Netrin-1 and the grade of atherosclerosis are inversely correlated in humans

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Arteriosclerosis, Thrombosis, and Vascular Biology

CLINICAL AND POPULATION STUDIES

Netrin-1 and the Grade of Atherosclerosis Are Inversely Correlated in Humans

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OBJECTIVE: Netrin-1 has been shown to play a role in the initiation of atherosclerosis in mice models. However, little is known about the role of Netrin-1 in humans. We set out to study whether Netrin-1 is associated with different stages of atherosclerosis.

APPROACH AND RESULTS: Plasma Netrin-1 levels were measured in different patient cohorts: (1) 22 patients with high cardiovascular risk who underwent arterial wall inflammation assessment using positron-emission tomography / computed tomography, (2) 168 patients with a positive family history of premature atherosclerosis in whom coronary artery calcium scores were obtained, and (3) 104 patients with chest pain who underwent coronary computed tomography angiography imaging to evaluate plaque vulnerability and burden. Netrin-1 plasma levels were negatively correlated with arterial wall inflammation (β, −0.01 [95% CI, 0.02 to −0.01] R^2, 0.61; $P<0.0001$), and concentrations of Netrin-1 were significantly lower when atherosclerosis was present compared with individuals without atherosclerosis (28.01 versus 10.51 ng/mL, $P<0.001$). There was no difference in Netrin-1 plasma concentrations between patients with stable versus unstable plaques (11.17 versus 11.74 ng/mL, $P=0.511$). However, Netrin-1 plasma levels were negatively correlated to total plaque volume (β, −0.09 [95% CI, −0.11 to −0.08] R^2, 0.57, $P<0.0001$), calcified plaque volumes (β, −0.10 [95% CI, −0.12 to −0.08] R^2, 0.53; $P<0.0001$), and noncalcified plaque volumes (β, −0.08 [95% CI, −0.10 to −0.06] R^2, 0.41; $P<0.0001$). Treatment of inflammatory stimulated endothelial cells with plasma with high Netrin-1 level resulted in reduced endothelial inflammation and consequently, less monocyte adhesion.

CONCLUSIONS: Netrin-1 plasma levels are lower in patients with subclinical atherosclerosis and in patients with arterial wall inflammation. Netrin-1 is not associated with plaque vulnerability; however, it is negatively correlated to plaque burden, suggesting that Netrin-1 is involved in some, but not all, stages of atherosclerosis.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: atherosclerosis ■ endothelium ■ inflammation ■ monocyte ■ Netrin-1 ■ risk

Despite major advances in our understanding of and therapeutic strategies for atherosclerosis, cardiovascular disease (CVD) and its complications remain a leading cause of mortality and morbidity.[1] Inflammation plays a crucial role throughout all stages of atherosclerosis, with transmigration of monocytes into the subendothelial space being an important process in the initiation of atherosclerosis. Subsequently, migrated monocytes trigger a local inflammatory response within the subendothelial compartment, promoting foam cell formation, which will ultimately lead to atherosclerotic lesion formation.[2] The ensuing process of plaque formation is a chronic process that goes unnoticed for decades in many subjects. However, upon rupture of the inflamed fibrous cap, occlusive luminal thrombosis is likely to occur, resulting in an acute cardiovascular event.[3]

The identification of patients at increased risk for cardiovascular events is critically important to implement effective preventive measures. Among the well-established risk factors for atherosclerosis are hypercholesterolemia, hypertension, smoking, diabetes mellitus, and obesity.[4,5] However, despite their value for cardiovascular

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Nonstandard Abbreviations and Acronyms

CAC	coronary artery calcification
CCTA	coronary computed tomography angiography
CRP	C-reactive protein
CT	computed tomography
HDL	high-density lipoprotein
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
MCP-1	monocyte chemoattractant protein 1
PET	positron emission tomography
TNF	tumor necrosis factor

risk assessment at large scale, these parameters lack specificity for prediction of individual coronary plaque burden or cardiovascular event risk.

Imaging techniques such as coronary artery calcification (CAC) scores with computed tomography (CT) are useful for risk categorization,[6] yet routine implementation in primary prevention is hampered by costs, lack of general availability, and the exposure to radiation.

In an effort to identify a useful plasma biomarker, we focused on neuroimmune guidance cues that are involved in the regulation of leukocyte trafficking. Netrin-1 is a neuroimmune guidance cue that was originally characterized for its role in axon-guiding cue in the developing nervous system. However, studies in the pancreas, lung, and mammary gland established that there is a lot more to Netrins than just wiring the brain.[7] Much preclinical research has been done to investigate a function for Netrin-1 in CVD. Netrin-1 is a soluble protein, expressed by both the endothelium and macrophages and can directly regulate leukocyte chemotaxis through the UNC5B (unc-5 netrin receptor B) receptor.[8–10] Endothelial Netrin-1 expression is increased by atheroprotective laminar flow, although decreased by inflammatory cytokines.[8,10] Regarding the regulation of endothelial Netrin-1 expression in vivo, mouse studies have observed a reduction in the levels of Netrin-1 within the vasculature of atherosclerotic mice.[9,11,12] In line with this, Netrin-1 has been shown to have an anti-inflammatory effect, both on the endothelial cells themselves,[8,13] as well as by inhibiting monocyte adhesion and migration.[8,9] Supporting evidence for this anti-inflammatory endothelial Netrin-1 is found in mouse models with acute lung inflammation due to *Staphylococcus aureus* infection, where Netrin-1 was found to be expressed in the luminal surface of lung endothelial cells, where is acted to block migration of monocytes.[14–16] Also in the atherosclerotic mouse model with low-density lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mice receiving overexpression of human Netrin-1 by adenovirus delivery show a reduction in plaque formation compared with sham-treated mice.[17]

The effect of Netrin-1 in humans is still unknown, and there is not much research done about Netrin-1 in the systemic circulation related to CVD. The current study aimed to investigate the association between ex vivo Netrin-1 levels and in vivo imaging of atherosclerosis in humans.

Highlights

- Netrin-1 is correlated with the extent of arterial wall inflammation in humans.
- Netrin-1 plasma levels are lower in individuals with subclinical atherosclerosis compared with individuals without atherosclerosis.
- Netrin-1 plasma levels are negatively correlated with plaque burden.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Patient Recruitment

All samples used for this study were collected in accordance with the ethical guidelines of the 1975 Declaration of Helsinki, and all protocols were approved by the institutional review board of the Amsterdam University Medical Center. Written informed consent was obtained from the participants in this study.

Cohort I: Arterial Wall Inflammation Cohort

For this cohort, a subset from the VISTA study (Arterial Wall Inflammation Measured With 18F-FDG PET/CT in Patients With Statin Intolerance Before and After Treatment With a PCSK-9 Inhibitor; https://www.trialregister.nl. Unique identifier: NTR6884) population was used.[18] The study population consisted of 50 statin-intolerant individuals who were at high risk for CVD. In these patients, the effect of the PCSK9 (proprotein convertase subtilisin/kexin type 9) antibody Alirocumab on arterial wall inflammation was assessed. In the current study, we included 22 sequential patients of whom baseline laboratory samples and scan results were available. The laboratory samples were collected before starting the therapy, and the extent of arterial wall inflammation was measured using ^{18}F-fluorodeoxyglucose positron-emission tomography (PET)/CT scans.[19] The target to background of the most diseased segment was used as a the readout marker for arterial wall inflammation[20] (Figure I in the online-only Data Supplement).

Cohort II: Asymptomatic High-Risk Cohort

The second cohort consisted of a selection of asymptomatic family members from the premature atherosclerosis biobank. In April 2017, this biobank contained a total of 1309 samples from all index patients with premature atherosclerosis as well as their family members who visited the outpatient clinic for premature

CVD in the Amsterdam UMC. We excluded all index patients and individuals who smoked or had diabetes mellitus.[21,22] We included all patients who underwent a coronary CT scan and of whom laboratory samples were available with a CAC >0; we subsequently created a control group 56 individuals with a CAC score of zero who were matched for age and sex from the same biobank. This resulted in a total study population of 168 individuals. Coronary CT imaging was performed as previously described,[23] and the CAC score was evaluated according to Agatston et al[24] (Figure II in the online-only Data Supplement).

Cohort III: Progressive Plaque Cohort

For the third cohort, samples from patients participating in the PACIFIC study (Prospective Comparison of Cardiac PET/CT, SPECT/CT Perfusion Imaging a CT Coronary Angiography With Invasive Coronary Angiography; http://www.clinicaltrials.gov. Unique identifier: NCT01521468) were used. Details regarding this study design have been reported previously.[25] In brief, the PACIFIC trial investigated the diagnostic performance of coronary CT angiography (CCTA), single-photon emission CT, and PET with invasively measured fractional flow reserve as reference standard, in a cohort of 208 patients with suspected coronary artery disease. Patients who smoked or had a history of diabetes mellitus or patients without atherosclerotic plaques were excluded. This resulted in a total study population of 104 patients.

Image acquisition of CCTA was performed as described previously.[25,26] Using semiautomated software (Comprehensive Cardiac Analysis; Philips Healthcare), all coronary segments with a diameter ≥2 mm were evaluated by an experienced reader. The coronary tree was evaluated using axial, multiplanar reformation, maximum intensity projection, and cross-sectional images (slice thickness 0.9 mm, increment 0.50 mm). Centerlines and vessel contours were automatically reconstructed, with the possibility of manual corrections. Total plaque volumes were calculated per patient by summing the lengths and volumes of separate plaques along the entire coronary tree. A scanner-specific threshold of 150 HU was used for distinguishing noncalcified from calcified plaque components, which subsequently was used to determine noncalcified and calcified plaque volumes. Furthermore, visual assessment of the presence of adverse plaque characteristics was assessed, that is, low-attenuation plaque, positive remodeling, spotty calcification, and napkin ring sign. Vulnerable plaques were defined by the presence of ≥2 adverse plaque characteristics (Figure III in the online-only Data Supplement).

Laboratory Parameters

Whole blood was collected in EDTA containing tubes, and plasma was collected after centrifugation for 10 minutes at 1500*g* at 4°C. Plasma samples were stored in cryovials at −80°C. Plasma total cholesterol, HDL (high-density lipoprotein) cholesterol, and triglyceride levels were analyzed with commercially available enzymatic methods. LDL (low-density lipoprotein) was calculated using the Friedewald formula.[27]

Cardiovascular Risk Definitions

Type 2 diabetes mellitus was defined as a fasting plasma glucose >7.0 mmol/L or the use of antidiabetic medication. Hypertension was defined as a systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg or the use of blood pressure–lowering drugs. A history of CVD was verified based on questionnaires. Untreated LDL-C (LDL cholesterol) values were (if unknown) calculated based on treated values and corrected for medication use.[28]

Netrin-1 Plasma Measurement

Wells were coated for 3 hours with 5 µg/mL of UNC5B recombinant protein (8869-UN-050; R&D Systems) in PBS and then blocked with 2% milk in PBS for 5 hours at room temperature. Plasma samples were diluted in a 1:2 ratio in PBS, loaded in duplicates in the UNC5B-precoated plates, and incubated overnight at 4°C. Accordingly, plates were incubated with sheep-anti-human Netrin-1 antibody (0.5 µg/mL, AF6419; R&D Systems) for 2 hours at room temperature, followed by a 1-hour incubation with horseradish peroxidase–conjugated donkey-anti-sheep IgG (1:1000, HAF016; R&D systems) in blocking buffer. To enable quantification of the horseradish peroxidase signal, ready to use 3,3′,5,5′-Tetramethylbenzidine solution (T4444; Sigma, Darmstadt) was added. After 30 minutes, the reaction was stopped with H_2SO_4, and absorbance at 450 nM was measured using a multiwell plate reader (SPECTRAmax M5, Molecular Devices). Plates were washed 3 to 5× with PBS and 0.05% Tween between each step. As a reference for quantification, a standard curve was established by a serial dilution of recombinant Netrin-1 (250–128 000 pg/mL, 6419-N1; R&D systems). If measurements exceeded the standard curve, samples were further diluted and measured again. The ELISA had an interassay of 7.3%, an intraassay coefficient of variability of 8.5%, sensitivity of 0.0920 ng/mL, and assay linearity range of 74% to 117% (Table I through IV and Figure IV in the online-only Data Supplement). Immunoblot validation of the Netrin-1 protein is added in the online-only Data Supplement (Figure V in the online-only Data Supplement). As well as immunoblot analysis of plasma of 4 patients from cohort 2 to confirm high and low concentrations of Netrin-1 in patient plasma. Two patient plasma's from subjects without subclinical atherosclerosis were compared with 2 plasmas of patients with subclinical atherosclerosis confirming lower Netrin-1 levels in patients with subclinical atherosclerosis compared with the patients without subclinical atherosclerosis (Figure VI in the online-only Data Supplement).

Endothelial Cells

Primary human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords obtained at the Leiden University Medical Center after written informed consent and ensuring that collection and processing of the umbilical cord was performed anonymously. The umbilical vein was flushed with PBS, using glass cannulas, to remove all remaining blood. Endothelial cells were detached by infusion of the vein with Trypsin/EDTA (1×, BE02-007E; Lonza) solution and incubation at 37°C for 15 minutes. After incubation, the cell suspension was collected and taken up in endothelial cell growth medium (Promocell; C222111 supplemented with C39211) with 1% antibiotics. After flushing the umbilical vein one more with PBS, to ensure all detached cells are collected, cells were pelleted by centrifugation at 1200 rpm for 7 minutes. Cell pellet was dissolved and maintained in EGM2 medium, and cells were cultured on gelatin (1%) coated surfaces.

THP1 Cells

The human THP-1 monocytic leukemia cell line was obtained from ATCC (THP-1 ATCC, TIB-202; Middlesex). Cells were

cultured in RPMI 1640 medium (22409; Gibco) supplemented with 10% FCS, 1% L-glutamine, 1% antibiotics (penicillin/streptomycin, 15070063; Gibco), and 25 nM β-mercaptoethanol.

Monocyte Adhesion to Endothelial Cells

Endothelial cells were grown to a confluent monolayer and stimulated with or without TNFα (tumor necrosis factor α, 10 ng/mL, H8916; Sigma) or patient plasma (250 µL) or recombinant Netrin-1 (500 ng/mL, 6419-N1; R&D Systems) for 24 hours. Plasma of 4 patients within the group with no subclinical atherosclerosis were used with a Netrin-1 concentration varying from 25.0 to 35.4 ng/mL as measured with ELISA. Also, plasma of 4 patients within the group with subclinical atherosclerosis was used with a Netrin-1 concentration varying from 3.1 ng/mL to 12.3 ng/mL as measured with ELISA. In some experiments, patient plasma or recombinant Netrin-1 was preincubated with UNC5B recombinant protein (500 ng/sample, 8869-UN-050; R&D Systems) 30 minutes before it was added to the endothelial cells. THP1 cells were labeled with Calcein AM (5 µg/mL, C3100MP; Molecular Probes Life Technologies) and incubated on top of a monolayer endothelial cells for 30 minutes at 37°C. Nonadhering cells were washed away by multiple washing steps with PBS, after which the cells were lysed in Triton-X 0.5% for 10 minutes. Fluorescence was measured with excitation wavelength of 485 nm and emission wavelength of 514 nm.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from endothelial cells using TRIzol and the RNeasy Mini Kit (74106; Qiagen) according to manufacturer's instructions. Total RNA was reverse transcribed using M-MLV Reverse Transcriptase Kit (M1701; Promega). Real-time polymerase chain reaction analysis was conducted using SYBR Select Master Mix (4472908; Applied Biosystems) and the forward and reverse primers, as indicated in Table V in the online-only Data Supplement. The polymerase chain reaction cycling conditions were: initial denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension step at 72°C for 10 minutes. mRNA expression was normalized to expression of GAPDH and expressed as copies per GAPDH.

Statistical Analyses

Data were analyzed by unpaired 2-tailed *t* tests. Normally was examined by means of inspection of histograms. When in doubt, the Shapiro-Wilk test was used to test for normality. In the case of skewed data, data were log transformed to allow for parametric testing. Linear regression was used to ascertain the predictive power of Netrin-1 levels. Multivariate analyses were performed with a linear regression and a logistic regression model. *P* values of <0.05 were considered statistically significant, and all data are presented as mean± SD or median with interquartile ranges. All statistical analysis was performed with SPSS version 25 and Graphpad Prism 8.

RESULTS

Negative Correlation Between Netrin-1 Plasma Levels and Arterial Wall Inflammation

Plasma Netrin-1 levels were measured in 22 patients in whom arterial wall inflammation was assessed using ^{18}F-fluorodeoxyglucose PET/CT. The demographic and clinical characteristics of the study population are summarized in Table 1. The mean target to background of the most diseased segment was 1.94 (range, 1.21–2.74 target to background of the most diseased segment). The mean circulating concentrations of Netrin-1 in plasma was 24.7 ng/mL (range, 16.1–40.4 ng/mL). A scatterplot of Netrin-1 plasma levels and target to background of the most diseased segment showed a negative correlation between Netrin-1 plasma levels and arterial wall inflammation (Figure 1 β, −0.01 [95% CI, −0.02 to −0.01;] R^2, 0.61; P<0.0001). According to multivariate analyses, only Netrin-1 plasma levels were a significant predictor of arterial wall calcification after adjustment for atherosclerosis-related factors (age, sex, body mass index, systolic blood pressure, untreated LDL-C, Lp[a] [lipoprotein (a)], and CRP [C-reactive protein]; Table VI in the online-only Data Supplement).

Inverse Association Between Netrin-1 Plasma Levels and Arterial Wall Calcification

To assess a potential association between Netrin-1 and subclinical atherosclerosis, Netrin-1 plasma levels were measured in 168 asymptomatic subjects with a positive family history of premature atherosclerosis.

Table 1. Baseline Characteristics Arterial Wall Inflammation Cohort

Variable	N=22
Sex, male	10 (45%)
Age, y	63±9
Body mass index, kg/m²	28±4
Systolic blood pressure, mm Hg	137±15
Diastolic blood pressure, mm Hg	82±7
Current smoking	5 (23%)
Type 2 diabetes mellitus	0 (0%)
Hypertension	15 (68%)
Family history of CVD	N/A
Total cholesterol, mmol/L	6.9±1.9
Triglycerides, mmol/L	1.9±1.4
HDL-C, mmol/L	1.4±0.3
Untreated LDL-C, mmol/L	4.6±1.6
Lp(a), mg/L	149.6±192.9
C-reactive protein, mg/L	4.4±8.9
Coronary artery calcium score (percentile)	N/A
Use of anti-inflammatory drugs	N/A
Netrin-1, ng/mL	24.67±6.6
Arterial wall inflammation (TBR-MDS)	1.94±0.5

Values are N±SD or N (%). CVD indicates cardiovascular disease; HDL-C, high density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); N/A, not applicable; and TBR-MDS, target to background of the most diseased segment.

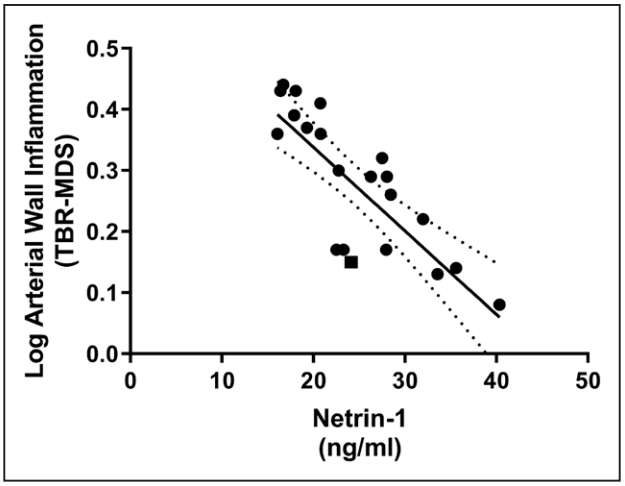

Figure 1. The correlation between Netrin-1 plasma levels and arterial wall inflammation.
In subjects with a high cardiovascular risk, there is a significant negative correlation with Netrin-1 plasma levels, measured by ELISA and arterial wall inflammation (β: −0.01 [95% CI −0.02 to −0.01] R^2, 0.61; $P<0.0001$). Arterial wall inflammation was assessed by ^{18}F-fluorodeoxyglucose positron-emission tomography computed tomography measured as target-to-background ratio (TBR) of the most diseased segment (MDS) of the index carotid. The MDS was determined by calculating the mean of the maximum TBR of the 3 adjacent slides with the highest TBR (MDS TBR). Individuals (N=22) are represented as thick dots, and the best-fitted line is displayed with 95% confidence bands. The square represents 2 individuals with a similar extent of arterial wall inflammation (Log 0.15 TBR-MDS) and a similar level of Netrin-1 (24.11 ng/mL and 24.19 ng/mL). Normality was examined by means of inspection of histograms, association between Netrin-1 and arterial wall inflammation was assessed with a linear regression model.

CT scans showed CAC score >0 correlating with calcified plaques in 112 subjects (67%) and CAC score =0 correlating with absence of calcified lesions in 56 subjects (33%).

The demographic and biochemical characteristics of the participants stratified by presence or absence of coronary atherosclerosis are presented in Table 2. There were no significant differences in baseline characteristics between the subjects with or without subclinical atherosclerosis, except for the difference in calcium score. The average concentration measured of Netrin-1 in plasma of subjects with CAC score =0 was 28.0 ng/mL (range, 0.4–114.6 ng/mL), whereas in plasma from patients with CAC score >0, Netrin-1 concentrations were significantly lower with a mean plasma level of 10.5 ng/mL (range, 0.2–72.1 ng/mL; Figure 2A, $P\leq0.0001$). According to multivariate analyses, only Netrin-1 plasma levels were a significant predictor of arterial wall calcification after adjustment for atherosclerosis-related factors (age, sex, body mass index, systolic blood pressure, untreated LDL-C, Lp[a], and CRP; Table VII in the online-only Data Supplement). Interestingly, no significant difference in the established acute phase reactant CRP levels was observed between groups (Figure 2B, $P=0.23$).

No Difference in Netrin-1 Plasma Levels in Patients With Different Stages of Plaque Vulnerability

Plasma samples were collected from 104 subjects with new-onset chest pain and suspected coronary artery disease who underwent CCTA imaging to identify coronary plaque morphology. Stable plaques were identified in 78 patients (75%) and unstable plaque in 26 (25%). The demographic and biochemical parameters stratified for the presence of either stable plaque or unstable plaque are presented in Table 3. Patients in the group with unstable plaques were more likely to be male, and HDL-cholesterol levels were significantly lower in the unstable plaque compared with the stable plaque group ($P<0.05$ for both). Mean circulating concentration of Netrin-1 in plasma were not significantly different between stable plaque (11.2 ng/mL; range, 2.2–24.5 ng/mL) versus patients with unstable plaques (11.7 ng/mL; range, 4.5–18.2 ng/mL; Figure 3; $P=0.511$).

Negative Correlation Between Netrin-1 Plasma Levels and Plaque Burden

In the cohort comprising 104 subjects with new-onset chest pain, we observed a negative an significant correlation between Netrin-1 plasma levels and total plaque volume (β, −0.09 [95% CI, −0.11 to −0.08] R^2, 0.57; $P<0.0001$), calcified plaque volume (β, −0.10 [95% CI, −0.12 to −0.08] R^2, 0.53; $P<0.0001$), and noncalcified plaque volume (β, −0.08 [95% CI, −0.10 to −0.06] R^2, 0.41; $P<0.0001$). Scatterplots depicted the relationship between Netrin-1 plasma levels and plaque volumes are displayed in Figure 4.

Netrin-1 in Patient Plasma Suppresses Expression of Vascular Adhesion Molecules and Inhibits Binding of Monocytes to Endothelial Cells

To investigate the biological function of Netrin-1 in plasma on the atherosclerotic process, we analyzed expression of adhesion molecules and cytokines by TNFα stimulated endothelial cells with and without treatment of Nertrin-1. Addition of recombinant Netrin-1 reduces the TNFα induces expression of ICAM-1 (intercellular adhesion molecule 1), IL (interleukin)-6, and MCP-1 (monocyte chemoattractant protein 1) with 30%, 55%, and 40%, respectively. Addition of the extracellular domain of human UNC5B fused to the Fc portion of human immunoglobulin G1 (UNC5B-Fc) to block the Netrin-1 reversed the anti-inflammatory effect of Netrin-1 in TNFα treated cells, but control immunoglobulin G (IgG) did not (Figure 5A through 5C). Furthermore, consistent with changes in cytokine and adhesion molecule expression, addition of TNFα enhanced the binding of monocytes by 8-fold, but addition of Netrin-1 to the TNFα stimulation of endothelial cells potently inhibited

Table 2. Baseline Characteristics Asymptomatic Individuals Cohort

	CAC=0; N=56	CAC >0; N= 112	*P* Value
Variable			
Sex, male	28 (50%)	65 (58%)	0.326
Age, y	53±7	52±10	0.374
Body Mass Index, kg/m^2	26±4	26 ±8	0.778
Systolic blood pressure, mm Hg	126±13	122±8	0.499
Diastolic blood pressure, mm Hg	76±8	74±21	0.631
Current smoking	0 (0%)	0 (0%)	N/A
Type 2 diabetes mellitus	0 (0%)	0 (0%)	N/A
Hypertension	16 (29%)	40 (36%)	0.358
Family history of CVD	54 (96%)	111 (99%)	0.219
Total Cholesterol, mmol/L	5.7±1.2	5.6±1.0	0.339
Triglycerides, mmol/L	1.7±2.6	1.3±0.8	0.095
HDL-C, mmol/L	1.5±0.4	1.4±0.4	0.149
Untreated LDL-C, mmol/L	3.9±1.1	3.8±0.9	0.596
Lp(a), mg/L	336±359	351±450	0.877
C-reactive protein, mg/L	1.9±2.8	2.8±4.7	0.237
Use of anti-inflammatory drugs	0 (0%)	0 (0%)	N/A
Calcium score (percentile)	0±0	75±22	<0.0001*
Netrin-1, ng/mL	28.01±27.22	10.51±12.56	<0.0001*

Values are N±SD or N (%). Patient characteristics of the whole cohort are displayed, stratified by the CACs-defined absence (CAC=0) and presence of atherosclerosis (CAC>0; *t* test, *P*<0.05). CAC indicates coronary artery calcification; CVD, cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); and N/A, not applicable.
*Statistically significant *P* value.

the adhesion of monocytes to endothelial cells, which could be blocked by UNC5B fused to the Fc portion of human immunoglobulin G1 and not by IgG (Figure VII in the online-only Data Supplement; Figure 5D).

To study the effect Netrin-1 in plasma derived from cases and controls on adhesion of monocytes and expression of adhesion molecules and cytokines, we stimulated human umbilical vein endothelial cells with either plasma of 4 patients within the group without subclinical atherosclerosis with a Netrin-1 concentration varying from 25.0 ng/mL to 35.4 ng/mL as measured with ELISA, or plasma of 4 patients within the group with subclinical atherosclerosis with a Netrin-1 concentration varying from 3.1 ng/mL to 12.3 ng/mL as measured with ELISA. In line with recombinant Netrin-1 protein, addition of patient plasma with high Netrin-1 concentrations reduced the TNFα induced expression of ICAM-1, IL-6, and MCP-1 with 50%, 70%, and 40%, respectively (Figure 5E through 5G). The same anti-inflammatory effect on monocyte adhesion was observed when endothelial cells were stimulated with plasma of patients with a high concentration of Netrin-1, whereas patient plasma with low Netrin-1 levels or plasma treated with UNC5B did not result in reduced monocyte adhesion (Figure 5H).

DISCUSSION

In the present study, we show that circulating Netrin-1 is significantly correlated with the extent of arterial wall inflammation and that Netrin-1 levels are lower in subjects with subclinical atherosclerosis as compared with subjects without atherosclerosis. Lower Netrin-1 plasma levels were also negatively correlated with plaque burden. However, Netrin-1 plasma levels were not statistically different between patients with stable and unstable plaques. Collectively, these results support the hypothesis that lower Netrin-1 plasma levels are associated with atherosclerosis initiation and progression in humans.

Inverse Correlation Between Netrin-1 and Arterial Wall Inflammation

Netrin-1 has been shown to have anti-inflammatory effects on the endothelium by preventing the activation

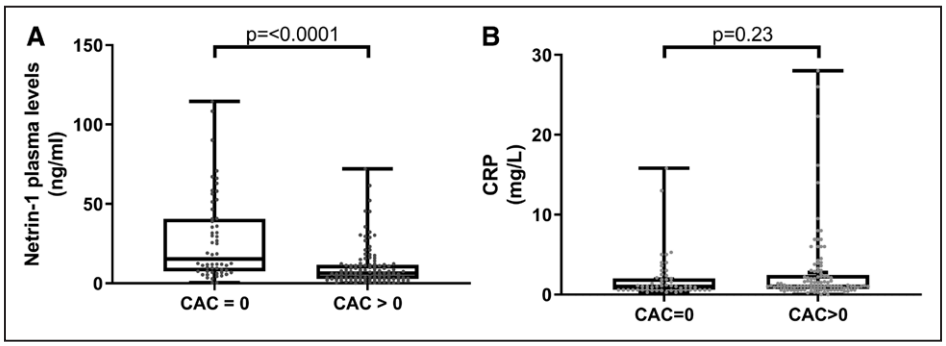

Figure 2. Plasma Netrin-1 and CRP (C-reactive protein) levels in subjects with and without atherosclerosis.
A, Plasma Netrin-1 levels, measured by ELISA, were significantly higher in apparently healthy individuals with no atherosclerosis (coronary artery calcification [CAC]=0, N=56) compared with apparently healthy subjects with subclinical atherosclerosis (CAC>0, N=112). **B**, Plasma CRP levels, measured using commercially available enzymatic method, did not differ between apparently healthy individuals with no atherosclerosis (CAC=0, N=56) and apparently healthy subjects with subclinical atherosclerosis (CAC>0, N=112). The threshold for a calcific lesion was set at a computed tomographic density of 130 Houndsfiend units and an area of ≥1 mm.^{2} A region of interest was placed around all lesions found within a coronary artery. A score for each region of interest was calculated by automated measurements. A total coronary calcium score was determined by adding up each of these scores. Individuals are represented as gray dots, and the whiskerplot represents the mean with minimum to maximum. Groups are compared by Students *t* test.

Table 3. Baseline Characteristics Progressive Plaque Cohort

	Stable Plaque N=78	Unstable Plaque N=26	*P* Value
Variable			
Sex, male	43 (55)	23 (89)	0.002*
Age, y	60±8	60±8	0.983
Body mass index, kg/m²	26.1±3.6	26.7±2.7	0.397
Systolic blood pressure, mm Hg	145±20	146±19	0.994
Diastolic blood pressure, mm Hg	84±12	84±12	0.616
Current smoking	0 (0%)	0 (0%)	N/A
Type 2 diabetes mellitus	0 (0%)	0 (0%)	N/A
Hypertension	40 (51%)	12 (46%)	0.654
Family history of CVD	43 (55%)	16 (62%)	0.572
Total cholesterol, mmol/L	4.6±1.0	4.4±1.2	0.252
Triglycerides, mmol/L	1.4±0.8	1.4±0.7	0.880
HDL-C, mmol/L	1.5±0.4	1.3±0.4	0.020*
Untreated LDL-C, mmol/L	3.7±1.2	3.7±1.2	0.814
Lp(a), mg/L	N/A	N/A	N/A
C-reactive protein, mg/L	0.8±1.9	1.1±2.9	0.542
Calcium score	84±18	84±16	0.859
Use of anti-inflammatory drugs	0 (0%)	0 (0%)	N/A
Netrin-1, ng/mL	11.17±4.4	11.74±3.8	0.511

Values are N±SD or N (%). Patient characteristics of the whole cohort are displayed, stratified by the presence of stable and vulnerable plaques (*t* test, $P<0.05$). CVD indicates cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein (a); and N/A, not applicable.

*Statistically significant *P* value.

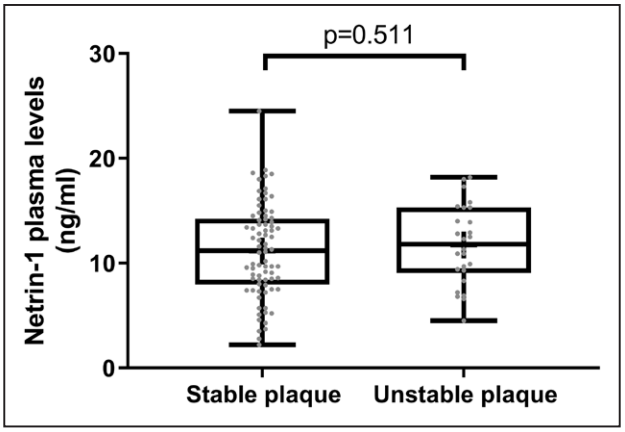

Figure 3. Plasma Netrin-1 plasma levels in subjects with stable and unstable plaque.
Plasma Netrin-1 levels, measured by ELISA, did not differ between individuals with stable plaques (N=78) compared with individuals with unstable plaques (N=26). Plaque stability was measured using semiautomated software (Comprehensive Cardiac Analysis, Philips Healthcare); all coronary segments with a diameter ≥2 mm were evaluated by an experienced reader. The coronary tree was evaluated using axial, multiplanar reformation, maximum intensity projection, and cross-sectional images (slice thickness 0.9 mm, increment 0.50 mm). Centerlines and vessel contours were automatically reconstructed, with the possibility of manual corrections. Visual assessment of the presence of adverse plaque characteristics was assessed, that is, low-attenuation plaque, positive remodeling, spotty calcification, and napkin ring sign. Vulnerable plaques were defined by the presence of ≥2 adverse plaque characteristics. Individuals are represented as gray dots, and the whiskerplot represents the mean with minimum to maximum. Groups were compared by Student *t* test.

of NF-κB (nuclear factor κB), causing impaired adhesion and influx of monocytes.[8] In the current study, we showed that Netrin-1 is negatively correlated with arterial wall inflammation, quantified by ^{18}F-fluorodeoxyglucose PET/CT.[19] The enhanced inflammatory activity in the arterial wall in patients with CVD is widely considered to be caused by continuous influx of circulating monocytes.[29] One of the crucial steps in the process of atherosclerosis is the migration of monocytes over the endothelial layer. Previous in vitro studies showed that Netrin-1 reduces leukocyte migration and recruitment into the atherosclerotic plaque by inhibiting adhesion of monocytes to the vessel wall.[10] We hypothesize that, as a consequence of lower Netrin-1 plasma levels, the inhibitory effect of Netrin-1 on monocyte migration decreases, which subsequently results in accelerated atherosclerotic plaque formation (Figure 6). This hypothesis is supported by the results of an in vivo study by Passacquale et al,[12] who treated *ApoE*$^{-/-}$ mice who were on a high-fat diet for 8 weeks, with intravenous either recombinant Netrin-1 receptor UNC5B, which blocks Netrin-1 activity, or a control protein 1 hour before infusing labeled monocytes. After 36 hours, an increased accumulation of monocytes was observed in the brachiocephalic artery in the mice treated with the blocking protein compared with the control mice,[12] clearly showing an in vivo effect of Netrin-1 on arterial wall inflammation. In line with this research, we observed the same effect on monocyte adhesion when endothelial cells were stimulated with TNFα and plasma of patients with either high or low Netrin-1 concentration. In patients with a high Netrin-1 concentration, TNFα-induced attachment of monocytes to the endothelium was prevented, but when endothelial cells were exposed to TNFα and plasma with low concentrations of Netrin-1, this inhibitory effect could not be observed.

Lower Netrin-1 Plasma Levels in Subjects With Coronary Calcified Atherosclerotic Lesions

In accordance with the inverse correlation between Netrin-1 and arterial wall inflammation, we observed a negative association between Netrin-1 plasma levels and the presence of subclinical atherosclerosis. This is in line with a previous article by Muñoz et al[16] that reported the negative correlation of Netrin-1 with subclinical atherosclerosis. When establishing our asymptomatic high-risk cohort, we excluded smokers and patients with diabetes mellitus. In 2016, both Kızmaz et al[21] and Ay et al[22] reported that plasma

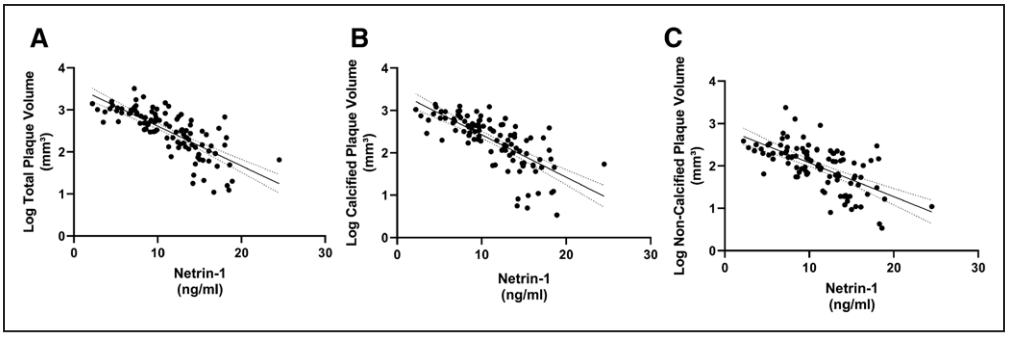

Figure 4. The correlation between Netrin-1 plasma levels and plaque burden.
In subjects with chest pain and undergoing a coronary computed tomography (N=104) there was a significant negative correlation between Netrin-1 plasma levels, measured by ELISA, and (**A**) total plaque volume (β, −0.09 [95% CI, −0.11 to −0.08] R^2, 0.57; $P<0.0001$), (**B**) calcified plaque volume (β, −0.10 [95% CI, −0.12 to −0.08] R^2, 0.53; $P<0.0001$), and (**C**) noncalcified plaque volume (β, −0.08 [95% CI, −0.10 to −0.06] R^2, 0.41; $P<0.0001$). Normality was examined by means of inspection of histograms, and correlation was assessed with linear regression. Total plaque volumes were calculated per patient by summing the lengths and volumes of separate plaques along the entire coronary tree. A scanner-specific threshold of 150 HU was used for distinguishing noncalcified from calcified plaque components, which subsequently was used to determine noncalcified and calcified plaque volumes. Individuals are represented as black dots, and the best-fitted line is displayed with 95% confidence bands.

Netrin-1 levels significantly increase in smokers and in patients with diabetic nephropathy. In the study by Muñoz et al,[16] smokers and patients with diabetes mellitus were included, and the proportion of smokers was significantly higher in the group with subclinical atherosclerosis compared with the group without subclinical atherosclerosis. This may have led to a potential spurious association, as smoking and diabetes mellitus could (per se) have resulted in both increased Netrin-1 levels and CVD risk.

We excluded smokers and patients with diabetes mellitus from our analysis for this reason and showed a similar result in an independent cohort, which can be regarded as a confirmation that Netrin-1 indeed is

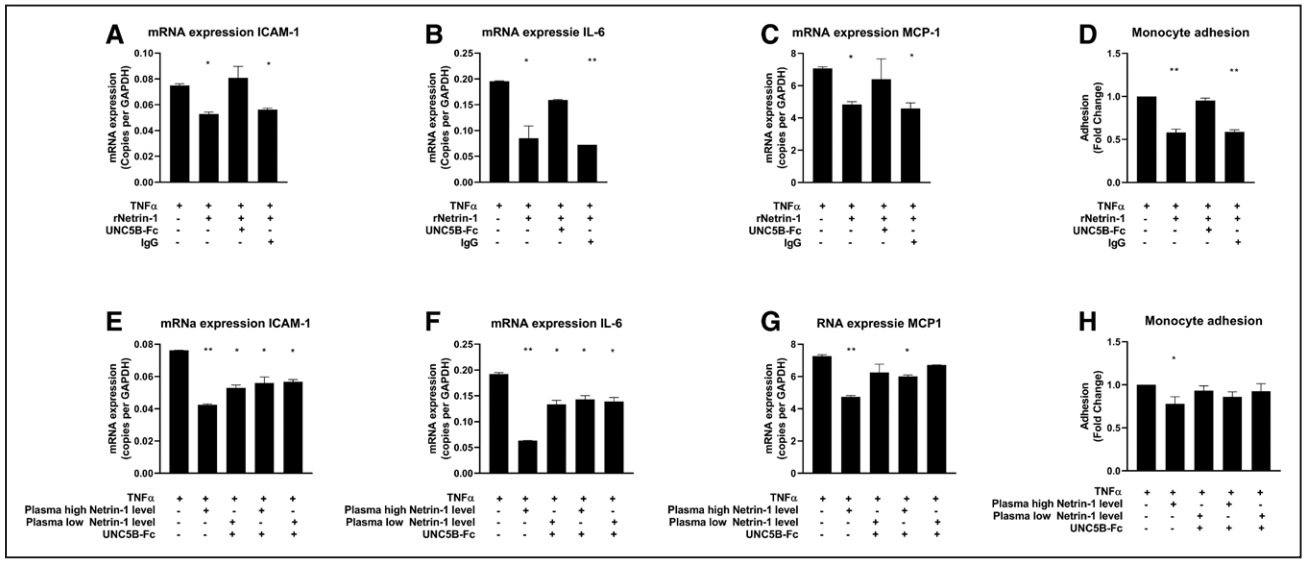

Figure 5. Netrin-1 prevents TNFα (tumor necrosis factor α)-induced attachment of monocytes to endothelial cells.
A–**C**, Quantitative polymerase chain reaction (PCR) analysis of ICAM-1 (intercellular adhesion molecule 1; **A**), IL (interleukin)-6 (**B**), or MCP (monocyte chemoattractant protein)-1 (**C**) mRNA in human umbilical vein endothelial cells (HUVECs) stimulated with TNFα (10 ng/mL) with or without recombinant Netrin-1 (500 ng/mL) treatment in the presence of UNC5B (unc-5 netrin receptor B) fused to the Fc portion of human immunoglobulin G1 (UNC5B-Fc) or IgG (control-Fc) for 24 h. Expression is presented as copies per GAPDH. **D**, Adhesion of THP1 (human monocytic cell line) monocytes to TNFα stimulated HUVECs with or without recombinant Netrin-1 (500 ng/mL) treatment, in the presence of UNC5B-Fc or IgG. Data presented relative to results with TNFα only stimulated cells, set as 1. **E**–**G**, Quantitative PCR analysis of ICAM-1 (**E**), IL-6 (**F**) or MCP-1 (**G**) mRNA in HUVECs stimulated with TNFα (10 ng/mL) with or without plasma of patients containing either high or low levels of Netrin-1 for 24 h. UNC5B-Fc or IgG was added to the plasma 30 min before addition of the plasma to the endothelial cells. Expression is presented as copies per GAPDH. **H**, Adhesion of THP1 monocytes to TNFα (10 ng/mL) stimulated HUVECs with or without plasma of patients containing either high (25.0–35.4 ng/mL) or low (3.1–12.3 ng/mL) levels of Netrin-1 for 24 h. UNC5B-Fc or IgG was added to the plasma 30 min before addition to the endothelial cells. Data presented relative to results with TNFα only stimulated cells, set as 1. **A**–**H**, Data are the mean±SEM, N=4. *$P<0.05$ compared with TNFα stimulated cells. **$P<0.001$ compared with TNFα stimulated cells.

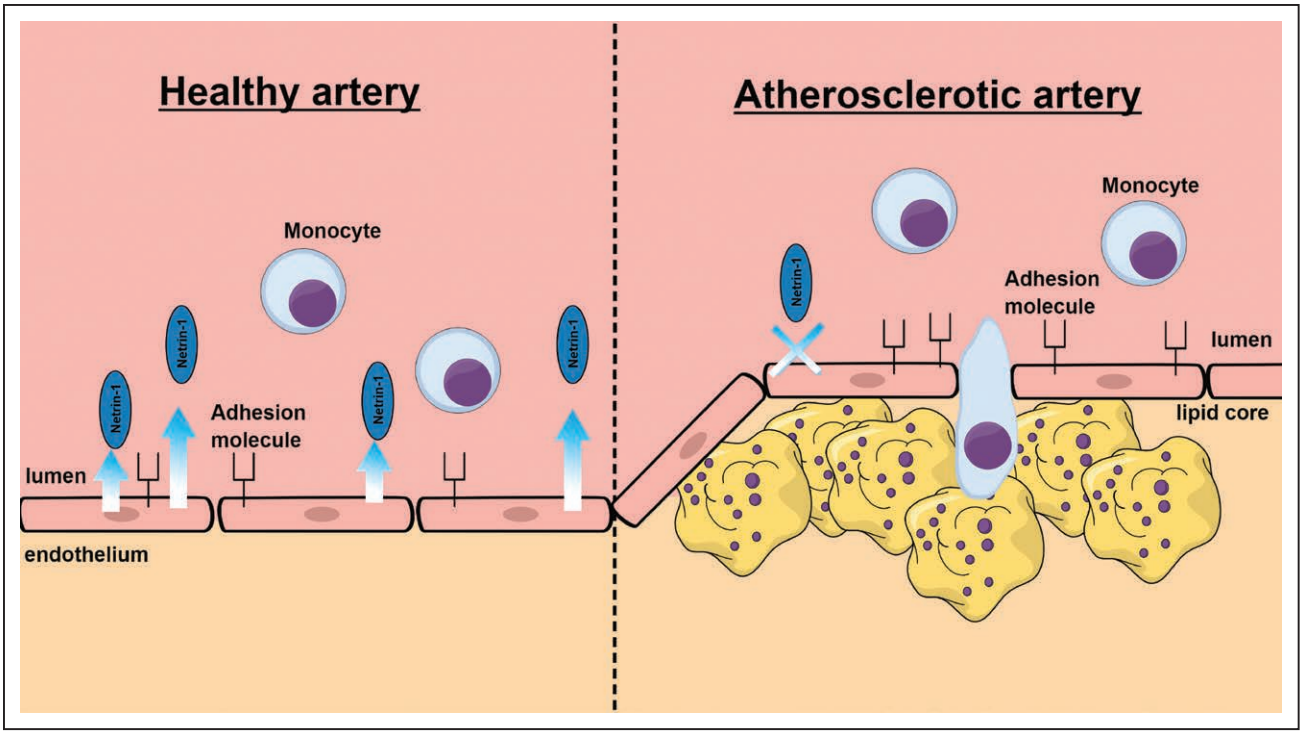

Figure 6. Graphic representation of Netrin-1 in the circulation and its effect on atherosclerosis development.

related to atherosclerosis, independent of 2 crucial risk factors (smoking and diabetes mellitus).

A second difference between the cohort of Muñoz et al[16] and our asymptomatic high-risk cohort is the level of risk for CVD. The cohort of Muñoz et al[16] comprised individuals who underwent a CT scan for noncardiovascular related reasons. Therefore, this cohort has a low a priori chance of coronary atherosclerosis. The individuals included in our cohort were selected based on the fact that they all had a first degree relative with premature atherosclerosis. As premature CVD is associated with substantially greater heritability than CVD at advanced age, this created a cardiovascular cohort we deem to be classified as substantially increased risk compared with the cohort enrolled in the study by Muñoz et al.[16] As plasma Netrin-1 is most likely produced by the endothelium,[14] our data implies that when atherosclerosis progresses, Netrin-1 expression by the (inflamed) endothelium is decreased, which leads to decreased Netrin-1 plasma concentrations. As discussed, the decrease in Netrin-1 could enhance monocyte recruitment into the vessel wall and accelerate the progression of atherosclerosis. A subanalysis in the cohort with asymptomatic high-risk individuals revealed that whereas Netrin-1 plasma levels were significantly lower in subjects with subclinical atherosclerosis, CRP was not different in patients with and without subclinical atherosclerosis. The absence of a difference in CRP levels between these groups is in line with data derived in the Dallas Heart Study, where CRP levels were also not found to be correlated with the extent of subclinical atherosclerosis.[30] The fact, however, that CRP levels add value to cardiovascular risk prediction algorithm, as observed in >40 large epidemiological studies,[31] suggests that Netrin-1 may also add value in future CVD event prediction tools. Measurement of plasma Netrin-1 levels in large prospective studies is warranted to address his question.

Netrin-1 and Plaque Morphology

Plasma levels of Netrin-1 were shown to be negatively correlated with CCTA-derived plaque volume in the current study. This lends further support to the hypothesis that when Netrin-1 excretion by the endothelium decreases, resulting in a lower plasma concentration in more advanced stages of atherosclerosis.

We anticipated that Netrin-1 levels would also lower in patients with vulnerable plaques, compared with patients with nonvulnerable plaques, as inflammation has been shown to have an impact on plaque stability.[32] In our study, however, we observed no differences in plasma Netrin-1 levels between patients with stable and vulnerable plaques, and Netrin-1 plasma levels thus seem not to be a suitable biomarker to distinguish between stable and unstable plaques. However, despite the clear predictive value of high-risk atherosclerotic lesion morphology for the occurrence of cardiovascular events, clinical studies demonstrated that not all plaque classified as being high risk of vulnerable actually cause clinical events,[33] which indicates that the currently used imaging classification is probably not optimal (yet). Given recent advances in noninvasive imaging of vulnerable plaques

using ^{18}F-sodium fluoride PET/CT and CCTA-derived pericoronary adipose tissue attenuation, further studies are warranted to investigate the predictive value of Netrin-1 for the presence of high-risk plaque using these novel techniques.

Limitations

Several limitations should be taken into account when interpreting the results of the current study. First, with the recent development of proximity extension assays, the simultaneous measurement of large numbers of proteins enables the use of proteomics in large clinical cohorts. Therefore, it seems vain to measure only one protein, as we did in the current study. However, a broad panel of neuroimmune guidance cues (let alone Netrin-1) has hitherto never been part of such assays. Other factors (such as MCP-1 and ICAM-1) are part of these assays. In our current study, we embark on the wide knowledge about the role of these chemotaxis-related proteins and specifically address the role of Netrin-1 in this pathway. The reason to focus on Netrin-1 was driven by observations in different models, supporting a role for Netrin-1 in atherosclerosis. In the current study, we have generated in vivo human data to suggest that indeed, Netrin-1 should be considered part of this broad panel, as we have clearly shown that plasma levels are associated in different stages of atherosclerosis. In future studies, we hope to be able to substantiate these findings using novel, integrated biomarker panels.

Second, the Netrin-1 plasma levels measured in our cohorts were higher than plasma levels published in other studies where Netrin-1 levels were measured with commercially available ELISAs. Notably, in some of the reported studies, plasma levels were reported that fall below the detection limit.[21,22] For the current study, we initially used the same assays. However, we measured Netrin-1 levels of 0 to 125 pg/mL, even when recombinant Netrin-1 was added in concentrations up to 2000 pg/mL, and we, therefore, produced an ELISA in house. The levels we measured with our ELISA are in line with the physiological and functional concentrations of Netrin-1 that were reported to vary between 50 and 150 ng/mL.[34–36] Our results were obtained in an observational study, and inferences about the causality and effect of Netrin-1 increase on atherosclerosis cannot be made. Although it is tempting to speculate about the potential role of Netrin-1 as a therapeutic target in the early stages of atherosclerotic disease, we stress that our studies preclude us from making any estimate about the likelihood that this scenario would result in cardiovascular risk reduction.

Lastly, it has been reported that Netrin-1 levels significantly increase in smokers and in patients with diabetic nephropathy.[21,22] We therefore excluded all those patients from our cohorts. In addition, it has been shown in both mice and human studies that acetylsalicylic acid increases Netrin-1 production by endothelial cells.[15] The cohorts used in the current study contained many patients with established CVD which invariably leads to inclusion of patients on these cohorts. However, if we would have been able to correct for the use of acetylsalicylic acid, we would probably have found an even greater difference in Netrin-1 levels between patients with and without CVD.

Conclusions

The current study investigated the relationship between Netrin-1 levels and atherosclerosis, and we show that Netrin-1 plasma levels are lower in patients with subclinical atherosclerosis. Moreover, plasma levels of Netrin-1 are correlated with the extent of arterial wall inflammation and plaque burden. As arterial wall inflammation and plaque burden are directly associated with CVD risk, these data lend supports for the hypothesis that Netrin-1 plays a role in atherosclerosis initiation and progression in humans. Additional studies are warranted to elucidate the exact role of Netrin-1 in human atherosclerosis.

ARTICLE INFORMATION

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Disclosures

G.K. Hovingh reports that his institution has received lecturing fees and advisory board from Regeneron, Pfizer, MSD, Sanofi, and Amgen. Until April 2019, G.V. Hovingh has served as Principal Investigator for clinical trials conducted with a.o. Amgen, Sanofi, Eli Lilly, Novartis, Kowa, Genzyme, Cerenis, Pfizer, Dezima, Astra Zeneca, and the institution received the investigational fees. Since April 2019, G.K. Hovingh is partly employed by Novo Nordisk and the Amsterdam UMC. E.S.G. Stroes reports that his institution has received lecturing fees and advisory board fees from Amgen, Inc, Regeneron, Sanofi, Akcea, Novartis and Athera. The other authors report no conflicts.

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