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ABSTRACT

It has been suggested that iron-deficient rats have lower bone mass than iron-replete animals, but a clear association between bone and iron repletion has not been demonstrated in humans. A growing body of evidences also suggest a relation between lipid oxidation and bone metabolism and between iron metabolism and LDL oxidation. Iron availability to cells also depends on haptoglobin (Hp) phenotypes. Hp has also important antioxidant properties according to its phenotype, hence we evaluate whether Hp phenotype could influence bone density, iron metabolism and lipid oxidation.

This cross-sectional study enrolled 455 postmenopausal women affected by osteoporosis (260) or not (195). Bone mineral density, markers of bone and iron metabolism, levels of oxidized LDL (oxLDL) and Hp phenotype were measured in all the subjects.

Hp 1.1 and 2.2 frequency was higher and Hp 2.1 was lower in the patients with fragility fractures (80) compared with the controls. We therefore evaluate different Hp phenotypes as risk or protective factors against fragility fracture: Hp 2.1 is a protective factor against fracture while 1.1 is an important and 2.2 a moderate risk factor for fragility fractures. Lower serum iron was associated with elevated transferrin in patients with Hp 1.1; moreover patients had relative iron deficiency compared with the controls and fractured patients had higher level of oxLDL. We found that both iron metabolism and oxLDL varies according to Hp phenotypes and are predictive of bone density.

Our data indicate that Hp 2.1 is a protective factor for fragility fractures, depending on its role on iron metabolism and its antioxidant properties.

Key words: iron; osteoporosis; oxidized LDL; haptoglobin.

INTRODUCTION

Postmenopausal osteoporosis stems from the lack of oestrogen function at menopause, combined with genetic factors, and alimentary and sedentary habits. Non-genetic factors heighten and prolong the rapid phase of bone loss characteristic of the early postmenopausal period. Several modifiable and unmodifiable risk factors promote postmenopausal bone loss and hence the development of osteoporosis. One such factor is an inadequate dietary intake of essential nutrients. Dietary calcium [1] and proteins [2] play active roles in bone metabolism, as well as phosphorus and vitamin D [3], while other vitamins and minerals are directly or indirectly needed for metabolic processes related to bone [4, 5]. For example it has been shown that iron-deficient rats have lower bone mass and mechanical strength than iron-replete animals [6-8]. A clear association between bone and iron status, however, has not been demonstrated in humans, though a trend between bone mineral density (BMD) at the radius and serum ferritin has been reported in adolescent females [9]. A growing body of evidences also suggest that increased oxidative stress favours bone loss [10, 11]. Oxidation of LDL occurs under the influence of reactive oxygen species and the catalytic activity of transition metals such as iron, initiating the alteration of its apolipoprotein B [12]. Iron availability to cells also depends on haptoglobin (Hp) phenotypes.

Hp is a tetramer glycoprotein formed of two α and two β chains, its major biological function is to bind and recycle free haemoglobin (Hb) and thus mediate haem iron recycling following physiological haemolysis [13]. In humans, three structurally different phenotypes (Hp 1.1, Hp 2.1, Hp 2.2) are the result of the expression of two alleles, designated Hp1 and Hp2, of the Hp gene located on chromosome 16q22 [14]. Hp 1.1 is

the most efficient haem binder and associated with the lowest levels of free serum iron [13]. Pro-oxidative forms of iron (Fe2+) and haem, derived from Hb, are able to generate hydroxyl radicals in the presence of H2O2 (Fenton reaction) which can initiate lipid oxidation causing the formation of reactive lipid decomposition products which can react with lysine residues of LDL-associated apolipoprotein B-100, resulting in the formation of oxidized LDL (oxLDL) [12, 15]. Hp plays a crucial role against Hb-induced oxidative stress by a mechanism thought to involve its high-affinity binding with Hb and prevents iron release from Hb. Hp itself has antioxidant activity [16]. Recently it has been suggested that different Hp phenotypes may influence the levels of oxLDL in humans [17].

The role of Hp phenotypes has been extensively studied in infectious [18], autoimmune [19] and degenerative [17, 20] diseases and has been related to differences in iron metabolism and the antioxidant properties of Hp.

In this study of a cohort of postmenopausal women we show that Hp 1.1 or 2.2 are associated with a greater risk of fractures with respect to Hp 2.1. We also suggest that the association between Hp and bone mass stems from its role in iron metabolism and antioxidant properties, since the serum iron levels of osteoporotic patients are lower than those of healthy controls, and patients had greater amount of oxLDL as compared to controls. Furthermore we show that iron metabolism parameter and levels of oxLDL are predictive of BMD.

METHODS

Subjects and bone density

Four hundred and fifty-five women in spontaneous menopause for at least one year who came to Department of Internal Medicine to perform a bone densitometry were enrolled in the study. Subjects with intestinal malabsorption diseases, other kind of deficient nutritional status or secondary osteoporosis were excluded.

Osteoporosis was determined according to the WHO criteria [21]. The study was approved by the human study review board of the Azienda Ospedaliera–Universitaria San Giovanni Battista in Torino and all the subjects signed an informed consent statement prior to their recruitment.

Bone mineral density (BMD) was measured by a Hologic QDR 4500 (Hologic Inc.) at lumbar spine or hip according to the women clinical feature. Wrist, femur and vertebra fragility fractures occurred over the age of fifty following minimal trauma were identified from the medical history and confirmed by inspection of the X-ray plates by a physician. Height and weight were registered and body mass index (BMI) was calculated

Biochemical parameters.

In all the subjects we measured serum levels of blood calcium and phosphorus, bone alkaline phosphatase (BAP), 25-hydroxyvitamin D, and PTH. Serum levels of osteocalcin (measured with an RIA technique - DiaSorin), blood iron, transferrin and ferritin were also evaluated with standard methods. The percentage of saturated transferrin was calculated using the following formula:

saturated transferrin = serum iron/(serum transferrin*1.42)*100.

A plasma sample for determination of Hp phenotype was frozen at -80° C and stored

until the measurements were done.

In order to evaluate the influence of Hp on lipid oxidation serum oxLDL concentration was measured by an enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden) [17]. Serum total cholesterol, HDL and triglycerides were evaluated enzymatically with Modular (Roche). LDL level was determined with Friedwald's formula.

Blood was drawn from an antecubital vein after an overnight fast of 10 or more hours and all the measurements were done from a single blood sample at a single time point per patient.

Risk factors for osteoporosis

All the women were asked to answer a questionnaire on the most substantial risk factors for osteoporosis: routine physical activity was classed as less than 30 min, 30-60 min and more than 60 min per day. Smokers were classified as: current (number of cigarettes recorded), past or never. Body mass index (BMI) was also registered. Eating habits were evaluated in a part of the cohort (173 women) with a previously validated semi quantitative weekly Food Frequency Questionnaire (FFQ).

Western blot

Hp phenotypes were identified by Western blot analysis in accordance with a previously standardized protocol. The following equipment was used: Biorad electrophoresis/Western blot system, 8% SDS-PAGE minigels, broad range molecular weight standards, and ECL chemiluminescence detection reagents (Amersham). For Hp detection, 5 µl of plasma (diluted 1:50) were used as previously described [22]. Hp was immunoprecipitated for 90 min at room temperature with a mouse anti-human Hp

antibody (Sigma), diluted 1:4000. Samples were layered onto the minigels along with the broad range standards. Western blot electrophoresis was run at 200 V for 45 min. The gels were then transferred to a nitrocellulose membrane (Hybond ECL, Amersham) for 80 min at 50 volts. Blots were incubated in HRP-conjugated secondary antibody followed by chemiluminescence substrate, developed, and exposed to X-ray film for 5 s (Fig. 1A). Protein concentration was determined with BCA reagents (Pierce), using bovine serum albumin as standard. In those cases with a questionable Western blot result the electrophoresis was repeated and, if the result was still not clear, the subject was excluded (2 cases).

Statistics

Three groups were formed to assess the influence of Hp phenotypes as osteoporosis and fracture risk factors: osteoporotic women with fractures, osteoporotic women without fractures, and healthy subjects. Hp phenotype distribution was analysed with the χ square test. The relative risk (RR) and corresponding confidence intervals (CI) were also calculated.

To look for selection bias, the distribution of other osteoporosis risk factors among the three groups and among patients with different Hp phenotypes was evaluated by one-way ANOVA for the numerical variables, and the χ square test for the non-parametric variables. The factors taken into account were: age, postmenopausal period, BMI, calcium intake and physical activity, as previously defined. One-way ANOVA was used to assess differences in calcium and iron metabolism between the Hp phenotypes. To analyze the differences among different Hp phenotypes and different group of subjects for serum levels of oxLDL we used a Kruskal-Wallis test as this variable was not

distributed according to a Gaussian curve. To evaluate correlations between iron, oxLDL and bone metabolism we used partial correlation after correction for age, time since menopause and BMI. To quantify the relation between BMD, oxLDL, iron metabolism and Hp phenotypes, we used a linear regression model with stepwise analysis.

All analyses were performed with the SPSS 15.0 for Windows, with $p < 0.05$ as the significance cut-off.

RESULTS

In our cohort 260 women were affected by postmenopausal osteoporosis (among them 80 had suffered from fragility fracture) and 195 women were not affected by osteoporosis and generally healthy. Patients and controls were not significantly different with regard to their baseline characteristics (Tab. 1).

Osteoporotic subjects have higher PTH levels.

Evaluation of calcium and phosphorus metabolism showed that PTH serum levels were higher in the patients. This suggests that their PTH secretion is at the top of the normal range in response to their lower blood calcium and phosphorus. Higher PTH may also explain their slight increase in osteocalcin values as an expression of greater skeletal turnover, whereas their BAP values were always within the normal limits (Tab. 2).

Iron metabolism and osteoporosis

Iron metabolism as expressed by blood iron, transferrin and ferritin values was within the normal limits in the osteoporotic patients. Their relative iron deficiency compared with the controls was confirmed by a significant increase of transferrin and a non-

significant reduction of ferritin (Table 2).

Iron metabolism was correlated with lumbar and femoral neck BMD, and with calcium and phosphorus metabolism (corrected for age, time since menopause and BMI), to look for direct correlations between iron deficiency, BMD and bone metabolism. Transferrin was significantly correlated with lumbar and femoral neck BMD (R=-0.2, p=0.015 and R=-0.34, p=0.000, respectively).

The slight alteration of calcium-phosphorus and iron metabolism values in the patients could be indicative of generic malnutrition/malabsorption compared with the controls. Differences in the dietary intakes of these two groups were sought from the answers given by 101 patients and 72 controls to an FFQ covering a period of one week. No significant differences emerged (Tab.3).

Hp 2.1 is a protective factor against fragility fractures.

Hp phenotypes were therefore evaluated as percentages in the patients (with or without fractures) and the controls to see whether the former carried a genetic predisposition to iron deficiency.

It was found that Hp 1.1 and 2.2 frequency was higher and Hp 2.1 was lower in the 80 patients with fractures compared with the controls (Fig. 1 B).

To determine whether the Hp phenotype is a risk factor for fragility fractures, we calculated the RRs and CIs for different Hp phenotypes comparing fractured patients with healthy controls and with subject without fractures regardless to their BMD, the data show that Hp 2.1 is a protective factor against fracture while 1.1 is an important and 2.2 a moderate risk factor (Fig. 2).

The influence of Hp phenotype on BMD was assessed by comparing BMD values in

non-fractured patients with different Hp phenotypes (Fig. 3A). Patients with Hp 1.1 tended to have a lower lumbar BMD than those with Hp 2.1 whereas there were no significant differences in the bone turnover levels (data not shown).

To rule out bias due to the possibly different distribution of other risk factors among the three phenotypes, we carried out a one-way ANOVA to look for phenotype-related differences in age, postmenopausal period, BMI, and dietary calcium intake. Smoking habits in the three phenotypes were analysed with the χ square test. No significant findings emerged (data not shown).

Hp phenotypes are predictive of iron metabolism.

Comparison of blood iron, ferritin, transferrin and saturated transferrin levels amongst the three phenotypes confirmed that lower serum iron was associated with elevated transferrin and reduced percentage of saturated transferrin in patients with Hp 1.1, as already reported [13, 14] (Fig 3B), whereas the reduction of their ferritin was not significant (data not shown). Confirmation of the role of Hp phenotype in iron metabolism was obtained by a multivariate regression model between iron metabolism parameters as dependent variables, age, menopausal period, BMI and Hp phenotypes as covariates, this model confirm that only Hp phenotype is significantly predictive of serum iron ($p=0.047$), of serum saturated transferrin ($p=0.015$), while it does not influence serum ferritin.

oxLDL level is higher in patients as respect to controls.

In order to determine whether the antioxidant properties of Hp play a role in bone loss we measured the level of oxidized LDL corrected for LDL and expressed as oxLDL/LDL ratio in 160 patients (80 with and 80 without fracture) and 80 healthy age matched

subject. The oxLDL/LDL ratio was significantly lower in subject with Hp 2.1, while it was higher in osteoporotic patients with fracture as respect to both controls and patients without fractures (Fig.4). This result confirm previous literature on the higher oxidative stress in osteoporotic patients [10, 11, 23].

Bone density, iron metabolism and oxLDL.

To determine whether iron metabolism parameter and/or oxidative stress are predictive of BMD variations, we constructed a linear regression model for age, period of menopause, BMI, iron metabolism, oxLDL/LDL and BMD. BMI, period of menopause, oxLDL/LDL and transferrin levels were predictive of 58-67% of lumbar BMD variations (R^2=0.58, p=0.000) and femoral neck BMD variations (R^2=0.67, p=0.000).

DISCUSSION

It is well known that postmenopausal bone loss is a physiological sequel to the cessation of ovarian function. Osteoporosis, however, does not always occur and is not always the cause of fractures.

It has recently been demonstrated that BMD alone accounts for 60 to 70% of bone strength [24]. Resistance to fracture, however, is also determined by bone architecture and density, and by extra-skeletal conditions such as propensity to fall.

The aim of our study was to look for differences (other than the known risk factors) between osteoporotic women and healthy controls, and find out whether there is a further difference between those with and those without fractures, with particular reference to iron deficiency and oxidative status. An assessment was therefore made of the best-known modifiable and unmodifiable risk factors, calcium-phosphorus metabolism, and iron metabolism in a cohort of menopausal women in function of their

BMD and the presence/absence of fragility fractures.

The first point of interest that emerged was the difference in calcium-phosphorus homeostasis between the patients and the controls. Serum calcium and phosphorus levels were lower in the patients and associated with increased PTH values. This difference was not due to reduced 25 OH vitamin D, nor to a dietary insufficiency of calcium or other nutrients. It was also found that the patients were iron-deficient compared with the controls. This reduced iron bioavailability could influence bone metabolism, since iron acts as a cofactor in enzymes involved in collagen bone matrix synthesis, as well as in 25 OH vitamin D hydroxylase [25], an enzyme involved in activating vitamin D and hence in calcium absorption. Partial correlations between transferrin and BMD, and analysis of a regression model provided evidence of the direct involvement of iron in human bone metabolism, as shown in the experimental animal [6, 7].

Since there was no substantial difference in the dietary intakes of the two groups of subjects, we identified the Hp phenotype of the patients to see whether they displayed a genetic predisposition to lower iron bioavailability. The distribution of the three Hp phenotypes indicated that Hp 2.1 is associated with lower fractures incidence , whilst Hp 1.1 and 2.2 are associated with higher fractures incidence. As Hp could also play a role in bone loss thanks to its antioxidant ability we measured lipid oxidation. Oxidative stress has been suggested as an important mediator of bone loss since deficiency of antioxidant vitamins has been found to be more common in the elderly osteoporotic patients [10, 11, 23]. In this study we found an increased lipid oxidation in osteoporotics as compared to controls, as according with previously reported, and a significant lower

ratio of oxLDL/LDL in Hp 2.1 as compared to Hp 2.2 and 1.1. The linear regression model confirmed the relation between oxLDL/LDL, iron metabolism, Hp phenotypes and BMD. In our preliminary work we suggested that Hp 1.1 and 2.1 are more common in osteoporotic as opposed to non-osteoporotic postmenopausal women [22]. In the present study we found a higher frequency of Hp 1.1 and 2.2 in osteoporotic patients with fragility fractures, and a lower frequency of Hp 2.1 in these patients. The present study considered a much larger cohort, including women with fragility fractures, and it is, therefore, more accurate. In the present study we also analysed iron and calcium-phosphorus metabolism, oxidative status and lifestyle-associated risk factors, including diet. Selection bias was thus ruled out, and support was found for the view that Hp phenotype could be associated with fractures via iron bioavailabity.

The evaluation of bone density in relation to phenotype showed that the lumbar spine BMD of women with Hp 1.1 was lower than that of those with Hp 2.1, while women with Hp 2.2 were not significantly different in terms of BMD as respect to women with Hp 2.1. The combined evidence of reduced iron bioavailability in osteoporotic women with Hp 1.1 as opposed to Hp 2.1, the inverse correlation between transferrin and BMD levels, and the demonstration that transferrin is a predictor of BMD combine to suggest that the Hp phenotype 1.1 is associated with lower lumbar BMD through differences in iron bioavailability; therefore, it is not an independent risk factor for fractures. The association of Hp 2.2 and fractures could be explained by the higher level of oxLDL/LDL in these phenotypes as respect to 2.1, as we found a clear association between the level of oxLDL and BMD.

In conclusion, our findings indicate that postmenopausal osteoporotic women with

fractures may be regarded as patients with a genetically determined, calcium-phosphorus and iron "malnourished" phenotype and an increased oxidative damage. The Hp phenotype is a genetic marker of iron metabolism and of oxidative stress, whereas a specific marker for calcium-phosphorus metabolism remains to be found. A candidate gene has been identified in vitamin D receptor polymorphisms [26].

Further work with higher number of patients is needed to determine the exact role of iron metabolism postmenopausal bone loss.

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TABLES

TABLE 1. Baseline characteristics of patients with osteoporosis and controls. P values were calculated by one-way ANOVA for continuous variables (age, years post-menopause, BMI and dietary calcium intake), and with the χ square test for non-continuous variables (smoking habits and physical activity). Continuous variables are expressed as means ± SD, non-continuous variables as percentages.

	PATIENTS			
	Never fractured (180)	**Fractured (80)**	**Controls (195)**	**p**
Age (yr)	65±9	67±10	66±11	NS
Time since menopause (yrs)	17±9	19±8	18±13	NS
BMI	23.4±3.1	24.1±3.6	24.2±3.3	NS
Calcium intake (mg/week)	5666.3±2675.2	6024.5±3062	5786.4±2570.9	NS
Active smokers (%)	10.9%	12%	11%	NS
Past smokers (%)	14.1%	15%	27.4%	NS
Never smoked (%)	75%	73%	61.6%	NS
Physical activity < ½ hour daily (%)	65.4%	65%	66.7%	NS
Physical activity between ½ hour and	17.8%	19%	18.3%	NS

1 hour				
Physical activity	16.8%	16%	15%	NS
more than 1 hour				
daily				

TABLE 2. Calcium-phosphorus and iron metabolism parameter values in patients and controls. Means and SD are shown; p values were calculated by one-way ANOVA. The references values are in parentheses.

	PATIENTS			
	Never fractured (180)	**Fractured (80)**	**Controls (195)**	**p**
Serum calcium (9-10.5 mg/dL)	9.58±0.4	9.22±0.6	10.4±0.9	**0.000**
Serum phosphorus (0.65-2.10 mMol/L)	1.22±0.47	1.20±0.57	1.56±0.82	**0.000**
PTH (10-65 pg/mL)	28.87±9.93	29.17±8.83	25.53±10.2	**0.037**
BAP (<21 UI/L)	15.80±6.45	16.2±3.44	14.37±9.45	NS
Osteocalcin (1.8-6.6 ng/mL)	9.21±2.13	9.55±3.13	4.2±2.97	**0.001**
25 OH Vitamin D (adequate> 37 nM/L)	45.69±41.5	46.58±30.2	49.04±9.03	NS
Serum iron (60-160 μg/dL)	92.92±25.2	91.88±22.2	99.3±25.54	**0.047**
Serum transferrin (200-300 mg/dl)	253.7±36.2	266.5±33.3	235.5±36.6	**0.000**
Serum ferritin (20-300 ng/dl)	75.6±51.26	74.2±31.34	84.83±66.5	NS
Saturated transferrin (15-50%)	28.33±0.89	28.44±0.87	28.94±1.05	NS

TABLE 3. Dietary intakes calculated from a food intake questionnaire. Mean and SD are shown in the Table. P values were calculated by one-way ANOVA.

	Patients (101)	**Controls (72)**	**p**
Protein intake (g/week)	405.5±114.1	432.4±119.7	NS
Fat intake (g/week)	230.5±114.1	432.4±80.9	NS
Carbohydrate intake (g/week)	1196.2±375.2	1261.8±387.3	NS
Cholesterol intake (g/week)	1.7±0.6	1.7±0.5	NS
Caloric intake (Kcal/week)	9657.2±2502	10309±268.1	NS
Alcohol intake (g/day)	10.2±12.5	11.5±13.4	NS

FIGURE LEGENDS

Figure 1. Western blot analysis of Hp phenotype: Hp 2.2 appears as a multiple band near the origin (400 KDa), Hp 1.1 as a single band at 200 KDa, Hp 2.1 as both bands combined (A). Hp phenotype is indicated on the top of the panel. Graph showing the distribution of Hp phenotype percentages in the patients (with and without fractures) and the controls (B). The p value was calculated with the χ square test.

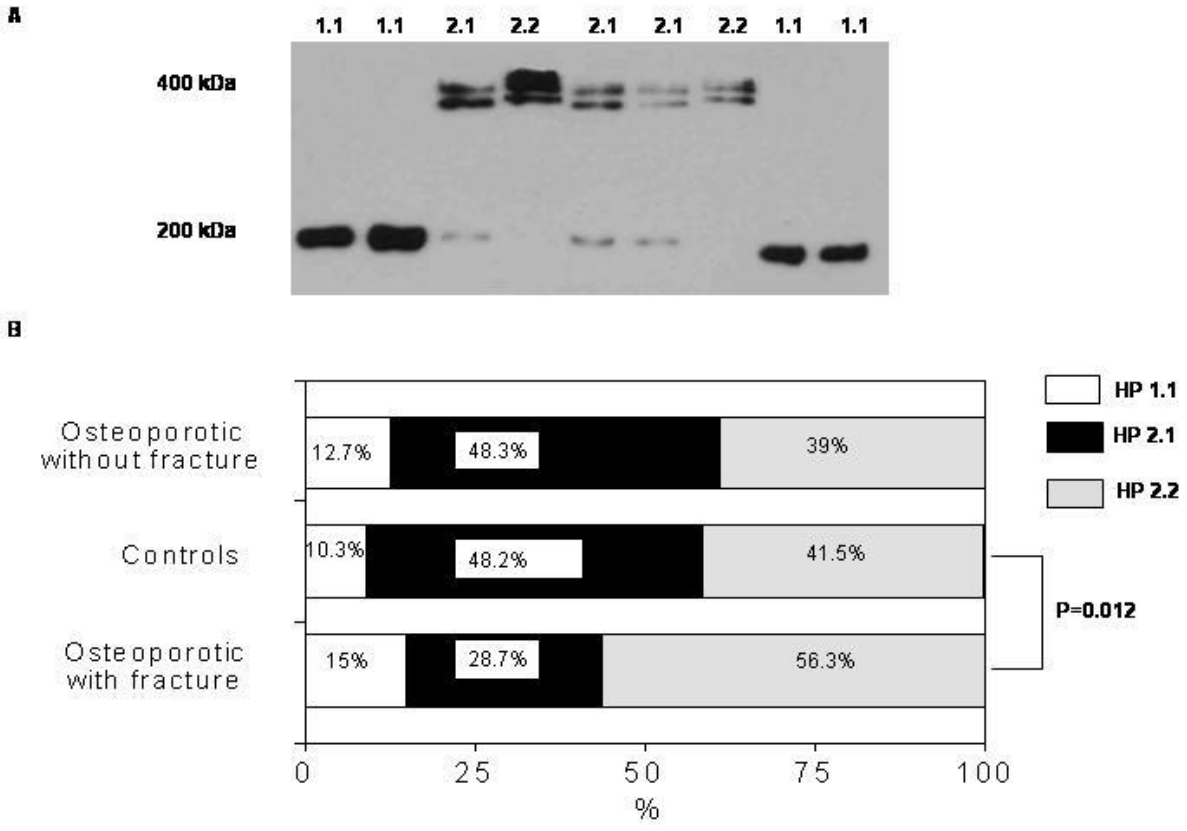

Figure 2. Graphs showing the relative risk (RR) and confidence intervals (CI) for different Hp phenotypes calculated comparing fractured patients vs healthy controls (A) or fractured patients vs non fractured subjects regardless to their BMD (B). The dotted line indicates the RR=1.

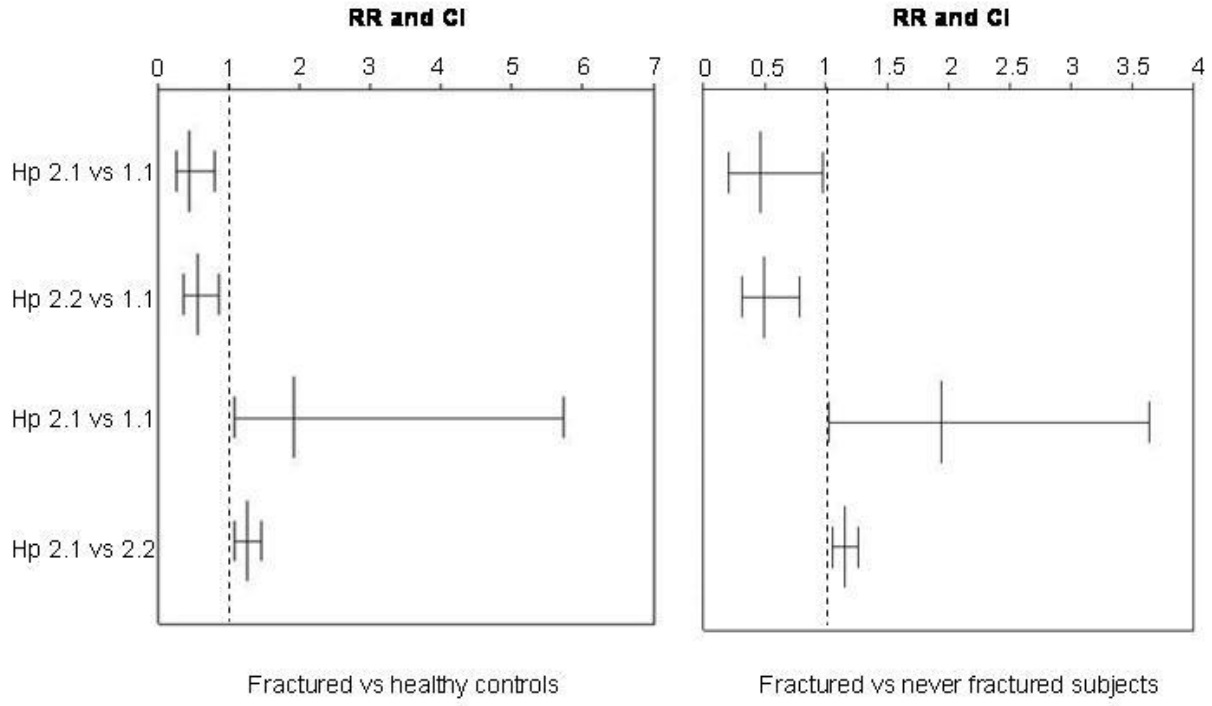

Figure 3. Graphs showing the distribution of lumbar and femoral neck BMD (A), serum iron and transferrin (B left), percentage of saturated transferrin (B right) according to different Hp phenotypes. The bars show the mean plus the SD, p values are calculated by one-way ANOVA.

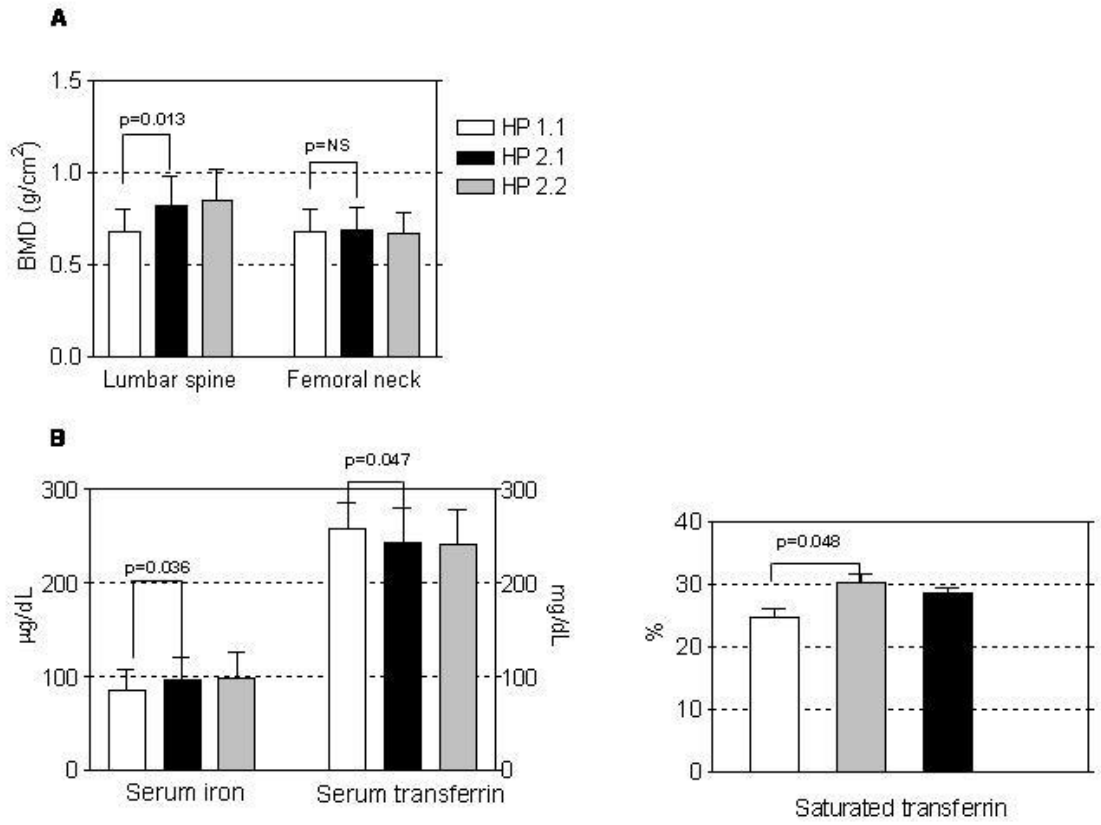

Figure 4. Graphs showing the levels of oxLDL/LDL and oxLDL among the three Hp phenotypes (A, B) and among fractured and non fractured osteoporotic patients and controls (C, D). The bars represent the median with the 25° and 75° percentile; p values were calculated with Kruskal-Wallis test.