Polymorphisms in the macrophage migration inhibitory factor gene and bone loss in postmenopausal women

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Abstract

Osteoporosis is a severe condition in postmenopausal women and a common cause of fracture. Osteoporosis is a complex disease with a strong genetic impact, but susceptibility is determined by many genes with modest effects and environmental factors. Only a handful of genes consistently associated with osteoporosis have been identified so far.

Inflammation affects bone metabolism by interfering with the interplay between bone resorption and formation, and many inflammatory mediators are involved in natural bone remodeling. The cytokine macrophage migration inhibitory factor (MIF) has been shown to affect bone density in rodents, and polymorphisms in the human *MIF* promoter are associated with inflammatory disorders such as rheumatoid arthritis. We investigated the association of polymorphisms in the *MIF* gene with bone mineral density (BMD) and bone loss in 1002 elderly women using *MIF* promoter polymorphisms MIF-$CATT_{5\text{-}8}$ and rs755622(G/C) located -794 and -173 bp upstream of the transcriptional start site. Bone loss was estimated both by the change in BMD over 5 years and by the levels of bone resorption markers in serum measured at four occasions during a 5 year period.

The MIF-$CATT_7$/rs755622(C) haplotype was associated with increased rate of bone loss during 5 years at the femoral neck ($p<0.05$) and total hip ($p<0.05$). In addition, the MIF-$CATT_7$/rs755622(C) haplotype carriers had higher levels of the bone turnover marker serum C-terminal cross-linking telopeptide of type I collagen (S-CTX-I, $p<0.01$) during the 5 year follow-up period. There was no association between MIF-$CATT_7$/rs755622(C) and baseline BMD at femoral neck, total hip or lumbar spine.

We conclude that *MIF* promoter polymorphisms have modest effects on bone remodeling and are associated with the rate of bone loss in elderly women.

Keywords

Osteoporosis, Genetics, Inflammation, Bone remodeling, Osteoimmunology

Introduction

Osteoporosis is a common disease, affecting one in three women during the course of their lifetime. It is characterized by reduced bone mass, micro-architectural deterioration of bone tissue and an increased risk of fractures; herein lies the clinical importance of the disease [1]. Osteoporosis is a complex disease with both genetic and environmental risk factors. Many genes are believed to contribute to the genetically conferred risk, with each gene having only a moderate effect. There are also gene-gene interactions and interaction between genes and the environment. Clinical risk factors will vary between individuals, thus each osteoporotic patient likely has a unique combination of genetic and environmental risk factors. It is known that some genes are specific to bone mineral density (BMD) while others may be shared not only with other bone related phenotypes, but also with other complex diseases such as obesity and diabetes [2]. The influence of genetic factors on osteoporosis risk is significant, affecting all aspects of the osteoporotic phenotype; *e.g.* BMD, bone quality, structure and strength. Twin- and family studies have shown that 60–80% of the population variance in BMD, which is the strongest clinical predictor of fracture risk, is genetically determined [3].

Inflammation can affect the balance between bone formation and resorption and patients with chronic inflammatory diseases seem to be at higher risk of developing osteopenia [4]. The interplay between the immune system and bone metabolism, or ‘osteoimmunology’, includes molecular and cellular interactions between osteoblasts, osteoclasts, lymphocytes and the monocyte-macrophage lineage [5]. The link between inflammation and bone turnover appears to depend mainly on the production of cytokines. As estrogen levels decline at menopause, there is an increase in the production of pro-inflammatory cytokines that may contribute to increased osteoclast activity and subsequent loss of bone density. However, cytokines have

dual roles in bone homeostasis, with different effects depending on the type of immune response [5-7].

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine mainly produced by immune cells (including T-cells, macrophages and monocytes), and it plays important roles in both the innate and acquired immune response. For instance, MIF contributes to the pathogenesis of acute, chronic and autoimmune inflammatory disorders and it counterbalances the immunosuppressive effects of glucocorticoids [8]. MIF also has been identified as an upstream regulator of TNFα, which has important effects in bone resorption [9]. In murine models, MIF has been shown to be highly expressed in osteoblasts and to induce expression of matrix metalloproteinases-9 and -13 in these cells [10]. Transgenic mice overexpressing MIF develop osteoporosis [11] while MIF-deficient mice are resistant to ovariectomy-induced bone loss [12]. The expression of MIF is also increased during fracture healing in rats [13]. Several experimental studies thus show that local MIF levels influence or reflect the extent of bone remodeling.

In human subjects, increased levels of MIF have been found in the joints of juvenile and adult rheumatoid arthritis patients [14-16], and polymorphisms in the promoter region of *MIF* have been shown to affect the level of gene transcription and to be associated to the pathogenesis of rheumatoid arthritis and several other inflammatory diseases [17-19]. The rs755622(C) allele located -173 bp upstream of *MIF* transcriptional start site creates an activator protein-4 response element that is associated with increased expression of *MIF* and poor clinical outcome of juvenile idiopathic arthritis [20-22]. MIF-$CATT_{5\text{-}8}$ is a tetranucleotide repeat polymorphism located -794 bp upstream of mRNA transcriptional start site and higher

number of repeats is associated with increased expression of *MIF* and disease severity in rheumatoid arthritis [17].

There are several reports on disease association for the MIF-$CATT_7$/rs755622(C) haplotype including inflammatory polyarthritis [23], scleroderma [24], atopy [25], prostate cancer [26], psoriasis [27], asthma [28], and systemic lupus erythematosus [29]. The risk haplotype MIF-$CATT_7$/rs755622(C) was also associated with increased MIF expression in inflammatory polyarthritis [23], while the low-expression allele MIF-$CATT_5$ was associated to mild asthma [28], arguing for functional effects of these, or closely linked, promoter polymorphisms. There is thus experimental evidence of an important role for MIF in bone turnover and polymorphisms in the promoter region of *MIF* have both been reported to affect transcription and be associated to inflammatory diseases. The aim of this study was to evaluate the effect of *MIF* promoter polymorphisms with regard to BMD and the rate of bone resorption.

The present study was performed in a population-based cohort of 1002 postmenopausal women aged 75 years at inclusion with information collected at the baseline visit and at the 1, 3, and 5-year follow-up visits. This is the first study to address genetic association of *MIF* to osteoporosis.

Materials and methods

Study subjects

The Malmö Osteoporotic Prospective Risk Assessment (OPRA) cohort is a population-based cohort of Caucasian women aged 75 years who were randomly selected from the Malmö city files between 1995 and 1999. No exclusion criteria were applied. A total of 1604 women were invited, 1044 (65%) chose to attend at baseline and 753 women returned for the 5 year follow-up visit. Phenotype data reported in this study include BMD at the lumbar spine, femoral neck and total hip at baseline and at the 5 year follow up visit. In addition, bone resorption markers were measured in serum at baseline and at the 1-, 3-, and 5-year follow-up visits. Information on medication, smoking and illness was collected by a questionnaire. The data reported in this analysis is based on women for whom MIF genotype data was available, corresponding to 1002 women at baseline and 753 women at 5-year follow up. Of the 1002 women at baseline, 138 were smokers, 198 were former smokers, 66 had diabetes and 115 were using estrogen (not including low-potency estriol) or bisphosphonates during the 5 year follow-up period. Clinical characteristics of the women attending the baseline visit are shown in Table 1.

Participants gave informed consent and the Lund University Ethics Committee approved the study. This study was performed according to the principles of the Helsinki declaration.

Genotyping

Total genomic DNA was isolated from blood using the QIAamp 96 DNA blood kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Genotyping of rs755622 (MIF-173G/C) was performed using Taqman SNP genotyping Assay (partnumber C_2213785_10, Applied Biosystems, Foster City, CA, USA). PCR was conducted in a Dual 384-well GeneAmp PCR system 9700 (Applied Biosystems). After PCR amplification an

endpoint plate read on ABI 7900HT (Appied Biosystems) using the SDS2.2.2 software, was performed. The success rate of genotyping of rs755622 was 99.4%.

The microsatellite MIF-$CATT_{5-8}$ (-794$[CATT]_{5-8}$) was genotyped by PCR from 20ng DNA for a 10 μl reaction using primers as follows: 5´TTG CAC CTA TCA GAG ACC-3´as a forward primer with 5´end labeled with 6-FAM and 5´-TCC ACT AAT GGT AAA CTC G-3´as a reverse primer. PCR was conducted in a 96-well GeneAmp PCR System 2700 (Applied Biosystems) and comprised 40 cycles 95°C 1 min, 54°C 1 min and 72°C 45 s followed by one cycle 72°C 7 min. Amplification was confirmed by 3% agarose gel electrophoresis before denaturated products were run with an internal sizing standard GeneScan™-400HD (Applied Biosystems) on a ABI Prism 3130 genetic Analyzer (Applied Biosystems). After the capillary electrophoresis, the alleles of MIF-$CATT_{5-8}$ were identified using Genemapper 3.0 software (Applied Biosystems). The success rate of genotyping of MIF-$CATT_{5-8}$ was 99.7%.

Bone density

The areal BMD at the femoral neck, total hip and lumbar spine (L2-L4) was measured by dual-energy x-ray absorptiometry (DXA) (Lunar DPX-L® Madison, USA). Analyses of scans were made with software versions 1.33 and 1.35 at baseline and 4.7b at 5 years. The total hip scans were all analyzed with version 4.7b.

The rate of bone loss represents the annual change in BMD (%) between the scan at baseline (BMD_{BL}) and the 5 year follow-up (BMD_{5Y}), and was calculated as [(BMD_{5Y}-BMD_{BL})/BMD_{BL}/years between scans × 100].

The rate of bone loss at the lumbar spine is not reported since previously in this cohort we showed that BMD increased, likely due to the presence of osteophytes, degenerative changes and compressive fractures common in women of this age [30].

Bone resorption markers

Serum C-terminal cross-linking telopeptide of type I collagen (S-CTX-I) and serum tartrate-resistant acid phosphatase 5b (S-TRACP5b) were analyzed. Non-fasting blood samples were collected between 08.00 and 13.00 and centrifuged within 2 hours after phlebotomy to isolate plasma. Samples were stored at -80°C. Storage times were up to 3.6 years apart since participants entered the study at 75 years of age but at different timepoints. This difference in storage time had only minor effects on the measures of bone resorption markers [30].

S-TRACP5b was determined with BoneTRAP® assay (SBA Sciences/Immunodiagnostic Systems IDS Inc., Bolton, UK) and S-CTX-I was determined with Elecsys β-CrossLaps immunoassay (Roche Diagnostics, Indianapolis, IN).

In the analyses of bone resorption markers, subjects that had received estrogen or bisphosphonate treatment anytime during the 5 year follow-up period were excluded since these medications have been shown to decrease S-CTX-I levels [31, 32]. Longitudinal measures of bone resorption markers at baseline and at the 1-, 3-, and 5-year follow-up visits were used. Longitudinal data from the four occasions was available from 537 subjects for S-TRACP5b and from 519 subjects for S-CTX-I. The log transformed mean value for the four visits were used for analysis. To compare women with high and low levels of bone turnover, subjects were classified as having consistently high (or consistently low) values of S-CTX-I or S-TRACP5b if they appeared in the higher (or lower) tertile at three or all four of the time points studied. All other were classified into the intermediate group [33].

Statistics

Deviation from Hardy-Weinberg equilibrium was calculated by the chi-square test. Marker-marker association between MIF-$CATT_{5\text{-}8}$ and rs755622 was analyzed with the EHplus software [34] based on genotype data from the OPRA cohort. Haplotypes were estimated by

PHASE version 2.02 [35]. Only individuals with a haplotype estimate >80% were used in haplotype analyses (n=1001). SPSS for Windows 17.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. The paired t-test was used to test for change in BMD and weight between baseline and the 5 year follow-up visit. Linear regression was used to identify confounding factors. ANOVA was used to determine associations between MIF genotypes, BMD and rate of bone loss. Bone resorption marker values were log-transformed prior to analysis due to non-normal distribution. For MIF association analyses to levels of bone resorption markers, the t-test was used for comparing mean S-CTX-I and S-TRACP5b values, and the Pearson Chi-Square test was used for resorption marker analyses between consistently high and low expression of S-CTX-I or S-TRACP5b.

Results

Genotype frequencies

There was no deviation from Hardy-Weinberg equilibrium for SNP rs755622 (p=0.78) or the microsatellite MIF-$CATT_{5\text{-}8}$ (p=0.30). The allele- and genotype frequencies for rs755622 and MIF-$CATT_{5\text{-}8}$ are shown in Table 2 and are similar to those reported in other Caucasian populations [36]. The most frequent MIF-$CATT_{5\text{-}8}$ allele was 6 repeats (61%), while 8 repeats was rare (0.1%). The markers were significantly associated (χ^2=743, p<0.000001), and are thus in linkage disequilibrium. Three haplotypes (MIF-$CATT_5$/rs755622(G), MIF-$CATT_6$/rs755622(G), MIF-$CATT_7$/rs755622(C)) accounted for 96% of the estimated haplotypes (Table 3).

Association to BMD

MIF-$CATT_{5\text{-}8}$, rs755622 alleles or constructed haplotypes were not associated to BMD at the femoral neck, total hip or lumbar spine in the 75-year old women at baseline or at the 5 year follow-up visit. Correction for confounders identified by linear regression was made for weight (at baseline), diabetes, smoking status and current use of estrogen or bisphosphonates at the baseline visit and for weight (at 5 years) at the 5 year follow-up visit.

Association to rate of bone loss

There was a significant decrease in both body weight and in BMD at the femoral neck and total hip between baseline and the 5 year follow-up visit (p<0.001).

The mean annual rate of bone loss between baseline and the 5-year follow-up visit was a -1.5% change in BMD for femoral neck and -1.2% for total hip (Table 1). Body weight at the baseline visit was used as covariate for analyses of rate of bone loss. Diabetes, smoking status and use of estrogen or bisphosphonates were not significant confounders for rate of bone loss.

The MIF-CATT$_7$/rs755622(C) haplotype, previously identified as being associated with increased expression of *MIF* [23], was associated to rate of bone loss both at the femoral neck (p=0.04) and total hip (p=0.038, Table 4).

The mean rate of bone loss for individuals carrying the MIF-CATT$_7$/rs755622(C) haplotype was 28% higher for femoral neck and 29% higher for total hip compared to the mean rate of bone loss for non-carriers of the haplotype (Table 4). There was no association between *MIF* haplotype and the 5-year change in body weight.

Association to bone resorption markers

The mean values for S-CTX-I and S-TRACP5b measures at baseline, 1, 3, and 5 years were highly correlated (Pearson correlation 0.52, p<0.001). Of the 105 individuals that were classified as having consistently high or low levels of S-CTX-I and S-TRACP5b, 41% were high and 48% were low for both markers.

Carriage of the MIF-CATT$_7$/rs755622(C) haplotype was compared between individuals with consistently high and consistently low levels of bone resorption markers S-CTX-I or S-TRACP5b at baseline, 1-, 3-, and 5-year follow-up. For subjects with inferred *MIF* haplotype data, the number of consistently high/low were 91/108 for S-TRACP5b and 120/117 for S-CTX-I. There were more MIF-CATT$_7$/rs755622(C) carriers in the groups with consistently high levels of bone resorption markers than in the groups with consistently low levels (S-CTX-I; p=0.01, S-TRACP5b; p=0.06, Table 5).

MIF-CATT$_7$/rs755622(C) haplotype carriers also had significantly higher mean S-CTX-I levels (5.79 nM) than non-carriers (5.67 nM, p=0.005). There was no significant difference in means for S-TRACP5b (1.32 U/L vs. 1.28 U/L, p=0.12).

Discussion

Reports from rodent experiments including transgenic mice argue for a strong effect of MIF on bone metabolism, particularly after ovariectomy and during fracture healing [11-13]. Numerous studies have identified functional effects for polymorphisms in the *MIF* promoter region that affect the level of gene expression and are associated with susceptibility or severity of diseases with an inflammatory component [17, 18, 20, 37]. Combining these findings creates an argument for studying the potential association of *MIF* promoter polymorphisms with human osteoporosis.

The *MIF* promoter polymorphisms MIF-$CATT_{5\text{-}8}$ and rs755622 were not observed to be associated with BMD at the femoral neck, total hip or lumbar spine in 75-year old women. The lack of association to baseline BMD suggests that other factors such as diet, medication and other genetic factors have more pronounced effects on BMD in women of this age. In contrast, there was a moderate association of the MIF-$CATT_7$/rs755622(C) haplotype to bone loss during the 5 year follow-up period (between age 75 and 80). Bone loss was assessed both by annual change in BMD, i.e. rate of bone loss, and by the levels of the bone resorption markers S-CTX-I and S-TRACP5b. Mean values of S-CTX-I from measures at baseline, 1-, 3-, and 5-year follow-up were significantly higher in MIF-$CATT_7$/rs755622(C) haplotype carriers, but since bone turnover markers vary over time we also identified individuals with consistently high or low levels of these markers at the four occasions. The strategy to classify individuals into groups with consistently high and low levels of bone resorption markers was recently shown to correlate well with the rate of bone loss and osteoporosis risk [33]. This method results in a more robust classification by minimizing the effects of variation ensuing from different measurement days (years) and from the intra-individual variation within each subject (seasonal, diet, exercise).

For both S-TRACP5b and S-CTX-I there were more MIF-$CATT_7$/rs755622(C) haplotype carriers, higher mean levels of the respective bone resorption markers and higher rate of bone loss in the "consistently high" compared to "consistently low" group.

Bone turnover markers can be used as predictive tools for women at high risk of fracture [38-40]. S-CTX-I is elevated upon increased degradation of bone collagen while TRACP5b is an enzyme expressed by osteoclasts, thus S-TRACP5b is a measure of both osteoclast number and activity [41, 42]. Recent data from the OPRA cohort suggests that elevated levels of S-TRACP5b and S-CTX-I are associated with increased fracture risk for up to a decade in the elderly women [43]. Genetic association to levels of bone resorption markers and bone loss as reported here for *MIF* promoter polymorphisms are thus clinically relevant. MIF could also be the target for pharmacological intervention. Several MIF inhibitors have been developed [44] but their potential clinical use in osteoporosis can currently only be speculated upon and requires more knowledge on the biological function of MIF in relation to bone. Sufficiently detailed information on duration and dosage of anti-inflammatory drugs was not available in the OPRA cohort and precluded investigation of interactions in relation to MIF haplotype.

Results obtained from a transgenic mouse model over expressing MIF and from a rat fracture model have pointed to a role for MIF in promoting bone resorption [11, 13]. On the other hand, studies in MIF-deficient or knock-out mice of different sexes have generated somewhat conflicting results. One study reported that *mif*$^{-/-}$ females were resistant to ovariectomy induced bone loss [12] while another study reported reduced trabecular bone volume in males, but with no changes in the number of osteoclast or osteoblasts [45]. MIF clearly plays a complex role in bone remodeling. *Ex vivo* cultures of bone marrow cells from *mif*$^{-/-}$ male mice have shown evidence of enhanced osteoclast formation while *in vitro* studies suggest

that exogenous MIF is a potent inhibitor of osteoclast formation at a late stage of the differentiation/fusion process [45]. Accordingly, the observations from mice over-expressing MIF and from mice in which the *mif* gene has been deleted are difficult to reconcile. Pertinent to the difficulties in comparing osteoclast formation in dissimilar *in vitro* and *in vivo* systems is the observation that osteoclast formation in bone marrow cultures is not necessarily representative of all skeletal sites. Furthermore, differences in the local microenvironment, MIF concentrations and cell-to-cell contacts may complicate comparisons between *ex vivo* and *in vivo* results. For example, a recent study found that although retinol is associated with decreased bone mass and increased fractures in humans, and to increased periosteal bone resorption (both *in vivo* and bone organ cultures) retinol is also a potent inhibitor of osteoclast formation in bone marrow and spleen cell cultures [46]. These and other observations [47, 48] indicate the existence of local differences in the regulation of bone resorption which must be taken into consideration when interpreting results from diverse model systems.

One of the bone resorption markers used in the present study, S-TRACP-5b is a marker for osteoclast number and bone resorption. There was suggestive evidence ($p=0.06$) for increased S-TRACP-5b levels in carriers of the MIF-$CATT_7$/rs755622(C) haplotype which could reflect increased number of osteoclasts. Another possibility, suggested from the results from MIF-transgenic mice, could be that *in vivo* the inhibitory effect on osteoclastogenesis is overridden by MIF-induced TNFα expression, which has stimulatory effects on osteoclasts [9].

In either case the observations in the present study suggest that elevated MIF levels are associated with increased bone resorption in post-menopausal women. While the mechanism is not clear, it may be mediated through the estrogen pathway, since estrogen is known to inhibit inflammatory responses in humans [49] and strongly inhibits inflammation mediated MIF production in mice [50]. This being the case, the MIF-$CATT_7$/rs755622(C) haplotype in

conjunction with estrogen depletion may synergistically contribute to bone loss in these women. Replication in pre-menopausal women or older women taking estrogen replacement therapy may prove interesting to study.

The finding that the rate of bone loss can itself be predictive of fracture makes it clinically relevant to study [51, 52]. Our preliminary results suggest that combining information on MIF haplotype and bone turnover markers may provide additional useful information in the prediction of bone loss and fracture risk. The p-values for MIF-$CATT_7$/rs755622(C) association to rate of bone loss and S-CTX-I are modest ($p<0.05$, $p<0.01$) and not corrected for multiple testing due to the non-independent relationship between resorption markers and between the skeletal sites studied; nonetheless the result is suggestive of association. The consistency between the measurements of bone loss (i.e. rate of bone loss and levels of bone resorption markers) point in the same direction i.e. that MIF-$CATT_7$/rs755622(C) haplotype increases the extent of bone resorption, leading to bone loss in elderly women.

To the best of our knowledge, the present report is the first study to address the association between *MIF* promoter polymorphisms and osteoporosis or bone loss. It was conducted in a large, well characterized cohort with relevant characteristics for the study of changes in bone, as the typical osteoporotic, fracture-prone patient is an elderly woman. In addition, we include data from baseline and three follow-up visits, allowing for the analyses on changes in bone over time. A limitation of this study was the lack of systemic inflammation markers. The OPRA cohort studied lacks complete data on the inflammatory status of the participants such as CRP levels and inflammatory disease. Biochemical measures and more detailed questionnaires regarding illness would have improved the ability to study *MIF* promoter polymorphisms with regard to both bone and inflammation.

In conclusion, the *MIF* promoter polymorphisms previously shown to be associated with increased MIF expression and inflammation are associated with bone loss measured by change in BMD and levels of bone resorption markers in elderly women, identifying MIF as a genetic risk factor for osteoporosis.

Conflict of interest statement

All the authors declare no conflict of interest. Dr. Bucala is a co-inventor on a patent application describing the potential prognostic utility of *MIF* polymorphisms in different inflammatory diseases.

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Table 1. Clinical characteristics of women in the OPRA cohort

Variable	Mean	SD	Min	Max
Age at baseline (years)	75.2	0.1	75.0	75.9
Age at 5-year follow-up (years)	80.2	0.2	79.6	80.9
Weight at baseline (kg)	67.8	11.5	41	110
Weight at 5-year follow-up (kg)	66.2	11.5	34	105
Height at age 20 (cm)	164.1	5.5	145	180
BMD (g/cm^2)				
Femoral neck at baseline	0.748	0.130	0.153	1.230
Total hip at baseline	0.849	1.149	0.498	1.416
Lumbar spine at baseline	0.993	0.195	0.518	1.855
Femoral neck at 5-year follow-up	0.713	0.128	0.150	1.480
Total hip at 5-year follow-up	0.800	0.140	0.260	1.390
Lumbar spine at 5-year follow-up	1.009	0.206	0.470	1.790
Rate of bone loss per year				
Femoral neck	-1.476	2.162	-13.89	7.70
Total hip	-1.230	1.868	-10.39	6.33

Number of individuals (N) for each phenotype: Age at baseline (BL): 1002, Age at 5 years (y): 753, Weight BL: 1002, Weight 5y: 702, Height at age 20: 958, Femoral neck (FN) BL: 923, Total hip (TH) BL: 902, Lumbar spine (LS) BL: 945, FN 5y: 678, TH 5y: 676, LS 5y: 693, Rate of bone loss (RBL) FN: 655, RBL TH: 638

Table 2. MIF allele and genotype frequencies in the OPRA cohort

MIF polymorphism	Allele	N	Frequency	Genotype	N	%
rs755622	G	1635	0.82	GG	671	67.2
	C	361	0.18	GC	293	29.4
				CC	34	3.4
				Total	998	
MIF-CATT$_{5-8}$	5	469	0.23	55	54	5.4
	6	1226	0.61	56	276	27.6
	7	304	0.15	57	83	8.3
	8	3	0.001	58	2	0.2
				66	388	38.8
				67	173	17.3
				68	1	0.1
				77	24	2.4
				Total	1001	

Table 3. Estimated MIF haplotype frequencies (N=1002)

MIF allele		MIF haplotype
$CATT_{5-8}$ repeats	**rs755622**	**Frequency (%)**
5	G	23.3
5	C	0.2
6	G	58.1
6	C	3.0
7	G	0.5
7	C	14.8
8	C	0.2

Table 4. Mean annual rate of bone loss (RBL) for carriers and non-carriers of the MIF-$CATT_7$/rs755622(C) haplotype

	MIF-$CATT_7$/rs755622(C)		
	Non-carriers	**Carriers**	**p-value[a]**
Femoral neck	n= 482	n= 173	
Mean RBL	-1.37	-1.76	0.04
SD	2.09	2.34	
Range	-8.54 to 7.7	-13.89 to 7.27	
Total hip	n= 468	n =170	
Mean RBL	-1.14	-1.47	0.04
SD	1.86	1.87	
Range	-7.15 to 6.33	-10.39 to 6.13	

[a]Univariate GLM-ANOVA

Table 5. Relative expression of S-CTX-I and S-TRACP5b in carriers and non-carriers of the MIF-$CATT_7$/rs755622(C) haplotype

	MIF-$CATT_7$/ rs755622(C)		
	Non-carriers (%)	**Carriers (%)**	**P-value[a]**
S-CTX-I			
Consistently high	75 (62.5)	45 (37.5)	0.01
Consistently low	90 (76.9)	27 (23.1)	
S-TRACP5b			
Consistently high	62 (68.1)	29 (31.9)	0.06
Consistently low	85 (78.7)	23 (21.3)	

[a] Chi square test