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Increased pro-MMP9 plasma levels are associated with neovascular age-related macular degeneration and with the risk allele of rs142450006 near *MMP9*

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Purpose: To evaluate the plasma levels of matrix metalloproteinase 9 (MMP9) and tissue inhibitors of metalloproteinase 3 (TIMP3) in neovascular age-related macular degeneration (nAMD) patients compared to controls, and to explore the potential effect of AMD-associated genetic variants on MMP9 and TIMP3 protein levels.
Methods: nAMD and control patients were selected from the European Genetic Database (EUGENDA) based on different genotypes of rs142450006 near *MMP9* and rs5754227 near *TIMP3*. Plasma total MMP9, active MMP9 and TIMP3 levels were measured using the enzyme linked immunosorbent assay (ELISA) and compared between nAMD patients and controls, as well as between different genotype groups.
Results: nAMD patients had significantly higher total MMP9 levels compared to controls (median 46.58 versus 26.90 ng/ml; p = 0.0004). In addition, the median MMP9 level in the homozygous genotype group for the AMD-risk allele (44.23 ng/ml) was significantly higher than the median for the heterozygous genotype group (26.90 ng/ml; p = 0.0082) and the median for the homozygous group for the non-risk allele (28.55 ng/ml; p = 0.0355). No differences were detected for the active MMP9. TIMP3 levels did not significantly differ between the AMD and control groups, nor between the different genotype groups for rs5754227.
Conclusions: The results of our MMP9 analyses indicate that nAMD patients have on average higher systemic MMP9 levels than control individuals, and that this is partly driven by the rs142450006 variant near *MMP9*. This finding might be an interesting starting point for further exploration of MMP9 as a therapeutic target in nAMD, particularly among individuals carrying the risk-conferring allele rs142450006.

Age-related macular degeneration (AMD) is a progressive multifactorial disease and the leading cause of vision loss in elderly populations [1,2]. Intermediate AMD, characterized by drusen formation between the retinal pigment epithelium (RPE) and Bruch's membrane, can progress into geographic atrophy (GA) or neovascular AMD (nAMD) [3]. The latter form of advanced AMD has the most severe impact on loss of visual acuity [2]. nAMD can be treated with intravitreal injections of anti-vascular endothelial growth factor (VEGF) antibodies, which slows down disease progression [3].

Studies have shown that regulation of the extracellular matrix (ECM) plays an important role in both subtypes of AMD [4,5]. Dynamic changes in the ECM during disease development have been linked to the activity of metalloproteinases and their inhibitors [4]. Genome-wide association studies (GWAS) have identified variants near the gene coding for ECM regulators, including matrix metalloproteinase 9 (*MMP9*) and the tissue inhibitor of metalloproteinases 3 (*TIMP3*), to be associated with AMD [6]. However, the functional effects of these variants on the disease process have yet to be described.

MMP9 is a zinc metalloproteinase that degrades collagens and multiple soluble and cell-surface proteins, thus playing a role in reproduction, growth and development, wound healing, inflammation and other physiologic processes [7,8]. Pathologic conditions such as neurodegenerative disease, cardiovascular disease, cancer and chronic inflammatory conditions have been associated with MMP9 levels and activity [9-17]. In AMD, the genetic association in the *MMP9* locus is the only association signal specific to nAMD, while all other genetic variants identified by GWAS associate with both nAMD and GA [6]. Interestingly, MMP9 is an important regulator of the angiogenic switch, which

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has been mainly described in the context of carcinogenesis [18,19]. This suggests that MMP9 may act in nAMD via its role in angiogenesis stimulation. A study in mice showed that MMP9 expression was upregulated during experimental choroidal neovascularization (CNV), and that the development of CNV was reduced in *MMP9* deficient mice [20].

Expression of MMP9 and other metalloproteinases is tightly regulated via transcriptional regulation, the activation of precursor proteins and binding to inhibitors to maintain tissue homeostasis [7]. The main inhibitors of MMPs in tissues are the tissue inhibitors of metalloproteinases (TIMPs), of which four family members are known. A common variant at the *TIMP3* locus has been associated with AMD via GWAS, while rare variants in *TIMP3* have been detected more frequently in AMD cases compared to controls [6]. Mutations in *TIMP3* cause Sorsby's fundus dystrophy (SFD), an inherited form of macular degeneration, which can present with choroidal neovascularization [21-23].

Plasma MMP9 and TIMP3 levels have been evaluated previously in relatively small case-control studies for AMD [24,25]. In both studies, plasma MMP9 levels increased in AMD patients compared to controls, although Krogh Nielsen et al., 2019, reported an effect in GA only and not in nAMD. However, these analyses did not distinguish between the pro- and active forms of MMP9. In one study, TIMP3 levels were shown to decrease in nAMD [25]. The effects of AMD-associated genetic variants on plasma MMP9 and TIMP3 levels were not evaluated.

In this study, we aim to extend these findings by measuring levels of pro- and active MMP9 and levels of TIMP3 in plasma samples of a larger cohort of nAMD patients and controls to investigate whether these proteins could be systemic biomarkers. Furthermore, we aim to explore whether the AMD-associated variants rs142450006 near *MMP9* and rs5754227 in the *TIMP3* locus influence MMP9 and TIMP3 levels, respectively.

METHODS

Study population: This study included 220 participants from the European Genetic Database (EUGENDA). EUGENDA is a multicenter database for the clinical and molecular analysis of AMD collected at the Radboud University Medical Center, Nijmegen, The Netherlands, and at the University Hospital of Cologne, Cologne, Germany. The study was performed in accordance with the tenets of the Declaration of Helsinki and the Medical Research Involving Human Subjects Act. Approval from the local ethics committees of both university hospitals was obtained, and written informed consent was acquired from all participants. All the individuals included in the study agreed to plasma measurements and genotyping. All participants were of European decent and older than 50 years. nAMD and control statuses were assigned by multimodal image grading according to the standard protocol of the Cologne Image Reading Center by certified graders [26].

Plasma samples of nAMD patients were selected based on the genotypes of rs142450006 (for MMP9 measurements) and rs5754227 (for TIMP3 measurements). They were also selected to represent each of the three genotypes: homozygous for the major (risk) allele, homozygous for the minor (non-risk) allele and heterozygous for both alleles. Controls were selected by genotype to have similar sized genotype groups and were age-matched to the cases. An overview of the study populations is presented in Table 1 and Table 2.

Genotyping: Genomic DNA was extracted from peripheral blood samples using standard procedures. After drawing the blood in EDTA tubes, samples were centrifuged and the cell pellets were used for DNA isolation within 72 h or otherwise stored at -80 ºC. Genomic DNA was extracted using Chemagen chemistry on a Hamilton robot. Part of the cohort was genotyped with a custom-designed HumanCoreExome array by Illumina (Illumina Inc., San Diego, CA) within the International AMD Genetics Consortium. All details regarding the design of the array, annotation, imputation and

Table 1. Subjects used for MMP9 measurements.

AMD status	Genotype rs142450006*	Number of samples	Average age (in years)	Number of samples under detection limit (active MMP9)
Control	T/T	19	73.6	7
Control	TTTTC/T	18	73.8	3
Control	TTTTC/TTTTC	52	74.0	8
nAMD	T/T	8	75.6	1
nAMD	TTTTC/T	21	75.1	4
nAMD	TTTTC/TTTTC	55	75.5	13

*TTTTC is the major and risk-conferring allele, T the minor non-risk allele (MAF 0.1).

Table 2. Subjects used for TIMP3 measurements.

AMD status	Genotype rs5754227*	Number of samples	Average age (in years)
Control	C/C	14	72.9
Control	T/C	13	72.5
Control	T/T	13	72.2
nAMD	C/C	4	80.5
nAMD	T/C	18	73.5
nAMD	T/T	18	74.1

*T is the major and risk-conferring allele, C the minor non-risk allele (MAF 0.1).

quality control of the genotypic data have been described elsewhere [6]. The other part of the cohort was genotyped using molecular inversion probes combined with next-generation sequencing [de Breuk et al. manuscript submitted].

Plasma MMP9 and TIMP3 quantification: Plasma was obtained by a standard centrifugation protocol, and within 1 h after collection the samples were stored at −80 °C. MMP9 levels were measured with the Human MMP-9 Activity Assay (Cat. Nr: QZBMMP9H; QuickZyme Biosciences, Leiden, The Netherlands). Pro- and active MMP9 were measured in separate wells. TIMP3 levels were quantified using the Human TIMP-3 ELISA Kit (Cat. Nr: EA100363; OriGene Technologies Inc., Rockville, MD). Analyses were performed according to the manufacturer's instructions. All samples were analyzed in duplicate. Samples of cases and controls were distributed randomly across plates. Out of the total 173, 36 (21%) of the active MMP9 measurements fell below the detection limit; this percentage was similar in the control group (18 out of 89) and the nAMD group (18 out of 84) (see Table 1). These values were set to half the value of the lowest detection point, as excluding these measurements would have introduced bias and imputation is challenging for non-normally distributed data [27].

Statistical analysis: Data analysis was performed using Graphpad Prism (version 5.03 for Windows, GraphPad Software, La Jolla CA,). Two-tailed Mann–Whitney U tests were used to determine possible associations between MMP9 or TIMP3 levels and gender or smoking. To test possible associations between MMP9 or TIMP3 levels and age or BMI, linear regression was used after the log transformation of the data. Two-tailed Mann–Whitney U tests were performed to assess a possible difference in MMP9 or TIMP3 levels between the controls and AMD patients and between each of the genotype groups. The TIMP3 measurements were performed in a different cohort than were the MMP9 measurements, so they were considered as separate experiments. For MMP9, differences between the AMD and control groups were considered significant if $p < 0.025$ because both the total and active MMP9 were tested and a Bonferroni correction was applied for multiple testing. A multivariate analysis of the total MMP9 was performed following the log transformation of the data to determine the effect of the AMD status corrected for genotype (and vice versa) using SPSS for Windows version 22 (SPSS IBM, New York, NY). Using the same approach, the TIMP3 data were corrected for age. Age was found to be significantly associated with TIMP3 levels.

Power calculation for TIMP3 measurements: A power calculation was performed for the TIMP3 measurements. The distribution of TIMP3 concentrations was retrieved in Krogh-Nielsen et al., 2019 [25]. Since the mean values are not shown in this publication, we used means from our own data set to calculate the population standard deviations (0.6 for healthy controls and 0.8 for AMD patients; average 0.7). The minimal sample size to obtain > 80% power ($\alpha = 0.05$) for distinguishing the two groups was calculated using SPSS for Windows version 22 (SPSS IBM).

RESULTS

Study participants: Plasma samples from 89 controls (average age 73.9 years) and 84 nAMD patients (average age 75.4 years) were selected for the MMP9 analyses (Table 1). Both the AMD cases and control groups were stratified for the three genotypes of rs142450006, and the lead variant at the *MMP9* locus was identified by GWAS ($p = 2.4 \times 10^{-10}$, OR = 0.85) [6]. For this variant, TTTTC was the major and risk-conferring allele. It was located in an intergenic region approximately 22 kb upstream of *MMP9*.

For TIMP3 measurements, plasma samples from a total of 40 controls (average age 72.5 years) and 40 nAMD patients (average age 74.5 years) were selected. All TIMP3 measurements were above the detection limit. The AMD cases and controls were stratified for the three genotypes of the lead AMD-associated variant at the *TIMP3* locus, rs5754227 ($p = 1.1 \times 10^{-24}$, OR = 0.77), for which T was the major and risk-conferring allele. Here, rs5754227 was located in an intron of *SYN3*, 90 kb upstream of *TIMP3*.

Association of MMP9 and TIMP3 levels with age, gender, BMI and smoking behavior: We measured levels of total MMP9, active MMP9 and TIMP3 in the plasma of all subjects. We first tested for associations between these protein levels and age, gender, BMI and smoking behavior (Appendix 1, Appendix 2, and Appendix 3). We observed a significant association between TIMP3 and age, where decreased TIMP3 levels were associated with increased age

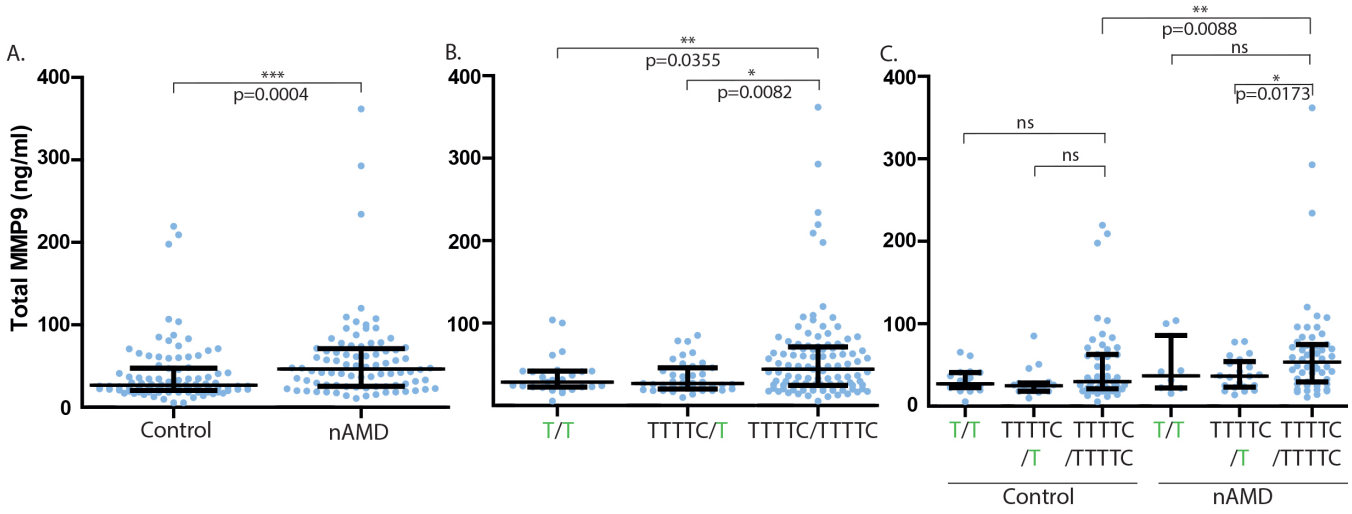

Figure 1. Total MMP9 levels in plasma samples of nAMD patients and controls. **A**: Comparison of total MMP9 levels in nAMD patients and controls. **B**: Total MMP9 levels in nAMD patients and controls mixed stratified by rs142450006 genotype. **C**: Total MMP9 levels in nAMD patients and controls separately and stratified by rs142450006 genotype. The non-risk allele of rs142450006 is depicted in green. Bars represent median values, and whiskers represent interquartile ranges.

(p = 0.011). TIMP3 measurements were corrected for age via multivariate analysis.

Association of MMP9 and TIMP3 levels with AMD and genotype: Total MMP9 levels (which included pro-MMP9 and active MMP9) were measured for the nAMD cases and controls. Patients had significantly higher MMP9 concentrations in plasma (median 46.58 ng/ml [25th to 75th percentiles 25.65–71.02] versus 26.90 ng/ml [25th to 75th percentiles 20.39–47.53]; p = 0.0004; Figure 1A). When evaluating the association of MMP9 levels with the rs142450006 genotype, we found a significant increase of MMP9 levels in carriers of the homozygous risk-conferring TTTTC/TTTTC genotype (median 44.23 ng/ml; 25th to 75th percentiles 24.34–71.28), compared to carriers of the heterozygous TTTTC/T genotype (median 26.90 ng/ml [25th to 75th percentiles 20.03–45.76]; p = 0.0082) and carriers of the homozygous low-risk T/T genotype (median 28.55 ng/ml; 25th to 75th percentiles 22.40–41.84; p = 0.0355; Figure 1B). Increases of MMP9 levels among carriers of the TTTTC/TTTTC genotype were seen in both the control and patient groups but was only significant when the carriers of the heterozygous genotype were compared to the carriers of the homozygous TTTTC/TTTTC genotype in the nAMD group (p = 0.0173; Figure 1C). A similar result was obtained when differences between the genotype groups were analyzed in the whole data set and corrected for AMD status. Here, only the TTTTC/TTTTC genotype group compared to the TTTTC/T group showed a significant difference (p = 0.004). This effect remained significant (p = 0.001) when analyzing the differences between the nAMD and control groups corrected for genotype effect, and when the nAMD was compared to the control in homozygous carriers for the major TTTTC allele only (p = 0.0088; Figure 1C).

Active MMP9 levels were evaluated in a similar way. None of the comparisons showed a significant difference, indicating that the nAMD and genotype were not associated with active MMP9 plasma levels (Figure 2).

In this study, none of the investigated groups had significant differences in plasma TIMP3 levels. This suggests that neither the AMD status nor genotype of rs5754227 are associated with systemic TIMP3 concentrations (Figure 3). To determine whether our study has sufficient power, we performed a power analysis using the findings of Krogh-Nielsen et al., 2019 [25]. Based on our power calculation, 35 controls and 35 AMD patients would be sufficient to detect a significant difference in TIMP3 levels between the two groups with 81.6% power.

DISCUSSION

In this study, we detected elevated plasma levels of total MMP9 in nAMD patients compared to controls, whereas the concentration of active MMP9 was similar in both groups. The risk-conferring genotype of rs142450006, TTTTC/TTTTC, resulted in higher total MMP9 levels compared to the heterozygous and non-risk T/T genotype. When we compared homozygous carriers for the major TTTTC allele only, the increase of MMP9 among nAMD patients remained significant. These results indicate that both nAMD and

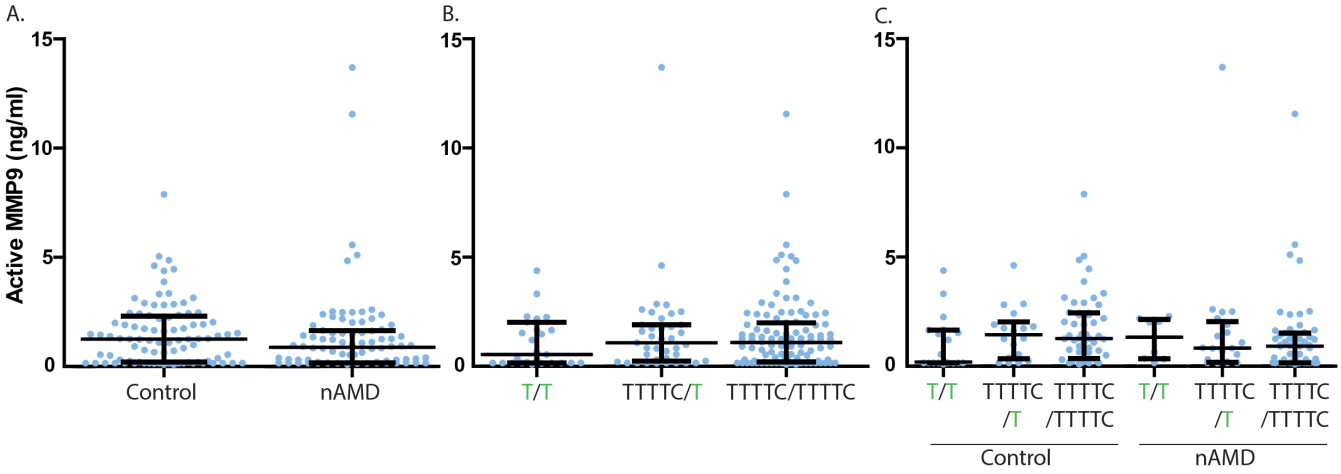

Figure 2. Active MMP9 levels in plasma samples of nAMD patients and controls. **A**: Comparison of active MMP9 levels in nAMD patients and controls. **B**: Active MMP9 levels in nAMD patients and controls mixed stratified by rs142450006 genotype. **C**: Active MMP9 levels in nAMD patients and controls separately and stratified by rs142450006 genotype. The non-risk allele of rs142450006 is depicted in green. Bars represent median values, and whiskers represent interquartile ranges.

genotype are associated with increased systemic pro-MMP9 levels independently of each other.

Increased MMP9 levels were found previously in AMD patients with and without CNV [24]. Krogh Nielsen et al., 2019, reported higher MMP9 levels in the plasma of patients with GA, but not in nAMD patients [25]. The lack of association in the latter group might be due to the small sample size (46 nAMD patients and 33 controls) of that study. Although these two studies both found increased MMP9 levels in GA patients, the genetic association of *MMP9* with AMD was exclusively described with nAMD [6]. The present study was the first to investigate and identify an association of this nAMD-associated variant with MMP9 protein levels.

MMP9 is expressed in a wide variety of cell types, including blood cells like neutrophils, platelets and macrophages, which release pro-MMP9 into the circulation [28-30]. The association signal near *MMP9* is located 22 kb upstream of the gene [6] but might still influence the gene expression of *MMP9* and other genes. Currently no expressed quantitative trait loci (eQTL) for variants in or near the *MMP9* gene are known in any of the tissues examined by the Genotype-Tissue Expression (GTEx) database (which does not include eye

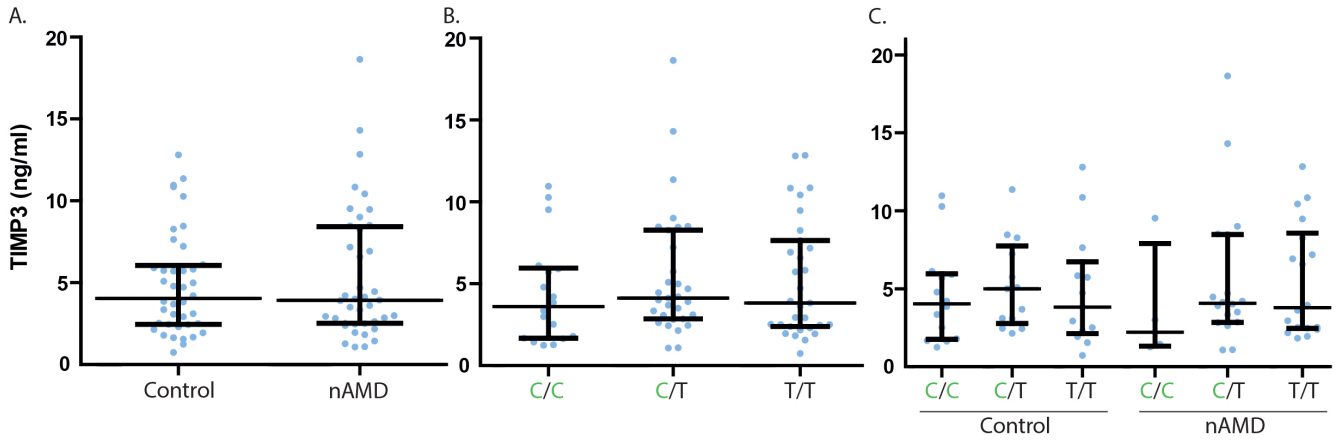

Figure 3. TIMP3 levels in plasma samples of nAMD patients and controls. **A**: Comparison of TIMP3 levels in nAMD patients and controls. **B**: TIMP3 levels in nAMD patients and controls mixed stratified by rs5754227 genotype. **C**: TIMP3 levels in nAMD patients and controls separately and stratified by rs5754227 genotype. The non-risk allele of rs5754227 is depicted in green. Bars represent median values, and whiskers represent interquartile ranges.

tissues) [31]. However, a recent transcriptomics analysis in the human eye revealed an eQTL in the *MMP9* locus in retinal tissue [32]. The strongest association was between rs3918251 (located at approximately 24 kb distance of rs142450006) and the expression of *SLC12A5-AS1*. This gene transcribes a long non-coding RNA, an anti-sense RNA for *MMP9*. Although we cannot explain the association between rs142450006 and MMP9 plasma levels by a direct regulatory effect of this variant on MMP9 RNA levels, neighboring variants within the *MMP9* locus might influence MMP9 expression via the transcriptional regulation of *SLC12A5-AS1*. This might lead to a local effect on MMP9 expression in the retina.

An increase in systemic MMP9 might reflect the altered regulation of the ECM. In various types of cancers, MMP9 has been shown to be a biomarker for metastasis and prognosis [17,33-35]. Upregulation of MMP9 in cancers is often accompanied with an increase in VEGF, which together stimulate angiogenesis that contributes to metastasis [18,36-40]. MMP9 can liberate VEGF sequestered to the ECM via cleavage of the heparan sulphates [40,41]. Interestingly, MMP9 has also been shown to be involved in angiogenic signaling in RPE cells. The upregulation and activation of MMP9 occurs in ARPE19 cells in response to complement activation, resulting in the release of VEGF from the ECM and creating a proangiogenic environment [42]. The upregulation of the MMP9 gene expression caused by hypoxia in cultured RPE cells also leads to increased VEGF expression and secretion [43].

In cancer cells and animal models, MMP9 inhibitors have been shown to have an anti-invasive effect [38,44-46]. Phase I, II and III clinical trials are currently ongoing to explore the effect of one MMP9 inhibitor, andecaliximab, on different cancers [47,48]. Given these examples from the cancer research field and the results of this study, MMP9 might be considered as a therapeutic target in nAMD, particularly for individuals carrying the risk-conferring allele of rs142450006. Currently, nAMD patients are treated with anti-VEGF. This improves visual acuity in many, but not all cases [49]. Inhibiting MMP9 might be even more effective because it acts further upstream in the pathway than does VEGF and could inhibit pathogenesis at an earlier stage.

In addition to its protease activity, MMP9 has non-enzymatic functions that can also be performed by pro-MMP9 [50]. Through its hemopexin (PEX) domain, pro-MMP9 can bind to membrane proteins at the cell surface (such as CD44, integrins and Ku protein). This can lead to the regulation of cell survival, migration and angiogenesis [50-52]. The increase of only pro-MMP9 levels in nAMD patients in this study might indicate that the non-catalytic activities of MMP9 are most important in AMD pathogenesis. In this case, inhibition of the PEX domain of MMP9 might be most beneficial for nAMD patients. A study in a tumor xenograft model showed that specific inhibition of the PEX domain of MMP9, while leaving the catalytic function intact, resulted in a decrease of tumor growth and lung metastasis via the inhibition of cell migration and proliferation [53]. Another study showed that human microvascular endothelial cells (HMECs) treated with the conditioned medium of glioblastoma cells transfected with the PEX domain of MMP9 resulted in reduced migration and capillary-like structure formation. These cells also exhibited decreased VEGF secretion and VEGF receptor-2 protein levels [52]. These results indicate that through these mechanisms, pro-MMP9 might be involved in regulating angiogenesis in nAMD patients. Further experiments are needed to evaluate whether targeting MMP9 at the PEX domain will be effective in nAMD.

A previous evaluation of plasma TIMP3 detected decreased levels in nAMD patients, but we did not detect any significant differences between nAMD cases and controls in the current study [25]. A power analysis based on the findings of Krogh-Nielsen et al., 2019, suggests that our study is not underpowered. Therefore, our study did not confirm the previous finding of lower TIMP3 levels in nAMD patients.

TIMP3 is an interesting protein to investigate in the context of AMD, especially because mutations in this gene are the cause of SFD, an inherited form of macular degeneration that can present with choroidal neovascularization [21,23]. However, the AMD-associated variant near *TIMP3* was not associated with plasma TIMP3 levels in our study. A potential effect of this variant might be difficult to detect with the current sample size, or it might not be reflected in the circulation.

In conclusion, the results of our MMP9 analyses indicate that nAMD patients have on average higher systemic MMP9 levels than do control individuals. This finding might be an interesting starting point for further exploration of MMP9 as a therapeutic target in nAMD, particularly for individuals who carry the risk-conferring allele of rs142450006.

APPENDIX 1.

To access the data, click or select the words "Appendix 1." Total MMP9 levels plotted against age (A), gender (B), BMI (C) and smoking (D). None of these factors are significantly associated with total MMP9 levels. For A and C; medians and errors are shown. For B and D; bars represent median values and whiskers represent interquartile range.

APPENDIX 2.

To access the data, click or select the words "Appendix 2." Active MMP9 levels plotted against age (A), gender (B), BMI (C) and smoking (D). None of these factors are significantly associated with active MMP9 levels. For A and C; medians and errors are shown. For B and D; bars represent median values and whiskers represent interquartile range.

APPENDIX 3.

To access the data, click or select the words "Appendix 3." TIMP3 levels plotted against age (A), gender (B), BMI (C) and smoking (D). Only the correlation between TIMP3 and age was significant (p=0.011). For A and C; medians and errors are shown. For B and D; bars represent median values and whiskers represent interquartile range.

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