

**An Association between Genetic Variants of Obesity  
Related Genes and Osteoporotic Fracture  
- Obesity and Osteoporosis : Friend or Foe?**

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Aging and Clinical Nutrition

**An Association between Genetic Variants of Obesity  
Related Genes and Osteoporotic Fracture  
- Obesity and Osteoporosis : Friend or Foe?**

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## 감사의 글

대학교에 들어온 지 20 년 만에 배움의 끝이라 할 수 있는 박사과정을 마치게 되었습니다. 강의 중심의 의과대학 학사와 보건학 석사 과정, 직접 선배로부터 도제식으로 배우는 전공의 수련 시절과는 달리 박사과정에서는 강의실뿐 아니라 실험실, 연구실 그리고 환자를 직접 치료하는 진료실 등 장소와 관계없이 통합적이고 창의적인 질문에 답하는 과정을 통해 지식이 습득이 되고 경험이 되는 것이었다 생각합니다. 이러한 질문이 가설을 만들고 가설을 증명하는 것이 연구이기에 그러한 연습을 하게 된 박사 과정은 배움의 끝이 아닌 시작임을 압니다.

한 사람의 미성숙한 연구자가 박사가 되어 가는 과정에는 많은 선생님들과 동료들의 가르침과 도움이 있었기에 가능합니다.

우선 제가 속해 있는 연세대학교의 노화과학 연구과정의 여러 교수님들에게 감사함의 말씀을 전합니다. 주심이신 장양수 교수님은 심혈관계 유전체학의 선구적인 연구를 주도하시는 분으로 교수님의 가르침과 탁월한 여러 논문들은 다소 어려운 분야를 시작하였던 제게 큰 지침이 되었습니다. 식품영양학과와 이종호 교수님께서는 보건학 석사를 마친 제게 이 과정을 소개해주신 분이고 제가 속한 병원과 여러 임상 연구를 같이 진행하면서 임상연구에 대해 체계를 알려주신 분입니다. 유전학과 단백질학을 가르쳐 주신 정지영 선생님, 지질 및 대사 증후군에 대해 탁월한 강의를 해주신 조홍근 선생님께도 감사를 드립니다. 신경균 선생님을 비롯하여 앞서 연구를 훌륭하게 마치신 노화과학 박사과정의 여러 선배님들과, 같이 수업을 듣고 서로의 연구를 격려 해주었던 동료, 후배들에게도 감사함을 전합니다. 바쁘신 가운데 부심을 맡아 주신

연세대학교 이은직 교수님과 골다공증 유전체학 분야에서 실제적인 조언을 많이 해주신 강북 삼성병원의 오기원 교수님께도 감사함을 드립니다.

미국 보스턴 Tufts 대학의 HNRCA (Human Nutrition Center on Aging)의 nutrition & genomics 연구실에서의 2 년간의 연구 경험은 nutrigenomics 를 비롯한 유전체학 전체를 이해하는데 큰 도움을 주었습니다. 그 탁월함과 세계적인 명성에 비해 언제나 유머로 연구원들을 격려해주실 뿐 아니라 세세하게 지도해주셨던 Jose. M. Ordovas 를 비롯하여 Larry (Laurence), Chao, Shen, Emmy 등 귀한 다국적 동료들에게도 감사함을 드립니다. 이들로부터 bioinformatics 지식을 배웠으며, 통계를 돌리고 결과를 만드는 법까지 많은 실제적 지식을 배웠습니다. 무엇보다 이들과 같은 국제적 network 를 갖게 된 것 자체가 귀한 경험이었습니다.

박사과정을 지난 4 년으로 국한하지 않고 그 전 배움과 경험의 연속이라 정의할 때, 제게 공부하는 즐거움을 알게 해주셨던 이해리 교수님께 감사함을 드립니다. 가정의학 의사로서 습관적인 처방이나 타성에 젖은 지식을 넘어 끊임없이 도전하는 것의 동기가 환자에 대한 사랑에서 임을 배운 것입니다. 또한 제게 늘 좋은 본이 되시는 이덕철 교수님 그리고 심재용 교수님께 감사함을 드립니다. 보건학 석사 과정 중 논문을 꼼꼼하게 쓰는 법을 알려주신 정우진 교수님, 그리고 제가 유전학에 대해 눈을 뜨게 해주신 지선하 교수님께도 감사를 드립니다. Human genome project 가 완성된 그 다음해 들었던 지선하 선생님의 ‘유전과 보건’의 수업이 유전학의 문외한이었던 제게 도전 정신을 불러일으켰기 때문입니다.

무엇보다 임상가인 제가 연구에 관심을 가지고 연구를 할 수 있도록 격려해주셨을 뿐 아니라 미국 연수 기회도 주신 노성일 이사장님께 감사를

드립니다. 이사장님은 늘 그 자리에 안주하지 말고 도전을 하라고 말씀해주셨고 그 말씀이 제게 큰 격려가 되었습니다. 같이 임상 연구를 진행하는데 큰 도움을 준 신동혁, 이세영 선생님 등에게도 감사함을 드립니다. 이런 좋은 동료와 같이 일하고 연구 하는 게 제겐 큰 행복입니다.

감사의 글을 마치기 전 그 어떤 분들보다 제가 박사를 마치는 데 가장 큰 격려가 되고 힘이 된 것이 있다면 그 것은 제 사랑하는 가족입니다.

아내는 늘 인생의 귀한 동반자이자 동역자였습니다. 석사까지면 충분하다는 저를 설득하여 박사를 지원하게 해주었고 같이 박사과정을 진행하면서도 늘 저를 먼저 배려해주었습니다. 논문 쓰는 데는 아무런 도움이 안된 듯한 우리 아이들 - 채은, 지원이는 사실 언제나 큰 힘이 되었습니다. 언젠가는 이 아이들이 자라 자신의 연구 분야에 매진할 때 그들의 성장기에 뒤늦은 공부를 하느라 잘 챙겨주지 못한 부모들을 이해해주길 바랄 뿐입니다.

마지막으로 사랑하는 어머니께 감사함을 드립니다. 석사 논문을 쓸 때 암이 발병되어 힘든 시기를 경험하였고 그로부터 불안한 4 년이 흘러 이번엔 박사 논문을 쓰는 이 때 암은 다시 재발되었습니다. 석사 논문 때처럼 박사 논문도 어머니의 병상 옆에서 마무리 되고 있습니다. 이번에는 다시 일어나기 힘든 것을 알기에 더욱 무거운 맘으로 글을 쓰고 있습니다. 제 인생에 가장 귀한 사랑의 원천이셨던 어머니와 갑작스러운 슬픔 속에 처한 아버지를 비롯한 가족 모두에게 저의 박사 논문과 학위 수여가 작은 기쁨과 격려가 되길 원하는 마음으로 이 논문을 마무리 합니다. 여기까지 인도하셨던 에벤에셀의 하나님을 찬양합니다.



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## **Abstract**

### **An association between Genetic variants of Obesity related Genes and Osteoporotic Fracture - Obesity and Osteoporosis : friend or foe?**

Kyong-Chol Kim.

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Yonsei University

Graduate Program in Science for Aging

#### **Back ground**

In contrast to our traditional belief that obesity is a protective factor for bone, recent epidemiologic studies show that body fat might be a risk factor of osteoporosis and bone fracture. Peroxisome proliferator-activated receptor-gamma (*PPAR- $\gamma$* ) and runt-related transcription factor 2 (*RUNX-2*) act as a “see-saw” which regulates a differentiation of mesenchymal stem cell to osteoblast or adipocyte. Two adipokines which are derived from the adipocyte

– leptin(*LEP*) and adiponectin (*ADIPOQ*) have a critical role in bone formation and bone resorption.

## **Methods**

907 postmenopausal healthy female subjects, age 60-79 years, recruited from Miz Medical hospital were measured for BMD, bone marker and adiposity and genotyped for 13 single nucleotide polymorphisms (SNP) from 5 genes (*PPARG*, *RUNX2*, *LEPR*, *LEP*, *ADIPOQ*). Finally 4 SPNS were selected to be analyzed (rs2938392 of *PPARG*, rs7771980 of *RUNX2*, rs8179183 of *LEPR*, rs1501299 of *ADIPOQ*).

## **Results**

The lumbar BMD was positively associate with body weight ( $p<0.001$ ) and negative with body % fat ( $p=0.0681$ ). Vertebral fracture risk also increased as body % fat increased; odd ratio 1.064 (1.003-1.029),  $p=0.0383$ . TC+CC genotype group of rs7771980 from *RUNX2* had a lower vertebral fracture risk than TT group ; odd ratio 0.55 (0.32-0.94),  $p=0.0297$  whereas in genotype group of rs 2938392 from *PPARG*, the prevalence of metabolic syndrome in AA group, AG group and GG group is 29.26%, 34.90% and 44.20% which gradually increased according to the genotype . (Odds ratio 1.39, 95% CI[1.13-1.71]  $p=0.0014$ ). For the *ADIPOQ* SNP, rs1501299, the prevalence of

osteoporotic fracture was 24.06% in GG group, 18.99% in GT group, 15.38% in TT group showing a gradual decrease according to the ordered genotype group (Odds ratio 0.76, 95% CI[0.58-0.99], p=0.0473). GG genotyped group of rs8179183 from *LEPR* had a relatively lower vertebral fracture risk (odd ratio 0.65, 95% CI[0.39-1.08], p=0.095) and a higher metabolic syndrome risk (odd ratio 1.46, 95% CI[0.96-2.09], p=0.076) in comparison with GC+CC group.

High Calcium intake(>1000mg/day) contributed to high BMD in GT+TT genotyped group of rs1501299 (p interaction = 0.0283)

### **Conclusion**

Some SNPs from adiposity-related genes were associated with BMD or fracture risk. Common genomic feature of these genes to two phenotypes - fat and bone give us a rationale to develop co-treatment drugs or nutrients to prevent obesity and osteoporosis together.

---

**Key words:** *PPARG*, *RUNX2*, *ADIPOQ*, *LEPR*, polymorphism, Osteoporosis, Obesity

## Abbreviations and Acronyms

ABI	Applied Biosystems
<i>ADIPOQ</i>	Adiponectin (Gene symbol)
BMD	Bone mineral density
BMI	Body mass index
DPD	Deoxypyridinoline
DXA	Dual energy x-ray absorptiometry
HWE	Hardy-Weinberg equilibrium.
LD	Linkage disequilibrium
<i>LEP</i>	Leptin (Gene symbol)
<i>LEPR</i>	Leptin receptor (Gene symbol)
NCBI	National Center for Biotechnology information
OC	Osteoclaclin
OPG	Osteoprotegerin
PPAR- $\gamma$	Peroxisome proliferator-activated receptor-gamma
<i>PPARG</i>	Peroxisome proliferator-activated receptor-Gamma (Gene symbol)
RANK	Receptor of nuclear factor-kB
RANKL	Receptor of nuclear factor-kB ligand

<i>RUNX2</i>	Runt domain family of transcription factors (Gene symbol)
rs#	Reference SNP number
SNP	Single nucleotide polymorphism
ucOC	Undercarboxylated osteocalcin

## 1. INTRODUCTION

In contrast to our traditional belief that obesity is a protective factor for bone, recent epidemiologic studies showed that body fat might be a risk factor of osteoporosis and bone fracture. As one ages, osteoblast in bone marrow is replaced by adipocyte, which makes bone weak eventually. There are several explanatory molecular mechanisms. Peroxisome proliferator-activated receptor-gamma (PPAR- $\gamma$ ) acts as a critical positive regulator of marrow adipocyte formation and as a negative regulator of osteoblast development from the same origin – mesenchymal stem cell. *In vivo*, increased PPAR- $\gamma$  activity leads to bone loss, similar to the bone loss observed with aging, whereas decreased PPAR- $\gamma$  activity results in increased bone mass. Two adipokines which are derived from adipocyte – leptin and adiponectin have a critical role of bone formation and bone resorption. However, few studies have been performed to assess the association between variants at those genes and BMD. The primary objectives are to identify genetic components underlying fat and bone interaction through PPAR- $\gamma$ , Leptin, Adiponectin's mechanisms. For this purpose, associations between single nucleotide polymorphisms (SNPs) and haplotypes at certain candidate genes and body composition and BMD will be evaluated. This will give us a rationale to develop co-treatment drugs or nutrients to prevent obesity and osteoporosis together.

## **2. BACKGROUND**

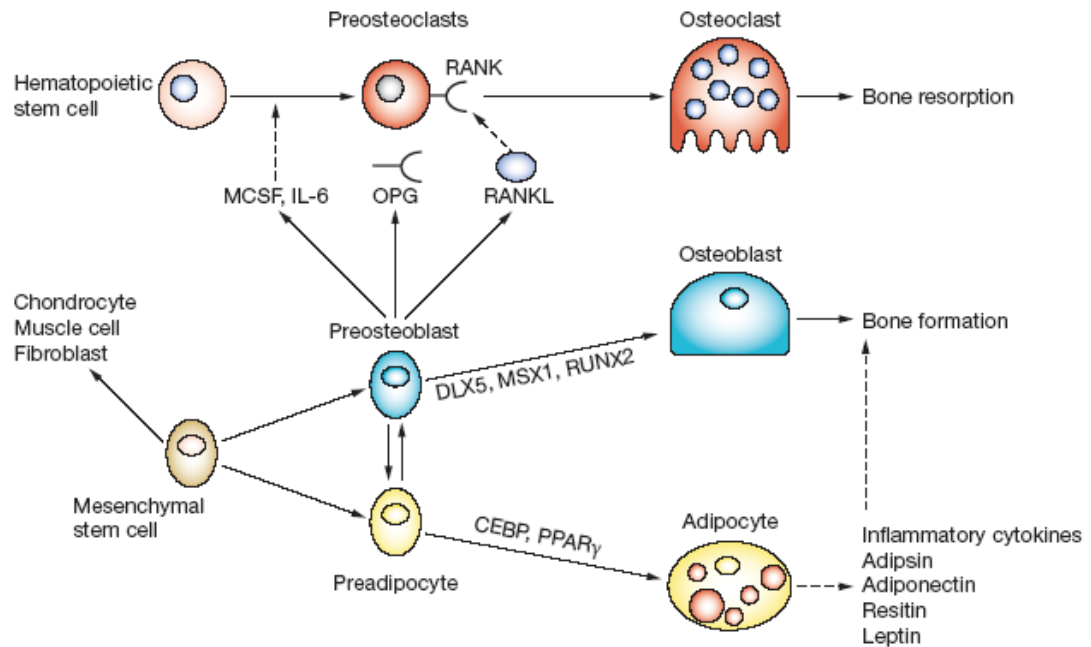
### **2.1 Obesity and Osteoporosis**

Many epidemiological data show that high body weight or BMI is associated with high bone mass, and moreover reductions in body weight may cause bone loss (1-3). The following mechanisms are thought to support these epidemiologic studies. First, it is generally accepted that a higher body mass enforce a greater mechanical loading on bone, and that it enhance the differentiation of osteoblast. Further, adipocytes are important sources of estrogen production in postmenopausal women, and estrogen is known to inhibit bone resorption and stimulate bone formation (4). Also, insulin resistance derived from body fat may alter and increases body sex hormone such as androgen and estrogen causing increasing bone mass (5).

In contrast, there is increasing evidence that fat mass is related to lower bone mineral density. To fully understand the relationship between obesity and bone mass, it is necessary to control for the mechanical loading effects of total body weight in the analyses. Zhao *et al* showed that when the mechanical loading effect of body weight on bone mass was adjusted, fat mass is correlated negatively with bone mass in both Chinese and Caucasians (6). Risks of osteoporosis, osteopenia, and non-spine fractures were significantly higher for subjects with higher percentage body fat independent of body weight (7). In

adolescence and young adults, fat mass, after accounting for lean mass, had a negative or no correlation with CT and DXA values for bone (8).

## 2.2 Biological action of fat (adipocytes) on Bone



**Fig 1) Lineage allocation in the bone-marrow milieu adapted from Rosen and Bouesein (9)**

Osteoblasts and adipocytes are derived from mesenchymal marrow stroma/stem cells (mMSC) (10). Therefore the marrow cavity is like a playground “see-saw” that can swing back and forth between bone and fat formation (11).

The milieu of intracellular and extracellular signals controls mMSC differentiation into osteoblast or adipocyte.

For a mesenchymal stem cell to become an osteoblast, activation of several key factors such as runt-related transcription factor 2 (RUNX-2), bone

morphogenetic protein 2 (BMP-2), transforming growth factor- $\beta$  (TGF- $\beta$ ), and transcription factor Sp7 (osterix), are necessary, although the precise sequence of events in this cascade has not been fully clarified. In contrast, to achieve full adipocytic differentiation, there are two groups of critical factors already present in mesenchymal stem cells that need to be activated: CCAAT/enhancer binding proteins (CEBP)  $\alpha$ ,  $\beta$  and  $\delta$ , and peroxisome proliferative activated receptors (PPAR)  $\alpha$ ,  $\gamma$ 2 and  $\delta$ .

This shift is characterized as an ‘either/or’ allocation; either the cell becomes a fat cell or it becomes a bone cell, but not both (9). Inflammatory cytokines can be released from adipocytes, and circulating hormones such as leptin, adipisin, adiponectin and resistin are also produced by fat cells (Fig 1).

Among these regulators, we selected RUNX2 and PPAR- $\gamma$  as key regulators of osteoblast (12) and adipocyte differentiation and further investigated two adipokines – leptin and adiponectin as a key linkage of fat and bone relationship.

### 2.3 PPAR- $\gamma$ 's action on bone

The adipocyte-specific transcription factor peroxisome proliferator-activated receptor-gamma (PPAR- $\gamma$ ) acts as a critical positive regulator of marrow adipocyte formation and as a negative regulator of osteoblast development (13). Activation of peroxisome proliferator-activated receptor-gamma inhibits the Runx2-mediated transcription of osteocalcin in osteoblasts (14).

*In vivo*, increased PPAR- $\gamma$  activity leads to bone loss, similar to the bone loss observed with aging, whereas decreased PPAR- $\gamma$  activity results in increased bone mass. Decreased PPAR- $\gamma$  activity in *PPAR- $\gamma$* -haplo insufficient mice or in mice carrying a hypomorphic mutation in the *PPAR- $\gamma$*  gene locus led to increased bone mass, due to increased osteoblastogenesis from bone marrow progenitors, but not due to effects on mature osteoblast activity or cells of the osteoclast lineage (15).

Transforming growth factor beta (TGF- $\beta$ ) which is an important regulator of bone formation by modulating osteoblastic cell proliferation and differentiation is an important modulator of adipocyte differentiation too (16). Manipulation of *PPAR- $\gamma$*  expression by exogenous TGF- $\beta$ 2 inhibits the exaggerated adipogenesis and corrects the balance between osteoblastogenesis and adipogenesis induced by unloading, leading to prevention of bone loss (17, 18). This indicates that TGF- $\beta$  is a negative regulator of PPAR- $\gamma$  and adipogenesis in unloaded rats (16).

### **2.3.1. Thiazolidinediones (enhancer of - PPAR $\gamma$ ) and Bone**

Activation of PPAR- $\gamma$  via the administration of rosiglitazone, an antidiabetic TZD (Thiazolidinediones), to rodents resulted in significant decreases in bone mineral density (BMD), bone volume, and changes in bone microarchitecture (19-22). Application of 10 mg/kg of BRL49653 (rosiglitazone) for 12 weeks resulted in enhanced bone loss (+31%; pQCT) and increased fat marrow volume (+117%; histomorphometry) compared to vehicle-treated OVX control (20). Rosiglitazone had no effect on the number of early osteoblast or osteoclast progenitors, or on osteoblast life span, but decreased the expression of the key osteoblastogenic transcription factors Runx2 and Osterix in cultures of marrow-derived mesenchymal progenitors (22). Decreased osteoblast number and activity due to increased apoptotic death of osteoblasts and osteocytes was apparent while osteoclast parameters and serum levels of osteocalcin, alkaline phosphatase activity, and leptin were unaltered by rosiglitazone treatment (21).

Some recent clinical studies support these in vitro and in vivo studies (23-30). Significantly more female patients who received rosiglitazone for 4-6 years experienced fractures than did female patients who received either metformin or glyburide in ADOPT (A Diabetes Outcome and Progression Trial) study (25, 30). 16 weeks use of pioglitazone, another Thiazolidinediones was followed by decrease BMD in lumbar spine and femur neck and decreased

measures of bone turnover in a premenopausal study population (26).

### **2.3.2. PPAR- $\gamma$ and osteoclastogenesis**

While the antiosteoblastic effect of PPAR- $\gamma$  on osteoblast differentiation is well established, its effect on osteoclast development is less clear (13).

In vitro, PPAR- $\gamma$  activation in osteoclast precursor cells inhibits their differentiation, whereas activation of PPAR- $\gamma$  in cells of mesenchymal lineage increases their support to osteoclastogenesis (31, 32). In vivo, and in contrast to other animal models, bone loss due to rosiglitazone administration to ovariectomized rats resulted from increased bone resorption, but not decreased bone formation (20). These results indicate that at least in some circumstances, bone loss due to PPAR- $\gamma$  activation may involve increased bone resorption.

These all findings provide a mechanistic explanation that PPAR- $\gamma$  activation is a negative regulator of bone mass and suggest that the increased production of oxidized fatty acids with age may indeed be an important mechanism for age-related osteoporosis in humans.(22)

### **2.3.3. Studies of PPAR- $\gamma$ gene polymorphism on insulin resistance, obesity**

Mutations of the PPAR- $\gamma$  gene (*PPARG*) are associated with an altered balance between bone and fat formation.

The Pro12Ala polymorphism is known to increase insulin sensitivity than

Pro12Pro genotype (33-36), therefore it is associated with type-2 diabetes (37) and early meta-analysis strongly suggested that pro Allele is a risk allele (38). The effect of Pro12Ala polymorphism on obesity is much complex which differ depending on race, fat depot, subject who have insulin resistance or diabetes.

The Pro12Ala polymorphism was significantly associated with adiposity in the biracial Coronary Artery Risk Development in Young Adults (CARDIA) cohort, a population-based sample of 5115 African Americans and whites (39). The association was different depending on race ; African Americans carrying the Ala12 allele had a 1.1 kg/m<sup>2</sup> lower body mass index (BMI) ( P = 0.02) and whites a 0.6 kg/m<sup>2</sup> higher BMI (P=0 .01), as compared to Pro12 homozygotes. Recent meta-analysis suggested similar results that in the Caucasian subjects, Ala allele genotype was associated with higher BMI although global comparison showed no difference in BMI (40). More focused on body fat, Kim *et al* demonstrated that PA/AA genotype of *PPARG* Pro12Ala (P12A) polymorphism is associated with increased subcutaneous/ visceral fat areas in overweight Korean female subjects.(41) González Sánchez *et al* also suggested that the Pro12Ala polymorphism of the *PPARG* promotes peripheral deposition of adipose tissue and increased insulin sensitivity for a given BMI (42). Data showed that higher BMI obese men carriers of the Ala12 allele had lower sagittal abdominal diameter than Pro12 homozygotes (p=0.01). These

are compatible to *in vivo* in human fat (43). The common Pro12Ala polymorphism of the *PPARG* has minor influence on mRNA expression of *PPARG* target genes in adipose tissue of obese subjects. Expression of both *PPARG* splice variants is dependent on fat depot: omental fat shows lower mRNA levels, compared with sub cutaneous fat depots (43).

#### **2.3.4. Studies of PPAR- $\gamma$ gene polymorphism on BMD**

In 1999, Ogawa *et al* studied an association between the restriction fragment length polymorphism (RFLP) of *PPARG* (exon 6 C/T silent mutation) and BMD and the possible involvement of this single nucleotide polymorphism (SNP) of postmenopausal osteoporosis in Japanese women. They demonstrated T allele genotype group has lower BMD than CC genotyping group in total BMD ( $p < 0.05$ ) (44).

However, Rhee *et al* showed no difference of BMD between two genotype group of SNP C161T and Pro12Ala except demonstrating lower mean serum OPG level in T allele Carriers of SNP C161T genotype (45) and in the Pro12Ala genotype group compared with the Pro12Pro genotype group (46).

While these two studies contradict one another, it must be remembered that first, the cohort size in these studies was very small and second, this is a silent polymorphism and is likely in LD with a more causative mutation (47).

Two studies have looked at associations between SNPs in the *PPARG* and

bone in larger human cohorts. A study of 6743 Chinese men and women examined a single SNP upstream of the first promoter of *PPARG* (rs2960422) and showed a modest increase in the risk of low BMD with the heterozygous state of this allele, but only in premenopausal women. No association was found in either men or postmenopausal women (48). A more comprehensive study of SNPs in *PPARG* and their association with aspects of bone density has been done in the Framingham Offspring cohort which consisted of 740 men and 776 women (49). Among eight SNPs constituting three LD blocks, only one coding SNP (rs1805192) which located in the universal exon one, codes for the substitution of an alanine (Ala) for the wild-type Proline (Pro) showed an association with BMD. Homozygosity for the more common Pro allele was associated with increased BMD at both the femoral neck and lumbar spine in women, when the data was adjusted for age and estrogen status. Conversely, men with this same allele had lower femoral neck and trochanter BMD (49).

Recently, Genome wide association with bone mass and geometry was done in the Framingham study. As a result, two SNPs - rs10510418 and rs2938392 in *PPARG* was observed to be associated with BMD and bone geometric trait. (FABT or GEE  $p < 0.05$ ) (50). This FHS (Framingham Heart Study) 100K SNP project offered an unbiased genome-wide strategy to identify new candidate loci and to replicate previously suggested candidate genes for osteoporosis.

## **2.4 *RUNX2* and Osteogenesis**

*RUNX2*, Runt domain family of transcription factors or *Cbfa1*, Core binding factor alpha 1 (Cbfa1) is essential for osteoblast development and proper bone formation (51). A member of the *RUNX2* binds specific DNA sequences to regulate transcription of numerous genes and thereby control osteoblast development from mesenchymal stem cells and maturation into osteocytes.

### **2.4.1 *RUNX2* and osteoblast differentiation**

The runt family transcription factor (*Cbfa1/RUNX2*) plays a critical role in formation of the mineralized skeleton during embryogenesis and differentiation of osteoblast cell (52). *RUNX2* determines bone maturity and turnover rate in postnatal bone development and is involved in bone loss in estrogen deficiency (53). Comparing to wild type mice (wt), *RUNX2* heterozygous knockout mice *RUNX2* (+/-) shows exacerbation in unloading-induced reduction in mineral apposition rate and bone formation rate in cortical bone as well as trabecular bone (54). In another mice study, Compared to wild-type mice, 6-week-old heterozygous *RUNX2* (+/-) had reduced trabecular bone volume (BV/TV%), cortical thickness, and bone mineral density (BMD), decreased osteoblastic and osteoclastic markers, lower bone formation rates, impaired osteoblast maturation of BMSCs in vitro, and significant reductions in mechanical properties. Homozygous *RUNX2* (-/-)

mice had a more severe reduction in BMD, BV/TV%, and, cortical thickness and greater suppression of osteoblastic and osteoclastic markers than *RUNX2* (+/-) mice (55).

#### **2.4.2. *RUNX2* polymorphism and BMD study**

To demonstrate the association between polymorphism of *RUNX2* and BMD, Bustamante *et al* (56) showed that the -1025 T/C polymorphism (rs7771980) in promoter 2 of *RUNX2* related to lumbar spine and femoral neck bone mineral density (BMD) in a cohort of 821 Spanish postmenopausal women. Because the high BMD allele had higher P2 promoter activity, the greater *RUNX2* P2 promoter activity is associated with higher BMD (57).

Moreover, allele of *RUNX2* is associated with BMD interacting by body weight (BMI) and menopause status in Scottish women. A alleles within the glutamine-alanine repeat were associated with Femur neck BMD in those above the median BMI ( $BMI > 25$ ), while no association was observed in thin/normal ( $BMI \leq 25$ ) postmenopausal women (58).

#### **2.4.3. *RUNX2* and adiposity**

Overexpression of *RUNX2* inhibited adipogenesis, as demonstrated by suppression of LPL and *PPARG* expression at the mRNA level and reduced lipid droplet formation. Moreover, adipose tissue-derived stem cells (ADSCs)

transduced with Ad-*RUNX2* underwent rapid and marked osteoblast differentiation as determined by osteoblastic gene expression, alkaline phosphatase activity and mineral deposition (59).

## **2.5 Leptin has a dual action on bone formation**

Leptin, a hormone secreted by adipocytes, acts on the hypothalamus to regulate appetite and neuroendocrine function. In the hypothalamus, both the arcuate nucleus and the ventromedial nucleus express leptin receptors (60, 61). Specific neurons in the arcuate nucleus regulate appetite and reproduction. In contrast, neurons in the ventromedial nucleus regulate bone mass (62). The melanocortin system is the downstream pathway for regulating appetite and neuroendocrine function (63). In contrast, the sympathetic nervous system is the downstream pathway for regulating bone mass (64, 65).

### **2.5.1. Leptin as an inhibitor of bone formation through central nerve system**

Ducy *et al.* demonstrated that obese mice deficient in leptin (*LEP*) (*ob/ob* mice) or the signaling form of its receptor (*LEPR*) (*db/ db* mice) have increased vertebral trabecular bone volume due to increased bone formation, despite having hypogonadism and hypercortisolism. Also intracerebroventricular infusion of leptin in both *ob/ob* and wild-type mice decreased vertebral trabecular bone mass (66).

Takeda *et al.* expanded to demonstrate that the effects of intracerebroventricular leptin are mediated by the sympathetic nervous system, that osteoblasts express  $\beta$ -adrenergic receptors, and that administration of  $\beta$ -

adrenergic agonists decreases trabecular bone volume by inhibiting bone formation (64). These studies identify that leptin is potent inhibitor of bone formation through the central nerve system. Recent data demonstrate that enhanced sympathetic activity also promotes bone resorption (67).

Furthermore, the  $\beta$  blocker propranolol increased bone formation in ovariectomized female rats (64) and adrenergic  $\beta_2$  receptor-deficient mice demonstrate a high bone mass due to an increase in bone formation and a decrease in bone resorption parameters (67-69).

Recent epidemiology studies showed that the association of current use of  $\beta$  blockers with low fracture risk is mediated, at least in part, by effects on BMD (68-71), cortical bone geometry and trabecular bone microarchitecture (72).

### **2.5.2. Leptin as dual action on bone**

In contrast to the negative action of leptin on bone cells, there are also many evidences for a direct and positive action on bone cells.

Using ob/ob mice, which are deficient in leptin, was observed to have a stimulatory effect of leptin on bone, with a dramatic increase in cortical bone formation in treated animals when compared with controls (73). In addition, systemic daily administration of leptin to sexually mature male mice significantly increased bone strength by more than 20% (74).

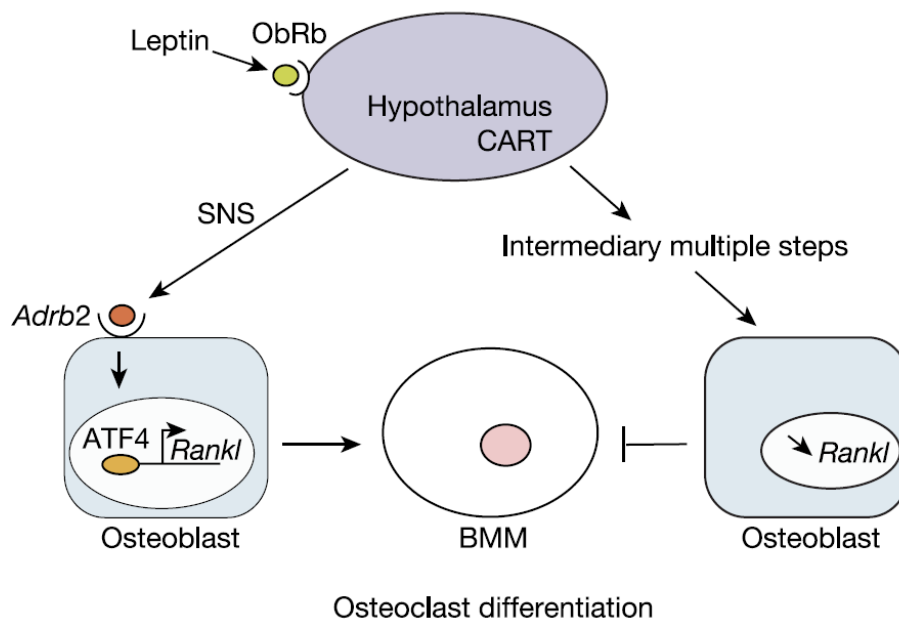
Hamlik *et al* showed that peripheral leptin treatment significantly decreased

bone marrow adipocyte size and number in *ob/ob* mice while increasing bone formation, BMC, and BMD (75). In another paper which recently published, he also demonstrated that injections of leptin into rat ventromedial hypothalamus increase adipocyte apoptosis in peripheral fat and in bone marrow (76). Farooqi *et al.* reported that after subcutaneous leptin therapy administered for up to four years, BMC, BMD and skeletal maturation increased normally, although weight and fat mass dramatically decreased, suggesting counteracting and beneficial effects of leptin therapy on the skeleton (77).

From conflicting or apparently contradictory data, Thomas *et al* hypothesize that leptin exerts dual effects depending on bone tissue, skeletal maturity and/or signaling pathway (78). Early life, leptin may stimulate bone through direct angiogenic and osteogenic effects on stromal precursor cells. Later, it may decrease bone remodeling in the mature skeleton, when trabecular bone turnover is high, by stimulating osteoprotegerin (OPG) expression. Leptin negative effects on bone formation effected through central nervous system pathway could counterbalance these peripheral and positive effects, the latter being predominant when the blood-brain barrier permeability decreases or the serum leptin level rises above a certain threshold (79).

Leptin acts via two antagonistic pathways to regulate bone remodeling. The

SNS pathway inhibits the differentiation of osteoblast and promotes the differentiation of osteoclasts. In contrast, CART (cocaine amphetamine regulated transcript), a neuropeptide expressed in the hypothalamus, inhibits differentiation of osteoclasts. The intermediate steps in the pathway and CART's receptor remain unknown to date (67) (fig 2).



**Fig 2) Leptin regulation of bone resorption by the sympathetic nervous system and CART. Adopted from Eleftheriou *et al* 2005 (67).**

### 2.5.3. Studies of Leptin and Leptin receptor gene polymorphism on obesity

The leptin gene (*LEP*) has been localized in humans on chromosome 7q32 and has three exons separated by two introns (80) and many polymorphisms.

Mostly one of these polymorphisms (A19G) has been studied. However, homozygosity for the G allele was found to have no association with obesity or BMI (81-83).

The leptin receptor gene (*LEPR*) which maps to chromosome 1p31.2 in human has at least 5 isoforms. In isoform 1 (*LEPR1*), several variants were studied; non-synonymous change of lysine to arginine at codon 109 in exon 4(K109R, rs1137100); non-synonymous change of glutamine to arginine at codon 223 in exon 6(Q223R, rs1137101); non-synonymous change of lysine to asparagine at codon 656 in exon 14(K656N, rs8179183) (84).

#### **2.5.4. Studies of Leptin and Leptin receptor gene polymorphism on bone**

There are no studies which demonstrate an association between *LEPR* polymorphism and BMD. Instead, Koh *et al* genotyped leptin receptor gene (*LEPR*) and demonstrated that the subjects carrying the Gln223 allele of *LEPR* had higher BMD at the lumbar spine compared with the subjects without this allele in 219 healthy volunteers aged 20–34 years (85). But Crabbe *et al* failed to demonstrate the association between the Gln223Arg *LEPR* polymorphism and BMD in both the cross section study and the longitudinal study (86). Recently, Fairbrother *et al* studied a large population of Caucasian postmenopausal women (87). He demonstrated that a heterozygote carrier in Gln223Arg genotyping has a lower BMD of both femoral and neck than other

genotyping groups.

## **2.6 Adiponectin : Complex mechanism for bone formation**

Recently adiponectin has emerged as an element in the regulation of bone metabolism (88-91) , but the regulation and detailed function of adiponectin in bone still remains obscure and inconclusive.

Oshima *et al* showed that adiponectin inhibited M-CSF- and RANKL-induced differentiation of mouse bone marrow macrophages and human CD14-positive mononuclear cells into osteoclasts and also suppressed the bone-resorption activity of osteoclasts and indicated that adiponectin increases bone mass by suppressing osteoclastogenesis and by activating osteoblastogenesis (90). However, many clinical data show that adiponectin exerts an independent negative effect on BMD in men or women and might have an unfavorable effect on bone metabolism (91-93).

Luo *et al* tried to explain how adiponectin has a dual action on bone (88, 94). Adiponectin induces human osteoblast proliferation and differentiation, and the proliferation response is mediated by the AdipoR/JNK pathway, while the differentiation response is mediated via the AdipoR/p38 mitogen-activated protein kinase (MAPK) pathway (88). However, adiponectin also increased osteoclast formation indirectly through stimulating RANKL and inhibiting OPG production in osteoblasts (94).

Moreover, Shinoda *et al* suggested three distinct adiponectin actions on bone formation: a positive action through the autocrine/paracrine pathway by

locally produced adiponectin, a negative action through the direct pathway by circulating adiponectin, and a positive action through the indirect pathway by circulating adiponectin via enhancement of the insulin signaling (95). These complex and even contradictory results demand further studies.

#### **2.6.1. Studies of adiponectin gene polymorphism on obesity**

Mostly two single nucleotide polymorphisms (SNPs) in the adiponectin gene (*ADIPOQ*), T45G in exon 2 and G276T in intron 2, have been reported to be associated with obesity (96-99).

The T45G polymorphism is a silent T to G substitution in exon 2, and G276T polymorphism is a G to T substitution in intron 2.

It was first reported by Stumvoll *et al.* that a T(45)G polymorphism (a synonymous mutation, Gly15Gly) in exon 2 was associated with body mass index (BMI) in nondiabetic subjects (96). The G allele was associated with higher BMI, waist to hip ratio (WHR), and body fat percentage in this German population.

In a prospective study involving 4,500 French Caucasians, the subjects with the GG genotype of T(45)G polymorphism had a greater increase in both BMI and WHR after 3 years (97). In addition to the T(45)G polymorphism, a nearby G (276)T in intron 2 was also investigated in several studies, which showed the different results (96-99).

Recently, the C allele of a promoter polymorphism, G (−11377)C, was also reported to associate with higher BMI among type II diabetes patients (100).

### **2.6.2. Studies of adiponectin gene polymorphism on bone mineral density**

First, Lee *et al* observed the association between adiponectin gene polymorphism and bone mineral density (101). In the female cohort, subjects with G alleles at the T45G locus had significantly lower lumbar spine BMD than those subjects with the TT genotype. Although BMD levels showed no association with the G276T locus, the GT genotype group showed significantly higher urine deoxypyridinoline levels than other genotype groups. In the male cohort, no association was observed between adiponectin genotypes and BMD levels.

Another SNP study was done by Zhang *et al* (102). Although no significant association was found between BMD and SNP in the adiponectin genes (T45G , G276T ) in both men and postmenopausal women, haplotype 2 (T-T) in the *ADIPOQ* was associated with lumbar spine BMD in postmenopausal women significantly.

### 3. HYPOTHESIS and SPECIFIC AIMS

**Hypothesis I** : Obesity is associated with osteoporosis in the manner of following; body weight is a protective factor whereas fat mass is a negative factor against osteoporosis.

**Aim 1** : To evaluate cross-sectional association between adiposity measurements including BMI, percent body fat and abdominal circumference and osteoporosis parameters including BMD (hip, spine), vertebral fracture, measure of bone turnover bone marker, Urine DPD (Deoxypyridinoline), Serum OC (Osteocalcin).

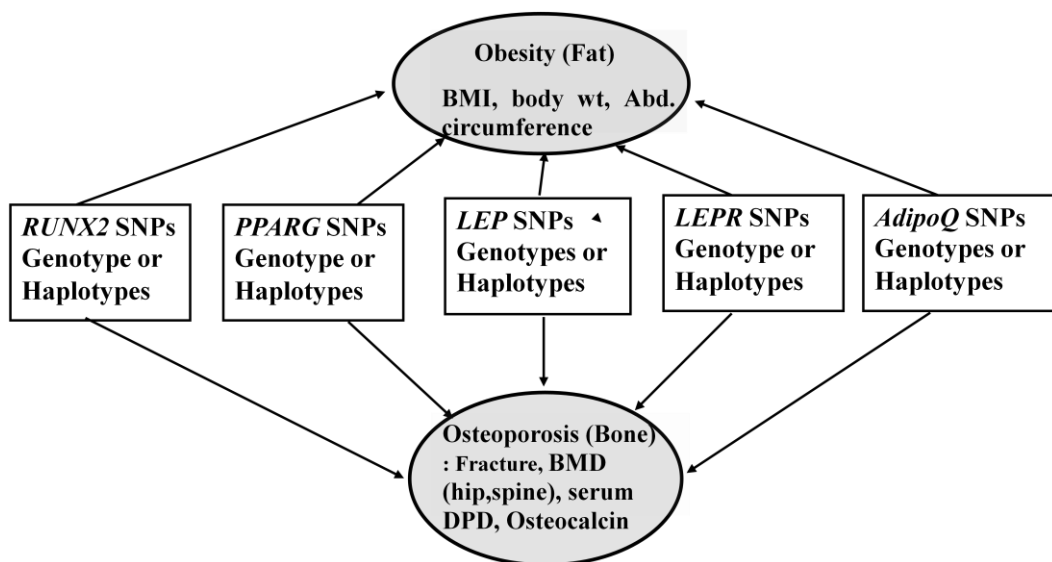
**Hypothesis II** :

Single nucleotide polymorphisms (SNP) in the *PPAR*, *RUNX2*, *LEPR* (*LEP*) and *ADIOPQ*, are associated with osteoporosis and obesity.

**Aim 2** : To evaluate cross-sectional association between SNPs in the *PPAR*, *RUNX2*, *LEPR* (*LEP*) and *ADIOPQ* gene with BMD (hip, spine), vertebral fracture, measure of bone turnover bone marker, Urine DPD (Deoxypyridinoline), Serum OC (Osteocalcin)

**Aim 3** : To evaluate cross-sectional association between in the *PPAR*, *RUNX2*, *LEPR* and *ADIPOQ* gene with adiposity measurements (bodyweight, BMI, percent body fat, abdominal circumference) and metabolic syndrome (lipid profile, serum glucose, blood pressure).

### Explanatory model of study design



**Fig 3) Explanatory model of study design**

## 4. SUBJECTS and METHODS

### 4.1. Study subjects:

Postmenopausal female subjects, age 60-79 years who meet general health inclusion criteria and exclusion criteria (as below) were recruited from two MizMedi hospital (west, south hospital)

#### 4.1.1 Inclusion Criteria

1. Generally healthy women  $\geq 60$  years of age but  $\leq 79$  years of age.
2. Ambulatory and community living.
3. **Vertebral Fracture Cases** Lateral radiographs of the thoracic and lumbar spine demonstrating the presence of vertebral fractures as interpreted by radiographic morphometry using Genant's semi-quantitative method.<sup>10</sup>
4. **Control Cases** Lateral radiographs of the thoracic and lumbar spine, obtained at the screening visit, demonstrating the absence of vertebral fractures as interpreted by radiographic morphometry using Genant's semi-quantitative method.
5. Written informed consent for blood and DNA collection, genotyping and radiographs when necessary.

#### **4.1.2. Exclusion Criteria**

1. History of co-morbidities known to affect bone metabolism such as cancer, inflammatory bowel disease, pituitary diseases, hyperthyroidism, primary hyperparathyroidism, renal failure, rheumatic disease or adrenal disease.
2. Use of glucocorticoids over the last 5 years.
3. Any history of HRT or selective estrogen receptor modulator (SERM), bisphosphonate and calcitonin use over 1 year.
4. Vertebral Fracture Cases: from known accidental trauma associated with diagnosis of vertebral fracture.

## **4.2 Measurements:**

### **4.2.1 Biochemical Markers**

Urine DPD (Deoxypyridinoline), Serum OC (Osteocalcin), Fasting lipid profile including: Total cholesterol, HDL-cholesterol, LDL-cholesterol, Triglycerides

### **4.2.2. Radiographs**

Presence of vertebral fractures, or lack of them for the control cases, found in lateral radiographs of the thoracic and lumbar spine as interpreted by radiographic morphometry using Genant's semiquantitative method (**Fig 4**). There were four Study Radiograph Examiners. Two Study Radiograph Examiners independently evaluated every study radiograph for vertebral fracture. If the two evaluations did not concur, a third Study Radiograph Examiner discussed results with other examiners and a confirmed diagnosis was done.

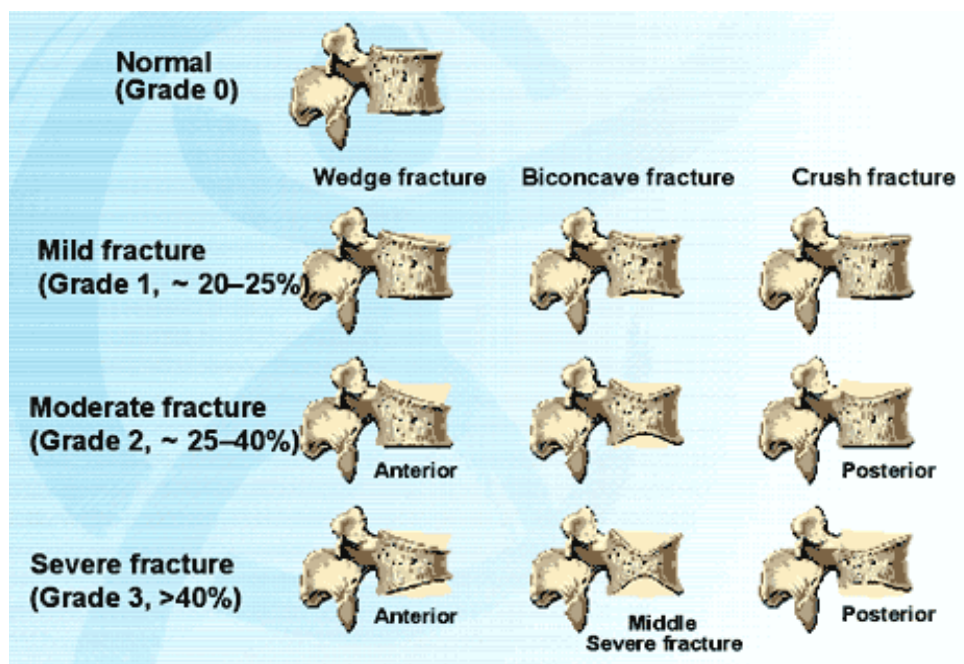


Fig 4) Genant's semiquantitative method

#### 4.2.3. Bone mineral density (BMD)

BMD determined at lumbar spine (L2-L4) was assessed using Dual Energy X-ray Absorptiometry (DEXA). The scanners used in study hospitals are Lunar (Madison, WI, USA) and Norland (Trumbull, CT, USA). The results recorded are Z score, T score and  $\text{g/cm}^2$ .

### **4.3 Genotyping analysis of polymorphisms in the *PPARG*, *RUNX2*, *LEP*, *LEPR* and *ADIPOQ*.**

#### **4.3.1 SNP Selection and Bioinformatic Techniques**

SNPs were chosen for genotyping if they fit one or more of the following criteria: SNPs within the chosen loci that have previously been reported in the literature to show an association with the phenotype of interest was chosen for genotyping. SNPs that may cause functional protein changes (such as non-synonymous amino acid changes, or SNPs that reside on splice sites) was considered for genotyping. In addition, SNPs residing in known or putative transcription factor binding sites and having the potential to alter gene expression was considered for genotyping. Finally, SNPs with good minor allele frequency (>1%) was preferentially chosen for genotyping. Furthermore, utilizing the Applied Biosystems SNP Browser software, Tagged SNPs can be determined in order to limit the number of SNPs required to study each gene. **Table 1)** lists the web-based databases that were used for SNP selection. **Fig 5)** shows LD block of each selected gene.

#### **4.3.2. DNA isolation**

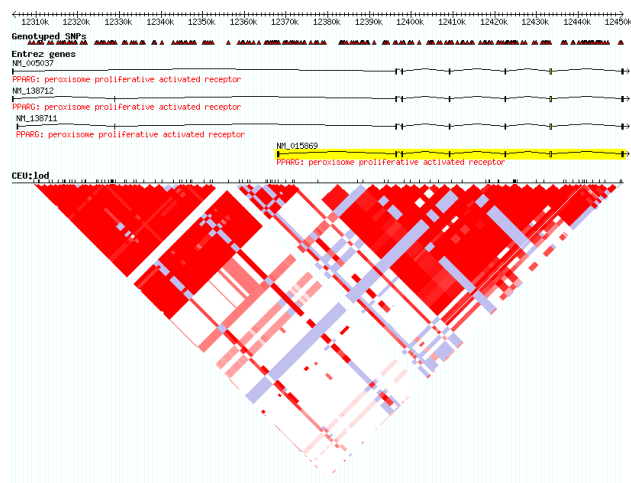
DNA was isolated from blood and purified for PCR analysis using the QIAamp DNA Mini Kit (Qiagen Inc., Chatsworth, CA). Whole blood and/or

buffy coat fraction was mixed with lysis buffer and incubated for 10 minutes at 70° C, before being mixed with isopropanol and loaded onto a QIAamp spin column for microcentrifugation. Column contents were washed and purified DNA was eluted in buffer (53).

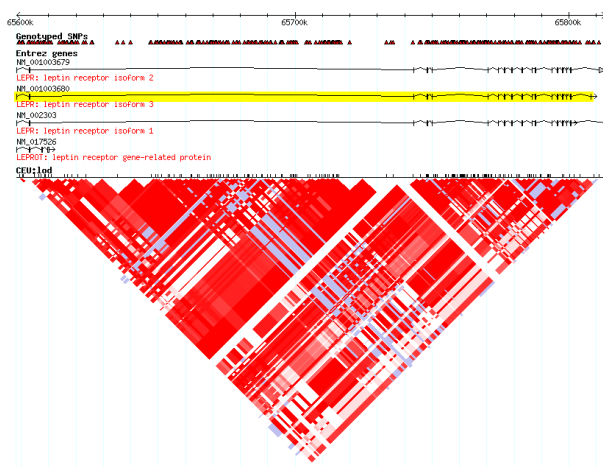
Table 1) summary of candidate gene and SNPs

Gene and Locus	NCBI SNP Cluster ID, Frequency and Function				
	dbSNP number	Alternative name	Allele frequency (Hapmap ; HCB)	function/location	Reference
<b>PPARG</b> 3p25.3	rs2960422		A(0.43)/G(0.57)	promoter	Hsu (49)
	rs1801282	Pro12Ala	C(0.956)/T(0.044)	Exon B : nonsynonymous Ala/Pro	Rhee.(46).
	rs10510418		A(0.867)/C(0.133)	Intron 2	Kiel.(50) (snp from GWA)
	rs2938392		C(0.349)/T(0.651)	Intron 5	Kiel.(50) (snp from GWA)
	rs3856806	His477His, 161C>T,1431C>T	C(0.767)/T(0.233)	Exon 6 : synonymous His/His	Rhee.(45)
<b>RUNX2</b> 6p21.2	rs7771980	-1025T>C	A(0.244)/G(0.756)	promoter2	Doecke (57) Bustamante (49)
	rs6921145	198A>G	No hapmap data	exon1	
<b>LEP</b> 7q32	rs7799039	-2548G>A.	A(0.744)/G(0.256)	promoter	
	rs2167270	19A>G	A(0.188)/G(0.812)	5' UTR	
<b>LEPR</b> 1p31.2	rs1137100	K109R, Lys109Arg	A(0.144)/G(0.856)	Exon 4 : nonsynonymous Lys/Arg	Tag SNP
	rs1137101	5193G > A, Q223R, Gln223Arg	A(0.111)/G(0.889)	Exon 6 : nonsynonymous Gln/ Arg	Koh.(85),(87) ; Tag SNP
	rs8179183	K656N, Lys656Asn	C(0.022)/G(0.978)	Exon 14: nonsynonymous Lys/Asn	
<b>ADIPOQ</b> 3q27	rs266729	-11377G>C	C(0.7)/G(0.3)	promoter	
	rs2241766	45T>G, Gly15Gly	No Hapmap data	Exon 2 : synonymous Gly/Gly	Lee.(101). Zhang.(102)
	rs1501299	276G>T	A(0.322)/C(0.678)	Intron 2	Zhang(102); Tag SNP

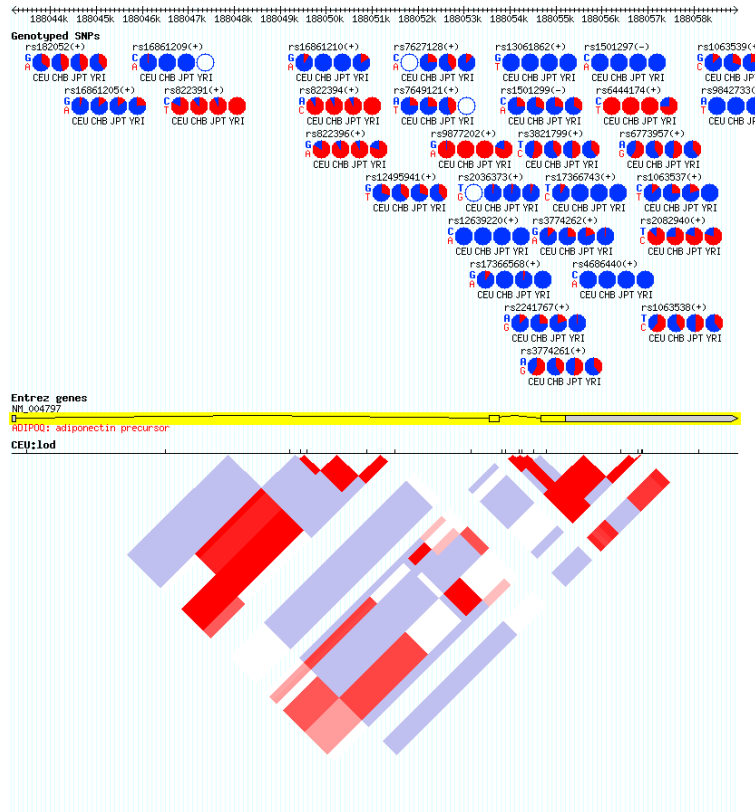
Fig 5) LD block of *PPARG* gene



LD block of *LEPR* gene



LD block of *AdiopQ* gene



### 4.3.3 Genotyping

Genotyping was carried out using the ABI prism 7900. SNP discrimination assays designed by Applied biosystem (ABI) were used. ABI has created over 50,000 pre-made SNP TaqMan 5' nuclease allelic discrimination assays.(103) In these assays, Taqman probe-based 5' nuclease assay chemistry unites PCR amplification and signal generation into a single step. In this system, a hybridization probe with fluorogenic and quencher tags was cleaved by the 5' nuclease activity of Taq DNA polymerase during PCR

amplification. Cleavage produces fluorescence by freeing the fluorogenic molecule from the quencher. By using two probes, one specific to each allele of the SNP and labeled with distinct fluorogenic tags, both alleles are specifically detected in a single tube (104). Genotyping information is transferred directly from the ABI7900 to spreadsheets containing all other data. Accuracy of genotype is tested by introducing 5% dummy duplicates and blank controls.

#### **4.4. Statistical analysis**

Statistical analysis was performed in SAS for Windows (v.9.1, Cary, NC, USA). Categorical variables were summarized using frequencies and percentages; continuous variables were summarized using sample size, mean, median, standard deviation, minimum and maximum. Significance was declared at a two-sided 0.05 level, unless otherwise specified. As actual treatment status is critical to studying the association with underlying genetic differences between individuals, the intent-to-treat analysis was not used in this study.

The analysis described below was carried out to assess all SNPs of interest in the genes and function. A genotype for each SNP was coded as a three-category variable: 1= homozygote for allele 1; 2= heterozygote; 3= homozygote for allele 2 (for example, A/A, A/G, G/G, respectively). The genotype frequencies for all studied polymorphisms were tested for Hardy-Weinberg equilibrium. Linkage Disequilibrium (LD) between the SNPs within each gene was estimated by Lewontin's  $|D'|$  and  $r^2$  (the square of the correlation between loci). Allele frequencies for each SNP were compared using a  $\chi^2$  test or one-way ANOVA test. If genotype group of homozygote variants was too small, it was combined with heterozygote genotype group and analyzed together. Groups that show significant difference in allele distribution was tested separately. We used an additive model to compare each three

genotype group if they are shown in order. For an additive model, we coded three-category variables of genotype groups as 1, 2 and 3 respectively and regarded them as orderly continuous variables. For adjustment of age, body weight, percent body fat, smoke, alcohol drink, total energy expenditure, total calcium intake, total fat and cholesterol intake, we used general linear model for continuous dependent variables (BMD, bone marker, lipid profile etc.) and logistic regression model for categorical dependent variables (Risk of osteoporotic fracture and metabolic syndrome). An association between genotype group and Risk of osteoporotic fracture and metabolic syndrome was calculated using Odds ratio and 95% CI (confidence interval).

The sample size for this study is limited to the subjects recruited. Assuming 80% power and  $\alpha = 0.05\%$ , the detectable mean differences between two genotype groups (recessive model) for allele frequencies ranging from 10% to 50% were calculated for hypotheses I and II. The detectable difference between genetic variant groups ranges from 0.31 standard deviations for a 50% minor allele frequency, to 1.41 standard deviations for a 10% minor allele frequency. These calculations are shown below in **Table 2**). Since a recessive model is the most conservative, any other mode of inheritance will provide greater statistical power.

**Table 2)** Power calculations for cross-sectional analysis

MAF <sup>1)</sup> %	N	Minor Allele Homozygote	Power %	Significance Level ( $\alpha$ )	Detectable Effect Size
10	400	4	80	.05	1.41
20	400	17	80	.05	0.69
30	400	37	80	.05	0.48
40	400	67	80	.05	0.37
50	400	104	80	.05	0.31

1) MAF (Minor Allele Frequency)

## 5. RESULTS

### 5.1 Preliminary genomic data

First, we genotyped selected 13 SNPs from 5 candidate genes with 48 subjects as a preliminary study. All Genotyped SNPs were satisfied by Hardy-Weinberg Equilibrium test ( $p>0.05$ ). These data are shown in **Table 3**) and **Table 4**). There were strong Linkage Disequilibrium (LD) between rs1051041 and rs2938292 of *PPARG* gene ( $r^2=0.35$ ,  $D=0.91$ ), rs1801282 and rs3856 of *PPARG* gene ( $r^2=0.164$ ,  $D=1$ ), rs2141766 and rs1501299 of *ADIPOQ* gene ( $r^2=0.16$ ,  $D=1$ ), rs7799039 and rs2167270 of *LEP* gene ( $r^2=0.94$ ,  $D=1$ ), rs1137100 and rs1137101 of *LEPR* gene ( $r^2=0.215$ ,  $D=0.6$ ). By using Mapper program (<http://snpper.chip.org/mapper/mapper-main>), some transcription factors (TF) were specified to match the region where SNP were located. An association between genotyped group of selected SNPs and phenotypes such as BMD, osteoporotic fracture and metabolic data is shown in **Table 3a**), **Table 3b**) as a p-value. Although this preliminary study had a limit of sample power, some genotype groups showed an association with bone parameter, obesity parameter and metabolic parameter. From these basic data, we selected 4 SNPs finally and genotyped remain 859 subjects. Although rs7771980 of

*RUNX2* gene had a small minor allele frequency (0.067) we included it as a final SNPs because of its possible key role.

**Table 3 a)** preliminary analyze of candidate SNPs (*PPARG* and *ADIPOQ* gene) and clinical data

Gene/SNPs	Gene	PPARG				ADIPOQ		
	SNP	-32648A/C	IVS5+357A/G	P12A	161C>T	-11377C>G	45T>G	276G>T
	rs number	rs10510418	rs2938292	rs1801282	rs3856806	rs266729	rs2241766	rs1501299
SNP analysis	Major	A	A	C	C	C	T	G
	Minor	C	G	G	T	G	G	T
	Freq Major	0.771	0.594	0.979	0.885	0.667	0.688	0.740
	Freq Minor	0.229	0.406	0.021	0.115	0.333	0.313	0.260
	LD block	$r^2=0.35$ D=0.91		$r^2=0.164$ D=1			$r^2=0.16$ D=1	
	HWE test(p=)	0.227	0.519	0.883	0.599	0.386	0.834	0.577
Bone parameter	TF binding	HMG-1Y,BRC-z4	Barbie box			sp-1		
	comment	GWA	GWA	non-syn	syn	promoter	syn	intron 2
	L BMD	0.8837	0.0632	0.7998	0.9928	0.9624	0.2561	0.8671
	F BMD	0.8021	0.1191	0.759	0.3832	0.5949	0.3464	0.2494
	Fracture	0.2145	0.7672	0.2474	0.9094	0.3484	0.6108	0.4283
	Osteocalcin	0.7357	0.9299	0.2838	0.8203	0.2706	0.2874	0.3757
Obesity parameter	DPD	0.6711	0.5244	.	0.5238	0.556	0.878	0.607
	waist	0.7754	0.9484	0.6134	0.4588	0.1979	0.6128	0.1058
	WHR	0.5186	0.9982	0.5643	0.801	0.149	0.943	0.187
	BMI	0.9553	0.495	0.7372	0.444	0.6357	0.342	0.0256
	% fat	0.9914	0.928	0.3103	0.1243	0.2299	0.6153	0.0467
	LBM	0.8366	0.1948	0.8551	0.9638	0.6996	0.6672	0.3186
Metabolic parameter	systolic bp	0.1622	0.3616	0.0465	0.704	0.1289	0.5641	0.496
	glucose	0.6144	0.763	0.3017	0.4814	0.9969	0.9553	0.7481
	TG	0.8481	0.2426	0.8305	0.499	0.8515	0.5698	0.2422
	HDL	0.9697	0.0068	0.5484	0.7249	0.2726	0.7106	0.1244
	MetS	0.1835	0.0888	0.4309	0.1486	0.6845	0.8523	0.4111

**Table 3 b)** preliminary analyze of candidate SNPs (*PPARG* and *ADIPOQ* gene) and clinical data

<i>Gene/SNPs</i>	Gene	RUNX2		LEP		LEPR	
	SNP	-1025T>C	-2548A/G	19G/A	R109K	R223Q	K656N
	rs number	rs7771980	rs7799039	rs2167270	rs1137100	rs1137101	rs8179183
SNP analysis	Major	T	A	G	G	G	G
	Minor	C	G	A	A	A	C
	Freq Major	0.933	0.760	0.771	0.745	0.833	0.906
	Freq Minor	0.067	0.240	0.229	0.255	0.167	0.094
	LD block	r2=0.94 D=1			r2=0.215 D=0.6		
	HWE test(p=)	0.632	0.550	0.214	0.961	0.729	0.474
Bone parameter	TF binding	PPARG,SPz1		AML-1,ChopCEBP			
	comment	promoter	promoter	5 UTR	non-syn	non-syn	non-syn
	L BMD	0.513	0.1901	0.1901	0.4253	0.9462	0.3151
	F BMD	0.7576	0.3615	0.3615	0.3184	0.2834	0.3463
	Fracture	0.2212	0.4846	0.4846	0.9873	0.8811	0.7672
	Osteocalcin	0.6995	0.1431	0.1431	0.3993	0.3301	0.9282
Obesity parameter	DPD	0.7594	0.4647	0.4647	0.6676	0.5833	0.4276
	waist	0.7233	0.366	0.366	0.0773	0.6434	0.0015
	WHR	0.2512	0.1891	0.1891	0.1329	0.5962	0.0076
	BMI	0.536	0.8755	0.8755	0.5183	0.8772	0.3202
	% fat	0.6793	0.6954	0.6954	0.0497	0.4213	0.0139
	LBM	0.467	0.4889	0.4889	0.5521	0.4242	0.3449
Metabolic parameter	systolic bp	0.2542	0.0252	0.0252	0.5364	0.4457	0.896
	glucose	0.3891	0.5051	0.5051	0.2202	0.9652	0.3303
	TG	0.0273	0.5424	0.5424	0.9303	0.6283	0.0127
	HDL	0.612	0.21	0.21	<b>0.1612</b>	0.2934	0.6314
	MetS	<b>0.1345</b>	0.5738	0.5738	0.7372	0.6769	<b>0.0097</b>

## 5.2. Demographic data

Main characters of 907 subjects are shown in **Table 4**). An average age of recruited patients which were distributed between 60 and 79 years was  $65.18 \pm 5.42$ .

**Table 4)** Main characters of subject and obesity, osteoporosis prevalence

variables	mean $\pm$ SD	n	%
Age (yr)	65.18 $\pm$ 5.42		
Body fat (%)	34.67 $\pm$ 2.95		
BMI(kg/cm <sup>2</sup> )	24.11 $\pm$ 2.68		
Obesity*	normal	302	33%
	overweight	264	29%
	obesity	341	38%
Waist(cm)	88.89 $\pm$ 7.59		
systolic BP	131.4 $\pm$ 16.19		
TG (mg/dl)	126.89 $\pm$ 71.83		
HDL (mg/dl)	54.72 $\pm$ 13.51		
Glucose (mg/dl)	87.26 $\pm$ 18.81		
Met SD	Yes	317	34%
	No	590	66%
LBMD (g/cm <sup>3</sup> )	0.84 $\pm$ 0.26		
Femur neck	0.67 $\pm$ 0.12		
Trochanter	0.54 $\pm$ 0.09		
Ward	0.45 $\pm$ 0.11		
Fracture	Yes	189	21%
	No	711	79%

Obesity\* (normal : BMI<25, overweight : 25<BMI<30, obesity : BMI>30)

Among 907 subjects, the number of people who having overweight was 302 (33%) and the number of people who having obesity is 341 (38%). An average of lumbar BMD (L BMD), Femur BMD (Neck, Trochanter, ward) was  $0.84\pm0.26$ ,  $0.67\pm0.12$ ,  $0.54\pm0.09$ ,  $0.45\pm0.11$  respectively. A prevalence of metabolic syndrome was 34% which is close to 32.6% of Korean population (2005, the Third Korea National Health and Nutrition examination survey (KNHANES III ) (105). Osteoporotic lumbar fracture prevalence rate was 21% in this study. Because there has not been official statistics of Osteoporotic vertebral fracture, this prevalence was not comparable.

### 5.3. Genomic data

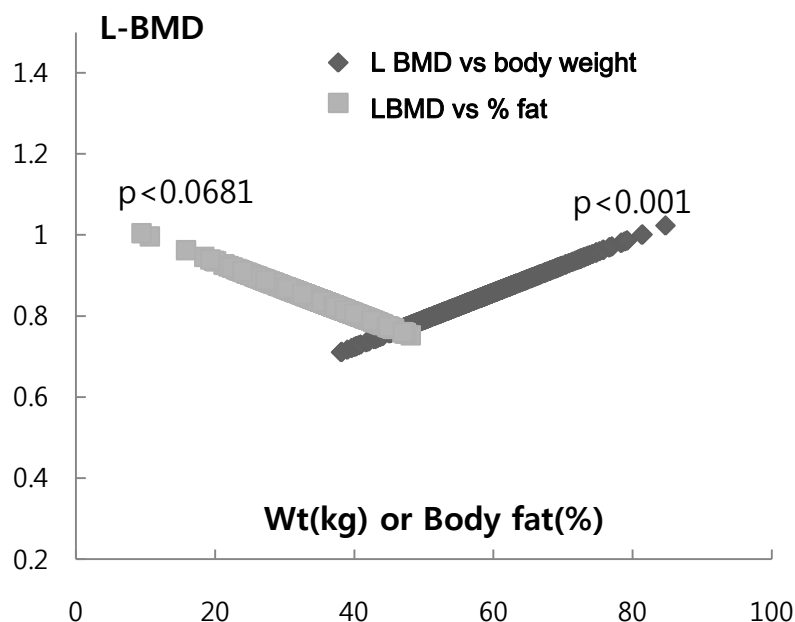
**Table 5)** shows data which we genotyped and analyzed from 907 subjects. Because rs7771980 of *RUNX2* gene had a small MAF (minor allele frequency) of 0.081%, we combined TC and CC group and analyzed them together. As rs8179183 (K656N) of *LEPR* gene also had a small MAF of 0.079, we combined GC and CC group together. All genotyped data were satisfied by Hardy-Weinberg Equilibrium test ( $p>0.05$ ).

**Table 5)** Genomic data of 4 candidate SNPs

Gene Symbol	RUNX2	PPARG	ADIPOQ	LEPR
SNP Name		IVS5+357G/A	276G>T	K656N
rs number	rs7771980	rs2938392	rs1501299	rs8179183
Major	T	A	G	G
Minor	C	G	T	C
Freq Major	0.919	0.547	0.69	0.921
Freq Minor	0.081	0.453	0.453	0.079
Genotype (%)	TT (84.53)	AA (30.06)	GT (47.34)	GG (84.93)
	TC (14.68)	AG (49.77)	GT (43.91)	GC (14.17)
	CC (0.01)	GG (20.15)	TT (0.08)	CC (0.01)
HWE (p-value)	0.52	0.74	0.26	0.21

#### 5.4. An association between obesity and osteoporosis

With a simple correlation test, both body weight (0.2947,  $p<0.001$ ) and body percent fat (0.1935,  $p<0.0001$ ) were associated with Lumbar BMD (L BMD) positively. However, after adjusting by age, smoke, alcohol drink, total calcium intake, total energy intake and body percent fat or body weight reciprocally, body percent fat was negatively associated with Lumbar BMD ( $p<0.0681$ ) whereas body weight was still positively associated with Lumbar BMD ( $p<0.001$ ) (**Fig 6**). With femur BMD, body percent fat was also negatively associated. (Femur neck  $p=0.0069$ , Trochanter  $p=0.0051$ , Ward  $p=0.0009$ . data are not shown).



**Fig 6)** An associate between body % fat, body weight and L BMD

**Table 6)** Metabole variables affecting on BMD

	Lumbar		neck		Trochanter		Ward	
	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
age (yr)	0.66	0.9193	-4.14	<b>&lt;0.001</b>	-2.79	<b>0.0053</b>	-5.25	<b>&lt;0.001</b>
wt (kg)	5.53	<b>&lt;0.001</b>	6.21	<b>&lt;0.001</b>	7.51	<b>&lt;0.001</b>	4.91	<b>&lt;0.001</b>
body fat (%)	-0.93	0.0966	-0.78	0.4327	-2.31	<b>0.0209</b>	-1.36	0.173
waist (cm)	-2.09	<b>0.037</b>	-5.07	<b>&lt;0.001</b>	-4.58	<b>&lt;0.001</b>	-5.01	<b>&lt;0.001</b>
glucose(mg/dl)	2.36	<b>0.016</b>	2.13	<b>0.0335</b>	2.65	<b>0.0082</b>	1.02	0.3096
HDLC(mg/dl)	1.57	0.1076	1.25	0.2123	2.09	<b>0.0366</b>	0.73	0.4652
TG(mg/dl)	1.98	0.0603	-0.2	0.8402	1.03	0.3025	-0.2	0.8439
Systolic BP	0.6	0.5927	-0.77	0.4388	-1.45	0.148	-1.26	0.2073

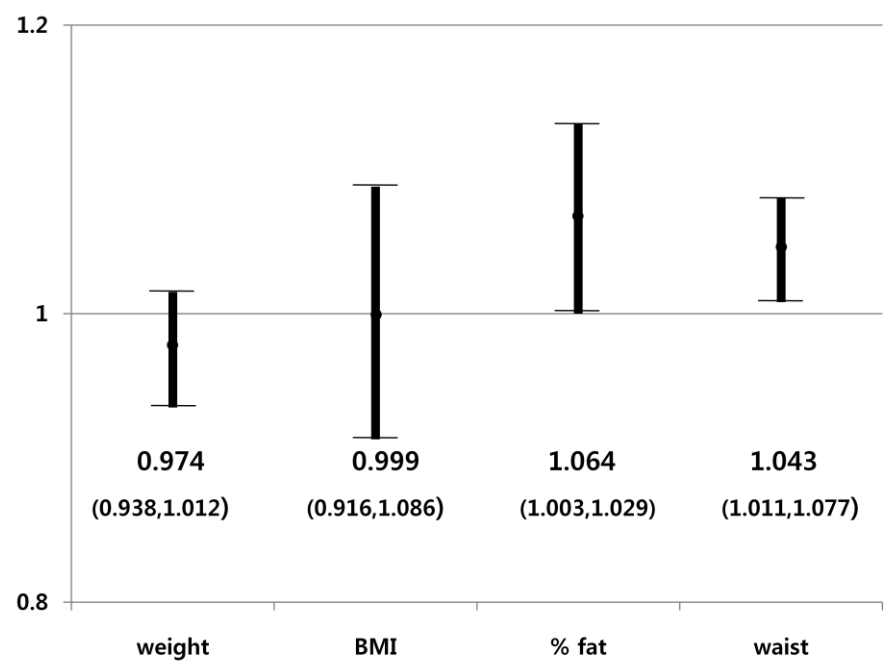
adjusted by age, smoke, alcohol, total calcium intake, total energy expenditure, total calorie intake

**Table 6)** shows different metabolic variables which affect on lumbar, femur neck, trochanter, ward BMD when it is adjusted by age, smoke, alcohol, total calcium intake, total energy expenditure, total calorie intake. Age was negative correlated with femur BMDs, but not with lumbar BMD. Body weight was positively associated with all sites BMDs. However percent body fat was negatively correlated with a trochanter BMD. Waist circumference representing intra-abdominal fat was also negatively correlated with all sites BMDs. Serum glucose level was a positively correlated with a lumbar BMD ( $p=0.016$ ), a femur neck BMD ( $p=0.0335$ ) and a femur trochanter BMD ( $p=0.0082$ ). Serum HDLC was only associated with a femur trochanter BMD ( $p=0.0366$ ). Serum TG and systolic BP were not associated with all sites BMD.

**Table 7)** demonstrates differences of independent variables between fracture and non-fracture group. The average age was higher in fracture group compared to non-fracture group (Odds ratio 1.077, 95% CI [1.042-1.114]). Body weight and BMI between two groups were not significantly different. However body percent fat and waist circumference were much bigger in fracture group compared to non-fracture group (Odds ratio 1.064 , 95% CI [1.003-1.129], Odds ratio 1.043 , 95% CI [1.011-1.077] respectively (**Fig 7**). Among other metabolic variables, serum HDLC in fracture group was lower than in fracture group (Odds ratio 0.982 , 95% CI [0.968-0.996]).

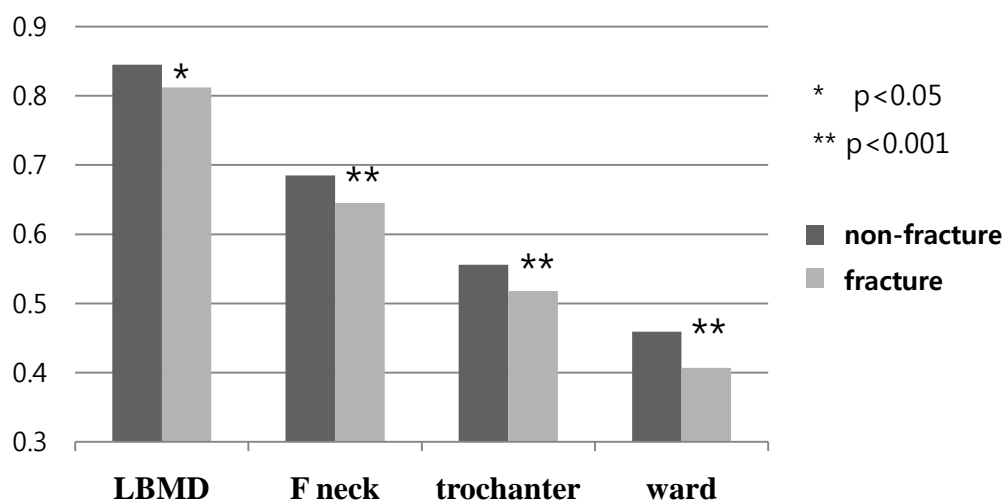
**Table 7)** Metabolic variables affecting Osteoporotic fracture

variables	Fracture	non-Fracture	OR (95% CI)	p-value
n (%)	189 (21%)	711 (79%)		
age (yr)	67.10±7.33	64.81±4.74	1.077(1.042,1.114)	<b>&lt;0.001</b>
wt (kg)	57.47±7.73	57.39±7.04	0.974 (0.938,1.012)	0.182
BMI (kg/cm <sup>2</sup> )	24.58±2.95	24.12±2.78	0.999 (0.916, 1.085)	0.9465
Body fat(%)	35.47±4.78	34.55±4.83	1.064 (1.003,1.129)	<b>0.0383</b>
waist (cm)	90.68±7.37	88.41±8.10	1.043 (1.011,1.077)	<b>0.0082</b>
Glucose(mg/dl)	86.62±19.14	87.47±18.54	0.995 (0.985,1005)	0.3104
HDLC (mg/dl)	52.14±11.99	55.38±13.74	0.982 (0.968,0.996)	<b>0.0110</b>
TG (mg/dl)	129.44±78.82	126.16±69.11	0.999 (0.996, 1.002)	0.4489
Systolic BP	133.34±17.20	130.92±15.90	1.001 (0.990, 1.011)	0.9198
Metabolic SD				
Yes	77 (24.37%)	239 (75.63%)		
No	112 (19.18%)	472 80.82%)	0.924 (0.570,1.498)	0.7481
adjusted by age wt % fat smoke alcohol total calcium intake				



**Fig 7) Odds ratio of osteoporotic fracture according to aiposity variables**

**Fig 8)** shows association between BMD and Osteoporotic Fracture. Lumbar BMD and Femur BMD (neck, trochanter, ward) were lower in fracture group.



**Fig 8)** An association between BMD and Osteoporotic Fracture

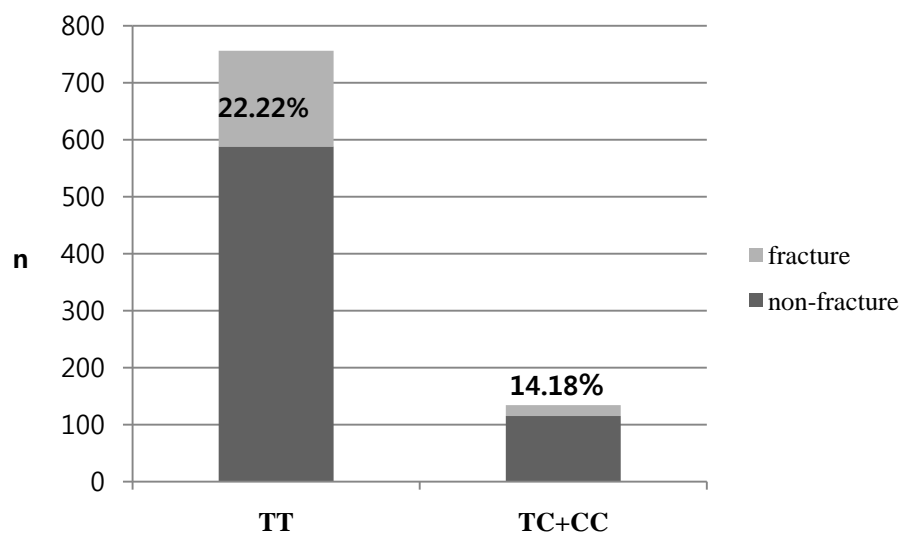
### **5.5. Genotype group of rs7771980 (*RUNX2*) distribution and association with obesity, metabolic syndrome and osteoporosis.**

The distribution of genotype group of rs7771980 is shown in **table 8**). TT homogygous genotype group was 84.53% and TC+CC (C carrier) genotype group is 15.47%. All significance which was shown in **table 8**) are adjusted by age, smoke, alcohol, total caloric intake, total energy expenditure, total calcium intake, total fat intake, weight, body percent fat. Obesity parameters including body percent fat, body weight, BMI, waist circumference were not different between TT and TC+CC group. Metabolic data (systolic BP, lipid profile, glucose) and a risk of metabolic syndrome were also not different between the two groups. Lumbar BMD in TT group was  $0.84 \pm 0.16 \text{ g/cm}^3$  which was a little higher than  $0.81 \pm 0.14 \text{ g/cm}^3$  in TC+CC group ( $p=0.058$ ). However, BMD of all the sites of femur (neck, trochanter, ward) showed no difference between two groups.

The risk of osteoporotic fracture in TC+CC genotype group was 45% lower than TT group (Odds ratio 0.55, 95% CI[0.32-0.94]  $p=0.0297$ ) (**Fig 9**). There was no statistical difference of bone marker (urine deoxypyridinoline, DPD and serum osteocalcin, OC) between the two groups.

**Table 8)** An association between genotype group of rs7771980 (*RUNX2*) and obesity, metabolic syndrome and osteoporosis

variables		Genotype		p-value
		TT	TC+CC	
n (%)		760 (84.53)	137 (15.47)	
age (yr)		65.21±5.54	65.84±5.05	p=0.3409
body %fat		34.76±4.88	34.51±4.62	p=0.2882
weight (kg)		57.44±7.14	56.71±7.26	p=0.7154
BMI		24.24±2.84	23.97±2.74	p=0.9913
waist (cm)		88.91±7.94	88.42±8.34	p=0.7051
systolic BP		131.42±16.21	131.74±16.48	p=0.8099
HDL(mg/dl)		54.53±13.34	55.98±13.91	p=0.2170
TG(mg/dl)		128.50±72.76	118.37±62.59	p=0.2057
glucose		87.15±18.81	87.41±15.75	p=0.7028
Met SD	no	500 (65.79)	83 (60.58)	OR 1.30 (0.88-1.92)
n (%)	yes	260 (34.21)	54 (39.42)	
LBMD(g/cm <sup>3</sup> )		0.84±0.16	0.81±0.14	p=0.058
Femur neck BMD		0.67±0.12	0.66±0.09	p=0.3450
Trochanter BMD		0.54±0.09	0.53±0.08	p=0.6915
Ward BMD		0.45±0.12	0.43±0.10	p=0.1854
Fracture	no	588 (77.78)	115 (85.82)	<b>OR 0.55 (0.32-0.94)</b>
n (%)	yes	168 (22.22)	19 (14.18)	
Osteocalcin		21.33±7.94	21.32±8.64	P=0.9213
Urine DPD		9.48±3.23	9.90±4.20	P=0.2766
adjusted by age, smoke, alcohol, total calorie intake, total energy expenditure, total calcium intake, total fat intake, wt, body percent fat.				



**Fig 9)** Difference of fracture risk among genotype group of rs7771980

## **5.6. Genotype group of rs2938392 (*PPARG*) distribution and association with obesity, metabolic syndrome and osteoporosis.**

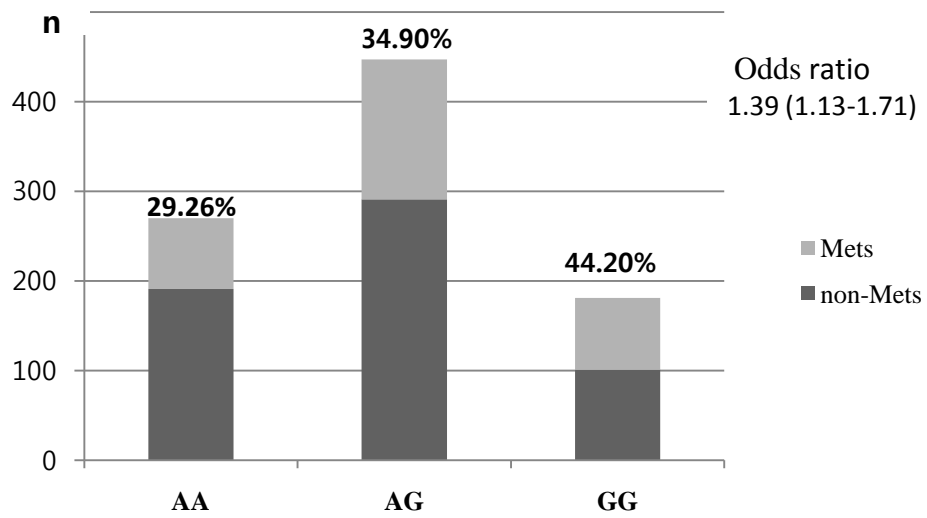
The distribution of genotype group of rs2938392 was shown in **table 9**). AA homogygous genotype group was 30.06% and AG heterogygous group was 49.71% and GG homogygous group is 20.15%. All the significance which was shown in **table 9**) was adjusted by age, smoke, alcohol, total calorie intake, total energy expenditure, total calcium intake, total fat intake, weight, body percent fat. Obesity parameters including body percent fat, body weight, BMI, waist circumference were not different between AA, AG and GG group. Metabolic data such as systolic BP, lipid profile, glucose showed no difference between the two groups , but the prevalence of metabolic syndrome in AA group, AG group and GG group was 29.26%, 34.90% and 44.20% which gradually increased according to the genotype. (Odds ratio 1.39, 95% CI[1.13-1.71] p=0.0014) (**Fig 10**).. Femur neck BMD in AA group, AG group and GG group was  $0.68 \pm 0.16 \text{ g/cm}^3$ ,  $0.67 \pm 0.10 \text{ g/cm}^3$  and  $0.66 \pm 0.10 \text{ g/cm}^3$  which gradually decreased BMD according to the genotype (p=0.056). However, lumbar BMD and other femur sites (trochanter, ward) showed no difference between the two groups. The risk of osteoporotic fracture among the three genotype groups was not different. There was no statistical difference of bone marker (urine

deoxypyridinoline, DPD and serum osteocalcin, OC) among the three genotype groups.

**Table 9)** An association between genotype group of rs2938392 (*PPARG*) and obesity, metabolic syndrome and osteoporosis

variables		Genotype			p-value
		AA	AG	GG	
n (%)		270 (30.06)	447 (49.71)	181 (20.15)	
age (yr)		65.72±5.18	65.05±5.81	65.38±5.02	p=0.4959
body %fat		34.93±4.66	34.53±5.06	34.97±4.94	p=0.4397
weight (kg)		57.40±6.87	57.10±7.50	57.89±6.77	p=0.6302
BMI		24.31±2.68	24.11±2.98	24.31±2.61	p=0.3379
waist (cm)		89.05±7.46	88.56±8.45	89.35±7.44	p=0.6679
systolic BP		131.15±15.19	131.64±16.40	131.70±17.21	p=0.4887
HDL (mg/dl)		54.46±13.44	55.36±13.28	53.39±13.83	p=0.5882
TG (mg/dl)			124.60±69.24	125.07±71.00	136.02±74.58
glucose		87.45±18.76	87.44±19.02	86.62±17.41	p=0.7602
Met SD	no	191(70.74)	291 (65.10)	101 (55.80)	<b>OR 1.39 (1.13-1.71)</b>
n (%)	yes	79 (29.26)	156 (34.90)	80 (44.20)	<b>p=0.0014</b>
LBMD(g/cm <sup>3</sup> )		0.82±0.14	0.84±0.17	0.83±0.14	p=0.4448
Femur neck BMD		0.68±0.16	0.67±0.10	0.66±0.10	<b>p=0.0566</b>
Trochanter BMD		0.54±0.10	0.54±0.09	0.54±0.09	p=0.8243
Ward BMD		0.45±0.15	0.45±0.11	0.43±0.10	p=0.1253
Fracture	no	210 (78.95)	352 (79.10)	140 (77.78)	<b>OR 1.05 (0.83-1.34)</b>
n (%)	yes	56 (21.05)	93 (20.90)	40 (22.22)	p=0.6409
Osteocalcin		21.15±7.92	21.14±8.31	22.15±7.64	p=0.3137
Urine DPD		9.91±3.62	9.27±3.12	±9.78±3.77	p=0.3137

adjusted by age, smoke, alcohol, total calorie intake, total energy expenditure, total calcium intake, total fat intake, wt, body percent fat.



**Fig 10)** Difference of metabolic syndrome risk among genotype group of rs2938392

### **5.7. Genotype group of rs1501299 (*ADIPOQ*) distribution and association with obesity, metabolic syndrome and osteoporosis.**

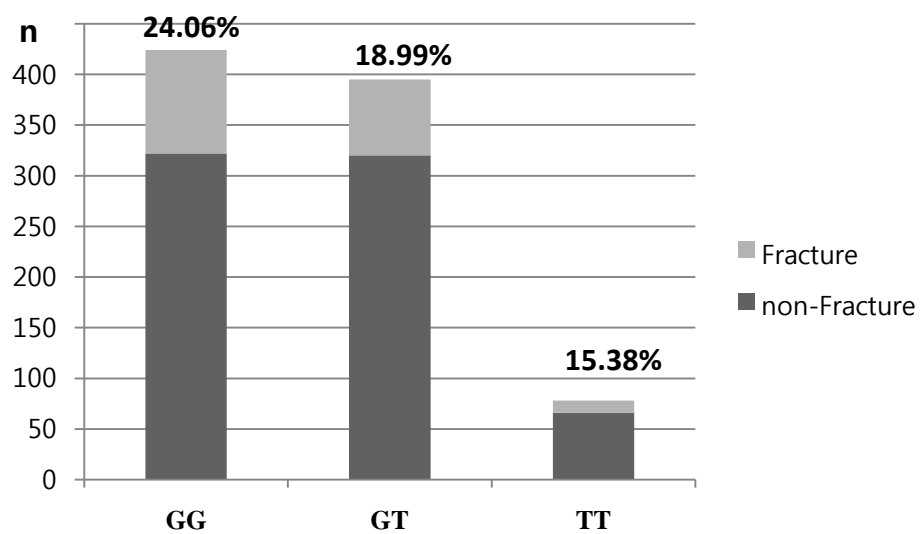
The distribution of genotype group of rs1501299 is shown in **table 10**). GG homogygous genotype group was 47.34%. and GT heterogygous group was 43.91% and TT homogygous group was 0.08%. All the significance which was shown in **table 10**) were adjusted by age, smoke, alcohol, total calorie intake, total energy expenditure, total calcium intake, total fat intake, weight, body percent fat. Obesity parameters including body percent fat, body weight, BMI, waist circumference were not different between GG, GT and TT group. Serum glucose level was  $85.68 \pm 15.38$ mg/dl in GG group,  $88.72 \pm 21.60$  in GT group,  $88.43 \pm 18.21$  in TT group with significant difference ( $p=0.0375$ ). Other metabolic data such as systolic BP, lipid profile and prevalence of metabolic syndrome among the three genotype group showed no difference. All BMD data (Lumbar, femur neck, trochanter, ward) did not show any difference among the three genotype groups, but the risk of osteoporotic fracture was different. The prevalence of osteoporotic fracture was 24.06% in GG group, 18.99% in GT group, 15.38% in TT group showing gradual decreased according to the ordered genotype group (Odds ratio 0.76, 95% CI[0.58-0.99],  $p=0.0473$ ) (**Fig 11**). There was no statistical difference of bone marker (urine

deoxypyridinoline, DPD and serum osteocalcin, OC) among the three genotype groups.

**Table 10)** An association between genotype group of rs1501299 (*ADIOPQ*) and obesity, metabolic syndrome and osteoporosis

variables		Genotype			p-value
		GG	GT	TT	
n (%)		428 (47.34)	397 (43.91)	79 (0.08)	
age (yr)		65.47±5.07	65.18±6.03	65.12±4.49	p=0.4114
body %fat		34.74±4.67	34.76±5.03	35.03±4.49	p=0.7363
weight (kg)		57.30±7.13	57.51±7.21	57.46±7.25	p=0.6357
BMI		24.19±2.78	24.23±2.84	24.40±2.82	p=0.2224
waist (cm)		88.88±7.77	88.87±8.34	89.49±7.18	p=0.6974
systolic BP		130.81±16.06	132.62±16.39	129.03±15.81	p=0.6712
HDL (mg/dl)		54.26±12.95	54.59±13.69	57.36±14.46	p=0.1036
TG (mg/dl)		127.47±65.77	125.14±73.51	123.91±86.20	p=0.4934
glucose		85.68±15.38	88.72±21.60	88.43±18.21	<b>p=0.0375</b>
Met SD	no	280 (65.42)	250 (62.97)	57 (72.15)	0.95 (0.76-1.19)
n (%)	yes	148 (34.58)	147 (37.03)	22 (27.85)	p=0.6482
LBMD(g/cm <sup>3</sup> )		0.83±0.14	0.84±0.18	0.83±0.12	p=0.8073
Femur neck BMD		0.67±0.14	0.67±0.09	0.68±0.13	p=0.6605
Trochanter BMD		0.54±0.10	0.54±0.08	0.56±0.10	p=0.9673
Ward BMD		0.45±0.14	0.44±0.09	0.45±0.13	p=0.5294
Fracture	no	322 (75.94)	320 (81.01)	66 (84.62)	<b>1.31 (1.00-1.71)</b>
n (%)	yes	102 (24.06)	75 (18.99)	12 (15.38)	<b>p=0.0473</b>
Osteocalcin		21.45±7.80	21.05±8.03	22.12±9.25	p=0.7295
Urine DPD		9.70±3.45	9.50±3.52	9.21±2.69	p=0.3386

adjusted by age, smoke, alcohol, total calrori intake, total energy expenditure, total calcium intake, total fat intake, wt, body percent fat.



**Fig 11)** Difference of metabolic syndrome risk among genotype group of rs2938392

## **5.8. Genotype group of rs8179183 (*LEPR*) distribution and association with obesity, metabolic syndrome and osteoporosis.**

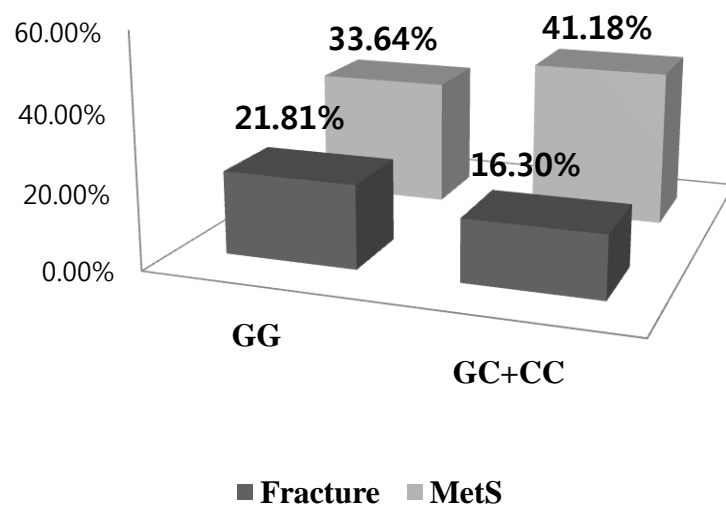
The distribution of genotype group of rs8179183 is shown in **table 11**). GG homogygous genotype group was 84.93% and GC+CC (C carrier) genotype group is 15.07%. All the significance which was shown in **table 11**) are adjusted by age, smoke, alcohol, total calorie intake, total energy expenditure, total calcium intake, total fat intake, weight, body percent fat. BMI in GG group was  $24.21 \pm 2.79$  (kg/cm<sup>2</sup>) which was a little higher than  $24.13 \pm 3.03$  (kg/cm<sup>2</sup>) in GC+CC group ( $p=0.0599$ ). Other obesity parameters including body percent fat, body weight, waist circumference were not different between GG and GC+CC group. HDL cholersterol was higher in GG group than GC+CC group ( $55.19 \pm 13.53$ mg/dl vs.  $52.22 \pm 12.58$  mg/dl,  $p=0.0115$ ) and serum glucose was a little lower in GG group than GC+CC group ( $86.83 \pm 18.49$ mg/dl vs.  $89.80 \pm 19.35$ ,  $p=0.0654$ ). The prevalence of metabolic syndrome in GC+CC group was 41.18%, relatively higher than 33.64% in GG group (Odds ratio 1.46, 95% CI[0.96-2.09]  $p=0.0768$ ) (**Fig 12**). All BMD data (Lumbar, femur neck, trochanter, ward) did not show any difference among the three genotype group, but the risk of osteoporotic fracture was different. The prevalence of osteoporotic frature in GC+CC genotype group was 16.30% , relatively lower than 21.84% in GG group (Odds ratio 0.65, 95% CI[0.39-1.08]  $p=0.095$ ) (**Fig**

**12).** There was no statistical difference of bone marker (urine deoxypyridinoline, DPD and serum osteocalcin, OC) between the two groups.

**Table 11)** An association between genotype group of rs8179183 (*LEPR*) and obesity, metabolic syndrome and osteoporosis

variables		Genotype		p-value
		GG	GC+CC	
n (%)		767 (84.93)	136 (15.07)	
age (yr)		65.39±4.97	64.89±7.68	p=0.5270
body %fat		34.72±4.84	34.78±4.82	p=0.6959
weight (kg)		57.32±7.20	57.59±7.14	p=0.8775
BMI		24.21±2.79	24.13±3.03	<b>p=0.0599</b>
waist (cm)		88.85±8.00	89.06±8.12	p=0.5057
systolic BP		131.36±16.14	131.42±16.66	p=0.8214
HDL(mg/dl)		55.19±13.53	52.22±12.58	<b>p=0.0115</b>
TG(mg/dl)		125.86±71.42	131.89±67.98	p=0.2276
glucose		86.83±18.49	89.80±19.35	p=0.0654
Met SD	no	509(66.36)	80 (58.82)	<b>OR 1.46 (0.96-2.09)</b>
n (%)	yes	258 (33.64)	56 (41.18)	
				<b>p=0.0768</b>
LBMD(g/cm <sup>3</sup> )		0.83±0.14	0.85±0.15	p=0.3032
Femur neck BMD		0.67±0.12	0.67±0.10	p=0.4165
Trochanter BMD		0.54±0.09	0.55±0.09	p=0.8961
Ward BMD		0.44±0.12	0.44±0.11	p=0.4037
Fracture	no	595(78.19)	113 (83.70)	<b>OR 0.65 (0.39-1.08)</b>
n (%)	yes	166 (21.81)	22 (16.30)	
				<b>p=0.095</b>
Osteocalcin		21.47±8.28	20.67±6.48	p=0.2513
Urine DPD		9.52±3.46	9.85±3.17	p=0.4245

adjusted by age, smoke, alcohol, total calorie intake, total energy expenditure, total calcium intake, total fat intake, wt, body percent fat.



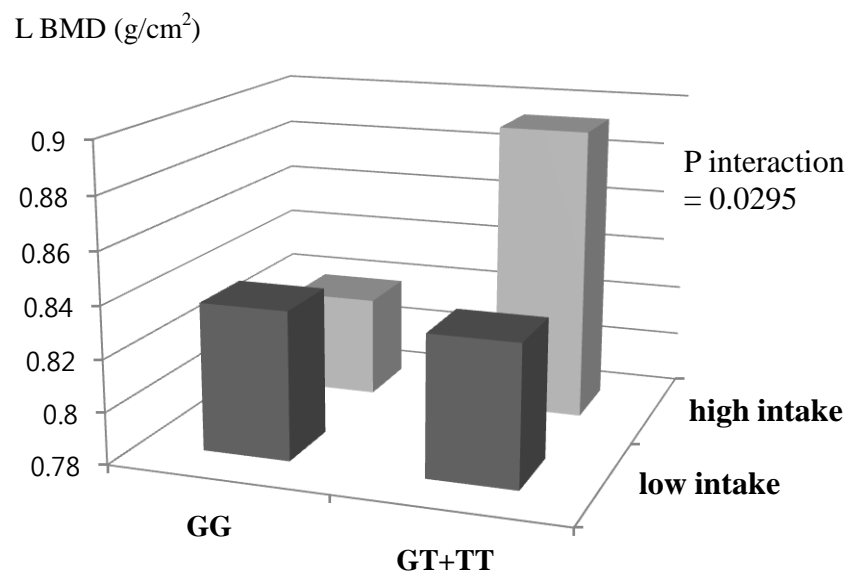
**Fig 12)** Difference of osteoporotic fracture and metabolic syndrome risk among genotype group of rs8179183

## 5.9. Gene-nutrient interaction analysis.

In addition, we investigated Gene-nutrient interaction. **Table 12)** shows *AdipoQ* gene-Calcium intake interaction to lumbar BMD (g/cm<sup>2</sup>). We defined high calcium intake as over 1,000mg of calcium intake a day. Lumbar BMD (g/cm<sup>2</sup>) was not significantly different between high and low calcium intake in GG group of rs1501299. However lumbar BMD(g/cm<sup>2</sup>) of high calcium intake group was higher than low calcium intake group in GT+TT group of rs1501299. (p=0.0264). There was an interaction between *AdipoQ* gene genotype group and calcium intake (p=0.0295) (**Fig 13**). Other genomic variants such as rs293892 (*PPARG*), 7771980 (*RUNX2*), rs8179183 (*LEPR*) didn't interact with calcium intake group.

**Table 12)** *AdipoQ*-Calcium interaction to lumbar BMD (g/cm<sup>2</sup>)

L BMD (g/cm <sup>2</sup> )				
Calcium	GG	GT+TT	p-value	p-interaction
low intake	0.837±0.145	0.834±0.168		
(n)	385	425	0.754	
high intake	0.819±0.122	0.893±0.242		
(n)	43	51	0.0603	
p-value	0.3714	<b>0.0264</b>		<b>0.0295</b>

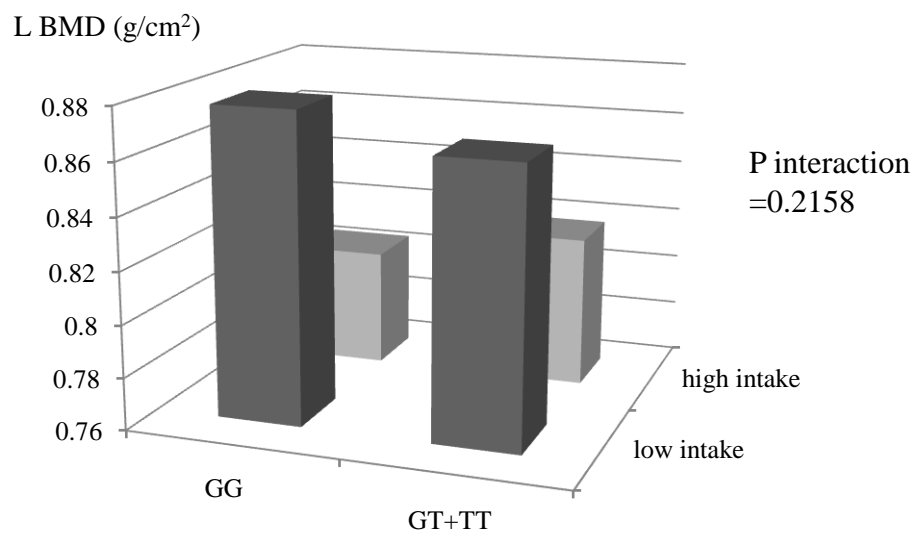


**Fig 13)** *AdipoQ*-Calcium interaction to lumbar BMD (g/cm²)

**Table 13)** showed *AdipoQ* gene-Fat intake interaction to lumbar BMD (g/cm<sup>2</sup>). We defined high Fat intake as over 45g of fat intake a day. Lumbar BMD (g/cm<sup>2</sup>) of low fat intake group was higher than high fat intake group in both GG and GT+TT group of rs1501299 respectively (p<0.001, p=0.0032). There was no interaction between *AdipoQ* gene genotype group and fat intake (p=0.2158) (**Fig 14**). Other genomic variants such as rs293892 (*PPARG*), 7771980 (*RUNX2*), rs8179183( *LEPR* ) didn't interact with fat intake and cholesterol intake.

**Table 13)** *AdipoQ*-Fat interaction to lumbar BMD (g/cm<sup>2</sup>)

L BMD (g/cm2)				
Fat	GG	GT+TT	p-value	p-interaction
low intake	0.878±0.145	0.865±0.134	0.3659	0.2158
(n)	179	219		
high intake	0.805±0.133	0.818±0.206	0.3716	
(n)	249	255		
p-value	<b>&lt;0.001</b>	<b>0.0032</b>		



**Fig 14)** *AdipoQ*-Fat interaction to lumbar BMD (g/cm<sup>2</sup>)

## 6. DISCUSSION

### 6.1. Obesity vs. Osteoporosis

Both obesity and osteoporosis are common disease affecting millions.

In the recent decades, the association between obesity and osteoporosis has been a matter of extensive clinical and basic investigation, and in fact, common pathogenic links have been recently proposed (1-3,6,(106).

It is generally accepted that obesity has a protective effect on bone tissue. On the other hand some authors present an opposite - the lack of beneficial effect of obesity on the development of osteoporosis fractures (107). The relation between obesity and osteoporosis varies depending on how we define obesity. If we define obesity as BMI or body weight, obesity might be a protective factor against bone mineral loss or osteoporotic fracture. If we define obesity as waist circumference or body percent fat, obesity might be an unfavorable factor against osteoporosis. Our data in **table 6), table 7)** and **Fig 6), Fig 7)** support that body weight is a protective factor against BMD loss or osteoporotic fracture whereas body percent fat or waist circumference is an unfavorable factor. This result is consistent with previous studies in Chinese (6-7). However some studies demonstrated not only lean mass but also fat

mass contribute positively to BMD (108-111). Cui *et al* demonstrate that fat mass appeared to contribute negatively to BMD at all sites in young Korean men, but contribute positively to BMD at the forearm and calcaneus in old Korean men (108). However they did not adjust for body weight to calculate fat mass effect on BMD. When we adjust for age only, even our data show that body percent fat is positively related with BMD at all sites except femur ward ( $p < 0.001$ , data not shown). But when we include body weight for adjustment, this association changes to an inverse relationship.

Metabolic syndrome has been studied in relation with osteoporosis. In the Rancho Bernardo Study, after adjusting for BMI, Metabolic syndrome (MS) was associated with lower, not higher BMD (112). Incidence of osteoporotic non-vertebral fractures was higher in participants with MS. MS may be another risk factor for osteoporotic fractures. Accumulating evidence indicates that the individual components of the metabolic syndrome such as hypertension, increased triglycerides, and reduced high-density lipoprotein cholesterol are also risk factors for low bone mineral density (113-115).

Clinical observation on diabetes patients suggests that hyperglycemia tend to reduce BMD and to increase fracture risk. Diabetes becomes one of risk factors of the osteoporotic fracture (116, 117). However, our data are contrary

to the studies mentioned above. Serum glucose is positively correlated with a lumbar, femur neck and femur trochanter BMD (**Table 6**). Unlike in the studies mentioned above, our participating subjects were basically healthy women and we excluded for diabetes. Because diabetes patients are prone to fall down due to neuropathy or retinopathy, high osteoporotic fracture rate in diabetes is an acceptable theory.

In postmenopausal women with primary hyperparathyroidism, serum glucose level was positively related to Z score of BMD at the lumbar spine and femoral neck; however this significance disappeared when fat mass was considered (118). Further investigation whether serum glucose affects bone mineral density and fracture is needed.

An association between lipid profile and BMD is also controversial.

Patients with vertebrae fractures had lower levels of total cholesterol, TG, LDL-C than the patients without vertebrae fractures in Turkish postmenopausal women (119). However some report that high lipid profile including total cholesterol and TG is associated with high BMD (120).

Paradoxically some studies demonstrated serum HDL is inversely associated with BMD in pre- and postmenopausal women (121-123). However our data shows high HDL is a protective factor of osteoporosis, increasing BMD and

lowering Odds ratio of osteoporotic fracture (**Table 6,7**). The mechanism how lipid profile affects directly is unclear and needs to be investigated.

In our results, overall prevalence of metabolic syndrome is not associated with osteoporotic fracture in our study (**Table 7**).

## 6.2. Genomics variants contributing Osteoporotic Fracture

Although *RUNX2* is a key molecule for osteoblast development, few studies has been published regarding genomic association study. In 821 Spanish postmenopausal women (56), individuals carrying the TC genotype of rs7771980 ( -1025 T/C) had higher mean adjusted Femur neck BMD values than those bearing the TT genotype whereas our results show no difference of femur neck BMD in both genotypes, but slightly higher lumbar BMD in TT group than TC+CC group (**Table 8**). One possible explanation of the difference between two groups of population comes from the difference of minor allele frequency (MAF). In Caucasian population C allele is a major allele (0.756, Hapmap), but in our subjects C allele is a minor allele (0.081). However, our data first demonstrate that genotype group in rs7771980 is associated with osteoporotic fracture (**Fig 9**). The reason that TT genotype group of rs7771980 has a higher lumbar BMD but more fracture rate than other group is needed to be investigated. The other region of *RUNX2* gene such as rs6921145 is deserved to be analyzed (124).

The most commonly studied variant of *PPARG* regarding obesity, insulin resistance and metabolic syndrome is rs1801282 (Pro12Ala) (28-34). However Rhee *et al* failed to demonstrate the association between genotype variants of

rs1801282 (Pro12Ala) and BMD in all sites (125). Instead, we selected rs2938392 (Intro 2) SNP which is one of candidate SNPs from one large scale whole genome wide association study (49). Compared with Caucasian population (C;0.349, T;0.651 Hapmap), genomics frequency of our subjects is 0.453 of G(C) and 0.547 of A(T) allele individually (**Table 5**). Although we failed to demonstrate the association between genotype variants of rs2938392 and osteoporotic fracture, lumbar BMD is weakly associated with genotype variants of rs2938392. Moreover, these genomic variants are associated with metabolic syndrome (**Table 9, Fig 10**). Only one study has been published to show that SNP of rs2938392 revealed significant association with BMI (126). This novel SNPs is needed to be investigated for an association with other phenotypes.

As adiponectin gene (*AdipoQ*) is expressed in adipose tissue exclusively, its polymorphism has been investigated mostly focusing on obesity or metabolic disease (96-99). Two SNPs – T45G(rs224176) and G276T(rs1501299) have extensively been analyzed to be associated with obesity. These two SNPs have been also chosen to demonstrate an association between genetic variants of *AIPOQ* and bone mineral density (101, 102). Because rs2241766 has no hapmap data, we selected rs1501299 as the final candidate SNP of *AdipoQ*. In comparison with hapmap data (A;0.322, C;0.678, **Table 1**), the allele

frequency of our subjects is 45.3% of T(A), 69% of G(C) (**Table 5**). Although our data failed to show a significant association between this SNP variant and obesity, serum glucose levels differ according to the three genotype groups (**Table 9**). As alterations in adiponectin-mediated pathways are known to be associated with glucose intolerance, insulin resistance (IR), obesity, and type 2 diabetes (T2D) mellitus, variants of *AdipoQ* gene have been widely investigated focusing on diabetes (127-131).

Interestingly, our data demonstrate that variants of rs1501299 (*AdipoQ*) are not associated with BMD in all sites, but associated with osteoporotic fracture (**Table 9**). Although our data demonstrated that BMDs in all sites are associated with fracture (**Fig 7**), BMD is not the best predictor of osteoporosis or osteoporotic fracture (132). A family history of osteoporotic fracture, a personal history of fracture as an adult, and a medical, surgical or therapeutic history that might be associated with accelerated bone loss or increased risk of fracture, medical conditions such as primary hyperparathyroidism and neurologic conditions that increase the risk of falling can explain osteoporotic fracture patients who have relatively high BMD. We also hypothesized fat mass contributes to bone quality which leads to ultimate bone fracture rather than to bone mineral density.

Although leptin is a key hormone which regulate appetite and osteoblast differentiation, polymorphism association studies regarding leptin gene (*LEP*) or its receptor gene (*LEPR*) have not widely investigated. A few studies were published to demonstrate an association between bone mineral density and variants of *LEPR* - mostly focused on rs1137101 (Gln223Arg) (85-87). Because preliminary data showed that only rs8179183 (K656N) is associated with metabolic syndrome among some candidate SNPs of *LEP* and *LEPR*, we finally chose rs8179183 to be analyzed (**Table 3b**). Minor allele frequency (MAF) is small (C; 0.079) which is similar to hapmap data (C; 0.022) (**Table 1**).

This SNP of rs8179183 (*LEPR*) is a novel SNP which has never been studied. Although we failed to demonstrate a significant association between its variants and metabolic syndrome ( $p=0.0768$ ) and fracture rate ( $p=0.095$ ) under  $p$  value of 0.005, genomic tendency to link both metabolic syndrome and fracture simultaneously provides us some idea (**Table 11**). As shown in **Fig 11**), patients who have GG genotype of rs8179183 have a higher prevalence of fracture but a lower prevalence of metabolic syndrome. This individual difference of genotype can explain the variety of phenotypes and ultimately provide the basic thought of personalized medicine (133, 134).

### 6.3. Nutrigenetic interaction on Osteoporotic Fracture

Numerous studies have indicated some nutrients including calcium, vitamin D, vitamin K, soybean, fat intake affect BMD and osteoporotic fracture (135-143). As those studies did not always provide conclusive results, we hypothesized that individual nutrient effect on bone mineral density might differ according to their genotype.

Although the beneficial effect of calcium intake on bone health is generally accepted, some author still questioned whether calcium intake is a protective factor against bone loss on aging (144, 145). These unexpected results require us to investigate the effect of calcium intake on BMD with calculation of genotypic difference. For example, interactions of interleukin-6 promoter polymorphisms with dietary calcium on bone mass are examined in both Framingham Osteoporosis Study (146) and in pre-menarche Chinese girl (147).

Our data in **Table 12**) demonstrat that calcium intake does not affect BMD in GG genotype group of rs1501299 (*AdipoQ*), but the BMD in high calcium group is much higher than low calcium intake group in GT+TT of rs1501299 (*AdipoQ*). (p interaction=0.0295). The mechanism of how calcium intake affects on BMD via adiponectin gene expression is unclear. Sun *et al* (148)

demonstrated that 1 $\alpha$ ,25-(OH)(2)-D(3) drives inflammatory cytokine expression; therefore suppression of 1 $\alpha$ ,25-(OH)(2)-D(3) by dietary calcium inhibits adipocyte-derived inflammation associated with obesity.

Several studies have examined the association between types of dietary fat and BMD in humans, but the results are conflicting. For example, saturated fat intake was found to be inversely associated with BMD in men and women in the NHANES III cohort study,(149) but Brownbill *et al* (120) could not find any association between saturated fat intake and BMD in a study of Caucasian postmenopausal women. Dietary conjugated linoleic acid (CLA) is positively associated with bone mineral density (BMD) in 136 Caucasian, healthy, postmenopausal women (150), whereas supplement with essential fatty acids did not affect on bone mineral density in healthy pre- and postmenopausal women (151). Our data showed that the dietary fat intake (g/day) is not associated with BMD in all sites (data not shown).

From these conflicting data, we suggest to investigate dietary fat and genomics interaction for osteoporosis. In Framingham offspring cohort, the interaction between SNPs (rs1151999, rs709150, rs1175381) of PPARG gene and dietary fat for phenotype of BMD were examined (152). In the same paper, they used a mouse model, the 6T congenic, to tease out a complicated gene (*PPARG*) by environment (dietary fat) interaction that impact on BMD (152).

Our data did not yield any interaction between dietary fat intake and rs2938392 of *PPARG* gene (data not shown).

Dietary fatty acids modulate eicosanoid presence, which have hormone-like activities in lipid metabolism regulation in adipose tissue, and may influence the expression of adipokines such as adiponectin and leptin (153). Moreover the effect of dietary fat on adiponkine response and insulin sensitivity differs according to adiponectin gene variants (154, 155). However, we failed to show an interaction between dietary fat intake and rs1501299 of *AdipoQ* gene on BMD (**Table 13, Fig 14**) and any obesity parameter (data not shown).

## 6.4. Limitation and further study

Our study has some limitations as followings.

First, to demonstrate whether body composition is associated with bone mineral density, we need to analyze body fat mass as visceral fat and subcutaneous fat. Peripheral fat mass is not correlated with bone mineral density (156) whereas visceral fat (intra-abdominal fat) mass might have an association with BMD (157).

WHR (waist/hip circumference) indicating central obesity (visceral fat mass) is associated with radius bone mineral density in postmenopausal obese women although BMI is not associated with BMD in same study (158). Carrasco *et al* (159) demonstrated that gastric bypass surgery (Bariatric surgery) in morbidly obese women induces a significant bone mineral density loss with decrease of visceral fat indicating that visceral fat might have an unfavorable effect on bone. Our data also indicate that waist circumference (cm) is associated with BMD in all sites (**Table 6**) and osteoporotic vertebral fracture (**Table 7**). However imaging analysis using CT scan is essential to evaluate exact distribution of visceral fat and subcutaneous fat and their effect on bone (157).

Second, due to the lack of budget, we did not examine serum adipokines such as adiponectin and leptin. Although serum adipokine data are much complex to be interpreted and sometimes provide to be an inconclusive data (160-163), it helps to link a mechanistic relationship between an expression of adipokine-related genes such as *AdipoQ* and *LEPR* and a phenotype including obesity and osteoporosis.

In preliminary studies(**Table 3a,3b**), we analyzed 13 SNPs of 5 candidate genes from 48 subjects. We will continue to analyze with these candidate SNPs from 907 whole subjects and to venture to investigate a gene-gene interaction if possible.

As this study was based on cross-sectional data, we further need to evaluate a longitudinal effect of BMD or osteoporotic fracture and obesity according to their genomic variants of obesity-related genes.

In conclusion, body weight or BMI is positively associated with BMD decreasing osteoporotic fracture whereas body percent fat or WHR is negatively associated with BMD increasing osteoporotic fracture.

Some SNPs (*RUNX2*, *ADIPOQ*) from adiposity-related genes were associated with BMD or fracture risk. Common genomic feature of these genes to two

phenotypes-fat and bone give us a rationale to develop co-treatment drugs or nutrients to prevent obesity and osteoporosis together.

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## 국문요약

### 비만관련 유전자의 변이와 골다공증 골절의 연관성

- 비만은 골다공증의 보호인자인가 위험인자인가?.

#### 배경

비만이 골다공증의 보호인자라는 전통적인 개념과는 달리 최근의 일부 역학 연구는 체지방이 골다공증과 골절의 위험인자임을 보여주고 있다.

Peroxisome proliferator-activated receptor-gamma (*PPAR-γ*) 와 runt-related transcription factor 2 (*RUNX-2*) 유전자는 골수의 조혈모세포에 서로 “시소” 같이 작용하여 각각 비만세포 및 골아세포의 분화에 영향을 미친다. 비만세포에서 분비되는 두 adipokines - leptin(*LEP*)과 adiponectin (*ADIPOQ*) 역시 골형성과 골파괴에 관여한다.

#### 방법

두 곳의 미즈메디 병원에서 자원한 60-79세 사이의 갱년기 이후의 여성 907명을 대상으로 골밀도, 골 표지자 검사, 비만 관련 지표 및 5개의 유전자 (*PPARG*, *RUNX2*, *LEPR*, *LEP*, *ADIPOQ*)와 관련한 13개의 유전자 다형성 (SNP)을 사전 조사하였으며 최종적으로 4개의 유전자 다형성을

분석하였다. (rs2938392;*PPARG*, rs7771980;*RUNX2*, rs8179183;*LEPR*, rs1501299;*ADIPOQ*).

## 결과

요추의 골밀도는 체중과 양의 상관관계 ( $p < 0.001$ ), 체지방과는 음의 상관관계를 보였으며 ( $p = 0.0681$ ) 척추 골질의 위험도도 체지방이 증가할수록 증가함을 보여 주었다 (odd ratio 1.064 95% CI [1.003-1.029],  $p = 0.0383$ ).

*RUNX2*의 유전자 다형성(rs7771980) 중 TC+CC 그룹은 TT 그룹 보다 낮은 골절율을 보였으며(odd ratio 0.55 [0.32-0.94],  $p = 0.0297$ ), *PPARG*의 유전자 다형성 (rs2938392) 중 AA 그룹(29.26%)은 각각 AG 그룹(34.90%), GG 그룹 (44.20% ) 보다 낮은 대사증후군 유병율을 보였다. (Odds ratio 1.39 95% CI [1.13-1.71]  $p = 0.0014$ ). *ADIPOQ*의 유전자 다형성 (rs1501299) 중 GG 그룹(24.06%)의 골다공증 골절율의 유병율은 각각 GT 그룹(18.99%), TT 그룹(15.38%) 보다 높았다 (Odds ratio 0.76 95% CI [0.58-0.99],  $p = 0.0473$ ). *LEPR*의 유전자 다형성 (rs8179183) 중 GG 그룹은 GC+CC 그룹에 비해 상대적으로 낮은 골절율과 대사증후군 유병율을 보였다 (odd ratio 0.65, 95% CI[0.39-1.08],  $p = 0.095$ , odd ratio 1.46, 95% CI[0.96-2.09],  $p = 0.076$ ) *ADIPOQ*의 유전자 다형성 (rs1501299) 중 GT+TT 그룹에서 고칼슘 섭취 그룹 ( $>1000\text{mg/day}$ )은 높은 골밀도를 보인 반면

G G 그룹에서는 관련이 없었다 (p interaction = 0.0283).

## 결론

일부 비만관련 유전자 다형성과 골다공증 골절과의 유의한 상관관계가 있었다. 이러한 비만세포와 골세포에 공통적으로 작용하는 유전체적 기전에 대한 이해는 비만과 골다공증에 대한 공통적 예방 및 치료제 개발에 도움이 되리라 생각된다 .

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**핵심어:** *PPARG*, *RUNX2*, *ADIPOQ*, *LEPR*, 유전자 다형성, 골다공증 , 비만