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Plaque calcification is driven by different mechanisms of mineralization associated with specific cardiovascular risk factors

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Abstract *Background and aims:* The aim of this study was to investigate possible associations among markers of mineralization, plaque instability and the main risk factors of atherosclerosis.
Methods and results: A Tissue MicroArray containing 52 samples of calcified carotid plaques from 52 symptomatic and asymptomatic patients were built. TMA serial sections were used to study the expression of inflammatory and mineralization markers (BMP-2, BMP-4, VDR, RANKL, Osteopontin, Sclerostin, β-catenin and calmodulin) by immunohistochemistry. Our data clearly demonstrated the expression of mineralization markers in atheromatic plaques. Indeed, with the exception of RANKL, all investigated markers were expressed in at least 60% of cases. Specifically, multivariate analysis displayed significant associations between both the expression of BMP-2 and the presence of unstable plaques as well as between the expression of β-catenin and the presence of stable plaques. We also found a significant inverse association between both a) the presence of hypertension and VDR and b) smoking habits and calmodulin expression. Finally, we noted a higher density of RANKL positive cells in plaques from diabetic patients as compared to non-diabetic ones and a significant positive association between hypertriglyceridemia and BMP-4 expression.
Conclusion: Our results support the hypothesis that the process of atherosclerotic plaque calcification presents a number of similarities with the physiological processes that occur in bone, involving both osteoblasts- and osteoclasts-like arterial cells. Finally, the present study suggests that risk factors, such as hypertension, cigarette smoke and diabetes, can cause the destabilization of the atheromatic plaque acting on calcification process as well as inflammation.

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Introduction

Plaque calcification is a common complication of atheromatous disease. Calcification has been long considered a passive, unregulated, degenerative process occurring at the end stage of atherosclerotic plaque formation [1]. However, recent evidences suggest that vascular calcification is an active, organized and regulated process with remarkable similarities to bone formation [1].

Plaque calcification resembles endochondral bone formation in long bones; its progression is driven by osteoblast-like cells and inflammatory factors such as cytokines produced by tissue macrophages and foam cells [2]. The conditions which predispose to vascular calcification are provided by two different mechanisms that involve apoptotic bodies derived from macrophages, and vascular smooth cells (VSMCs) trans-differentiation [1]. Apoptotic bodies and necrotic debris serve as nucleating sites for calcium crystals deposition, mainly hydroxyapatite (HA). It is known that this mechanism results in a positive feedback that increases calcification and inflammation since the early stages of atheromatous disease [1]. The other mechanism affects pericytes and/or VSMCs, which can undergo cellular re-programming to chondrocyte-like and osteoblast-like cells, causing mineralization and bone tissue formation [3]. However, it is not well established if these two processes both lead to calcification or, conversely, if they occur independently.

The process of ectopic calcification, as previously demonstrated in some tissues such as breast and prostate, appears to be orchestrated by molecular mechanisms similar to those that occur during physiological mineralization [4–7].

In a previous study [8], by using energy dispersive X-ray microanalysis (EDX) technology we demonstrated the presence of two different types of calcification in carotid atheromatous plaques: HA and calcium oxalate (CO). In particular, we observed an association between HA and plaque instability, while CO was detected mainly in stable plaques [8]. However, it is currently not clear if calcification occurring in vulnerable plaques is driven by different mechanisms of mineralization as compared to stable plaque. Starting from these considerations, new molecular imaging analyses have been developed to both study the mechanisms of plaque calcifications assess cardiovascular risk associated with the presence of calcifications. Specifically, recent clinical studies proposed that positron emission tomography (PET) imaging with sodium fluoride can identify the presence of calcific deposits in early phases of the formation of plaque calcifications, hence potentially highlighting high-risk plaques [9,10].

In this context, numerous studies have shown that some risk factors such as diabetes and hypertensions, were significantly associated with the presence of arterial calcification [11–14]. Still no biomechanical evidence has yet been put forward.

Since plaque calcification is a well-recognized prognostic factor for coronary and cerebrovascular disease [15–20], the study of in situ expression of biomarkers that characterize the physiological mineralization and their association with classical major risk factors could provide information about the plaque calcification process.

Therefore, the aim of this study was to investigate the possible associations among markers of mineralization, plaque instability and main risk factors of atherosclerosis.

Methods

Cases selection

WE employed a Tissue MicroArray (TMA) made with 52 samples of calcified carotid plaques from 52 symptomatic (major stroke or TIA) and asymptomatic patients submitted to surgical carotid endarterectomy (CEA) at the Hospital of University of Tor Vergata (Rome, Italy) from January to December 2018. All asymptomatic patients had a carotid stenosis >60%, assessed by echography or CT angiography. For symptomatic patients, the time interval between symptoms onset and CEA was between 1 and 3 months. Patients who underwent by CEA were excluded from the study if (1) they had a possible source of cardiac embolization (arrhythmia, stenosis, prolapse or calcification of mitral valve, mechanical cardiac valves, endocarditis, recent myocardial infarction with left ventricular thrombus, atrial myxoma, dilated cardiomyopathy, patent foramen ovale) and (2) the plaque was fragmented. We only included intact calcified carotid plaques from patients submitted to CEA, for whom complete clinical and laboratory assessment of major cardiovascular risk factors was available. The present study includes some cases that have been used in our previous investigation [8].

As all symptomatic patients showed unstable plaques and all asymptomatic patients displayed stable plaques, patients were divided in two subgroups: (a) symptomatic patients with unstable plaques and (b) asymptomatic patients with stable plaques.

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the IRBs of our Institution.

TMA construction

For Tissue MicroArray (TMA) construction, we utilized fragments from the plaque shoulder, identified in

corresponding hematoxylin–eosin (H & E)-stained sections and marked on the donor paraffin block. Areas with calcifications were excluded to allow the correct construction of the recipient master block. Indeed, the presence of calcifications compromise the stability of the matrix. A 2 mm-thick core of the donor block was placed in the recipient master block of the Galileo TMA CK2500 (Brugherio, Milan, Italy). The analysis methods have been previously reported [21].

Histology

From the TMA 12 serial sections (5 mm thick) were cut. Two sections were stained with H & E and Movat pentachrome stains. Plaques were classified into stable and unstable according to the modified American Heart Association atherosclerosis classification [22]. Unstable plaques included (a) thrombotic plaques associated with rupture or erosion of the cap and (b) vulnerable plaque or thin-cap fibro-atheroma (TCFA) characterized by a fibrous cap less than 165 μm thick heavily infiltrated by macrophages, CD68 positive (>25 per high magnification field), without plaque rupture. Plaques not included in this group were considered as stable. Histopathologic examination was performed by two different pathologists (AM, LA). Inter-observer reliability was >98%.

Immunohistochemistry

Ten TMA serial sections were used to characterize the *in-situ* expression of the main mineralization markers reported in Table 1 by immunohistochemistry. Also, inflammatory infiltrates were evaluated by CD68 and CD3 immunostaining. Immunohistochemical reactions were evaluated by counting the number of cells/mm^2. Briefly, antigen retrieval was performed on 3-μm-thick paraffin sections using EDTA citrate pH 7.8 buffers for 30 min at 95 °C. Sections were then incubated for 1 h at room temperature with the primary antibodies reported in Table 1. Reactions were revealed by HRP – DAB Detection Kit (UCS Diagnostic, Italy). To assess the background of immuno-staining we included a negative control for each reaction by incubating the sections with secondary antibodies (HRP) and detection system (DAB). Reactions have been set-up by using specific control tissues as indicated in the data sheets. For each mineralization marker, were considered as positive only plaques with more than 10% of cells with strongly positive signal.

Risk factors

Categorical, dichotomic Risk factors were defined as follows: (a) hypertension: systolic BP ≥ 140 mmHg and/or a diastolic BP ≥ 90 mmHg corresponding to stage 2 of 2017 ACC/AHA guidelines [23] or taking antihypertensive treatment at the time of carotid endarterectomy; (b) diabetes mellitus: patients with fasting blood glucose > 126 mg/dL and/or following oral treatment or insulin therapy; (c) tobacco dependence (patients were categorized as smokers and former smokers). Former smokers who had stopped smoking for less than five years were considered smokers and patients who had not smoked for >5 years were considered non-smokers; (d) hypercholesterolemia: patients with total cholesterol level > 200 mg/d; (e) patients with low HDL-C: < 40 mg/dL in men or <50 mg/dL in women; (f) hypertriglyceridemia: patients with serum triglycerides levels ≥ 150 mg/dL.

Statistical analysis

Data were analyzed using SPSS version 16.0 (SPSS Inc, Chicago, Ill) software. Continuous variables were expressed as the mean ± SD or SE. The Shapiro–Wilk test

Table 1 Immunohistochemical antibodies used in order to characterize the in situ expression of the mineralization markers and inflammation.

Antibody	Characteristics	Target
RANK-L	Rabbit monoclonal clone 12A668; AbCam, Cambridge, UK	Control bone regeneration and remodeling by osteoclast activation
Calmodulin	Mouse monoclonal, clone 2D1; ThermoFisher Scientific, Waltham, MA, USA	Calcium-binding protein involved in bone mineralization
Osteopontin	Mouse monoclonal, clone 1B20; Novus Biologicals, Littleton, CO, USA	Non-collagenous proteins present in bone matrix and involved in the assembly of hydroxyapatite
Sclerostin	Rabbit monoclonal, clona N/A; AbCam, Cambridge, UK	Glycoprotein secreted by osteocyte that inhibit osteoblastogenesis
β-catenin	Mouse monoclonal, clone 14, Ventana, Tucson, AZ, USA	Protein involved in both osteoblastogenesis and osteoclastogenesis
VDR	Rabbit polyclonal clone NBP1-19478; Novus Biologicals, Littleton, CO, USA	vitamin D receptor involved in the uptake of calcium
BMP-2	Mouse monoclonal, clone 1A11; Novus Biologicals, Littleton, CO, USA	Osteoblast induction factor
BMP-4	Rabbit polyclonal clone AB39973; AbCam, Cambridge, UK	Osteoblast induction factor
CD68	Rabbit Monoclonal, clone KP-1; Ventana, Tucson, AZ, USA	Anti-human monocytes – macrophages
CD3	Rabbit Monoclonal, clone 2GV6; Ventana, Tucson, AZ, USA	Anti-human T cell

was used to statistically assess the normal distribution of the data.

In order to assess the univariate differences in continuous variables when stratifying the cohort according to plaque stability (stable and unstable) we employed the Student t-test for independent samples or the Mann Whitney-U test (if the distribution was not normal). Categorical data were analyzed using the chi square test. Association between density (number $\times$ mm^2) of monocytes/macrophages cells and that of T-lymphocytes in the carotid plaques was analyzed by linear regression and the degree of association was evaluated in terms of R^2 as well as in terms of the p-value associated with the regression coefficient.

Finally, we employed multivariate logistic regression to identify independent markers which displayed significant associations with the stability of plaques (stable vs unstable). In detail, the model included all immunohistochemical markers as well as age and gender (as nuisance covariates) as independent variables, and plaque stability/instability as dependent variable. Similarly, in order to speculate about the mechanisms by which each risk factor may influence plaque calcification, we employed separate general linear models which included each immunohistochemical marker as dependent variable and the presence or absence of a single risk factor as well as age and gender (as nuisance covariates) as independent variables. A 2-tailed p value <0.05 was considered statistically significant.

Results

Clinical data

The main clinical characteristics of our patient population are reported in Table 2. The age of the patients included in the study was 68.69 $\pm$ 7.59, min 54 max 80 years. In particular, the average age of patients with stable plaques was 70.35 $\pm$ 6.55 y, whereas the average age of patients with unstable plaques was 66.24 $\pm$ 7.59 y. No significant differences in the age ($p = 0.06$) and gender ($p = 0.20$) were found between stable and unstable plaque groups. Also, 75.0% of patients were hypertensive (39/52) while 53.8% were hypercholesterolemic (28/52). The other risk factors considered in the study showed an incidence in the range of 23.7% (hypertriglyceridemia) to 46.2% (smoking habit). No significant differences were observed between patients with stable and unstable plaques.

Markers of plaque calcification

In the atheromatic plaques, mineralization markers showed variable expression. Among these, sclerostin and osteopontin were expressed in all plaques, with a ratio sclerostin/osteopontin ~2:1 (30.11 $\pm$ 2.23 vs 15.83 $\pm$ 1.11 positive cells/mm^2). Both sclerostin and osteopontin were expressed by cells with macrophage/osteoblast-like phenotype.

RANKL (Receptor activator of nuclear factor kappa-B ligand) expression was observed only in 18 cases (34.6%), in which it was expressed only by cells showing a macrophage/osteoblast-like phenotype in less than 10% of analyzed cells. β-Catenin and Vitamin D Receptor (VDR) were respectively positive in the 61.5% and 86.5% of all plaques.

Among positive plaques, the abundance of positive cells to the different markers analyzed was variable: VDR and Bone Morphogenetic Protein (BMP) 4 were frequently expressed in both stable and unstable plaque, whereas a small number of cells expressed β-catenin (8.38 $\pm$ 1.20 positive cells/mm^2) and calmodulin (9.44 $\pm$ 1.48 positive cells/mm^2).

The results from logistic regression using plaque stability as a dependent variable are reported in Table 3. This analysis revealed a significant association between plaque instability and the expression of BMP-2 and β-catenin. In detail, higher BMP-2 was positively related with plaque instability ($p = 0.04$) whileβ-catenin was significantly more expressed among stable plaques ($p = 0.01$). In regression analysis between inflammation markers, a significant association was found between density (number $\times$ mm^2) of monocytes/macrophage cells and of T-lymphocytes in carotid plaques ($r^2 = 0.30$, $p < 0.01$). At univariate analysis, the number of monocytes/macrophages and that of lymphocytes was significantly higher in unstable plaques as compared to stable one ($p < 0.01$ in both cases). No other significant results were found at univariate analysis.

Association between molecular markers and risk factors

We employed separate general linear models which included each immunohistochemical marker as dependent variable and the presence or absence of a single risk factor as well as age and gender (as nuisance covariates) as independent variables. In detail, the average number of VDR positive cells was lower in patients affected by hypertension, irrespective of age and gender (36.15 $\pm$ 4.19 positive cells/mm^2 in patients affected by hypertension vs 57.54 $\pm$ 9.13 positive cells/mm^2 in patients without hypertension; $p < 0.0001$). Also, average RANKL value was higher in the presence of diabetes, irrespective of age and gender (5.80 $\pm$ 6.56 positive cells/mm^2 in diabetic patients vs 1,92 $\pm$ 4.27 positive cells/mm^2 in non diabetic patients; $p = 0.047$). No other significant associations between biomarkers and risk factors were found.

Discussion

Our study clearly demonstrates the expression of mineralization markers in atheromatic plaques. Indeed, excluding RANKL, all investigated markers were expressed in at least 60% of cases.

The presence of calcium mineral depositions in the tissues is frequently associated to degenerative processes involved in several pathological conditions such as cancer, chronic inflammation and atherosclerosis [4–6,8]. As

Table 2 Clinical characteristics of patients.

	All patients (N = 52)	Asymptomatic patients with stable plaques (N = 31)	Symptomatic patients with unstable plaques (N = 21)	p
Age (mean ± SD)	68.69 ± 7.59	70.35 ± 6.55	66.24 ± 7.59	0.05
Gender				
Male	37 (71.2%)	20 (64.5%)	17 (81.0%)	
Female	15 (28.8%)	11 (35.5%)	4 (19.0%)	0.20
Risk factors				
- Hypertension	39 (75.0%)	22 (71.0%)	17 (81.0%)	0.41
- Diabetes	15 (28.8%)	9 (29.0%)	6 (28.6%)	0.97
- Smoking habit	24 (46.2%)	14 (45.2%)	10 (47.6%)	0.86
- Hypercholesterolemia	28 (53.8%)	16 (51.6%)	12 (57.1%)	0.69
- Low HDL	16 (30.8%)	7 (22.6%)	9 (42.9%)	0.12
- Hypertriglyceridemia	17 (23.7%)	9 (29.0%)	8 (38.1%)	0.49
- Obesity (BMI > 30)	10 (19.2%)	6 (19.4%)	4 (19.2%)	0.98
Previous cardiovascular diseases				
- Myocardial infarction	11 (21.2%)	6 (19.4%)	5 (23.8%)	0.70
- Obstructive arteriopathy	5 (9.6%)	3 (9.7%)	2 (9.5%)	0.98
- Abdominal aneurism	2 (3.8%)	1 (3.2%)	1 (4.8%)	0.78
Previous therapy				
- Statins	26 (50.0%)	15 (48.4%)	11 (52.4%)	0.78
- Anti-hypertensive drugs	35 (67.3%)	20 (64.5%)	15 (71.4%)	0.60

concern the atherosclerosis, calcium crystals are often detected into atheromatic plaques by radiological investigation due to their presence can predict a higher risk of cardio and cerebrovascular diseases and death [24]. In this scenario, the needed to know how plaque structure, composition, and stability lead to developing cardiovascular events has accelerated the search about the biological mechanisms of the formation of plaque calcifications. Recent studies support the notion that the process of calcification involves the participation of arterial osteoblasts and osteoclasts mimicking the physiological process of mineralization that occurs in bone.

Interestingly, osteopontin, a protein that actively participates in the assembly of HA [25], was detected in 100% of analyzed plaques. This evidence, as already proposed by Ling and colleagues [26], underlines the connection between bone metabolism markers and vessel atherosclerosis disease], providing a scientific rationale for the comprehension of specific mechanisms involved in the formation of calcification into the atheroma.

It is interesting to note that multivariate analysis displayed significant associations between a) the expression of BMP-2 and unstable plaques and b) the expression of β-catenin and stable plaques. BMP-2 is a protein belonging to the TGFβ superfamily involved in several biological processes such as morphogenesis, cell differentiation, cell growth, epithelial to mesenchymal transition and apoptosis [27]. In the bone environment, it is considered to be one of the most powerful osteoblast differentiation factors [27]. BMP-2 is able to recruit and to differentiate the mesenchymal stem cells into mature osteoblasts. In light of this, we can speculate that plaque instability related to massive formation of HA crystals could be induced by the BMP-2 over-expression.

In a previous study, we demonstrated by EDX analysis that in nodular calcification that both micro and macro calcifications were made of HA [8]. Nodular calcification is an unstable plaque characterized by of an eruptive calcified mass protruding into the lumen with an irregular surface and discontinuity of the thin fibrous cap and an overlying luminal thrombus [11,22]. Indeed, as reported by Hutcheson et al., the deposition of calcifications in the fibrous cap of atherosclerotic plaques lead to considerable stress accumulation the destabilizing the plaque [28]. The presence of mineral depositions in the fibrous cap creates a mismatch in tissue properties and large stress

Table 3 Summary of associations between mineralization markers and plaque stability.

	All plaques	Positive cases	Stable plaques A	Unstable plaques B	Multivar. analysis A vs B p-value
RANK-L	3.03 ± 0.73	18 (34.6%)	3.71 ± 1.02	2.05 ± 1.00	0.85
Calmodulin	9.44 ± 1.48	33 (63.5%)	9.97 ± 1.77	8.67 ± 2.63	0.92
Osteopontin	15.83 ± 1.11	52 (100%)	15.38 ± 1.36	16.48 ± 1.91	0.67
Sclerostin	30.11 ± 2.23	52 (100%)	29.90 ± 3.06	30.42 ± 3.29	0.21
β-catenin	8.38 ± 1.20	32 (61.5%)	10.29 ± 1.68	5.67 ± 1.48	0.01
VDR	41.50 ± 4.05	45 (86.5%)	39.26 ± 5.71	44.81 ± 5.54	0.27
BMP-2	22.27 ± 2.84	39 (75.0%)	18.61 ± 3.21	27.67 ± 5.51	0.04
BMP-4	38.44 ± 4.72	42 (80.8%)	34.51 ± 5.89	44.24 ± 7.83	0.30

Results are reported as positive cells/mm^2 ± SE.

concentrations at the interface between cap and calcifications, and may lead to sudden rupture of the fibrous cap [20,29,30].

Conversely, β-catenin has a role in both osteoblastogenesis and osteoclastogenesis [31]. Specifically, several scientific evidences indicated that β-catenin signals in osteoclast precursors were required for their proliferation and differentiation, whereas the sustained activation of β-catenin signals inhibited osteoclast formation favoring the osteoblastogenesis. The higher expression of β-catenin in stable plaques rather than unstable one could reflect this double role of β-catenin. Indeed, it is possible to hypothesize that the activation of both osteoblast and osteoclast-like cells prevents the massive deposition of calcium minerals into the plaques in a process similar to bone remodeling. Despite the limited number of analyzed samples, our study also showed that some mechanisms involved in plaque calcification can be specific for the different cardiovascular risk factors analyzed. In this context, we found several significant associations between in situ biomarker expression and the presence of the main cardiovascular risk factors. In detail, we found a significant negative association between hypertension condition and the VDR expression in the plaques. VDR is a member of the nuclear receptor family. Upon activation by activated vitamin D, the VDR forms a heterodimer with the retinoid-X receptor and binds to hormone response elements on DNA, resulting in expression of genes involved in numerous physiological process mainly calcium uptake and metabolism [32]. Data about VDR could reflect the association between high blood pressure and hypovitaminosis D frequently detected in aged patients [33]. Thus, the expression of VDR in atheromatic plaques could be related to the 25-hydroxyvitamin D serum level rather than taking part in the plaque calcification process. This hypothesis is supported by the fact that we found no significant differences in VDR expression between stable and unstable plaques. Interestingly, we found an positive association between RANKL expression and the presence of diabetes. Many studies have already shown that diabetes is a condition that causes a reduction of bone quality, especially by increasing the osteoclasts activity [34]. RANKL represents one of the most important activators of osteoclasts therefore the increase of RANKL expression in plaques of diabetic patients may reflect a systemic condition frequently associated to the occurrence of osteoporosis.

Study limitations

In order to speculate about the mechanisms by which each risk factor may influence the plaque calcification, we employed separate general linear models which included each immunohistochemical marker as dependent variable and the presence or absence of a single risk factor as well as age and gender (as nuisance covariates) as independent variables. We therefore did not examine possible interactions between risk factors. However, the significant associations that we found between in situ biomarkers expression and the main cardiovascular risk factors, can form the basis for explaining how hypertension, diabetes, hypercholesterolemia as well as other risks factors may induce plaque calcification. In particular, the associations between risk factors and in situ biomarkers may help to better identify differential mechanisms involved in the formation of plaque calcifications, which should be addressed in *ad-hoc* future studies. Finally, it should be noted that several significant statistical associations reached a nominal significant p value that may not survive adjustment for multiple comparison, and therefore these results will require further validation and confirmation.

Conclusion

The in situ expression of the main mineralization markers confirms the hypothesis that the process of atherosclerotic plaques calcification presents many similarities with the physiological process that occurs in bone, involving both osteoblasts- and osteoclasts-like arterial cells. Plaque calcification has been demonstrated to be a predictor of future vascular events as it represents an important factor of plaque destabilization, favoring its rupture and thrombosis. Our results suggest that risk factors cause the destabilization of the atheromatic plaque, favoring not only the inflammation, but influencing the calcification process. These scientific evidences can lay the foundation for the development of new in vivo diagnostic analyses based on the identification of different calcification type into atheromatic plaques. In this context, molecular imaging investigations, based on the use of radiotracers incorporated in the structure of HA, could provide essential information to discriminate calcifications actively produced by osteoblast-like cells, and linked to plaque instability, from those related to necrotic degenerative process.

Conflicts of interest

There are no potential conflicts of interest relating to the manuscript (for each authors), and there were no extramural sources supporting this research (excluding sources already declared). The study is original and the manuscript has not been published yet and is not being considered for publication elsewhere in any language either integrally or partially except as an abstract. All authors have agreed with the submission in its present (and subsequent) forms.

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No relevant found to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2019.08.009.

References

[1] Sakamoto A, Virmani R, Finn AV. Coronary artery calcification: recent developments in our understanding of its pathologic and clinical significance. Curr Opin Cardiol 2018 Nov;33(6):645–52.

[2] Aikawa E, Nahrendorf M, Figueiredo JL, Swirski FK, Shtatland T, Kohler RH, et al. Osteogenesis associates with inflammation in early-stage atherosclerosis evaluated by molecular imaging in vivo. Circulation 2007 Dec 11;116(24):2841–50.

[3] Durham AL, Speer MY, Scatena M, Giachelli CM, Shanahan CM. Role of smooth muscle cells in vascular calcification: implications in atherosclerosis and arterial stiffness. Cardiovasc Res 2018 Mar 15; 114(4):590–600.

[4] Bonfiglio R, Scimeca M, Toschi N, Pistolese CA, Giannini E, Antonacci C, et al. Radiological, histological and chemical analysis of breast microcalcifications: diagnostic value and biological significance. J Mammary Gland Biol Neoplasia 2018;23(1–2):89–99.

[5] Scimeca M, Bonfiglio R, Varone F, Ciuffa S, Mauriello A, Bonanno E. Calcifications in prostate cancer: an active phenomenon mediated by epithelial cells with osteoblast-phenotype. Microsc Res Tech 2018 Jul;81(7):745–8.

[6] Scimeca M, Bonfiglio R, Montanaro M, Bonanno E. Osteoblast-like cells in human cancers: new cell type and reliable markers for bone metastasis. Future Oncol 2018 Jan;14(1):9–11.

[7] Tesauro M, Mauriello A, Rovella V, Annicchiarico-Petruzzelli M, Cardillo C, Melino G, et al. Arterial ageing: from endothelial dysfunction to vascular calcification. J Intern Med 2017 May;281(5):471–82.

[8] Bischetti S, Scimeca M, Bonanno E, Federici M, Anemona L, Menghini R, et al. Carotid plaque instability is not related to quantity but to elemental composition of calcification. Nutr Metab Cardiovasc Dis 2017 Sep;27(9):768–74.

[9] Bailey G, Meadows J, Morrison AR. Imaging atherosclerotic plaque calcification: translating biology. Curr Atheroscler Rep 2016 Aug; 18(8):51.

[10] Zhang Y, Li H, Jia Y, Yang P, Zhao F, Wang W, et al. Noninvasive assessment of carotid plaques calcification by 18F-sodium fluoride accumulation: correlation with pathology. J Stroke Cerebrovasc Dis 2018 Jul;27(7):1796–801.

[11] Cardellini M, Rovella V, Scimeca M, Anemona L, Bischetti S, Casella S, et al. Chronic kidney disease is linked to carotid nodular calcification, an unstable plaque not correlated to inflammation. Aging Dis 2019 Feb 1;10(1):71–81.

[12] Odink AE, van der Lugt A, Hofman A, Hunink MG, Breteler MM, Krestin GP, et al. Risk factors for coronary, aortic arch and carotid calcification; the Rotterdam Study. J Hum Hypertens 2010;24: 86–92.

[13] Moe SM, Chen NX. Pathophysiology of vascular calcification in chronic kidney disease. Circ Res 2004;95:560–7.

[14] Patil SR. Prevalence of carotid artery calcification in postmenopausal women and its correlation with atherogenic risk factors. J Nat Sci Biol Med 2015;6:S1–6.

[15] Kwee RM. Systematic review on the association between calcification in carotid plaques and clinical ischemic symptoms. J Vasc Surg 2010 Apr;51(4):1015–25.

[16] Hunt JL, Fairman R, Mitchell ME, Carpenter JP, Golden M, Khalapyan T, et al. Bone formation in carotid plaques: a clinicopathological study. Stroke 2002 May;33(5):1214–9.

[17] Nandalur KR, Baskurt E, Hagspiel KD, Finch M, Phillips CD, Bollampally SR, et al. Carotid artery calcification on CT may independently predict stroke risk. Am J Roentgenol 2006 Feb; 186(2):547–52.

[18] Sangiorgi G, Rumberger JA, Severson A, Edwards WD, Gregoire J, Fitzpatrick LA, et al. Arterial calcification and not lumen stenosis is highly correlated with atherosclerotic plaque burden in humans: a histologic study of 723 coronary artery segments using nondecalcifying methodology. J Am Coll Cardiol 1998 Jan;31(1): 126–33.

[19] Alexopoulos N, Raggi P. Calcification in atherosclerosis. Nat Rev Cardiol 2009 Nov;6(11):681–8.

[20] Mauriello A, Servadei F, Zoccai GB, Giacobbi E, Anemona L, Bonanno E, et al. Coronary calcification identifies the vulnerable patient rather than the vulnerable plaque. Atherosclerosis 2013 Jul;229(1):124–9.

[21] Scimeca M, Giannini E, Antonacci C, Pistolese CA, Spagnoli LG, Bonanno E. Microcalcifications in breast cancer: an active phenomenon mediated by epithelial cells with mesenchymal characteristics. BMC Canc 2014 Apr 23;14:286.

[22] Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2000 May;20(5):1262–75.

[23] Whelton PK, Carey RM, Aronow WS, Casey Jr DE, Collins KJ, Dennison Himmelfarb C, et al. 2017 ACC/aha/aapa/abc/acpm/ags/APhA/ash/aspc/nma/pcna guideline for the prevention, detection, evaluation, and management of high blood pressure in adults: executive summary: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. J Am Coll Cardiol 2018 May 15;71(19): 2199–269.

[24] Tarkin JM, Dweck MR, Evans NR, Takx RA, Brown AJ, Tawakol A, et al. Imaging atherosclerosis. Circ Res 2016 Feb 19;118(4): 750–69.

[25] Hunter GK. Role of osteopontin in modulation of hydroxyapatite formation. Calcif Tissue Int 2013 Oct;93(4):348–54.

[26] Ling Y, Wang Z, Wu B, Gao X. Association of bone metabolism markers with coronary atherosclerosis and coronary artery disease in postmenopausal women. J Bone Miner Metab 2018 May; 36(3):352–63.

[27] Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. Growth Factors 2004 Dec;22(4):233–41.

[28] Hutcheson JD, Maldonado N, Aikawa E. Small entities with large impact: microcalcifications and atherosclerotic plaque vulnerability. Curr Opin Lipidol 2014 Oct;25(5):327–32.

[29] Richardson PD, Davies MJ, Born GV. Influence of plaque configuration and stress distribution on fissuring of coronary atherosclerotic plaques. Lancet 1989;2:941–4.

[30] Maldonado N, Kelly-Arnold A, Vengrenyuk Y, Laudier D, Fallon JT, Virmani R, et al. A mechanistic analysis of the role of microcalcifications in atherosclerotic plaque stability: potential implications for plaque rupture. Am J Physiol Heart Circ Physiol 2012; 303:H619–28.

[31] Kobayashi Y, Uehara S, Koide M, Takahashi N. The regulation of osteoclast differentiation by Wnt signals. Bonekey Rep 2015 Jul 1; 4:713.

[32] Franczyk A, Stolarz-Skrzypek K, Wesołowska A, Czarnecka D. Vitamin D and vitamin D receptor activators in treatment of hypertension and cardiovascular disease. Cardiovasc Hematol Disord Drug Targets 2014;14(1):34–44.

[33] Mehta V, Agarwal S. Does vitamin D deficiency lead to hypertension? Cureus 2017 Feb 17;9(2):e1038.

[34] Gilbert MP, Pratley RE. The impact of diabetes and diabetes medications on bone health. Endocr Rev 2015 Apr;36(2):194–213.