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Integrative Studies Implicate Matrix Metalloproteinase 12 as a Culprit Gene for Large-Artery Atherosclerotic Stroke

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ABSTRACT

Background: Ischemic stroke and coronary heart disease are important contributors to the global disease burden and share atherosclerosis as the main underlying cause. Recent evidence from a genome-wide association study (GWAS) suggested that single nucleotide polymorphisms (SNP) near the *MMP12* gene at chromosome 11q22.3 were associated with large-vessel ischemic stroke. Here we evaluated and extended these results by examining the relationship between *MMP12* and atherosclerosis in clinical and experimental studies.

Methods and Results: Plasma concentrations of MMP-12 were measured at baseline in 3 394 subjects with high-risk for cardiovascular disease (CVD) using the Olink ProSeek CVD I array. The plasma MMP-12 concentration showed association with incident cardiovascular and cerebrovascular events (130 and 67 events respectively over 36 months) and carotid intima-media thickness progression ($p=3.6 \times 10^{-5}$). A GWAS of plasma MMP-12 concentrations revealed that SNPs rs499459, rs613084 and rs1892971 at chr11q22.3 were independently associated with plasma MMP-12 ($p<5 \times 10^{-8}$). The lead SNPs showed associations with mRNA levels of MMP-12 and adjacent MMPs in atherosclerotic plaques. MMP-12 transcriptomic and proteomic levels were strongly significantly increased in carotid plaques compared with control arterial tissue and in plaques from symptomatic *vs.* asymptomatic patients. By combining immunohistochemistry and proximity ligation assay, we demonstrated that MMP-12 localises to CD68+ macrophages and interacts with elastin in plaques. MMP-12 silencing in human THP-1 derived macrophages resulted in reduced macrophage migration.

Conclusions: Our study supports the notion that *MMP12* is implicated in large-artery atherosclerotic stroke, functionally by enhancing elastin degradation and macrophage invasion in plaques.

Key words: atherosclerosis, carotid intima-media thickness, matrix metalloproteinase, plaque rupture, stroke, vascular disease

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INTRODUCTION

Atherosclerosis is a major cause of coronary heart disease (CHD) and along with cardioembolism contributes significantly to ischemic stroke (IS). The disease involves gradual accumulation of lipids and inflammatory cells encapsulated by fibrotic tissue in the intima of larger arteries [1]. Erosion or rupture of the fibrous cap covering the atherosclerotic plaque exposes plaque content to circulating blood and triggers thrombosis that may lead to myocardial infarction (MI) and stroke. The more recent view of plaque rupture and erosion suggests that atherosclerotic lesions undergo circles of remodelling, involving both stabilisation and destabilisation events, rather than being gradually weakened in a continuous manner [2].

Plaque remodelling involves breakdown of extracellular matrix (ECM), which is a process partly controlled by matrix metalloproteinases (MMPs), a family of 20 secreted or membrane-associated endoproteinases and their inhibitors [3, 4]. Evidence for a role of MMPs in cardiovascular disease (CVD) has been obtained from studies in transgenic disease models and in studies investigating some MMPs as biomarkers for clinical events [5-10]. In contrast, few studies have evaluated the role of MMPs in CVD using human genetics integrated with mRNA and protein expression in carotid tissue. Robust genetic association between the MMP gene cluster (chromosome 11q22.3) and large artery stroke was recently demonstrated in genome-wide association study (GWAS) (13). In addition, association between SNPs at the 11q22.3 locus and COPD has been demonstrated [11-13]. The lead SNP for large-artery stroke, rs660599, is located near the *MMP12* gene and it was shown that the MMP-12 mRNA expression in disease tissue is increased compared to healthy controls, which suggested that MMP-12 drives the genetic association signal for stroke observed at 11q22.3 [13].

It is well established that identification of the causal gene of GWAS loci is challenging, mainly due to high degree of linkage disequilibrium across large regions of the genome but also because distal genomic regulation of genes is common. The 11q22.3 region is no exception as it harbours several other members of the MMP family (*MMP7, MMP20, MMP8, MMP10, MMP1, MMP3* and *MMP13*), of which many have been suggested to play a role in atherosclerosis. Therefore, additional evidence is required to establish the role of MMP-12 in large-artery stroke. To address this question, we applied an integrative approach and investigated MMP-12 protein and gene variants in relation to carotid intima-media thickness (cIMT) progression, incident CVD, transcriptomic and proteomic levels in carotid plaque tissue, followed-up with detailed *in situ* and *in vitro* studies (design shown in Fig. 1).

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MATERIAL AND METHODS

The IMPROVE study

The IMPROVE study is a multicentre, longitudinal, observational study, which recruited 3 711 subjects with at least three cardiovascular risk factors but who were asymptomatic for CVD. Subjects were recruited from Finland, France, Italy, the Netherlands, and Sweden. Detailed cIMT measurements were performed at baseline, after 15 months and at 30 months. The Fastest-$IMT_{max\text{-}progr}$ parameter, herein referred to as cIMT progression, was previously developed and evaluated in the IMPROVE study and found to independently predict new CVD events [14]. Incident CVD end points were defined as occurrence of myocardial infarction, angioplasty, diagnosis of angina pectoris, angioplasty, coronary artery bypass grafting and/or sudden cardiac death, ischemic stroke, transient ischemic attack, peripheral revascularization, and/or diagnosis of intermittent claudication during a median follow-up of 36 months. Further design features and definitions of the study are described in the online supplement and in previously published articles [14, 15].

Plasma MMP-12 concentrations were measured at baseline using the Olink ProSeek CVD array I (Olink Proteomics, Uppsala, Sweden), according to the standard protocol [16]. The inter-plate coefficient of variation for the MMP-12 assay was 14 %, as estimated using a pooled plasma control across all plates. The measurements were carried out in 3 394 IMPROVE participants in whom genotype information post quality control (QC) was also present. Plasma MMP-12 activity was measured in duplicates in 20 plasma samples from the IMPROVE cohort. Ten samples were randomly chosen from the 75-95th percentile of plasma MMP-12 concentration and ten from the 5-25th percentile. Activity was measured with the Fluorimetric Sensolyte 520 MMP-12 Assay Kit (Anaspec), which specifically detects elastin degradation products (desmosin).

Genotyping in the IMPROVE study was performed using the Illumina CardioMetabochip and the Immunochip arrays [17, 18]. The combined SNP genotyping data on both platforms were merged and subjected to the following QC using PLINK 1.7, standard programming tools and manual proof-reading. SNPs were excluded for probe to genome mismatch, incorrect assignment of allelic variants in the array design, failed Hardy-Weinberg Equilibrium test at $1x10^{-6}$, call rate <95% or failed Illumina genotype calling QC. Samples were excluded that showed evidence of gender mismatch, abnormal inbreeding coefficient, failed cryptic relatedness test or had an overall sample call rate <95%. After QC and filtering out of SNPs with a minor allele frequency of <0.5%, a total number of 260 484 unique SNPs in 3 403 subjects remained for analysis.

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The BiKE study

Patients undergoing surgery for symptomatic (S) or asymptomatic (AS), high-grade (>50% NASCET) carotid stenosis at the Department of Vascular Surgery, Karolinska University Hospital, Sweden were consecutively enrolled in the study and clinical data recorded on admission [19]. Symptoms of plaque instability were defined as transitory ischemic attack (TIA), minor stroke (MS) and amaurosis fugax (retinal TIA; AF). Patients without qualifying symptoms within 6 months prior to surgery were categorised as AS and indication for carotid endarterectomy based on results from the Asymptomatic Carotid Surgery Trial (ACST) [20]. Carotid endarterectomies (carotid plaques, CP) and blood samples were collected at surgery and retained within the Biobank of Karolinska Endarterectomies (BiKE). This study involved 3 non-overlapping sub-cohorts of patients, where one Affymetrix microarray dataset was generated by profiling n=127 atherosclerotic plaques (of which n=87 were from S+40 from AS patients) and n=10 normal arteries (further referred to as the 'large dataset') and the other by profiling n=50 plaques (n=40 from S+10 from AS patients) and n=5 normal arteries (further referred as the 'small dataset'). DNA genotyping by Illumina chips was carried out on patients from the 'large' sub-cohort, and used for expression quantitative trait loci (eQTL) analyses. The third sub-cohort of n=18 BiKE plaques (n=9 from S+9 from AS patients, matched for gender, age and statin medication) were analysed using LC-MS/MS as previously described [21]. For these proteomic analyses, a central portion of the plaque corresponding to the maximum stenosis was separated from the respective downstream peripheral end (adjacent tissue) of the plaque and used in comparisons [22, 23]. Details of patient demographics, sample processing, microarray and proteomic analyses have been described previously and the study was approved by the regional Ethics Committee [24-27].

In this study CHD [28] was defined as stable and unstable angina, nonfatal MI and coronary death and large artery stroke as thrombosis or embolism due to atherosclerosis of a large artery (internal carotid-, middle cerebral-, vertebral- or basilar arteries) [29]. The study cohorts and workflow are presented in Fig. 1.

Statistical analyses

Association between plasma MMP concentrations, SNPs and cIMT at baseline and progression (fastest-$IMT_{max\text{-}progr}$) and incident CVD were tested using linear or logistic regression models with one basic model adjusting for recruitment centre, age, gender and another advanced model including also Framingham risk score parameters i.e. smoking, diabetes, systolic blood pressure, HDL-C and total cholesterol. Survival analysis of plasma MMP-12 tertiles was performed using Cox-regression. A total number of 67 cerebrovascular and 130 coronary endpoints were observed after 36 months in the subset of 3 394 IMPROVE subjects with complete baseline clinical data, genetic information and

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plasma MMP-12 measurements. A total of 260 484 SNPs from the combined Illumina Immunochip and Metabochip genotyping arrays were tested as determinants for plasma MMP-12 using the Plink 1.9 Wald-test of residuals derived from a linear regression model that adjusted for recruitment centre, age, gender, smoking, diabetes, hypertension, BMI and Olink ProSeek analysis batch [30]. The standard p-value threshold for genome-wide significance at $5x10^{-8}$ was used in the discovery of novel SNPs linked to plasma MMP-12 concentrations. Microarray and proteomic dataset analyses of BiKE samples were performed as reported previously [21, 24]. Correlation between mRNA or protein levels was assessed using Spearman whereas ANOVA was used for multi-group comparisons, with $p<0.05$ as threshold for statistical significance. In the *in vitro* studies, cells were automatically counted using the "Cell Counter" plugin of the ImageJ software. Differences between the groups were tested using Student's t-test.

Additional methods are available in the online Supplemental Material.

RESULTS

Plasma MMP-12 levels associate with cIMT progression and incident cardiovascular events

MMP-12 plasma concentrations were positively correlated with age, smoking and diabetes and inversely with HDL-C in the IMPROVE study (Table 1), whereas no or weak correlations were observed with gender, systolic blood pressure or total cholesterol. Plasma MMP-12 at baseline was tested as potential predictor of cIMT both at baseline and in progression, using a basic model and an advanced model with additional adjustment for Framingham risk score baseline parameters. As previously shown by Goncalves et al [10], plasma MMP-12 was associated with cIMT at baseline, with the average thickness across all measured segments showing the strongest association (Table 2). The plasma MMP-12 concentration was also positively associated with faster cIMT progression, which persisted after adjustment for Framingham score parameters (Table 3). The association between plasma MMP-12 concentration and cIMT progression persisted even after additional adjustment for IMT_{max} at baseline (standardized beta=0.052, p=0.008). Plasma MMP-12 levels were also associated with incident CVD at 36 months (n=197) after adjustment for study site, age and gender (odds ratio 1.26, 95% confidence interval (1.03-1.55), p=0.028). The odds ratio for cerebrovascular events (n=67) was 1.45, 95% confidence interval (1.03-2.05), p=0.034. After adjustment for Framingham risk score parameters, associations with both all CVD events and with cerebrovascular events only were attenuated (odds ratio 1.13, 95% confidence interval (0.91-1.40), p=0.26 and odds ratio 1.25, 95% confidence interval (0.87-1.81), p=0.22, respectively). A survival analysis of plasma MMP-12 tertiles

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suggested that patients in the highest tertile progressed more rapidly to cerebrovascular endpoints compared to the middle and lowest tertile (Cox-regression p=0.022, Supplementary Fig I) After adjustment for covariates, the association was no longer significant (p=0.15).

Of note, MMP-1, MMP-3, MMP-7 and MMP-10 (present on the Olink CVD I assay) were also tested for association with cIMT at baseline, cIMT progression and incident CV events, but did not pass significance levels.

SNPs at 11q22.3 and plasma MMP-12 concentrations

Next, we aimed to assess whether SNPs at 11q22.3 were linked to plasma MMP-12 concentration using the IMPROVE study. Of a total number of 260 484 SNPs, 12 SNPs exceeded the standard threshold for genome-wide significance at $5x10^{-8}$ for association with plasma MMP-12 concentration (Supplementary Fig II). All associated variants mapped to the MMP gene cluster region on chromosome 11q22.3. SNP rs660599, previously associated with risk of stroke in the METASTROKE study by Traylor et al (13), was associated with plasma MMP-12 at $p=8.8x10^{-35}$. To identify potential additional association signals at this locus, conditional analyses were performed by adding the lead variant as covariate in linear regression models of adjusted plasma MMP-12. After three rounds of conditioning, including in order rs499459, rs1892971 and rs613804, no association signal at $5x10^{-8}$ remained (Supplementary Table I). The r^2 measure for linkage disequilibrium (LD) was less than 0.01 for any combination of these SNPs, suggesting that the effects of rs499459, rs1892971 and rs613804 on plasma MMP-12 concentration are independent from one another (Supplementary Table II). Finally, for the 3 variants emerging from IMPROVE analyses (rs499459, rs1892971 and rs613804) and the 1 from METASTROKE study (rs660599), we also stratified the association with plasma MMP-12 levels, mean and max cIMT and plaque area by each allele (Supplementary Table III). Interestingly, our results show that the A allele of rs660599, which was previously associated with stroke risk in the METASTROKE study, here associated with higher cIMT for the different intima-media measures, however the same allele also associated with lower plasma MMP-12 concentration. Similarly, the A allele of rs499459 associated with higher cIMT measurements and lower plasma MMP-12 levels.

SNPs in the *MMP12* region affect its mRNA expression in atherosclerotic plaque tissue

In order to evaluate potential associations of SNPs rs499459, rs613804, and rs1892971 with plaque MMP-12 mRNA levels in patients with carotid atherosclerosis, eQTL analyses were performed using microarray data from the BiKE cohort. A tentative association was found for rs1892971 with MMP-

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12 mRNA levels in plaques (p=0.079) (Fig. 2A). To investigate whether the effect of rs1892971 was specific for *MMP12* or related also to adjacent genes in the *MMP* gene cluster, we tested its association with expression of *MMP1, MMP3* and *MMP13* in plaques, but no significance could be shown (Fig. 2A). A proxy for rs499459 (rs566125, r^2=0.92; D'=1) showed no association with *MMP12* plaque expression in the whole cohort, but when the association was stratified according to the patient phenotype, this variant showed significance particularly in plaques from asymptomatic patients (Supplementary Fig III). However, it was also significantly associated with expression of *MMP1, MMP3* and marginally with *MMP13* (p=0.00048, p=0.02 and p=0.045, respectively, Fig. 2B). No significance was obtained by similar eQTL analyses for rs613804. In addition, we sought to evaluate whether the reported lead SNP for large-artery atherosclerotic stroke, rs660599, was also related to *MMP12* expression levels in plaques. A perfect proxy for rs660599, rs615098 (R^2=1, D'=1) showed association with *MMP12* mRNA expression in plaques generally (p=0.0039), as well as in plaques from asymptomatic patients (p=0.005) and symptomatic ones (p=0.049) separately (Supplementary Fig III). Interestingly, this variant also showed strong associations with both *MMP1* and *MMP3* levels in plaques (p=0.0016 and p=0.0025, respectively, Fig. 2C). Other *MMPs* in this region *(MMP7, MMP8, MMP10, MMP20* and *MMP27)* were also examined for association with these variants, but showed no significance.

Further analysis of LD in the associated genomic region using the SNIPA tool [31] with data from the European part of the 1000 genomes project, showed that rs499459 is located in a broader region of high LD with multiple other SNPs (including the lead SNP for stroke rs660599), that span *MMP1, MMP3* and *MMP12.* In contrast, rs613804 is located in a region of high LD near and some distance downstream of the *MMP12* gene. SNP rs1892971 (and those in high LD) is located in a narrow intergenic region upstream of *MMP12* and downstream of *MMP13* (Supplementary Fig IV).

Of note, MMP-12 mRNA expression levels found in plaques from these eQTL analyses, were several fold higher than levels of any other interrogated MMP in this region.

MMP12 is highly elevated in carotid plaques from symptomatic patients

Indeed, on the transcriptomic level we observed strong upregulation of the MMP-12 mRNA expression in two independent microarray datasets comparing plaques vs. normal arteries (Fig. 3A). In the large dataset, the difference between these groups was log mean±SD=8.14±0.17, p<0.0001 whilst in the smaller dataset it was log mean±SD=5.20±0.22, p<0.0001. Further upregulation was noted by stratifying patients into symptomatic vs. asymptomatic category (large dataset log mean±SD=0.74±0.33, p=0.03), and a trend towards upregulation in symptomatic patients was noted also in the smaller dataset (not shown). A strong enrichment of MMP-12 was confirmed on the

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proteomic level, with mean difference ±SD=0.90±0.10, p=0.04 comparing central plaques vs. adjacent tissue and mean difference±SD=1.00±0.07, p=0.03 by comparing plaques from symptomatic vs. asymptomatic patients (Fig. 3A).

To evaluate the previous publications reporting high expression of MMP-12 in macrophages [32-35], we next investigated the correlation between MMP-12 expression and macrophage cell markers in plaques (Fig. 3B). MMP-12 transcript was strongly significantly correlated with that of macrophage markers CD163 (Spearman r=0.67, p<0.0001), CD36 (r=0.81, p<0.0001) and MAC2 (r=0.72, p<0.0001), and similarly positive significant correlations of MMP-12 with macrophage markers were confirmed on the protein level.

MMP-12 localizes to macrophages and interacts with elastin in plaques

In continuation, we sought to trace the MMP-12 protein and its major substrate elastin in human carotid arteries by immunohistochemistry. No signal for MMP-12 was present in a normal artery whereas elastin was abundantly expressed in the media, as expected (Fig. 4A, arrows). In plaques we localized total MMP-12 mostly to macrophage-rich regions in the necrotic core by co-staining with an antibody against macrophage marker CD68 (Fig. 4B, third panel, arrows and Supplementary Fig VA). We also detected preserved, orderly packed elastin fibres sub-intimally at the plaque periphery, where no MMP-12 was expressed (Fig. 4B, second panel, arrows). However, towards the necrotic core, staining for elastin appeared gradually disarranged and co-localized with MMP-12 (Fig. 4B, second panel, enlarged inset), and was lost in consecutive sections of areas near the fibrous cap with condensed MMP-12^{+} macrophages (Supplementary Fig VA, enlarged insets). Signal for active MMP-12 was observed diffusely in the extracellular matrix in the lesions, where elastin fibres were disarranged or absent (Supplementary Fig VB, arrows). Furthermore, by proximity ligation assay (PLA) we showed that MMP-12 and elastin proteins co-interact in plaques *in situ*, particularly in regions with moderate to strong MMP-12 expression (Fig. 4C, enlarged insets).

MMP-12 levels correlate with elastin breakdown in plasma and plaques from atherosclerotic patients

We also assessed the relationship between MMP-12 concentration and elastin degradation in plasma from the IMPROVE cohort, by measuring the level of the released degradation product desmosin. With equal elastin substrate amount available, we observed on average a 22% elevated elastin degradation in plasma samples with high MMP-12 levels compared to those with low MMP-12 levels (mean 56.1±5.84 vs. 46.0±9.20 μM desmosin, p=0.15, Supplementary Fig VIA). Similarly, in plaques

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we observed a strongly significant inverse correlation between MMP-12 and elastin protein levels (r= -0.47, p=0.004, Supplementary Fig VIB). Together with immunostainings for active MMP-12 in plaques, our results confirm an association between higher MMP-12 levels and increased elastolytic activity both in plasma and plaques from atherosclerotic patients.

MMP-12 silencing leads to reduced macrophage recruitment and ECM invasion capacity

In vitro experiments were performed to evaluate the previous studies suggesting that MMP-12 influences macrophage migration and degradation of ECM proteins [34]. MMP-12 mRNA expression in THP-1 derived human macrophages was effectively inhibited by siRNA and the reduction was estimated to 70% by qPCR analysis (Supplementary Fig VII). Western blot demonstrated that MMP-12 mRNA downregulation was associated with ~25% reduction in active MMP-12 protein in macrophages (Supplementary Fig VII). Our results showed that macrophages with reduced MMP-12 levels exhibited ~30% lower invasion capacity compared to controls (%invasion=45±13 vs. 64.5±12, p=0.01). We then examined the macrophage invasion capacity upon lowering of the MMP-12 availability with an MMP-12-specific antibody, in both the control and the silenced cells. MMP-12 inhibition by antibody blocking resulted in ~31% less macrophage invasion compared to controls (%invasion=16.5±4.2 vs. 23.9±5.2, p=0.04). Furthermore, we observed a reduction in the total number of macrophages invading through the ECM once the antibody was introduced. The mean reduction was 63%, p=0.02 for cells with suppressed MMP-12 levels and 62%, p=0.02 for those with normal MMP-12 levels (Fig. 5A).

In addition, we sought to identify whether MMP-12 expression levels in macrophages affected the recruitment of other macrophages. We observed that the migration of new macrophages was 20% lower towards macrophages with reduced MMP-12 expression. Post migration, 28.5% (mean=430.5±27 cells) of the total cell count in the Transwell chamber originated from macrophages that migrated towards those with suppressed MMP-12, whereas 35.4% (mean=743.5±81 cells) originated from the population of macrophages that had migrated towards those with unchanged MMP-12 expression (p=0.01, Fig. 5B).

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DISCUSSION

We used an integrative approach based on genetics, transcriptomics and proteomics in clinical and experimental samples to demonstrate that i) MMP-12 is regulated at the mRNA and protein level by SNPs in the MMP gene cluster, ii) its plasma concentration is linked to cIMT progression as well as cerebrovascular events in high-risk subjects, and iii) it is highly enriched in human atherosclerotic plaques where it interacts with elastin and enhances macrophage invasion. Moreover, we link a SNP near *MMP12* that was previously shown to be associated with stroke by Traylor et al. [13] with plasma MMP-12 concentrations and mRNA expression of MMP-12, but also with other MMPs in carotid plaques. Therefore, the present investigation in combination with the recent article by Traylor et al. suggests that MMP-12 may be causally implicated in large-artery stroke.

We found that three SNPs, rs499459, rs613804 and rs1892971, which are all located in chromosome 11q22.3 region near the genes *MMP1, MMP3, MMP12* and *MMP13*, were independently associated with plasma MMP-12 concentration. No other signals at genome-wide significance were observed in our study, although we acknowledge that SNPs outside of chromosome 11q22.3 with smaller effects on plasma MMP-12 may still exist. The finding links chromosome 11q22.3 to plasma MMP-12, but does not provide conclusive evidence that the associations are mediated via the *MMP12* gene itself, as other genes in the region may also play a role in determining the plasma MMP-12 concentration. To further investigate whether the SNPs associated with plasma MMP-12 levels were specifically involved in the regulation of MMP-12 or had effects on neighbouring MMPs too, we performed eQTL analyses in carotid plaques. The results revealed that rs1892971 is marginally linked to MMP-12 mRNA expression, whereas rs499459 is an eQTL also for MMP-1 and MMP-3. Based on the LD analysis and the eQTL results, one may speculate that rs1892971 is more distinctly related to the *MMP12* gene whereas rs499459 and SNPs in LD such as rs660599 may influence a broader set of MMPs in the region. Analysis also indicated that rs499459 and rs660599 variants may have the potential to highlight a subgroup of patients that are clinically silent at the time of surgery (asymptomatic), but that may confer a higher risk of stroke, based on association with MMP-12 expression in their plaques.

In the IMPROVE study, we showed that increased plasma MMP-12 concentration at baseline was robustly associated to baseline cIMT. This result is well in line with the previous report by Goncalves et al. In addition, we observed that plasma MMP-12 at baseline predicted a higher rate of cIMT change over time, regardless of the baseline cIMT, and an increased risk of suffering a cerebrovascular event within 36 months from baseline. We noted that the association of plasma MMP-12 with prospective CVD events was attenuated by adjustment for Framingham risk score

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parameters, which implies that MMP-12 in plasma reflects presence of one or several of the Framingham score risk factors. Indeed, positive correlations between plasma MMP-12 and systolic blood pressure, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) have previously been reported [10], which limits the implications of the reported association of plasma MMP-12 with cIMT progression for CHD. Importantly, since MMP-12 concentrations measured with our plasma assay may not necessarily reflect the proteinase activity of MMP-12, we addressed this issue by a complementary experiment where we confirmed that subjects with higher plasma MMP-12 concentrations showed an overall higher concentration of the elastin degradation product desmosin.

We also assessed MMP-12 expression in a large biobank of human carotid endarterectomy samples and found that it was strongly enriched at both transriptomic and proteomic levels in plaques compared to undiseased arteries, as well as in plaques from patients that suffered stroke compared to asymptomatic ones, which not only validates but also extends previous publications about MMP-12 [32]. Regarding the proteomic enrichment of MMP-12 in central vs. distal segments of plaques, these areas have been identified as areas of severe plaque complications vs. stable growth respectively. It has been recognized that the central part is associated with all components of a complicated lesion (fibrous cap, necrotic core, shoulder region) and increased incidence of cap rupture, while the adjacent peripheral end was associated with higher SMC content and represents the fibrotic intima [36-38]. Taken together with our genetic data, these findings underscore the significance of MMP-12 in prevalent as well as incident stroke.

Interestingly, Scholtes et al. earlier showed that patients having a high proportion of MMP-12 expressing macrophages in their carotid plaques are more likely to suffer subsequent stroke and MI [33]. In our study, MMP-12 protein was localised to macrophages and shown to interact with elastin *in situ*, especially in areas of degraded ECM. It has been previously demonstrated that MMP-12 has broad substrate specificity for a range of ECM proteins, including type IV collagen, fibronectin, laminin, vitronectin and basement membrane proteins, while potential regulators of MMP-12 in the atherosclerotic environment include several pro-atherosclerotic inflammatory cytokines and growth factors released by various cells in the atherosclerotic milieu, such as macrophages, smooth muscle and endothelial cells [39]. Considering that MMP-12 activates MMP-2 and MMP-3 [40], we suggest that besides direct effects, higher expression of MMP-12 could also lead to activation of these other MMPs in plaques, which would further increase degradation of the fibrous cap and promote plaque instability. Considering that plaque rupture frequently occurs over a macrophage-rich area near the shoulder of the plaque [41], it is interesting to hypothesize that macrophages expressing MMP-12 may particularly contribute to thinning and rupture of the fibrous cap and subsequent adverse events. It is worth noting here that the BiKE cohort comprises only advanced lesions (AHA grade IV and V), and therefore cannot provide information about disease progression. Also, the phenotyping of patients

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based on the presence or absence of cerebral symptoms was likely insufficient to completely exclude overlap between symptomatic and asymptomatic groups, since lesions from asymptomatic patients may vary in morphological features of plaque instability [27]. In addition, since atherosclerosis presents different features in different vascular beds, we limit our findings about MMP-12 to carotid arteries.

Important studies that have helped to expand the understanding of MMP-12 in atherosclerosis were published by Liang et al [42], who demonstrated that rabbits overexpressing human MMP-12 in tissue macrophages developed more extensive atherosclerosis in large arteries compared to controls, and Johnson et al, who showed that a murine specific MMP-12 inhibitor significantly reduced atherosclerotic plaque area in Apo-E knockout mice fed a Western diet [43]. The MMP-12 inhibitor treatment induced additional beneficial changes, such as attenuated macrophage invasion and apoptosis. Our *in vitro* results support the notion that MMP-12 influences macrophage invasion, as we were able to validate these previous studies by showing markedly decreased migration through the ECM upon MMP-12 suppression [34]. Moreover, we demonstrated that MMP-12 suppression not only attenuated macrophage invasion, but also reduced the recruitment of other macrophages. One may speculate that the invasion-promoting effects of MMP-12 contribute to a vicious cycle where the rate of new macrophage recruitment into plaques is amplified by increased production of MMP-12 in plaque macrophages and a systemic release of MMP-12 to the circulation. Therefore, suppression of MMP-12 could promote double protective effects on the progression of disease: in reducing the invasion capacity of macrophages and prohibiting further macrophage recruitment, as well as in ameliorating the overall inflammation. One limitation of our invasion assay with the secondary macrophage migration setup could be the degradation level of the ECM coating for the newly invading macrophages. This could be dependent not only on the primary macrophages in the culture (control or siMMP-12), but also the number of the available cells. Thus, a less degraded ECM present in the control macrophage culture might provide an easier access for migration and affect the secondary invasion rate. However, these effects are likely to be reflective of the pathophysiological situation in plaques as well, and *in vitro* may be considered less significant due to the presence of potent chemo-attracting properties of the medium environment in the bottom well.

Altogether, in the present study we show that MMP-12 transcriptomic and proteomic levels are strongly significantly increased in plaques compared with control tissue and in plaques from symptomatic vs. asymptomatic patients. MMP-12 localises to CD68+ macrophages in plaques, where it interacts with elastin and its silencing results in reduced macrophage migration. These studies clearly link higher MMP-12 mRNA and protein levels in plaques to increased atherosclerotic disease. In contrast, it appears that genetically lower plasma MMP-12 is significantly associated with increased cIMT measurements and risk of stroke. Our study does not aim to disentangle this seemingly complex relationship, but as one potential explanation we suggest is the locus pleiotropy,

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as it is possible that the *MMP12* SNPs (in addition to plasma MMP-12) are also associated with other yet unknown factors, which may be responsible for their observed protective effects. It may also be that MMP-12 expression has differential effects during early atherogenesis compared to advanced stages, and even the differential local vs. systemic effects should be taken into account. We speculate that plasma MMP-12 may have a protective function, which it can exert on the growing plaque in the vasculature by 'surveying' and minimizing the plaque deposits especially during early atherogenesis, while the MMP-12 secreted by macrophages within the late stage plaques degrades the tissue rendering it more prone to rupture.

Despite encouraging pre-clinical data, the design and development of selective MMP inhibitors for therapeutic use in CVD still remains at an early stage. The failure of the first generation of broad-spectrum inhibitors undermined continued evaluation of MMPs as disease targets, and the main focus recently has been to design inhibitors targeting detrimental MMPs, without interfering with those that have an important role in preventing the progression of disease [44]. To achieve this, a greater understanding of the general MMP functions remains essential, including potential differences in how MMP-12 particularly contributes to carotid vs. coronary vascular disease. In conclusion, our study strengthens the support for MMP-12 as a causal factor in large-artery atherosclerotic stroke and highlights its role in macrophage invasion and elastin degradation.

Conflict of Interest Disclosures

AM is employed by Pfizer and holds stock in Pfizer Inc. None of the other authors have disclosures to report.

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TABLE AND FIGURE LEGENDS

Fig 1. Flow-chart of the study cohorts and design. Analyses of plasma MMP-12 concentrations in relation to cIMT progression, future CVD events and SNPs were conducted in the IMPROVE study, which recruited 3,397 subjects with high CVD risk but without manifest CVD at baseline. These investigations were followed by eQTL analyses of the associated SNPs in relation to MMP-12 mRNA expression in atherosclerotic plaque specimens from 127 patients undergoing carotid endarterectomy in the BiKE study. Furthermore, MMP-12 mRNA and protein expression levels were examined in atherosclerotic plaques compared to control tissues (non-atherosclerotic arteries or adjacent distal plaque tissue). Finally, MMP-12 protein localisation, activity and interaction with elastin and the effects of MMP-12 silencing were investigated using molecular biology methods in plaques, plasma and functional assays *in vitro*.

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Table 1. Association of plasma MMP-12 and Framingham risk parameters. Partial correlations with correction for study recruitment site were calculated to assess the relationship between plasma MMP-12 and Framingham score parameters. R indicates the correlation coefficient.

Table 2. Association of plasma MMP-12 with baseline cIMT. CCA indicates common carotid artery, standardised beta indicates the standard deviation change per standard deviation of the cIMT measure. R2 model indicates the variability explained by linear regression model adjusted for age, gender, smoking, total cholesterol, HDL cholesterol, diabetes, systolic blood pressure and study centre.

Table 3. Association of Framingham score parameters and plasma MMP-12 with cIMT progression. cIMT progression was defined as previously described (Baldassarre D *et al. J Am Coll Cardiol* 2012; **60:** 1489-99.). The left side of the Table shows correlation coefficients and p-values for single-parameter correlations with correction only for study site. The right side of the Table shows results from a multiple linear regression model that included all parameters shown. The beta-values are given as standard deviation per standard deviation of cIMT progression.

Fig 2. eQTL analysis of *MMP12* associated SNPs in atherosclerotic plaques. *MMP12* variant rs1892971 was found to be tentatively associated to MMP-12 mRNA expression in carotid plaques. This variant was also un-associated to the expression of other MMPs from the same locus in plaques **(A).** Variant rs566125 was strongly associated to the expression levels of MMP-1 and marginally also MMP-3 and MMP-13, but not MMP-12 **(B)**. SNP rs615098 (a perfect proxy for METASTROKE *MMP12* associated variant rs660599) was marginally associated to MMP-12 expression in plaques, and significantly to the expression of both MMP-1 and MMP-3 **(C).** Plots show median±standard deviation (SD).

Fig 3. Expression analyses of MMP-12 in carotid plaques. MMP-12 mRNA is upregulated in plaques (cp) compared with normal arteries (na) and in plaques extracted from symptomatic (s) compared with asymptomatic (as) patients. Plots show log2 mean±SD (upper panels). Similarly, MMP-12 protein levels are enriched in plaques compared with adjacent arterial tissue, and in plaques from symptomatic vs. asymptomatic patients. Plots show mean relative levels with SD **(A)**. MMP-12 transcript and protein are significantly correlated with macrophage markers (CD163, CD36 and MAC2) in BiKE plaques **(B)**.

Fig 4. Localisation of MMP-12 in plaques. In the normal artery no MMP12 immunoreactivity was detected, while elastin (green signal) was abundant in the media (A, arrows). In consecutive plaque sections (B), MMP-12 was localised to the $CD68^{+}$ macrophage-rich regions in the necrotic core (third panel, arrows and enlarged inset). Intact elastin fibres were present at the periphery of the plaques (middle panel, green staining, arrows) where no MMP-12 (red signal) was expressed, but they

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appeared gradually disarranged closer towards the necrotic core where MMP-12 was enriched (enlarged inset). By proximity ligation assay (PLA) in consecutive plaque sections, positive signal for MMP-12 and elastin co-interacting proteins was detected (enlarged inset, middle panel), specifically in regions with moderate to strong MMP-12 expression (C). As positive control PLA probe directed to MMP-12 antibody was used, showing areas of intense MMP-12 expression in the necrotic core (arrows, third panel). No PLA signal was observed in the negative control (enlarged inset, first panel).

Fig. 5. MMP-12 RNA silencing and antibody blocking reduce macrophage migration. Panel in A (left) shows THP-1 derived macrophages migrated through a Transwell chamber without bottom coating. Middle panel shows macrophages migrated through a Transwell with MaxGel coating. Right panel shows macrophages incubated with 0.25mg MMP-12 antibody prior to migration through a Transwell with MaxGel coating. All chambers had 8mm diameter holes at the bottom of the top Transwell, cells were stained with the nuclear stain DAPI (blue). In (B) macrophage nuclei stained red (DRAQ5) represent the primary invaded cells in control or siMMP12 conditions, while macrophage nuclei in blue (DAPI) represent the migrated cells in both conditions. Purple coloured nuclei are those double stained for DRAQ5/DAPI. On average, 70% (±16.2% SD, $p=0.011$) more macrophages migrated towards the control macrophages than towards siMMP12 macrophages. The quantification is represented as a bar graph in the last panel.

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FIGURES AND TABLES

Figure 1.

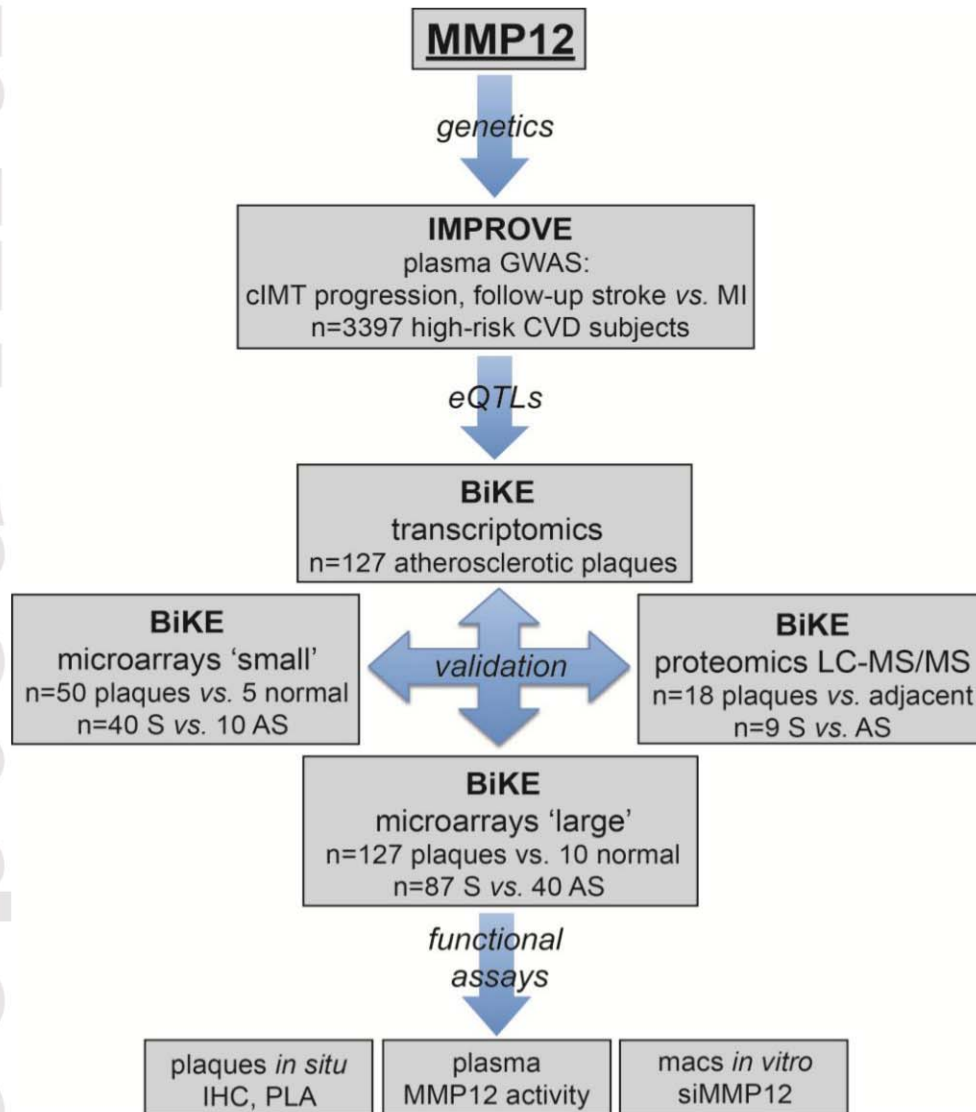

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Table 1.

	Plasma MMP-12	
	R	P-value
Age	0.24	8.7E-45
Female gender	-0.03	0.097
Smoking	0.19	4.2E-29
Total cholesterol (mmol/L)	0.01	0.633
HDL cholesterol (mmol/L)	-0.13	9.3E-15
Diabetes	0.05	0.002
Systolic blood pressure (mm Hg)	0.04	0.024

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Table 2.

	Standardised beta	p-value	R2 model
Mean CCA IMT	.091	5.6E-08	0.190
Mean Bulb IMT	.088	3.3E-07	0.151
Mean IMT	.112	5.4E-12	0.242
Max IMT	.100	6.9E-09	0.155
Plaque area	.099	5.4E-10	0.268

Table 3.

	Correlations		Multiple Linear Regression	
	R2	p-value	Standardised Beta	p-value
Age	0.04	0.027	0.069	0.001
Female gender	-0.08	2.0E-05	-0.074	2.0E-05
Smoking	0.07	3.1E-04	0.038	0.042
Total cholesterol (mmol/L)	0.04	0.021	0.053	0.008
HDL cholesterol (mmol/L)	0.02	0.297	-0.052	0.011
Diabetes	-0.07	2.9E-04	0.038	0.051
Systolic blood pressure (mm Hg)	0.06	1.6E-03	0.042	0.04
Plasma MMP-12 (arbitrary units)	0.11	1.1E-09	0.074	5.0E-05

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Figure 2.

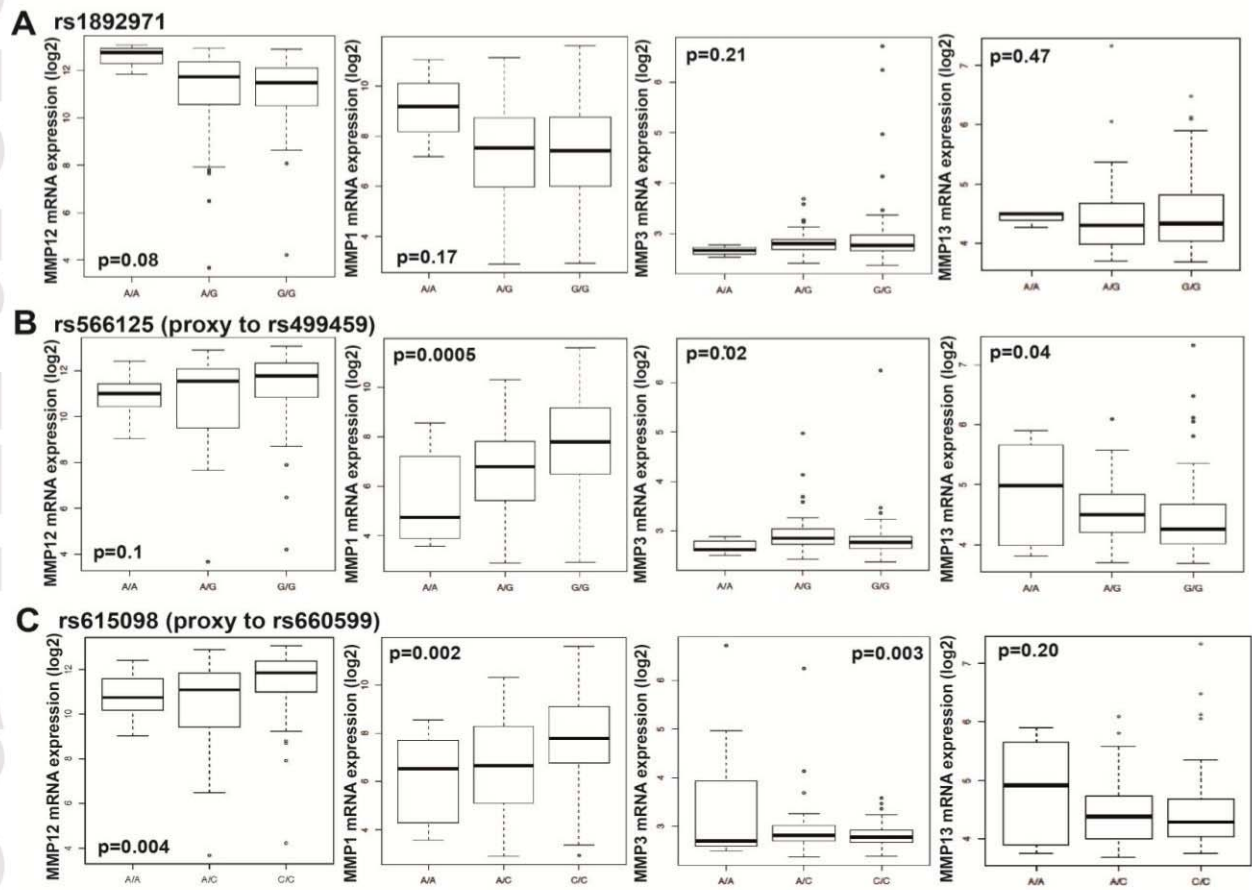

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Figure 3.

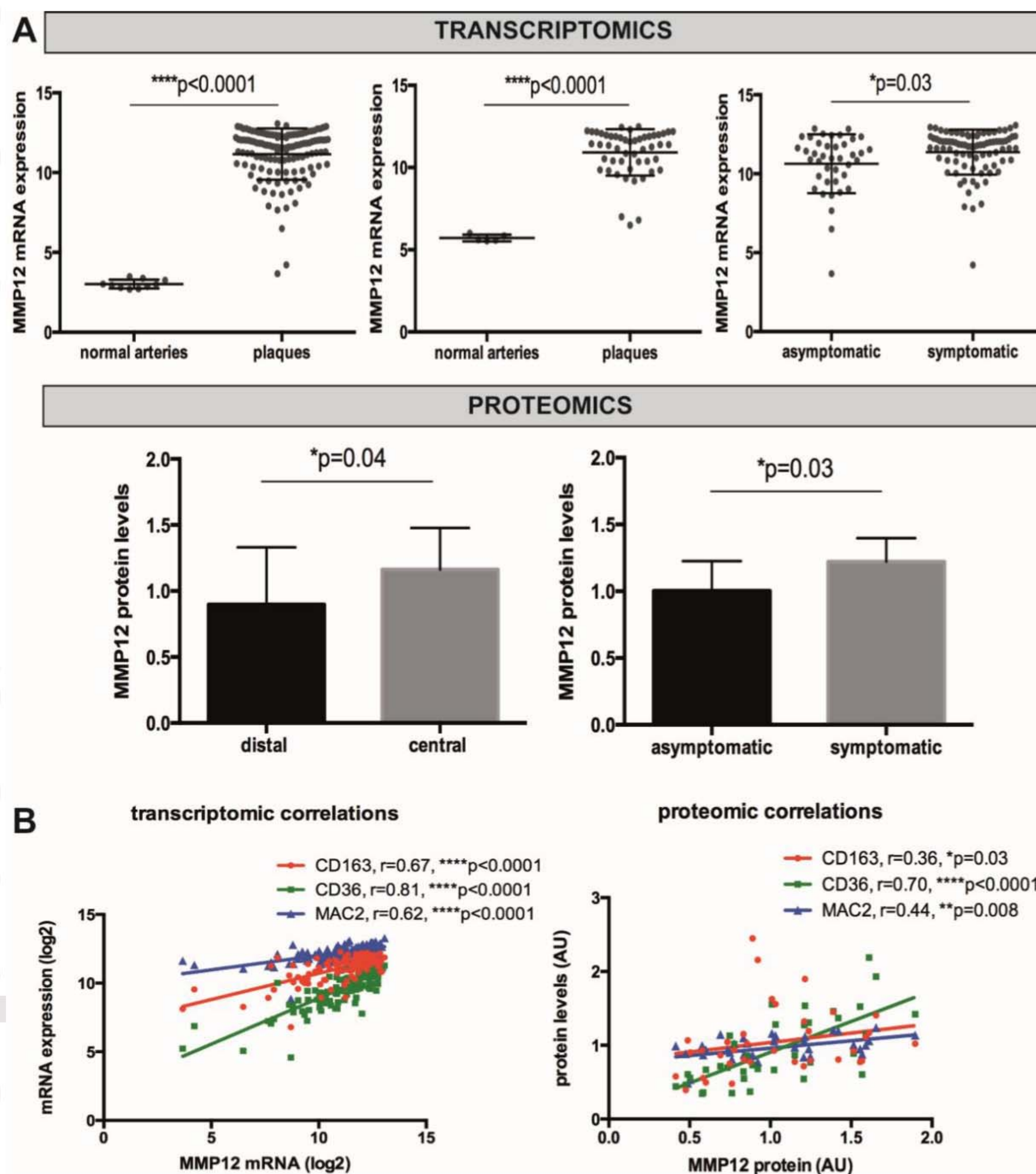

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Figure 4.

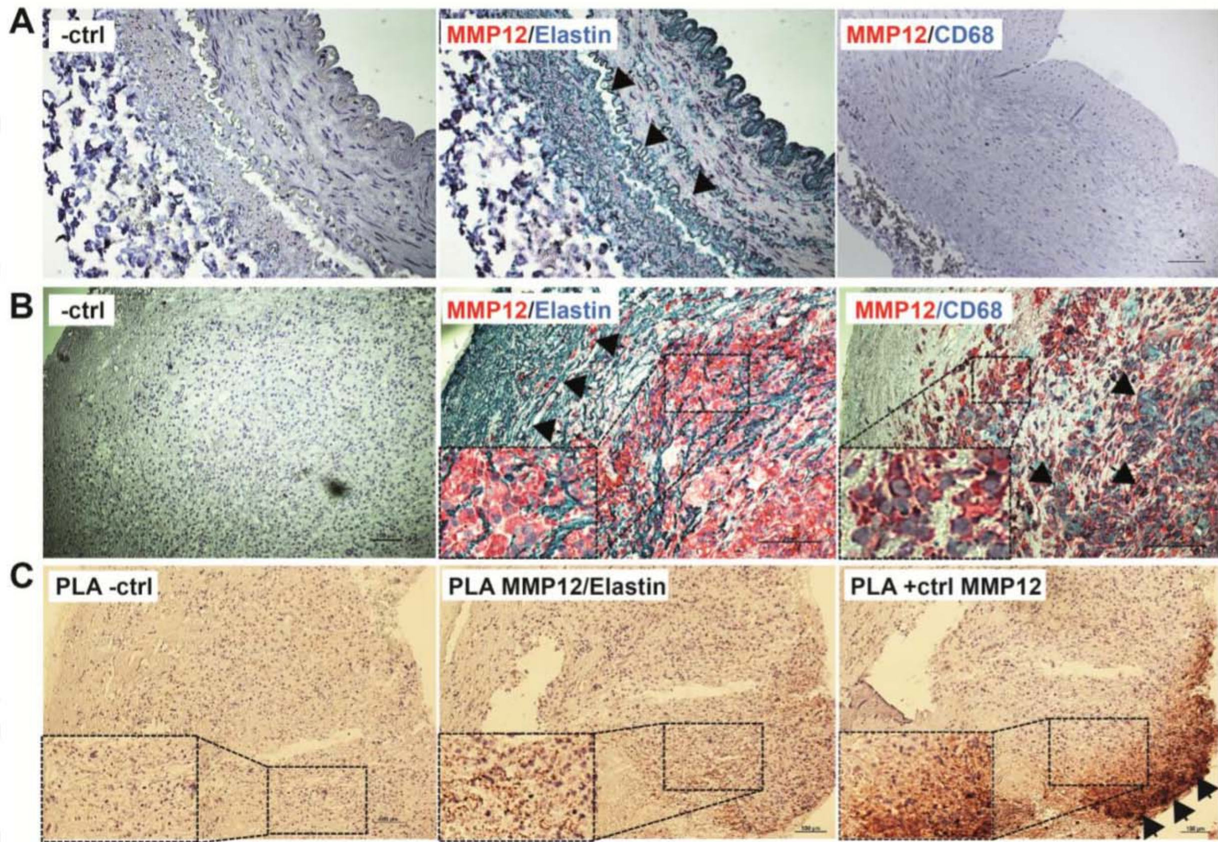

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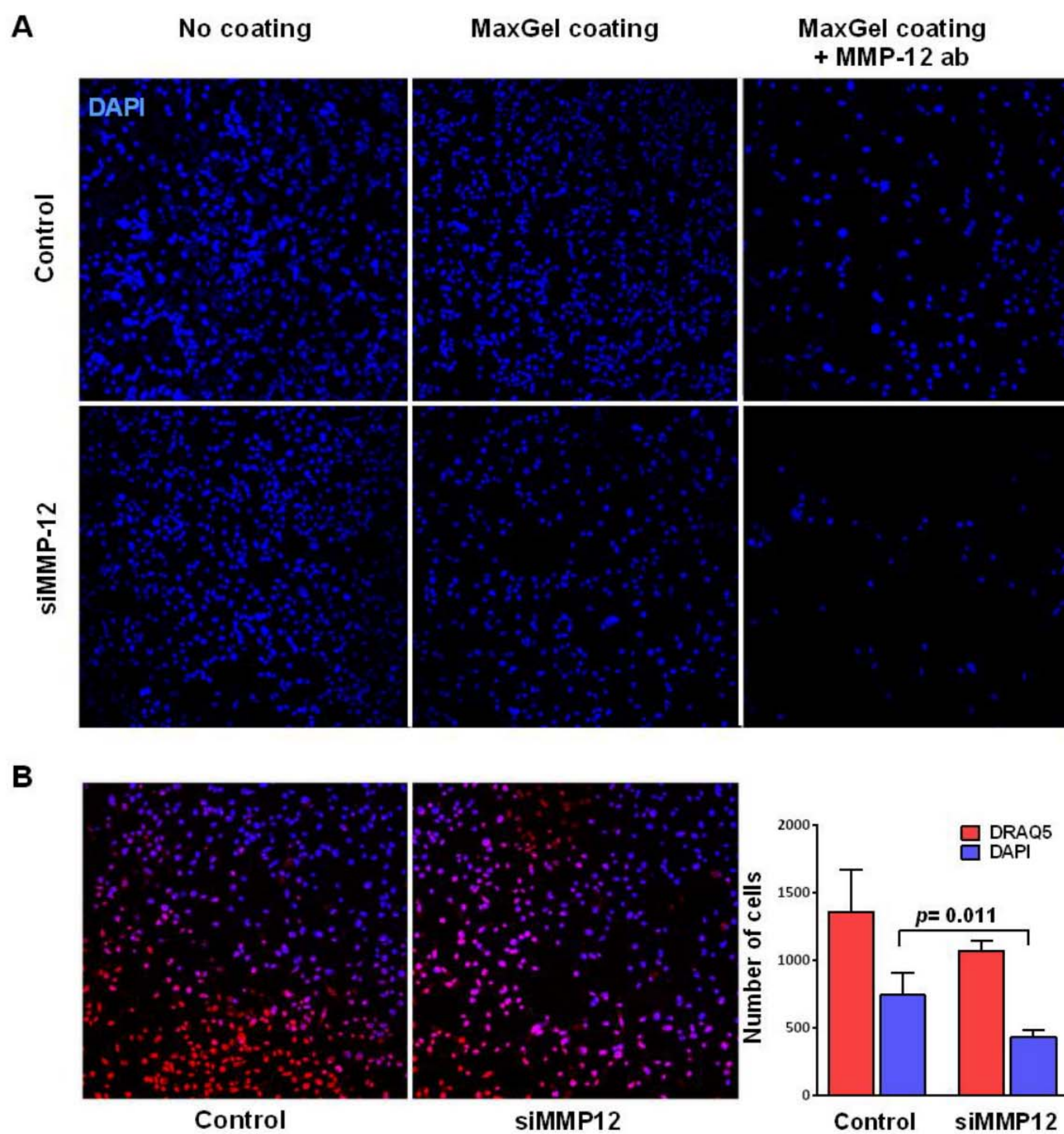
Figure 5.



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