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J. Renwick Beattie

Diane Feskanich

M. Clare Caraher

Mark R. Towler
Missouri University of Science and Technology, mtowler@mst.edu

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A Preliminary Evaluation of the Ability of Keratotic Tissue to Act as a Prognostic Indicator of Hip Fracture Risk

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J Renwick Beattie[1], Diane Feskanich[2], M Clare Caraher[3] and Mark R Towler[4]

[1]J Renwick Beattie Consulting, Causeway Enterprise Agency, Ballycastle, UK. [2]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA. [3]ICON plc, Dublin, Ireland. [4]Department of Mechanical and Industrial Engineering, Ryerson University, Toronto, ON, Canada.

ABSTRACT: Studies have shown that Raman spectroscopic analysis of fingernail clippings can help differentiate between post-menopausal women who have and who have not suffered a fracture. However, all studies to date have been retrospective in nature, comparing the proteins in nails sourced from women, post-fracture. The objective of this study was to investigate the potential of a prospective test for hip fracture based on spectroscopic analysis of nail tissue. Archived toenail samples from post-menopausal women aged 50 to 63 years in the Nurses' Health Study were obtained and analysed by Raman spectroscopy. Nails were matched case-controls sourced from 161 women; 82 who underwent a hip fracture up to 20 years after nail collection and 81 age-matched controls. A number of clinical risk factors (CRFs) from the Fracture Risk Assessment (FRAX) tool had been assessed at toenail collection. Using 80% of the spectra, models were developed for increasing time periods between nail collection and fracture. Scores were calculated from these models for the other 20% of the sample and the ability of the score to predict hip fracture was tested in model with and without the CRFs by comparing the odds ratios (ORs) per 1 SD increase in standardised predictive values. The Raman score successfully distinguished between hip fracture cases and controls. With only the score as a predictor, a statistically significant OR of 2.2 (95% confidence interval [CI]: 1.5-3.1) was found for hip fracture for up to 20 years after collection. The OR increased to 3.8 (2.6-5.4) when the CRFs were added to the model. For fractures limited to 13 years after collection, the OR was 6.3 (3.0-13.1) for the score alone. The test based on Raman spectroscopy has potential for identifying individuals who may suffer hip fractures several years in advance. Higher powered studies are required to evaluate the predictive capability of this test.

KEYWORDS: Clinical risk factors, fracture risk assessment, Nurses' Health Study, osteoporosis, Raman spectroscopy, toenail

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CORRESPONDING AUTHOR: J Renwick Beattie, J Renwick Beattie Consulting, Causeway Enterprise Agency, Ballycastle, UK. Email: rene@jrenwickbeattie.com

Introduction

Osteoporosis is a skeletal disorder characterised by compromised bone strength predisposing a person to an increased risk of fracture. The current reference standard for the diagnosis of osteoporosis is the measurement of bone mineral density (BMD) using dual-energy X-ray absorptiometry (DXA). Although low BMD is among the strongest known risk factors for fragility fractures, it is not robust.[1,2] For example, in the Rotterdam Study, only 44% of the women and 21% of the men with a non-vertebral fracture had a BMD below the threshold for osteoporosis.[1] To improve its predictive performance, there has been a trend towards the use of clinical risk factors (CRFs) in combination with BMD measurements.[3] One such clinically accepted risk calculator is the Fracture Risk Assessment Tool (FRAX).[4] Although the use of FRAX may be an advance over BMD alone, there is still room for improvement. In a recent systematic review and meta-analysis on the performance of FRAX in predicting 10-year risk of major osteoporotic fractures, a substantial number of patients who developed fractures were missed by the baseline FRAX assessment.[5] Furthermore, FRAX includes BMD as an input in predicting fracture risk, and assessment of BMD by DXA or other instruments requires direct patient measurement by highly trained staff and regular equipment calibration, making it unsuitable for widespread use. With an anticipated rising worldwide population burden of osteoporosis and fragility fractures,[6] the World Health Organization has identified a need for improved prognostic indicators and alternatives to BMD-based diagnostic tools to assess fracture risk.[7,8] The ideal test for bone fragility would be amenable to high sample throughput operation in clinical reference labs and would work in conjunction with known CRFs to enable the screening of a broader population base and aid preventative treatment decisions. Such a test that is capable of accurately assessing long-term fracture risk would be a significant advance in osteoporosis diagnosis and fracture prevention.

The use of nail tissue, rather than bone, could possibly provide a simpler means of assessing fracture risk. Previous studies by the authors have hypothesised a relationship between human nail structure, osteoporosis, and fracture risk[9–14] due to common exposure to systemic factors for bone and nail. These studies have used Raman spectroscopy, an optical analytical technique for obtaining semi-quantitative and qualitative information on complex samples. The spectrum of a sample is

a linear combination of the spectra of all the molecules in the sample and can be regarded as an optical molecular fingerprint of the sample.[15] Keratin and type I collagen are the 2 key proteins in nail and bone, respectively, and they both undergo post-translational modifications, which can be identified using Raman spectroscopy.[16–18] Recently, these 2 proteins as measured in the bone and claw of rats have been shown to exhibit parallel disordering under systemic changes induced by loss of ovaries.[19] In humans, loss of ovaries leads to a decline in oestrogen production (and therefore systemic circulation) is prominent and is associated with increased fracture risk.[20] In our most recently published clinical study, Fracture Risk Assessment by Nail (FRAN), nail clippings were sourced from 633 postmenopausal British and Irish women from 6 clinical sites, of whom 42% had experienced a fragility fracture.[13] Results from the test set showed that a novel algorithm, combining spectroscopy data with clinical data, provided area under the curve (AUC) of 74% compared with an AUC of 62% from a reduced QFracture Score[21,22] (a clinically accepted risk calculator, similar but not identical to FRAX) and 60% from the DXA *T* score. Spectroscopic interpretation revealed that the test was discriminating those at risk of fracture based on a pattern of differences in amino acid composition and concomitant differences in the higher levels (secondary to quaternary) of structure of the keratin protein.[14] As promising as the outcomes of this study were, it was not designed to test the predictive ability of the tool.

The study reported here was designed as a preliminary prospective test to understand the prospects of using Raman spectroscopic measurement of nails to predict risk of future fracture. The objective was to assess the ability of Raman spectroscopic analysis of nail samples in post-menopausal women less than 65 years to discriminate between those who have and have not suffered a hip fracture over a time period up to 20 years after sample collection in a nested case-control study. The nail samples were sourced as matched case-control pairs from those archived in the Nurses' Health Study (NHS), a long-term cohort study of women. Alongside testing the prospective power of Raman spectroscopy to identify those who would go on to suffer a hip fracture, the study also explored whether superior performance could be obtained with the Raman-based result in combination with CRFs over the CRFs alone.

Methods

Nurses' Health Study

The NHS is a cohort study which commenced in 1976 with 121 700 female registered nurses aged between 30 and 55 years and living in one of the 11 most populous US states. Approximately 96% of the participants are white. The women have been followed up by questionnaire every 2 years. The CRF data from the biennial questionnaire immediately prior to toenail collection that are part of FRAX include race, smoking status, weight and height (from which body mass index [BMI] was calculated), thyroid hormone use, rheumatoid arthritis, diabetes, wrist fracture, osteoporosis diagnosed by a physician, age at menopause, and alcohol consumption. Information on femoral neck BMD and other FRAX CRFs, ie, glucocorticoid use, parental history of hip fracture, and some secondary causes of osteoporosis, were not available. Diagnoses of stroke and cancer and use of post-menopausal hormones were also assessed and used to identify the study population.

With respect to hip fracture outcomes, the women were asked to report all previous fractures in 1982 and subsequent fractures were recorded on later questionnaires. Report of hip fracture can be expected to be accurate in a population of trained nurses. Validity was examined in a 1986 study that confirmed in 30 reports of hip fracture that were all present in the medical records.[23] A number of studies focused on hip fracture risk have already been conducted in this cohort and have shown that activity, BMI, and vitamin D are associated with lower risk and that abdominal obesity is associated with higher risk.[24–27]

Archived toenail clippings

Toenail clippings were collected from 62 865 participants in the NHS cohort between December 1982 and July 1984 when the participants were between 36 and 63 years of age (median = 48.9). Participants were asked to send clippings from all 10 toenails. The clippings were stored in dust and lightproof envelopes in a dry environment at room temperature following collection. The nails were originally collected to investigate the relationship between selenium levels in the nail and cancer risk[28,29] and have since been used in a number of published studies.[30,31] The toenail samples used in these investigations were often destroyed in processing; therefore, 49 820 samples remained in storage.

Study population and design

A nested case-control design was selected to establish the ability of the Raman-based analysis to differentiate between women with a history of hip fracture and control subjects. The study population from which the sample was drawn consisted of the women who, at the time of their toenail return, were post-menopausal, aged 50 to 63 years, not currently using post-menopausal hormones, and without a hip fracture, stroke, or cancer history (n = 13 312). From this population, we identified 279 cases who had a hip fracture from 3 to 20 years after toenail return (median = 14.5 years). Hip fractures due to traumatic events (eg, motor vehicle accident, skiing, horse riding) were excluded. Of the remaining women in the study population who did not report a hip fracture through 2004, we randomly selected 1 control per case matched on month and year of birth.

For this study in which we developed the Raman analytic algorithm, we randomly selected 82 case-control pairs from the available 279 pairs to ensure that we would have sufficient

remaining toenails for a later validation study. One sample envelope proved to be empty; therefore, 163 samples were available for analysis. Approval for the study was obtained from the Institutional Review Board of the Brigham and Women's Hospital. The subjects provided informed consent when the nail samples were collected, and the study was conducted in accordance with the Declaration of Helsinki.

Nail spectral measurement and processing

The instrumentation used was a Sierra Reader (Snowy Range Instruments, Laramie, WY, USA) using 785-nm excitation with 50 mW power at the sample. Measurements were conducted by 3 operators blind to clinical details, based at one location (C-TRIC, Altnagelvin Hospital, Londonderry, UK), and using one instrument. Triplicate, spatially separated measurements, each lasting 1 minute, were conducted on each sample. The nails were inspected by the naked eye to confirm that they were free of visible contamination, then the nails were placed so that the upper surface of the nail faced the laser exit aperture. No further sample preparation was undertaken. The Raman data collected from the nails were processed using models created in a previous study.[13] Briefly, this was a singular-value decomposition–based background removal[32–34] modelled on an archive of 1500 nails (excluding all nails used in test set for this study), with linear interpolation of the loadings using 12 points. The data were then normalised to the first PC score, using MATLAB 2013a (Natick, MA, USA).[35,36] Spectra were acquired from 400 to 1800 cm^{-1}, and this full spectral range was used for the data processing and analysis (see below).

Prediction model development

Prediction models were generated from paired toenail samples for increasing time periods between sample collection and onset of fracture (in the matched case). The smallest time period that could be investigated was up to 10 years due to the small number of samples available at shorter periods being insufficient to build useful models. Further periods increased by 1-year increments up to a maximum of 20 years. Within each increment, we aimed to assign approximately 75% to develop the model and withhold 25% to test the model. As each increment had relatively few samples, the rounding effect resulted in an overall 80/20 split in the development and test sets. For example, at 10 years, 14 case-control pairs were assigned to the development set and 5 pairs to the test set. The next 1-year increment added 4 pairs to the development set and 1 to the test set so that the total for the 11-year period was 18 and 6, respectively. The counts at the maximum 20-year period were 67 and 15 pairs, respectively.

For each period, the spectra were subject to a principal component analysis, whose scores were filtered by significance on a *t* test (between cases and controls) to reduce the dimensionality of the data. The 4 PC scores selected were then input into linear discrimination analysis (LDA) to develop a predictive multivariate statistical model.[37] The data reduction and filtering reduced the number of input variables for the LDA from 776 to 4, reducing the risk of overfitting. For clarity, any reference to 'scores' in the article refer to the LDA discriminant scores unless indicated otherwise.

The Raman score and risk of hip fracture

In the test set of toenails, we examined the association between the Raman scores and the risk of hip fracture for increasing time periods in unconditional logistic regression models controlled for age.

The performance of the Raman scores alone, the CRFs alone, and the score in combination with the CRFs were evaluated. Only CRF data available at the time of the nail sample collection were used as inputs into the models to avoid biasing the analysis. Only the CRFs with an estimated (based on prevalence in the training set) 1 positive sample present in both groups in test set were included in the models. To compare the models which contain both continuous and categorical data, the data were standardised to a distribution with mean = 0 and SD = 1 and predictive values were generated by generalised linear model for a binomial reference variable (fracture vs non-fracture). The odds ratio (OR) per 1 SD increase was used to evaluate the performance of the linear model.

Partial subtraction spectra

When comparing spectra, it is common to simply scale the spectra either to the mean intensity or to a standard peak. In complex spectra, neither solution is entirely satisfactory as the large number of overlapping bands can make the resulting difference spectrum difficult to interpret. As a visual aid, we have prepared partial subtraction spectra, where we have scaled the subtrahend such that the minuend has no negative peaks. This point is decided by subtracting until negative bands appear then reducing the scaling factor to the point just before any negative features are observed. We also show the traditional subtraction spectrum (spectra were already normalised as described above so no further scaling adjustment applied) in Figure 1 so that those more familiar with the traditional subtraction spectrum can compare.

Results

Characteristics of the study population

Characteristics of the hip fracture cases and controls at the time of toenail collection are shown in Table 1. As cases and controls were matched on month and year of birth, the mean age (58 years) was identical in both groups. None of the cases or controls had a diagnosis of rheumatoid arthritis when the toenails were collected; therefore, this factor could not be considered in further analyses. About 99% of both cases and controls

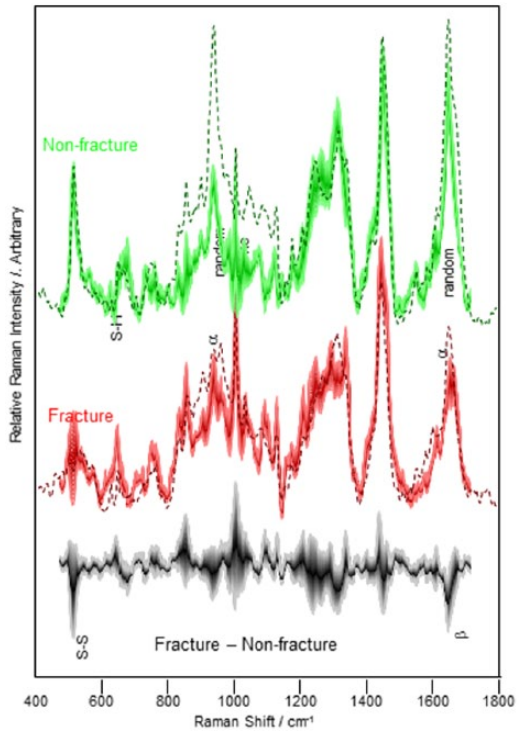

Figure 1. Raman spectra for the Nurses' Health Study 4-year fractures (dark red, dark green, and blue) compared with FRAN fractures (light green, bright red, and black). Most of the same features are visible. This is the shortest interval between collection and fracture so would be expected to be most similar to FRAN. FRAN indicates Fracture Risk Assessment by Nail.

Table 1. Clinical risk factor at time of toenail collection in hip fracture cases and controls in the Nurses' Health Study.

	CASES (N = 82)	CONTROLS (N = 81)
Age[a], y (mean ± SD)	57.6 ± 3.2	57.6 ± 3.3
BMI, kg/m^2 (mean ± SD)	24.4 ± 4.3	25.5 ± 4.6
Current smoker (n)	28	21
Osteoporosis (n)	2	4
Wrist fracture (n)	19	18
Rheumatoid arthritis (n)	0	0
Diabetes type 1 (n)	1	0
Age at menopause <45 y (n)	8	8
Thyroid hormone user (n)	16	5
≥3 alcoholic drinks/d (n)	1	3
White (n)	81	80
African American (n)	1	0
Asian (n)	0	1

[a]Controls were matched to cases on month and year of birth.

were white, with 1 African American subject among the cases and 1 Asian subject among the controls. The cases had a higher number of smokers and thyroid hormone users, whereas controls had a higher mean BMI and were more likely to report a diagnosis of osteoporosis and high alcohol intake.

Spectral analysis. Figure 1 illustrates the mean spectra grouped into case and control groups with an increment of 4 years between collection and fracture, with subtractions scaled to the point just before negative features appear in the spectra (plus the standard subtraction in black). These are superimposed on the equivalent spectra observed in our previous clinical study.[13] Our previous clinical study was comparing existing fracture (increment <0) and no samples available for this study had a delay shorter than 4 years, so these spectra represent those closest to the conditions of this previous study.

The spectral features correspond well to the spectral features identified in our previous studies,[9–13] with the disulphide stretching mode 510 cm^{-1} very prominent in the non-fracture controls, whereas the fracture cases exhibit a weaker broader peak shifted to 525 cm^{-1}. The fracture cases also exhibit a strong doublet at 625 and 645 cm^{-1}, a pair of peaks characteristics of free S-H bonds. In the amide I region, sensitive to protein secondary structure, the fracture cases exhibit elevated intensity around 1653 cm^{-1}, whereas the non-fracture controls exhibit elevated intensity around 1660 cm^{-1}.

Figure 2A shows the distribution of samples by increment between collection and occurrence of fracture. Most of the fractures occurred beyond 15 years after collection, with the mode being a fracture occurring between 17 and 18 years after collection. Figure 2B shows how the difference in mean Raman score between cases and controls varies with the time to fracture. Between 4 and 8 years, the difference is relatively consistent, but between 8 and 15 years, the contrast between cases and controls gradually declines. From 15 years and beyond a plateau is evident where the mean difference is 1/5 of the difference between 4 and 8 years.

Figure 3 compares average non-fracture and fracture spectra at the 2 plateaus in Figure 2B and in the middle of the slope. Many features consistently appear in the same set of donors, including the disulphide mode (510 cm^{-1}), tyrosine modes (810 and 830 cm^{-1}), and the bands sensitive to secondary structure (amide I: 1630-1680 cm^{-1}, amide III: 1220-1320 cm^{-1}, and the C-C stretching modes: 920-1000 cm^{-1}).

Statistical analysis: hip fracture prediction

The Raman score from hip fracture cases and controls up to 20 years after toenail collection achieved an OR of 2.2 (95% confidence interval [CI]: 1.5-3.1) in the test set (Table 2). The OR based on the CRFs alone was similar, and the highest predictability was achieved for the Raman score and CRFs together (OR = 3.8, 95% CI: 2.6-5.4).

Results are also shown for Raman scores from hip cases and controls up to 13 years after toenail collection, a period that was selected because it represents the approximate midpoint of the

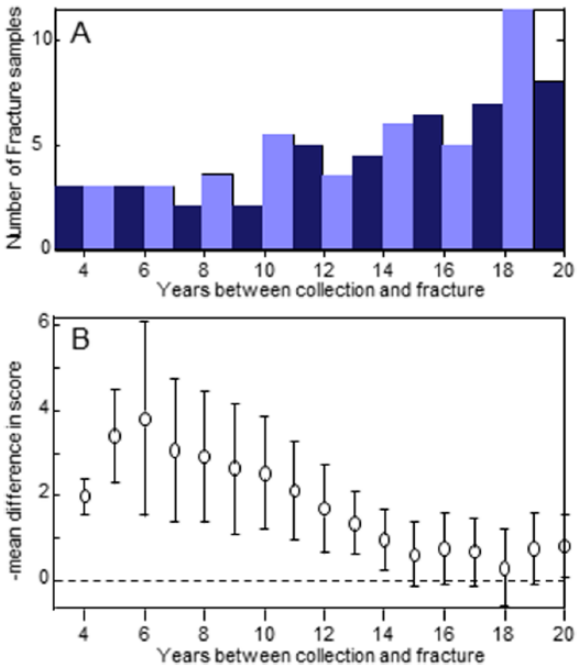

Figure 2. (A) Sample distribution by increment between collection and fracture. (B) Mean difference in Raman score for fracture vs non-fracture for donors with different increments between collection and fracture (predictions made using model derived from donors with fractures up to 13 years after collection but applied to all data).

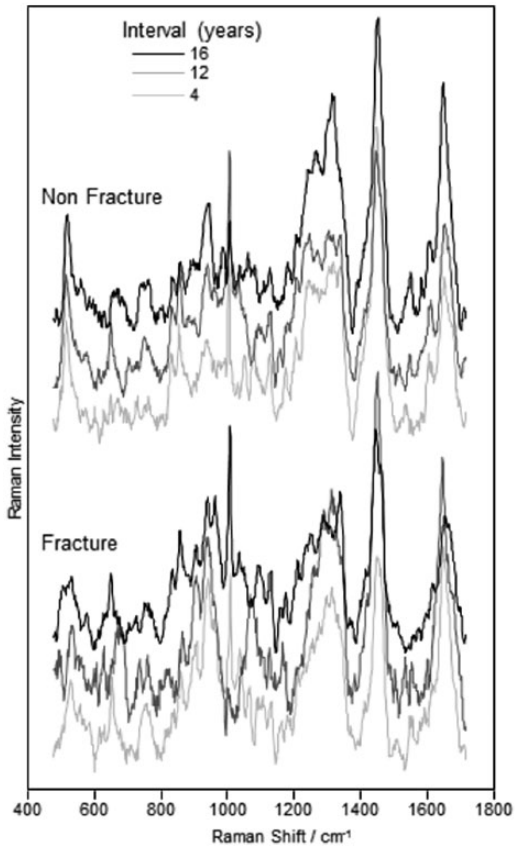

Figure 3. Scaled subtraction spectra of non-fractures and fractures at increments between collection and fracture occurrence (in age-matched sample for controls) of 4, 12, and 16 years. These increments represent points in Figure 2B at the 2 plateaus and the middle of the slope.

slope in Figure 2B and provided sufficient samples to create a representative model. The OR of 6.3 (95% CI: 3.0-12.6) was higher than for the 20-year period. In contrast, a model based on only the cases and controls beyond 13 years did not provide a predictive model, giving an OR = 2.1 (95% CI: 0.8-6.1). The CRFs did not contribute any benefit to the Raman score in the model up to 13 years.

Discussion

Anecdotally, patients diagnosed with osteoporosis have reported loss of fingernail resilience (D. Lyons, Personal Communication, April 2002) with disease progression. Previous studies by the authors have suggested a relationship between human nail structure, osteoporosis, and fracture risk, providing preliminary supporting evidence for a new biomarker.[9–13] Taking advantage of archived nail samples from post-menopausal women 50 to 63 years of age, this study tested the ability of Raman spectroscopy of nail samples to identify hip fracture risk over periods of 4 to 20 years in age-matched cases and controls. We developed a predictive model using a subset of the study sample and tested the Raman model score in the remaining samples. We found that risk of hip fracture was more than double per 1 SD change in Raman score for the full 20-year period. We also demonstrated that the contrast between cases and controls improved as the period between collection and fracture shortens, such that risk was increased more than 6-fold when limited to a 13-year period after nail collection. This prediction period is in line with CRF-based tools such as FRAX and Q-fracture, which focus on predicting fractures up to 10 years in advance. The Raman score provided discriminatory power greater than the available CRFs, a comparable result to that seen with our retrospective data sourced from the 633 subjects in the FRAN study[13] which suggests that the Raman-based score is providing new information and could provide a significant performance enhancement. Other methods have been investigated for assessing nails as predictors or osteoporosis and fracture risk, including laser-induced breakdown spectroscopy, which showed some correlation with BMD,[38] and cation measurements, for which there have been conflicting reports.[39,40]

In perimenopausal women 45 to 54 years of age followed for an average of 10 years, Barr et al[41] reported hazard ratios of 1.9 (95% CI: 1.5-2.3) for osteoporotic fractures per 1 SD decrease in spine BMD and 1.8 (95% CI: 1.4-2.2) per 1 SD decrease in femoral neck BMD when measured by DXA. Most of the osteoporotic fractures in that population were wrist fractures, a less serious and costly outcome than the hip fractures assessed in this study. Results were similar in the subgroup with BMD measurements using quantitative ultrasound (QUS), and various other studies have also demonstrated that QUS has a relative risk of fracture comparable with DXA-based measurements over short time frames.[42–44] If similar results to those shown in this study are found when the Raman-based score is validated

Table 2. Prediction of hip fracture by Raman score and CRF[a] in the test set from the Nurses' Health Study.

	CASES/CONTROLS	OR (95% CI)[B]
Total sample	15/14	
Raman score		2.2 (1.5–3.1)
CRF		2.0 (1.4–2.9)
Raman score + CRF		3.8 (2.6–5.4)
≤13 y to hip fracture	7/7	
Raman score		6.3 (3.0–13.1)
CRF only		1.9 (1.0–3.5)
Raman score + CRF		6.3 (3.0–13.1)
>13 y to hip fracture	8/7	2.14 (0.8–6.1)

Abbreviations: CI, confidence interval; CRF, clinical risk factor; OR, odds ratio.
[a]Clinical risk factors (assessed at toenail collection) included all factors with a minimum of 5 samples (body mass index, osteoporosis diagnosis, thyroid hormone user, and current smoker).
[b]OR in the test set and 95% CI for risk of hip fracture per 1 SD increase in standardised predicted values of factors in the model.

in a larger independent sample, its performance in combination with CRFs would be at least comparable with those reported by Barr et al for DXA and QUS.

The use of archived nails for this type of analysis is based on the assumption that they do not substantially change from collection at baseline, and this is valid according to the literature. A comparison of 500-year-old mummified baby nails showed little variation in key structures compared with the nails of modern-day babies.[45] What differences did occur were attributed to changes in the hydration level of the keratin. The literature suggests that Raman spectra can be measured decades after the original sample was stored without degradation.[46] The nails in the NHS have been kept for more than 25 years in a manner consistent with these findings.

Collagen and keratin are fibrous proteins that serve structural and mechanical roles in the body, providing a framework for support of cells and tissues.[47] Both proteins consist of polypeptide chains formed by amino acid condensation[47] and express the same characteristic bands (CH_2 and amide I) in key regions of Raman spectra collected from biological samples.[11,47] Byproducts of bone remodelling are evident in serum and urine; therefore, it is conceivable that these markers of changes in bone chemistry could also be detected in nails, a continually growing material, more stable than serum and urine, which is in direct contact with the periosteum of the phalangeal bone during its growth.[48]

There have been calls to develop population-based screening protocols for osteoporosis and fracture risk that could be implemented around time of the menopause so that earlier treatment could improve outcomes.[1,41] In contrast to DXA or QUS, tests amenable to high-throughput testing by clinical reference labs could facilitate screening a broader population base in combination with CRF-based screening tools. A number of markers of bone resorption are offered as central lab tests including those based around hydroxyproline, hydroxylysine glycosides, pyridinoline, deoxypyridinoline, aminoterminal, carboxyterminal, etc,[49,50] although to-date clinical studies have not provided information on the biochemical marker fracture prediction performance in women less than 65 years with long follow-up periods. The simplicity of preparing, transporting, and testing nail clippings may enable central lab testing for fracture risk assessment. A simple-to-use analysis, which could accurately identify high-risk women who would most benefit from increased monitoring and treatment at an earlier stage, would be a significant public health advancement.

This preliminary study suggests that Raman spectroscopy of the human nail is a promising tool for identifying postmenopausal women less than 65 years of age who are at increased risk of hip fracture over a period of up to 13 years. The preliminary results compare favourably with existing QUS, DXA, and CRF-based technologies and support the view that the prediction model provides a platform for a confirmatory study in an independent population to validate the prognostic marker. Further work is needed to validate the predictive ability of Raman spectroscopy for the assessment of future fracture risk.

Limitations of the study

1. Underpowered for reliably evaluating predictive performance of Raman spectroscopy for periods up to 10 years after nail collection. This means that overall spectroscopic trends associated with variable period between collection and fracture can be examined and investigated but no confident assertions about predictive performance can be made.
2. Hip fractures were self-reported and no clinical (ie, X-ray or other radiographic) evidence was collected to validate the reports. However, all women in the cohort are nurses and should be capable of accurate reporting.
3. Difficult to compare the predictive power of CRFs that form part of FRAX when combined with the Raman score in isolation as all these CRFs were not assessed or in comparison with the previously published FRAN study, which used CRFs from QFracture.
4. The study used predominantly white women, so no conclusion can be made on the effect of race nor sex.
5. Further work to understand the underlying mechanism of action linking bone and nail is also required.

Author Contributions

DF and MRT contributed to the study design. JRB and MCC conducted the study. DF and MCC contributed to data collection. JRB analysed the data. JRB, DF, MCC, and MRT contributed to data interpretation and approved the final manuscript. JRB drafted the manuscript. DF, MCC, and MRT revised the manuscript. JRB takes responsibility for integrity of the data analysis.

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