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Measuring Inflammatory Marker Levels to Determine Risk of Bone Loss and Fractures in Older Women

KE Barbour, PhD[1], **JA Cauley, DrPH**[2]

[1]Arthritis Program, Division of Population Health, CDC, Atlanta, GA, USA

[2]Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA, USA

Elevated levels of pro-inflammatory markers are associated with an increased risk of a number of chronic conditions[1-6] and death[7]. Furthermore, there is evidence that high levels of inflammatory markers may contribute to faster rates of bone loss.[8-10] More recently, we have demonstrated that greater inflammatory burden (measured primarily using cytokines and their soluble receptors) is associated with a greater fracture risk.[11,12] Additionally, studies have found that high levels of high sensitivity C-reactive protein (hs-CRP) (a generic marker of systemic inflammation that increases in response to greater inflammation) also predict incident fractures.[13-15]

Researchers have identified evidence of 2 biological mechanisms that may explain this increased bone loss and fracture risk among those with high levels of inflammatory markers.[16-18] In the first, cytokines bind to mesenchymal stem cells and increase the expression of receptor-activator of NF-κβ ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) and decrease osteoprotegerin production, which effectively increases activation of osteoclasts (cells responsible for resorption of bone tissue).[16] In the second, cytokine-mediated osteoclast activation is augmented in the presence of estrogen deficiency.[17,18]

Extensive bone loss can result in the development of osteoporosis. Osteoporosis is defined as a systemic bone disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a subsequent increase in bone fragility and susceptibility to fracture.[19] The World Health Organization defines osteoporosis as having a sex-specific bone mineral density (BMD) of less than or equal to 2.5 standard deviations (SDs) below the mean BMD of a young adult.[20] The burden of osteoporosis in women is high. In the US, the prevalence of osteoporosis is estimated to range from 17-20% among women ages 50 years or older.[21]

Osteoporosis can result in osteoporotic fractures (i.e., hip, spine, humerus, forearm), some of the most common causes of disability and a major source of medical costs.[22] An estimated

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All authors have no conflict of interest to declare

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60 to 70% of osteoporotic fractures occur in women.[23,24] In the US, the 2005 incidence of osteoporotic fractures among women was estimated to be over $1.4 million. The direct annual cost associated with these osteoporotic fractures was over $12 billion, and projected to rise to over $18 billion by 2025.[24]

Among all osteoporotic fractures, hip fractures have the most serious consequences with significant impact on morbidity and mortality.[25] In the US (among patients 65 years or older, and between 1986 and 2005), the annual mean number of hip fractures per 100,000 was 957 (95% CI: 922-993) for women and 414 (95% CI, 402-427) for men.[26] The burden of hip fractures is particularly high among women, and increases exponentially with age. Women comprise roughly 70% of all hip fractures[23]. The lifetime risk of a hip fracture in white women is estimated to be 1 in 6[22], and even greater among white women with osteoporosis (between 40 to 50%).[27,28] Black and Asian women have about half the rate of hip fracture when compared to white women.[22] Additionally, hip fractures comprise an estimated 35.6% of osteoporotic fractures in women ages 80-85.[29] The number of disability-adjusted life years (DALYs) lost globally due to hip fractures is almost two times greater in women (1.53 million) than men (0.82 million).[23] Furthermore, approximately 1 in 5 women will die within a year of a hip fracture.[30,31]

Several risk factors for bone loss and fractures have been identified in older women. Lower weight, greater weight loss, current smoking, lower serum estradiol, and higher serum adiponectin comprise some of the risk factors for bone loss.[32-34] Additionally, meta-analyses have identified BMI[35], and prior smoking[36] as predictors of hip fracture independent of BMD. Likewise, prior fracture[37], and corticosteroid use[38] have been implicated as risk factors for hip fractures, osteoporotic fractures, and any fractures.

This report will focus on the laboratory methods used to measure concentrations of cytokines, cytokine soluble receptors, and hs-CRP. We will also discuss inflammatory markers and the risk of bone loss and fractures in older women.

Lab Procedures for Inflammatory Marker Measurement

In biomedical research, Enzyme-Linked Immuno-Sorbant Assay (ELISA) is the most commonly used method for measuring concentrations of inflammatory markers, especially low-abundance markers such as cytokines.[39] ELISA uses an antibody “sandwich”, with one antibody to specifically detect the cytokine or receptor of interest that is fixed to a plastic well, while the second antibody is linked to an enzyme that acts as an amplification factor to enable colorimetric or chemiluminescent detection and quantitation.

However, there are documented methodological limitations that coincide with using ELISA to quantify inflammatory marker concentrations. First, for very low-abundance markers (i.e., tumor necrosis factor-alpha (TNF-α)), the ELISA can require a relatively large volume of serum for analysis (e.g., 200 – 200 uL), and many studies fall short of the required threshold. Second, the cost of individual ELISAs for each of several markers can add up to prohibitive costs for researchers who lack the adequate funds to conduct such measurements.

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Recently, multiplex arrays (which have the ability to estimate levels of several inflammatory marker in one assay) have been developed which, when compared to traditional ELISAs, requires smaller sample volume, are less expensive, and more time efficient.[39] The most widely used multiplex array for measuring inflammatory markers is based on flow cytometry technology. Flow cytometric multiplex arrays use microscopic beads with several pre-defined colors; beads of each color are coated with antibodies specific for one cytokine, which form the capture site for that specific cytokine. The beads can then be mixed together in "panels" in which each of the differently colored bead sets represents a different cytokine, and a single serum or plasma sample is added to the "panel" of beads. Subsequently, fluorescence or streptavidin labeled detection antibodies attach to the cytokine of interest on each the differently-colored bead sets. The flow cytometer uses the color of the beads to keep track of which cytokine is being measured, and fluorescent signals are used to estimate the amount of cytokine detected. Multiplex arrays using chemiluminescence or electrochemiluminescence technology have also been developed for measuring inflammatory marker concentrations. Although the technology offers great promise, more studies are needed to evaluate the performance of multiplex assays relative to accepted ELISAs, and address or confirm some of the putative limitations. For example, complications may arise because of the different range in concentrations of various antigens being assayed together; also there may be discordance between serum and plasma measurements,[40] and greater sensitivity to high levels of circulating proteins in serum or plasma samples. Finally, quality control of multiplexed assays is considerably more complicated,[41] and manufacturers have found it more difficult to maintain constancy in sensitivity and specificity when preparing multiplexed reagents.[42]

In epidemiological studies, most hepatic inflammation biomarkers, such as CRP, fibrinogen, serum amyloid A and others, are measured using either nephelometry or immunoturbidimetry. Historically, nephelometry was the assay of choice, because of its high sensitivity, however latex-enhanced immunoturbidimetry has produced comparable sensitivity. To estimate the concentration of CRP, immunoturbidimetry measures the turbidity of a sample, and nephelometry the scattering of light, upon application of a beam of light. Assay reagent is added to the sample resulting in a formation of an antibody-antigen complex. Immunoturbidimetry measures the intensity of the light absorbed by the now-turbid sample. In contrast, nephelometry measures the intensity of the light scattered. CRP concentrations are then estimated by using a calibration curve. ELISA can also be used to measure CRP.

As an example of research practice, consider how we measured inflammatory markers in our studies.[11,12] Blood samples were obtained after approximately 12 hours of fasting, and stored at −80°C using strict control procedures until assay.[43] Subsequently, the stored serum samples were sent to testing laboratories for measurements. Cytokines and soluble cytokine receptor levels were measured in duplicate using Solid-Phase Sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) at the University of Vermont. The detectable limits for the cytokines Interleukin 6 (IL-6) (using the HS600 Quantikine kit) and TNF-α (using HSTA50 kit) were 0.10 and 0.18 pg/ml, respectively. The detectable limits for the soluble receptors of IL-6 (IL-6 SR) (using the DR600 kit), Interleukin 2 (IL-2 SR) (using Q2000B kit), and TNF-α (TNF SR1 using the DRT100kit, and TNF SR2 using the DRT200kit)

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were 6.5, <10, 3.0, and 1.0 pg/ml, respectively. Hs-CRP was also measured in duplicate by ELISA based on purified protein and polyclonal anti-CRP antibodies.[44] The hs-CRP assay was standardized according to the World Health Organization's First International Reference Standard, with a sensitivity of 0.08 μg/ml. The interassay coefficient of variation (CV) is a measure of the reliability between assays using the ratio of the standard deviation to the mean, with a lower interassay CV suggesting higher reliability. Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision. The CVs of IL-6, TNF-α, IL-6 SR, TNF SR1, TNF SR2, and hs-CRP were 10.3%, 15.8%, 12.5% to 14.8%, 6.7% to 10%, 5.6% to 6.2%, and 8%, respectively.

In summary, inflammatory marker measurement using ELISA remains the standard assay for epidemiological studies. Future research should consider whether multiplex arrays can be used as a practical alternative to ELISA for the measurement of inflammatory markers.

Inflammatory markers and the risk of bone loss and incident fractures

Based on a comprehensive review of the literature we identified 8 epidemiological studies that evaluated the association of bone loss and incident fractures according to levels of these inflammatory markers. Most studies have focused on older women (Tables 1-3).

Observational studies which have examined if high inflammatory marker levels increase the rate of bone loss have shown some evidence of an association.[8-10] The main limitation of these studies is the relatively short follow-up (1 to 3.3 years) and sample sizes (137-242) (Table 1). Studies are needed that examine bone loss in a larger cohort and over a longer period of time (i.e., 5 years). Furthermore, studies in men and premenopausal women are needed to understand if the effect of inflammation on bone loss is independent of hormone levels (i.e., estradiol).

The effect of inflammatory marker levels on risk of incident fractures has been examined in several studies.[11-15] Two different methods to classify inflammation for these fracture studies have emerged. Our studies have used a composite variable[11,12] which combines the number of cytokines and/or their soluble receptors in the highest quartile as the exposure, whereas the other studies have limited the exposure to hs-CRP[13-15] only. We created a composite measure of inflammation based on studies suggesting that measuring one biomarker is unlikely to capture an accurate level of inflammation or risk.[45,46]

As an example of research practice, consider how we examined risk in our studies.[11,12] The characteristics and findings of our two studies[11,12] are summarized in slightly more detail below (Table 2). The epidemiological study design and selected study population differed by study. The earlier study[11] was a cohort study using participants from the Health ABC study which included men and women as well as whites and blacks, while the more recent study[12] was a nested case-control study within the Women's Health Initiative observational cohort and was limited to primarily white women (Table 2). A nested case-control study is a case-control study within a cohort study. We opted for a nested case-control study to substantially reduce the costs associated with assaying 39,795 baseline serum samples for the total cohort. Instead, we randomly selected 400 incident hip fractures cases and 400

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controls from the remaining cohort members without hip fracture matched by age, race, and date of blood draw. We assigned inflammatory marker quartile levels based on the distribution observed in the controls, which should provide the expected concentrations of inflammatory markers in the population that gave rise to the cases. The follow-up times and age of participants in the 2 studies were similar (Table 2). Study outcomes differed with the earlier study using non-traumatic fractures (fractures occurring spontaneously or from modest trauma) and the subsequent study using hip fractures. Both studies accounted for a large number of potential confounders (i.e., weight, cigarette smoking, corticosteroids, and diabetes) while the most recent study adjusted for several potential mediators (factors that are likely to be in the causal pathway between inflammation and fracture) (Table 2). Findings for both studies were consistent when examining the effect of single inflammatory biomarkers on fractures. For instance in both studies, IL-6 SR was not associated with fractures, whereas participants in the top quartile of TNF SR2 had an increased risk of fracture. Among single inflammatory markers (i.e., IL-2 sR, TNF SR1, and TNF SR2) that were significantly associated with an increased risk of fracture the magnitude of effect (i.e., hazard ratio or relative risk) was between 1.48 and 1.73. Using the composite variable, we showed that participants with the highest burden of inflammation (3 or more markers in the highest quartile) had an almost 3-fold risk of fractures (non-traumatic and hip fractures) compared with those with the lowest inflammation burden (0 or 1 inflammatory marker in the highest quartile) (Table 2). Analyses from the earlier study were limited by statistical power (i.e., low number of hip fractures and low fracture rates among non-white women), whereas the most recent study was unable to account for BMD and estimate person-time risk (Table 2). As a result, these findings are primarily generalizable to white postmenopausal women.

Obviously, two well conducted observational studies are not enough to conclude that there is a causal link between inflammatory marker levels and risk of fracture. We are limited by only one measure of inflammation per participant, and measurements over time are needed to better quantify long term inflammation. Other factors (i.e., age, BMI, diabetes, and frailty) are strongly correlated with inflammation, although we have accounted for these and other important measures in our analyses. We hope to continue to evaluate how inflammatory markers effect fracture risk in different cohorts to determine if these findings remain consistent across studies, and address some of the limitations of prior studies.

Finally, we have summarized some of the key findings from the 3 cohort studies we identified that focused mainly on the association between hs-CRP and risk of incident fractures (Table 3).[13-15] All 3 studies used the prospective cohort design with the vast majority of participants followed for 5 years or more. The study populations consisted predominantly of postmenopausal women of either Caucasian or Japanese descent. Findings were mostly consistent across studies, showing that higher levels of hs-CRP are associated with an increased risk of fracture. In fact, Schett et al. reported that participants in the highest versus lowest tertile group of hs-CRP had over 9 times the risk of non-traumatic fracture. On the other hand, it is worth noting, that we found no association between hs-CRP and incident non-traumatic fractures[11], furthermore, Pasco et al. reported a rather modest albeit significant association[14].

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In summary, an elevated level of one inflammatory marker may or may not significantly increase the risk of fracture. However, the association with fractures appears the strongest when inflammatory markers are combined into a composite variable, suggesting that inflammatory burden may be an important biological risk factor. Future research should confirm these associations in men and pre-menopausal women.

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Table 1.

Characteristics of prior studies showing the association between inflammatory markers and risk of bone loss

Ref #	Study Design	Study Population	Major Finding(s)
8	Cohort study with participants followed for 2.9 years	168 participants (mean 63 years, range 52–78, 48% female	Il-6 was the strongest most consistent predictor for bone loss.
9	Cohort study with 1 year follow-up	242 postmenopausal women (mean age= 54.4 ± 3.3 years)	Inflammatory markers accounted for 1.1-6.1% of the variance to the observed 12-mo changes in bone mineral content (BMC) and BMD.
10	Cohort study with follow-up mean of 3.3 years	137 postmenopausal German women, 52–80 years old at baseline	Serum IL-6 was a predictor of postmenopausal bone loss, and the effect appeared to be most relevant in the first postmenopausal decade.

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Table 2.

Characteristics of our studies showing the association between inflammatory markers and risk of incident fracture

Ref #	Study Design	Study Population	Potential Confounders	Mediators	Major Finding(s)	Main Limitations
11	Cohort study followed participants for mean ± SD =5.8 ± 1.6 years	2985 white and black women and men (42%, black; 51%, women) 70–79 years of age	Age, race, site, sex, height, weight, cigarette smoking, alcohol, weight gain 5 lbs in the past year, weight loss 5 lbs in the past year, physical activity, corticosteroids, statins, NSAIDS, osteoporosis medications, calcium supplements, diabetes, lung disease, osteoarthritis, heart disease, stroke.	Physical function, falls, BMD	The relative risk of fracture (95% CIs) for subjects with the highest inflammatory markers (quartile 4) compared with those with lower inflammatory markers (quartiles 1, 2, and 3) was 1.34 (0.99, 1.82) for CRP; 1.28 (0.95-1.74) for IL-6; 1.28 (0.97-1.70) for TNF-α; 1.52 (1.04-2.21) for IL-2 sR; 1.33 (0.90-1.96) for IL-6 sR; 1.73 (1.18-2.55) for TNF sR1 and 1.48 (1.01-2.20) for TNF sR2. In subjects with three or more (out of seven) high inflammatory markers, the relative risk of non-traumatic fracture was 2.65 (1.44–4.89) in comparison with subjects with no elevated markers (p trend=0.001).	Unable to examine hip fractures alone (underpowered analysis, n=39). Most fractures occurred in white women, thus, unable to perform stratified analyses.
12	Nested case-control study with 7.1 years of median follow-up.	Primarily white women (mean age=71 ± 6.2 years). 400 hip fracture cases matched to 400 controls on age race, and date of blood draw.	Health status, physical activity, parental history of hip fracture, history of fracture, smoking, alcohol use, NSAID use, treated diabetes, RA, corticosteroid use, and total calcium and vitamin D intake.	Physical function, falls, bioavailable estradiol and testosterone, SHBG, cystatin-C, PINP, CTX, and 25(OH)D.	The risk of hip fracture for subjects with the highest levels of inflammatory markers (quartile 4) compared with those with lower levels (quartiles 1, 2, and 3) was 1.43 (95% confidence interval [CI], 0.98-2.07) for interleukin-6 (IL-6) soluble receptor (SR), 1.40 (95% CI, 0.97-2.03) for tumor necrosis factor (TNF) SR1, and 1.56 (95% CI, 1.09-2.22) for TNF SR2. In subjects with all three inflammatory markers in the highest quartile, the risk ratio of fracture was 2.76 (95% CI, 1.22-6.25) in comparison with subjects with 0 or 1 elevated marker, (p trend==0.018).	Unable to account for BMD. Cannot estimate person-time risk.

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Table 3.

Characteristics of studies showing the association between hs-CRP and risk of incident fractures

Ref #	Study Design	Study Population	Major Finding(s)
13	Cohort study with participants followed up to 15 years	906 men and women (about a 1:1 ratio). Age range from 40-79 years.	The adjusted relative risk (95% confidence interval) of nontraumatic fracture in the highest vs lowest tertile group for hs-CRP was 9.4 (3.6-24.8) (P<0.001).
14	Cohort study. The median (IQR) follow-up period was 5.5 (3.8-6.2) years	444 women (median (IQR) age=77 (71.1-82.3)) years	A 23% increased significant risk of fracture associated with each SD increase in ln-hsCRP was not explained by BMD or other covariates.
15	Cohort study with participants followed up to 6 years.	751 Japanese women aged 69 years or older.	The adjusted HRs of fracture for the medium and highest quartiles of hsCRP levels, compared to the lowest quartile, were 2.22 (95% CI,1.02–4.84) and 2.40 (95% CI, 1.10–5.24), respectively.