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# Matrix metalloproteinase-10 deficiency delays atherosclerosis progression and plaque calcification

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## HIGHLIGHTS

- MMP10 is expressed and secreted by human atherosclerotic plaques.
- Atherosclerosis is delayed in *Apoe*$^{-/-}$*Mmp10*$^{-/-}$ mice.
- Local and systemic inflammation is reduced in *Apoe*$^{-/-}$*Mmp10*$^{-/-}$mice.
- *In vitro* calcification is reduced in *Mmp10*$^{-/-}$ VMSCs.

## ARTICLE INFO

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## ABSTRACT

*Background and aims:* Matrix metalloproteinases (MMPs) have been implicated in atherosclerosis and vascular calcification. Among them, we reported that MMP10 is present in human atheroma, associated with atherosclerosis. However, it remains unclear whether MMP10 is involved in atherogenesis and vascular calcification.
*Methods:* MMP10 was measured in serum from patients with subclinical atherosclerosis and analyzed in carotid endarterectomies by immunostaining. ApoE-deficient mice (*Apoe*$^{-/-}$) were crossed to MMP10-deficient (*Mmp10*$^{-/-}$) mice and followed up to 20 months. Plaque area and composition were assessed by histology and immunohistochemistry. Inflammatory markers were measured in atherosclerotic plaques by RT-qPCR, and leukocyte subpopulations were analyzed by flow cytometry. *In vitro* calcification assays were performed in aortic vascular smooth muscle cells (VSMC).
*Results:* MMP10 serum levels were associated with coronary calcification in subjects with subclinical atherosclerosis. Immunostaining revealed MMP10 expression in human atheromas, spatially associated with calcification areas, and complicated plaques released higher amounts of MMP10 than non-diseased segments. Interestingly, vascular MMP10 expression was confined to the atherosclerotic lesion in *Apoe*$^{-/-}$ mice, and *Apoe*$^{-/-}$*Mmp10*$^{-/-}$ showed a substantial reduction in atherosclerotic lesion size, macrophage content and plaque calcification. Reduced local and systemic inflammatory markers could be demonstrated in *Apoe*$^{-/-}$*Mmp10*$^{-/-}$ by gene expression and flow cytometry analysis. Calcium phosphate deposition and vascular calcification markers were downregulated in VSMC from *Apoe*$^{-/-}$*Mmp10*$^{-/-}$ mice.

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*Conclusions:* Delayed plaque progression and altered cellular composition in the absence of MMP10 suggests that MMP10 plays a role in atherosclerosis, favoring inflammation, development and complication of the plaque.

## 1. Introduction

An imbalance between proteases and their inhibitors has been hypothesized to be involved in the growth, destabilization, and eventual rupture of atherosclerotic plaques [1]. Matrix metalloproteinases (MMPs) are a group of more than 20 zinc-containing endopeptidases that are either secreted or expressed at the cell surface. MMPs can act on a wide range of extracellular proteins, including several components of the extracellular matrix (ECM) [2]. There is substantial evidence indicating that MMPs play a relevant role in various physiological and pathological processes such as organ development, tissue remodelling and inflammation, particularly in atherosclerosis and its ischemic clinical manifestations such as myocardial infarction and stroke [2]. Several MMPs are locally expressed within human atherosclerotic lesions in vulnerable rupture-prone sites [3] and have also been associated with vascular calcification [4], involving phenotypic changes of vascular smooth muscle cells (VSMC) and expression of bone-related proteins [5,6]. Macrophage infiltration and proinflammatory cytokines are essential in early stages of plaque development leading to calcification [7].

MMP10 (stromelysin-2) has been proposed to participate in physiological processes like bone growth [8] and wound healing [9], while its overexpression has been reported in various carcinomas and tumours [10], and it has been suggested to be involved in vascular pathology [11]. MMP10 expressed by macrophages in response to acute infection has been shown to play a beneficial role by moderating their proinflammatory response [12]. Our group has shown that MMP10 is induced in response to injury or inflammatory stimuli [13–15], it is present in atherosclerotic lesions at rupture-prone sites and increased serum proMMP10 is associated with clinical and subclinical atherosclerosis [13,16,17]. However, little is known on its pathophysiological role in atherothrombosis, particularly in the development of calcified lesions.

We explored MMP10 expression in human and murine atherosclerosis [apolipoprotein E deficient (*Apoe*$^{-/-}$) mouse], and studied the effect of functional MMP10 deficiency on atherogenesis. We found that MMP10 was associated with vascular calcification in human atherosclerotic plaques and in patients with cardiovascular (CV) risk. *Apoe*$^{-/-}$*Mmp10*$^{-/-}$ mice displayed reduced atherosclerosis, decreased macrophage content and calcification. Moreover, MMP10 inactivation was associated with lower expression of pro-calcifying genes in murine aortic VSMC. Taken together, our results support a relevant role for MMP10 in modulating progression of atherosclerosis and plaque calcification.

## 2. Materials and methods

A detailed description is presented in the Supplementary Data.

### 2.1. Patients

A computed tomography (CT) scan for coronary artery calcium scoring was performed in 136 apparently healthy subjects (90% men, mean age 59 ± 8 years), free from overt vascular disease, recruited at the time of attending the outpatient clinic for vascular risk assessment at Clínica Universidad de Navarra (Pamplona, Spain).

Human carotid endarterectomy samples (n = 52), obtained from patients undergoing surgery at the Centre Cardiologique du Nord (Saint-Denis, France), were collected and dissected as described previously [18,19], separating the culprit area of each plaque (CP) from its adjacent non-culprit plaque (NCP). MMP10 release from atherosclerotic plaques was measured by ELISA (DuoSet, R&D Systems) in conditioned media from human endarterectomy samples incubated for 24 h in RPMI medium. MMP10, smooth muscle α-actin (SMA) and CD68 were analyzed by immunohistochemistry in atherosclerotic plaques from patients undergoing carotid endarterectomy (n = 10). Calcium deposition was assessed by Alizarin red staining. Study protocols conform to the ethical guidelines of the 1975 Declaration of Helsinki, were approved by the Institutions' ethics committees, and written informed consent was obtained from each patient included in the study.

### 2.2. Experimental animals

Animal experimentation was performed with wild type, *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Mmp10*$^{-/-}$ mice (all in C57Bl/6 background). *Mmp10*$^{-/-}$ mice (B6.129P2-Mmp10$^{tm1Jkmg}$) were crossbred with *Apoe*$^{-/-}$ mice (B6.129P2-Apoe$^{tm1Unc}$/J; Charles River Laboratories, L'Arbresle Cedex, France) to obtain *Apoe*$^{-/-}$*Mmp10*$^{-/-}$ mice. Animals were maintained on standard chow diet throughout the experiment until sacrifice. Blood cell populations were analyzed by flow cytometry. All experiments were conducted according to the European Community guidelines for ethical animal care and use of laboratory animals (Directive 86/609), and were approved by the University of Navarra Animal Research Review Committee.

### 2.3. Analysis of plaque area and composition

Aortic *en face* preparations of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Mmp10*$^{-/-}$ mice were used to quantify the surface area occupied by atherosclerosis. Total plaque area in the brachiocephalic artery and in the aortic root was measured on van Gieson-stained tissue sections, and calcification was assessed by Alizarin red staining. Sirius red staining was used to quantify collagen under polarized light. VSMC and macrophage content in the aortic root and brachiocephalic artery were analyzed by immunohistochemistry.

### 2.4. In vitro studies

Primary cultures of aortic VSMC were established from 8-12 week-old *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Mmp10*$^{-/-}$ mice and *in vitro* calcification assays were performed. The expression of vascular calcification-related genes [alkaline phosphatase (*ALPL*), bone morphogenetic protein-2 (*BMP2*), Fetuin-A, matrix GLA protein (*MGP*), Osterix (*Sp7*) and *Runx2*] was assessed by reverse transcription and real time-quantitative PCR.

### 2.5. Statistical analysis

Continuous variables were expressed as mean ± standard deviation or median (interquartile range), unless otherwise stated. Data normality was assessed through Shapiro-Wilk's test. Serum MMP10 concentration was transformed logarithmically and its relationship with coronary calcium was analyzed by ANCOVA. Association between two variables was assessed by Pearson's correlation test (normal distribution) or Spearman's rank correlation test (non-parametric distribution). Differences between two groups were analyzed by Student's t-test when following a normal distribution or Mann-Whitney *U* test in case of non-parametric distribution. When comparing more than two groups, one factor ANOVA with post hoc test was used, in the case of parametric distribution, or Kruskal Wallis test followed by Mann-Whitney test with Bonferroni correction, in the case of non-parametric. The Pearson's $\chi^2$ -