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

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HIGHLIGHTS

- In a family with premature atherosclerosis a Netrin-1 variant was identified.
- Patient Netrin-1 (c.1769G > T, *mut*Netrin-1) affects atherogenesis on multiple levels.
- *mut*Netrin-1 fails to exert anti-inflammatory effects on endothelial cells.
- *mut*Netrin-1 inhibits smooth muscle cell and macrophage migration compared to *wt*Netrin-1.
- Reduced binding of *mut*Netrin-1 to DCC mediates the extra inhibition on migration.

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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 (*wt*Netrin-1) or the patient observed variation (*mut*Netrin-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 *mut*Netrin-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 *mut*Netrin-1 to the receptors UNC5B, DCC and β3-integrin and an increased binding capacity to neogenin, heparin and heparan sulfate compared to *wt*Netrin-1. Exposure of endothelial cells to *mut*Netrin-1 resulted in enhanced monocyte adhesion and expression of IL-6, CCL2 and ICAM-1 compared to *wt*Netrin-1. In addition, *mut*Netrin-1 lacks the inhibitory effect on the NF-κB pathway that is observed for *wt*Netrin-1. Moreover, the presence of *mut*Netrin-1 diminished migration of macrophages and smooth muscle cells. Importantly, UNC5B or DCC specific knockdown showed that *mut*Netrin-1 is unable to act through DCC resulting in enhanced inhibition of migration.

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

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.

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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 [5–8]. For CVD, Netrin-1 has been shown to play an important role in atherosclerosis and ischemia-reperfusion injury by acting as a cardioprotective agent [9–12]. 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).

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).

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* [23–25] 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-

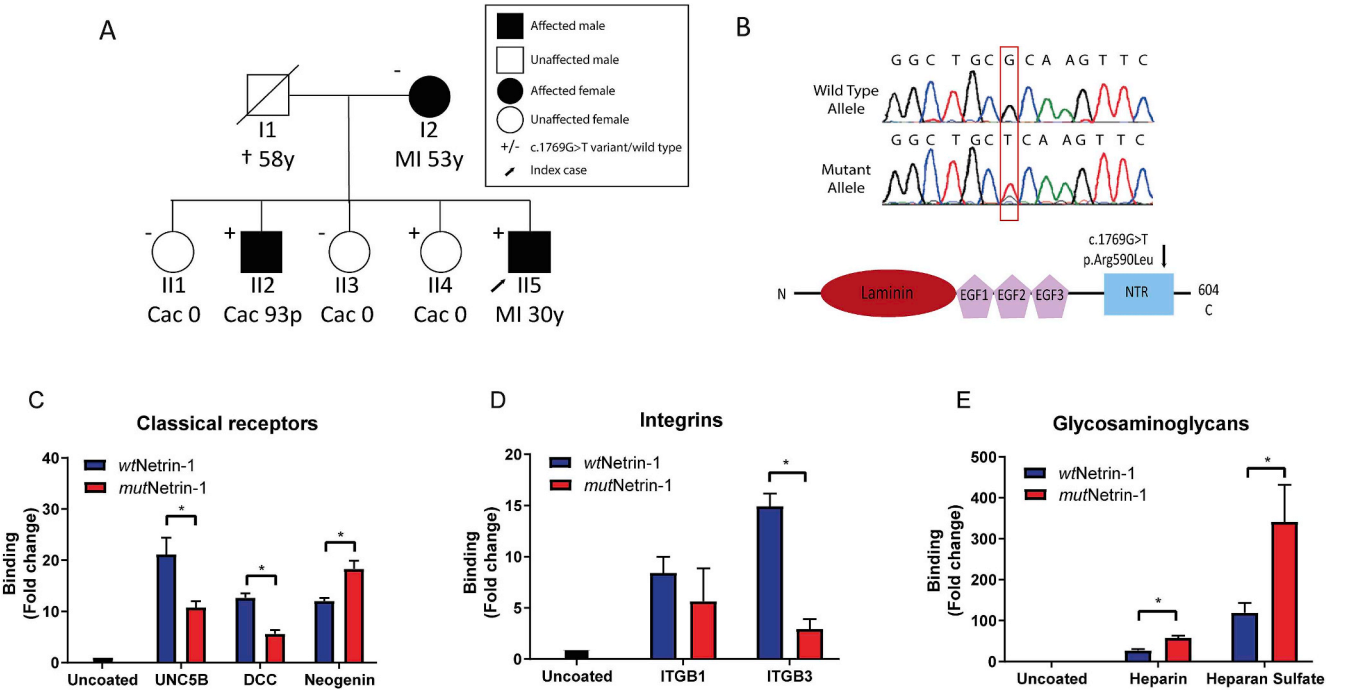

Fig. 1. Identification of c.1769G > T variation in Netrin-1 and the effect on binding capacity.
(A) Pedigree with premature atherosclerosis. The Arabic number identifies each individual, whereas the generation is marked with a roman number. MI = myocardial infarction, CAC = coronary calcium score. (B)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

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.

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

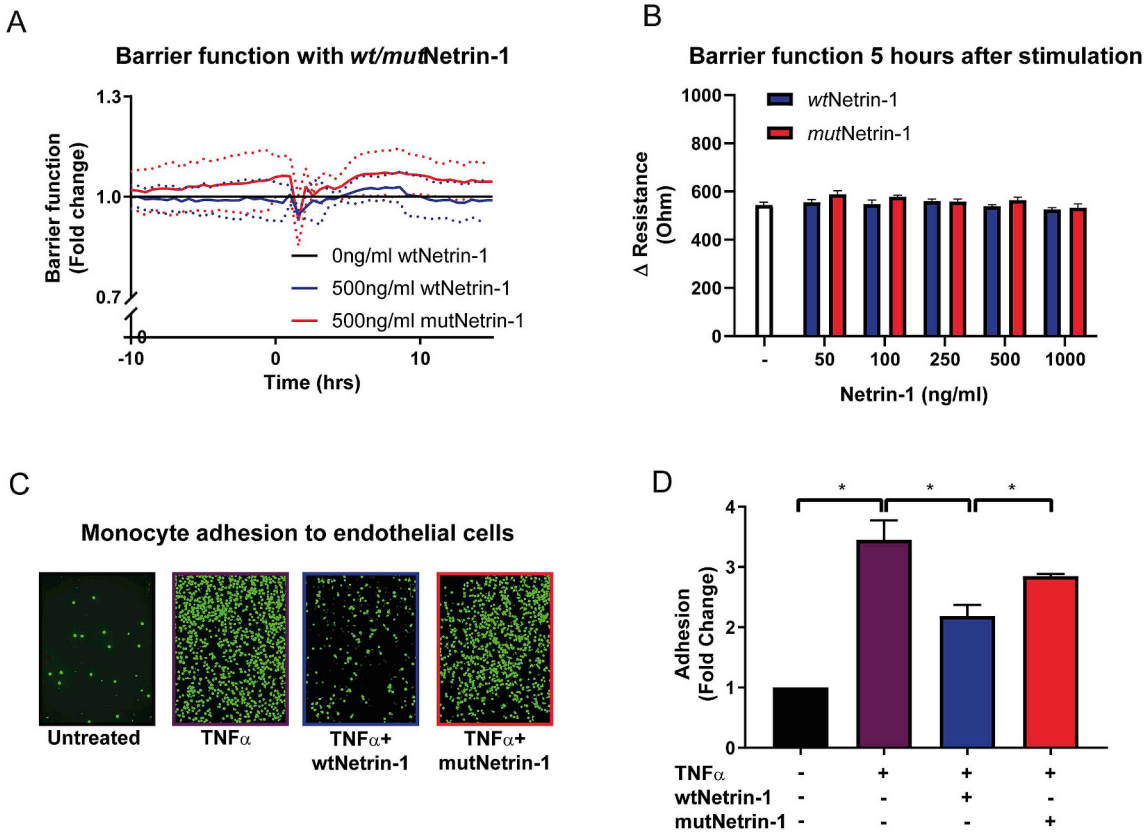

Fig. 2. Effect of *mut*Netrin-1 on endothelial function.
(A and B)Transendothelial electrical resistance of endothelial cells. At time = 0 cells were treated with *wt/mut*Netrin-1 (500 ng/ml). The dotted line represents SD of n = 3. (A) Real-time barrier function is presented relative to unstimulated cells, set at 1. (B) Trans-endothelial electrical resistance of endothelial cells 5 h after stimulation. (C and D) Adhesion of labelled monocytes to 24 h unstimulated, TNFα (10 ng/ml) stimulated, or TNFα + *wt/mut*Netrin-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, $^{*}p < 0.05$.

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.

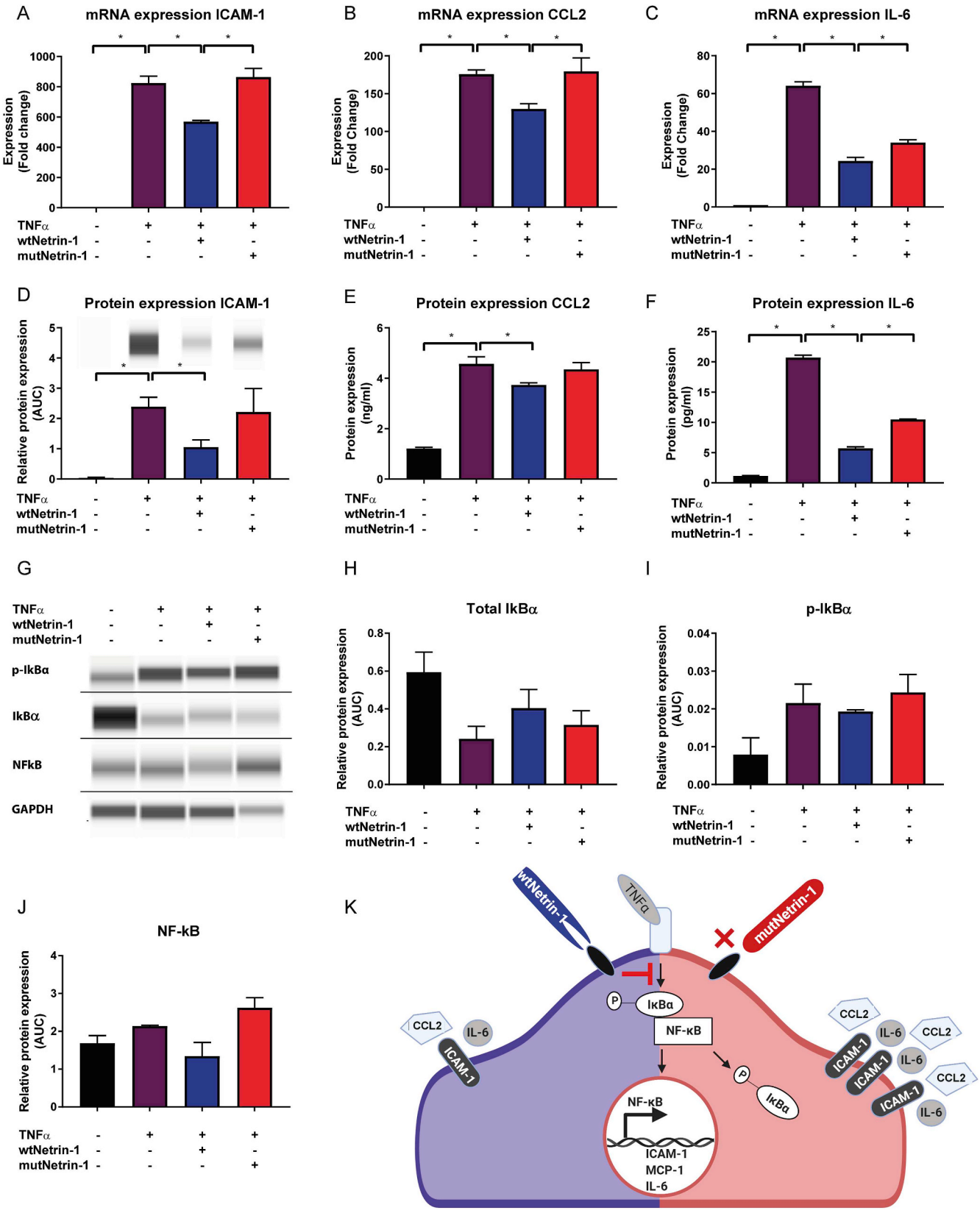

Fig. 3. Altered TNFα-induced expression of adhesion molecules and NF-κB pathway activation by Netrin-1.
(A–J) Endothelial monolayers were unstimulated or stimulated with 10 or 1 ng/ml TNFα (A-F, G-J respectively), TNFα + 500 ng/ml *wt/mut*Netrin-1 for 24 or 6 h (A-F, G-J respectively). (A–C) mRNA expression 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.

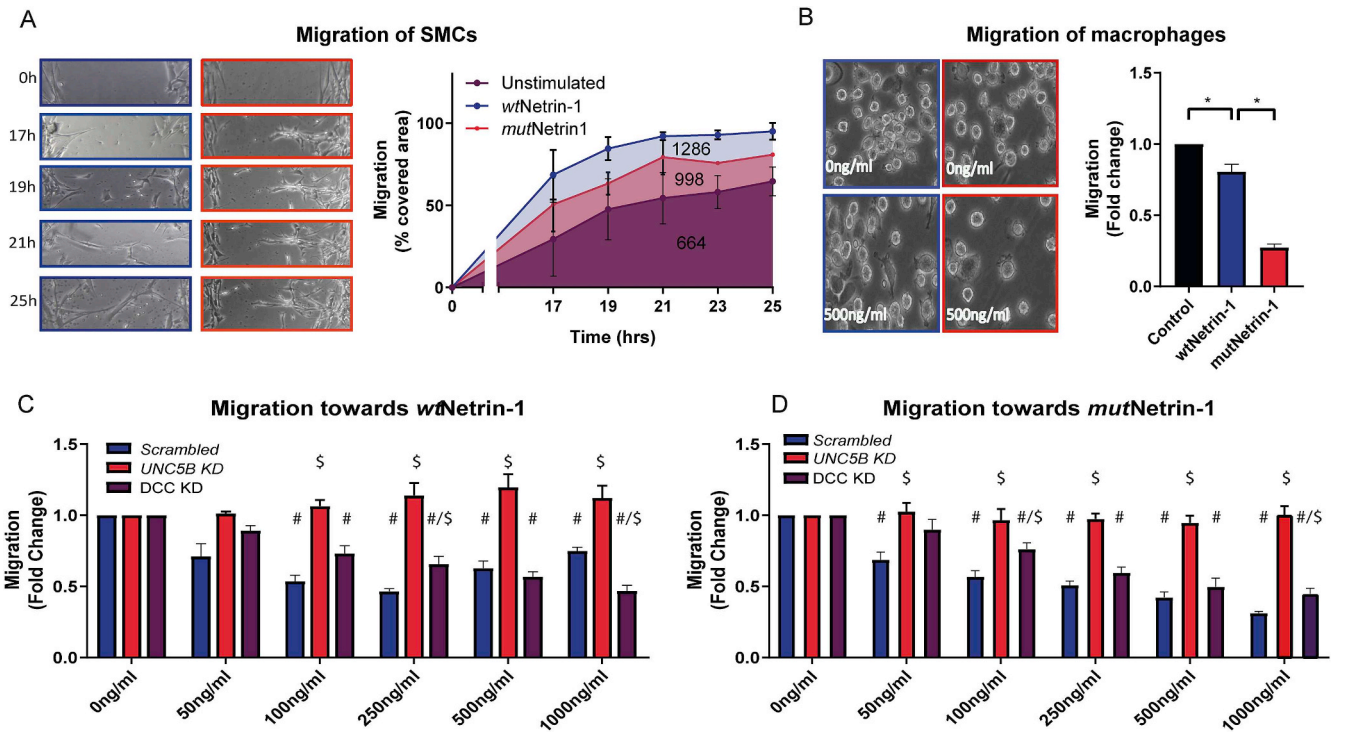

Fig. 4. Inhibition of migration with *mut*Netrin-1.
(A) Migration of SMCs stimulated with *wt*Netrin-1 or *mut*Netrin-1. Migration is presented as percentage covered area. Mean ± SEM quantification of the AUC is depicted for each curve. (B) Migration of human macrophages towards CCL5 (10 ng/ml) with 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.

4.1. 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

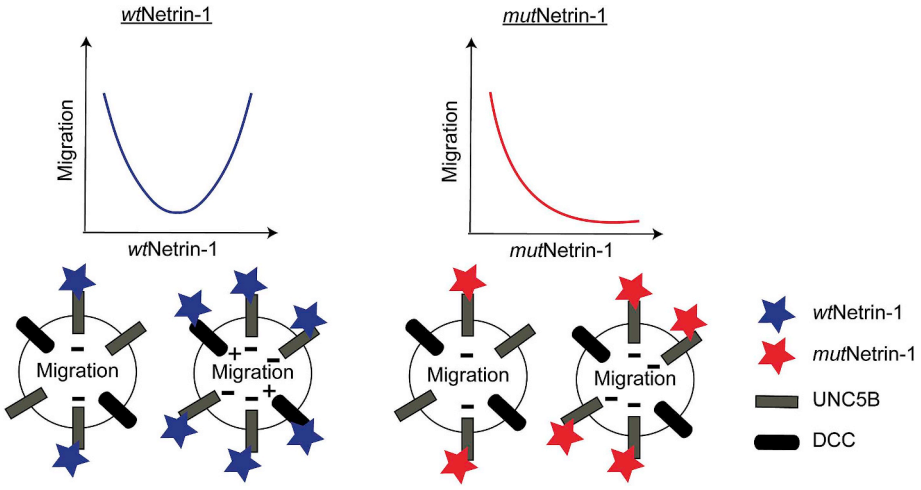

Fig. 5. Impact of UNC5B and DCC activation on leukocyte migration.
(Top) Graphical representation of the effect of low and high dose of *wt/mut*Netrin-1 on leukocyte migration. (Bottom) Migration is mediated by the cumulative effect of migration-inhibiting UNC5B and migration-promoting DCC signalling.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2020.02.015.

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