Neuroimmune guidance cues in vascular (patho)physiology

Vreeken, D.

Chapter 6

The identification and function of a Netrin-1 mutation in a pedigree with premature atherosclerosis

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Abstract

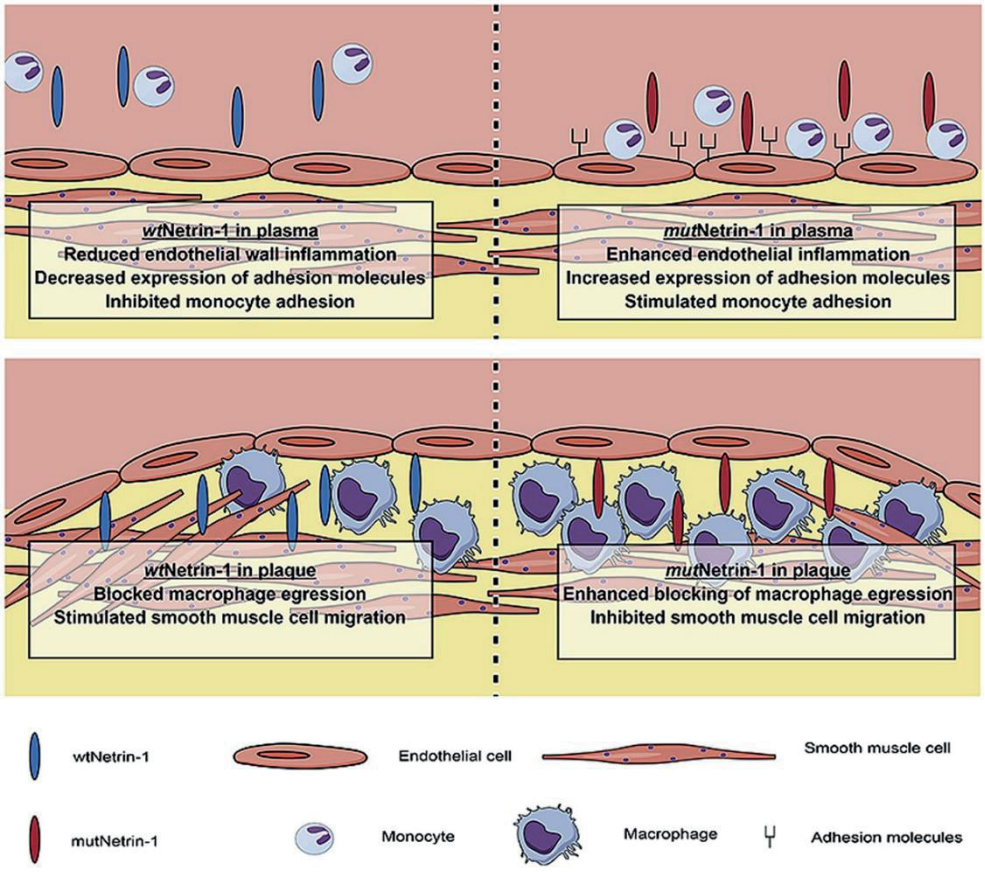
Background and aims: Neuroimmune guidance cues have been shown to play a role in atherosclerosis, but their exact role in human pathophysiology is largely unknown. In the current study, we investigated the role of a c.1769G > T variant in Netrin-1 in (premature) atherosclerosis.

Methods: To determine the effect of the genetic variation, purified Netrin-1, either wild type (wtNetrin-1) or the patient observed variation (mutNetrin-1), was used for migration, adhesion, endothelial barrier function and bindings assays. Expression of adhesion molecules and transcription proteins was analyzed by RT-PCR, Western blot or ELISA. To further delineate how mutNetrin-1 mediates its effect on cell migration, lenti-viral knockdown of UNC5B or DCC was used.

Results: Bindings assays revealed a decreased binding capacity of mutNetrin-1 to the receptors UNC5B, DCC and β3-integrin and an increased binding capacity to neogenin, heparin and heparan sulfate compared to wtNetrin-1. Exposure of endothelial cells to mutNetrin-1 resulted in enhanced monocyte adhesion and expression of IL-6, CCL2 and ICAM-1 compared to wtNetrin-1. In addition, mutNetrin-1 lacks the inhibitory effect on the NF-κB pathway that is observed for wtNetrin-1. Moreover, the presence of mutNetrin-1 diminished migration of macrophages and smooth muscle cells. Importantly, UNC5B or DCC specific knockdown showed that mutNetrin-1 is unable to act through DCC resulting in enhanced inhibition of migration.

Conclusions: Our data demonstrates that mutNetrin-1 fails to exert anti-inflammatory effects on endothelial cells and more strongly blocks macrophage migration compared to wtNetrin-1, suggesting that the carriers of this genetic molecular variant may well be at risk for premature atherosclerosis.

Graphical abstract



1. Introduction

Despite improvements in percutaneous coronary intervention and drug treatment, cardiovascular disease (CVD) remains a leading cause of death in economically developed countries (1). Atherosclerosis, characterized by the accumulation of inflammatory cells in the vascular wall (2, 3), is a leading underlying pathophysiological substrate for CVD events and develops secondary to a state of chronic systemic inflammation (4). The exact dynamics of this inflammatory process are unknown. In recent years, it became increasingly clear that members of the Netrin, Semaphorin, Ephrin and Slit families of neuroimmune guidance cues, proteins known from directing cell and axon migration during neural development, also play a central role in (pathological) immune responses, including atherosclerosis in mouse models (5, 6, 7, 8). For CVD, Netrin-1 has been shown to play an important role in atherosclerosis and ischemia-reperfusion injury by acting as a cardioprotective agent (9, 10, 11, 12). Netrin-1 expression is increased by atheroprotective laminar flow, while decreased by inflammatory cytokines (7, 13). An important known anti-atherogenic function of Netrin-1 is its anti-inflammatory action on the endothelium reducing the adhesion and migration of monocytes (7, 11, 13, 14). In contrast, Netrin-1 produced by plaque-resident macrophages can lead to the retention of macrophages in atherosclerotic plaques, elucidating also an atheroprone function for Netrin-1 (9).

Netrin-1 acts through a repertoire of receptors, including deleted in colorectal cancer (DCC), neogenin, and the UNC5 family. The amino-terminal domains V and VI of Netrin-1 are homologous to the laminin amino-terminal domains, and bind to DCC, neogenin and UNC5 receptors (15, 16). The remaining C-domain of Netrin-1 is known as the Netrin-like (NTR) domain (15, 16). The functional significance of the NTR module in Netrin-1 is largely unidentified. Upon whole exome sequencing in a pedigree comprising 2 generations of 7 family members who suffered from premature atherosclerosis, we found a rare variant located in the *NTN1* gene, c.G1769T (p.R590L). Using multiple functional assays, we demonstrated pro-atherosclerotic functional consequences for this variation in Netrin-1.

2. Materials and methods

Detailed materials and methods are provided in the Supplementary Materials.

2.1 Patient characterization

The index case in our study is a male patient who suffered from a myocardial infarction at the age of 30 years. He and his family members were referred to the outpatient clinic of the Amsterdam UMC, Academic Medical Centre for evaluation of CVD risk factors.

Blood was obtained and all family members without a medical history of CVD were invited to undergo a CT-scan of the coronary arteries to assess the extent of coronary artery calcification. The study is in compliance with the Declaration of Helsinki and the protocol was approved by the Institutional Review Board of the Amsterdam UMC, location Academic Medical Centre (METC-2004_236). All participants provided written informed consent.

2.2 Exome sequencing and mutation analysis

Genomic DNA extraction, whole exome sequencing and candidate variant selection were done as previously described (17). Based on a PubMed search, variants were appointed as being athero-associated or not (Supplemental Table 1). The *NTN1* variant was confirmed in other family members by Sanger sequencing (18).

2.3 Netrin-1 protein purification

Using the Q5 site-directed mutagenesis kit, a plasmid containing the c.1769G > T variant was generated from a plasmid containing wild type HIS-tagged human *Netrin-1*. The wild type variant (*wt*Netrin-1) or the p.R590L variant of Netrin-1 (*mut*Netrin-1) was collected from supernatants of HEK293F cells (19).

2.4 Simple Western protein analysis

Protein quantification from lysed cells was performed with Simple Western according to manufacturer's instructions.

2.5 Real time PCR

RT-PCR analysis was conducted using SYBR Select Master Mix and the forward and reverse primers as indicated in Supplemental Table 2. mRNA expression is normalized to *GAPDH*.

2.6 ELISA

C–C motif chemokine ligand 2 (CCL2) and interleukin-6 (IL-6) levels were measured by enzyme linked immunosorbent assay according to the manufacturer's instructions.

2.7 Primary cells, cell lines and media

2.7.1 Human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells were isolated from human umbilical cords obtained from the Leiden University Medical Center, with informed consent, and

collection and processing of the umbilical cord were performed anonymously. Cells were grown and cultured in EGM2 on gelatine coated surfaces.

2.7.2 Macrophages

Freshly isolated $CD14^+$ peripheral blood mononuclear cells were cultured in RPMI 1640, supplemented with FCS, L-glutamine, antibiotics and M-CSF to differentiate them to a macrophage phenotype.

2.7.3 Smooth muscle cells

Human internal thoracic C6 cells were isolated from fragments of human internal thoracic artery as described previously (20). Cells were grown in M199 supplemented with FCS.

2.7.4 THP1 cells

THP1 cells were obtained from ATCC. Cells were cultured in RPMI 1640 medium supplemented with FCS, antibiotics, L-glutamine and β-mercaptoethanol. For UNC5B and DCC knockdown shRNA against the coding region of UNC5B or DCC respectively, was used. As a control scrambled shRNA was used.

2.8 Bindings assay

UNC5B, DCC, NEO1, ITGB1, ITGB3, heparin or heparan sulfate coated plates were incubated with *wt*Netrin-1 or *mut*Netrin-1, followed by an anti-HIS antibody incubation. HRP conjugated secondary antibody was added for 1 h after which TMB solution was added. The reaction was stopped with H_2SO_4 and read at 450 nM.

2.9 Trans-endothelial electrical resistance measurement

Endothelial barrier function analysis was performed with impedance-based cell monitoring using the electric cell-substrate impedance sensing system (ECIS) (21).

2.10 THP-1 adhesion to endothelial cells

A confluent monolayer of endothelial cells (ECs) was stimulated with TNFα and/or *wt*/*mut*Netrin-1 for 24 h. Labelled THP1 cells were incubated on top of the ECs and adhering cells were quantified by measuring fluorescence.

2.11 Migration assay

Chemotaxis of human macrophages or THP1 cells was measured using Boyden chambers. C–C chemokine ligand 5 (CCL5) for macrophages, CCL2 for THP1 cells and/or different concentrations of *wt*/*mut*Netrin-1 were added to the lower chamber. After 16 or 4 h, migrated cells were resuspended and quantified by cell count.

Migration of SMCs was determined using Ibidi culture inserts. Medium enriched with *wt*/*mut*Netrin-1 was added and migration was monitored over time. Migration was quantified by measuring the gap size at different time points.

2.12 Statistical analyses

All data is presented as mean ± SEM or SD and was analyzed with unpaired two-tailed t-tests for two groups or with ANOVA and *post-hoc* t-tests for multiple groups. All statistical analysis were performed with SPSS version 24 or Graphpad Prism 8.

3. Results

3.1 Family with premature atherosclerosis

The index case (Fig. 1A, II5) was a non-diabetic male patient who suffered from an acute myocardial infarction (AMI) at 30 years of age. The patient was a smoker (7 pack/years), whose plasma cholesterol levels were within normal range, did not suffer from hypertension and had a BMI of 27.7 kg/m^2 (Supplemental Table 3). The notion that the patient suffered from an AMI at young age in the absence of relative abundance of classical risk factors, was a reason for referral to the outpatient clinic of the Amsterdam UMC, for further analysis of the cardiovascular status of his family. The mother of the index case (I2) had suffered from premature atherosclerosis, with a myocardial infarction at the age of 53 years. A coronary artery calcium CT scan was performed in an asymptomatic brother (II2), which showed a score at the 93rd percentile for age and sex, while the calcium score was zero in the three sisters (II1, II3-4). Exome sequencing in the index case revealed no mutations in any of the known genes associated with atherosclerosis (*LDL-R, ApoB* or *PCSK9*). However, 20 protein altering variants were revealed with a minor allele frequency (MAF) < 0.05 and a combined annotation dependent depletion (CADD) score >30 (variants shown in Supplemental Table 2). Of these, only the *NTN1* (Netrin-1) gene had been described to be associated with atherosclerosis. Both the brother (II2) and his younger sister (II4) were found to be heterozygous carriers of the same variant in *NTN1* (Fig. 1A/B, Supplemental Table 3).

3.2 The p.Arg590Leu variant has altered binding capacity

The c.G1769T variant was not annotated in any of the public available genomic datasets. Using the CADD tool (22), this c.G1769T/p.Arg590Leu variant is predicted to be highly deleterious, with a score of 34 (ranging: 1–40). The arginine on position 590, which is a highly conserved positively charged amino acid, is thereby replaced by the hydrophobic amino acid leucine. This variant is located in the NTR domain (Fig. 1B).

Since the *NTN1*: c.G1769T variant is likely to have a deleterious effect, we hypothesize that this variant contributes to the premature atherosclerosis phenotype in this family.
In order to investigate the functional impact of the p.Arg590Leu Netrin-1 variant, wild-type Netrin-1 protein (*wt*Netrin-1) and the mutated Netrin-1 protein (*mut*Netrin-1) were purified (Supplemental Fig. 1).
First, we assessed the binding of *wt*Netrin-1 and *mut*Netrin-1 to the various binding molecules of Netrin-1. Compared to *wt*Netrin-1, binding of *mut*Netrin-1 to the receptor neogenin was increased by 2-fold and binding to the receptors UNC5B and DCC was significantly reduced by 50% (Fig. 1C). *mut*Netrin-1 did not bind differently to integrin beta chain beta 1 (ITGB1), but the binding capacity to integrin beta chain beta 3 (ITGB3) was reduced with 80% compared to *wt*Netrin-1 (Fig. 1D). Furthermore, binding of *mut*Netrin-1 to both heparin (2-fold) and heparan sulfate (3-fold) was increased compared to *wt*Netrin-1 (Fig. 1E).

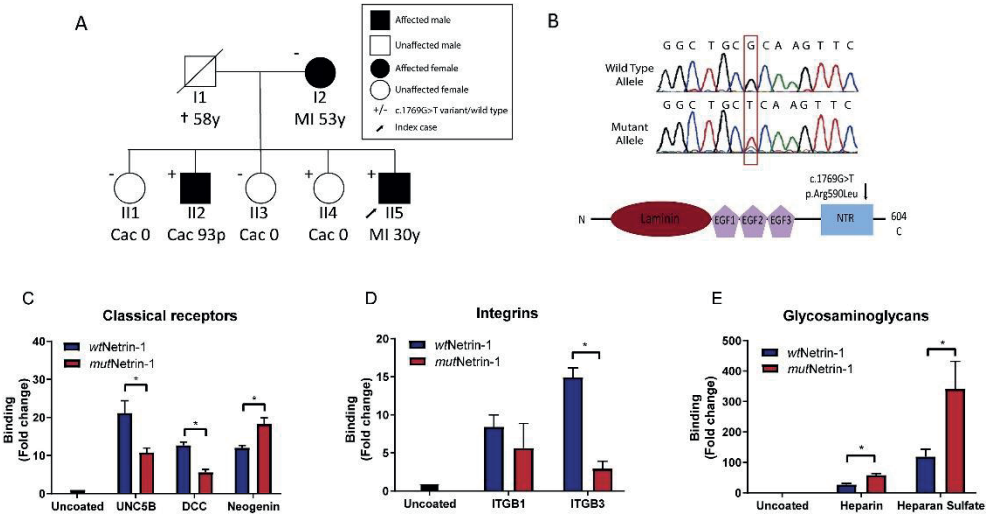


Figure 1 Identification of c.1769G > T variation in Netrin-1 and the effect on binding capacity. (A) Pedigree with premature atherosclerosis. The Arabic number identifies each individual, whereas the generation is marked with a roman number. MI = myocardial infarction, CAC = coronary calcium score. (B) Sanger sequencing chromatogram showing the heterozygote c.G1769T NTN1 variant and a schematic overview of the Netrin-1 protein. (C–E) Binding of wt/mutNetrin-1 to uncoated wells or wells coated with the classical Netrin-1 receptors (C), integrins (D), and glycosaminoglycans (E).

3.3 The p.Arg590Leu variant stimulates monocyte adhesion

Next, we tested the effect of the *wt*Netrin-1 and *mut*Netrin-1 on endothelial barrier function. ECs were seeded on gelatine-coated culture plates with electrodes in the growth area to enable electrical resistance measuring by ECIS (21). Either *wt*Netrin-1

or *mut*Netrin-1 was added in different concentrations to a stable monolayer of ECs. No differences in barrier function of the endothelial monolayer were observed after stimulation with *wt*Netrin-1 or *mut*Netrin-1 protein (Fig. 2A and B).

Acknowledging that Netrin-1 has an anti-inflammatory effect on ECs (13), we measured monocyte adhesion to an endothelial monolayer stimulated with TNFα in combination with *wt*Netrin-1 or *mut*Netrin-1. Addition of TNFα enhances the binding of monocytes by 3.5-fold compared to unstimulated ECs. The binding of monocytes to ECs stimulated with TNFα in combination with *wt*Netrin-1 was decreased by approximately 40% compared to only TNFα, while monocytes adherence decreased by only 15% when ECs were stimulated with *mut*Netrin-1 and TNFα (Fig. 2C and D).

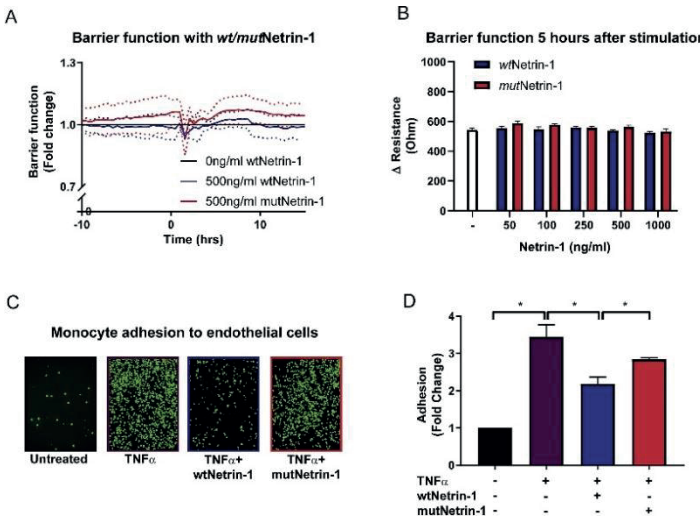


Figure 2 Effect of *mut*Netrin-1 on endothelial function. (A, B) Transendothelial electrical resistance of endothelial cells. At time = 0 cells were treated with wt/mutNetrin-1 (500 ng/ml). The dotted line represents SD of n = 3. (A) Real-time barrier function is presented relative to unstimulated cells, set at 1. (B) Trans-endothelial electrical resistance of endothelial cells 5 h after stimulation. (C, D) Adhesion of labelled monocytes to 24 h unstimulated, TNFα (10 ng/ml) stimulated, or TNFα+wt/mutNetrin-1 (500 ng/ml) stimulated ECs. (C) Representative images of adhered monocytes. (D) Quantification of adhered monocytes. Results are relative to unstimulated cells, set at 1. Mean ± SEM of n = 3, *p < 0.05.

3.4 The p.Arg590Leu variant loses anti-inflammatory effect on endothelial cells

Further analysis of the anti-inflammatory effects on ECs revealed that addition of *wt*Netrin-1 reduced TNFα induced gene expression of intercellular adhesion molecule 1 (*ICAM-1*), *CCL2* and *IL-6* with 30%, 25% and 60% respectively, while the addition of *mut*Netrin-1 did not have this effect, or not as strong (Fig. 3A–C). The effects of Netrin-1 on *ICAM-1, CCL2* and *IL-6* mRNA expression were confirmed at protein level (Fig.

3D–F). Since the activation of the transcription factor NF-κB induces a pro-inflammatory cascade involving the transcription of *ICAM-1, IL-6* and *CCL2* (23, 24, 25) and ultimately promoting human atherosclerosis (26), the regulatory role of *wt*Netrin-1 and *mut*Netrin-1 on the levels of IκBα and NF-κB was tested. Stimulation of ECs with TNFα induced a 3-fold increase of phosphorylated IκBα (Fig. 3G/I), a 75% decrease in total levels of IκBα (Fig. 3G/H) and a 2-fold increase in NF-κB (Fig. 3G/J). The addition of *wt*Netrin-1 during TNFα stimulation suppressed the phosphorylation of IκBα of 10% while, in the meantime, total IκBα degradation was prevented (Fig. 3G–I). The addition of *mut*Netrin-1 could not suppress TNFα activation of the NF-κB pathway as seen for *wt*Netrin-1 (Fig. 3G/J). These results are consistent with the changes in monocyte adhesion and the expression of ICAM-1, CCL2 and IL-6 and propose a diminished anti-inflammatory effect of *mut*Netrin-1 (Fig. 3K).

3.5 Reduced binding to DCC mediates enhanced blocking of directed migration of macrophages

Since Netrin-1 is considered a chemoattractant for smooth muscle cells (SMCs) facilitated by the neogenin receptor (9, 27), we next assessed the effect of *mut*Netrin-1 on SMCs. SMCs stimulated with *wt*Netrin-1 showed a 2-fold increase in migration compared to unstimulated SMCs, while *mut*Netrin-1 was only able to induce migration by 1.5-fold compared to unstimulated cells (Fig. 4A). In addition, Netrin-1 has been shown to block migration of macrophages facilitated by the UNC5B receptor (9). Using the Boyden chambers, we found that chemotaxis of human macrophages towards CCL5 and *wt*Netrin-1 was inhibited with 20% compared to chemotaxis towards just CCL5. Chemotaxis of human macrophages towards CCL5 and *mut*Netrin-1 was inhibited with 70% compared to chemotaxis towards CCL5 alone (Fig. 4B).

We postulated that the receptors UNC5B and DCC mediate the diminished macrophage migration in the presence of *mut*Netrin-1. To assess the role of UNC5B and DCC in this process, the expression in THP1 monocytes was abrogated using shRNAs (Supplemental Fig. 2A). Neither UNC5B nor DCC knockdown affected migration of THP1 cells towards CCL2 (Supplemental Fig. 2B). However, when scrambled-treated THP1 cells migrated towards different concentrations of *wt*Netrin-1, a U-shape could be observed with a maximum inhibition of migration at 250 ng/ml (Fig. 4C, blue). This U-shape was not observed when cells were exposed to *mut*Netrin-1 (Fig. 4D, blue) or in THP1 cells with low expression levels of UNC5B or DCC (Fig. 4C/D, red and purple). DCC knockdown in THP1 cells resulted in a dose dependent inhibition of migration towards both *wt*Netrin-1 and *mut*Netrin-1, while UNC5B knockdown resulted in a modest increase in migration towards *wt*Netrin-1 and no change in migration towards *mut*Netrin-1.

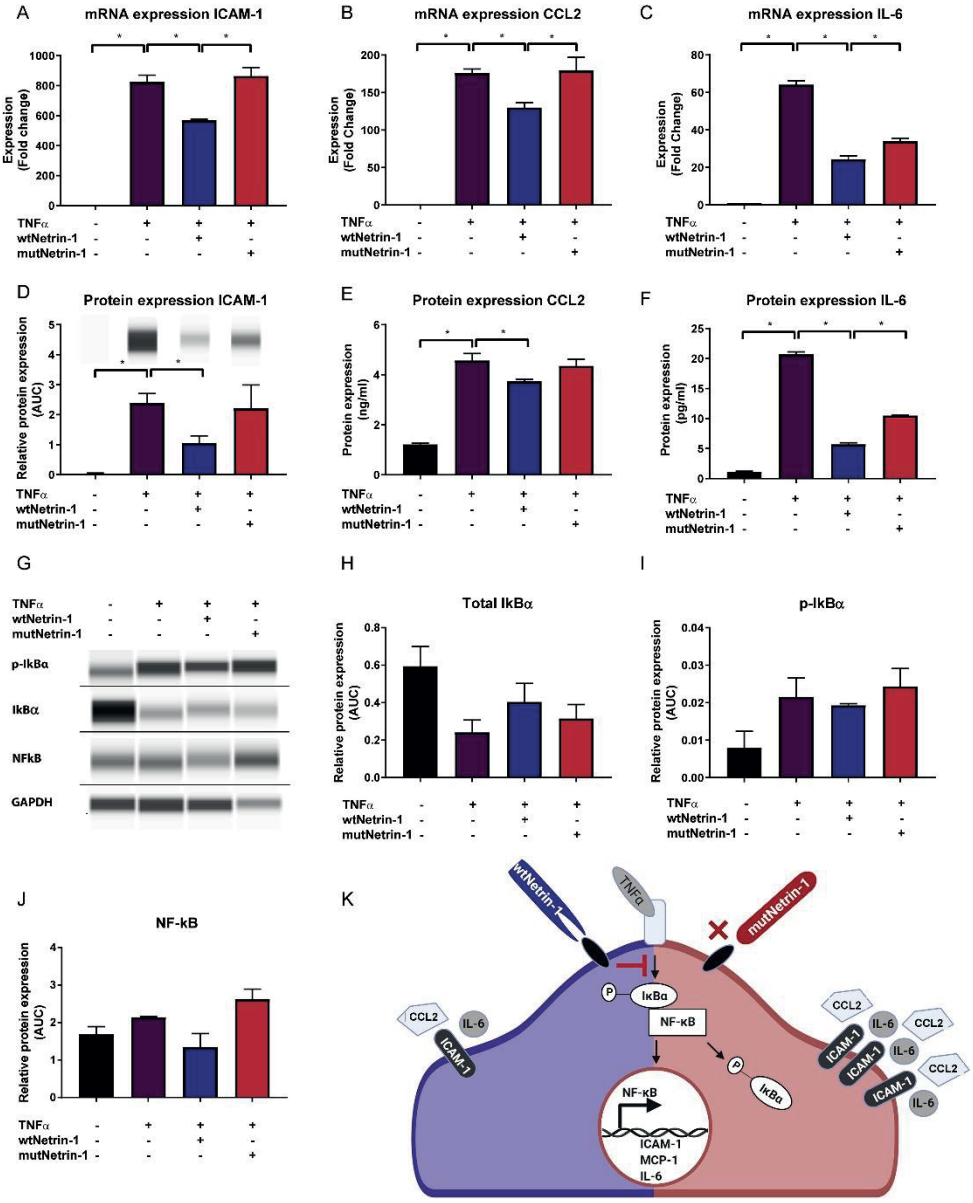


Figure 3 Altered TNFα-induced expression of adhesion molecules and NF-κB pathway activation by Netrin-1. (A–J) Endothelial monolayers were unstimulated or stimulated with 10 or 1 ng/ml TNFα (A-F, G-J respectively), TNFα+500 ng/ml *wt*/*mut*Netrin-1 for 24 or 6 h (A-F, G-J respectively). (A–C) mRNA expression of *ICAM-1* (A), *CCL2* (B) and *IL-6* (C). (D–F) Protein expression of ICAM-1 (D), CCL2 (E) and IL-6 (F) measured with WES™(D) or ELISA (E–F). Results are relative to unstimulated endothelial cells, set at 1. (G–J) Immunoblot analysis of total IκBα (H), p- IκBα (I), NF-κB (J) and GAPDH (G). Results presented relative to GAPDH. Mean ± SEM of n = 3, *$p < 0.05$. (K) Graphical summary of the (anti-)inflammatory effect of *wt*/*mut*Netrin-1.

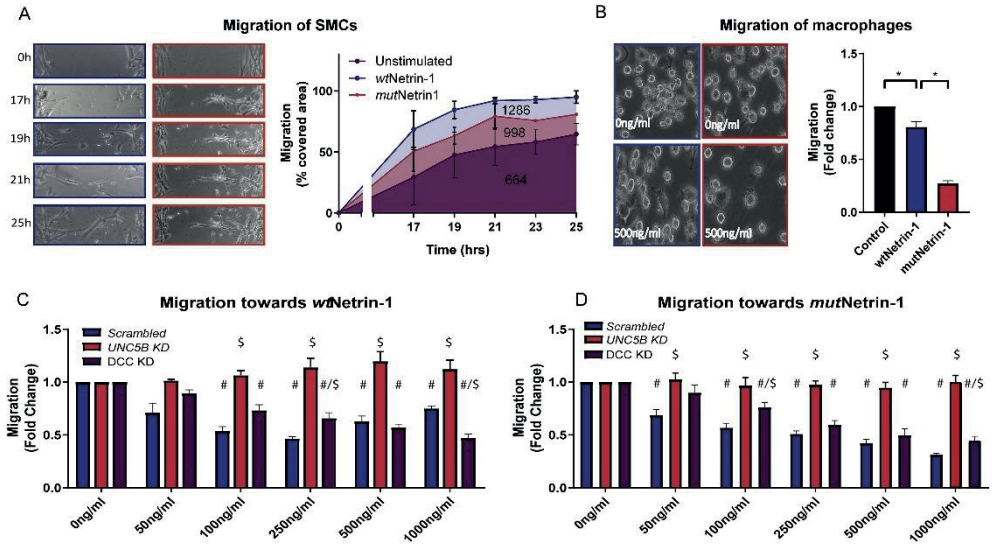


Figure 4 Inhibition of migration with *mut*Netrin-1. (A) Migration of SMCs stimulated with *wt*Netrin-1 or *mut*Netrin-1. Migration is presented as percentage covered area. Mean ± SEM quantification of the AUC is depicted for each curve. (B) Migration of human macrophages towards CCL5 (10 ng/ml) with or without *wt*/*mut*Netrin-1 protein (500 ng/ml). Results are presented relative to migration towards CCL5, set at 1. (C–D) Migration of control, UNC5B or DCC knockdown cells towards CCL2 (10 ng/ml) in the presence of increasing concentrations of *wt*Netrin-1 (C) or *mut*Netrin-1 (D). Results are relative to migration towards CCL2 alone, set at 1. Mean ± SEM of n = 3, **p* < 0.05, #*p* < 0.05 significantly different from 0 ng/ml *wt*/*mut*Netrin-1, $ *p* < 0.05 significantly different from scrabbled control cells.

4. Discussion

In the current study, we show that a genetic variant in Netrin-1 (*NTN1*), identified in a family with unexplained premature atherosclerosis, impacts on different aspects of the atherosclerotic process. In line with previous data derived from animal models, our data supports the hypothesis that Netrin-1 plays a role in atherogenesis (9, 10). Using *in vitro* models, we have revealed that the patient variant of Netrin-1 acquired altered receptor-binding properties and reduced anti-inflammatory properties resulting in enhanced monocyte adhesion, diminished SMC migration, and decreased macrophage egression compared to wild type Netrin-1. Together, the patient Netrin-1 variant shows a phenotype that theoretically leads to increased vascular inflammation and reduced plaque stability.

The Netrin-1 genomic variant at position 1769 results in an arginine to leucine amino acid change (*NTN1* c.1769G > T; p.Arg590Leu). This arginine residue is highly conserved among different species, rendering it important for protein structure and function. The variant is located in the protein NTR domain. While this domain does not appear to be

required for axon chemoattraction (16), it does play a role in axon guidance (28), but its exact role is currently unknown (29). The NTR domain in Netrin-1 could also mediate its function by the Arginyl-glycyl-aspartic acid (RGD) motif (29), which mediates integrin binding to Netrin-1 (30). We indeed observe Netrin-1 binding to ITGB1, but also to ITGB3. The Netrin-1 variant binds less to ITGB3, even though the point mutation is not located in the RGD domain. In addition, the NTR domain can bind heparan sulfate proteoglycans, which have been suggested as co-ligands for Netrin-1 (31, 32) and thereby might mediate Netrin-1 signalling. The arginine to leucine substitution in our variant induces a loss of positive charge, but enhances binding to heparin and heparan sulfate. We speculate that the gain of a hydrophobic leucine residue in the *mut*Netrin-1 affects the folding of the protein. The NTR domain is attached with a flexible linker to the rest of the elongated rigid Netrin-1 protein (33). Due to the variant in Netrin-1, the NTR domain might be orientated differently and could thereby affect the function of the other domains in Netrin-1, such as reduced binding to DCC, which can bind at the end of the rigid structure of the Netrin-1 protein just before the linker to the NTR domain (33).

Monocyte trafficking across the arterial wall is an important contributor to arterial wall inflammation (34). Activation of arterial wall ECs, by atherogenic factors such as oxidized lipids, adverse hemodynamic environment, or inflammation, results in NF-κB driven transcription of multiple cytokines and adhesion molecules facilitating the adhesion and migration of monocytes into the arterial wall (35). Consistent with previous studies (13), we observed that *wt*Netrin-1 has an anti-inflammatory effect on the endothelial wall, by reducing activity of the NF-κB pathway and subsequently the expression of CCL2, IL-6 and ICAM-1. Importantly, the mutated variant of Netrin-1 has less of this anti-inflammatory ability. Multiple signalling pathways are indicated to participate in the inhibitory effects of Netrin-1 on the NF-κB cascade. Liu et al. have shown that Netrin-1 can inhibit phosphate oxidase isoform 4 (NOX4), which is upregulated by inflammation and can activate the NF-κB cascade in ECs (36). Another possible mechanism is the upregulation of endothelial nitric oxide synthase (eNOS) activity by Netrin-1 (37), as eNOS is inactivated by inflammation resulting in increased activity of NF-κB in ECs (38). Since inhibition of the NF-κB pathway is mediated by the Netrin-1 receptor UNC5B (13), we speculate that *mut*Netrin-1 cannot sufficiently inhibit the NF-κB pathway due to the diminished binding capacity between *mut*Netrin-1 and UNC5B we have observed.

Netrin-1 in the circulation cells exerts an atheroprotective function, and we have shown that the effect of *mut*Netrin-1 on the endothelial cell layer has a more atheroprone character. It is very interesting that Netrin-1 produced by accumulated macrophages

within the plaque has an opposite effect by inhibiting macrophage efflux from the plaque and supporting chemo-attraction of coronary artery smooth muscle cells (39). As plaque stability is determined by macrophage content and thickness of the SMC containing fibrous cap (40), this makes Netrin-1 within the plaque atheroprone.

Macrophages and SMCs contribute to plaque (in)stability through the secretion of extracellular matrix- degrading proteases and cytotoxic factors. Migration of SMCs and their ability to synthesize collagen within the plaque maintain the integrity of the plaque's fibrous cap (40). Netrin-1 is a chemoattractant for SMCs (9, 41) and we have found that the migration of SMCs is still induced by *mut*Netrin-1, but not as potent as by the wild type protein. Previous studies indicate that the Netrin-1 receptor neogenin mediates the chemoattractive effect of Netrin-1 on SMCs (9, 41). As *mut*Netrin-1 has a higher binding capacity to neogenin than *wt*Netrin-1, the Netrin-1-neogenin interaction cannot explain the effect of Netrin-1 on SMC migration. However, binding capacity of *mut*Netrin-1 is also altered for ITGB3, heparin and heparan sulfate, which can also affect SMC proliferation and migration (42, 43), indicating that probably additional intracellular and/or extracellular signalling pathways are at play here.

Another factor of plaque stability is the amount of macrophages in the atherosclerotic lesion (40). Macrophages within the plaque express Netrin-1, which immobilizes the macrophages and thereby prevents their egression from the plaque (9). In our current study we observed that *mut*Netrin-1 blocks macrophage migration even more than *wt*Netrin-1, suggesting that production of *mut*Netrin-1 within the plaque potently blocks the egression of macrophages, leading to a high macrophage content resulting in larger and unstable plaques.

While previous studies have indicated that the Netrin-1 receptor UNC5B mediates the inhibition on leukocyte migration (7, 9), the observed additional inhibition of *mut*Netrin-1 on macrophage migration cannot be explained by this interaction as we observed a decreased binding affinity of *mut*Netrin-1 for UNC5B. Netrin-1 can induce both attraction or repulsion of cells (44) depending on 1) expression levels of repulsive (DCC and neogenin) and/or attractive receptors (UNC5B), 2) receptor affinity along with Netrin- 1 concentrations and 3) presence of additional intra- or extracellular signalling molecules (45). Here we suggest a role for DCC to counteract the inhibition of macrophage migration by UNC5B. We indeed see that UNC5B mediated the inhibitory effect of Netrin-1 on macrophage migration. However, when Netrin- 1 concentration exceed 250 ng/ml the DCC receptor counteracts this effect. As *mut*Netrin-1 binds less to DCC, this compensatory mechanism on macrophage migration at higher Netrin-1 concentrations is lost thereby reinforcing the immobilization of macrophages (Fig. 5). In addition, as the NTR domain has been shown to be needed for DCC recruitment to

the plasma membrane (46), the altered NTR domain in our variant could add to the persistent inhibitory effect on macrophage migration by the *mut*Netrin-1.

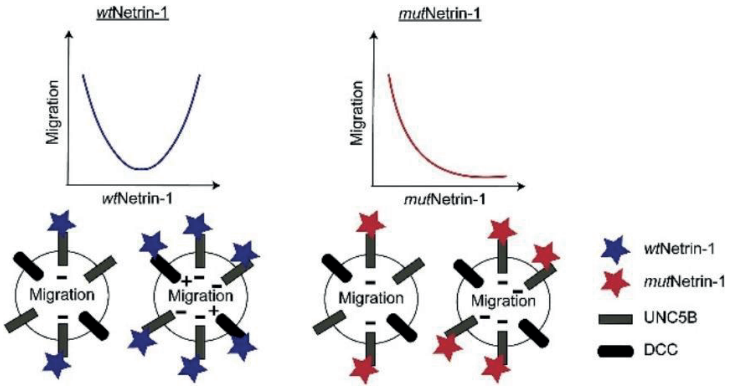


Figure 5 Impact of UNC5B and DCC activation on leukocyte migration. (Top) Graphical representation of the effect of low and high dose of wt/mutNetrin-1 on leukocyte migration. (Bottom) Migration is mediated by the cumulative effect of migration-inhibiting UNC5B and migration-promoting DCC signalling.

4.1 Limitations

The small number of carriers of the variant does preclude us from establishing a firm confirmation on the exact role of the variant in the studied pedigree. Moreover, no other atherosclerosis related variants in the family were shown, nor was this specific variant found in any of the 88 probands with premature atherosclerosis within our medical center. Multiple common variants with an MAF>5% were found to be associated with CVD in genome-wide association studies in recent years (47). In these studies, 10% of the total CVD risk is attributed to common genetic variations, which suggests that low frequency variants are likely to play a role in the so-called 'missed heritability' (48, 49). Family studies have been proven to be extremely instrumental to investigate this and identified culprit genetic defects in families with extreme phenotypes (17, 50, 51). Therefore, we have taken our pedigree as a model to study Netrin-1 in great detail. The identification of the extremely rare variant in Netrin-1, which cannot be found in large CVD databases and the confirmation that the mutated protein promotes monocytes adhesion, blocks smooth muscle cell migration and inhibits macrophage egression does suggest that this variation contributes to the premature atherosclerotic phenotype. Therefore, this study provides novel insights into the importance and mechanisms of Netrin-1 in human atherosclerosis.

4.2 Conclusion

In summary, we have identified a variant in the NTR-domain of Netrin-1 in a family with premature atherosclerosis. The variant results in an inflamed arterial wall leading to increased adhesion of monocytes. Moreover, the p.Arg590Leu variant blocks egression of macrophages and migration of SMCs, resulting in unstable plaques that are more prone to rupture. Our observations confirm previous findings that Netrin-1 plays a role in the initiation and progression of atherosclerosis.

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CRediT authorship contribution statement

Caroline S. Bruikman: Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. Dianne Vreeken: Investigation, Writing - review & editing. Huayu Zhang: Investigation. Marit J. van Gils: Investigation. Jorge Peter: Data curation. Anton Jan van Zonneveld: Funding acquisition, Writing - review & editing. G. Kees Hovingh: Funding acquisition, Supervision, Writing - review & editing. Janine M. van Gils: Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplemental material

Supplemental material and methods

Patient characterization

The index case in our study is a male patient (Fig. 1A) who suffered from a myocardial infarction at the age of 30 years. Because of his positive family history of cardiovascular disease (CVD) and in near absence of traditional risk factors for CVD, he and his family members were referred to the outpatient clinic of the Amsterdam UMC, location Academic Medical Centre. At this outpatient clinic patients and family members are evaluated to identify and, where possible, treat CVD risk factors. Blood was obtained in EDTA containing tubes after an overnight fast. Whole blood was analyzed for levels of total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) and triglycerides according to the local hospital protocol. Plasma was stored at -80 degrees Celsius after centrifuging at 1750xg. The number and severity of CVD risk factors were evaluated in all family members. Hypertension was defined as a systolic blood pressure >140 mmHg, and/or a diastolic blood pressure >90 mmHg or the use of blood pressuring lowering drugs. Diabetes Mellitus was defined as a fasting glucose >7.0 mmol/L and/or the use of antidiabetic medication. All family members over the age of 30, without a medical history of clinical cardiovascular events were invited to undergo low dose CT-scan of the coronary arteries to assess the extent of coronary artery calcification. Coronary CT scans were performed as described previously (1) . The study is in compliance with the Declaration of Helsinki and the protocol was approved by the Institutional Review Board of the Amsterdam UMC, location Academic Medical Centre (METC-2004_236). All participants provided written informed consent.

Exome sequencing and mutation analysis

Genomic DNA was extracted using a Autopure LS system according to the manufacturer's protocol (Gentra Systems, Minneapolis, USA). Whole exome sequencing was performed with the Agilent SureSelect 38Mb exome chip with >100x coverage on the Illumina GAII platform (Illumina, Little Chesterford, UK). For the selection of candidate variants only protein altering variants were included (I.e. missense, nonsense, splice donor or acceptor alterations and frameshift variants causing insertions or deletions). All variants were filtered for a minor allele frequency (MAF) <0.05, based on Exome Variant Server (EVS, https://evs.gs.washington.edu/EVS/), the ExAC database (2) and the Genome of the Netherlands (GO-NL http://www.nlgenome.nl) database. Functional consequences of the selected variants were evaluated with the Combined Annotation Dependent

Depletion (CADD) software tool (3) . Based on a Pubmed search, variants were appointed as being athero-associated or not (Supplemental table 1). The Netrin-1 variant was confirmed in other family members by Sanger sequencing (4).

Netrin-1 protein purification

We generated a plasmid containing the c.1769G>T variant by PCR in a plasmid containing wild type human NTN1 with an HIS-tag (Vector builder, Catalog#: VB171030-1105gzm) using the Q5 site-directed mutagenesis kit (NEB, E0554S). Mutagenesis was done according manufacturer's instructions using the primer pair: forward 5'-CGGCGGCTGCtCAAGTTCCAGC -3' and reverse 5'- CGCCCACGTGTCCCGCCA -3'. The resulting mutated plasmids were verified by Sanger Sequencing. Supernatants of HEK293F cells (Thermo Fisher, R79007) (5) transfected with a plasmid containing either the wild type variant (wtNetrin-1) or the p.R590L variant of Netrin-1 (mutNetrin-1) were collected and used for protein purification. His-tagged Netrin-1 protein was extracted from supernatants by affinity chromatography using a NiCl column and further purified by size exclusion chromatography. Protein concentrations were determined using NanoDrop Spectrophotometer.

Immunoblot analyses

Proteins were denatured using DTT and heating at 95°C for 10 minutes followed by size separation on a 10% Mini-PROTEAN gel (Biorad, 4561033) and transferred to PVDF membranes (Biorad, 1704156), using Trans-Blot Turbo system (Biorad), and blocked in TBST-5% milk or TBST-5% BSA (Sigma, A2058). Primary antibodies against humanNetrin-1 (0.5µg/ml, R&D Systems, AF6419), or the HIStag (1:1000, Cell signaling, 12698S) were incubated overnight at 4 degrees Celsius. Horseradish peroxidase (HRP-) conjugated secondary antibodies (1:1000, R&D, HAF008/HAF016) and Western lighting ECL (PerkinElmer, NEL103001EA) were used to visualize protein bands with the ChemiDoc Touch Imaging System (Biorad).

Real time PCR

Total RNA was isolated from HUVECs using TRIzol and the RNeasy Mini Kit (Qiagen 74106) according to manufacturer's instructions. Total RNA was reverse transcribed using M-MLV Reverse Transcriptase Kit (Promega, M1701). RT-PCR analysis was conducted using SYBR Select Master Mix (Applied Biosystems, 4472908) and the forward and reverse primers as indicated in Supplemental table 2. The PCR cycling conditions were: Initial denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final

extension step at 72°C for 10 minutes. mRNA expression was normalized against expression of GAPDH and expressed as fold change compared to untreated samples.

ELISA

Il-6 and CCL2 levels were measured by enzyme linked immunosorbent assay (ELISA) purchased from PeliPair (Amsterdam, The Netherlands, 2904161453) and R&D systems (Minneapolis, the US, DCP00), respectively, according to manufacturer's instructions.

Primary cells, cell lines and media

Human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords obtained from the Leiden University Medical Center with informed consent and 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. We used Trypsin/EDTA (1x) (Lonza, BE02-007E) solution to detach the endothelial cells from the vein. The cell suspension was collected and taken up in endothelial cell growth medium (EGM2 medium, Promocell C22111) with 1% antibiotics (penicillin/streptomycin, Gibco, 15070063). The cells were pelleted by centrifugation at 1200rpm for 7 minutes and dissolved and maintained in EGM2 medium and cultured on gelatin (1%) coated surfaces.

Macrophages

Peripheral blood mononuclear cells were isolated from buffy coats from 3 individual healthy subjects by density gradient separation using Ficoll. Magnetic separation of CD14 positive monocytes was done with CD14 Microbeads (Miltenyi Biotec, 130-050-201) and LS columns (Miltenyi Biotec, 130-042-401). Isolated cells were kept in RPMI 1640 medium (Gibco, 22409) supplemented with 10% FCS, 1% L-glutamine, 1% antibiotics and 20 ng/ml M-CSF (Miltenyi Biotec, 130-093-963) for 7 days to differentiate them to a macrophage phenotype. For migration experiments macrophages were detached by incubation with cell dissociation reagent (StemPro Accutase A1110501) for 5-10 minutes.

Smooth muscle cells

Human internal thoracic C6 (HITC6) cells were isolated from fragments of human internal thoracic artery as described previously6 . Cells were grown in M199 media supplemented with 10% FCS.

THP1 cells

THP1 cells were obtained from ATCC (THP1 ATCC® TIB-202™) Middlesex, United Kingdom. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1% antibiotics, 1% Lglutamine and 25nM β-mercaptoethanol. To achieve a knockdown of

UNC5B or DCC, THP1 monocytes were transduced with lentiviral particles encoding a shRNA against the coding region of UNC5B, DCC or scrambled (MISSION library Sigma-Aldrich, TRCN0000011158, TRCN0000011160). Selection of transduced cells was achieved using puromycin (2.0 µg/ml). Knockdown was validated for several receptors and Netrin-1 (Supplemental figure 2).

Bindings assay

96-well plates were coated with recombinant protein (UNC5B 180 µg/ml R&D systems, 8869- UN-050, DCC 200 µg/ml R&D systems, 8637-dc, Neogenin 200 µg/ml R&D systems, 9699-ne, ITGB1 20 µg/ml Abnova H00026548-P01, ITGB3 100 µg/ml Abnova H00023421-P01, Heparin 20 µg/ml or Heparan Sulfate 20µg/ml, AMS biotechnology 370255-S) for 7 hours. Wells were then blocked with 2% milk in PBS overnight at 4 degrees Celsius. wtNetrin-1, mutNetrin-1 (200 ng/well in 200µl) were loaded in duplicates and incubated for 1 hour at room temperature, followed by 2 hour incubation with an anti-HIS antibody (1:1000 Cell signaling, 12698S) in blocking buffer at room temperature. HRP-conjugated rabbit anti-HIS IgG (1:1000, R&D systems, HAF016 R&D) in blocking buffer was added for 1 hour at room temperature. Plates were washed 3-5 times with PBS/0.05% Tween after each step. After final washing the plates were incubated with TMB solution (Sigma-Aldrich T4444) for a maximum of 30 minutes. The reaction was stopped with 2N H_2SO_4 and the plate was read at 450 nM using a multi-well plate reader (SPECTRAmax M5, Molecular Devices).

Transendothelial electrical resistance measurement

Endothelial barrier function analysis was performed with impedance-based cell monitoring using the electric cell-substrate impedance sensing system (ECIS Zθ, Applied Biophysics). ECIS plates (96W20idf PET, Applied Biophysics) were pretreated with L-Cystein and coated with 1% gelatin. Endothelial cells were added after a baseline measurement over approximately 1 hour. The multiple frequency/time mode was applied for the real-time assessment of the barrier. When a stable barrier was formed after approximately 24 hours, endothelial cells were stimulated with different concentrations of recombinant wt/mutNetrin-1.

THP-1 adhesion to endothelial cells

Endothelial cells were grown to confluence and stimulated with TNFα (10 ng/ml) and/or wt/mutNetrin-1 (500 ng/ml) for 24 hours. THP1 cells were labelled with 5 µg/ml Calcein AM (Molecular Probes Life Technologies, C3100MP) and incubated on top of the stimulated monolayers of endothelial cells for 30 minutes at 37°C. Non-adhering 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 of adhered cells was measured at λex 485nm and λem 514nm.

Simple Western protein analysis

HUVEC cells were washed in cold PBS and lysed in cold RIPA buffer supplemented with 1 mg/ml protease and phosphatase inhibitor cocktail (Roche). Protein concentrations were determined with the BCA protein assay kit Thermo Scientific, 23225) and equalized. Protein quantification was performed with Simple western (ProteinSimple, WES). Using a 12-230kDa Wes Separation module with 25 capillaries (ProteinSimple, SM-W004), primary antibodies against ICAM-1 (Cell signaling 4915S, 1:500), NF-κB (Cell signaling 8242S, 1:400), IkBα (Cell signaling 4814S, 1:400), pIkBα (Cell signaling 9246S, 1:100) and GAPDH (Novus Biologicals NB300-324, 1:25) and the antirabbit or anti-mouse Detection Module (ProteinSimple, DM-0012 or DM-002) Simple Western was performed according manufacturer's instructions. Expression was normalized to GAPDH.

Migration assay

Chemotaxis of human macrophages and THP1 cells were measured using 24-well Boyden chambers with a 8 μm (macrophages) and 5 μm (monocytes) pore size filter (Corning, 734- 1574/1573). Recombinant RANTES protein for macrophages (CCL5, R&D Systems, 278-RN-010, 10 ng/ml), recombinant monocyte chemoattractant protein-1 for THP1 cells (CCL2, R&D Systems, 279-MC, 10ng/ml) and/or different concentrations of wt/mutNetrin-1 were added to the lower chamber as chemoattractant. After 16 hours (macrophages) or 4 hours (monocytes), cells in the lower chamber were resuspended and counted in randomly selected fields for each well to determine the number of cells that had migrated.

The migration of smooth muscle cells (SMCs) was performed using 2 well culture inserts (Ibidi, 80209). SMCs were cultured inside the wells. After a confluent layer was formed inserts were removed revealing a cell-free gap in which the cell migration can be visualized and measured with a Leica microscope. Medium without FCS, but enriched with wt/mutNetrin-1 (500 ng/ml) was added. Pictures of the wells were taken at different time intervals (0, 17, 19, 21, 23 and 25 hours) to measure closure of the gap. Migration was quantified by measuring the size of the gap at different time points, compared to baseline.

Supplemental tables and figures

Supplemental table 1: Primers used in this paper.

Gene	Forward primer	Reverse primer
Netrin-1	GGGTGCCCTTCCACTTCTAC	GCGAGTTGTCGAAGTCGTG
DCC	AGCCTCATTTTCAGCCACACA	TTCCGCCATGGTTTTTAAATCA
Neogenin	ACCTTCCTCAGTTTATGCTGGG	ACTTTCCACTACGCAGCGATA
UNC5B	CAAGAGTCGCCGAGCCTAC	GCACTGCAGGAGAACCTCAT
UNC5C	CCGCCACCCAGATCTTTTCA	CTTCCCGGACAATGAGACC
DSCAM	TTGCGGTCTTCAAGTGCATTA	TGCAGCGGTAGTTATACATCCA
ICAM-1	GGCCGGCCAGCTTATACAC	TAGACACTTGAGCTCGGGCA
CCL2	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
IL-6	AAGCCAGAGCTGTGCAGATGAGTA	AACAACAATCTGAGGTGCCCATGC
GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG

Supplemental table 2: Variants found in index case after selection based on, MAF (30). SNV: single nucleotide variant.

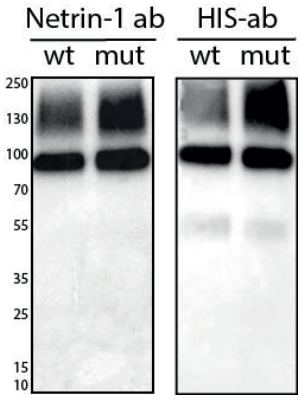
Chr.	Start	Ref	Alt	Gene	Exonic variant function	Gene change	CADD score	Athero related in literature
chr3	190026140	G	A	CLDN1	stopgain	c.C562T	35	No
chr5	140237650	C	T	PCDHA10	stopgain	c.C2017T	35	No
chr5	160061466	G	A	ATP10B	stopgain	c.C1276T	37	No
chr6	43014020	C	T	CUL7	nonsynonymous SNV	c.G2614A	34	No
chr6	143806309	C	T	PEX3	nonsynonymous SNV	c.C962T	34	No
chr7	150761721	G	A	SLC4A2	nonsynonymous SNV	c.G326A	32	No
chr9	18928640	G	A	SAXO1	stopgain	c.C835T	38	No
chr9	116136445	C	T	HDHD3	nonsynonymous SNV	c.G190A	34	No
chr9	140007817	G	A	DPP7	nonsynonymous SNV	c.C617T	32	No
chr11	57087817	C	T	TNKS1BP1	nonsynonymous SNV	c.G464A	33	No
chr11	92569867	C	T	FAT3	nonsynonymous SNV	c.C10222T	35	No
chr13	42385421	C	T	VWA8	nonsynonymous SNV	c.G2003A	34	No
chr14	91700389	C	A	GPR68	stopgain	c.G1006T	37	No
chr16	56377842	C	T	GNAO1	nonsynonymous SNV	c.C1045T	35	No
chr16	81298282	C	T	BCO1	nonsynonymous SNV	c.C509T	32	No
chr16	89598369	G	A	SPG7	nonsynonymous SNV	c.G1045A	31	No
chr17	9143239	G	T	NTN1	nonsynonymous SNV	c.G1769T	34	Yes
chr17	61557841	G	A	ACE	nonsynonymous SNV	c.G799A	31	No
chr19	372680	C	A	THEG	stopgain	c.G586T	37	No
chr19	49113215	G	A	FAM83E	nonsynonymous SNV	c.C676T	35	No

Supplemental table 3: Clinical characteristics of the premature atherosclerosis family.

Patient	Gender	Type of CVD	Age of CVD	Netrin-1	Medi-cation	DM	Hyper-tension	Smo-king	Untreated LDL-c mmol/L	BMI
I2	Female	AMI	53	c.1769G	Simva40	No	No	No	3.47	34.1
II1	Female	None	N/A	c.1769G	No	No	Yes	No	4.07	34.8
II2	Male	CAC93p	43	Heterozygous c.1769G>T	No	No	No	No	3.15	25.6
II3	Female	None	N/A	c.1769G	No	No	No	No	2.87	26.6
II4	Female	None	N/A	Heterozygous c.1769G>T	No	No	No	Yes	1.68	29
II5	Male	AMI	30	Heterozygous c.1769G>T	Simva40	No	No	Yes	3.41	27.7

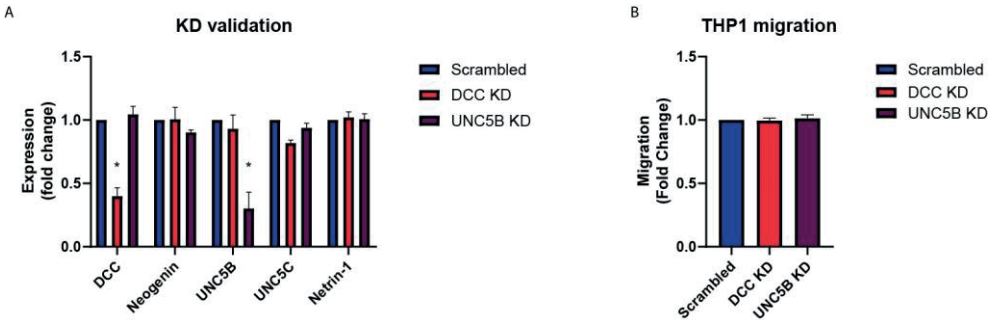
Clinical characteristics of the pedigree with premature atherosclerosis. AMI=acute myocardial infarction, CAC(X)p=coronary calcium score with X the percentile of coronary artery calcification on CT corrected for age and gender, BMI=body mass index (kg/cm2), N/A=not applicable, DM=diabetes mellitus, LDL-c = low-density-lipoprotein cholesterol.

Supplemental Figure 1: Western blot validation for purified wtNetrin-1 and mutNetrin-1 protein.



Immunoblot validation of purified wild type (wt) and mutated (mut) protein with a human Netrin-1 antibody and a HIS antibody.

Supplemental Figure 2: Validation of knockdown.



(A) mRNA expression of DCC, neogenin, UNC5B, UNC5C, and Netrin-1 in THP1 cells treated with a mock shRNA (blue), a shRNA against DCC (red) or a shRNA against UNC5B (purple). Results are relative to mock shRNA, set at 1. Mean ± s.e.m. of n = 3, $p<0.05$. (B) Migration of control, UNC5B knockdown and DCC knockdown cells towards CCL2 represented relative to migration of scrambled cells towards CCL2, set at 1.

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