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Unsaturation Level Decreased in Bone Marrow Fat of Postmenopausal Women with Low Bone Density Using High Resolution Magic Angle Spinning (HRMAS) ^{1}H-NMR Spectroscopy

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Abstract

There are increasing evidences suggesting bone marrow adiposity tissue (MAT) plays a critical role in affecting both bone quantity and quality. However, very limited studies that have investigated the association between the composition of MAT and bone mineral density (BMD). The goal of this study was to quantify MAT unsaturation profile of marrow samples from post-menopausal women using ex vivo high-resolution magic angle spinning (HRMAS) proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, and to investigate the relationship between MAT composition and BMD. Bone marrow samples were obtained by iliac crest aspiration during surgical procedures from 24 postmenopausal women (65–89 years) who had hip surgery due to bone fracture or arthroplasty. Marrow fat composition parameters, in particular, unsaturation level (UL), mono-unsaturation level (MUL) and saturation level (SL), were quantified using HRMAS 1H-NMR spectroscopy. The patients were classified into three groups based on the DXA BMD T-scores: controls, osteopenia and osteoporosis. Marrow fat composition was compared between these three groups as well as between subjects with and without factures using ANOCOVA, adjusted for age. Subjects with lower BMD (n=17) had significantly lower MUL (P = 0.003) and UL (P = 0.039), and significantly higher SL (P = 0.039) compared to controls (n = 7). When separating lower BMD into osteopenia (n=9) and osteoporosis (n=8) groups, subjects with osteopenia had significantly lower MUL (P = 0.002) and UL (P = 0.010), and significantly higher SL (P = 0.010) compared to healthy controls. No significant difference was observed between subjects with osteopenia and osteoporosis. Using HRMAS ^{1}H-NMR, significantly lower

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Conflicts of Interest

None.

unsaturation and significantly higher saturation levels were observed in the marrow fat of subjects with lower BMD. HRMAS ^{1}H-NMR was shown to be a powerful tool for identifying novel MR markers of marrow fat composition that are associated with bone quality and potentially fracture, and other bone pathologies and changes after treatment. A better understanding of the relationship between bone marrow composition and bone quality in humans may identify novel treatment targets, and provide guidance on novel interventions and therapeutic strategies for bone preservation.

Keywords

Marrow adipose tissue; marrow fat composition quantification; high-resolution magic angle spinning NMR spectroscopy; bone mineral density

1. Introduction

There is an increasing recognition that bone marrow adiposity plays a critical role in affecting both bone quantity and quality, rather than just “space filler” when there is a bone loss with aging or pathologies (1, 2). Some animal and cell models suggested that, with aging and with certain pathologies and medications, mesenchymal stem cell (MSC) allocation favors adipocytes over osteoblasts, leading to reduced bone density with increased marrow adipose tissue (MAT) (3, 4). In addition, MAT may have important direct effects on the bone marrow microenvironment by secreting an array of factors with potential autocrine and paracrine effects. Some evidence suggests that bone marrow adipocytes inhibit osteoblast activity and may promote osteoclast differentiation (5–7). Clinically, increased marrow fat measured by magnetic resonance imaging (MRI) or ^{1}H-MR spectroscopy (^{1}H-MRS) has been reported in subjects with decreased bone mineral density (BMD) (8–11), as well as in other pathologies including anorexia nervosa and diabetes, which were nicely reviewed in references (12–14).

However, very limited studies in the literature that have investigated the association between the composition of MAT and BMD, have shown mixed results. Using in vivo ^{1}H-MRS, Yeung et al observed increased marrow fat contents and decreased unsaturation level in osteopenic and osteoporotic women compared to controls (9); Bredella et al reported that high saturation level or lower unsaturation level of marrow fat were correlated with low BMD in women with anorexia nervosa (15). Two studies using ex vivo specimens and gas chromatography methods for lipid analysis, however, reported no correlation between marrow composition and BMD (16, 17). Previous studies also suggested that less unsaturated and more saturated fatty acids in MAT, as measured by in vivo MRS, were associated with type-2 diabetes (18), and associated with fractures in postmenopausal women (19). To better understand the association between MAT composition with bone quality will enhance our understanding of the mechanisms that determine the level and composition of marrow adiposity and its impact on bone metabolism.

Nuclear magnetic resonance (NMR) spectroscopy has been suggested as a powerful tool for quantifying lipid composition (20–26). Compared to gas chromatography (GC) and mass spectrometry (MS), which are commonly used to determine the fatty acid composition of

biological samples (27–30), NMR is a non-destructive technique and has the unique advantage that the same sample can be used for direct comparison between the biochemical profile of the tissue and a downstream application such as histology or gene expression after NMR experiment. Furthermore, by spinning the sample at a very fast rate (≈2 kHz) and a specific angle ($\theta = 54.7°$), magic angle spinning (MAS) techniques dramatically reduce chemical shift anisotropy and dipole-dipole interactions such that solution-like spectra with narrow line-widths can be obtained. High-resolution MAS NMR (HRMAS-NMR) can provide high-resolution spectrum from intact tissue, without the need for extensive extraction steps (e.g., chloroform-methanol extraction for lipids) typically used prior to NMR analyses.

To date, studies using HRMAS ^{1}H-NMR for MAT composition are very limited. Zhang et al. showed the feasibility of quantifying marrow fatty acid composition using the whole femur in mice using HRMAS ^{1}H-NMR (31). The goal of this study was to quantify MAT unsaturation profile of marrow samples from post-menopausal women using ex vivo HRMAS ^{1}H-NMR spectroscopy, and to investigate the relationship between MAT composition and BMD and fractures.

2. Material and Methods

2.1. Subjects

Twenty-four postmenopausal women (65–89 years) who had hip surgery due to bone fracture or arthroplasty were recruited for the study at the Trauma Section of Hospital Sótero del Río, Santiago, Chile. All donors considered themselves healthy, except for the surgery, and were not under glucocorticoid or estrogen replacement therapy. Samples from osteoporotic patients with fracture were obtained after 2–7 days after hip fracture. BMD at the lumbar spine (L2–L4) was measured for each subject within 4 weeks of surgery using dual-energy X-ray absorptiometry (DXA) (LUNAR, Prodigy, General Electric Medical Systems, Madison, WI, USA). The study was approved by our institutes. All participants provided written informed consent.

2.2. Sample Collection and Preparation

Bone marrow samples were obtained by iliac crest aspiration during surgical procedures. Bone marrow supernatant fluid was obtained after spinning the bone marrow–aspirated sample (~2 mL) for 5 minutes at 600 × g. Approximately 500 to 800 μL of bone marrow supernatant fluid was collected and kept at −20°C or −80°C before they were shipped on dry ice to UCSF for NMR experiments.

2.3. HRMAS ^{1}H-NMR Data Acquisition

HRMAS ^{1}H-NMR spectra were acquired using a 11.7T (500 MHz for ^{1}H) Varian INOVA spectrometer (Varian Inc., Palo Alto, CA, USA) equipped with a 4 mm gHX nanoprobe with HRMAS capabilities. In addition to allowing the use of magic angle spinning, a nanoprobe was preferred to a “liquids” probe in order to reduce sample size and provide sufficient water suppression and consequently minimize baseline distortions that could in turn affect metabolite quantification. A custom-designed 20 μL leak-proof zirconia rotor containing

oblate spheroid geometry to improve the magnetic field homogeneity across the sample was used. Approximately 3 μL of deuterium oxide containing 0.75 wt % 3-(trimethylsilyl)propionic-2,2,3,3-*d4* acid (D_2O + TSP; Sigma-Aldrich) was added to achieve a field-frequency (D_2O) lock signal and to establish a frequency reference (TSP). The weight of both D_2O and the tissue samples were measured before acquiring data.

The data was acquired at a temperature of 1°C to minimize tissue degeneration during data acquisition. A spin rate of 2250 Hz was used as the spinning side bands generated from the water resonance at this spin rate did not overlap with metabolite resonances at 11.7 T. First order shimming was employed when necessary to ensure that the average line-width at half maximum of the water resonance was 7–8 Hz. A standard single 90° RF pulse was used, which was preceded by a water pre-saturation pulse to attenuate the water signal at 4.8 ppm. 124 signal averages were acquired with a total acquisition time of 12 minutes and a spectral bandwidth of 20 kHz. To quantify the metabolite signal, a synthesized RF pulse was added based on the Electronic Reference To access In-vivo Concentrations (ERETIC) method (8) during the acquisition period to generate a signal in the NMR spectrum with an offset frequency to −0.5 ppm. Reproducibility was evaluated using two samples with each sample being scanned three times.

2.4. HRMAS ^{1}H-NMR Data Processing

The resulting HRMAS spectra were processed using ACD Labs 1D NMR processor (ver. 9.0). The 1D FID's were zero-filled to 131K points, apodized with an exponential function, Fourier transformed, phase corrected, baseline corrected and referenced to TSP at 0ppm. The resonances were assigned to protons of fatty acid chains and glycerol based on studies in the literature (23, 32) and were indicated as letters A-L, as shown in Figure 1. Quantitation was achieved using the peak area under each peak of interest and normalized to ERETIC peak and tissue mass. The triglyceride composition was subsequently calculated as proposed in (23):

$$Unsaturation\ Level\ (UL) = A/L * 3/2 \quad (1)$$

$$Double-unsaturation\ level\ (DUL) = G/L * 3/2 \quad (2)$$

$$Mono-unsaturation\ level\ (MUL) = UL - DUL\ (assuming\ little\ poly-unsaturated\ lipids) \quad (3)$$

$$Saturation\ level\ (SL) = 1 - UL \quad (4)$$

The factor 3/2 was used due to the different numbers of protons associated with resonances of A (two protons), G (two protons), and L (three protons) respectively. The calculation is a simplified quantification of marrow fat unsaturation profile by assuming negligible poly-unsaturated lipids.

A few previous studies used in vivo MRS for evaluating bone marrow fat composition (9, 15, 18, 19). Using in vivo MRS, normally four peaks were able to be resolved: the olefinic, double bond –CH=CH– protons at 5.31 ppm (A in Figure 1), water protons at 4.65 ppm, the CH_2 methylene protons α- to a double bond (–CH=CHCH_2–), at 2.03 ppm (I), and the bulk CH_2 methylene protons at 1.3 ppm (K). The unsaturation level was calculated in previous in vivo MRS studies primarily as: $I_{5.31ppm}/(I_{1.3ppm} + I_{2.03ppm} + I_{5.31ppm}) \times 100\%$. With an attempt to relate NMR measures with the unsaturation level measurement using in vivo MR spectroscopy, we calculated a Pseudo Unsaturation Level (pseudoUL) using peaks that are potentially visible in in vivo MRS as:

$$pseudoUL = A/(A+K+I) \times 100\% \quad (5)$$

2.5. Statistical Analysis

The patients were classified into three groups based on the DXA BMD T-scores with Controls having BMD T-score ≥ −1.0, Osteopenia subjects having −2.5 < BMD T-score < −1.0, and Osteoporosis subjects having BMD T-score ≤ −2.5. ANOCOVA was used to examine the difference in marrow fat composition between these three groups, adjusted for age. If a significant difference from ANOCOVA was observed, unpaired t-tests were used to compare between each two groups, adjusted for age. Unpaired t-tests were also performed between controls and subjects with low BMD (BMD < −1.0, combining subjects with Osteopenia and Osteoporosis), adjusted for age. Secondly, the patients were groups into fracture vs. non-fractured groups and the marrow fat composition was compared between the two groups using unpaired t-tests, adjusted for age.

3. Results

3.1. Study Subjects

Table 1 shows the age, BMI and DXA BMD T-scores of the patients in each group. Four subjects had hip fractures, with one subject from the osteopenia group and three subjects from the osteoporosis group. No significant differences in age and BMI were found between groups, although subjects with osteoporosis tended to be older than control subjects (P = 0.13) and subjects with fracture tended to be older than subjects without fracture (P = 0.07). The BMD T-scores were significantly different between controls, osteopenia and osteoporosis group by the group definition, and the subjects with fracture had significantly lower BMD T-scores than those without fractures.

3.2. Marrow Fat Composition in Groups with different BMDs

The root-mean-square coefficients of variation (RMS-CV) was 4.86%, 2.10%, 3.08%, 3.43%, 0.91% for MUL, DUL, UL, SL and pseudoUL quantification respectively.

Significant differences were found in mono-unsaturation level (MUL), unsaturation level (UL) and saturation level (SL) between groups with different BMD, Table 2. Subjects with lower BMD (n=17) had significantly lower MUL (P = 0.003) and UL (P = 0.039), and significantly higher SL (P = 0.039) compared to controls (n = 7). When separating lower BMD into osteopenia (n=9) and osteoporosis (n=8) groups, subjects with osteopenia had significantly lower MUL (P = 0.002) and UL (P = 0.010), and significantly higher SL (P = 0.010) compared to healthy controls. No significant difference was observed between subjects with osteopenia and osteoporosis. Similar results were obtained when subjects with fracture (n=4) were excluded from analyses. No significant differences were found in marrow fat composition between subjects with (n=4) and without (n=20) fracture, Table 2.

The pseudoUL derived using similar equation as previous in vivo studies were correlated with UL significantly (R = 0.61, P = 0.0016), although pseudoUL was systematically lower than UL, Figure 2. pseudoUL was significantly lower in subjects with low BMD (P = 0.024) and in subjects with osteopenia (P = 0.037) compared to controls, Table 2.

4. Discussion

To our best knowledge, this was the first study correlating HRMAS ^{1}H-NMR measured marrow composition with BMD using human bone marrow supernatant fluid. We demonstrated that the unsaturation level decreased and the saturation level increased in marrow samples from subjects with low BMD. This result suggests not only the amount but more importantly the MAT composition changes during the disease such as osteoporosis. MAT composition quantification will help identify non-invasive and novel markers for bone quality and potentially for fracture.

High field ^{1}H-NMR is a useful ex vivo tool for characterizing biochemical profile of biological tissues. The NMR measures were highly reproducible with coefficients of variation (CVs) < 5% for quantifying unsaturation and saturation levels in the present study and a previous study (24). Studies that have applied the ex vivo NMR technique in bone marrow fat composition analysis, however, are very limited. Yeung et al. examined the MAT composition in extracts of marrow lipids (using deuterated chloroform) from 10 human vertebral body samples (24). Significant correlations were obtained between the NMR and (GC) results for polyunsaturated (R=0.88), monounsaturated (R=0.94) and saturated fatty acid (R=0.94). These results suggest NMR spectroscopy is a reliable method to quantify fatty acid composition for bone marrow.

The magic angle spinning techniques can further reduce the linewidth caused by short T2/T2* component in the biologic tissues and allow direct spectroscopic analysis of intact tissues, instead of extracts. Combining the high field strength and MAS techniques enable resonances to be resolved to a degree that allows identification of chemical markers that may facilitate subtle distinctions between normal and pathologic tissues. Using HRMAS ^{1}H-NMR, we were able to quantify marrow fat composition directly from marrow samples without lipid extraction in this study. The unsaturation level quantified using HRMAS ^{1}H-NMR showed a large range from 15.3% to 51.8%. This unsaturation level was in the range, but with lower average values as compared to previous studies that quantified marrow fat

composition using gas chromatography (GC) (16, 17). This under estimate of unsaturation level of NMR methods as compared to GC was consistent with the report from Yeung et al (24).

Our study showed that the HRMAS ^{1}H-NMR measured unsaturation level was significantly lower and the saturation level was significantly higher in subjects with lower BMD as compared to controls. These results were consistent with previous studies using in vivo MRS that showed the unsaturation level was significantly lower in vertebral marrow of subjects with lower BMD (9) and of subjects with history of fracture, with or without type-2 diabetes (19) compared to their respective matched controls. These results suggested that the marrow fat composition plays an active and significant role in affecting bone quality. The potential mechanism of how marrow fat composition affects bone metabolism, however, is far from being well understood. Some previous in-vitro and in-vivo studies showed that fat, particularly long chain polyunsaturated fatty acids (PUFA), can positively or negatively influence bone remodeling (5, 6, 33–35). PUFA may modulate cellular activity of osteoblasts and osteoclasts (36, 37), and calcium metabolism (38, 39). Very interestingly, human studies have suggested that dietary intake of n-3 PUFA and the n-3/n-6 PUFA ratio were associated with BMD and hip fracture risk (40–43). However, the relationship between the dietary intake of fatty acids and marrow fatty acids are unknown and worth of future investigation.

Our result showing the association between NMR measured unsaturation level with BMD was in contrast to two previous studies that found no significant difference in marrow fat composition in subjects with different BMD using gas chromatographers (16, 17). This difference in experimental findings may result from differences in the quantification methods, different sensitivity and reproducibility of the techniques, differences in specimen location and preparation, and differences in study cohort.

Specifically with specimen collection, marrow supernatant fluids were examined in this study, which is the same as the study by Miranda et al (17), but different from the marrow samples that contain adipocytes as used in the study by Griffth et al (16). The lipid composition in the marrow fluid gives account of lipids in the interstitial compartment surrounding cells in the bone marrow. It does not denote directly adipocytes, nor plasma circulating lipids composition (17). The relative levels of unsaturated and saturated fatty acids compares well to those observed in the human marrow fat tissue, implying a distinct lipid content in marrow supernatant fluids which replicate relevant fatty acids derived from the activity of adipocytes and other marrow cells, rather than those provided by blood plasma composition (17). Previously, such bone marrow supernatant fluids were evaluated and distinctive differences were reported between non-osteoporotic and osteoporotic elderly women (44, 45), substantiating that measurement in the bone marrow supernatant fluid reliably reflects the physiologically significant concentrations in the bone cell microenvironment, albeit dissimilar from circulating plasma or whole blood.

Previous studies also showed mixed results regarding relationship between marrow fat composition and fracture. Using in vivo ^{1}H-MRS, Patsch et al reported subjects with historical fractures had significantly lower unsaturation level and higher saturation level

compared to those without factures (19). Miranda et al, however, using gas chromatographer coupled with a mass spectrometer, reported increased unsaturation and decreased saturation levels in specimens harvested from subjects with hip fractures compared to those without fractures (17). The authors contributed the different observations to different tissues (in vivo intact marrow vs harvested bone marrow supernatant fluid, and different time period after fracture (historical vs several hours after fractures). In our study, no significant differences were found in fat composition between subjects with and without fractures due to very small sample size of the subjects with fractures, although we observed a trend towards decreased monunsaturation levels in subjects with recent hip fractures. Clearly, more studies comparing NMR methods with gas chromatographer/mass spectroscopy methods for bone marrow samples, and the correlation of both methods to BMD and other bone measures from the same subjects are warranted.

In most in vivo MRS spectra in the literature, due to the low spectral resolution, only two peaks (water and saturated lipids with bulk CH_2 methylene protons at 1.3ppm), or sometimes four peaks (previous two peaks plus unsaturated lipids –CH=CH– protons at 5.31 ppm and the CH_2 methylene protons α- to a double bond (–CH=$CHCH_2$–) at 2.03 ppm) were quantified. In this study, a Pseudo Unsaturation Level (pseudoUL) using peaks that are potentially visible in in vivo MRS was calculated using equation (5). We observed a significant correlation between pseudoUL, and the unsaturation level (UL), although the former was consistently less than the latter. The pseudoUL measured from ex vivo NMR in this study ranged from 3% to 11%, corresponding very well with previous in vivo MRS measurements (9, 15, 18, 19). The pseudoUL was significantly lower in subjects with lower BMD as compared to controls, which is also consistent with previous in vivo MRS studies (9). This result suggests that, despite the limited spectral resolution of in vivo MRS data, the in vivo pseudoUL may serve as a useful marker for marrow fat composition evaluation.

There are several limitations of the study. Although HRMAS-NMR can help with minimizing pre-processing of samples such as chloroform-methanol extraction for lipids, it shall be noted that using samples without lipid extraction may introduce potential contaminations from other molecules to lipid signals. In such cases, advanced spectral quantification techniques such as triglyceride fitting models (46, 47) may help with improving the quantification robustness. The sample size of this study was small, in particular for subjects with fractures, and consequently no multi-comparison correction, nor multivariable linear regression including other potential confounding factors such as BMI, was performed during statistical analysis. Furthermore, no in vivo MRS data was available from the same subjects for a more direct comparison with the ex vivo NMR measures. Indeed, a larger-scale study that will collect in vivo MRS data of marrow fat, ex vivo NMR experiments of specimens harvested from the same location, followed by gas or lipid chromatography coupled with mass spectroscopy, would be ideal for advancing the understanding of marrow fat composition quantification, and potentially to help with translating novel markers identified from ex vivo techniques to in vivo measures. HRMAS ^{1}H-NMR can serve as a powerful tool in such studies.

5. Conclusions

Using HRMAS ^{1}H-NMR, significantly lower unsaturation and significantly higher saturation levels were observed in the marrow fat of subjects with lower BMD. HRMAS ^{1}H-NMR was shown to be a powerful tool for identifying novel MR markers of marrow fat composition that are associated with bone quality and potentially fracture, and other bone pathologies and changes after treatment. A better understanding of the relationship between bone marrow composition and bone quality in humans may identify novel treatment targets, and provide guidance on novel interventions and therapeutic strategies for bone preservation.

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Abbreviations

MAT	marrow adipose tissue
BMD	bone mineral density
DXA	dual-energy X-ray absorptiometry
UL	unsaturation level
MUL	mono-unsaturation level
SL	saturation level
HRMAS	high-resolution magic angle spinning
NMR	nuclear medicine resonance

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Highlights

- HRMAS NMR is a powerful tool for quantifying unsaturation profile of bone marrow
- Significantly lower unsaturation levels in marrow fat of subjects with lower BMD
- Better understanding marrow fat/bone interaction to identify novel treatment targets

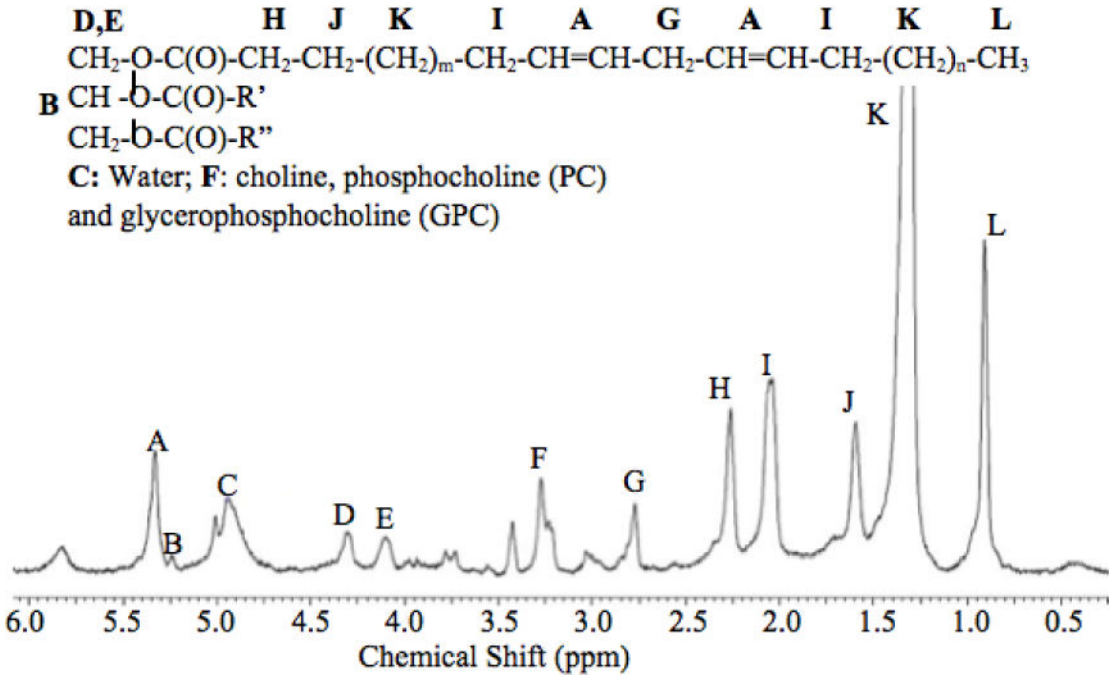

Figure 1.

A representative HRMAS ^{1}H-NMR spectrum of bone marrow specimens. The resonances were assigned to protons on the fatty acid chains and glycerol as indicated by letter A–L.

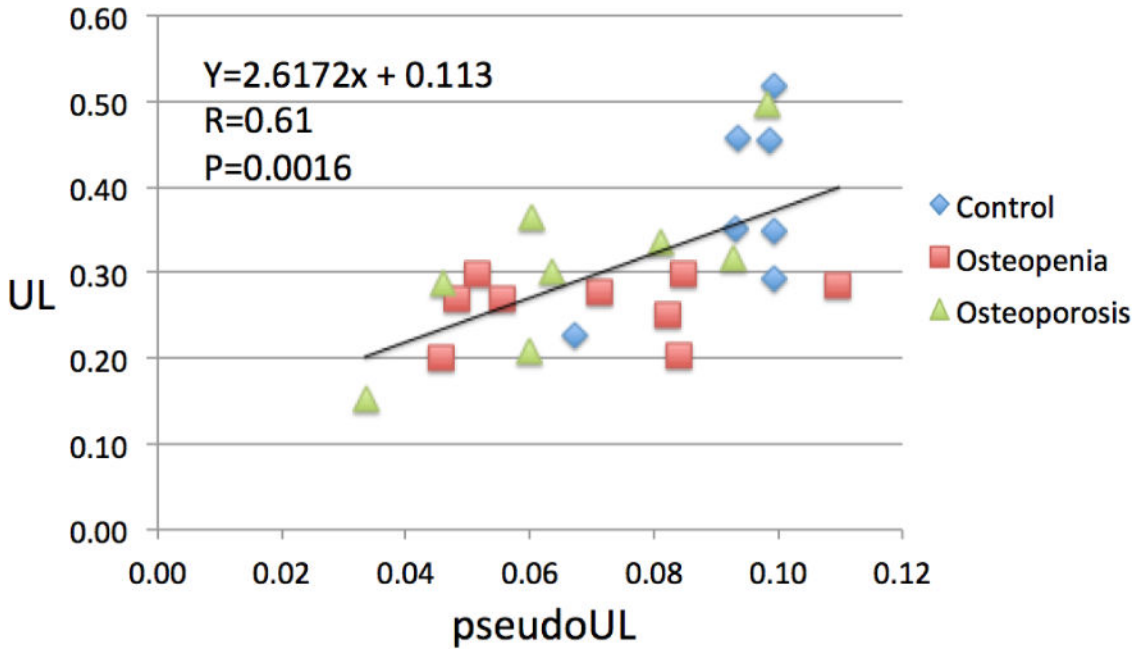

Figure 2.

The pseudoUL derived using equations suggested in previous in vivo studies were correlated with the unsaturation level (UL) significantly ($R = 0.61$, $P < 0.05$), although pseudoUL was systematically lower than UL.

Table 1

Age, BMI and BMD T-scores of subjects.

	Age (years)	BMI (kg/m^2)	DXA BMD T-score in L2–L4
Control (n=7)	70.7± 5.1	27.4 ± 5.0	0.6 ± 1.5
Osteopenia (n=9)	72.2 ± 5.6	26.5 ± 3.2	−1.7 ± 0.3
Osteoporosis (n=8)	77.1 ± 9.0	24.2 ± 3.0	−3.1 ± 0.5
P (control vs. Osteopenia)	0.58	0.68	0.007
P (control vs. Osteoporosis)	0.13	0.17	0.0004
P (Osteopenia vs. Osteoporosis)	0.23	0.16	0.00002
Non-Fracture (n=20)	71.6 ± 5.8	26.5 ± 3.8	−1.3 ± 1.7
Fracture (n=4)	81.0 ± 7.2	24.5 ± 4.4	−3.0 ± 1.0
P (Non-Fracture vs. Fracture)	0.07	0.45	0.03

Table 2

Bone marrow fat composition as measured by HRMAS NMR HRMAS of control, osteopenia and osteoporotic samples.

	MUL	DUL	UL	SL	pseudoUL
Control (n=7)	26.7% ± 7.2%	11.2% ± 3.7%	37.8% ± 10.3%	62.2% ± 10.3%	9.3% ± 1.2%
Osteopenia (n=9)	15.4% ± 4.5%	11.5% ± 3.9%	26.9% ± 3.3%	73.1% ± 3.3%	7.7% ± 2.0%
Osteoporosis (n=8)	15.8% ± 8.4%	15.0% ± 8.3%	30.8% ± 10.3%	69.2% ± 10.3%	6.7% ± 2.2%
P (Control vs. Osteopenia)	**0.002**	0.753	**0.010**	**0.010**	**0.037**
P (Control vs. Osteoporosis)	0.083	0.234	0.549	0.549	0.066
P (Osteopenia vs. Osteoporosis)	0.830	0.177	0.183	0.183	0.979
P (control vs. low BMD)	**0.003**	0.627	**0.039**	**0.039**	**0.024**
Non-Fracture (n=20)	19.5% ± 7.7%	11.6% ± 5.5%	31.1% ± 8.6%	68.9% ± 8.6%	7.7% ± 2.1%
Fracture (n=4)	16.0% ± 10.8%	15.4% ± 7.1%	31.4% ± 14.4%	68.6% ± 14.4%	6.7% ± 2.8%
P (Non-Fracture vs. Fracture)	0.441	0.250	0.951	0.951	0.440