



**UNIVERSITY OF SIENA**  
**PhD PROGRAM IN BIOMEDICINE AND IMMUNOLOGICAL**  
**SCIENCES**  
**CYCLE XXIV**

**THE ROLE OF OSTEOPROTEGERIN, RANKL**  
**AND MATRIX-GLA PROTEIN ON THE**  
**CAROTID ATHEROSCLEROSIS IN NORMAL**  
**AND TYPE 2 DIABETIC SUBJECTS**

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**Academic Year 2011-2012**

## **TABLE OF CONTENTS**

Vascular calcification	p 2
Atherosclerosis and vascular calcification in diabetes	p 7
Vascular calcification and cardiovascular risk	p 13
Pathobiology of arterial calcification	p 15
Matrix GLA protein	p 19
Bone morphogenetic protein	p 21
OPG, RANK, RANKL	p 23
Aim of the study	p 31
Methods	p 32
Results	p 39
Conclusions	p 44
References	p 46

## **INTRODUCTION**

### **Vascular calcification**

Vascular calcification consists of calcium salt precipitates, mostly in apatite form, similar to the hydroxyapatite found in bone.

Several risk factors which are associated with the presence or progression of vascular calcification have been identified (1, 2) such as age, african descent, total cholesterol, current smoking, hypertension.

Calcification of the arteries is usually detected with plain X-ray or CT. Prevalence is highly dependent upon the studied population. In a population-based cohort of over 100.000 men and women who had chest X-ray for screening purpose, prevalence of calcification in the aortic arch was 1.9% in male patients and 2.6% in female patients (1).

In the past vascular calcification was seen as an inert end-point of atherosclerosis, however, recently it has become clear that it is a actively regulated process already occurring in the early stages of atherosclerotic lesions (3-5).

Vascular calcification is frequently seen in 1 of 2 forms, atherosclerotic lesion calcification and medial calcification (also referred to as media sclerosis or Mönckeberg disease).

Calcific vasculopathy can be categorized by its location in the neointima (that is, within atherosclerotic plaque) or in the tunica media (that is, the medial smooth muscle layer). Calcification of both locations often occurs in the same patient and even in the same arterial site.

Calcific vasculopathy can also be distinguished by its histological appearance as either amorphous (lacking tissue architecture) or chondro-osseus (having tissue architecture of cartilage or, when microvascular invasion occurs, even bone).

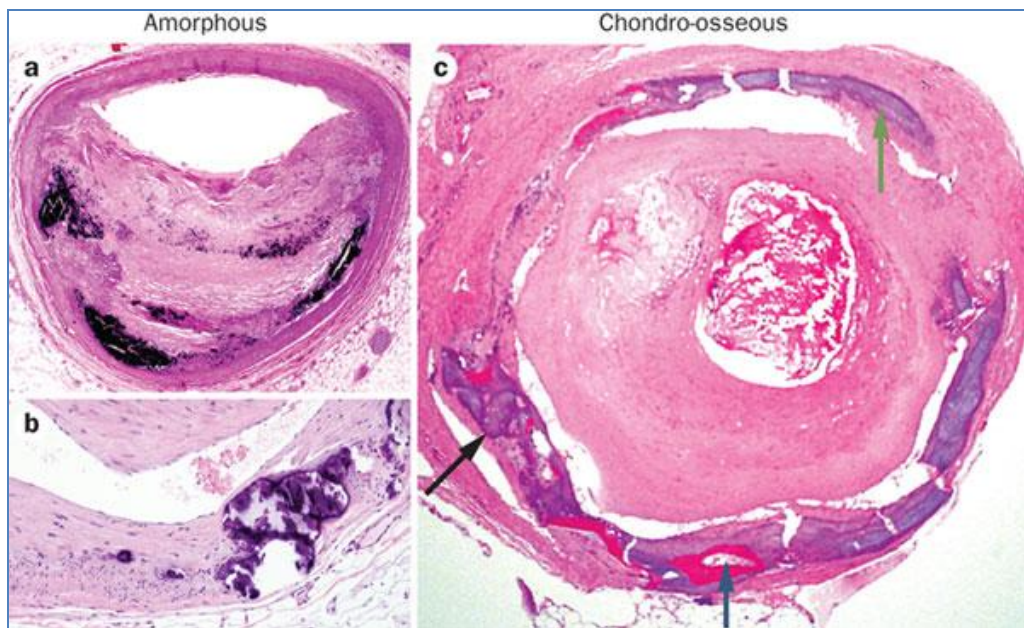
The histological characteristics of both of these forms of vascular calcification are illustrated in Figure 1. Amorphous calcified matrix is far more common than the chondro-osseus form, but latter often seems to emerge from the former, which may represent a precursor lesion.

Neointimal calcium deposits are found in a patchy distribution throughout the vascular tree, co-localizing with atherosclerotic lesions.

Calcification was previously thought to occur only in the most-advanced, end stage plaques in the elderly.

However, small hydroxyapatite mineral crystals arise in elderly lesions in the third decade of life, and coronary calcium deposition is now used as a quantitative marker for atherosclerotic plaque burden, even in early, subclinical stages.

Figure 1. Histological sections of human vascular calcification. Amorphous calcification is shown in a) the neointima of an atherosclerotic human artery and b) the medial layer of a nonatherosclerotic human artery. Chondro-osseous calcification is shown in c) the tunica media of a human artery (6)



Medial calcium deposits are distributed more diffusely throughout the vascular tree, at sites with or without atherosclerosis, and are more often circumferential at a given site owing to the close association with elastic laminae. Medial calcification is primarily associated with chronic kidney disease and diabetes (7).

Monckeberg's sclerosis leads to vessel stiffening, which is characterized by increases in pulse pressure and pulse wave velocity and is associated with increased cardiovascular risk (8).

Both types of calcification involve activation of osteogenic cell differentiation (9).

## **Atherosclerosis and vascular calcifications in diabetes**

Diabetes mellitus is associated with severe cardiovascular complications, including vascular calcification and accelerated atherosclerosis, leading to increased morbidity and mortality in diabetic patients. (10-12).

Coronary artery disease (CAD) causes much of the serious morbidity and mortality in patients with diabetes, who have a 2- to 4-fold increase in the risk of CAD (13).

In one population-based study, the 7-year incidence of first myocardial infarction (MI) or death for patients with diabetes was 20% but was only 3.5% for nondiabetic patients (14).

Regardless of the severity of clinical presentation, patients who have diabetes and coronary events experience increased rates of MI and death.



Patients with diabetes also have an adverse long-term prognosis after MI, including increased rates of reinfarction, congestive heart failure, and death (15).

A Finnish study (16) on the trends of MI showed that diabetes increased 28-day mortality by 58% in men (hazard ratio [HR], 1.58; 95% confidence interval [CI], 1.15-2.18) and 160% for women (HR, 2.60; 95% CI, 1.71-3.95). In fact, the 5-year mortality rate following MI may be as high as 50% for diabetic patients—more than double that of nondiabetic patients (17)

Epidemiological evidence confirms an association between diabetes and increased prevalence of peripheral arterial disease (PAD).

Individuals with diabetes have a 2- to 4-fold increase in the rates of PAD (18), more often have femoral bruits and absent pedal pulses (19).

The duration and severity of diabetes correlate with incidence and extent of PAD (20). Diabetic patients more

commonly have infrapopliteal arterial occlusive disease and vascular calcification than nondiabetic cohorts (20).

The Hoorn study (21) examined the rates of PAD among groups ranging from patients with normal glucose tolerance to those with diabetes requiring multiple medications. Patients with diabetes more commonly develop the symptomatic forms of PAD, intermittent claudication and amputation (22).

In the Framingham cohort, the presence of diabetes increased the risk of claudication by 3.5-fold in men and 8.6-fold in women (23).

Diabetes adversely affects cerebrovascular arterial circulation, akin to its effects in the coronary and lower extremity vasculature. Patients with diabetes have more extracranial atherosclerosis (24).

In patients undergoing dental panoramic radiographs, diabetic patients had 5-fold excess prevalence of calcified carotid atheroma (25)

The frequency of diabetes among patients presenting with stroke is 3 times more than that of matched controls (26).

The risk of stroke is increased 150% to 400% for patients with diabetes, (27-29) and worsening glycemic control relates directly to stroke risk.

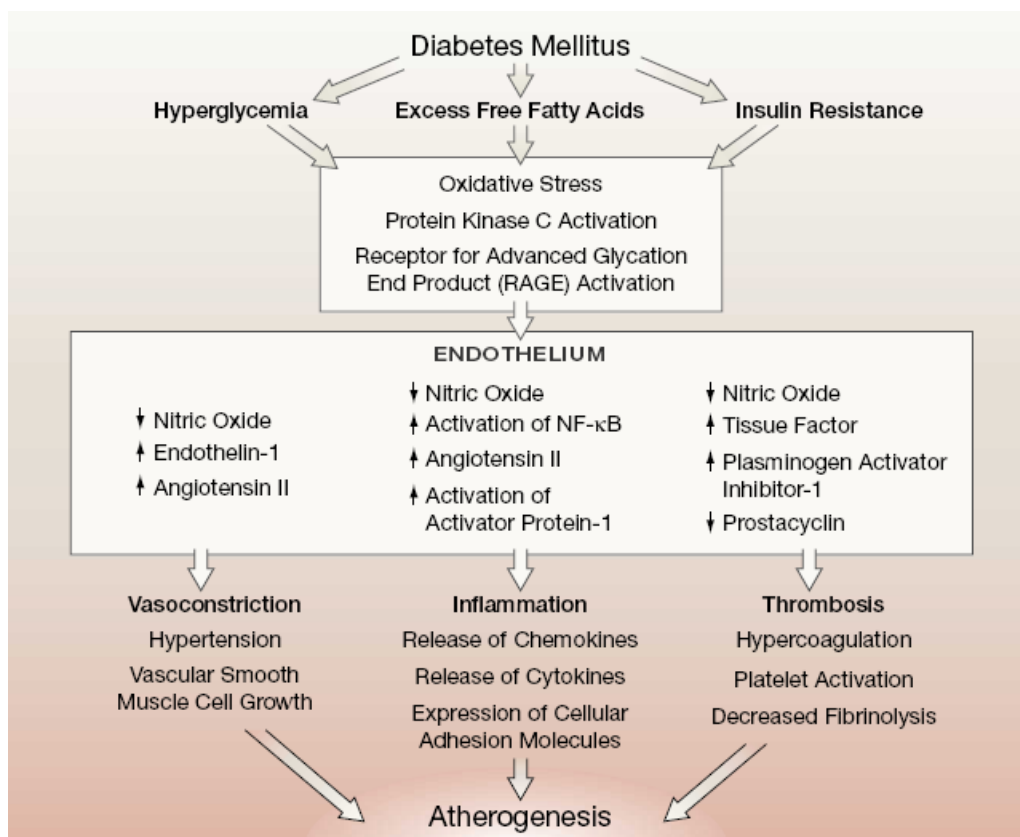
The abnormal metabolic state that accompanies diabetes causes arterial dysfunction.

Relevant abnormalities include chronic hyperglycemia, dyslipidemia, and insulin resistance. These factors render arteries susceptible to atherosclerosis.

Diabetes alters function of multiple cell types, including endothelium, smooth muscle cells, and platelets, indicating the extent of vascular disarray in this disease (figure 2).

At the cellular level, advanced glycation end products associated with diabetes promote also mineralization in cultures of microvascular pericytes.

Fig. 2 Endothelial Dysfunction in Diabetes (30)



In diabetes, hyperglycemia, excess free fatty acid release, and insulin resistance engender adverse metabolic events within the endothelial cell. Activation of these systems impairs endothelial function, augments vasoconstriction,

increases inflammation, and promotes thrombosis. Decreasing nitric oxide and increasing endothelin-1 and angiotensin II concentrations increase vascular tone and vascular smooth muscle cell growth and migration. Activation of the transcription factors nuclear factor kB (NF-kB) and activator protein 1 induces inflammatory gene expression, with liberation of leukocyte-attracting chemokines, increased production of inflammatory cytokines, and augmented expression of cellular adhesion molecules. Increased production of tissue factor and plasmin activator inhibitor creates a prothrombotic milieu, while decreased endothelium derived nitric oxide and prostacyclin favors platelet activation (30).

Even though a number of signaling pathways have been implicated in diabetic vasculopathy (6, 31-33), the links between hyperglycemia and vascular disease are still incompletely understood.

## **Vascular calcification and cardiovascular risk**

In 2009, a meta-analysis of 30 prospective cohort studies demonstrated the consistent finding that presence of vascular calcification poses an increased risk of cardiovascular and all-cause mortality (34).

In coronary arteries, calcified plaque independently predicts a 1.7 fold increase mortality (35). When coronary calcification is extensive, mortality is increased 60-fold (35).

Calcification of peripheral arteries also independently predicts mortality as well as risk of amputation (36).

Recent research showed changes in the mechanical properties of the atherosclerotic lesion and increased inflammation in response to calcification, which may increase the risk of plaque rupture.

Calcium deposits might affect plaque stability by introducing compliance mismatch at the interface of the rigid mineral with the more distensible artery wall tissue. Under mechanical stress, this interface has an increased risk of

mechanical failure (plaque rupture)(37). Such plaque rupture is believed to cause most myocardial infarction and stroke events. The net effect of calcium deposition on risk of rupture depends on the anatomic orientation of the calcium deposits relative to plaque and any necrotic core.

Aortic rigidity, the most insidious effect of vascular calcification, results in hypertension, left ventricular hypertrophy, ischemia, heart failure, amputation and death (6).

More than a century ago, investigators recognized vascular calcification as a form of extraskeletal ossification. This concept was forgotten in the past century, when cholesterol dominated the field: vascular calcium deposits became regarded as a passive, inevitable, unregulated, and degenerative consequences of aging.

In the past decade, however, the prevalence, significance, and regulatory mechanisms of vascular calcification have gained recognition among investigators and clinicians.

## **Pathobiology of arterial calcification**

Vascular calcification recapitulates embryonic osteogenesis. Pathologists in the 19<sup>th</sup> century recognized the presence of bone-like issue within atherosclerotic arteries, with lamellar structure, osteoblast-like cells and hemopieric elements. Yet, for most of the 20<sup>th</sup> century, vascular calcification has been regarded as a passive, unregulated, degenerative process occurring within advanced atherosclerotic plaques. The concept of regulated ossification as the mechanism behind vascular calcification has re-emerged only in the past decade.

Ossification has been identified histologically in 60% of restenotic aortic valves after ballon valvuloplasty. Approximately 15% of carotid atherosclerotic plaque specimens and calcified cardiac valve tissue have ossification. Vascular calcification may include both osteogenic and chondrogenic differentiation. Although osteoblasts and chondrogenic are distinct cell types, they have substantial

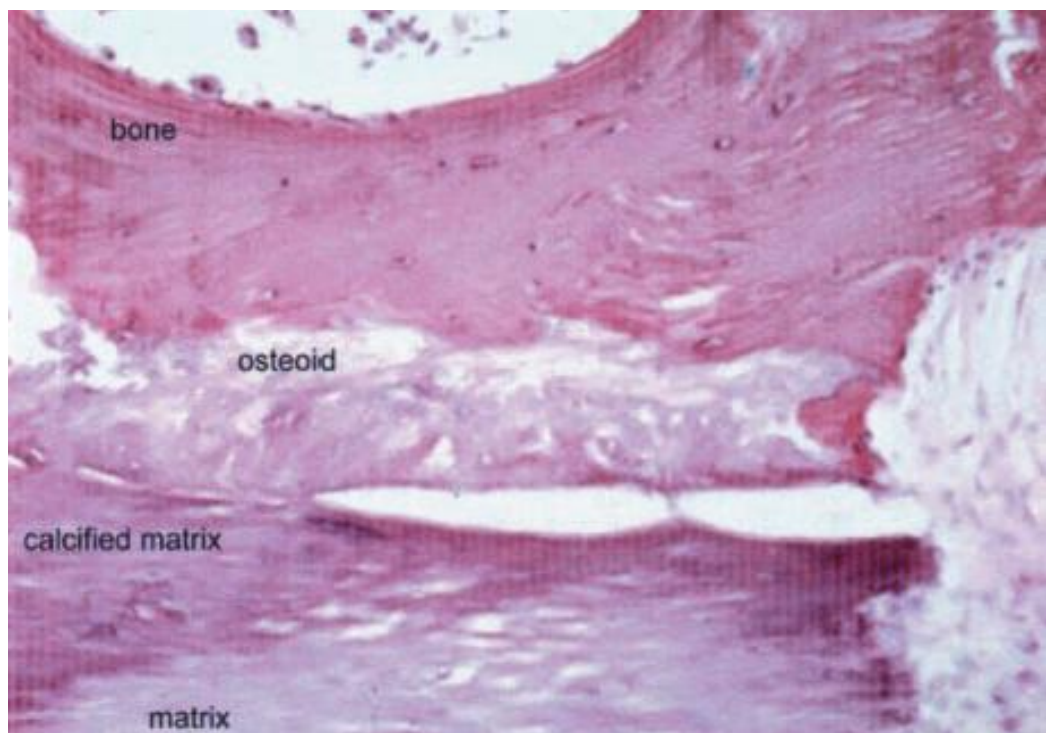


overlap in mineralization mechanism and gene expression, including alkaline phosphatase, Cbfa-1 and osteopontin.

Vascular smooth muscle cells (VSMc), migrated from the media to the intimal layer of the vasculature lose their contractile phenotype and change into so-called synthetic VSMc. When these VSMc become apoptotic in the atherosclerotic lesion they may form the nidus for calcification. (39). Moreover, VSMc can change their phenotype upon calcification and develop features of osteoblast or chondrocyte-like cells with respect to gene expression (40).

The progression from amorphous mineral to ossification follows the same stages as embryonic endochondral ossification. The earliest stage is an acellular, mineralized matrix. This matrix is partially replaced with osteoid, which undergoes remodeling as neoangiogenic vessels invade. Finally mature bone tissue forms as the osteoid mineralizes.

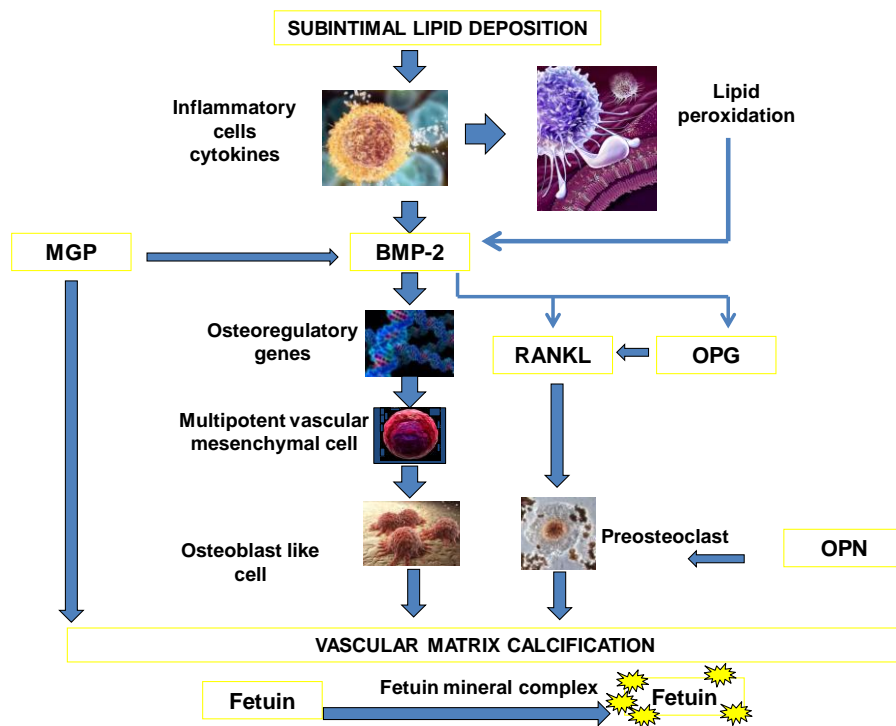
Figure 3. Transitional stages in vascular calcification recapitulate embryonic endochondral ossification, including acellular matrix (matrix), amorphous mineralized matrix (calcified matrix), remodeling (osteoid), and, after ingrowth of angiogenic vessels, complete bone tissue (bone) (3).



A variety of proteins have been identified as inhibitors of calcification, whereas others promote vascular calcification.

Below we discuss the role of matrix Gla protein, Bone morphogenetic protein and OPG/RANKL/RANK axis in the arterial calcification.

Figure 4. The different protein and their role in the calcification process.



## **Matrix GLA protein**

MGP is a vitamin K-dependent protein, produced by VSMc and chondrocytes (41).

There is an active and an inactive form depending on whether or not the protein has been carboxylated (activated) by a vitamin K-driven  $\gamma$ -glutamyl carboxylation. Blocking carboxylation of MGP (i.e. with coumarins) or low vitamin K levels (i.e. deficient intake) results in excessive calcification (42-43).

The function of MGP is believed to be a regulator of bone morphogenetic protein type 2 (BMP2), but it can also bind directly to calcium crystals in the vascular matrix, thereby preventing further calcification growth. (44).

Animal studies show that a deficiency or impairment of MGP (blocking vitamin K action by coumarins) lead to rapid and extreme calcification on the vascular matrix (45).

In the human “Keutel Syndrome”, an autosomal recessive disorder in which patients lack mature MGP, excessive calcification of large arteries is seen (46).

Circulating uncarboxylated MGP levels are inversely proportional to coronary calcification and were significantly lower in patients who underwent PTCA versus a healthy control population (47).

Several experimental studies suggest that MGP, produced in the vascular matrix, is transported to plasma in combination with fetuin-A (another inhibitor of vascular calcification), forming the fetuin-A-mineral complex. Whether uncarboxylated MGP levels are a reliable reflection of the calcification process in the vascular wall is not fully clear, although from the previously mentioned studies it seems that patients with high cardiovascular risk have lower serum MGP levels.

## **Bone Morphogenetic protein**

BMPs are members of the transforming growth factor (TGF)  $\beta$  superfamily, and play key signaling roles in the maintenance and repair of bone and other tissue in adult. Their role in vascular calcification is complex. When VSMC change their phenotype from contractile to synthetic, they enter a state of proliferation in which the expression of smooth muscle markers is diminished. Additionally, they produce large amounts of extracellular matrix proteins and may become osteoblast-like cells. This reduction in smooth muscle marker expression is thought to be crucial in the pathogenesis of atherosclerosis and Monckeberg's sclerosis. The loss of smooth muscle markers can be influenced by BMPs.

Two BMPs, BMP 2 and BMP 7, have been extensively studied in relation to vascular calcification (48).

Expression of BMP2 is found in atherosclerotic lesions, in peri-adventitial myofibroblasts and tunica media cells.

Induction of BMP2 in the vasculature is related to oxidative stress, inflammation, oxidized lipids and hyperglycemia (49). Increased expression of BMP2 stimulates the osteoregulatory gene MSX-2. Then core binding factor-1 (Cbfa-1 or RUNX2) and osterix, both transcription factors, stimulate differentiation of multipotent vascular mesenchymal cells into 'osteoblast-like' cells capable of bone formation and increased intramembranous bone formation in the artery wall (48). The effect of BMP-2 on bone formation is suggested to be modulated by MGP. Diminished VSMC expression of MGP or inactive MGP may lead to unopposed BMP-2 action and hence vascular calcification.

## **OPG, RANK, RANKL**

OPG is a cytochine of the tumor necrosis factor (TNF) receptor superfamily and is classed as an osteoclatogenesis inhibitory factor.

Biochemically, OPG is a basic secretory glycoprotein composed of 401 amino acid residues with 7 distinct structural domains. It exists as either a monomer of 60 Kd or a disulfide bond linked homodimer of 120 kDa (50).

The homodimeric form of OPG is biologically more active than the monomeric form. Although initial studies were done with monoclonal antibodies detecting only the homodimeric form of OPG, more recent studies use the enzyme-linked immunosorbent assay technique to detect total serum OPG levels-monomeric, homodimeric, and OPG bound to its ligands in the serum.

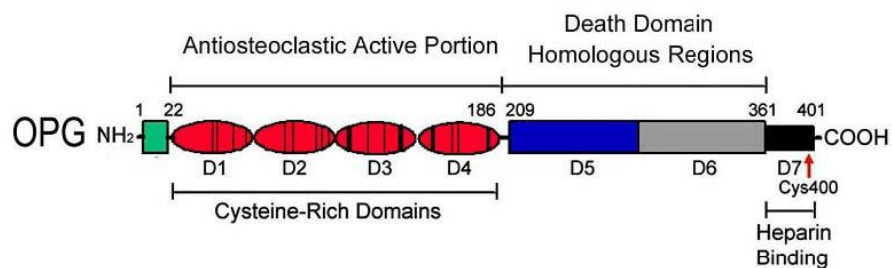
The amino terminal domains 1 through 4 are cysteine rich and confer osteoclastogenesis inibithory proprieties (50).



Domain 5 and 6 at the carboxy terminal end of the protein contain apoptosis-mediating death domain homologous region (50).

Domain 7 contain a heparin-binding region as well as a free cysteine residue required for disulfide bond formation and dimerization (50) (Figure 3).

Figure 5. Schematic representation of the structure of OPG. Main domains and their biochemical and/or functional properties are indicated. NH<sub>2</sub> indicates amino-terminus; COOH, carboxy-terminus



In human, the OPG gene is a single-copy gene extending over 29 kB of the genome in chromosome 8 and contain 5 exons. One major transcription initiation region is located upstream of the initiation ATG codon, and 2 other minor regions are noted further upstream.

OPG is expressed *in vivo* by endothelial cells (ECs), vascular smooth cells (VSMc), and osteoblasts.

Within ECs, OPG is associated with von Willebrand factor within secretory granules called Weibel-Palade bodies.

Upon stimulation with TNF- $\alpha$  or interleukin-1 $\beta$  *in vitro*, the OPG-von Willebrand factor complex is secreted into the surrounding growth medium. This complex is also noted in human serum, indicating EC activation by proinflammatory cytokines as one of the possible sources of circulating OPG in patients with active atherosclerosis (51).

OPG act a decoy substrate to RANKL and competes with RANK, inhibiting RANKL-RANK interactions (52).

RANKL is expressed *in vivo* by osteoblasts, stromal cells, and T lymphocytes. RANK is expressed on the surface

of osteoclast precursor cells such as monocytes, macrophages, and dendritic cells. Interaction of RANKL with RANK activates nuclear factor  $\kappa$  (a transcription factor) by degradation of I $\kappa$ B protein by I $\kappa$ B kinase; this degradation of I $\kappa$ B protein frees the nucleus initiating transcription of specific genes required for differentiation of osteoclasts.

OPG acts a decoy substrate to RANKL and competes with RANK, inhibiting RANKL-RANK interactions (52).

Binding of OPG to RANKL prevents the proliferation and differentiation of osteoclasts and consequently bone resorption. OPG/RANK/RANK also play an important role in vasculature biology and adaptative immunity.

OPG has been demonstrated in normal arteries, whereas RANK, RANK and osteoclasts have been identified mainly in calcified arteries (52).

A steady balance between RANKL and OPG prevents disorders in bone remodeling and vascular calcification.

Selective deletion of OPG in mice results in early-onset severe osteoporosis as well as significant medial

calcification of the aorta and renal arteries (53). When compared with OPG +/+ mice, OPG -/- mice display increased calcification of the aortic media, particularly when given a high dose of phosphate or vitamin D3.

Contrary to the apparent protective role of OPG observed in the animal models, there seems to be a distinct relationship between serum levels of OPG and severity of atherosclerosis in human studies.

A few studies have explored the association between OPG levels and traditional cardiovascular risk factors (54-55). The only consistent association seems to be with increasing age and duration of diabetes (55).

There is no consensus on the association of other established cardiovascular risk factors such as body mass index, serum low-density lipoprotein, high-density lipoprotein, or systolic hypertension.

In human studies, a significant association between levels of circulating OPG and vascular calcification has been described.

Clinically, high serum levels of OPG are associated with atherosclerosis or risk factors for atherosclerotic disease indicating a compensatory increase in OPG levels in response to progressive atherosclerosis and thus OPG may lessen vascular calcification.

Clancy et al. (56) reported such an association with the presence of abdominal aortic calcification, a known risk factor in the development of abdominal aortic aneurism (AAAs).

In a cross-sectional study, Ziegler et al (57) reported a positive correlation between serum levels of OPG and severity of peripheral artery disease, but there was no statistically difference in OPG levels between patients with documented peripheral artery disease and healthy controls.

There have been also a number of studies which have attempted to elucidate the relationship between serum OPG and insulin sensitivity/resistance.

In type 2 diabetes patients, serum levels of OPG appear to be higher when compared to non-diabetic controls, but it

is not clear whether this is simply due to the hyperglycaemic state or a marker of diabetes-related vascular complications.

In a prospective study Anand et al. demonstrated the association between atherosclerotic plaque burden and OPG levels. In this study, 510 asymptomatic diabetic patients had coronary artery calcium (CAC) scans and were followed over a period of  $18 \pm 5$  months after which a repeat scan was performed. Increased CAC scores were significantly associated with plasma levels of OPG in a multivariate model adjusted for other risk factors, statin use, and duration of diabetes (adjusted odds ratio: 2.84,  $p < 0.01$ ). OPG levels were also significantly higher in subjects who experienced a cardiovascular event during the follow-up ( $p < 0.0001$ ). In contrast, high-sensitivity C-reactive protein and interleukin-6 levels neither correlated with CAC score nor predicted cardiovascular events in the short term (55).

In a prospective observational follow-up study on 283 type 2 patients, followed for a median of 16.8 years, high versus low levels of OPG predicted all-cause mortality

(hazard ratio 1.81, CI 1.21-2.69). In addition, elevated levels of OPG were associated with an increased risk of cardiovascular mortality. The effect was shown to be independent of conventional cardiovascular risk (58).

In relation to RANKL there are inconsistent results. Serum RANKL has been reported to be associated with increased (59) and decreased (60) risk of cardiovascular disease while serum levels appear to be similar in healthy and type 2 diabetes patients.

### *Aim of the study*

Recent observations have suggested that several cytokines involved in bone formation may also be implicated in diabetic vasculopathy. Among these osteoprotegerin (OPG)/RANKL/RANK axis and vitamin K dependent matrix GLA protein (MGP) have been reported to play an important role.

This study aimed to evaluate the relationships between OPG, RANKL and MGP serum levels and carotid atherosclerosis in normal and type 2 diabetic subjects (DM2).



## ***Methods***

### **Study participants**

A total of 340 subjects, 190 normals and 150 with type 2 diabetes (DM2), (mean age:  $56.6 \pm 7.5$  yrs), were recruited to participate in the study.

Participants were excluded if they had malignancy, renal impairment, type 1 diabetes, pregnancy and disorder of calcium metabolism, previous diagnosis of osteoporosis and disorder of calcium metabolism.

All procedures were approved by the Ethics Committees of Siena.

All participants provided written informed consensus.

A full clinical history and physical examination was performed on all study subjects.

Height and weight were measured and body mass index (BMI) was calculated as the weight (kg) divided by the square of the height (m<sup>2</sup>).

## **Blood collection and measurements**

All study participants presented, after an overnight fast, at 8.00 in the morning for serum sampling.

In all subjects we measured total, HDL and LDL cholesterol using colorimetric methods (Autoanalyzer, Falcor 350 Menarini).

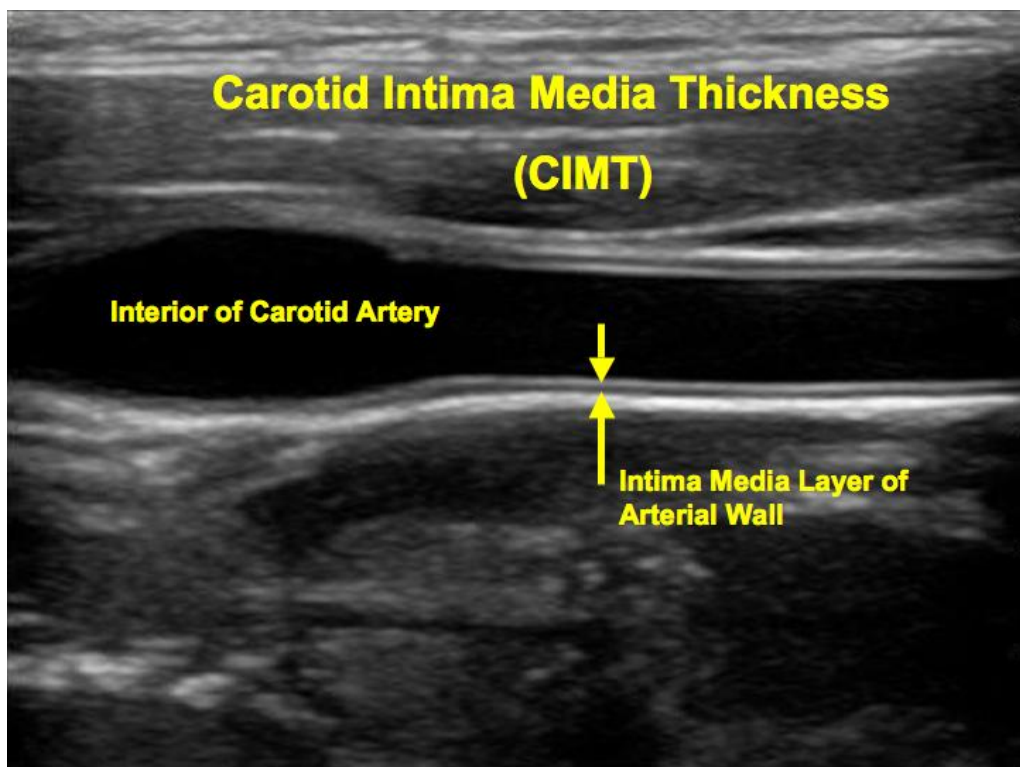
We measured also OPG, RANKL and MGP serum levels, using an ELISA methods (Enzyme-linked immunoassay): OPG (Osteoprotegerin, Biomedica Gruppe, Wien, Austria, intra and interassay coefficients of variation 7% and 7.5% respectively); RANKL (Ampli sRANKL human, Biomedica Gruppe, Wien, Austria, intra and interassay coefficients of variation 8.5% and 5% respectively); MGP (Human MGP Matrix Gla Protein, Biomediac Gruppe, Wien, Austria intra and interassay coefficients of variation 6.5 and 8% respectively).

## **Carotid examination**

An ultrasound examination of carotid vessels was performed to assess intima-media-thickness (IMT), presence of plaque and the degree of calcification.

Carotid intima-medial thickness (CIMT), measured by B-mode ultrasound, is a marker for atherosclerosis. CIMT is correlated with the known risk factors for cardiovascular disease and is an independent predictor of myocardial infarction and ischemic stroke. Consequently, CIMT is used as an outcome measure in intervention trials and has recently been promoted as a method for assessing cardiovascular risk in individual patients in clinical practice (61-64).

Figure 6. Carotid intima media thickness



The subjects were examined in the supine position with the head slightly tilted to the opposite side.

The examination were performed by one experienced examiner, and with the use of a Toshiba power vision ultrasound scanner equipped with a linear array 7.5 MHz transducer.

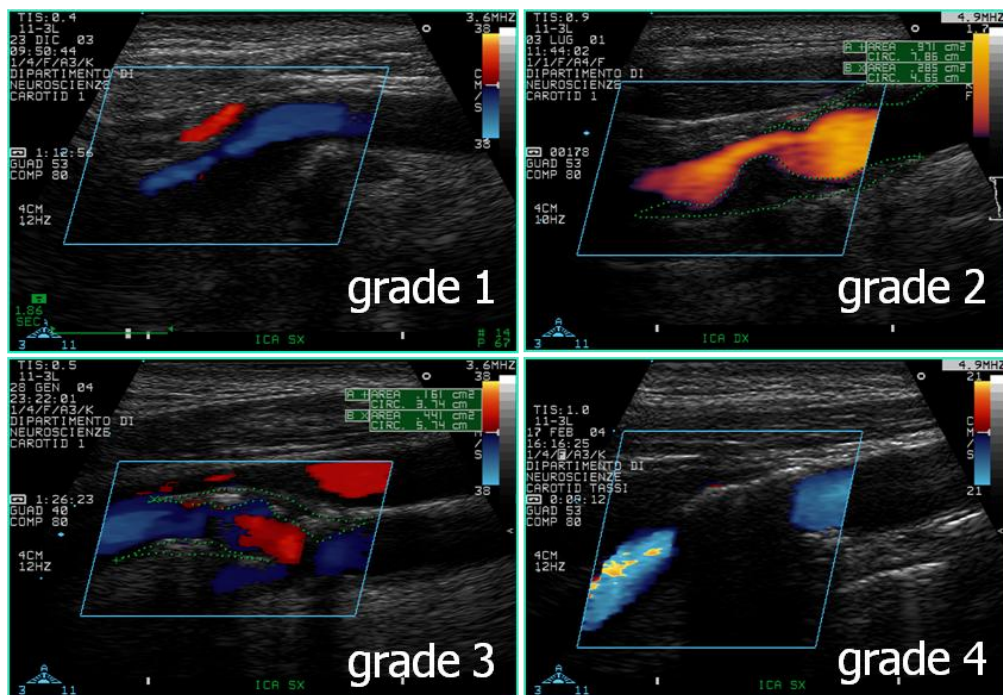
In brief, IMT was measured on the common carotid artery, carotid bifurcation, and internal carotid artery of the left and right carotid arteries. On a longitudinal, two-dimensional ultrasound image of the carotid artery, the anterior (near) and posterior (far) walls of the carotid artery are displayed as two bright white lines separated by a hypoechoic space. The distance between the leading edge of the first bright line of the far-wall (lumen-intima interface) and the leading edge of the second bright line (mediaadventitia interface) indicates the intima-media thickness. When an optimal longitudinal image was obtained, it was frozen and the frozen images were digitized. The beginning of the dilatation of the distal common carotid

artery served as a reference point for the start of measurement. The average of the intima-media thickness of each of the three frozen images was calculated.

We attempted to identify and record atherosclerotic plaques from six segments of the carotid artery: a plaque was defined as a localized protrusion of the vessel wall into the lumen.

Plaque echogenicity was graded from 1 to 4, where grade 1 denotes low echogenicity or echolucency (defined as a plaque appearing black or almost black as flowing blood), and grade 4 denotes strong echogenicity (defined as a plaque appearing white or almost white, similar to the far wall media-adventitia interface) (Fig 4). Plaques that were difficult to classify because of echo-shadowing from calcifications in near wall plaques, calcifications just below the surface of a far wall plaque hiding substantial parts of the rest of the plaque, or unsatisfactory imaging quality were defined as unclassifiable.

Figure 7. Plaque echogenicity



## ***Results***

Clinical characteristics of the two different patient categories of the study population are presented in Table 1.

The diabetic group had diabetes for  $6.78 \pm 6.46$  years and had glucose control with mean glycated haemoglobin (HbA1c) of  $7.1 \pm 1.2\%$ .

As expected weight and BMI were significantly higher in diabetic subjects compared to the healthy non diabetic controls.

Table 1. Clinical parameters

	<b>Normal Subjects</b>	<b>Diabetic subjects</b>
N (M/F)	190 (82/108)	150 (72/78)
Age (yrs)	$57.12 \pm 7.58$	$58.15 \pm 6.61$
Weight (Kg)	$72.8 \pm 14.0$	$81.5 \pm 18.2$ **
BMI (Kg/m <sup>2</sup> )	$27.0 \pm 4.71$	$29.3 \pm 5.6$ *
Duration diabetes (yrs)	-	$6,78 \pm 6.46$
HbA1c	-	$7.1 \pm 1.2$

\*p<0.05, \*\*p<0.01



IMT, plaque prevalence and stenosis percentage were significantly higher in patients with type 2 diabetes when compared to controls.

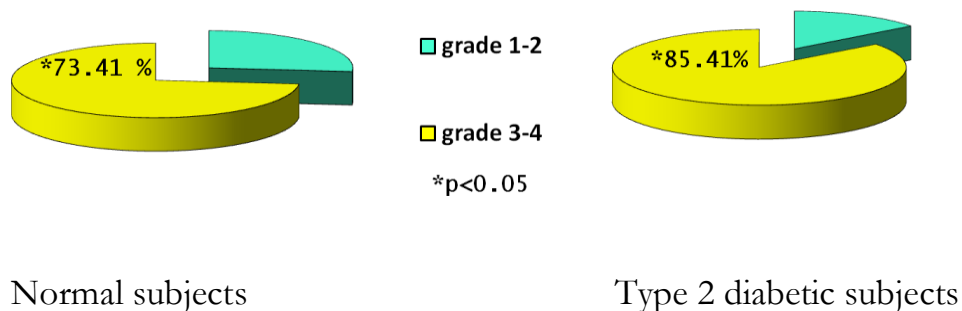
Table 2. Vascular parameters

	Normal Subjects	Diabetic subjects
IMT (mm)	0.89 ± 0.29	1.04 ± 0.35 *
Plaque prevalence (%)	30,7	48,4 **
Stenosis %	9.17 ± 15.8	16.6 ± 19.2 **

\*p<0.05, \*\*p<0.01

The prevalence of more calcified carotid plaques (grade 3 and 4) resulted significantly higher in the patients with type 2 diabetes respect to controls (Figure 8).

Figure 8. Prevalence of the different grade of carotid plaque in the two groups.



MGP resulted significantly lower in DM2 patients than in normal subjects ( $7,39 \pm 2,2$  nmol/l vs  $12,9 \pm 6,4$  nmol/l;  $p < 0.01$ ), whereas RANKL resulted significantly higher in DM2 patients ( $0.11 \pm 0.12$  pmol/l vs  $0.09 \pm 0.14$  pmol/l).

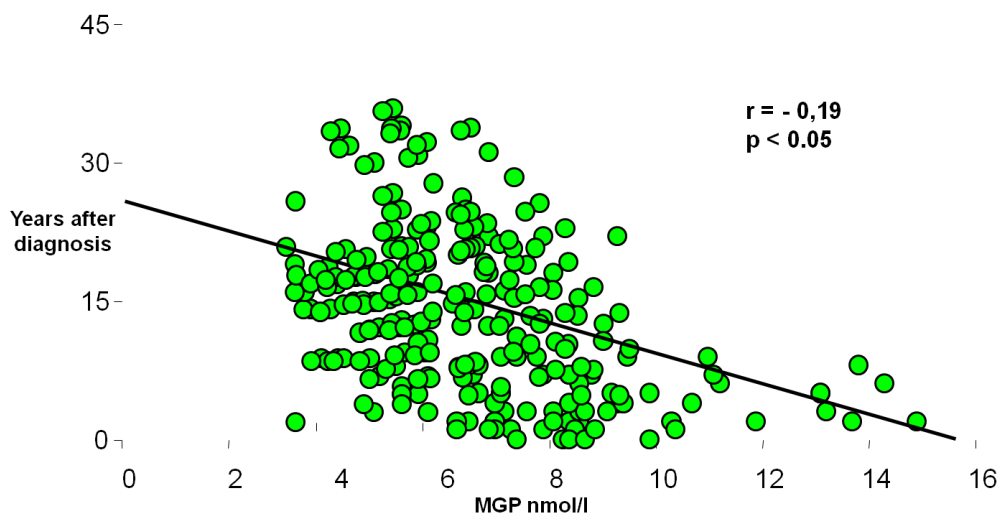
OPG resulted higher in DM2 patients than in normal subjects, but the difference was not statistically significant.

Table 3. Biochemical parameters

	Normal Subjects	Diabetic Subjects
Glycemia (mg/dl)	$99.8 \pm 13.1$	$130.5 \pm 32.7$
Total Cholesterol (mg/dl)	$213.0 \pm 42.9$	$189.5 \pm 38.6$ *
HDL Cholesterol (mg/dl)	$55.6 \pm 13.8$	$56.0 \pm 22.6$
LDL Cholesterol (mg/dl)	$129.0 \pm 37.2$	$101.9 \pm 32.7$ *
Triglycerides (mg/dl)	$127.8 \pm 77.7$	$148.3 \pm 80.3$
Uric Acid (mg/dl)	$5.1 \pm 1.3$	$4.6 \pm 1.6$
OPG (pmol/l)	$4.42 \pm 2.17$	$4.82 \pm 2.20$
RANKL (pmol/l)	$0.09 \pm 0.14$	$0.11 \pm 0.12$ *
MGP (nmol/l)	$12.91 \pm 6.41$	$7.39 \pm 2.25$ **

The serum levels of MGP were also inversely correlated with the years after diagnosis of diabetes ( $R = -0.19$ ,  $p < 0.05$ ).

Figure 9. Correlation between MGP and years after diabetes diagnosis



In both normal subjects and DM2 patients MGP was inversely associated with carotid stenosis ( $r = -0,15$ ;  $p < 0,05$  and  $r = -0,17$ ;  $p < 0,05$ ). A significant correlation between MGP and IMT was found only in DM2 patients ( $r = -0,17$ ;  $p < 0,05$ ).

Table 4. Correlation between carotid parameters and biological markers in normal subjects

	IMT	Stenosis %
OPG (pmol/l)	0.06	0.07
RANKL (pmol/l)	0.01	-0.03
MGP (nmol/l)	0.02	-0.15 *

\* p<0.05

Table 5. Correlation between carotid parameters and biological markers in diabetic subjects

	IMT	Stenosis %
OPG (pmol/l)	0.08	0.03
RANKL (pmol/l)	0.03	0.12
MGP (nmol/l)	-0.17 *	-0.17 *

\* p<0.05

## ***Conclusions***

The results of the our study confirm the relationship between carotid atherosclerosis and type 2 diabetes.

In fact, in the diabetic population, the IMT, the stenosis percentage and the prevalence of the carotid plaques were founded statistically significant higher with respect to control subjects.

Other there, the type 2 diabetic subjects presented more frequently type 3-4 plaques (higher echogenicity).

About serum level parameters, MGP resulted significantly lower in DM2 patients, whereas RANKL resulted higher in DM2 patients with respect to the control subjects.

Between serum markers studied, we founded that only MGP was associated with carotid atherosclerosis: in both normal subjects and DM2 patients, MGP was inversely associated with carotid stenosis and only in DM2 patients there was a significant correlation between MGP and IMT.

In conclusion, our findings seem to demonstrate that several factors known to be involved in the process of bone formation, such as OPG/RANK and MGP, play an important role in the carotid atherosclerotic process, so confirming the presence of links between mineral metabolism and cardiovascular diseases, especially in the diabetic population.

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