Published in final edited form as:

Osteoporos Int. 2016 February ; 27(2): 815–820. doi:10.1007/s00198-015-3269-9.

FIBROSIS MARKERS, HIP FRACTURE RISK, AND BONE DENSITY IN OLDER ADULTS

Joshua I. Barzilay, MD¹, Petra B. Žková, PhD², Jorge R. Kizer, MD³, Luc Djoussé, MD⁴, Joachim H. Ix, MD⁵, Howard A. Fink, MD⁶, David S. Siscovick, MD⁷, Jane A. Cauley, PhD⁸, Kenneth J. Mukamal, MD⁹

¹Kaiser Permanente of Georgia, Division of Endocrinology and the Division of Endocrinology, Emory University School of Medicine, Atlanta, GA.

²Department of Biostatistics, University of Washington, Seattle, WA.

³Department of Medicine and the Department of Epidemiology and Population Health, Albert Einstein College of Medicine, Bronx, NY.

⁴Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA.

⁵Division of Nephrology, University of California San Diego, San Diego VA Healthcare System, San Diego, CA.

⁶Geriatric Research Education & Clinical Center, Veterans Affairs Medical Center, Minneapolis, Minnesota.

⁷New York Academy of Sciences, New York, NY.

⁸Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA.

⁹Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA.

Abstract

Purpose: Transforming growth factor beta one (TGF- β 1) serves several roles in bone formation and resorption. A consequence of TGF- β 1 activation is production of procollagen type III N-terminal propeptide (PIIINP), a marker of collagen III deposition. Here we explore whether these two biomarkers are related to incident hip fracture and bone mineral density (BMD) and whether their associations are modified by systemic inflammation, as measured by C-reactive protein (CRP) levels.

Methods: Participants were from the Cardiovascular Health Study (mean age 78 years; mean follow-up 8.3 years). We included 1,681 persons with measured levels of TGF- β 1 (149 hip fractures), and 3,226 persons with measured levels of PIIINP (310 hip fractures).

Corresponding authors: Joshua Barzilay M.D., 3650 Steve Reynolds Blvd, Duluth, GA 30096. Tel 770 931 6094, Fax 770 931 6365, joshua.barzilay@kp.org.

Joshua I. Barzilay, Petra B. Žková, Jorge R. Kizer, Luc Djoussé, Joachim H. Ix, Howard A. Fink, David S. Siscovick, Jane A. Cauley, and Kenneth J. Mukamal declare that they have no conflicts of interest.

Results: Among women, higher TGF- β 1 levels were associated with lower hip fracture risk (HR, per doubling, 0.78 [95% CI, 0.61, 0.91]). Among men, TGF- β 1 levels were associated with hip fracture risk in a non-linear manner, but among those with elevated CRP levels, doubling was associated with increased risk of fracture (HR 2.22 [1.20, 4.08]) ($p=0.02$, interaction between low and high CRP and TGF- β 1 on fracture risk). TGF- β 1 levels had no significant association with total hip or total body BMD in women, but were significantly associated with lower BMD in men. There were no associations of PIIINP levels with hip fracture risk or BMD in men or women.

Conclusions: TGF- β 1 levels appear to be associated with bone-related phenotypes in a sex-specific manner. The reasons for these differences between men and women regarding TGF- β 1 levels and hip fracture risk and bone density require further investigation.

MINI-ABSTRACT

We examined whether blood levels of two markers of fibrosis (TGF- β 1 and PIIINP) are related to hip fracture risk and to bone mineral density (BMD). TGF- β 1 levels were associated with lower hip fracture risk in women and with lower BMD in men. PIIINP levels were not associated with either outcome.

Keywords

TGF- β ; PIIINP; hip fracture; bone mineral density

INTRODUCTION

Transforming growth factor beta-1 (TGF- β 1) is a connective tissue cytokine that plays varying roles in bone physiology. During periods of bone formation, TGF- β 1 attracts osteoprogenitor cells and regulates the synthesis of extracellular matrix proteins (1). During bone resorption, TGF- β 1 recruits osteoclasts for the removal of old bone (2). Given its pleiotropic effects, it is not surprising that disorders of TGF- β 1 action, secretion, or function can result in bone disease (2). For example, in a transgenic mouse model, in which TGF- β 2 is overexpressed by osteoblasts, progressive bone loss is observed, phenotypically resembling bone abnormalities seen in osteoporosis (3). To our knowledge, no prospective studies have examined serum TGF- β 1 levels with fracture risk or bone mineral density (BMD).

In the process of bone remodeling and collagen deposition, collagen byproducts such as procollagen type III N-terminal propeptide (PIIINP) are released into the circulation (4). Circulating levels of PIIINP may serve as a marker of bone turnover activity. They too have not been examined prospectively for fracture risk or BMD.

Here we explore the prospective association of baseline TGF- β 1 and PIIINP levels with incident hip fracture risk and their cross-sectional association with BMD. We also seek to determine whether any adverse effects of TGF- β 1 may be amplified in the presence of systemic inflammation (as measured by C-reactive protein [CRP] levels), similar to what we observed previously for total mortality (5). The cohort for this study is drawn from the Cardiovascular Health Study (CHS), a population based study of individuals with a mean age of 78 years at the time of biomarker measurement.

METHODS

The CHS is a prospective, observational population-based cohort study of 5,888 Medicare-eligible adults ≥ 65 years in four US communities (6). In the original cohort, 5,201 eligible men and women were enrolled during 1989–1990. In the second recruitment, during 1992–1993, an additional 687 predominantly black men and women were enrolled. Clinic examinations were performed at study baseline and at annual visits through 1998–1999, and again in 2005–2006. Participants were contacted by telephone annually between exams, and twice per year during 2000–2004 and 2007, when no clinic examinations occurred. All participants signed informed consent upon study entry.

The cohort for this analysis was taken from the 1996–1997 examination. By that point, 1180 of the original 5888 participants had died, and 295 were lost to follow-up or refused further visits. An additional 116 participants with medical record codes consistent with hip, leg, arm, wrist, and shoulder fractures within one year of the blood draw were excluded so as to avoid reverse causation due to an increase in TGF- β 1 levels related to bone healing (7). Of the remaining 4297 participants, 1681 had TGF- β 1 levels measured and 3226 had PIIINP levels measured. 1,071 persons had both analytes measured.

Measurement of Hip Fracture:

Incident hip fracture was identified using International Classification of Diseases, Ninth Revision (ICD-9) codes from hospitalization records from the time of the 1996–1997 visit through June 30, 2008. CHS prospectively gathers all hospitalization data, including discharge summaries, from participants every six months. To ensure completeness of hospitalization records, data were checked against Medicare claims data to identify any hospitalizations that were not reported by the participant. Hip fracture was defined as ICD-9 code of 820.xx. Admissions for pathologic fractures (ICD-9 code 773.1x) and motor vehicle accidents (E810.xx-E825.xx) were excluded.

Measurement of TGF- β 1 and PIIINP:

TGF- β 1 and PIIINP levels were measured in stored plasma samples from the 1996–1997 visit, which is the baseline for these analyses. TGF- β 1 was measured using an enzyme-linked immunosorbent assay (Human TGF-beta 1 Quantikine ELISA Kit, R&D Systems, Minneapolis, Minnesota). PIIINP was measured using a radioimmunoassay kit (UniQ PIIINP RIA; Orion Diagnostics, Fountain Hills, Arizona). Inter- and intra-assay coefficients of variation were 1.9%–2.9% and 6.4%–9.3%, respectively, for TGF- β . For PIIINP, inter- and intra-assay coefficients of variation were both less than 7.2%.

Platelets are a significant source of TGF- β 1, and platelet contamination in plasma samples can artificially elevate TGF- β 1 measurements (8). We conducted pilot studies that confirmed probable platelet contamination of EDTA plasma samples collected at 2 of 4 clinic sites. Hence, we *a priori* measured TGF- β 1 levels only in samples taken at the 2 remaining sites. Participant characteristics (including PIIINP measurements) did not differ substantially between sites that were included and excluded (5).

Bone Mineral Density Measurement:

A subset of the cohort from two field centers (n=1315) underwent BMD scanning 1–2 years before the plasma samples for biomarker levels were obtained. Total hip BMD in grams/cm² was measured by DXA (QDR 2000 or 2000±; Hologic, Inc, Bedford, MA). All scans were completed using the array beam mode. Scans were read blindly at the University of California, San Francisco reading center with Hologic software version 7.10. The coefficient of variation for total hip BMD was <0.75%. Z scores for the total hip were computed based on the means and SD of male and female non-Hispanic whites and on age (60–69, 70–79, >80 years) from the NHANES III (9).

Statistical Analysis:

Owing to differences in the natural history of hip fractures between men and women, we *a priori* categorized our analyses by gender. Descriptive characteristics were analyzed using standard statistical methods. Time to event was calculated as the interval in years from the baseline visit in 1996/1997 to the earliest date of first incident hip fracture, death, loss to follow-up, or end of follow-up on June 30, 2008. Cox proportional hazards models were used to estimate the hazard ratio (HR) of incident fracture associated with quartiles and, after transformation to the log(base 2), per doubling of TGF-β1 and PIIINP. We adjusted for age (continuous), black race, current smoking, alcohol consumption (3 categories: 0 is reference, 1–7 drinks/week, >7 drinks/ week), waist circumference, diabetes, kcal expenditure (continuous), falls, thiazide use, estrogen use, glucose (continuous), prevalent CHD, eGFR based on cystatin C levels (continuous), hypertension, log₂(CRP), and log₂(albumin:creatinine ratio [ACR]). These results were further stratified by elevated (≥ 3 mg/L) or non-elevated CRP levels to examine effect modification by inflammation using cut-points we have used previously (5); we also report p-values for interaction using CRP as a continuous variable. In the sub-cohort with BMD measurements, linear regression analysis was used to estimate the association between TGF-β1 or PIIINP with total hip or total body BMD values.

We explored the functional form of the continuous TGF-β1 (log₂ scale) with generalized additive models in both Cox regression for hip fracture and linear regression for BMD. We found the association of TGF-β1 and hip fracture in men to be non-linear. To address the overall association of TGF-β1 and hip fracture in men, we performed a fixed span size permutation test for significance of the difference in deviance between models including and excluding the smoothed (LOESS) TGF-β1 term using 1000 permutations (p<0.01) (10).

Analyses were conducted using R (R Development Core Team (2009) (11).

RESULTS

The median ages of the TGF-β1/ PIIINP cohort at baseline were 77.7±4.5 years among women and 78.0±4.7 years among men. The median follow-up time was 9.7 (5.2, 11.5) years. There were 681 men and 1000 women who had measured TGF-β1 values; there were 1295 men and 1931 women who had PIIINP levels measured. The median TGF-β1 value was 3532 (1098, 6066) ng/L for men and 3463 (1998, 6307) ng/L for women. The

associations of TGF- β 1 and of PIIINP levels with baseline characteristics are shown in Appendix 1.

We documented 149 hip fractures in the sub-cohort with TGF- β 1 measurements (50 in men, 99 in women), for an incidence rate of 1.07 per 100 PY (0.94 and 1.15 per 100 PY for men and women, respectively). The overall association of TGF- β 1 with fracture risk differed by gender (p interaction = 0.0096). Among men, there was a non-linear association of risk, with significantly higher risk in the third quartile and numerically higher risk in the second and fourth quartiles. Among women, risk was numerically lower in the second to fourth quartiles, with a significant inverse trend (Table). When TGF- β 1 values were log (2) transformed, each doubling of values was associated with a significant 22% lower fracture risk among women (HR 0.78 [0.61, 0.91]), but no significant association was present for men (1.13 [0.84, 1.54]).

When we stratified participants by the presence or absence of elevated CRP levels (≥ 3 mg/L), men with high CRP values had a significantly increased risk of fracture for every doubling of TGF- β 1 (HR 2.22 [1.20, 4.08]) (Figure). In contrast, in the low CRP group, higher TGF- β 1 tended to be associated with lower risk (adjusted HR 0.87 [0.57, 1.33]; p interaction=0.02), similar to that observed in women. The association between TGF- β 1 and hip fracture among women did not differ significantly by CRP (p interaction=0.32).

There were no associations of PIIINP levels with fracture risk when examined by quartiles or log transformation, in either men or women (Table).

Bone Mineral Density:

Among women, there was no significant association of TGF- β 1 values with total hip or total body BMD. Among men, there was a significant inverse association – the higher the level of TGF- β 1, the lower was the BMD in grams/cm² (total hip BMD: β = -0.29 [-0.05, -0.01]; total body BMD β = -0.19 [-0.04, -0.01]). The associations of TGF- β 1 with BMD were similar irrespective of CRP levels (p interactions all >0.26). For PIIINP, there were no significant adjusted associations with total hip or total body BMD as a whole or in either gender (Table).

Our results were similar when we examined the associations of TGF- β 1 and PIIINP with Z-scores for BMD, rather than absolute BMD.

DISCUSSION

In this exploratory study, we found a linear relationship between higher levels of TGF- β 1 with a lower risk of hip fracture in women. Among men, higher TGF- β 1 levels were associated with an increased non-linear risk of fracture, at least among those with higher levels of CRP.

These inconsistent results between men and women and between those with high or low CRP levels are difficult to explain. One possible explanation is that these findings arose by chance. While this is possible, a prior cross-sectional study of women (12) showed a positive correlation between TGF- β 1 levels and BMD, consistent with the lower fracture risk of

increasing TGF- β 1 in women in our study (although we could not confirm a similar association with BMD). A second explanation could be that since the etiology of bone fragility differs between men and women, the impact of TGF- β 1 on bone may also differ. As one example, testosterone down-regulates expression of genes in the TGF- β -signaling pathway, such as Smad (13). Defects in TGF- β -Smad signaling can cause bone-related disorders such as osteoporosis and increased fractures (14). Third, TGF- β 1 arises from multiple sites, not only bone (15), although this has uncertain relationship with the sex-specificity seen.

We note that the highest quartile of TGF- β 1 was not associated with higher or lower risk in men or women. We speculate that the pro-fibrotic and anti-inflammatory effects of TGF- β 1 may lead to competing effects, with differing effects at one level and other effects at a high level.

With regard to the differing associations of TGF- β 1 with fracture risk when categorized by CRP levels, TGF- β 1 is reported to have an anti-inflammatory effect. TGF- β 1-deficient mice die of systemic inflammation (16). As such, elevated CRP levels in the face of elevated TGF- β 1 levels may signal a strong inflammatory environment that may enhance bone fragility, particularly in men with otherwise less fragile bones.

We observed no association of PIIINP levels with hip fracture risk or BMD in the total cohort or in either gender. PIIINP is a marker of new collagen formation that arises in response to growth factors during growth, fracture healing, use of medications impacting bone turnover, or chronic illnesses. It has been positively associated with a number of outcomes in CHS (5), and we had a large number of incident fractures in the sub-cohort with measures of PIIINP (n=310). Nonetheless, it appears that markers of ongoing fibrosis when measured late in life are unlikely to be associated with hip fractures in the oldest old.

Advantages of this study include a well characterized cohort in whom adjustments were made for multiple factors associated with fracture risk and low BMD. Participants with recent fractures were excluded to avoid the effect of these fractures on TGF- β 1 and PIIINP levels. The hip fracture rate reported here was similar to that reported recently for U.S adults, ages 75–84 years (17). Limitations include lack of data on biochemical factors that are associated with bone fragility, such as testosterone. Also, we examined only TGF- β 1 and not other isomers of TGF- β that have been implicated in fracture risk (18).

In conclusion, these findings suggest that TGF- β 1 levels may be associated with risk of hip fracture in older adults and that such associations are complex and influenced by gender and inflammation. Further studies are needed to elucidate these relationships, and specifically to explain the different effects associations in women and men.

Acknowledgments

This research was supported by contracts HHSN268201200036C, HHSN268200800007C, N01 HC55222, N01HC85079, N01HC85080, N01HC85081, N01HC85082, N01HC85083, N01HC85086, and grant HL080295 from the National Heart, Lung, and Blood Institute (NHLBI), with additional contribution from the National Institute of Neurological Disorders and Stroke (NINDS). This work was also funded in part by R01 HL094555 from the National Heart, Lung, and Blood Institute (to Drs. Djoussé, Ix, Kizer, and Mukamal). A full list of principal CHS investigators and institutions can be found at www.CHS-NHLBI.org.

APPENDIX

Baseline characteristics of the cohort by whether the TGF- β 1 and PIIINP levels are above the median level. Results are categorized by sex.

MALE				
	TGF β 1 Below Median	TGF β 1 Above Median	PIIINP Below Median	PIIINP
N	336	345	598	697
Median Age (years)	77(75–81)	77 (74–81)	77 (75–80)	78 (75–82) **
African American (%)	15.5	22.3 *	13.4	14.5
Current Smoker (%)	8.7	11.2	10.2	7.1
Waist Circumference (cm)	97(92–103)	97(92–103)	97.5(92–102.5)	98.8(92.5–106) *
Diabetes (%)	13.2	22.9 **	15.1	19.8 *
Hypertension (%)	55.3	53.4	59.7	57.2
CRP (mg/L)	1.93(1.02–4.16)	2.34(1.01–5.71) *	1.96(0.95–4.16)	2.35(1.14–5.33) *
Cystatin eGFR (ml/min/1.73m ²)	69.3 (57.3–79.8)	67.1 (54.8–80.5)	71.6(60.7–83.6)	65.7 (52.5–76.7) **
FEMALE				
N	504	496	1031	900
Median Age (years)	77(74–81)	77 (74–80)	76 (74–80)	77 (75–82) **
African American (%)	21.6	28.0 *	13.8	22.3 **
Current Smoker (%)	8.1	8.6	7.1	6.4
Waist Circumference (cm)	95.5 (85.4–105.6)	94.0(86.5–105.0)	94 (85–103.7)	97.5(88.0–107.5) **
Diabetes (%)	62.2	63.4	11.1	15.7 **
Hypertension (%)	62.2	63.4	62.7	69.7 **
CRP (mg/L)	2.65(1.19–5.41)	2.75(1.26–85.73)	2.41(1.05–5.13)	2.84(1.27–5.67)
Cystatin eGFR (ml/min/1.73m ²)	73.2(63.1–86.0)	72.4 (60.7–85.7)	75.8(65.0–88.0)	67.8 (54.8–78.6) **

* p < 0.05

** p < 0.01

REFERENCES

1. Erlebacher A, Filvaroff EH, Ye JQ, Derynck R (1998) Osteoblastic responses to TGF-beta during bone remodeling. *Mol Biol Cell* 9(7):1903–1918. [PubMed: 9658179]
2. Tang SY, Alliston T (2013) Regulation of postnatal bone homeostasis by TGF β . *Bonekey Rep* 2:255. doi: 10.1038/bonekey.2012.255. [PubMed: 24404376]
3. Erlebacher A, Derynck R (1996) Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype. *J Cell Biol* 132(1–2):195–210. [PubMed: 8567723]
4. Sundström J, Vasan RS (2006) Circulating biomarkers of extracellular matrix remodeling and risk of atherosclerotic events. *Curr Opin Lipidol* 17(1):45–53. [PubMed: 16407715]
5. Agarwal I, Glazer NL, Barasch E, Biggs ML, Djousse L, Fitzpatrick AL, Gottdiener JS, Ix JH, Kizer JR, Rimm EB, Sicovick DS, Tracy RP, Mukamal KJ (2014) Fibrosis-related biomarkers and incident cardiovascular disease in older adults: The Cardiovascular Health Study. *Circ Arrhythm Electrophysiol* 7 (4): 583–589. [PubMed: 24963008]
6. Fried LP, Borhani NO, Enright P, Furberg CD, Gardin JM, Kronmal RA, Kuller LH, Manolio TA, Mittelmark MB, Newman A (1991) The Cardiovascular Health Study: design and rationale. *Ann Epidemiol* 1 (3): 263–276. [PubMed: 1669507]

7. Fisher DM, Wong JM, Crowley C, Khan WS (2013) Preclinical and clinical studies on the use of growth factors for bone repair: a systematic review. *Curr Stem Cell Res Ther* 8(3):260–268. [PubMed: 23317434]
8. Grainger DJ, Mosedale DE, Metcalfe JC (2000) TGF-beta in blood: a complex problem. *Cytokine Growth Factor Rev* 11(1–2):133–145. [PubMed: 10708961]
9. www.cdc.gov/nchs/data/series/sr_11/sr11_251.pdf
10. Young RL, Weinberg J, Vieira V, Ozonoff A, Webster TF (2011) Generalized Additive Models and Inflated Type I Error Rates of Smoother Significance Tests. *Comput Stat Data Anal* 55 (1): 366–374. [PubMed: 20948974]
11. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria ISBN 3-900051-07-0, URL <http://www.R-project.org>
12. Xie GQ, Lei DD, He HB, Gong JJ, Chen C, Chen P, Zhang H, Luo XH, Liao EY, Wu XP (2013) Relationship between serum TGF- β 1, OPG levels and osteoporotic risk in native Chinese women. *Clin Chim Acta* 423:116–121. [PubMed: 23643930]
13. Braga M, Bhasin S, Jasuja R, Pervin S, Singh R (2012) Testosterone inhibits transforming growth factor- β signaling during myogenic differentiation and proliferation of mouse satellite cells: potential role of follistatin in mediating testosterone action. *Mol Cell Endocrinol* 350(1):39–52. [PubMed: 22138414]
14. Borton AJ1, Frederick JP, Datto MB, Wang XF, Weinstein RS (2001) The loss of Smad3 results in a lower rate of bone formation and osteopenia through dysregulation of osteoblast differentiation and apoptosis. *J Bone Miner Res* 16(10):1754–1764. [PubMed: 11585338]
15. Blobel GC, Schiemann WP, Lodish HF (2000) Role of transforming growth factor beta in human disease. *N Engl J Med* 342 (18): 1350–1358. [PubMed: 10793168]
16. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, et al. (1992) Targeted disruption of the mouse transforming growth factor- β 1 gene results in multifocal inflammatory disease. *Nature* 359 (6397): 693–699. [PubMed: 1436033]
17. Levant S, Chari K, DeFrances CJ. Hospitalizations for Patients Aged 85 and Over in the United States, 2000–2010. *NCHS Data Brief*. 2015; 182:1–8.
18. Cho TJ, Gerstenfeld LC, Einhorn TA (2002) Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. *J Bone Miner Res* 17 (3):513–520. [PubMed: 11874242]

Figure 1:
Generalized additive models (splines) for $\log_2(\text{TGFB})$. Quartiles are blue. Low CRP left, high CRP right.

Table:

Biomarker associations with hip fracture and bone mineral density in the Cardiovascular Health Study.

A. Adjusted hazard ratios with 95% CI of hip fracture by quartiles of TGF-β and PNIIP and per doubling. The lowest quartile is the reference group.		
	MEN	WOMEN
HIP FRACTURE		
TGF-β 1		
Quartile 2	1.70 (0.67, 6.10)	0.55 (0.30, 1.00)
Quartile 3	2.49 (1.01, 6.10)	0.33 (0.16, 0.68)
Quartile 4	1.55 (0.57, 4.26)	0.63 (0.34, 1.16)
Per Doubling	1.13 (0.84, 1.54)	0.78 (0.61, 0.91)
PIIIP		
Quartile 2	0.90 (0.47, 1.73)	0.88 (0.58, 1.32)
Quartile 3	0.85 (0.44, 1.65)	0.94 (0.63, 1.42)
Quartile 4	1.18 (0.63, 2.22)	0.76 (0.47, 1.21)
Per Doubling	0.99 (0.74, 1.32)	0.95 (0.66, 1.37)
B. Adjusted increments in BMD (g/cm²) per doubling of TGF-β or PNIIP (β [95% CI]).		
HIP MINERAL DENSITY		
TGF- β	-0.29 (-0.05, -0.01)	-0.005 (-0.02, 0.01)
PIIIP	-0.003 (-0.04, 0.03)	0.013 (-0.01, 0.04)
TOTAL BODY MINERAL DENSITY		
TGF- β	-0.19 (-0.04, -0.01)	-0.01 (-0.01, 0.01)
PIIIP	-0.011 (-0.04, 0.02)	0.01 (-0.01, 0.03)