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Relationship between Serum Gamma-Glutamyltransferase (GGT) Activity and Indices of Iron Metabolism with Bone-Mineral Parameters in Orthogeriatric Patients

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Abstract

Purpose: To examine serum levels of GGT activity and biomarkers of iron metabolism in relation to parameters of bone and mineral metabolism in elderly patients with and without osteoporotic bone fractures in the absence of overt liver diseases.

Methods: In a cross-sectional study of 416 older (>60 years) patients (168 subjects with hip fracture, 89 with other fractures of the peripheral skeleton and 160 without any fracture; mean age 78.9 ± 8.7 years; 282 women) we simultaneously measured serum levels of two bone formation markers (osteocalcin, OC; procollagen type 1 N-terminal propeptide, P1NP), bone resorption marker (β -isomerised carboxy-terminal cross-linking telopeptide of type I collagen, β CTX), their ratios (PINP/OC, PINP/ β CTX, OC/ β CTX), 25(OH) vitamin D, PTH, calcium, phosphate, magnesium, GGT activity, other liver function tests and indices of iron metabolism (serum ferritin, iron, transferrin and transferrin saturation [TSAT]).

Results: Multivariate regression analyses demonstrated significant bidirectional links between P1NP and GGT, P1NP/OC ratio and GGT, P1NP and TSAT, OC and ferritin, GGT and ferritin. GGT, ferritin and TSAT were independent indicators of β CTX, while ferritin and TSAT were also independent predictors of the P1NP/OC ratio, and TSAT was an independent predictor of the P1NP/ β CTX ratio. In multivariate regression, P1NP, β CTX, P1NP/ β CTX ratio, ferritin, magnesium and age were independent indicators of fracture.

Conclusions: The study highlighted the bidirectional links between serum GGT activity, indices of iron and bone metabolism and the role of GGT and iron homeostasis in maintaining bone health and identified indicators for osteoporotic fractures. The GGT- and iron-related factors contributing to bone integrity warrant further investigations.

Keywords: Bone turnover markers; GGT; Iron metabolism; Fracture; Elderly

Introduction

The interest in gamma-glutamyltransferase (GGT; E.C.2.3.2.2), a heterodimeric glycosylated protein embedded into outer surface of the plasma membrane of numerous cells and strongly associated with many major non-skeletal diseases[1], in osteology has recently increased considerably owing to several reports. GGT-deficient (GGT^{-/-}) mice exhibit severe osteoporosis caused by decreased osteoblast activity and increased osteoclast number and activity [2]. GGT induces osteoclast formation in vitro [3], accelerates bone resorption causing osteopenia/osteoporosis in transgenic mice [4,5], and increases in urinary excretion of GGT correlate with urinary bone resorption markers in osteoprotegerin (OPG)-deficient osteoporotic mice as well as in postmenopausal osteoporotic women [6]. In older Korean men (>50 years) higher serum GGT levels were associated with incident osteoporotic fractures over a mean 3-year follow-up period [7]. Iron, an essential trace element in all three kingdoms of living organisms, is recognized as an important determinant of bone metabolism [8-10]. However, there is disagreement as to whether iron overload or iron deficiency lead to osteopenia/osteoporosis and whether the iron-related bone loss is due to reduced bone formation or to increased resorption of bone.

Studies in humans without haematological disorders on relationship between markers of iron metabolism and bone status are scarce and controversial. A significant association between higher serum ferritin level and lower bone mineral density (BMD) was reported in healthy Koreans aged >40 years [11], especially in women \geq 45 years of age [12], in postmenopausal Chinese women with fragility fractures [13], and in astronauts during space flights [14], whereas in other studies, increased serum ferritin levels were inversely correlated with BMD (of lumbar spine) only in premenopausal women, but not in postmenopausal women (either at the femur or lumbar spine) [15]. No significant association between ferritin levels and

osteoporosis has been observed in Turkish postmenopausal women [16]. Italian postmenopausal women with osteoporotic fractures were found to have higher transferrin levels and lower ferritin levels than controls [17] and a positive association between serum ferritin and BMD (in both femoral neck and lumbar spine). A positive ferritin-BMD correlation was also observed in adolescent girls [18] and in elderly Korean men (>65 years) [19]. Furthermore, numerous nutritional studies suggested that iron deficiency, rather than excess, may lead to osteoporosis [10].

Both GGT [20] and iron [21] are implicated in generating oxidative stress, which plays a significant role in bone pathophysiology [22,23], but these relationships remain unclear. Despite accumulating data suggesting importance of both GGT and iron in bone health, and the central role of liver in iron storage and regulation, no analysis of the potential links between serum GGT activity, markers of iron and bone-mineral metabolism in humans has been reported to date. The aim of this study was to examine serum levels of GGT and biomarkers of iron metabolism in relation to parameters of bone and mineral metabolism in elderly patients with and without osteoporotic bone fractures in the absence of overt liver diseases. We simultaneously measured serum levels of two bone formation markers (osteocalcin, OC, and N-terminal propeptide type 1 collagen, P1NP), bone resorption marker (β -isomerised C-terminal cross-linking telopeptide of type I collagen, β CTX), and their ratios (P1NP/OC, P1NP/ β CTX, OC/ β CTX), 25-hydroxyvitamin D (25(OH)D), intact parathyroid hormone (PTH), calcium, phosphate, magnesium in relation to serum GGT activity (and other liver function parameters) and indices of iron metabolism (serum ferritin, iron, transferrin and transferrin saturation).

Materials and Methods

Patients

We conducted a cross-sectional study of 493 older (>60 years) patients admitted to the orthopaedic unit of our hospital between 1 January 2010 and August 2011. After excluding patients with high-trauma fracture, acute or known chronic hepatobiliary diseases, iron metabolism-related diseases (hemochromatosis, thalassemia), acute infection, primary hyperparathyroidism, and Paget's disease, or who lacked adequate laboratory data, 416 patients (282 women, 134 men) were evaluated for the study.

The study was conducted according to the standards of the Declaration of Helsinki and was approved by the local Health Human Research Ethical Committee. Informed consent from each patient or carer was obtained.

Laboratory measurements

In each patient venous blood samples were collected in the morning following an overnight fast, usually within 24 h after arrival.

Markers of bone and mineral metabolism

The following serum indicators of bone and mineral metabolism were measured: bone formation markers (P1NP and OC), bone resorption marker (β CTX), PTH, 25(OH)D, calcium, phosphate and magnesium concentrations. The serum concentrations of P1NP, OC and β CTX were measured using an electrochemiluminescent immunoassay (Elecsys 2010, Roche Diagnostics, Ltd Corp., IN, USA). Intra- and inter-assay coefficients of variation (CV) for P1NP were 2.6% and 4.1%, respectively; for OC 3.6% and 6.6%, respectively, and for β CTX 3.2% and 6.5%, respectively. Serum 25(OH)D level was measured by a radioimmunoassay (Dia Sorin, Stillwater, MN, USA) and intact PTH was determined by a two-site chemiluminescent enzyme-linked immunoassay on DPC Immulite 2000 (Diagnostic Products Corp., Los Angeles, CA, USA); the intra- and inter-assay CV ranged from 2.1% to 12.7%. Serum calcium concentrations were corrected for serum albumin. For bone turnover markers, we used reference ranges and data provided by the manufacturers. Vitamin D status was defined as deficient for circulating 25(OH)D concentration <25 nmol/L, and as insufficient for 25–50 nmol/L. Secondary hyperparathyroidism (SHPT) was defined as elevated serum PTH (>6.8 pmol/L, the upper limit of the laboratory reference range).

Liver function tests

The serum levels of GGT, alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured enzymatically using the Abbott Architect CI16200 automatic analyser, total bilirubin was measured using diazonium salt, albumin was analysed using bromocresol green, and total protein by a Biuret method. The mean inter-assay and intra-assay CV for these tests were within 1.1% – 6.6%. For liver enzymes two times upper normal limit (UNL) cut-offs were used to define abnormal tests.

Indicators of iron status

Serum indices of iron metabolism were analysed on the Abbot Architect CI16200 (Abbott Laboratories, IL 60064, USA). Serum iron was assessed by direct colorimetric determination, serum ferritin concentration was measured by a two-step chemiluminescent microparticle immunoassay and transferrin was measured using an immunoturbidimetric procedure. The mean inter-assay and intra-assay CV for these tests were between 1.6% and 3.7%. Transferrin saturation (TSAT) was calculated using the IFCC protein standards (1 mg of transferrin carries 1.49 μ g of iron) as follows: TSAT (%) = iron (μ mol/L) \times 3.8 / transferrin (g/L); reference range 18-46%.

In addition, in all patients routine hematological and biochemical assessments (complete blood count, urea, creatinine, fasting blood glucose, haemoglobin A1c (HbA1c, only in diabetic patients), thyroid function tests (thyroid-stimulating hormone, TSH, and thyroxine, T4), C-reactive protein (CRP) were also performed by standardized methods on autoanalyzers. Estimated glomerular filtration rate (eGFR) was determined using the serum creatinine equation. Chronic kidney disease (CKD) was defined as eGFR <60 ml/1.73 m² (CKD \geq 3 stage).

Statistics

Statistical analyses were performed using Stata software version 10 (StataCorp., College Station, TX, USA). For summary statistics, data were untransformed and presented as mean \pm standard deviation (SD) for continuous variables and as numbers (or percentages) for categorical variables. For Pearson correlations and regressions, values of all laboratory parameters were logarithmically transformed to account for the skewed nature of most of these variables. Sidak adjustments for multiplicity were performed. Linear regressions involving the predictive value of GGT, and indices of iron metabolism on bone turnover markers were performed accounting for age, sex, other liver function tests, 25(OH)D, PTH, eGFR, T4, TSH, CRP, white cell count (WCC), alcohol consumption, smoking status, presence of diabetes mellitus (DM), cardiovascular disease (CVD), hip fracture (HF) or any fracture. Similar models were used to assess independent determinants of GGT, ferritin, TSAT, HF or any fracture. When the dependent parameter was stratified by level, odds ratios with 95% confidence intervals (CI) were measured in multivariate logistic regression models. The significance of multicollinearity phenomena in multivariate regression analyses was assessed by the variance inflation factor. Two tailed tests were used and results were considered statistically significant if $P < 0.05$.

Results

Patient demographic, clinical and laboratory characteristics

Among 416 studied patients there were 168 subjects with a HF (52.2% cervical, 44.1% trochanteric and 3.7% subtrochanteric), 89 patients with a non-HF fracture of the peripheral skeleton and 160 subjects without any fracture; 67.8% of the entire cohort were women. Hypertension was diagnosed in 62.7%, coronary artery disease (CAD) in 18.3%, atrial fibrillation (AF) in 17.1%, history of stroke or transient ischemic attack in 12.3%, peripheral vascular disease in 5.0%, chronic cardiac failure (CCF) in 10.8%, DM in 19.5%, CKD (eGFR < 60 ml/min/1.73 m²) in 19.2%, dementia in 17.6%, Parkinson's disease in 3.6%, chronic obstructive airway disease (COPD) in 11.1%, and osteoarthritis in 46.8%. At least one CVD and/or DM was found in 76.0% of the cohort; 18.9% of the patients consumed alcohol > 3 times a week, 19.3% were ex-smokers and 9.1% were current smokers, 19.9% of the patients were admitted from long-term residential care facilities.

The demographic and biochemical characteristics of the study cohort are shown in **Table 1**. Patients with fractures, as could be expected, were older, had significantly lower mean levels of P1NP, transferrin, hemoglobin, serum calcium (corrected for albumin) and magnesium, and higher concentrations of PTH, β CTX, and ferritin than patients without fractures; accordingly, P1NP/ β CTX and OC/ β CTX ratios were also significantly lower in the fracture group. In addition, patients with HF compared to

the non-fracture subjects had lower serum phosphate and iron concentrations. Patients with fractures had a higher prevalence of vitamin D deficiency (11.7% vs. 5.7%, $p = 0.043$) and SHPT (43.1% vs. 34.2%, $p = 0.044$).

Low hemoglobin levels (< 120 g/L) were present in 331 (79.6%) patients. Both serum ferritin level and TSAT were in the normal range in 14.6% of our patients. High serum ferritin (> 370 μ g/L, upper limit of reference range) was found in 32.5% of patients, low TSAT ($< 18\%$, lower limit of reference range) in 78.9%, including 52.9% with ferritin in the normal range and 26.0% with a "paradoxical" combination (i.e. relatively high serum ferritin but low TSAT); only 4 (0.96%) patients demonstrated TSAT $> 46\%$ (upper limit of reference range). Compared to the rest of the cohort, patients with low TSAT ($< 18\%$) had significantly lower mean P1NP/OC (8.5 ± 8.9 vs. 12.2 ± 18.5 , $p = 0.009$) and P1NP/ β CTX (123.4 ± 96.9 vs. 156.5 ± 118.7 , $p = 0.007$) ratios, while subjects with elevated serum ferritin levels (> 370 μ g/L) had lower OC concentrations (6.5 ± 3.8 vs. 7.7 ± 4.5 μ g/L, $p = 0.009$) and higher P1NP/OC ratios (11.2 ± 16.2 vs. 8.3 ± 8.4 , $p = 0.018$).

Relationship between serum GGT activity and indices of bone-mineral and iron metabolism

When the subjects were grouped according to the GGT tertiles patients with lower GGT activity tended to be older (80.4 ± 8.4 years in the first tertile vs. 77.6 ± 9.3 years in the third tertile, p for trend 0.027). Increasing tertile of GGT was significantly associated with higher P1NP (46.1 ± 39.5 vs. 64.0 ± 70.7 μ g/L, $p = 0.028$), ferritin (262.6 ± 218.0 vs. 522.5 ± 542.1 μ g/L, $p = 0.000$), and calcium levels (2.31 ± 0.13 vs. 2.37 ± 0.13 mmol/L, $p = 0.001$), while 25(OH)D concentrations showed a downward trend (64.4 ± 27.3 vs. 55.9 ± 24.4 nmol/L, $p = 0.029$).

Pearson's correlation coefficients of logarithmically transformed variables adjusted for age and sex revealed that logGGT correlated positively with logP1NP ($r = 0.136$, $p = 0.006$), P1NP/OC ratio ($r = 0.181$, $p = 0.000$), P1NP/ β CTX ratio ($r = 0.096$, $p = 0.052$), logPTH ($r = 0.119$, $p = 0.015$), logcalcium ($r = 0.194$, $p = 0.000$), logphosphate ($r = 0.108$, $p = 0.028$), as well as with logferritin ($r = 0.182$, $p = 0.000$), logiron ($r = 0.103$, $p = 0.037$) and logTSAT ($r = 0.107$, $p = 0.030$), and negatively with log25(OH)D ($r = -0.126$, $p = 0.010$). Although logP1NP, as expected, correlated with both logOC ($r = 0.424$, $p = 0.000$) and log β CTX ($r = 0.484$, $p = 0.000$), and logOC correlated with log β CTX ($r = 0.353$, $p = 0.000$), no significant correlation between logGGT and logOC or log β CTX was observed.

Serum GGT activity and indices of iron metabolism as predictors of bone turnover markers

Stepwise multivariate linear regression analyses (**Table 2**) showed that both logGGT and logTSAT were significant independent determinants of logP1NP, log β CTX (negatively by logTSAT), P1NP/OC and P1NP/ β CTX (borderline significant by GGT, $p = 0.068$) ratios. Logferritin was inversely associated with

both logOC and log β CTX, and positively with P1NP/OC ratio independently of multiple potential confounders.

Table 1 Demographic and biochemical characteristics of the study patients overall and according to the presence of fracture.

Characteristic	Total, n=416	Without fracture, n=160 (1)	With hip fracture, n=168 (2)	With any fracture, n=256(3)	P Value	P Value
					(2 -1)	(3-1)
Age, years	78.9 \pm 8.7	75.6 \pm 8.1	82.8 \pm 7.9	81.0 \pm 8.5	0.000	0.000
Women, n (%)	282 (67.8)	101(63.1)	116(69.1)	181(70.0)	0.257	0.108
GGT, U/L	50.3 \pm 57.7	53.4 \pm 64.3	43.1 \pm 42.6	48.4 \pm 53.2	0.088	0.391
*Calcium, mmol/L	2.35 \pm 0.13	2.38 \pm 0.12	2.33 \pm 0.13	2.34 \pm 0.14	0.001	0.001
Phosphate, mmol/L	0.92 \pm 0.24	0.96 \pm 0.21	0.90 \pm 0.28	0.91 \pm 0.26	0.039	0.067
Magnesium, mmol/L	0.77 \pm 0.11	0.79 \pm 0.10	0.75 \pm 0.13	0.76 \pm 0.12	0.004	0.023
25(OH)D, nmol/l	60.2 \pm 26.4	60.9 \pm 24.1	60.5 \pm 28.9	59.7 \pm 27.8	0.878	0.645
PTH, pmol/L	7.0 \pm 4.7	6.4 \pm 3.5	8.0 \pm 6.0	7.4 \pm 5.3	0.004	0.036
OC, μ g/L	7.3 \pm 4.3	7.5 \pm 4.4	7.1 \pm 4.5	7.1 \pm 4.3	0.37	0.372
P1NP, μ g/L	57.6 \pm 61.1	65.4 \pm 70.1	55.1 \pm 57.5	52.7 \pm 54.3	0.144	0.039
β CTX, μ g/L	0.50 \pm 0.33	0.44 \pm 0.26	0.58 \pm 0.39	0.53 \pm 0.36	0.000	0.006
OC/ β CTX ratio	18.9 \pm 16.6	21.2 \pm 19.9	15.3 \pm 9.5	17.4 \pm 14.1	0.000	0.023
P1NP/ β CTX ratio	130.0 \pm 102.5	156.3 \pm 117.3	112.4 \pm 94.5	113.8 \pm 88.6	0.000	0.000
P1NP/OC ratio	9.3 \pm 11.7	10.3 \pm 12.3	9.5 \pm 13.4	8.7 \pm 11.3	0.589	0.178
haemoglobin, g/L	106.5 \pm 16.9	108.6 \pm 15.3	103.7 \pm 17.3	105.1 \pm 17.8	0.007	0.040
Iron, μ mol/L	5.8 \pm 4.7	6.1 \pm 4.1	4.9 \pm 5.0	5.6 \pm 5.0	0.018	0.333
Ferritin, μ g/L	436.1 \pm 572.9	334.8 \pm 404.3	592.0 \pm 405.8	499.3 \pm 526.6	0.001	0.019
Transferrin, g/L	1.7 \pm 0.43	1.8 \pm 0.43	1.6 \pm 0.41	1.7 \pm 0.42	0.000	0.000
Transferrin sat, %	12.5 \pm 9.5	12.5 \pm 8.2	11.6 \pm 9.6	12.6 \pm 10.2	0.377	0.919
Urea, mmol/L	6.3 \pm 3.6	6.2 \pm 2.8	6.8 \pm 4.5	6.4 \pm 4.0	0.110	0.489
Creatinine, μ mol/L	79.1 \pm 49.5	78.5 \pm 31.3	84.7 \pm 69.5	79.5 \pm 58.1	0.306	0.848
eGFR, ml/min/1.73 m ²	80.3 \pm 26.4	79.0 \pm 25.5	79.8 \pm 28.7	81.1 \pm 26.9	0.801	0.437
T4, pmol/L	16.2 \pm 3.1	16.3 \pm 3.0	16.0 \pm 3.2	16.1 \pm 3.2	0.340	0.379
TSH, mU/l	1.7 \pm 3.1	1.7 \pm 3.7	1.7 \pm 2.9	1.7 \pm 2.6	0.990	0.891

Values are means \pm SD. GGT: Gamma-Glutamyl Transferase; 25(OH)D: 25 Hydroxyvitamin D; PTH: Parathyroid Hormone; OC: Osteocalcin; P1NP: Amino-Terminal Propeptide of Type 1 Procollagen; β CTX: Crosslinked Carboxy-Terminal Telopeptide of Type I Collagen; TSAT: Transferrin Saturation; eGFR: Estimated Glomerular Filtration Rate; T4: Free Thyroxine; TSH: Thyroid Stimulating Hormone.*Albumin-corrected.

Besides, log β CTX was also predicted by logiron (positively) and logtransferrin (negatively). All three bone turnover markers were also independently predicted by logPO4 (positively) and logeGFR (negatively). LogPTH was inversely associated with logP1NP and P1NP/ β CTX ratio, while logMg was inversely associated with logP1NP and P1NP/OC ratio.

Presence of any fracture demonstrated significant negative correlations with logP1NP, P1NP/OC and P1NP/ β CTX ratios, while presence of HF was an independent indicator for log β CTX, P1NP/ β CTX and OC/ β CTX ratios (negative associations with both ratios).

Taken together, these findings show that both serum GGT activity and indices of iron metabolism are specific and important factors influencing bone turnover, which is highly relevant to the mechanical properties of bone and fractures.

Parameters of bone-mineral and iron metabolism as indicators of serum GGT activity

Multivariate logistic regression analysis (**Table 3**) revealed the following factors as independently and significantly associated with GGT activity: logP1NP, P1NP/OC ratio, logferritin (all positively), log25(OH)D and age (both inversely); logPTH levels demonstrated borderline significance (p=0.059).

In other words, increasing age and higher 25(OH)D are independently associated with lower GGT, whereas higher P1NP, P1NP/ OC ratio, ferritin and possible PTH are indicators of higher GGT.

Table 2 Serum GGT activity and indices of iron metabolism as independent determinants of bone turnover markers (stepwise multivariate linear regression analyses).

	Log P1NP	Log OC	Log β CTX	P1NP/OC	P1NP/ β CTX	OC/ β CTX
Log GGT	0.095 (0.019)		0.073 (0.043)	1.829 (0.008)	10.809 (0.068)	
*Log Cac		0.588 (0.003)				
Log PO4	0.421 (0.000)	0.154 (0.047)	0.358 (0.000)	2.695 (0.099)		-4.537 (0.057)
Log Mg	-0.489 (0.048)			-17.63 (0.000)		
Log PTH	-0.198 (0.004)				-22.532 (0.014)	
Log Ferritin		-0.116 (0.000)	-0.086 (0.036)	1.533 (0.016)		
Log Fe			0.347 (0.033)			
Log Transferrin			-0.740 (0.001)			8.501 (0.0.10)
Log TSAT	0.136 (0.012)	-0.356 (0.027)	3.237 (0.000)	27.535 (0.000)		
LogeGFR	-0.344 (0.000)	-0.303 (0.000)	-0.349 (0.000)			
HF			0.185 (0.004)		-20.749 (0.044)	-5.476 (0.002)
Any fracture	-0.145 (0.038)			-2.312 (0.045)	-41.184 (0.000)	
R ²	0.371	0.185	0.270	0.269	0.186	0.070

Data represented as the regression coefficient and associated significance level (in brackets) if $p < 0.100$. The R-squared value for the overall regressions are also shown. Abbreviations: GGT: Gamma-Glutamyl Transferase; P1NP: Amino-Terminal Propeptide of Type I Procollagen; OC: Osteocalcin; Bctx: Crosslinked Carboxy-Terminal Telopeptide of Type 1 Collagen; PO4: Phosphate; Mg: Magnesium; 25(OH) D: 25-Hydroxyvitamin D; PTH: Parathyroid Hormone; Fe: Iron; eGFR: Estimated Glomerular Filtration Rate; HF: Hip Fracture. *Calcium: Corrected for Albumin. In each model the bone turnover parameter was the dependent variable. Independent variables included age, gender, logGGT, logALT (alanine aminotransferase), logALP (alkaline phosphatase), logbilirubin, logalbumin, log25(OH)D, PTH, logcalcium(corrected for albumin), logphosphate, logmagnesium, logeGFR, alcohol overuse (>3 drinks/week), smoking status (current smoker, ex-smoker), presence of hip fracture or any fracture (in separate models, i.e. the model included presence of hip fracture or presence of any fracture), diabetes mellitus and cardiovascular disease.

Serum bone-mineral parameters and GGT activity as indicators of iron metabolism

Both ferritin and TSAT, the two most informative and commonly used indices of iron metabolism, were independently positively predicted by logP1NP and logALT, and negatively by logOC and eGFR (**Table 4**). In addition, higher logGGT, CRP, male gender and presence of HF or any fracture correlated positively and DM correlated negatively with logferritin levels.

CRP correlated negatively with TSAT, and there was an inverse correlation between blood white cell count (WCC) and TSAT of marginal statistical significance ($p=0.076$).

The divergent results regarding inflammatory markers (CRP and WCC) are in line with the well known fact that serum ferritin is as a positive acute phase reactant, whereas transferrin is considered a negative one.

Variables independently associated with hip fracture or any fracture

The following 6 variables were independently associated with presence of any fracture (**Table 5**): log β CTX, logferritin and age (all three positively), logP1NP, P1NP/ β CTX ratio and logMg (the last three variables inversely).

When HF patients were considered alone, the statistically significant independent associates were also logGGT and logPO4 (both inverse). In other words, the risk of HF increases with every increment of one unit of log β CTX by 127%, of one unit of logferritin by 67%, and with one year of age by 13%, while increases in logP1NP, P1NP/ β CTX ratio, logGGT, logPO4 and logMg have significant protective effects.

Similarly, the risk of any fracture increases with an increase in one unit of log β CTX by 91%, in one unit of logferritin by 30%, with one year of age by 8%, whereas increases in logP1NP, P1NP/ β CTX ratio and logMg demonstrate protective effects.

Discussion

Main findings

Our data shows a complex interplay between serum GGT activity, iron status and bone metabolism (**Figure 1**). Bone remodelling is regulated by GGT and iron status, and specific bone-derived molecules (P1NP and OC) affect both the GGT activity and iron homeostasis, while GGT and ferritin are also interrelated.

The bidirectional links between P1NP and GGT (1), P1NP/OC ratio and GGT (2), P1NP and TSAT (3), OC and ferritin (4), GGT and ferritin (5) suggest that these molecules and factors act as

feedback loop signals participating in the complex homeostatic, adaptive and compensatory mechanisms.

Furthermore, serum GGT activity, ferritin and TSAT are independent indicators of β CTX, while ferritin and TSAT are also independent predictors of the P1NP/OC ratio, and TSAT is an independent predictor of the P1NP/ β CTX ratio.

Table 3 Parameters of bone - mineral and iron metabolism and related variables as independent determinants of serum logGGT activity (stepwise multivariate linear regression analysis).

	β	95%CI	P value
LogP1NP	0.131	0.026; 0.235	0.015
P1NP/OC ¹	0.01	0.003; 0.016	0.003
Logferritin	0.331	0.226; 0.435	0.000
Logtransferrin	0.359	-0.039; 0.757	0.077
Logiron	0.112	-0.009; 0.233	0.069
Log25(OH)D	-0.207	-0.367; -0.046	0.012
LogPTH	0.139	-0.005; 0.283	0.059
Age	-0.019	-0.029; -0.009	0.000
HF	-0.147	-0.320; 0,026	0.096
R ²	0.195		

Results with $p < 0.100$ are shown. Abbreviations: P1NP: Amino-Terminal Propeptide of Type 1 Procollagen; 25(OH) D: 25-Hydroxyvitamin D; PTH: Parathyroid Hormone; HF: Hip Fracture. logGGT was the Dependent Variable and the Independent Variables Were: Age, Gender, LogP1NP, LogOC, Log β CTX, P1NP/OC, P1NP/ β CTX, OC/ β CTX, Log25(OH)D LogPTH, Logcalcium(corrected for albumin), Logphosphate, Logmagnesium, LogALT, LogALP, Logbilirubin, Logalbumin, Logegfr, Logcrp, Logwcc, Alcohol Overuse (>3 Drinks/Week), Smoking Status (Current Smoker: Ex-Smoker), Presence of Hip Fracture or Any Fracture, Diabetes Mellitus and Cardiovascular Diseases. ¹P1NP and OC (as independent variables) were not included in the model examining P1NP/OC ratio; similarly, P1NP and β CTX were not included in the model examining P1NP/ β CTX ratio and OC and β CTX were not included in the model examining OC/ β CTX ratio.

The study provides evidence that GGT and iron status indices (even within normal or mildly-moderately abnormal range) act as independent determinants of different aspects of bone metabolism, and are, in turn, regulated by bone-derived peptides. Remarkably, both osteoblast-specific secreted

molecules - OC (well documented previously [24,25]) and P1NP (as described, for the first time, in this study) – exert multiple extra-skeletal metabolic functions. The biological and clinical relevance of our results, which confirm and extend a number of previous studies are further discussed.

Serum GGT activity

In our multivariate adjusted models serum GGT activity was an independent determinant of P1NP and β CTX levels, as well as the P1NP/OC ratio. On the other hand, higher serum GGT activity was independently predicted by higher P1NP, P1NP/OC ratio, ferritin levels, lower 25(OH)D concentrations and younger age.

These results are consistent with:

- Hxperimental data indicating important (patho)physiological role of GGT in bone turnover state involving both bone formation and bone resorption [2-6].
- Human studies which reported as (i) Increased urinary excretion of bone resorption markers in parallel with urinary GGT levels in postmenopausal women [6], (ii) Correlation between serum GGT and ferritin levels in the adult population [26], as well as in patients with chronic hepatitis C [27] and DM [28], and (iii) An inverse association between 25(OH)D and GGT activity [29].

The results from the present study show that age-related decreases in GGT activity, a well known phenomenon [30,31], is associated with suppression of bone formation (indicated by lower P1NP and P1NP/OC ratio), as well as bone resorption (lower β CTX), but more affecting the former process.

On the other hand, both lower serum GGT and P1NP levels are associated with lower ferritin concentrations which, in turn, correlate inversely with markers of bone differentiation (higher OC) and bone resorption (higher β CTX).

Lower GGT activity is also a significant independent indicator of HF. These findings suggest a complex/dual effect of lower GGT on bone turnover, and indicate that GGT and iron operate as integral components of a complex homeostatic network that regulates bone metabolism.

Table 4 Serum bone-mineral parameters, GGT activity and related variables as independent determinants of indices of iron metabolism (stepwise multivariate linear regression analyses).

	Log ferritin			Log TSAT		
	β	95%CI	P value	β	95%CI	P value
LogOC	-0.328	-0.487; -0.169	0.000	-0.143	-0.256;-0.032	0.012
LogP1NP	0.173	0.053; 0.294	0.005	0.126	0.045; 0.207	0.002
LogGGT	0.171	0.066; 0.277	0.002			
LogALT	0.217	0.093; 0.340	0.001	0.137	0.064; 0.210	0.000
CRP	0.003	0.002; 0.004	0.000	-0.003	-0.004; -0.002	0.000
WCC				-0.014	-0.029; 0.001	0.076

LogeGFR	-0.26	-0.424; -0.330	0.022	-0.2	-0.336; -0.64	0.004
DM	-0.229	-0.451; -0.046	0.016			
HF	0.328	0.166; 0.489	0.000			
Any fracture	0.202	0.032; 0.372	0.020			
Sex(m)	0.306	0.143; 0.469	0.000			
R ²		0.293			0.398	

Results with $p < 0.100$ are presented. The dependent variables were logferritin and TSAT and the independent variables were age, gender, logP1NP, logOC, log β CTX, log25(OH)D, PTH, logcalcium (corrected for albumin), logphosphate, logmagnesium, logGGT, logALT, logALP, logbilirubin, logalbumin, logeGFR, CRP, WCC, alcohol overuse (>3 drinks/week), smoking status (current smoker, ex-smoker), presence of hip fracture or any fracture, DM, cardiovascular diseases. Abbreviations: TSAT: Transferrin Saturation; P1NP: Amino-Terminal Propeptide of Type 1 Procollagen; OC: Osteocalcin; Bctx: Crosslinked Carboxy-Terminal Telopeptide of Type 1 Collagen; PTH: Parathyroid Hormone; Mg: Magnesium; ALT: Alanine Aminotransferase; ALP: Alkaline Phosphatase; eGFR: Estimated Glomerular Filtration Rate; HF: Hip Fracture; DM: Diabetes Mellitus; CRP: C-Reactive Protein; WCC: White Cell Count.

Iron metabolism

Our logistic regression analyses after adjusting for demographics, liver, renal, mineral and inflammatory markers, smoking and alcohol consumption status, presence of DM, CVD and fracture indicated independent positive associations of TSAT with P1NP, P1NP/OC and P1NP/ β CTX ratios, and negative with β CTX, while ferritin was a negative regulator of both OC and β CTX and a positive determinant of the P1NP/OC ratio. On the other hand, both P1NP and OC independently predicted TSAT, as well as ferritin.

These findings suggest that:

- Higher iron status (as indicated by higher TSAT and/or ferritin levels) is associated with suppression of osteoclast function and bone resorption (lower β CTX).
- Bone formation (P1NP) increases when TSAT is higher, but higher ferritin suppresses the process of bone differentiation (lower OC and higher P1NP/OC ratio).
- Iron-related markers have different effects on specific aspects of osteoblast function; feedback signals between molecules involved in iron and bone metabolism generate a positive loop with osteoblast formation (TSAT – P1NP) and a negative loop with osteoblast differentiation (ferritin – OC).

Thus, the bidirectional links TSAT-P1NP and ferritin-OC, as well as the effects of both ferritin and TSAT on β CTX, of TSAT on P1NP/OC and P1NP/ β CTX ratios should be considered as components of homeostatic systems that maintain structural and functional integrity in the bone (as well as in different other tissues).

Experimental studies linked osteoporosis/osteopenia with both iron overload [32-34] and iron deficiency [35-37]. Few studies in humans that examined the relation between parameters of iron status and bone turnover markers have produced inconclusive results.

An inverse association between serum ferritin and OC was reported in subjects aged 55-80 years with CVD [38]. In postmenopausal women with HF, serum ferritin correlated

positively and transferrin correlated negatively with both P1NP and β CTX [13].

Table 5 Associations between serum markers of bone-mineral and iron metabolism and GGT activity with hip fracture or any fracture. Clearly, contradicting results on associations between indices of iron and bone metabolism at least in part may be explained by the heterogeneity of the study populations, effects of age, gender, ethnicity, hormonal and environmental factors, different definitions of iron status, analytical variability of assays and adjustments only for some confounding factors.

	Hip fracture			Any fracture		
	OR	95%CI	P value	OR	95%CI	P value
Log P1NP	0.59	0.39; 0.92	0.019	0.51	0.36; 0.73	0.000
*P1NP/ β CTX	0.43	0.29; 0.82	0.008	0.44	0.33; 0.71	0.000
Log β CTX	2.27	1.36; 3.79	0.002	1.91	1.25; 2.91	0.003
Log PO4	0.29	0.94; 0.89	0.030			
Log Mg	0.08	0.01; 0.48	0.006	0.17	0.03; 0.81	0.027
Logferritin	1.67	1.20; 2.31	0.002	1.3	1.01; 1.68	0.040
LogGGT	0.6	0.40; 0.88	0.010			
Age	1.13	1.09; 1.17	0.000	1.08	1.05; 1.11	0.000
R ²	0.311			0.176		

Multivariate linear regression analyses of the dependent variables (hip fracture or any fracture – top row) and the significant ($p < 0.05$) independent variables (left column) shown. Adjustments: age, sex, logP1NP, logOC, log β CTX, P1NP/ β CTX (in a model without LogP1NP and log β CTX), log25(OH)D, logPTH, logcalcium (corrected for albumin), logphosphate, logmagnesium, logGGT, logALT, logALP, logbilirubin, logalbumin, logeGFR, alcohol overuse (>3 drinks/week), smoking status (current smoker, ex-smoker), presence of diabetes mellitus and/or cardiovascular diseases.

In young women negative correlations were found between log-ferritin and a urinary bone resorption marker (log-NTx), and

between transferrin and P1NP [10], while serum ferritin concentration was not correlated with serum bone resorption marker (tartrate-resistant acid phosphatase type 5b) in patients with sickle cell disease [39]. In astronauts, both ferritin and TSAT positively correlated with biochemical markers of bone resorption [14].

Our observation of the inverse relationship between ferritin and OC as well as between ferritin and β CTX concur with some previous clinical observations [10,38] and is in line with animal studies which showed lower serum OC concentration in iron-deficient rats [36]. The positive associations of TSAT with P1NP and P1NP/OC ratio in this study is consistent with lower P1NP

levels in premenopausal women with iron deficient anemia [10] and decreased bone matrix formation in anemic rats [35]. Notably, in our study, three iron indices - TSAT, ferritin and transferrin - were independently and inversely associated with β CTX, suggesting that iron deficiency results not only in decreased bone formation but also in increased bone resorption, a finding consistent with significantly elevated levels of urinary resorption marker in iron deficient women [10], and data of increased bone resorption in anaemic rats [35]. Other researchers found both reduced bone formation and resorption in iron-deficient anaemic rats but bone resorption remained greater than bone formation, resulting in overall bone loss [36].

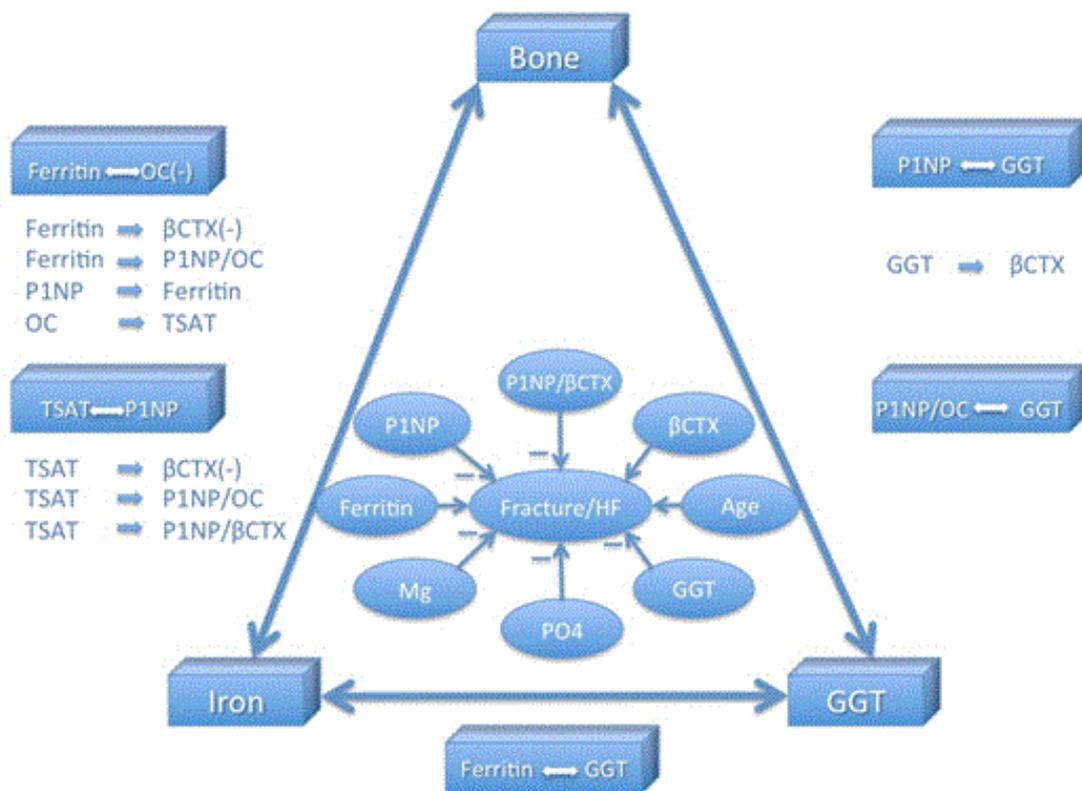


Figure 1 Schematic representation of the complex interactions between serum gamma-glutamyl transferase activity, markers of iron and bone-mineral metabolism in the elderly; factors independently associated with bone fractures are shown in ovals. Abbreviations: GGT: Gamma-Glutamyl Transferase; TSAT: Transferrin Saturation; P1NP: Amino-Terminal Propeptide of Type 1 Procollagen; OC: Osteocalcin; β CTX: Crosslinked Carboxy-Terminal Telopeptide of Type 1 Collagen; PO4: Phosphate. Bidirectional links are underlined; (-) indicates an inverse association; arrows indicate the direction of affect. Serum GGT activity is an independent determinant of P1NP, β CTX, P1NP/OC ratio and ferritin, which, in turn, is an independent determinant of OC, β CTX, and P1NP/OC ratio, while TSAT is an independent predictor of P1NP, β CTX, P1NP/ β CTX and P1NP/OC ratios. The bidirectional links between ferritin and GGT, ferritin and OC, GGT and P1NP, GGT and P1NP/OC ratio, TSAT and P1NP indicate feedback signals involved in the complex homeostatic mechanisms. Higher serum concentrations of β CTX and ferritin, lower P1NP, P1NP/ β CTX ratio, PO4 levels and advanced age are independent indicators of any fracture; lower GGT activity is an additional independent indicator of HF.

An important point to note is that ferritin demonstrated a bidirectional inverse link with OC, whereas TSAT showed a bidirectional positive association with P1NP, despite the fact that P1NP is correlated with OC. It appears, therefore, that the contribution of ferritin to bone metabolism may reflect an effect mediated by mechanism (-s) other than increased iron stores. Furthermore, despite the strong inverse bidirectional link between ferritin and OC, neither serum iron, nor TSAT (additional indicators of body iron stores, transport and availability) independently predicted OC in our and other studies [28].

These observations are in keeping with the existing evidence that elevated serum ferritin is a multifactorial metabolic marker which reflects in addition to body iron stores other conditions such as inflammation, oxidative stress, chronic kidney, liver and autoimmune disorders; elevated ferritin levels do not exclude underlying iron deficiency. Indeed, 78.9% of our patients had low TSAT, and 26.0% demonstrated a “paradoxical” combination of elevated ferritin levels with low TSAT, indicating functional iron deficiency [40], a condition typical for anemia of chronic disease (anemia of inflammation) and chronic kidney disease. In the present study ferritin was independently predicted by CRP (positively) and eGFR (negatively).

Although hydroxylation of 25(OH)D to 1,25(OH)D requires iron [36] and low vitamin D levels were linked with iron deficient anemia in females [10], in our study, 25(OH)D was not an independent determinant of TSAT or ferritin.

Homeostatic mechanisms

Our data indicate a negative-feedback loop ferritin – OC and a positive-feedback loop TSAT – P1NP, whereas both P1NP and P1NP/OC ratio demonstrate positive-feedback loops with GGT. These observations reflect the complexity of homeostatic mechanisms and are in line with the growing body of evidence suggesting oxidative stress as an integrative (patho)physiologic mechanism that links both GGT activity and iron status with bone integrity. Higher level of GGT, the main determinant of extracellular hydrolysis of glutathione, and elevated serum ferritin concentrations leading to release of more free iron, could facilitate glutathione catabolism, increase production of reactive oxygen species and subsequent oxidative stress, which in turn, affects bone metabolism and causes bone loss [22,23,41]. The hypothesis that oxidative stress is involved in the pathogenesis of bone loss is supported by animal studies showing that treatment with the antioxidant N-acetyl-L-cysteine, precursor of glutathione, reversed osteopenia in GGT-deficient mice [2] and prevented the development of bone abnormalities in iron-overloaded mice [33]. In ours and other studies, GGT and ferritin correlated with each other. On the other hand, these variables demonstrated associations with β CTX and presence of HF, which is in line with the dual role of reactive oxygen species (positive at moderate levels and damaging at elevated levels) in bone metabolism, as in a number of other pathological conditions. Interrelated serum GGT and ferritin levels appear as an important homeostatic bridge between metabolic, oxidative and bone status.

Taken together, the existing literature (although results are mixed) and our observations provide evidence that regulation of both osteoblastic and osteoclastic differentiation and function requires an optimal iron status: iron levels should be sufficiently high to facilitate bone formation and suppress bone resorption, but not so high that they affect bone differentiation and increase GGT activity. Importantly, these factors are in a state of mutual influence: feedback mechanisms involving both osteoblast-specific proteins (P1NP and OC) participate in maintaining iron homeostasis and GGT activity.

Biomarkers of osteoporotic fractures

In multivariate regression, P1NP, β CTX, P1NP/ β CTX ratio, ferritin, magnesium and age were independent indicators of HF or any fracture; GGT and phosphate levels were additional independent negative indicators of HF. Multivariate regression analyses revealed also that presence of HF was an independent positive predictor for logferritin, log β CTX, and a negative predictor for both P1NP/ β CTX and OC/ β CTX ratios. Interestingly, neither serum 25(OH)D, nor PTH were independently associated with fractures, and no association between serum 25(OH)D and any of serum bone markers was found, but logPTH was an independent negative predictor of logP1NP and P1NP/ β CTX ratio.

The literature on the association between fracture risk and bone turnover markers, as well as serum 25(OH)D and PTH concentrations is controversial. Our data are in agreement with previous studies showing that: (1) higher serum and urinary bone resorption markers predict risk for HF, vertebral and non-vertebral fracture [42], (2) in women low serum levels of P1NP (independent of BMD) predict increased risk of HF [43], (3) serum concentrations of PTH correlated negatively with P1NP, while there was no correlation between 25(OH)D and any bone markers [43,44].

Although P1NP and CTX were recommended as osteoporotic markers by the Bone Marker Standards Working Group [45], the P1NP/ β CTX ratio has not been evaluated previously. In our study, the P1NP/ β CTX ratio that combines two different markers reflecting both bone formation and bone resorption appears as an independent and a better indicator of fracture than absolute P1NP and β CTX values.

Practical considerations

First, the fact that abnormal iron status, a preventable and curable factor, contributes to the occurrence of osteoporotic fractures, signifies the importance of screening for and treating all elderly persons with altered iron metabolism and/or anemia for osteoporosis and vice versa.

Second, in the elderly iron deficiency, absolute (low iron stores) or functional (impaired iron mobilization from storage and transportation to target tissues despite normal or abundant body iron stores) is prevalent and multifactorial (renal insufficiency, sex hormone deficiency, limited iron intake, blood loss). Since ferritin levels are not specific for iron overload (serum ferritin is almost equally both a measure of iron stores and an inflammatory marker), this parameter has to be

interpreted with caution. Moderately elevated ferritin levels, as in our study, especially if combined with a low TSAT (an index of the amount of iron molecules available in the serum that are bound to transferrin), may reflect multiple systemic processes. Therefore, the recommendation of lowering “iron overload” (based on serum ferritin levels only) by chelation therapy [9] or by a biologically active form of hepcidin [8] for the treatment of osteoporosis seems premature if not inappropriate, specifically in the elderly, in whom osteoporosis, iron-deficiency, chronic inflammation and renal impairment are common. Therapeutic manipulation of iron balance in this population is challenging; to be effective and safe it should be based on detailed assessment of pathogenic mechanisms and individualised.

Third, because low BMD, currently the main diagnostic criterion of fracture risk, has a poor sensitivity (about half of low-trauma fractures occur in subjects with non-osteoporotic BMD), our observations together with literature reports indicate that bone turnover markers, especially the P1NP/ β CTX ratio, may be useful in detection of increased risk of osteoporotic fracture and in selection of the most suitable type of antiosteoporotic treatment in a given patient (i.e., anabolic versus antiresorptive medications in individuals with low P1NP). A combined approach with both indices of bone turnover and BMD could possibly improve fracture prediction and management.

Limitations

A number of limitations of this study need to be considered. First, because it is a cross-sectional study, it cannot prove causality. Second, a single-point measurement of the biomarkers may be subject to within-individual variation, although the majority of biochemical tests, including serum GGT, ferritin, iron, OC, P1NP, indices of mineral metabolism are relatively stable and have no circadian and between-day variations. Third, although in multivariate models we attempted to adjust for a series of potential confounders (laboratory, clinical, behavioural), the possibility of residual or unknown confounding cannot be excluded. Fourth, as we did not measure hepcidin, soluble transferrin receptor, and undercarboxylated OC, our results should be interpreted cautiously. Finally, this study was conducted at a single centre and as the cohort was almost entirely Caucasians, our findings may not necessarily translate to other centres and be applicable to other ethnic and racial populations. The strength of this study includes comprehensive clinical and laboratory evaluations and simultaneously measuring of multiple parameters of liver, iron and bone metabolism in a moderately large sample size, whereas most of previous clinical studies assessed a limited number of variables and often did not control for important factors. In all our models the variance inflation factor ranged between 1.03 and 1.17 indicating that the amount of multicollinearity was not significant.

Conclusions

The study highlighted and specified the bidirectional interconnections between serum GGT activity, indices of iron

metabolism and bone-derived factors, the role of GGT and iron homeostasis in maintaining bone health in the elderly, reinforced the need to individualize prophylactic and therapeutic strategies based on alterations in individual biochemical profiles, and identified the serum bone turnover markers, especially P1NP/ β CTX ratio, as indicators for osteoporotic fractures. The clinical significance and ways of modulation of GGT and iron-related factors warrant further investigations.

Conflict of interests

The authors declare that they have no conflict of interests.

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