Osteogenesis Associates With Inflammation in Early-Stage Atherosclerosis Evaluated by Molecular Imaging In Vivo

Elena Aikawa, MD, PhD; Matthias Nahrendorf, MD; Jose-Luiz Figueiredo, MD; Filip K. Swirski, PhD; Timur Shtatland, PhD; Rainer H. Kohler, PhD; Farouc A. Jaffer, MD, PhD; Masanori Aikawa, MD, PhD; Ralph Weissleder, MD, PhD

Background—Arterial calcification is associated with cardiovascular events; however, mechanisms of calcification in atherosclerosis remain obscure.

Methods and Results—We tested the hypothesis that inflammation promotes osteogenesis in atherosclerotic plaques using in vivo molecular imaging in apolipoprotein E⁻/⁻ mice (20 to 30 weeks old, n=35). A bisphosphonate-derivatized near-infrared fluorescent imaging agent (excitation 750 nm) visualized osteogenic activity that was otherwise undetectable by x-ray computed tomography. Flow cytometry validated the target specifically in osteoblast-like cells. A spectrally distinct near-infrared fluorescent nanoparticle (excitation 680 nm) was coinjected to simultaneously image macrophages. Fluorescence reflectance mapping demonstrated an association between osteogenic activity and macrophages in aortas of apolipoprotein E⁻/⁻ mice ($R^2=0.93$). Intravital dual-channel fluorescence microscopy was used to further monitor osteogenic changes in inflamed carotid arteries at 20 and 30 weeks of age and revealed that macrophage burden and osteogenesis concomitantly increased during plaque progression ($P<0.01$ and $P<0.001$, respectively) and decreased after statin treatment ($P<0.0001$ and $P<0.05$, respectively). Fluorescence microscopy on cryosections colocalized near-infrared fluorescent osteogenic signals with alkaline phosphatase activity, bone-regulating protein expression, and hydroxyapatite nanocrystals as detected by electron microscopy, whereas von Kossa and alizarin red stains showed no evidence of calcification. Real-time reverse-transcription polymerase chain reaction revealed that macrophage-conditioned media increased alkaline phosphatase mRNA expression in vascular smooth muscle cells.

Conclusions—This serial in vivo study demonstrates the real-time association of macrophage burden with osteogenic activity in early-stage atherosclerosis and offers a cellular-resolution tool to identify preclinical microcalcifications.

Key Words: atherosclerosis • calcification • inflammation • imaging

Calcification is a characteristic feature of atherosclerosis and is predictive of cardiovascular events. Clinico-pathological studies suggest that atherosclerotic plaques are prone to rupture at interface areas between high- and low-density tissue, particularly in the superficial nodules of calcium deposition. In addition, microcalcification in the thin fibrous cap may cause microfractures that lead to plaque rupture and acute cardiovascular events. Furthermore, calcification impacts clinical outcome not only by complicating atherosclerosis but also by impairing the movement of aortic valve leaflets, increasing arterial stiffness, which in turn affects cardiac function, or by causing plaque fracture during angioplasty.
and sequential phenotypic differentiation. Moreover, accumulating data suggest that proatherogenic stimuli promote phenotypic conversion of vascular and valvular myofibroblasts into osteoblastic cells, promote expression of bone-regulating proteins (eg, alkaline phosphatase, osteopontin, osteocalcin, osteonectin, and collagen types I and II) and transcription factors (eg, Runx2/Cbfα1 and Osterix), and eventually promote calcification. However, the precise cellular and molecular mechanisms that lead to ectopic calcification remain incompletely understood. The inability to spatially and temporally resolve and quantify dynamic pro-osteogenic molecular mechanisms in vivo also accounts for the limited knowledge in the field. In vivo also accounts for the limited knowledge in the field. In vivo evidence remains scant. Novel imaging technologies allow detection in vivo of the expression and activity of proinflammatory and pro-osteogenic molecules. We therefore tested the specific hypothesis that atherosclerotic plaque inflammation, determined as macrophage infiltration, triggers osteogenic activity. Serial imaging studies in apolipoprotein (apo) E–deficient (apoE) mice provide evidence that atherosclerotic inflammation precedes osteogenic activity and promotes calcification, which possibly explains the epidemiological link between inflammation, hypercholesterolemia, and calcification. The results of the present study provide new insights into the biology of inflammation-triggered osteoblastic activity in early stages of atherosclerosis and aid the exploration of novel, more refined therapeutic strategies to combat calcific cardiovascular disease.

**Methods**

An expanded Methods section is available online as a Data Supplement.

**Animal Protocol**

We studied osteogenic changes in carotid arteries of apoE mice (20 to 30 weeks old [n=35] and 72 weeks old [n=5]) that consumed an atherogenic diet (Teklad TD 88137: 42% milk fat, 0.2% total cholesterol, Harlan, Indianapolis, Ind) from 10 weeks of age. Mice were randomized either to continue with the atherogenic high-cholesterol diet (n=15, sequential imaging group; n=5, cathepsin K group; n=5, histological control) or to consume the high-cholesterol diet admixed with a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (n=10, statin group, 0.01% wt/wt atorvastatin, Pfizer, Groton, Conn). Age-matched wild-type C57BL6 mice (n=5, Jackson Laboratory, Bar Harbor, Me) and apoE mice lacking probe injection (n=5) served as controls. The Subcommittee on Research Animal Care at Massachusetts General Hospital approved all procedures.

**Intravitral Laser Scanning Fluorescence Imaging and 3D Reconstruction**

Mice received imaging agents or saline via intravenous injection 24 hours before imaging. We performed multichannel fluorescence imaging using an intravitral laser scanning fluorescence microscope specifically developed for imaging small experimental animals. Excitation at 633 and 748 nm and image collection of the different channels was done serially to avoid cross talk between channels. Image stacks were processed and analyzed with ImageJ software (version 1.38a, Bethesda, Md). For 3-dimensional (3D) reconstruction, image stacks were split into individual channels and imported into Amira software (version 3.1, Mercury Computer Systems, Chelmsford, Mass).

**Macroscopic Fluorescence Reflectance Imaging Ex Vivo**

After mice were euthanized, aortas were perfused with saline, dissected, and imaged to map the macroscopic near-infrared fluorescence (NIRF) signals elaborated from each imaging agent by use of a fluorescence reflectance imaging system equipped with multichannel filter sets (Omega Optical, Brattleboro, Vt).

**Molecular Imaging Agents**

**Macrophage-Targeted Fluorescent Nanoparticles**

We used a cross-linked iron oxide fluorescent nanoparticle, an agent that elaborates fluorescence detectable through the NIRF window (excitation/emission 673/694 nm), for in vivo detection of macrophage accumulation.

**Calcification**

We used bisphosphonate-conjugated imaging agent (OsteoSense750/OS750, VisEn Medical Inc, Woburn, Mass), which elaborates fluorescence detectable through the NIRF window (excitation/emission 750/780 nm), to detect osteogenic activity.

**Cathepsin K Activity**

Protease-activatable imaging agent detects the activity of cathepsin K in atherosclerotic lesions. The cathepsin K probe consists of the backbone of a cathepsin K–cleavable peptide substrate that contains a fluorochrome. After enzymatic cleavage, the fluorochromes separate, which results in amplification of the signal. This agent elaborates fluorescence detectable through the NIRF window (excitation/emission 673/694 nm).

**Statistical Analysis**

An expanded statistical analysis section is available in the online-only Data Supplement. Statistical analyses for comparison of multiple groups used 1-way ANOVA followed by the Tukey post hoc test, performed with GraphPad Prism software (version 4.0, GraphPad Software, San Diego, Calif). The ΔΔCt (change in cycle threshold) of real-time reverse-transcription polymerase chain reaction data for alkaline phosphatase mRNA was used in the Mann–Whitney U test. Data are presented as mean±SEM. Probability values <0.05 were considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

**Results**

**Topographic Association Between Macrophage Burden and Osteogenic Activity in the Aorta of ApoE Mice**

Fluorescence reflectance imaging was used to map osteoblastic activity and macrophage burden in aortas of apoE mice (n=15). We administered a bisphosphonate-derivative NIRF imaging agent (OsteoSense, excitation 750 nm; plaque target-to-background ratio 5.4±0.5 versus 1.7±0.2 in uninjected controls; P<0.01) via tail-vein injection 24 hours before imaging to monitor osteogenesis. To visualize macrophages, we coinjected mice with a spectrally distinct NIRF nanoparticle (excitation 680 nm; plaque target-to-background ratio 6.8±0.9 versus 1.4±0.4 in uninjected controls; P<0.01). Fluorescence reflectance imaging in apoE mice yielded strong macrophage-
targeted, fluorescent nanoparticle–derived signals at the level of aortic root, aortic valve, arch, and abdominal aorta that correlated with osteogenic activities in the same regions ($R^2=0.93$; Figure 1A and 1B). These results suggest that calcification associates with sites of macrophage accumulation.

### Osteoblast-Like Cells but Not Cells of Myeloid Origin Are the Source of the Osteogenic Signal in Atherosclerotic Aortas

The cellular source of OsteoSense signal was examined with flow cytometry. We identified OsteoSense-positive cells in aorta of apoE$^{-/-}$ mice but not in wild-type control mice (Figure 1C). Spleens of injected apoE$^{-/-}$ mice and wild-type controls were OsteoSense negative. The present data show that OsteoSense colocalized preferentially with osteopontin-positive cells in lesion-rich aortas but not with monocytes/macrophages or dendritic cells in any tissue tested, which suggests their osteoblastic phenotype (Figure 1D).

### Early Atherosclerotic Lesions Contain Hydroxyapatite Nanocrystals

Electron microscopic analysis revealed early stages of calcium deposition in aortic lesions of 20- to 30-week-old apoE$^{-/-}$ mice (Figure 1E). Both apoptotic bodies and matrix vesicles contained electron-dense needlelike structures compatible with nanocrystals of hydroxyapatite.

### Active Processes of Early Osteogenesis Occur in Inflamed Atherosclerotic Plaques Before Development of Advanced Calcification

Macrophage-rich atherosclerotic plaques (Mac3-positive area 23.4±4.8%) of 20- to 30 week-old mice had increased osteogenic activity detected as alkaline phosphatase activity (alkaline phosphatase–positive area 17.1±3.1%), whereas von Kossa staining showed negligible calcification (von Kossa–positive area 0.7±0.5%; Figure 2A). In contrast, calcified lesions (von Kossa–positive area 21.8±2.5%) of...
Aged mice had decreased macrophage accumulation (0.8±0.2%) and alkaline phosphatase activity (12.2±1.9%; Figure 2B). Notably, early plaques showed no evidence of microscopic calcification, whereas fluorescence microscopy detected an OsteoSense-positive area (von Kossa-positive area 0.4±0.2% versus OsteoSense-positive area 3.2±0.6%; Figure 2C and 2D), which suggests that OsteoSense enhanced the areas of osteogenesis that were not detected by von Kossa staining. Cross sections through advanced plaques demonstrated prominent calcification as detected by von Kossa staining (left) that correlated with NIRF signal (right). E, Cross sections through advanced lesion demonstrated prominent calcification as detected by von Kossa staining (left) that correlated with NIRF signal (right). E, Cross sections through advanced lesion demonstrated prominent calcification as detected by von Kossa staining (left) that correlated with NIRF signal (right).

Changes in Osteogenic Activity During Plaque Progression and Antiinflammatory Treatment

To monitor the dynamic changes in inflammation and osteogenesis in atherosclerotic plaques, we used intravital multi-channel, high-resolution laser scanning fluorescence microscopy. At 20 weeks, mice were randomized either to continue consumption of an atherogenic high-cholesterol diet or to consume a high-cholesterol diet admixed with statin (Figure 3A). We performed 50 sequential intravital microscopy sessions on carotid arteries of untreated (n=15) and statin-treated (n=10) mice at 20 and 30 weeks of age. At 20 weeks, macrophages correlated with little if any osteogenic activity as shown by imaging and histology (Figure 3B); however, by 30 weeks, macrophage accumulation increased in association with advanced osteogenic signal (Figure 3C). Statin treatment prevented progression of macrophage burden and osteogenesis (Figure 3D). Quantitative analyses further demonstrated that macrophage-derived (680 nm) and osteoblast-derived (750 nm) NIRF signal intensities increased over time concurrently (P<0.01 and P<0.001, respectively) and decreased after antiinflammatory statin treatment (P<0.0001 and P<0.05, respectively; Figure 3E). Areas of inflammation and calcification also increased during plaque progression in parallel (P<0.001) and decreased with statin treatment (P<0.001 and P<0.05, respectively; Figure 3F), which supports our hypothesis that inflammation promotes osteogenic activity.

3D Evaluation of Calcium and Macrophage Burden in Atherosclerotic Plaques

Using the same model of in vivo calcification in atherosclerotic carotid arteries, we evaluated further the temporal and spatial associations of cellular inflammation and calcification (Figure 4A). Moreover, defining colocalization as the degree of overlap between 2 different fluorescent labels (green signifying inflammation and red, calcification) within the same image, we demonstrated that microcalcifications and inflammation evolve within close proximity of one another and overlap at the border regions (colocalized pixels appear white; Figure 4B). Overlap (r=0.62 at 20 weeks versus r=0.81 at 30 weeks) and Pearson’s coefficients (r=0.14 at 20 weeks versus r=0.46 at 30 weeks) increased over time. To assess whether the degree of inflammation and calcification can be monitored over time, 3D reconstructed images were derived from Z-stack data sets at 20 and 30 weeks of age. In addition, we displayed color-coded sequential images of the carotid artery at 0°-90°-180° at 20 and 30 weeks and thereby visualized the progression of calcification and inflammation in living mice (Figure 4C). Image acquisition through a portion of carotid plaque in 3-μm steps allowed assessment of the 3D distribution of calcium and macrophage burden (online-only Data Supplement Movie; Figure 2). We observed an overall increase in volume of inflammation of 160% (green=0.13 mm³ at 20 weeks versus yellow=0.34 mm³ at 30 weeks) and in volume of calcification of 25% (red=0.15 mm³ at 20 weeks versus blue=0.19 mm³ at 30 weeks) in the 10-week-interval between 2 imaging sessions.

Aged ApoE−/− Mice Exhibit Increased Osteogenic Signal Spatially Distinct From Macrophages

We next analyzed atherosclerotic plaques in aged (72 weeks old, n=5) apoE−/− mice. Gross morphology, intravital microscopy (Figure 5A), and fluorescence microscopy (Figure 5B) demonstrated that calcification in more advanced plaques in aged mice appeared spatially distinct from macrophage accumulation. Osteogenic NIRF signal intensities increased with age (P<0.05), whereas macrophage-derived inflamma-
Figure 3. Sequential intravital fluorescence microscopy was used to monitor changes in inflammation and osteogenesis in mouse carotid atherosclerotic plaques. A, At 20 weeks, mice were randomized either to continue with the high-cholesterol diet or to consume the high-cholesterol (High-chol.) diet admixed with a statin for 10 more weeks. Intravital microscopy (IVM), fluorescence reflectance imaging (FRI), and histopathological analyses were performed at 20 and 30 weeks in treated and untreated mice. IVM was done sequentially at 20 weeks (B), 30 weeks (C), and 30 weeks on statin diet (D). Image stacks simultaneously visualized 2 different biological processes: inflammation (green fluorescence) and osteogenesis (red fluorescence). Bar=50 μm. Representative hematoxylin-and-eosin (H&E) images were used to correlate IVM with histopathological changes (right panels). L indicates lumen; arrows depict internal elastic lamina. Bar=50 μm. E, Quantification analyses demonstrated that macrophage- and osteoblast-derived NIRF signal intensities increased over time and decreased after statin treatment. F, Areas of macrophage accumulation and calcification increased from 20 to 30 weeks. Statin treatment reduced areas of both inflammation and calcification at 30 weeks. At 72 weeks, areas of inflammation decreased, but calcification increased significantly compared with 30 weeks. AU indicates arbitrary units; stat, statin.

Cathepsin K Activity Colocalizes With Calcified Areas in Early Atherosclerosis

We further investigated the relationship between cathepsin K activity and calcified areas in early atherosclerotic lesions. Intravital microscopy of carotid atherosclerotic plaques was performed 24 hours after injection of the cathepsin K (excitation 680 nm) and OsteoSense750 agents (n=5). A significantly enhanced NIRF signal was observed in mice injected with the cathepsin K activatable agent (plaque target-to-background ratio 3.2±0.7 versus 1.1±0.4 in un.injected controls, P<0.05). Cathepsin K activity preceded osteogenic activity at 20 weeks and colocalized with areas of calcification in 30-week-old apoE/−/− mice (Figure 7).

Organic Culture of Human Carotid Plaques Shows Spatial Distribution of Macrophages and Osteoblast-Like Cells

To determine the relationship between inflammation and calcification in human atherosclerotic plaques, we used serial fluorescence reflectance imaging of surgically obtained carotid atheroma specimens (n=6) incubated with macrophage-targeted fluorescent nanoparticle and OsteoSense750. NIRF signal derived from both agents evolved over 24 hours (Figure 8A). Correlative histological analysis of cryosections...
demonstrated colocalization of fluorescent nanoparticle–associated signal with macrophages (CD68) and of OsteoSense-derived NIRF signals with immunoreactive osteopontin and osteocalcin (not shown) and calcium deposits detected by hematoxylin-and-eosin and von Kossa staining (not shown; Figure 8B).

Macrophage-Conditioned Media Increase Alkaline Phosphatase mRNA Expression in Human Vascular Smooth Muscle Cells

To address further whether macrophages promote an osteogenic phenotype in vascular cells, we examined alkaline phosphatase expression by human primary smooth muscle cells treated with culture media from human primary macrophages. Real-time reverse-transcription polymerase chain reaction showed that macrophage-conditioned media produced a statistically significant increase in alkaline phosphatase mRNA expression by smooth muscle cells in 6 hours compared with the control cell-free media (mean 3.1±0.7-fold increase, P<0.05; n=4; Figure 8C).

Discussion

The present study provides in vivo evidence that macrophage infiltration precedes osteogenic activity in the atherosclerotic microenvironment, thus extending the paradigm that arterial calcification is an inflammatory disease. Key findings documented here (1) demonstrate that arterial calcification is associated with macrophage burden, as detected by simultaneous mapping of spectrally distinct NIRF signals amplified by calcification- and inflammation-targeted imaging agents; (2) unravel in vivo processes of mineralization, which were undetectable by conventional histological and imaging approaches; (3) show the progression of plaque calcification in apoE-deficient mice from 20 to 72 weeks of age; (4) demonstrate that conditioned media from macrophages induce the osteogenic potential of vascular smooth muscle cells; (5) show that early introduction of statin therapy retards calcification; and (6) quantify the dynamic pro-osteogenic molecular processes at the initial stages of atherosclerosis. These results concur with our hypothesis on inflammation-triggered osteogenic activities and therefore suggest that antiinflammatory treatment can prevent the progression of arterial calcification. Because atherosclerosis and aortic valve stenosis share similar mechanisms and epidemiological risk factors, the present findings also apply to calcific aortic valve disease29–31 and suggest that cellular-resolution molecular imaging can identify microcalcifications and subclinical valvular lesions and potentially predict risk for devastating clinical complications in patients.

Pioneering work by Demer and colleagues19–22 demonstrated in vitro that macrophage-derived cytokines such as
calcification in atherosclerosis and further suggest that calcification in the proinflammatory microenvironment. The in vivo data documented here provide comprehensive understanding and mechanistic insights into the initiation of calcification in atherosclerosis and further suggest that calcification is a complex yet predictable and therefore preventable process that responds to mechanical signals, as well as local and systemic factors that regulate vascular cell differentiation and function.

Previous histopathological studies have shown that calcification occurs reproducibly in advanced atherosclerotic lesions of aged apoE−/− mice.17,32,33 Such studies have provided a model of advanced arterial calcification that assessed the presence of osteogenic cells and elucidated the factors that participate in differentiation of osteoblastic/chondroblastic cells within the setting of fibrosclerotic lesions. The present study explored further the pathogenesis of atherosclerotic plaques and identified the initial stages of arterial calcification in early atherosclerosis. In addition, we investigated the progression of plaque development and aging at cellular resolution in vivo. Intravital imaging showed that in early-stage atherosclerosis, inflammation precedes calcification, which suggests that macrophages promote the proinflammatory milieu and send specific signals to vascular wall cells to initiate osteogenic differentiation. Then, both processes developed in parallel and within close proximity. This stage of “microcalcification” may cause plaque rupture and microfractures that may result in the acute clinical events predicted by the theoretical model of Vengrenyuk and colleagues.5 Once equilibrium in the arterial wall shifts toward calcification, deposition of hydroxyapatite could progress quickly, as shown in the present study. Microcrystals of calcium phosphate may elicit proinflammatory responses from macrophages, which suggests a positive-feedback amplification loop of calcification and inflammation that drives disease progression.34,35 In addition, macrophages and smooth muscle
cells may undergo apoptotic changes, providing new foci for calcium deposition. The eventual appearance of acellular fibroatheromatous plaques with prominent mineralization is associated with the classic view of calcification as a degenerative and geriatric disease.

The balance of osteoblastic and osteoclastic activities regulates osteogenesis. The cathepsins are among the most potent elastases characterized to date. Elastolysis induced by inflammation in the atherosclerotic plaques may alter smooth muscle cell phenotype and promote their osteogenic differentiation. Moreover, cathepsin K is a major protease expressed during bone resorption. Cathepsin K deficiency results in accumulation of bone matrix and development of ectopic calcification. The present study demonstrated that spectrally distinct NIRF signal derived from enzymatically active cathepsin K preceded osteoblastic activity in early atherosclerosis and colocalized later with prominent calcification, which suggests that osteoblastic and osteoclastic activities evolve in parallel.

Previous studies used histological methods to analyze static conditions in advanced atherosclerotic lesions at the time of death, when calcification had already become prominent and irreversible. Not surprisingly, limited resolution of imaging technologies failed to recognize initial osteogenesis or cellular-level microcalcifications. The present study used imaging techniques for which the resolution exceeds that of conventional imaging modalities (eg, MRI, intravascular ultrasound, and optical coherence tomography). Furthermore, the NIRF imaging agent binds to nanocrystals of hydroxyapatite elaborated by vascular smooth muscle cells that undergo vesicle-mediated calcification or differentiation into osteoblast-like cells, thereby producing images undetectable by earlier approaches. In the present study, for example, molecular agent–based intravital fluorescent microscopy but not x-ray CT detected early osteogenesis and microcalcifications.

The increased risk of mortality and morbidity associated with cardiovascular calcification has driven the development of new therapeutic strategies to prevent and even reverse this process. Although recent clinical trials failed to show the reduction of calcific aortic stenosis, a growing body of research indicates that statins may have therapeutic advantages in cardiovascular calcification. Previous studies on hypercholesterolemic rabbits indicated that in addition to their effect on inflammation, statins may inhibit osteogenic pathways in myofibroblasts. The present study demonstrates that statin treatment in early-stage atherosclerosis reduces inflammation and osteogenic activity concomitantly, hence supporting the concept that early pharmacological modification of proinflammatory processes retards the progression of arterial calcification. Therefore, clinical molecular imaging of microcalcifications and osteogenic activity ("precalcification") may identify high-risk atherosclerotic plaques and aortic valve lesions while the disease is still silent and may enable the monitoring of osteogenic activity during therapeutic interventions, thereby providing a powerful tool for personalized preventive cardiovascular medicine.
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Disclosures
Dr Weissleder is a shareholder of VisEn Medical in Woburn, Mass. The remaining authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

Arterial calcification may trigger acute coronary events. Although conventional structural imaging modalities can identify prominent late-stage calcification, they cannot detect and quantify the dynamic pro-osteogenic processes in vivo at the earlier stages of subclinical disease. Our innovative functional imaging strategies not only monitor plaque initiation and progression but also provide robust evidence for coregistration of osteogenic processes with inflammation, thus extending the paradigm that cardiovascular calcification is an inflammatory disease. The present serial in vivo study demonstrates further that statin treatment concomitantly inhibits arterial inflammation and osteogenic activity in early-stage atherosclerosis, which suggests that early pharmacological modification of proinflammatory pathways can retard the progression of cardiovascular calcification. Because atherosclerosis and aortic valve stenosis share similar mechanisms and epidemiological risk factors, our findings also apply to calcific aortic valve disease that currently has no therapeutic options other than surgical valve replacement. Development of catheter-based fluorescence sensors and tomographic fluorescence imaging would enable simultaneous imaging of different biological processes such as inflammation and osteogenesis in patients before severe complications occur. Therefore, clinical molecular imaging of microcalcifications and osteogenic activity (“precalcification”) may identify high-risk atherosclerotic plaques and aortic valve lesions while the disease is still silent and may enable the monitoring of osteogenic activity during therapeutic interventions. In the near future, the combined use of molecular imaging, novel biomarkers, and genetics will identify subclinical lesions, providing a powerful tool for personalized preventive cardiovascular medicine.
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