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Raman Spectroscopy of the Human Nail: A Potential Tool for Evaluating Bone Health?

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Raman spectroscopy of the human nail: A potential tool for evaluating bone health?

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Abstract Dual X-ray absorptiometry (DEXA) is the current gold standard for the diagnosis of osteoporosis. However, patients can suffer osteoporotic fractures despite normal bone mineral density, partly because of unmeasured influences of both the protein and mineral phases of bone that are affected in osteoporosis. There is currently no clinically applicable method of evaluating the health of the protein phase. The proteins in human nail (keratin) and bone (collagen) require sulphation and disulphide bond (S–S) formation for structural integrity and disorders of either sulphur metabolism or cystathione beta-synthase can lead to structural abnormalities in these tissues. Raman protein spectra provide a method of non-invasive measurement of the degree of sulphation of structurally related proteins that may be indicative of bone health. Raman spectroscopy was used to evaluate the disulphide (S–S) content of fingernails. The nail samples came from from 169 women (84 pre- and 85 post-menopausal), of which 39 had a history of osteoporotic fracture. BMD was measured by DXA at the spine. Analyses included parametric and non-parametric tests, dependent on the distribution of the test variable. Mean disulphide content of the nail reduced with age and was slightly higher in pre-, compared to post-menopausal women ($P = 0.187$). Significantly lower disulphide content was observed in nails obtained from subjects with a history of fracture ($P = 0.025$). When either disulphide content or BMD was used as a predictor, the odds ratio of these two measures were found to be comparable predictors for fracture status. This suggests that measurements of change in the protein phase of structural proteins such as keratin in the human nail may be correlated with clinically relevant changes in bone proteins that are important in fracture risk.

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1 Introduction

Dual X-ray Absorptiometry (DXA) scanning is the current standard for the diagnosis of osteoporosis, allowing determination of a fracture risk at the measured site [1, 2], in association with other risk factors. DXA is limited in its ability to detect individuals who will fracture [3]. It measures bone mineral density (BMD) but cannot measure the micro-architecture of bone, the crystal organisation, size and shape, the connectivity of the trabecular network, ability to repair micro-cracks, and the structure of the bone proteins [4–7]. In a previous study the authors used both Raman spectroscopy and nano-indentation to examine the protein phase of nail sourced from women undergoing treatment for osteoporosis. The mean moduli of fingernails from patients with low BMD were lower than those with normal BMD. The

mean difference in mean modulus between the groups was found to be 0.996 ($P = 0.15$ between groups). The spectroscopy data also showed differences between the two sets of nails. The disulphide bond content of the nails sourced from osteoporotic patients, as evaluated by the width half maxima (WHM) method, was lower than that from healthy patients ($P = 0.06$ between groups) [8]. These data led us to further investigate the possible link between the protein phase of the nail and bone health.

The mechanical strength of the nail protein keratin is determined, in part, by the content of the sulphur-containing amino acids that form disulphide linkages within its tertiary structure. In this respect a change in the disulphide content of the protein phase of nail may affect the mechanical integrity that the protein phase provides [9]. Raman protein spectra can be used to determine the S–S vibrational band originating from the amino acids methionine, cysteine and cystine in biological tissues, e.g. nail and bone [10, 11]. The C–S and S–S bands of the Raman spectra obtained from these tissues reflect the cysteine and cystine content since methionine is negligible.

As with the nail protein keratin, cysteine incorporation and disulphide bonding also occurs in the protein matrix of bone. Disulphide bonding is a feature of the C-propeptide and N-propeptide domain of procollagen during folding and of inter-chain disulphide bonding in mature collagen [12]. In addition, bone contains cysteine-rich matrix proteins such as osteonectin (secreted protein acid rich in cysteine, SPARC; BM-40) and the family of proteins of the transforming growth factor-B (TGF-B) superfamily of signalling proteins that are critical to bone remodelling, bone architecture and the maintenance of bone mass [13, 14].

In this paper we describe an innovative, non-invasive approach to study change in the composition of structural proteins. Using Raman spectroscopy, the disulphide content of the human nail obtained from pre- and post-menopausal women was compared and analysed against BMD and history of fracture.

2 Materials and methods

2.1 Subjects

With the approval of the University of Limerick Research Ethics Committee (ULREC 03/62) and informed consent, females aged 18–67 years ($n = 169$) volunteered to participate in this study.

2.2 Analysis of nail

Two nail clippings were taken from each subject from the free edge of the nail plate and stored in sealed specimen jars prior to analysis. Raman spectroscopy (Inspector Raman Instrument, Delta Nu, Wyoming, USA) was performed on the underside of the fingernail. Excitation was by HeNe laser operating at 633 nm. Spectra were recorded (400–1800 cm^{-1}) by securing the nail to the analysis port and performing 10 scans, to improve the signal-to-noise ratio, each with a laser exposure time of 5 s. To evaluate the intensity of the S–S bonding in the sample, the previous method of evaluating the intensity of the S–S bond, WHM, was replaced with a peak height method, following discussions with the Raman manufacturers. Using the peak height method, the stretching vibration of the disulphide bond (S–S) at 510 cm^{-1} was measured relative to the methylene (CH_2) deformation band at 1450 cm^{-1} (Fig. 1).

Normalising the S–S peak to the CH_2 peak addressed concerns regarding variable nail thickness. The average of 10 scans was taken as one determination. Two determinations were performed upon each of the two nails. The result was recorded as the mean (S–S) peak height of the four readings.

2.3 Measurement of BMD

Bone mineral density was measured by DXA (Norland, NY, USA) with strict adherence to recommended quality assurance practice. Precision error of measurement was 0.9% at the lumbar spine (L_2–L_4).

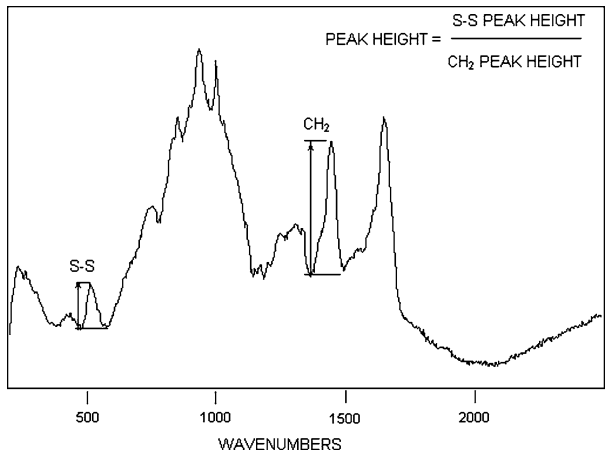

Fig. 1 Raman spectrogram of a human nail

2.4 Statistical analysis

Dependent variables were tested for normal distribution (Kolmogorov–Smirnov test). Peak height of the S–S peak of the nail was not normal at its extremes and was analysed by ANCOVA and logistic regression as well as non-parametric testing using SPSS software.

3 Results

3.1 Subjects

Details of the physical characteristics of the subjects are provided in Table 1. In total, 39 (23%) of the 169 subjects reported a history of fracture (mainly wrist, ankle and arm fractures), 18 of these were in the pre-menopausal and 21 in the post-menopausal group.

3.2 Raman spectroscopy and disulphide content of the nail

Figure 1 shows a typical Raman protein spectrum of a human nail. All the major spectral peaks of human nail [10, 11] were found. The height of the S–S peak was found to vary between subjects (mean(SE) 0.745(0.013); median 0.73; min–max 0.4–1.17), approximate to a normal distribution (Fig. 2) and to be unrelated to the subjects' age (Fig. 3).

The height of the S–S peak of the nail was greater for pre-menopausal than post-menopausal women but this difference was not statistically significant ($P = 0.187$; Fig. 4). Independent of menopausal status, the median height of the S–S peak was found to be significantly greater ($P = 0.025$ Mann–Whitney test; Fig. 4) for the no-fracture (median = 0.73) than the fracture (median = 0.63) group. Adjusting for age and menopausal status the height of the S–S peak at mean age (45.3) was also significantly greater for the no-fracture group than for the fracture group ($P = 0.044$). When adjusted for age only, the height of the S–S peak was significantly different between fracture groups ($P = 0.047$).

Table 1 Physical characteristics of the subjects and incidence of fracture

		Age	BMI	Fracture
Pre-menopausal ($n = 84$)	Mean	33.2	24.7	
	SD	11.6	4.5	
	(Min–max)	18–53	15.5–37.4	$n = 18$
Post-menopausal ($n = 85$)	Mean	57.5	25.8	
	SD	5.5	3.6	
	(Min–max)	43–67	18.2–34.3	$n = 21$

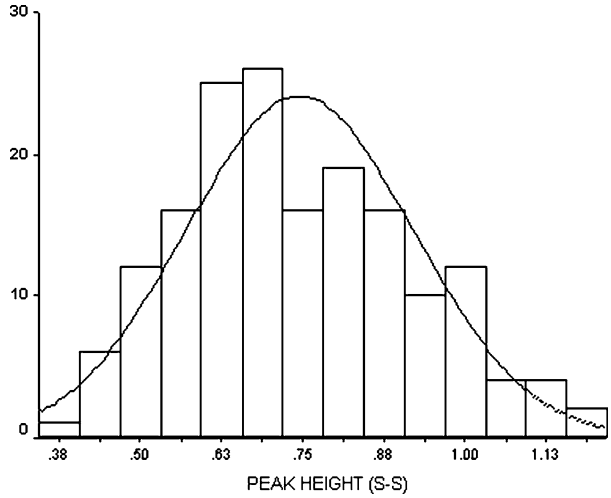

Fig. 2 Histogram of disulphide content of nails sourced from women subjects ($n = 169$)

3.3 Relationship between height of the S–S peak, BMD and fracture risk

Figure 5 depicts the relationship between height of the S–S peak and BMD for the lumbar spine for all subjects. Regression analysis indicated only a weak association ($R^2 = 0.1521$) between these two variables.

Logistic regression analysis was carried out in an attempt to predict fracture risk. Height of the S–S peak gave an odds ratio of 0.188 95% CI (0.115, 0.307) ($P < 0.0005$) when a constant term was not included in

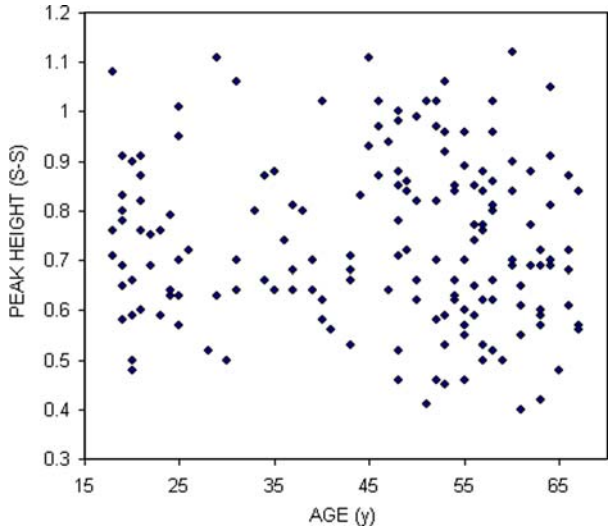

Fig. 3 Disulphide content of nails sourced from women subjects in relation to age ($n = 169$)

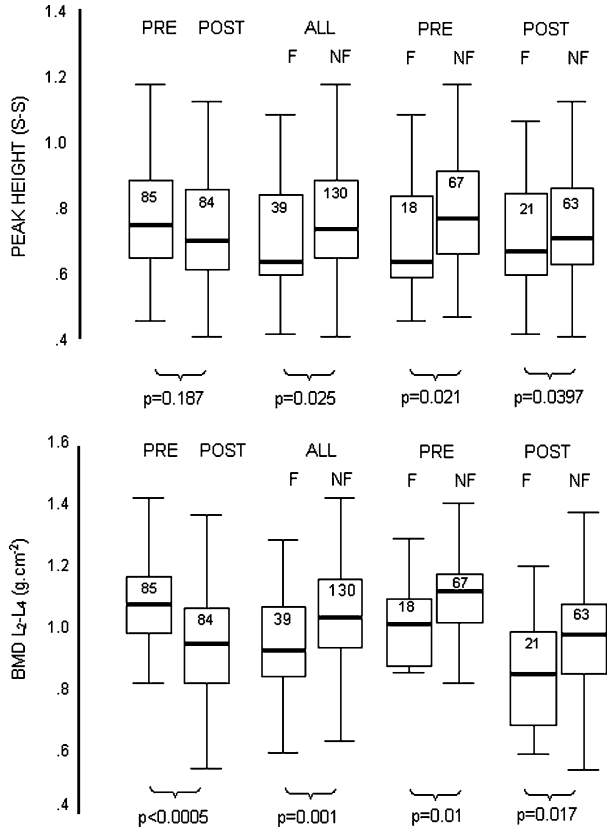

Fig. 4 Boxplot of the disulphide content of the nail and BMD (L_2–L_4) of pre- and post-menopausal women and in relation to non-fracture (NF) and fracture (F)

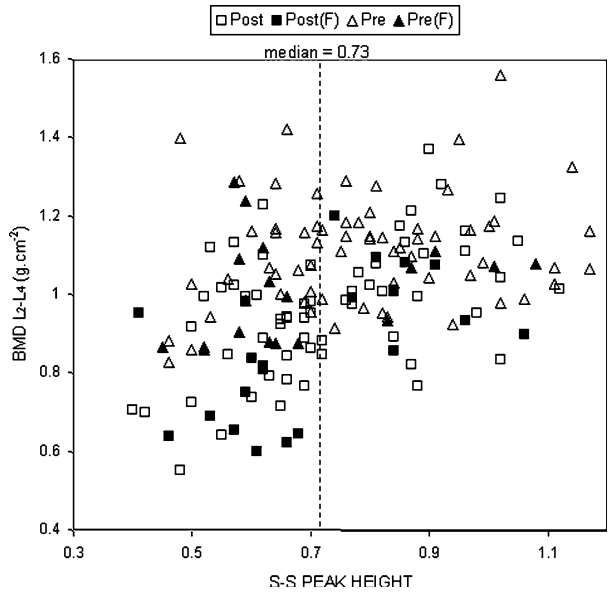

Fig. 5 Relationship between disulphide content, BMD and fracture in pre- and post-menopausal women ($n = 169$). Filled symbols represent subjects with history of fracture

the model. When BMD was used as a predictor the odds ratio was 0.282 95% CI (0.197, 0.405) ($P < 0.0005$) indicating that these two measures are comparable predictors for fracture status using this model.

4 Discussion

Raman protein spectra of the S–S vibrational band originating from sulphur-containing amino acids was used to determine changes in the protein composition of the fingernail. The main finding was a distinct difference in the disulphide content of nails sourced from pre- and post-menopausal women and the lower disulphide content in nails sourced from women with a history of fracture independent of menopausal status. These data support our previous observation of an association between reduced disulphide content and an increase in the brittleness of the nail in women undergoing treatment for osteoporosis [8].

In this study significantly lower disulphide content in subjects with a history of fracture was observed. It is argued that the disulphide content in the nail protein keratin may reflect changes in the disulphide content of the protein phase of similar matrix proteins present in bone. In osseous tissue the placement of cysteine residues within structural proteins and consequently disulphide bonding results in structures with varied functional properties. Cysteine incorporation and a degree of sulphation are common to many bone proteins and cysteine residues with disulphide bonding are also a feature of non-collagenous bone proteins. Of these, the main cysteine-containing bone matrix proteins appear to be osteonectin (secreted protein acid rich in cysteine, SPARC; BM-40) and a family of proteins of the transforming growth factor-B (TGF-B) superfamily of signalling proteins. Proteins of the TGF-B superfamily are disulphide-linked dimers composed of two polypeptide chains, each containing seven highly conserved cysteine residues. The crystal structure of some of these proteins e.g. BMP-2, contain a cysteine-knot with two finger-like double strands [15] indicating that the cysteine residues are important for the three-dimensional structure of the protein [16]. Osteonectin, a glycoprotein, is critical in the support of bone remodelling maintenance of bone mass and bone architecture [13]. Human trabecular bone has 20- to 40-fold more osteonectin than cortical bone, making trabecular bone more sensitive to osteonectin content. Given the progressive decline in trabecular bone in adults, osteonectin is likely to be linked to the

maintenance/decline of adult bone in ageing and/or osteoporosis.

The authors propose that the change in disulphide content of the nail could act as a surrogate marker of a similar change in related proteins of osseous tissue. As explained above, loss of cysteine would be expected to affect, both indirectly and directly, collagenous and non-collagenous proteins. Moreover, there is an in vivo exchange between inorganic and organic sulphate chiefly due to the synthesis and breakdown of sulphated glycosaminoglycans, which form the ground substance of bone matrix. The expression of sulphur-containing bone matrix proteins is also considered to be important in bone fracture repair as promoters and regulators of bone remodelling.

DXA is limited in its ability to detect individuals who will fracture [4]. DXA measures bone mass but cannot measure the micro-architecture of bone, the crystal organisation, size and shape, the connectivity of the trabecular network, ability to repair micro-cracks or the structure of the bone proteins [5–7]. Equally, the overlap in BMD between individuals who have sustained fractures and those who have not would indicate that low BMD is not the only cause of fragile bones. Thus, while the degree of mineralisation is the current standard by which osteoporosis is diagnosed, DXA is unable to detect bone fragility due to deficiency in the protein matrix. This study found only a weak association between Raman measurement of disulphide content and bone mineral density as measured by DXA suggesting that, whilst both the protein and mineral phases of bone both influence fracture risk they do so to different degrees. In this respect it is interesting to observe the distribution of subjects with history of fracture in relation to BMD and disulphide content as depicted in Fig. 5. This analysis shows that 26 of the 39 subjects (67%) with history of fracture recorded values below the median value of disulphide content of the nail as measured by Raman spectroscopy.

5 Conclusion

Fingernails sourced from a cohort 169 healthy women revealed lower disulphide content in women post-menopause. In addition, the disulphide content of nails from women with a history of fracture was significantly lower than those with no history of fracture. These data support earlier observations linking the physical properties of the nail to its disulphide content. It is proposed that disulphide content of the nail may act as a surrogate marker of similar change in related structural matrix proteins in osseous tissue that could compliment existing measures used to identify those at risk of fracture.

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