Arteriosclerosis, Thrombosis, and Vascular Biology

CLINICAL AND POPULATION STUDIES

COMP (Cartilage Oligomeric Matrix Protein) Neoepitope

A Novel Biomarker to Identify Symptomatic Carotid Stenosis

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OBJECTIVE: COMP (cartilage oligomeric matrix protein) is abundantly expressed in the cardiovascular system, cartilage, and atherosclerotic plaques. We investigated if the total COMP (COMPtotal) and COMP neoepitope (COMPneo) with other cardiovascular markers and clinical parameters could identify symptomatic carotid stenosis.

APPROACH AND RESULTS: Blood samples were collected from patients with symptomatic carotid stenosis (stenosis, n=50), patients with stroke without carotid stenosis but small plaques (plaque, n=50), and control subjects (n=50). COMPtotal and COMPneo were measured using an ELISA. Ninety-two cardiovascular disease markers were measured by the Olink CVD kit. The presence of native COMP and COMPneo was determined by immunohistochemistry. The concentration of COMPneo was higher and COMPtotal was lower in the stenosis group. When the concentration was compared between the stenosis and control groups, IL-1ra (interleukin-1 receptor antagonist protein), IL6 (interleukin-6), REN (Renin), MMP1 (matrix metalloproteinase-1), TRAIL-R2 (tumor necrosis factor-related apoptosis-inducing ligand receptor 2), ITGB1BP2 (integrin beta 1 binding protein 2), and COMPneo were predictive of stenosis. Conversely, KLK6 (kallikrein-6), COMPtotal, NEMO (nuclear factor-kappa-B essential modulator), SRC (Proto-oncogene tyrosine-protein kinase Src), SIRT2 (SIR2-like protein), CD40 (cluster of differentiation 40), TF (tissue factor), MP (myoglobin), and RAGE (receptor for advanced glycation end-products) were predictive of the control group. Model reproducibility was good with the receiver operating characteristic plot area under the curve being 0.86. When comparing the plaque group and stenosis group, COMPneo, GAL (galanin), and PTX3 (pentraxin-related protein PTX3) were predictive of stenosis. Model reproducibility was excellent (receiver operating characteristic plot area under the curve 0.92). COMPneo was detected in smooth muscle-, endothelial-, and foam-cells in carotid stenosis.

CONCLUSIONS: Degradation of COMP may be associated with atherosclerosis progression and generation of a specific COMP fragment—COMPneo. This may represent a novel biomarker that together with COMPtotal and other risk-markers could be used to identify symptomatic carotid stenosis.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ biomarker ■ carotid stenosis ■ extracellular matrix proteins ■ plasma ■ stroke

Stroke is a significant cause of mortality and morbidity in industrialized countries, and it is the fifth leading cause of death in the United States.[1] Most cases of stroke are of the ischemic type, where small or large vessel atherosclerosis accounts for the majority of cases. For large vessel atherosclerosis, the most common cause is carotid stenosis.[1] A fast and accurate diagnosis of the underlying cause of stroke is important for its subsequent

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For Sources of Funding and Disclosures, see page 1227.

Nonstandard Abbreviations and Acronyms

AUC	area under the curve
CD40	cluster of differentiation 40
COMP	cartilage oligomeric matrix protein
COMPneo	COMP neoepitope
COMPtotal	total COMP
ECST	European Carotid Surgery Trial
GAL	galanin
IL-1ra	interleukin-1 receptor antagonist protein
IL-6	interleukin-6
ITGB1BP2	integrin beta 1 binding protein 2
KLK6	kallikrein-6
LDL	low-density lipoprotein
MB	myoglobin
MMP1	matrix metalloproteinase-1
OPLS-DA	orthogonal partial least square discriminant analysis
PCA	principal component analysis
SRC	proto-oncogene tyrosine-protein kinase Src
PTX3	pentraxin-related protein PTX3
REN	renin
ROC	receiver operating characteristic
SIRT2	SIR2-like protein
TF	tissue factor
TRAIL-R2	tumor necrosis factor-related apoptosis-inducing ligand receptor 2

Highlights

- Total cartilage oligomeric matrix protein (COMPtotal) and a COMP neoepitope together, with other cardiovascular markers and clinical parameters can be used to identify symptomatic carotid stenosis.
- IL-1ra (interleukin-1 receptor antagonist protein), IL-6 (interleukin-6), REN (renin), MMP1 (matrix metalloproteinase-1), TRAIL-R2 (tumor necrosis factor-related apoptosis-inducing ligand receptor), iITGB1BP2 (integrin beta 1 binding protein 2), and COMP neoepitope were predictive of stenosis.
- The degradation of native COMP may be associated with atherosclerosis progression with the generation of a specific COMP fragment.

treatment. Current diagnosis is mostly based on clinical examination and neuroimaging, which may lack accuracy, is resource intensive and takes a significant amount of time. Thus, there is a great need for novel biomarkers to enhance current diagnostic procedures.

Atherosclerosis involves low-grade inflammation with proinflammatory cytokines during the early stages and disease progression,[2] which leads to fragmentation of the extracellular matrix molecules. COMP (cartilage oligomeric matrix protein), also known as thrombospondin-5, was first described as a matrix protein in cartilage; however, it has subsequently also been found in the extracellular matrix of the arterial wall and in human atherosclerotic plaques.[3–5]

We recently discovered a unique COMP neoepitope (COMPneo) with cleavage site S^{154} (SGPTH), a highly conserved sequence that is identical across several species.[6] The release of COMPneo in the synovial fluid and serum in horses with acute osteoarthritis indicates its role as a biomarker for cartilage degradation.[7,8] We hypothesize that degradation of COMP in atherosclerotic plaques results in increased levels of COMPneo in the blood similar to what has been described in osteoarthritis and that the COMPneo may serve as a biomarker for advanced carotid atherosclerosis.

The aim of the present study was to investigate whether COMPneo is released in the blood of humans with and without symptomatic carotid atherosclerotic lesions. We also wanted to investigate whether the total COMP (COMPtotal) and COMPneo in combination with other biochemical and clinical risk markers could be used to create models for predicting symptomatic carotid stenosis. To elucidate the link between the levels of COMPneo in plasma and carotid atherosclerosis, the localization of native COMP and COMPneo were determined in samples from patients undergoing a carotid endarterectomy.

MATERIALS AND METHODS

Owing to the sensitive nature of the data collected for this study, this dataset cannot be made publicly available. However, access may be granted to qualified researchers trained in human subject confidentiality protocols by contacting the corresponding author.

All other supporting data are available within the article and in the Data Supplement. An extended material and methods section is available in the Data Supplement including information regarding clinical chemistry analyses, COMPtotal and COMPneo analyses, protein expression profiling, immunohistochemistry, statistical methods used in Tables in the Data Supplement and sample size considerations.

Study Population

The study subjects were recruited from the Western Region Initiative to Gather Information on Atherosclerosis database[9] which includes patients with stroke at Sahlgrenska University Hospital and volunteers without previous stroke identified through official registers and invited for screening for carotid artery atherosclerosis (controls). From the Western Region Initiative to Gather Information on Atherosclerosis database, stratified and randomly selected groups with nonsignificant

carotid lesions (plaque group, n=50) and controls (n=50) were included. From the Gothenburg Atheroma Study Group Biobank at the Sahlgrenska University Hospital,[10] samples from symptomatic patients with high-grade carotid stenosis were available (stenosis group, n=50). High-grade carotid stenosis was defined as ≥70% stenosis according to the ECST (European Carotid Surgery Trial) criteria. A detailed description of the diagnostic criteria has been published previously.[9] There was no significant stenosis present in the other groups. The individuals included in the study were matched for age and sex. The study was approved by the Regional Ethical Review Board in Gothenburg. All the subjects provided written informed consent before participation.

Blood samples from the patients were analyzed for COMPtotal and COMPneo, a panel of 92 proteins related to cardiovascular disease as well as some routine chemistry analyses. Carotid endarterectomy samples for immunohistochemical analysis were obtained from the stenosis group (n=8).

Plasma Protein Quantification and Immunohistochemical Analysis

COMPneo was measured by an in-house ELISA.

The COMPneo ELISA was valid for plasma measurement with a detection limit of 125 ng/mL and an intraassay variation of 10.2% and an interassay variation of 23.8%. COMPtotal was measured by a commercial ELISA. Protein profiling with 92 proteins was performed using the OLINK Proseek Multiplex CVD kit (catalog number 94200; Olink Bioscience, Uppsala, Sweden). Immunohistochemistry against COMPtotal and COMPneo was carried out on de-paraffinized serial sections from endarterectomy samples after heat-induced epitope retrieval.

Statistical Analyses

Univariate analyses of COMPtotal, COMPneo, and ratio COMP neo/total on all study subjects were carried out by 1-way ANOVA with post hoc analysis by Tukey's multiple comparisons test using GraphPad Prism version 7.00 (GraphPad Software, La Jolla, CA). $P \leq 0.05$ was considered to be statistically significant.

Multivariate analyses were carried out using SIMCA v.15.0.2 (Umetrics, Umeå, Sweden). Data were log transformed before statistical analysis. Variables were centered and scaled to unit variance. Goodness-of-fit was used to assess the explained variation (R2), while the reproducibility of the model was assessed by cross validation (Q2) as well as fitting the model on a validation dataset as described below. R2 and Q2 values of 1 represent a model that perfectly explains all the variations and that is perfectly reproducible.

Principal component analysis (PCA) was used to explore the segregation of patients based on all laboratory variables (chemistry-, protein-, and COMP-analyses) in an unbiased way. To further explore the predictive capacity of these variables, the dataset was randomly subdivided into training and validation datasets using a customized script in the R software (R Core Team [2018], https://www.R-project.org/). Of all cases, 75% and 25% in each group were selected for the training and validation datasets, respectively. The training dataset was used to create predictive orthogonal partial least square discriminant analysis (OPLS-DA) models. The results are shown as a variable importance in the projection plot, predictive and plots of calculated regression coefficients. Variable importance in the projection plot, predictive gives an estimate of the importance of the different variables for the predictive model. A higher value indicates a higher degree of importance. Regression coefficients show the predictive direction of the different variables, for the group indicated on the *y* axis. Variables with coefficients >0 are positively associated with the indicated group, whereas variables with coefficients <0 are negatively associated. Error bars are displayed for coefficients at the 95% confidence level, estimated by a jack-knifing procedure. Coefficients where the error bars did not cross zero were considered most reliable.

To create submodels including the most important variables, only those variables that had a variable importance in the projection plot, predictive value >1, and with reliable regression coefficients (ie, error bars that did not cross zero) were selected.

Predictive models were evaluated on the validation dataset. These results are presented as receiver operating characteristic (ROC) curves and misclassification tables, where observations were assigned to the nearest group. With the exception of Figure III in the Data Supplement, no threshold was used in the misclassification tables. Fisher's exact test results are included to show the probability of the observed table to occur by chance.

RESULTS

The clinical characteristics of the study subjects are presented in Table I in the Data Supplement. Notably, LDL (low-density lipoprotein) and total cholesterol were significantly lower in both, the plaque and stenosis groups. As this most likely was the effect of more intensive lipid-lowering treatment in these groups (that may have been initiated due to the clinical event that was an inclusion criterion for the present study), lipid analyses were excluded in subsequent multivariate analyses.

Plasma Levels of COMPtotal, COMPneo, and the COMPneo/COMPtotal Ratio

Plasma levels of COMPneo were significantly higher in the symptomatic patients with advanced carotid atherosclerotic lesions (stenosis) than in the control subjects (controls; Figure 1A). COMPneo levels were also significantly higher in the stenosis group compared with patients with stroke with less advanced carotid atherosclerotic lesions (plaque). The levels of COMPtotal were lower in the patients with stenosis than in the control subjects (Figure 1B). There was also a significant difference between the plaque and stenosis groups. The COMPneo/COMPtotal ratio was higher in the plasma of patients with stenosis than in the control or the plaque group (Figure 1C).

Multivariate Characterization of the Study Subjects Using Unsupervised PCA

An unsupervised PCA was used to explore whether a combination of chemistry analyses, COMP measurements,

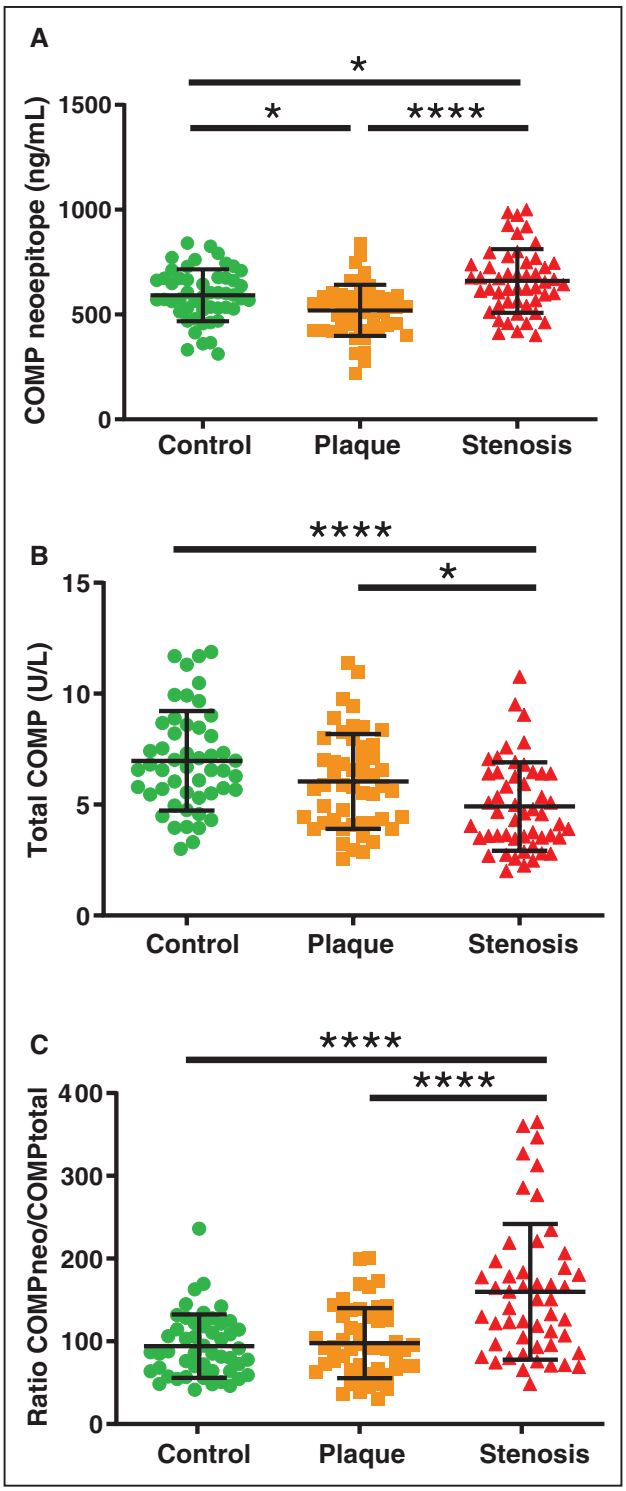

Figure 1. COMP (cartilage oligomeric matrix protein) neoepitope (COMPneo) and total COMP (COMPtotal) plasma-levels in the control, plaque, and stenosis groups.
Lines in figures indicate mean±SD. **A**, COMPneo levels. **B**, COMPtotal levels. **C**, COMPneo/COMPtotal ratio.

and cardiovascular disease markers would result in segregation of the different study groups (Figure 2). A PCA model with 4 significant components was obtained. While the first 2 PCA components did not reveal any clear segregation of the different study groups (Figure 2A), components 3 and 4 showed a clear segregation of the control and the stenosis groups (Figure 2B and 2C). Conversely, the control and the plaque groups seemed to overlap to a large extent. To further elucidate which aspects that PCA components 1 and 2 may represent, these were color coded against various clinical parameters (Figure I in the Data Supplement); however, no clear pattern was revealed.

A Predictive Model for the Stenosis Versus Control Group Using OPLS-DA Revealed Several Potential Biomarkers

For all the predictive models, the dataset was first randomly subdivided into training and validation datasets. As the unsupervised PCA model showed the clearest segregation of the control and stenosis groups, these 2 groups were included in an OPLS-DA predictive model to determine which parameters had the best predictive performance for carotid stenosis (Figure 3). Both clinical and laboratory parameters were included. The OPLS-DA model including all parameters (Figure 3A through 3C) showed good predictive performance when applied to the validation dataset as determined by the ROC plot (area under the curve [AUC] 0.91) and misclassification table (Figure 3C). When a new OPLS-DA model with the most important variables was created (Figure 3D), only a slight decrease in predictive performance was noted when the model was applied to the validation dataset (Figure 3D, ROC plot and misclassification table, AUC 0.86). Notably, the majority of the selected variables were significantly different between the control and stenosis groups also when doing group-wise comparisons using standard parametric/nonparametric hypothesis tests and adjustment for multiple testing according to the Benjamini & Hochberg procedure (Table IIA and IIB in the Data Supplement).

A high concentration of IL-1ra (interleukin-1 receptor antagonist protein), IL-6 (interleukin-6), REN (renin), MMP1 (matrix metalloproteinase-1), TRAIL-R2 (tumor necrosis factor-related apoptosis-inducing ligand receptor), ITGB1BP2 (integrin beta 1 binding protein), and COMPneo in the plasma were found as predictive factors for the stenosis group when compared with the control group. Additional traditional risk factors such as smoking, smoking pack-years, diabetes, and hypertension were all predictive of stenosis.

Conversely, high concentrations of KLK6 (kallikrein-6), COMPtotal, NEMO (nuclear factor-kappa-B essential modulator), SRC (proto-oncogene tyrosine-protein kinase Src), SIRT2 (SIR2-like protein), CD40 (cluster of differentiation 40), TF (tissue factor), MB (myoglobin), and RAGE (receptor for advanced glycation end-products) were predictive of the control group (Figure 3D). OPLS-DA models, based on only clinical parameters and

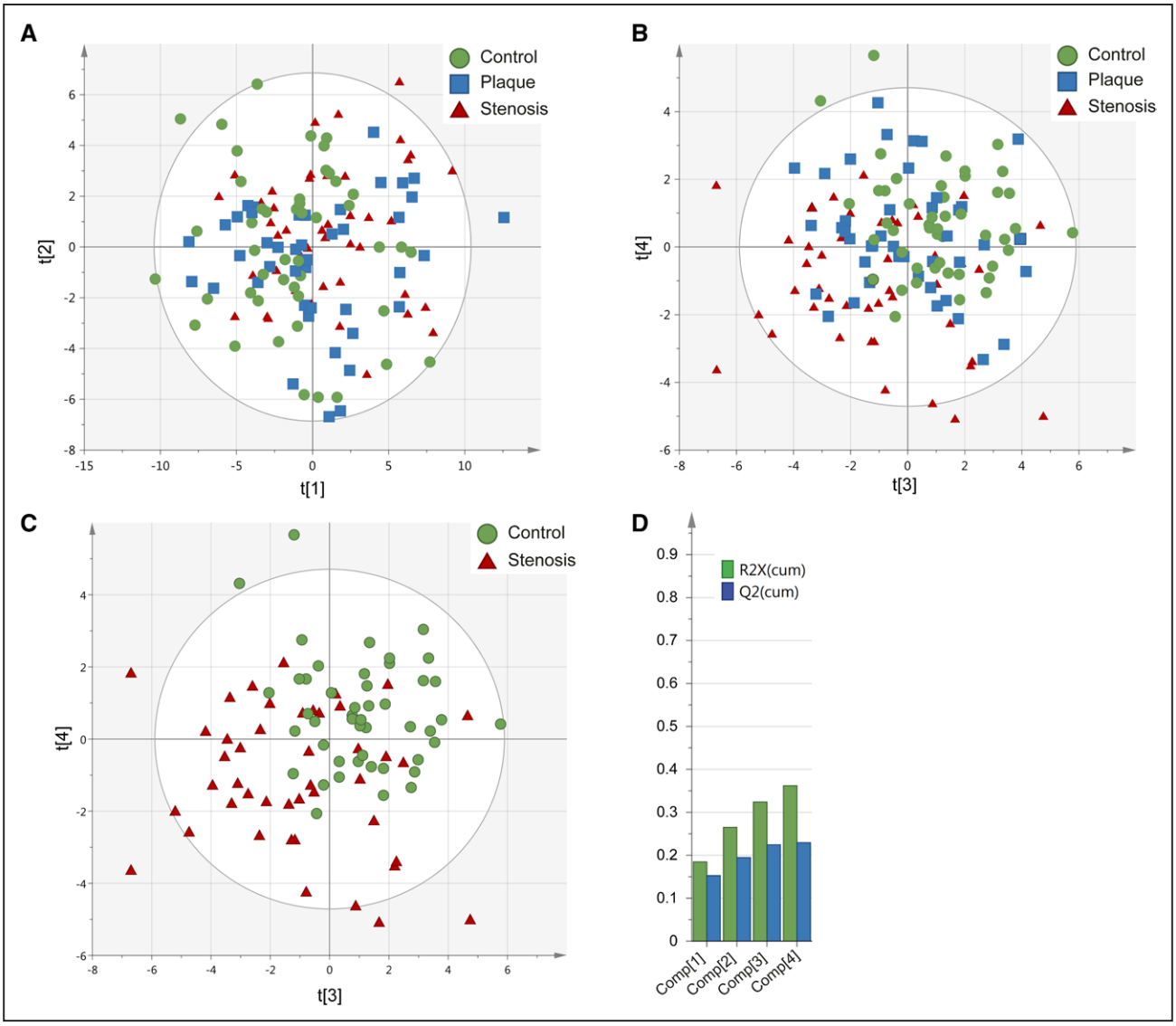

Figure 2. Principal component analysis (PCA) of chemistry-, COMP (cartilage oligomeric matrix protein), and OLINK-analyses. A model with 4 significant components was obtained. **A**, PCA score plot of the first 2 components. **B**, PCA score plot of components 3 and 4, where a certain degree of clustering of the different groups are observed. The control and stenosis groups were most clearly separated, as shown in **C** where the plaque group is omitted. **D**, Model performance estimated by cross-validation.

laboratory parameters, showed similar predictive performance when plotted on a ROC curve (Figure IIA and IIB in the Data Supplement, AUCs, 0.86 and 0.84, respectively). However, the model with only laboratory parameters demonstrated the best classification performance when shown as a misclassification table.

A Predictive Model Using OPLS-DA Could Efficiently Identify Plaque and Stenosis Subjects

Since it would be of both clinical and scientific value to identify markers that are predictive of symptomatic carotid stenosis versus stroke without significant stenosis, a predictive OPLS-DA model was created between these 2 groups (Figure 4). By including all the parameters (Figure 4A through 4C), a model with very high predictive performance was obtained, as determined by fitting the model to the validation dataset (Figure 4C, ROC-plot, and misclassification table, AUC 0.96). However, a rather low Q2 value was observed by cross-validation (Figure 4C), which was probably a result of many of the variables being less important and displaying a high degree of variation (ie, high error bars in the coefficients plot, Figure 4B).

When only selecting the most important and significant variables, a model with only 7 variables was obtained (Figure 4D). One of these variables, TF had a coefficient close to 0 and thus did not contribute much to the model. Interestingly, this new model displayed a predictive performance very close to the full model with only a slight decrease in AUC (0.92) when fitted to the validation dataset (Figure 4D, ROC-Plot).

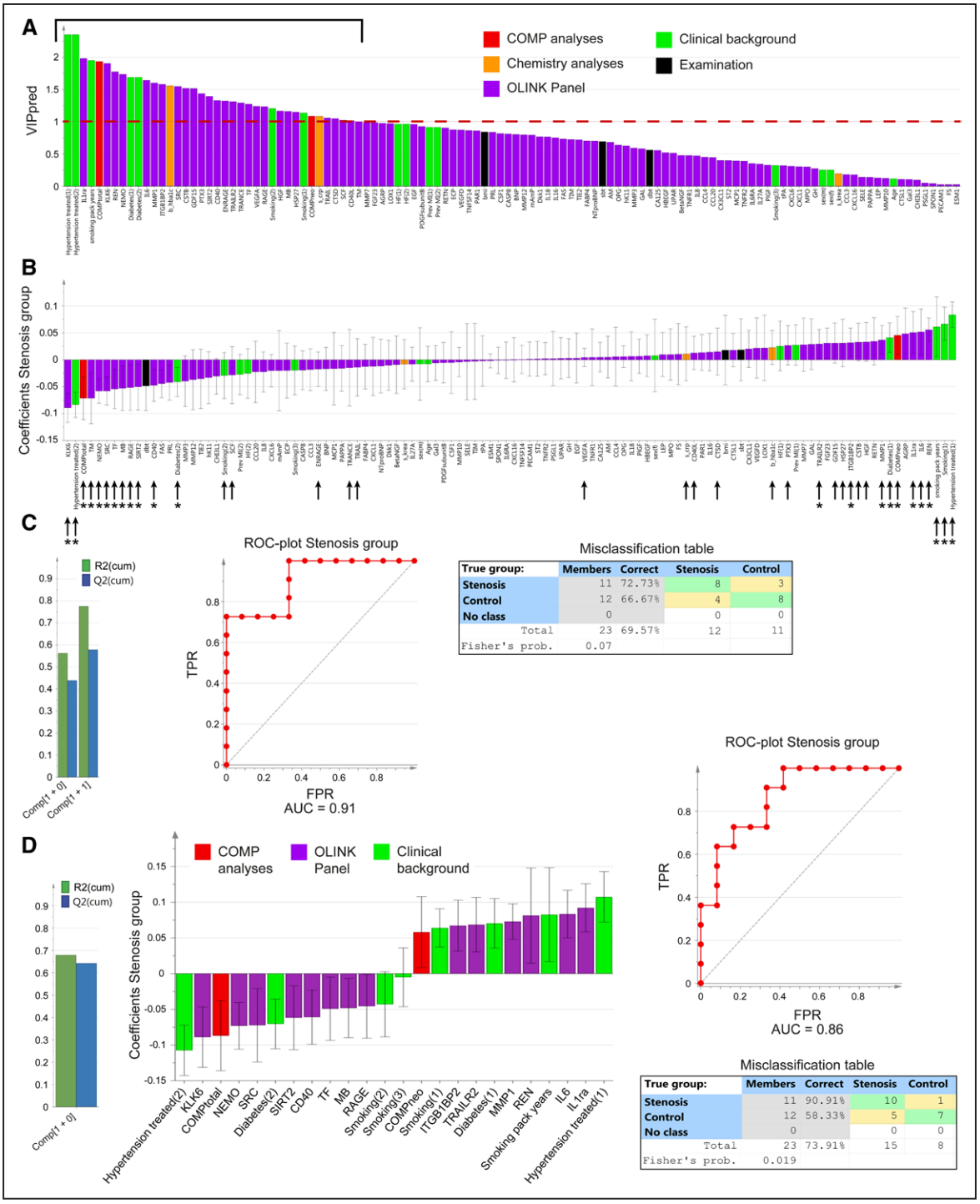

Figure 3. Comparison of control and symptomatic carotid stenosis (stenosis) groups.
A predictive orthogonal partial least square discriminant analysis (OPLS-DA) model was fitted, including both, laboratory and clinical variables. **A**, Shows variable importance plot predictive (VIPpred) for all variables. The red dotted line indicates the threshold for selecting variables for a submodel of the most important variables. **B**, Regression coefficients plots. Variables selected based on a VIPpred >1 are indicated by arrows. Variables that were considered significant based on error bars estimates are marked with asterisks. **C**, Validation of the model with cross-validation on the training dataset (Q2 [cum], R2 [cum]) as well as fitting of the model on the validation dataset. For evaluating the classification performance based on the validation dataset, a receiver operating characteristic (ROC) plot and misclassification table are shown. **D**, New model calculated using only the most important variables. To the left, model fit determined by cross-validation on training dataset and a coefficients plot. To the right, results from fitting the new model on the validation dataset, shown by a ROC plot and misclassification table. 1=yes, 2=no, 3=previous. AUC indicates area under the curve; CD40, cluster of differentiation 40; COMP, cartilage oligomeric matrix protein; f, female; FPR, false positive rate; IL-1ra, interleukin-1 receptor antagonist protein; IL6, interleukin-6; ITGB1BP2, integrin beta 1 binding protein 2; KLK6, kallikrein-6; m, male; MB, myoglobin; MMP1, matrix metalloproteinase-1; NEMO, nuclear factor-kappa-B essential modulator; RAGE, receptor for advanced glycation end-products; REN, rennin; SIRT2, SIR2-like protein; SRC, proto-oncogene tyrosine-protein kinase Src; TPR, true positive rate; and TRAIL-R2, tumor necrosis factor-related apoptosis-inducing ligand receptor 2.

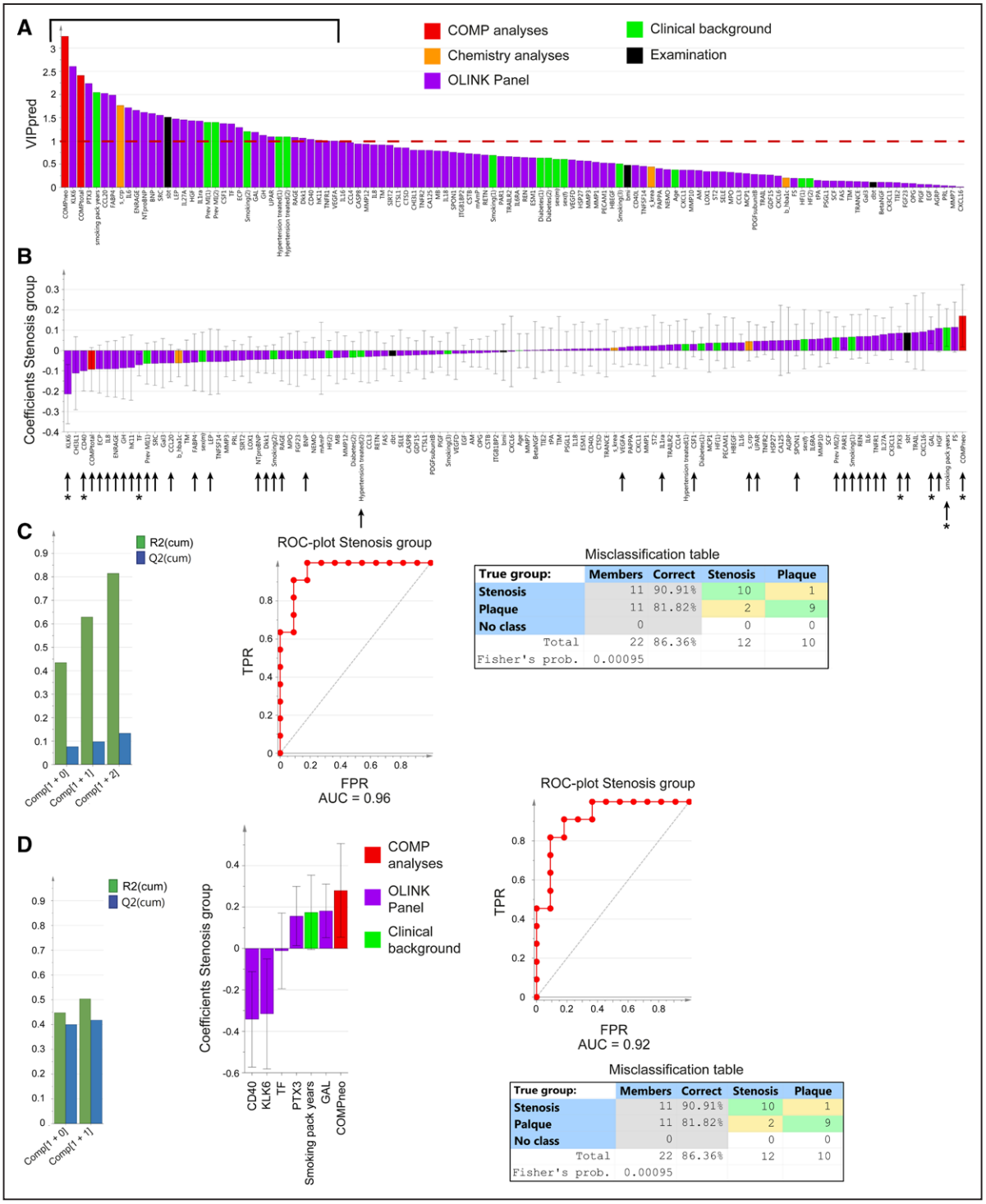

Figure 4. Comparison of the plaque and stenosis groups.
A predictive orthogonal partial least square discriminant analysis (OPLS-DA) model was fitted, including both laboratory and clinical variables. **A**, Shows variable importance plot predictive (VIPpred) for all variables. Red dotted line indicates threshold for selecting variables for a submodel of the most important variables. **B**, Regression coefficients plots. Those variables selected based on a VIPpred >1 are indicated by arrows. Variables that were considered significant based on error bars estimates are marked with asterisks. **C**, Validation of the model with cross-validation on the training dataset (Q2 [cum], R2 [cum]) as well as fitting of the model on the validation dataset. For evaluating classification performance based on the validation dataset, a receiver operating characteristic (ROC) plot and misclassification table are shown. **D**, New model calculated using only the most important variables. To the left, model fit determined by cross-validation on training dataset and a coefficients plot. To the right, results from fitting the new model on the validation dataset shown as ROC plot and misclassification table. 1=yes, 2=no, 3=previous. AUC indicates area under the curve; CD40, cluster of differentiation 40; COMP, cartilage oligomeric matrix protein; f, female; FPR, false positive rate; GAL, galanin; KLK6, kallikrein-6; m, male; PTX3, pentraxin-related protein PTX3; TF, tissue factor; and TPR, true positive rate.

Additionally, cross-validation, as expected, showed a much higher degree of reproducibility as determined by a higher Q2 value. In the reduced model, smoking pack-years and the following laboratory parameters were predictive for stenosis; a high concentration of COMPneo, GAL (galanin), and PTX3 (pentraxin-related protein PTX3) in plasma. Predictive parameters for the plaque group were increased concentrations of CD40 and KLK6 (Figure 4D). When doing group-wise comparisons using standard parametric/nonparametric hypothesis tests (Table IIIA and IIIB in the Data Supplement), COMPneo and KLK6 were significantly different between the plaque and stenosis groups after adjustment for multiple testing.

Importantly, when fitting an OPLS-DA model using only clinical parameters, poor predictive performance was observed compared with an OPLS-DA model with only laboratory parameters (Figure IIC and IID in the Data Supplement).

To investigate the predictive performance of a model including all 3 study groups, an OPLS-DA model was created for this scenario (Figure III in the Data Supplement). As expected from the PCA analysis (Figure 1), the control and stenosis groups showed high AUCs in the ROC plot (0.81–0.90) when fitted to the validation dataset, whereas the plaque group was the most difficult to classify correctly and displayed a lower AUC in the ROC plot (0.70).

COMPneo Staining Was Associated With Atherosclerotic Lesions

COMPneo and native COMP staining largely followed the same pattern of immunoreactivity, suggesting that the polyclonal antibody directed against native COMP also recognizes its cleaved fragments. Immunohistochemical analysis revealed a predominantly intracellular localization in and around the atherosclerotic lesions.

Smooth muscle cell staining was localized adjacent to atherosclerotic plaques, whereas cells further from the lesions were unstained (Figure 5A and 5B). Positive staining in smooth muscle cells for COMPneo, occurred both intracytoplasmatically and intranuclearly (Figure 5C and 5D). A population of these positive cells had a morphology suggestive of a motile phenotype with irregular shape and protrusions. Furthermore, foam cells had a consistent cytoplasmatic staining of both native COMP and COMPneo; however, consistent intranuclear staining was only visible with COMPneo (Figure IVA and IVB in the Data Supplement). The endothelial cells and leukocytes showed a more variable staining pattern (Figure IVC and IVD in the Data Supplement). A summary of the immunohistochemical results is presented in Table IV in the Data Supplement.

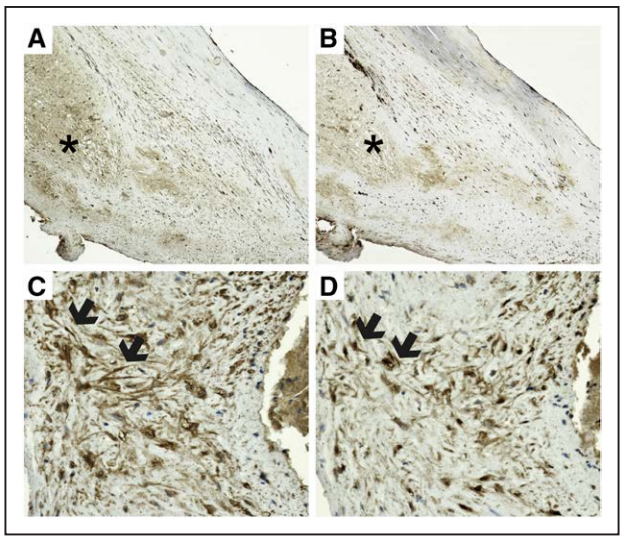

Figure 5. Immunoreactivity against native COMP (cartilage oligomeric matrix protein) and neoepitopes in human carotid plaques.
*Intralesional plaques. Arrows toward cells with smooth muscle cell (SMC) morphology. Overview of the lesion in the common carotid artery (×100), native COMP (**A**), and neoepitope (**B**). Staining of SMC native COMP (**C**) and neoepitope (**D**) in the common carotid artery (×400).

DISCUSSION

The present study is the first to show that plasma levels of COMPtotal and a unique COMPneo in patients with symptomatic carotid stenosis are significantly different when compared with both, patients with stroke with milder carotid lesions and to control subjects. The stenosis group displayed the highest concentrations of COMPneo and lowest concentration of COMPtotal. Furthermore, COMPtotal and COMPneo together with other cardiovascular risk markers could predict the presence of carotid stenosis with high accuracy. Finally, COMPneo was found in human carotid atherosclerotic lesions, indicating its potential functional role in the progression of atherosclerosis.

COMP has previously been shown to be involved in atherosclerosis progression[3,11] as well as the progression of osteoarthritis.[12] The low-grade inflammation present in atherosclerosis and osteoarthritis[2,13] leads to fragmentation of extracellular matrix molecules and the formation and release of unique neoepitopes. In the present study, increased levels of COMPneo were detected in patients with carotid stenosis, supporting an increased degradation of COMP in severe atherosclerotic disease.

When the stenosis group was compared with the control subjects in the multivariate analysis, several clinical background factors were found to be highly predictive for the stenosis group. All these are previously well-known risk factors for the development of atherosclerosis and carotid stenosis.[14] Interestingly, blood pressure was not found to be a discriminating

variable. This apparent paradoxical finding was most likely because these patients received sufficient treatment for hypertension.

When patients with stroke were compared with patients with symptomatic stenosis, only smoking pack-years was found to be a clear discriminating variable among the clinical background variables. This suggested that while other clinical variables are important for the development of atherosclerotic disease, smoking exposure has the strongest association to large vessel atherosclerotic disease. This is in accordance with a population-based study where current smoking was identified as the strongest risk factor for carotid artery atherosclerosis.[15] Our study further extends these results by showing that cumulative smoking exposure rather than the presence of smoking is most important for predicting carotid stenosis.

Several of the predictive factors for the stenosis group when compared with those of controls (ie, IL-1ra, IL-6, MMP1, TRAIL-R2, ITGB1BP2, REN, and COMPneo) have previously been associated with inflammation. Elevated levels of proinflammatory cytokines such as IL-6 are associated with all stages of atherosclerosis.[16] MMP1 is also upregulated by inflammation and contributes to atherosclerosis progression by tissue remodeling and paracrine effects.[17] TRAIL-R2 belongs to the tumor necrosis factor receptor superfamily and participates in the regulation of the adaptive immune response.[18]

The finding of IL-1 receptor antagonist protein as a predictive factor for stenosis may seem paradoxical, as this is a natural inhibitor of the proinflammatory cytokine IL-1 and has been linked to reduced atherosclerosis in animal models.[19] This could be attributed to the fact that IL-1 receptor antagonist protein is upregulated as a negative feedback mechanism by concurrent high levels of IL-1. Unfortunately, IL-1 was not included in the Olink cardiovascular disease panel, which precludes us from evaluating the balance between IL-1 and the IL-1 receptor antagonist protein.

In summary, most predictive factors for carotid stenosis were associated with the immune response, inflammation, and extracellular matrix degradation, supporting the role of systemic inflammation in chronic atherosclerosis.

Conversely, KLK6, COMPtotal, NEMO, SRC, SIRT2, CD40, TF, MB, and RAGE were parameters predictive of the control group.

Both KLK6 and SIRT2 have previously been shown to have prosurvival or anti-apoptotic effects. SIRT2 has been linked to decreased atherosclerotic plaque,[20] whereas KLK6 to our knowledge has not been previously described in the context of atherosclerosis.

CD40 is a receptor expressed by macrophages, which also exists in a soluble form. Soluble CD40 has been reported to limit CD40-CD40 ligand interaction and confer protection against atherosclerosis.[21] Data on soluble CD40 in cardiovascular disease are limited. However, one study, in contrast to our results, reported increased levels in coronary heart disease.[21]

When the stenosis and plaque groups were compared, fewer predictive markers were found. The reason for this may be that the stenosis and plaque groups had more similar comorbidities, including atherosclerotic disease in other locations in the arterial tree, compared with the stenosis and control groups. With the exception of smoking pack-years, none of the clinical background factors showed high predictive performance, underlining the need for biomarkers to separate the stenosis and plaque groups. The plasma concentration of COMPneo, GAL, and PTX3 were predictive factors for stenosis.

COMPneo was found to be the most important predictive factor when the stenosis group was compared with the plaque group. Notably, it was also found to be a predictive factor for stenosis when the stenosis group was compared with the control group—indicating that it may be the most clearly associated marker with atherosclerotic disease severity in the present study.

GAL is involved in the regulation of energy homeostasis. In a mouse model, it was shown that increased levels of GAL resulted in a phenotype similar to metabolic syndrome with insulin resistance and a lipid profile with high triglyceride levels.[22] To our knowledge, it has not been previously associated with atherosclerosis or carotid stenosis.

PTX3 is an acute phase protein belonging to the same family as C-reactive protein. PTX3 has previously been associated with atherosclerotic disease in a number of studies, including one on carotid plaques where high levels of PTX3 were predictive of plaque vulnerability.[23]

CD40 and KLK6 were predictive for stroke without carotid stenosis (plaque group). Notably, these markers were also predictive of the control group when compared to the stenosis group and are discussed in further depth in this section. This could be interpreted as these markers being most clearly negatively correlated with the severity of atherosclerotic disease.

The location of native COMP and the COMPneo in atherosclerotic artery tissue was investigated in endarterectomy samples. Native COMP has previously been detected in the medial layer of normal internal arteries, in vascular smooth muscle cells with normal contractile phenotype, and in the extracellular matrix of arteries with intimal thickening.[3] The presence of COMP degradation products has not been previously investigated in atherosclerotic lesions. In our study, there was a clear overlap between the immunohistochemistry staining for native COMP and COMPneo in the atherosclerotic lesions. An explanation could be that the polyclonal antibody against the native form of COMP recognizes the intact molecule as well as various COMP fragments while the neoepitope antibody is specific to the cleavage site.[8] The similar staining suggests that most of the COMP

is degraded in advanced atherosclerotic lesions. Interestingly, we detected cells with the motile morphology of smooth muscle cells that stained positive for COMPneo in the cytoplasm and in the nucleus. This observation suggested that signaling conveyed by neoepitopes may have a role in de-differentiation of vascular smooth muscle cells.

Some limitations of the present study should be acknowledged. The cases with symptomatic carotid stenosis were included from a different patient cohort compared with the plaque group. This resulted in slightly different inclusion criteria, where the symptomatic carotid stenosis cohort either suffered from stroke, transient ischemic attack, or amaurosis fugax, whereas all the subjects in the plaque group suffered from stroke. Although we cannot exclude its effect on the results, when the OPLS-DA discriminatory model was recalculated on only stroke cases, the pattern of variables was similar to the original model. Furthermore, when the model with only the most important variables presented in the study was evaluated on only stroke cases in the validation dataset, the predictive performance was similar to when all the cases were included (AUC 0.97, data not shown). Another general limitation of the study was that blood samples were not drawn at the time of the clinical event, but rather when patients were included in the study. In most cases, this was within a few days after the clinical event; however, in some cases, this happened up to a few weeks after the event. Finally, the study had a small sample size and the results thus need to be confirmed in a larger patient cohort.

In summary, our data suggest that degradation of COMP may be associated with inflammation in atherosclerosis progression with the generation of a specific COMP fragment. In blood, this COMPneo was found to represent a novel biomarker that, together with additional biomarkers and risk factors, efficiently identified patients with symptomatic carotid stenosis. Our results could potentially be used to develop a screening tool for patients with stroke with the highest risk of carotid stenosis for early carotid artery screening to reduce time delay to endarterectomy surgery. However, a further validation study on a larger cohort is necessary. There is also a need for further mechanistic studies to elucidate the role of COMP and COMP degradation products including the COMPneo in the progression of atherosclerotic disease.

ARTICLE INFORMATION

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Disclosures

E. Skiöldebrand is a shareholder in SGPTH Life Science AB, which currently holds a patent with the title "Comp peptide and antibodies thereto for diagnosing osteoarthritis". The international publication number is WO 2017/216289 A1, published on December 21, 2017.

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