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# Association of biochemical parameters of bone metabolism with progression and/or development of new aortic calcifications

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## Summary

***Objetive:*** Biochemical parameters continue to be the most widely used option for the follow-up of patients with bone metabolic disorders. The objective of our study was to assess the association of some biochemical markers of bone metabolism with the appearance and progression of aortic calcifications.

***Material and methods:*** In this study, 624 men and women older than 50 years were selected at random. The participants completed a questionnaire and underwent two lateral dorsal-lumbar x-rays and bone densitometry. Four years later, the same studies were repeated in 402 subjects along with a biochemical study.

***Results:*** Age and the proportion of men were higher in those who had "global progression" of aortic calcification (progression of the existing ones plus new ones). The serum levels of calcium and calcitriol were significantly higher and those of osteocalcin significantly lower in which "global progression" of aortic calcification was observed. Multivariate analysis showed that only osteocalcin was independently associated with "global progression" of aortic calcification, with an 18% decrease for each 1 ng/mL increase in osteocalcin levels (odds ratio (OR)=0, 82; 95% confidence interval (95% CI): 0.71-0.92). The categorization of osteocalcin into tertiles showed that the subjects of the first tertile (<4.84 ng/mL) were associated with a higher proportion of new aortic calcifications: (OR=2.45; 95% CI: 1.03-3, 56) with respect to the third tertile (>6.40 ng/mL).

***Conclusion:*** Serum levels of osteocalcin could be a biochemical marker to evaluate the appearance and/or evolution of aortic calcification. However, it is necessary to determine with greater precision how it could exert this protective effect in the process of vascular calcification.

**Key words:** osteocalcin, vascular calcification, biochemical markers, bone mineral density.

## Introduction

Atherosclerosis, arteriosclerosis, vascular calcification and osteoporosis are common age-related disorders associated with high morbidity and mortality[1,2]. Due to the increased life expectancy in the Spanish population, these disorders are expected to become more and more frequent in the coming decades. Although recent work has been carried out on the development of non-invasive techniques for the early detection of vascular calcifications, such as pulse wave velocity and non-contrast carotid ultrasound, serum biochemical parameters continue to be the most widely used option for monitoring patients with bone metabolic disorders[3-5].

Having easily accessible non-invasive tools such as biochemical markers allow for the adoption of therapeutic measures in order to mitigate the deleterious effect of bone loss. Taking into account that osteoporosis and vascular calcification share etiopathogenic mechanisms[6,7], some biochemical parameters used to study bone metabolism could serve as possible markers of vascular cal-

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cification. Therefore, this study aims to assess the association of some biochemical markers of bone metabolism with the appearance and progression of aortic calcifications.

## Material and methods

This study used data from a European project designed to determine the prevalence of vertebral fracture (European Vertebral Osteoporosis Study - EVOS)[8], in which the Bone and Mineral Metabolism Service of the Central University Hospital of Asturias took part.

For this work, 308 men and 316 women over 50 years of age were selected at random from the municipal registry of Oviedo. Our protocol involved patients' filling out a questionnaire on risk factors related to osteoporosis, two lateral dorsal-lumbar x-rays and a bone densitometry (DXA) (the radiographic study was not completed in only 2 cases), and collecting anthropometric measurements such as height and weight to determine body mass index (BMI). All subjects had sufficient stamina to go up two floors without an elevator and 99% lived in their own home.

After four years, they were invited to repeat the radiological study, bone densitometry, anthropometric measurements and respond to a questionnaire on risk factors for osteoporosis and a biochemical study. In the second control, 402 subjects participated (213 women and 189 men), of whom 335 agreed to carry out the biochemical study. A total of 67 subjects (16.7%) were excluded from the analysis because they had been treated for osteoporosis or their renal function was impaired with serum creatinine greater than 0.8 mg/dL in women and 1.1 mg/dL in men, respectively. All data were available at baseline and at 4 years in 262 subjects.

### Evaluation of the progression of vascular calcification

Abdominal aortic calcification was evaluated by two independent investigators, and was defined and classified as grade 0 (absent), grade 1 (mild-moderate), and grade 2 (severe). Isolated punctate calcifications, a visible linear calcification in less than 2 vertebral bodies, or a dense calcified plaque were defined as mild-moderate calcification[9]. The presence of a visible linear calcification along at least two vertebral bodies and/or the presence of two or more calcified dense plaques was defined as severe calcification. The degree of intra- and inter-observer concordance in the analysis of the x-rays was 92% and 90%, respectively, with a Kappa coefficient of 0.78 and 0.73, data that indicate good reproducibility[9].

The progression of aortic calcification was determined by comparing the x-rays taken at baseline with those at 4 years. It was defined as "global progression" of aortic calcification when an increase in the magnitude of baseline aortic calcification coexisted with the appearance of new calcifications, comparing the x-rays at the outset with those done 4 years later.

### Densitometric evaluation

Bone mineral density (BMD) was measured with a Hologic® QDR-1000 DXA densitometer (Hologic Inc., Waltham, Massachusetts, USA). In all cases, the anteroposterior lumbar spine (L2-L4) and the proximal extremity of the right femur were analyzed. For the evaluation of lumbar BMD, 4 subjects with marked degenerative osteoarthritis were excluded. The coefficients of variation (CV) were 1.2% and 1.9%, respectively[9]. The precision and quality control was performed daily with a lumbar spine phantom, with which a CV of 0.0±0.1% was obtained. In the fourth year, BMD was determined in the same areas used in the first study, and the percentage of change between both measurements was used to evaluate changes in BMD.

### Biochemical analysis

In the baseline study, no biochemical study was carried out. At 4 years, a fasting blood and urine sample was taken from each subject participating in the study. Once the serum was separated, the latter and the urine were kept frozen at -80ºC until quantification. Serum calcium, creatinine, phosphorus, total alkaline phosphatase, and acid resistant tartrate phosphatase were measured using an autoanalyzer (Hitachi Mod. 717, Ratigen, Germany). The serum levels of calcidiol (25OHD) were determined by prior extraction with acetonitrile (IDS, Ltd., Bolton, United Kingdom), whose intra- and inter-assay coefficients of variation (CV) were 5.2% and 8.2%, respectively.

Levels of 1,25-dihydroxyvitamin D were measured by radioimmunoassay (IDS, Ltd.); intra- and interassay CVs were 6.5% and 9%, respectively. The intact PTH and total osteocalcin levels were measured by radioimmunoassay (Nichols Institute, San Juan Capistrano, California, USA). Intra- and inter-assay CV values were 2.6% and 5.8% for PTH and 4.5% and 5.1% for osteocalcin, respectively.

All the tests carried out followed the principles set forth in the Declaration of Helsinki and were formally approved by the Committee for Clinical Trials of the Principality of Asturias.

### Statistic analysis

Data analysis was carried out using SPSS version 17.0 for Windows. The quantitative variables were analyzed by Student's t test and the qualitative variables by chi-square.

Multivariate analysis was performed using logistic regression adjusting for age, sex and BMI, in those serum or urinary markers in which the univariate analysis was significantly associated with progression and/or appearance of new abdominal aortic calcification.

Pearson correlations were performed between those biochemical parameters that, at the multivariate level, showed a significant association with the percentage of change in BMD between both cross-sectional studies.

## Results

The mean age of those who had "global progression" of aortic calcification (progression of existing vascular calcifications plus new vascular calcifications) was higher than the age of those in whom this situation was not observed (Tables 1 and 2). However, there were no age differences in those who only presented a new aortic calcification as a change in the control at 4 years (Table 3). The BMI was similar in those with aortic calcification, both in those in which the calcification progressed, as in those with new calcifications, or considering both variations together (Tables 1-3).

Male sex was significantly more frequent in those in whom progression of existing aortic calcifications and/or new aortic calcifications was observed. In contrast, there were no differences in the smoking habit (Tables 1-3).

**Table 1. Clinical and anthropometric variables and biochemical markers of bone and mineral metabolism in the presence or absence of "global progression" of vascular calcification (CV)**

| Progression more new CV | Aortic calcification (n=118) | No aortic calcification (n=144) | P value |
|---|---|---|---|
| Male gender | 77 (72.3%) | 61 (42.4%) | **<0.001** |
| Smoker | 23 (19.5%) | 18 (12.5%) | 0.121 |
| Age (years) | 69.6 ± 7.7 | 66.4 ± 8.9 | **0.002** |
| BMI (kg/cm$^2$) | 28.0 ± 3.8 | 28.4 ± 4.2 | 0.367 |
| PTH (pg/mL) | 54.0 ± 27.1 | 51.8 ± 20.5 | 0.460 |
| Alkaline phosphatase (IU/L) | 177 ± 89 | 175 ± 55 | 0.817 |
| Calcium (mg/dL) | 9.46 ± 0.30 | 9.35 ± 0.34 | **0.011** |
| Phosphorus (mg/dL) | 3.45 ± 0.44 | 3.44 ± 0.48 | 0.964 |
| Calcidiol (ng/mL) | 15.5 ± 7.5 | 17.4 ± 9.8 | 0.092 |
| Calcitriol (pg/mL) | 43.9 ± 17.3 | 39.4 ± 14.4 | **0.025** |
| Osteocalcin (ng/mL) | 5.42 ± 1.76 | 6.22 ± 2.15 | **0.002** |
| FATR (U/L) | 2.02 ± 0.65 | 2.13 ± 0.64 | 0.212 |
| Ca/creatinine urine | 0.18 ± 0.11 | 0.17 ± 0.10 | 0.675 |

The variables are expressed in number (percentage) and in mean ± standard deviation; BMI: body mass index; PTH: parathormone; FATR: tartrate-resistant acid phosphatase.

**Table 2. Clinical and anthropometric variables and biochemical markers of bone and mineral metabolism in the presence or absence of progression of vascular calcifications (VC)**

| CV progression | Aortic calcification (n=62) | No aortic calcification (n=144) | P value |
|---|---|---|---|
| Male gender | 40 (64.5%) | 61 (42.4%) | **0.003** |
| Smoker | 13 (21.0%) | 18 (12.5%) | 0.119 |
| Age (years) | 70.6 ± 8.2 | 66.4 ± 8.9 | **0.002** |
| BMI (kg/cm$^2$) | 27.8 ± 4.0 | 28.4 ± 4.2 | 0.319 |
| PTH (pg/mL) | 54.7 ± 29.2 | 51.8 ± 20.5 | 0.428 |
| Alkaline phosphatase (IU/L) | 175 ± 51 | 175 ± 55 | 1.000 |
| Calcium (mg/dL) | 9.48 ± 0.26 | 9.35 ± 0.34 | **0.009** |
| Phosphorus (mg/dL) | 3.42 ± 0.45 | 3.44 ± 0.48 | 0.774 |
| Calcidiol (ng/mL) | 14.8 ± 7.6 | 17.4 ± 9.8 | 0.082 |
| Calcitriol (pg/mL) | 42.8 ± 17.2 | 39.4 ± 14.4 | 0.154 |
| Osteocalcin (ng/mL) | 5.46 ± 1.87 | 6.22 ± 2.15 | **0.019** |
| FATR (U/L) | 2.09 ± 0.58 | 2.13 ± 0.64 | 0.692 |
| Ca/creatinine urine | 0.17 ± 0.10 | 0.17 ± 0.10 | 0.891 |

The variables are expressed in number (percentage) and in mean ± standard deviation; BMI: body mass index; PTH: parathormone; FATR: tartrate-resistant acid phosphatase.

Regarding the biochemical markers of bone metabolism, serum levels of calcium and calcitriol were significantly higher and those of osteocalcin significantly lower in those subjects in whom "global progression" of aortic calcification was observed (new calcifications plus progression of vascular calcification ) (Table 1).

The logistic regression analysis adjusted for age, sex and BMI showed that the only biochemical marker that was independently associated with "global progression" of aortic calcification was osteocalcin, showing that increases of 1 ng/mL were associated with a decrease in 18% in the progression of aortic calcification (odds ratio (OR)=0.82; 95% confidence interval (95% CI): 0.71-0.95) (Table 4). Age and male sex were also significantly associated with progression of vascular calcification (OR=1.05; 95% CI: 1.01-1.08 and OR=2.06; 95% CI: 1.20-3.54, respectively) (Table 4).

In the univariate analysis, serum osteocalcin levels were significantly lower and calcium levels significantly higher in those subjects in whom aortic calcification had progressed (Table 2). The logistic regression analysis adjusted for age, sex and BMI confirmed that osteocalcin was the only parameter that showed a significant association:

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**Table 3. Clinical and anthropometric variables and biochemical markers of bone and mineral metabolism in the presence or absence of new vascular calcifications (VC)**

| New CV | Aortic calcification (n=56) | No aortic calcification (n=144) | P value |
|---|---|---|---|
| Male gender | 37 (66.1%) | 61 (42.4%) | **0.004** |
| Smoker | 10 (17.9%) | 18 (15.0%) | 0.327 |
| Age (years) | 68.5 ± 7.1 | 66.4 ± 8.9 | 0.082 |
| BMI (kg/cm$^2$) | 28.2 ± 3.6 | 28.4 ± 4.2 | 0.691 |
| PTH (pg/mL) | 53.3 ± 24.8 | 51.8 ± 20.5 | 0.676 |
| Alkaline phosphatase (IU/L) | 180 ± 119 | 175 ± 55 | 0.717 |
| Calcium (mg/dL) | 9.43 ± 0.34 | 9.35 ± 0.34 | 0.166 |
| Phosphorus (mg/dL) | 3.47 ± 0.44 | 3.44 ± 0.48 | 0.694 |
| Calcidiol (ng/mL) | 16.3 ± 7.3 | 17.4 ± 9.8 | 0.463 |
| Calcitriol (pg/mL) | 45.2 ± 17.4 | 39.4 ± 14.4 | **0.022** |
| Osteocalcin (ng/mL) | 5.36 ± 1.65 | 6.22 ± 2.15 | **0.009** |
| FATR (U/L) | 1.95 ± 0.73 | 2.13 ± 0.64 | 0.103 |
| Ca/creatinine urine | 0.18 ± 0.11 | 0.17 ± 0.10 | 0.579 |

The variables are expressed in number (percentage) and in mean ± standard deviation; BMI: body mass index; PTH: parathormone; FATR: tartrate-resistant acid phosphatase.

increases of 1 ng/mL were associated with a 16% increase in the progression of aortic calcifications (OR=0.84; 95% CI: 0.70-0.99) (Table 4). Sex (OR=1.95; 95% CI: 1.01-3.76) and also age (OR=1.06; 95% CI: 1.02-1.10) were associated in this multivariate model (Table 4).

When only those subjects who presented a new aortic calcification were analyzed, serum levels of osteocalcin were found to be significantly lower and those of calcitriol, significantly higher (Table 3). The logistic regression analysis adjusted for age, sex and BMI confirmed that only osteocalcin showed a significant association: increases of 1 ng/mL were associated with a 20% appearance of new aortic calcifications (OR=0.80; 95% CI: 0.67-0.97) (Table 4). Male sex (OR=2.30; 95% CI: 1.15-4.59), but not age, was associated in this multivariate model (Table 4).

The categorization of serum osteocalcin levels into tertiles showed that the lowest tertile (osteocalcin <4.84 ng/mL) was the one that showed the highest proportion of new aortic calcifications (22; 42.3%). The second tertile (osteocalcin between 4.84 and 6.40 ng/mL) showed the same trend, but in a lower proportion (18; 34.6%), while the third tertile (osteocalcin >6.4 ng/mL) ) showed the lowest proportion (12; 23.1%). A logistic regression analysis adjusted for age, sex and BMI showed that the subjects of the first tertile were associated with a higher proportion (2.45 times) of new aortic calcifications: (OR=2.45; 95% CI: 1.03-3.56). There were no differences with those of the second tertile (OR=1.48; 95% CI: 0.611-3.56).

The bi-variate correlations between the percentage of change in BMD at the lumbar and femoral neck level and the serum levels of osteocalcin showed a negative and significant correlation. Higher values of osteocalcin were associated with a lower loss of BMD, while lower values of osteocalcin were associated with greater losses of bone mass (Figure 1A and 1B).

## Discussion

Our results confirm that, of the biochemical markers analyzed, osteocalcin was the only marker associated with the appearance and progression of aortic calcifications independently of age, sex and BMI. A 1 ng/mL increase in osteocalcin decreased the "global progression" of aortic calcification by 18%, a protection equivalent to being 3-4 years younger.

Osteocalcin, a vitamin K-dependent protein, is the most abundant non-collagen component in the mineralized matrix of bone. It is not only produced by bone, but also by vascular smooth muscle cells that show a phenotype similar to osteoblasts[10]. It inhibits the precipitation of calcium phosphate and shows a strong affinity for hydroxyapatite[11]. Initially, it was thought that osteocalcin inhibited the growth of hydroxyapatite crystals[12] and limited bone formation[13].

Experimental studies have shown that decarboxylated osteocalcin can up-regulate nitric oxide synthesis in human endothelial cells with a protective effect against endothelial dysfunction. These findings support the opinion that decarboxylated osteocalcin is the biologically active form of the protein, with a protective function on the vasculature independent of its metabolic role, although more studies are required to confirm this fact[14].

Osteocalcin has been detected to a greater degree in calcified plaques and aortic valves than in healthy non-calcified vessels[15,16]. The level of osteocalcin mRNA reportedly increases between 8 and 14 times in calcified aortic plaques compared to healthy aortas[17]. The increase in total osteocalcin may occur as a result from the development of an osteogenic phenotype in atherosclerotic plaques[18]. However, this requires further validation. Recently, osteocalcin has been found to play a crucial role in arterial calcification mediated by Wnt/β-catenin signaling through increased oxidative phosphorylation, and this finding may have clinical implications[19].

**Table 4. Multivariate analysis of the independent variables significantly associated in the univariate analysis with the progression and/or presence of new aortic calcifications. The odd ratio (OR) and the 95 confidence interval (95% CI) are represented. Significant values are shown in bold**

| Dependent variable | Independent variables | OR | IC 95% | P value |
|---|---|---|---|---|
| Global progression of calcification (progression and new) | Age (every year) | **1.05** | **1.01 - 1.05** | **0.007** |
| | Gender (male) | **2.06** | **1.20 - 3.54** | **0.009** |
| | Calcium (each mg/dL) | 1.87 | 0.79 - 4.42 | 0.152 |
| | Calcitriol (each pg/mL) | 1.02 | 0.99 - 1.03 | 0.068 |
| | Osteocalcin (each ng/mL) | **0.82** | **0.71 - 0.95** | **0.007** |
| Progression of aortic calcification | Age (every year) | **1.06** | **1.02 - 1.10** | **0.005** |
| | Gender (male) | **1.95** | **1.01 - 3.76** | **0.046** |
| | Calcium (each mg/dL) | 2.61 | 0.90 - 7.55 | 0.077 |
| | Osteocalcin (each ng/mL) | **0.84** | **0.70 - 0.99** | **0.040** |
| New aortic calcifications | Age (every year) | 1.02 | 0.98 - 1.07 | 0.250 |
| | Gender (male) | **2.30** | **1.15 - 4.59** | **0.018** |
| | Calcium (each mg/dL) | 1.51 | 0.56 - 4.03 | 0.415 |
| | Calcitriol (each pg/mL) | 1.02 | 0.99 - 1.04 | 0.073 |
| | Osteocalcin (each ng/mL) | **0.80** | **0.67 - 0.97** | **0.024** |

Significant values are shown in bold.

However, studies in patients are inconclusive. A relatively recent meta-analysis included 46 studies that examined the association between osteocalcin and atherosclerosis[20]. Of the studies that analyzed the association between osteocalcin and carotid intima-media thickness (CIMT), four reported that higher levels of osteocalcin were associated with greater CIMT, four reported that higher levels of osteocalcin were associated with a lower CIMT, and three did not find any correlation. However, studies that examined mononuclear cells positive for osteocalcin or histological staining for osteocalcin showed that higher levels of osteocalcin were associated with an increase in markers of atherosclerosis and calcification[20]. Thus, it is suggested that osteocalcin could be a marker of the calcification process.

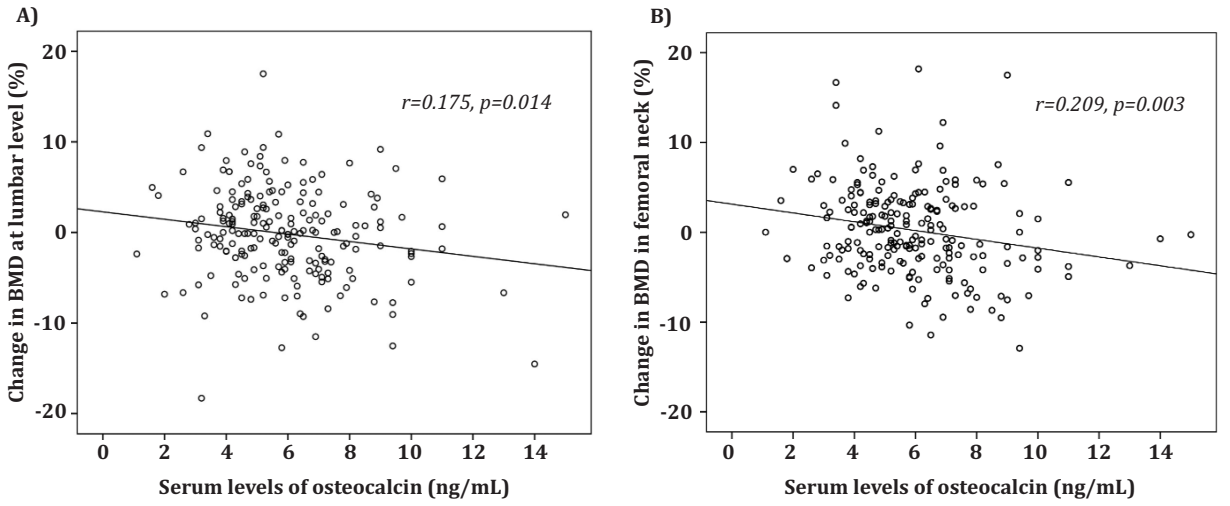
Our results show that, in the 4-year period between both cross-sections, both the presence of new aortic calcifications and their progression were associated with lower levels of osteocalcin, regardless of age, sex and BMI. It is noteworthy that the lowest tertile of osteocalcin (<4.84 ng/mL) was associated with a significant increase in new aortic calcifications: 2.45 (1.03-3.56) compared to subjects with serum osteocalcin levels higher than 6.4 ng/mL.

Kim et al. found similar results in Asian women to those in our study, with an inverse correlation between osteocalcin and vascular calcification measured by the Agatston score, even after adjusting for age[21]. Similar results have also been shown in other cross-sectional[22,23] and longitudinal studies, such as ours, in which raised levels of osteocalcin are found to be associated with less progression of abdominal aortic calcification[24]. These authors suggest that osteocalcin could be involved in the aortic calcification process indirectly by its action on insulin and insulin resistance. Fusaro et al. have recently observed in a population on dialysis that those diabetic patients with a higher prevalence of vascular calcification had lower serum levels of total and decarboxylated osteocalcin[25]. In fact, in a secondary analysis of our study, analyzing osteocalcin levels in those subjects diagnosed with diabetes, it was observed that the presence of diabetes (n=23) was associated with significantly lower levels of osteocalcin than those without diabetes (n=241) (4.89±1.80 ng/mL compared to 5.96±2.14 ng/mL; p=0.020).

It could also be conjectured that low levels of osteocalcin are associated with vascular calcification (VC) due to less bone remodeling, which could be a VC risk factor[26,27]. However, this possibility would not be supported by the results of this study, since the subjects with lower levels of osteocalcin and higher VC were those with lower BMD, which would be more indicative of high remodeling than low remodeling[28].

On the other hand, the usefulness of osteocalcin as a serum marker remains controversial. There is still a long way to go to define whether osteocalcin can be used as a diagnostic or detection tool in the appearance of VC. It is noteworthy that no study has differentiated between forms of osteocalcin when it comes to VC. Consequently, it is necessary to study the effect that carboxylated and decarboxylated osteocalcin could have in this environment, as well as to consider the mechanisms associated with the increase of osteocalcin in calcified tissue[5].

**Figure 1. Bi-variate correlations between changes in BMD in percentage A) at lumbar level and B) femoral neck with serum levels of osteocalcin**




This study presents several limitations. First, osteocalcin determination was only carried out in the second cross section, which limits the associations found. Second, intact or total osteocalcin was determined without differentiating between carboxylated or decarboxylated. On the other hand, the evaluation of vascular calcification was carried out by simple X-ray imaging and not by more sensitive techniques. It is also possible that some of the people who attended the second check-up after 4 years would have done so because they were in a worse physical condition compared to those who did not attend it, although no clear selection biases were found[29].

Despite these limitations, the study also has important strengths, such as the adequate response of the subjects who participated in the study, both at baseline (50%)[30] and at 4 years of the follow-up period (70%). The degree of reliability among observers for the assessment of vascular calcification supports its use as a diagnostic criterion. Finally, unlike other studies, this study was prospective, and not cross-sectional like most of those cited. This reinforces the validity of the results found and their greater degree of association.

Thus, although new studies are needed to confirm these results, this study seems to indicate that serum levels of osteocalcin could be a promising biochemical marker associated with the appearance and/or development of aortic calcification.

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