Table 1. There was no influence of age on bone mass, microarchitecture, matrix and mechanics parameters. No effect of sex was found except for BMC, which was significantly higher in men than in women (7.63 ± 2.03 g vs. 5.62 ± 1.32 , $p = 0.031$).

No correlation was found between the bone matrix characteristics and bone mass or microarchitecture (Table 2). The BMD and Tb.BV/TV were positively correlated with failure load, stiffness and work to failure ($r = 0.56$ to 0.69 ; $p = 0.002$ to 0.019) (Table 2). The SMI was negatively correlated with failure load, stiffness and work to failure ($r = -0.81$, -0.58 and -0.64 ; $p = <0.0001$, 0.014 , 0.005 , respectively) (Table 2). In addition, the parameters of thickness (i.e., Tb.Th and Ct.Th) were positively correlated with work to failure ($r = 0.53$ and 0.61 ; $p = 0.027$ and 0.009 , respectively) (Table 2). The mineral maturity, the mineralization and the crystallinity index were not correlated with whole vertebrae mechanics (Table 2). However, the collagen maturity was positively correlated with whole vertebra failure load and stiffness ($r = 0.64$; $p = 0.005$ and $r = 0.54$; $p = 0.025$, respectively) (Table 2).

Using forward stepwise multiple regression models to explain mechanical behavior with the following equation:

$$\begin{aligned} \text{mechanical behavior (i.e. failure load, stiffness or work to failure)} = & \\ & \text{bone mass} \\ & \text{(i.e. BMC, BMD or Tb.BV/TV)} \\ & + \text{microarchitecture} \\ & \text{(i.e. SMI, Tb.Th, DA, Ct.Th, Ct.Po or Ct.Curv)} \\ & + \text{bone matrix properties} \\ & \text{(i.e. mineralization or crystallinity index, or mineral or collagen maturity),} \end{aligned}$$

only the combination of BMC (1st step, $p = 0.001$), Tb.Th (2nd step, $p = 0.007$) and collagen maturity (3rd step, $p = 0.012$) was significant for the whole vertebra stiffness ($R = 0.84$, $p = 0.001$).

Discussion

Beside geometry, mass and microarchitecture, bone strength is influenced by the intrinsic material properties of the bone tissue, i.e., the organic and inorganic bone matrix. Quality-related bone markers such as enzymatic and non-enzymatic collagen cross-links have been used to reflect the organic bone matrix and were intensively studied in vivo as well as ex vivo [9–11]. Indeed, patients with osteoporosis (OP), characterized by a low BMD and prevalent fractures, exhibited significant reductions in enzymatic cross-links and an accumulation of advanced glycation end-products (AGEs) in bone, without alteration of collagen content, compared to age-matched healthy subjects [9–11,20,21]. Particularly, the extent of non-enzymatic glycation cross-links (AGEs) in the bone matrix has been shown to constitute a major and independent predictor of bone fragility, significantly associated with microdamage accumulation and alterations in trabecular microarchitecture resulting in a reduced fracture resistance [20,21]. In addition, other studies using physicochemical degradation of cortical and trabecular bone have emphasized the role of the organic bone matrix to fracture mechanisms [22,23]. These suggest that fragility of osteoporotic bone is related to collagen changes [22,23]. However, beside these studies, this contribution is not a straightforward analysis to be explored directly at the whole-bone level, and it remains poorly understood in humans. Therefore, comprehensive ex-vivo studies are needed to address precisely the direct contribution of bone tissue constituents to the whole material mechanical properties.

In the current study, we demonstrated that collagen maturity was an independent predictor of the whole vertebra failure load and stiffness explaining respectively 41% and 29% of their variability, independently of bone mass and microarchitecture. In addition, collagen maturity in combination with bone mass and microarchitecture improved prediction of the whole vertebra stiffness, explaining 71% of its variability. Interestingly, we found no significant contribution of mineralization parameters, evaluated by FTIRM, to whole vertebrae mechanical behavior. Overall, at the microscopic level, both mineral and organic matrix contributions are assumed to explain 100% of the mechanical properties. At this scale, mineralization was demonstrated to be a major determinant of elasticity, whereas organic matrix conferred bone toughness, its ability to deform during energy absorption without cracking [13]. At the whole organ level, mineral and organic contributions become minor parameters to explain the mechanical behavior, compared to bone mass and microarchitecture; particularly in such a cohort of elderly subjects characterized by a low BMC and BMD. Using forward stepwise analyses, we demonstrated that, among the major constituents of bone, collagen maturity played the third most important role in whole bone strength after bone mass and microarchitecture, respectively.

Collagen maturity, initially described by Paschalis et al., is related to the Pyridinoline (PYD)/dihydroxylysinoxonoleucine (DHLNL) ratio, i.e. mature over immature enzymatic cross-links, and is calculated as the ratio of 2 sub-bands ($1660/1690 \text{ cm}^{-1}$ ratio) in the amide I band [19]. The amide I band in IR spectroscopy results from the stretching vibration of the peptide carbonyl group ($\text{C}=\text{O}$), and is highly related to the secondary structure of proteins [24,25]. In bone tissue, this band is related to the organic matrix composed by 90% type I collagen and 10% of non-collagenous proteins. Intrinsically, several events occur simultaneously during bone maturation. After being synthesized by osteoblasts, the organic matrix is rapidly mineralized. Simultaneously, organic matrix dehydration occurs with bone mineralization process [26–28]. Along with augmentation in the bone mineral content and improvement of apatite crystal size/perfection, enzymatic and non-enzymatic cross-links (PYD, deoxypyridinoline and AGEs) increase, and non-collagenous proteins (osteopontin and osteocalcin) decrease [9,29–32]. This results in

Table 1
Descriptive statistics (mean $\pm$ SD) for bone mass, microarchitecture, matrix and mechanics.

	Mean $\pm$ SD
Bone mass	
BMC (g)	6.69 ± 1.98
BMD (g/cm^2)	0.62 ± 0.13
Bone microarchitecture	
Tb.BV/TV (#)	16.71 ± 4.41
SMI (#)	1.77 ± 0.20
Tb.Th (mm)	0.23 ± 0.04
DA (#)	0.43 ± 0.03
Ct.Th (mm)	0.71 ± 0.36
Ct.Po (%)	3.15 ± 3.44
Ct.Curv (mm)	33.52 ± 17.33
Bone matrix	
Mineralization index	2.67 ± 0.35
Crystallinity index	25.50 ± 0.81
Mineral maturity	1.54 ± 0.15
Collagen maturity	3.97 ± 0.40
Bone mechanics	
Failure load (N)	2596.30 ± 1147.68
Stiffness (N/mm)	3032.79 ± 1676.27
Work to failure (N.mm)	1651.94 ± 1117.96

Table 2

Pearson's coefficients of correlation between bone mass, microarchitecture, matrix and mechanics. Bold characters: significant correlations.

	BMD	BMC	Tb.BV/TV	SMI	Tb.Th	DA	Ct.Th	Ct.Po	Ct.Curv	Mineralization index	Crystallinity index	Mineral maturity	Collagen maturity	Failure load	Stiffness
BMC	0.90^a														
Tb.BV/TV	0.84^a	0.63^b													
SMI	−0.62^b	−0.45	−0.74^b												
Tb.Th	0.53^c	0.55^c	0.37	−0.16											
DA	−0.26	−0.24	−0.30	−0.25	−0.23										
Ct.Th	0.48^c	0.55^c	0.30	−0.42	0.76^a	0.13									
Ct.Po	0.31	0.43	0.16	−0.47	0.46	0.33	0.83^a								
Ct.Curv	−0.60^c	−0.57^c	−0.38	0.02	−0.41	0.25	−0.36	0.02							
Mineralization index	−0.15	−0.07	−0.04	0.26	0.15	−0.11	−0.17	−0.21	0.21						
Crystallinity index	0.09	0.10	0.12	0.24	0.10	−0.28	−0.31	−0.42	−0.10	0.84^a					
Mineral maturity	0.10	0.03	0.27	−0.21	−0.03	0.20	−0.20	−0.19	0.20	0.71^b	0.66^b				
Collagen maturity	0.25	0.06	0.45	−0.47	−0.09	0.36	−0.02	−0.03	0.08	0.04	0.09	0.64^b			
Failure load	0.65^b	0.48^c	0.69^b	−0.81^a	0.07	0.08	0.24	0.25	−0.10	−0.36	−0.26	0.14	0.64^b		
Stiffness	0.56^c	0.44	0.57^c	−0.58^c	−0.21	0.05	−0.18	0.03	0.01	−0.12	0.04	0.37	0.54^c	0.75^a	
Work to failure	0.58^c	0.40	0.61^b	−0.64^b	0.53^c	−0.01	0.62^b	0.45	−0.27	−0.41	−0.38	−0.20	0.32	0.73^a	0.18

^a $p < 0.0001$.^b $p < 0.001$.^c $p < 0.05$.

modifications of the collagen secondary structure with a tighter packing of collagen with maturation of bone [28,33,34]. Therefore, this simultaneous evolution of several parameters during maturation leads to a complex interpretation of the amide I band in bone tissue assessed by IR spectroscopy [18,19]. Farlay D et al. demonstrated that there was no significant difference in the 1660/1690 cm^{-1} ratio in bone from lathyritic rats characterized by a massive decrease in the formation of collagen enzymatic cross-links due to the inhibition of lysyl oxidase when compared to control rats [18]. This study also demonstrated that the destruction of PYD cross-links using ultraviolet photolysis performed on bovine bone slices did not change the 1660/1690 cm^{-1} ratio when compared to control slices [18]. Such results suggested that this ratio reflected probably more changes in organic matrix maturation and hydration than changes in enzymatic cross-link content [18]. Therefore, our results demonstrated that a more mature and dehydrated organic matrix, with higher collagen packing, was associated with improved bone mechanical behavior of whole vertebrae (i.e., failure load and stiffness). Similarly, in biomaterials science, nanofibers in nanocomposite materials are glued to each other in the absence of water, due to molecular level interactions resulting in a strong and rigid material [35]. However, when water is added, water molecules unglue the nanofibers and the material becomes softer [35]. Indeed, using nanoindentation ex vivo, Bembey AK et al. observed that a hydration increase was associated with decrease in bone tissue stiffness, while a hydration decrease resulted in increased stiffness [36]. In addition, Nyman JS et al. showed that removal of water from the bone matrix using drying conditions impaired significantly mechanical properties of human cortical bone [37]. Therefore, the collagen and its level of maturity and hydration were demonstrated to be important determinants of bone strength independently of mineralization. Our data support the conclusion that disorganization of the organic matrix has a major role in the fracture risk. This observation can be illustrated in the pathophysiology of osteogenesis imperfecta (OI). That is, despite the absence of bone microarchitectural abnormalities, and even with an increased matrix mineralization, the OI bones are highly brittle due to a mutation in the collagen-type 1 triple-helix molecule, resulting in low bone matrix stiffness due to disorganization of the organic matrix [38,39].

This study presented with five main limitations. The first limitation is related to our limited sample size that might underpower this study, particularly regarding the absence of correlation with mineralization parameters. Second, the average age of our subjects was 76 years, so the results might not be representative of the general population, but rather of an elderly population. However, the elderly population is the most susceptible to fragility fracture. Third, whole vertebra mechanics were assessed using quasi-static uniaxial compressive loading. Other loading

conditions that are relevant for vertebral fracture such as cyclic fatigue, bending or shearing may have different associations with bone mass, microarchitecture and matrix properties. Fourth, bone matrix properties and mechanical behavior were measured on different though adjacent vertebrae since both of these analyses induced damage to the vertebra. Finally, this study was focused on FTIRM analysis of the bone matrix and did not evaluate other aspects of the matrix such as enzymatic and non-enzymatic collagen cross-links, collagen content or degree of mineralization.

In conclusion, collagen maturity was an independent predictor of whole vertebra failure load and stiffness, and explained more than two-third of the stiffness variability in combination with bone mass and microarchitecture. No relationship between mineralization parameters and whole vertebra mechanics was found in this study focused on an elderly patient cohort.

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All authors state that they have no conflict of interest that might bias this study.

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