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DOI:

[10.1177/2048004020959574](https://doi.org/10.1177/2048004020959574)

Document Version

Peer reviewed version

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Citation for published version (APA):

Claro, V., & Ferro, A. (Accepted/In press). Netrin-1: focus on its role in cardiovascular physiology and atherosclerosis. *JRSM cardiovascular disease*. <https://doi.org/10.1177/2048004020959574>

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Netrin-1: focus on its role in cardiovascular physiology and atherosclerosis

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Short title: Netrin-1 in cardiovascular physiology and disease

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Manuscript category: Review

Word count: 4249

1 **Abstract**

2 The netrins form a family of laminin-related proteins which were first described as modulators
3 of cell migration and axonal guidance during fetal development. Netrin-1 is the most
4 extensively studied member of this family and, since its discovery, non-neural roles have been
5 associated with it. Together with its receptors, DCC/neogenin and UNC5, netrin-1 has been
6 shown to be involved in the regulation of angiogenesis, organogenesis, cancer and
7 inflammation. An NF- κ B-dependent truncated isoform of netrin-1 has also been shown to be
8 produced in endothelial and some types of cancer cells, which both accumulates in and affects
9 the function of the nucleus. In atherosclerosis, conflicting roles for netrin-1 have been reported
10 on plaque progression via its receptor UNC5b. Whereas endothelial-derived netrin-1 inhibits
11 chemotaxis of leukocytes and reduces the migration of monocytes to the atherosclerotic
12 plaque, netrin-1 expressed by macrophages within the plaque plays a pro-atherogenic role,
13 promoting cell survival, recruiting smooth muscle cells and inhibiting foam cell egress to the
14 lymphatic system. In contrast, there is evidence that netrin-1 promotes macrophage
15 differentiation to an alternative activated phenotype and induces expression of IL-4 and IL-13,
16 while downregulate expression of IL-6 and COX-2. Further work is needed to elucidate the
17 precise roles of the two isoforms of netrin-1 in different cell types in the context of
18 atherosclerosis, and its potential as a putative novel therapeutic target in this disease.

1 **1. Introduction**

2 Atherosclerosis, the principal pathophysiology underlying ischaemic heart disease and stroke,
3 is caused by progressive accumulation of cholesterol in the arterial wall. In recent years, it has
4 become clear that there is an important inflammatory component to atherosclerosis, which
5 progresses over a long period of time. Atherosclerosis is initially triggered by an accumulation
6 of low-density lipoproteins (LDL) in the arterial wall, between the endothelium and the tunica
7 intima, and mostly in artery segments where endothelial integrity is compromised, such as
8 curvatures or branching points, due to local turbulence of blood flow.¹ Once inside the arterial
9 wall, LDL undergoes a chain of chemical and enzymatic reactions which ultimately results in
10 an oxidized form of LDL, changing the biological function of its membrane phospholipids and
11 creating degradation products, which promote oxidative stress.² Oxidized LDL (oxLDL)
12 activates the endothelium and induces the expression of chemokines and adhesion
13 molecules, such as MCP-1 and VCAM-1, leading to the recruitment of leukocytes, especially
14 monocytes.³ In the plaque, monocytes differentiate into macrophages and recognise oxLDL
15 as a pathogen-associated molecular pattern, through its scavenger and toll-like receptors.
16 This recognition leads to macrophage shifting into a pro-inflammatory phenotype, increasing
17 the expression of pro-inflammatory cytokines and ultimately promoting atherogenesis.⁴
18 Macrophages take up cholesterol from the sub-intimal layer and transform into foam cells,
19 losing their ability to egress to the lymphatic system and contributing to the development of
20 necrotic plaque core and thinning of the fibrous cap, the plaque thereby becoming more
21 vulnerable to erosion or rupture with resultant superadded arterial thrombosis giving rise
22 to cardiovascular events.⁵ Netrins and semaphorins have been studied as strong candidates
23 for causing the loss of mobility observed in foam cells.^{6,7} Nonetheless, more research is
24 required to completely understand how these molecules modulate atherosclerosis.

25 Recent research has focussed on novel anti-inflammatory approaches which may offer
26 benefits over and above standard cardiovascular therapies such as lipid-lowering and anti-
27 platelet drugs. In this review, we will focus on the role of netrin-1 in normal physiology and in
28 the modulation of inflammation, and we will discuss specifically how this may relate to the
29 pathophysiology of inflammation in atherosclerosis and how this may pave the way to novel
30 therapeutic approaches in this disease.

31 32 **2. The netrin family of proteins**

33 Netrins are a family of laminin-like proteins, first described in the early 1990s as axonal
34 guidance cues during embryonic development. In humans, four different netrins have been

1 described (netrin-1, -3, -4 and -5: Figure 1), presenting a secondary structure which is highly
2 conserved between species. Netrin-1 to -4 are composed of one N-terminal domain VI, a
3 positively charged C-terminal domain and three laminin V-type epidermal growth factor (EGF)
4 domains with the same characteristic number and spacing of Cys residues as observed in
5 laminins.^{8,9} Unlike the structures of netrin-1, -3, and -4, full length netrin-5 comprises three V-
6 type EGF and the C-terminal domains but lacks the N-terminal laminin VI domain (Figure 1).¹⁰
7 While netrin-1 and 3 present similar V- and VI-type domains to those observed in laminin- γ 1,
8 netrin-4 amino-terminal domains are most similar to the amino terminus of the laminin- β 1
9 chain.^{11,12} The C-terminal domain is the part which varies the greatest between species. The
10 C-terminal sequences are rich in basic amino acids, which are binding sites for membrane
11 glucolipids and proteoglycans, thereby allowing interaction with cell surface components and
12 extracellular matrix.⁹

13 Most of the members of the netrin family are secreted proteins and they are bifunctional, acting
14 as both short- and long-range signals.¹³ They bind to different receptors due to the different
15 homologies between them.

16 Although netrins were first defined due to their role in the development of the central nervous
17 system, more recently other physiological and pathological roles have been ascribed to these
18 proteins. There is evidence that netrins play important roles in different types of cancer and in
19 the morphogenesis of various tissues;¹⁴ nonetheless, the only netrin that has been described
20 in the cardiovascular system to date is netrin-1, and therefore we will concentrate on this family
21 member in this review.

22

23 **3. Netrin-1**

24 Netrin-1 is the most extensively studied of all the netrins. In humans, it is encoded
25 by the gene NTN1 in chromosome 17 and encodes a highly conserved 604 amino acid
26 protein. It is expressed by different types of cells and can be found in most tissues. Relative
27 quantification of mRNA shows high expression of netrin-1 in brain, heart, kidney and lungs,
28 and low expression in the liver, intestine and spleen.¹⁵ Netrin-1 possesses two receptor-
29 binding sites in the two ends of an elongated flower-shaped structure (Figure 2), which interact
30 with two different receptor molecules.¹⁶

31 The functions of netrin-1 were initially identified as mediation of axonal orientation, axonal
32 outgrowth and neuronal migration.¹⁷ The ability of netrin-1 to repel neuronal cells makes it a
33 potential candidate for the regulation of inflammatory cell migration. Netrin-1 has been
34 identified as having roles in angiogenesis,¹⁸ tumorigenesis,¹⁹ organogenesis²⁰ and

1 inflammation, suggesting that it regulates cell migration in a broad context, as described
2 below.

3 Netrin-1 has been reported as an angiogenic agent in both physiological and pathological
4 contexts, acting either as promoter or as inhibitor of angiogenesis. It is well established that
5 netrin-1 participates in both angiogenesis and morphogenesis of the vasculature during fetal
6 life and as well as in adult homeostasis. Similar to what is observed in axon formation, the
7 specific effects of netrin-1 depend on which receptors it binds to. Park et al. showed that netrin-
8 1 not only promotes angiogenesis itself, but also enhances the angiogenic activity of vascular
9 endothelial growth factor (VEGF) both *in vitro* and *in vivo*.¹⁸ Pro-angiogenic effects were also
10 observed in pathological angiogenesis, where it promoted the creation of new vessels in
11 conditions such as cancer. Shimizu et al. and Akino et al. respectively showed that netrin-1
12 promotes the creation of new vessels in glioblastoma and paediatric medulloblastoma, leading
13 to a more aggressive pathology.^{21,22}

14 Netrin-1 has also been classified as an oncogene due to its various roles in tumorigenesis,
15 including inhibition of macrophage recruitment, promotion of cell survival, stimulation of
16 invasiveness and of pathological angiogenesis. Several groups have shown that netrin-1 is
17 upregulated in glioblastoma, medulloblastoma and other malignancies, and its overexpression
18 leads to poorer prognosis.^{21,22} The ability of netrin-1 to inhibit apoptosis is related to binding to
19 its specific receptors, which trigger apoptosis in the absence of the ligand. Cancers producing
20 excessive amounts of netrin-1 will therefore have their apoptosis program inhibited. This
21 interaction between netrin-1 and its receptors will be described in more detail in the following
22 section.

23 In addition to axonal guidance and angiogenesis, the role of netrin-1 and its receptors in
24 organogenesis have been studied in terms of its regulation of migration, differentiation and
25 branching morphogenesis. The importance of this molecule in the development of tissues,
26 such as lungs, pancreas or mammary glands, has been revealed in the last couple of
27 decades.^{20,23,24} Dalvin et al. showed that netrin-1 controls epithelial-mesenchymal interaction,
28 which is a key process in lung organogenesis.²³ Furthermore, De Breuck et al. demonstrated
29 that netrin-1 is involved in pancreatic morphogenesis and tissue remodelling *in vivo*.²⁴
30 Together, these data show that netrin-1 and its receptors play an important role in the
31 morphogenesis of branched tissues.

32 As mentioned above, there is evidence that netrin-1 mediates inflammation due to its effect
33 on immune cells, having a role in their recruitment and motility, as well as in their stimulation
34 and production of cytokines. Nonetheless, it is controversial whether a high expression of
35 netrin-1 is beneficial or detrimental. Some studies show that netrin-1 has a protective effect in

1 pathological models. *In vivo* work performed by Mirakaj et al. showed that expression of netrin-
2 1 was repressed in acute inflammation in lung injury and peritonitis. Moreover, this group
3 showed that treatment with exogenous netrin-1 dampened the inflammation and reduced
4 tissue damage.^{25,26} In accordance with this, Grenz et al. demonstrated that acute kidney injury
5 was aggravated in a mouse model with partial netrin-1 deficiency, and treatment with
6 exogenous netrin-1 attenuated the effects observed, reducing tissue injury.²⁷ In contrast,
7 Mediero et al. have proposed a pro-inflammatory role for netrin-1, which is required for the
8 development of particle-induced osteolysis, hypothesising that it leads to the recruitment,
9 retention and enhancement of osteoclast differentiation and activity.²⁸ Van Gils and colleagues
10 have provided evidence that netrin-1 promotes atherosclerosis due to its effect on inhibiting
11 the emigration of macrophages and increasing recruitment of smooth muscle cells to the
12 plaque.²⁹ Ramkhalawon et al. showed that adipose tissue produces high amounts of netrin-1,
13 promoting macrophage retention, chronic inflammation and insulin resistance.³⁰ The pro- and
14 anti-inflammatory effects of netrin-1 therefore appear to depend on the model being studied.

15

16 **4. Netrin-1 receptors**

17 The function of netrin-1 in regulating neuronal navigation has been associated with two main
18 types of receptors, termed dependence receptors: DCC (Deleted in Colorectal Carcinoma)
19 and its orthologue neogenin, and UNC5 (UNCoordinated protein 5) receptors (UNC5a-d).
20 These are receptors that play a dual role triggering different cellular responses in the presence
21 or absence of ligand. On the one hand cell proliferation, migration and survival are promoted
22 in the presence of netrin-1 whilst, on the other hand, apoptosis is induced in the absence of
23 the ligand. Moreover, relative expression levels of both families of receptors will mediate the
24 attraction or repulsion effects observed in neuronal development, which will be further
25 explored below.^{31,32}

26 DCC and neogenin are receptor homologues which share nearly half of their amino acid
27 identity and display the same secondary structure.³³ They are single-pass transmembrane
28 receptors with an extensive extracellular region, which comprises four immunoglobulin (Ig)-
29 like domains in a horseshoe configuration,³⁴ followed by six fibronectin type III domains (FnIII).
30 The intracellular portion of the receptor contains three conserved motifs, which are responsible
31 for signalling (P1, P2 and P3) (Figure 3A).³⁵ Biochemical studies suggest that FnIII4, FnIII5
32 and FnIII6 are the DCC domains responsible for the binding of netrins.^{36,37}

33 UNC5 receptors are also single-pass transmembrane receptors with an extracellular region
34 consisting of two Ig-like motifs, followed by two thrombospondin I modules (TSP).³⁸ On the

1 cytosolic side, UNC5 receptors have a long tail which consists of a ZU5 domain, a DCC-
2 binding motif (UPA) and a death domain (DD) (Figure 3A).³⁹ Both Ig-like domains may be
3 responsible for the primary binding of netrins and it is suggested that the second motif (Ig2)
4 contributes more to the binding than Ig1.³⁶

5 Different signalling pathways have been proposed for these receptors and receptor
6 complexes. In the development of the central nervous system, netrin-1 exerts a bifunctional
7 effect, depending on which combination of receptors it binds to. Netrin-1 can promote
8 attraction if it binds to a DCC-DCC complex, leading to the activation of several cascades of
9 events which promote directional axonal outgrowth. The opposite effect occurs, namely
10 repulsion, when netrin-1 binds exclusively to an UNC5 receptor or UNC5 and DCC
11 simultaneously, promoting short- and long-range repulsion respectively (Figure 3B).^{32,40}

12 DCC was originally characterised as a suppressor of colorectal cancer when a pronounced
13 reduction of its expression was observed in several colorectal carcinoma cell lines, due to a
14 deletion in chromosome 18q21.⁴¹ Many studies have established the importance of
15 DCC/neogenin and netrin-1 in different pathologies. For example, Reyes-Mugica et al. and
16 Koren et al. showed that loss of DCC expression is associated with poor prognosis in patients
17 with glioma and breast cancer respectively.^{42,43} In the immune system, the DCC/neogenin
18 receptor family mediates a pro-migration response to netrin-1. Boneschansker et al. reported
19 that netrin-1 plays an important role inducing migration of leukocytes via its receptor neogenin.
20 Netrin-1 is an inducer of CD4⁺ T cells, and its interactions with neogenin augmented the
21 migration of these leukocytes in an *in vitro* model. The same group also reported that
22 neogenin-expressing T cells are recruited in pro-inflammatory environments where netrin-1 is
23 expressed. Using a humanized SCID mouse model engrafted with human skin, injection of
24 netrin-1 led to an increase in T cell infiltration compared to vehicle injection.⁴⁴

25 Apoptosis and cell survival have also been identified as downstream effects dependent on
26 netrin-1 and its receptors. Mehlen et al. showed that, in the absence of netrin-1, DCC induces
27 apoptosis. Moreover, this receptor is a caspase substrate which loses its pro-apoptotic effect
28 if there is a mutation at the site where caspase-3 cleaves DCC (Figure 3C).⁴⁵ Cleavage of
29 UNC5 by caspase-3 similarly induces cell apoptosis, through its intracellular death domain
30 (Figure 3D). However, contrary to that which is observed in DCC, mutation of the caspase-3
31 cleavage site of UNC5 does not inhibit apoptosis; on the other hand, deletion of the
32 myristoylation signal peptide, which is localized in the C-terminus of the receptor, prevents its
33 induction of apoptosis.³¹

34 Netrin-1 has been shown to play a key role in modulating the migration of leukocytes through
35 its UNC5b receptor.¹⁵ Although the precise signalling pathway is not yet understood, there is

1 strong evidence that this event is an important modulator in the recruitment of leukocytes and
2 inflammation; this will be described further below, in the context of the role of netrin-1 in
3 atherosclerosis.

4 CD146, an adhesion molecule expressed by endothelial cells, mostly in the vasculature, has
5 also been described as a netrin-1 receptor. The molecular mechanisms associated with this
6 receptor are angiogenesis, vessel permeability and leukocyte transendothelial migration.⁴⁶
7 CD146 has been described as a pro-angiogenic mediator, counterbalancing the UNC5b anti-
8 angiogenic signal. Further studies are needed to clarify which signalling pathways it utilises in
9 leukocytes to modulate their migration.

10 Down syndrome cell adhesion molecule (DSCAM) has more recently been proposed as a
11 novel receptor for netrin-1. Its collaboration with DCC has been shown to be essential for
12 commissural axons to grow towards and across the midline.⁴⁷ There is no evidence to date
13 that this receptor plays a role in cardiovascular disease, and therefore it will not be explored
14 further in this review.

15

16 **5. Netrin-1 isoforms**

17 In the last decade, a number of studies have shown the existence of two isoforms of netrin-1
18 (Figure 4), which may help to explain some of the apparently conflicting effects reported for
19 this protein.^{19,48,49} Delloye-Bourgeois et al. first described a new netrin-1 isoform which is
20 truncated and abundantly expressed in a neuroblastoma cell line model.¹⁹ This isoform is
21 produced by an alternative promoter, localised in intron I of the netrin-1 DNA sequence, which
22 is NF- κ B dependent.⁴⁸ They reported that the C-terminus of netrin-1 mediates its nucleolar
23 localisation and the N-terminus prevents it, the latter instead promoting its secretion from the
24 cell. As a result, the truncated netrin-1, which lacks part of the N-terminal domain, stays
25 localised within the nuclei.¹⁹ Truncated netrin-1 has been shown to be expressed in a range
26 of types of cancer, and high expression of this isoform appears to associate with poorer patient
27 prognosis.^{19,49} This truncated isoform affects nucleolar function; for instance, in cancer cells,
28 overexpression of this isoform promotes ribosome synthesis, tumour growth and cell
29 proliferation.¹⁹

30 Passacuale et al. showed that endothelial cells also express the truncated isoform of netrin-
31 1. TNF- α treatment disturbs the balance of expression between the two isoforms, promoting
32 upregulation of the truncated one and downregulation of the secreted (full length) isoform.
33 They also demonstrated that the two isoforms are regulated differently in endothelial cells. As
34 previously described by Delloye-Bourgeois et al. in neuroblastoma cells, the truncated isoform

1 promoter was found to be NF- κ B dependent, so that treating endothelial cells with an NF- κ B
2 inhibitor suppressed TNF- α -induced expression of the truncated isoform, both at the
3 transcriptional and protein levels. They also showed that the induction of secreted full-length
4 netrin-1 is NF- κ B-independent, but on the other hand is dependent on histone H3 acetylation,
5 which in turn could be induced by aspirin treatment of endothelial cells.⁴⁸

6 The effects of the nucleolar isoform in other cell types, such as leukocytes, has not been
7 determined as yet. Further work is required to clarify whether this isoform is expressed in
8 inflammatory conditions, such as atherosclerosis, and what its contribution to the modulation
9 of such inflammatory pathologies might be.

10

11 **6. Netrin-1 in atherosclerosis**

12 As described earlier, atherosclerosis is a chronic inflammatory disease that progresses over
13 many years and often is only detected when the patient develops symptoms such as
14 myocardial ischaemia. Monocytes are recruited to the arterial wall in areas of developing
15 atherosclerotic plaque, where they differentiate into macrophages and take up oxLDL,
16 becoming immobile foam cells, increasing the cellular burden and instability within the plaque.

17 The reported effects of netrin-1 in atherosclerosis have been conflicting, having been shown
18 to play both pro- and anti-inflammatory roles in the development of the disease. This conflicting
19 evidence may be explained by the temporal and spatial expression of netrin-1: endothelial-
20 derived netrin-1 has been reported as playing a protective role, whilst netrin-1 secreted within
21 the atherosclerotic plaque by macrophages appears to exert a pro-atherogenic effect. These
22 are described in detail below.

23

24 **6.1 Endothelial-derived netrin-1**

25 Endothelial cells secrete full length netrin-1, leading to a reduction of monocyte chemotaxis
26 into the arterial wall (Figure 5A), as first demonstrated by Passacquale et al. In ApoE^{-/-} mice,
27 they found that UNC5b blockade inhibited the effects of netrin-1 with a consequent increase
28 in accumulation of monocytes in the brachiocephalic artery.⁴⁸ Furthermore, Lin et al. and
29 Bruikman et al. showed that netrin-1 reduces the expression of adhesion molecules induced
30 by the pro-inflammatory cytokine TNF- α . In an *in vitro* model of human aortic endothelial cells
31 stimulated with TNF- α , a significant reduction in the expression of VCAM-1, ICAM-1 and E-
32 selectin was observed when cells were co-treated with netrin-1, and high concentrations of
33 netrin-1 completely abolished the expression of these adhesion molecules.⁵⁰ Significant

1 downregulation of ICAM-1, IL-6 and MCP-1 has also been observed in endothelial cells from
2 patients with underlying atherosclerosis, this effect being abolished by UNC5b blockade.⁵¹
3 Furthermore, Lin et al. also showed that netrin-1 suppresses endothelial-derived cytokine
4 production induced by TNF- α in a concentration-dependent manner (Figure 5B and 6). They
5 demonstrated that netrin-1 selectively suppresses toll-like receptor 4 (TLR4) and inhibits the
6 NF- κ B pathway by suppressing TNF- α -induced IKK and I κ B α activation in the cytoplasm.
7 They also showed that netrin-1 affects the nuclear NF- κ B subunit p65 by reducing its
8 accumulation in the nuclei. While stimulation with TNF- α led to an increase of approximately
9 3.4 fold of p65 in the nuclei compared with basal levels, treatment with 200 and 400ng/ml of
10 netrin-1 reduced its nuclear accumulation by 25% and 50%, respectively. Moreover, netrin-1
11 suppressed NF- κ B promoter activation in endothelial cells after TNF- α stimulation: TNF- α
12 increased the promoter activity by 70-fold, and this was suppressed by 30% and 60% after
13 treatment with 200 and 400ng/ml of netrin-1 respectively.⁵⁰

14 From a different perspective, Passacuale et al. showed that pro-inflammatory stimuli cause
15 downregulation of endothelial-derived netrin-1 expression.⁴⁸ Thus, it would be expected that
16 the protective effect of netrin-1 will decrease as the atherosclerotic process proceeds, thereby
17 further accelerating its progression. Indeed, Bruikman and colleagues showed that netrin-1
18 plasma levels are inversely correlated with arterial wall inflammation as well as total plaque
19 volume.⁵¹

20

21 **6.2 Macrophage-derived netrin-1**

22 Macrophages can also express netrin-1.^{15,29,30,52} Nonetheless, what effects macrophage-
23 derived netrin-1 may exert on the ability of macrophages to migrate in response to chemokines
24 or on modulation of their phenotype remain entirely unclear. Tadagavadi et al. and
25 Komatsuzaki et al. reported, in terms of netrin-1 receptors, that only UNC5b is expressed in
26 monocytes, granulocytes and lymphocytes, using immunohistochemistry and quantitative RT-
27 PCR.^{15,53} We will therefore concentrate on the possible effects of netrin-1 on macrophages on
28 the basis that it interacts with UNC5b only.

29 Ly et al. showed that, in infection and pro-inflammatory models, the levels of netrin-1 decrease
30 and the recruitment of leukocytes to the site is promoted. They hypothesised that netrin-1 is
31 an immunomodulator, which keeps leukocyte influx in check, preventing aberrant tissue
32 destruction. This inhibitory effect on migration was verified *in vitro* and *in vivo* in response to
33 a range of chemoattractive stimuli, whilst not affecting other functions, for example the
34 production of superoxide.¹⁵ Moreover, Taylor et al. demonstrated that netrin-1 is also capable

1 of inhibiting macrophage chemotaxis to non-chemokine attractants⁵². This group showed that
2 the chemotaxis generated by the complement component C5a can be partially inhibited by
3 netrin-1, through UNC5b.

4 In contrast, other workers describe netrin-1 as a pro-inflammatory mediator. Van Gils and
5 colleagues showed that netrin-1 can promote atherosclerosis, inhibiting the migration of the
6 foam cells to the lymph nodes, becoming trapped in the atherosclerotic plaque and thereby
7 contributing to its development and instability. In accordance to what was reported in cancer
8 cell models, they also reported that netrin-1 promotes cell survival in the atherosclerotic
9 plaque. Deficiency of netrin-1 in the macrophages reduced atherosclerosis and allowed
10 egress of foam cells to the lymphatic system.²⁹

11 Similarly, Ramkhelawon et al. showed that netrin-1 promotes the retention of macrophages in
12 adipose tissue, enhancing metabolic dysfunction and chronic inflammation. In an experiment
13 where mutant mice, whose macrophages do not express netrin-1, and wild-type mice were
14 fed a high fat diet, they found that recruitment of macrophages to the adipose tissue was
15 similar in both groups. However, on the 14th day, the retention of macrophages was
16 significantly lower in the mutant mice compared with the wild-types, consistent with the
17 hypothesis that netrin-1 traps macrophages in the site and promotes chronic inflammation.
18 Furthermore, adipose tissue macrophages isolated from the mutant mice exhibited lower
19 mRNA expression of pro-inflammatory markers and cytokines and a higher expression of M2
20 macrophage markers, compared with the wild-type.³⁰

21 Regarding macrophage phenotypes, Ranganathan et al. and Mao et al. showed the opposite
22 to what was observed in the Ramkhelawon et al. study. In their experiments, they found that
23 higher expression of netrin-1 is protective, enhancing the shifting of macrophage phenotype
24 towards M2, with concomitant upregulation of anti-inflammatory markers such as IL-4 and IL-
25 13 and downregulation of IL-6 and cyclooxygenase-2 (COX-2).^{54,55} Both groups showed that
26 netrin-1 activates anti-inflammatory pathways that are known to regulate macrophage
27 polarization, such as peroxisome proliferator-activated receptors (PPAR). PPAR mediate a
28 variety of inflammatory mechanisms, which include repressing the activities of pro-
29 inflammatory transcription factors such as NF- κ B or STATs.⁵⁶ Netrin-1 led to a decrease of in
30 M1 polarization and cytokine production induced by IFN- γ and suppressed ischaemia-
31 reperfusion injury, effects which were inhibited by PPAR antagonists.⁵⁴ Further work by
32 Ranganathan et al. suggests that netrin-1 regulates inflammation and cell migration through
33 suppression of NF- κ B, leading to downregulation of COX-2-mediated PGE2 and thromboxane
34 A2 production, both *in vitro* and *in vivo*.⁵⁷

1 The current data suggest that the immunomodulatory effects of netrin-1 depend on which cell
2 type expresses it and in which environment. Macrophage-derived netrin-1 in the plaque may
3 be detrimental because it stops macrophage egress to lymph nodes, whereas endothelial-
4 derived (systemic) netrin-1 may be essential to suppress levels of inflammation.

5

6 **7. Conclusion**

7 Netrin-1 plays different roles in a range of pathologies, and its effects vary depending on which
8 type of cells it acts upon and which receptors it binds to. In the context of atherosclerosis, its
9 effects may be both pro- and anti-inflammatory, the balance depending on where it is produced
10 (endothelial versus macrophage-derived), their relative production, and the relative expression
11 of the truncated versus the full length isoform. Despite netrin-1 isoforms having been studied
12 and characterised in endothelial cells, information is currently lacking as to how their
13 expression varies in macrophages under different inflammatory conditions. To fully
14 understand the relevance of netrin-1 in atherosclerosis, it will be important for expression of
15 its isoforms to also be characterised in the different macrophage phenotypes. As described
16 previously, endothelial-derived netrin-1 expression is suppressed under inflammatory
17 conditions,⁴⁸ but further work is needed to establish what effect restoration of endothelial-
18 derived netrin-1 expression would have on foam cell function and motility within the plaque.

19 Whether the netrin-1 system presents a worthwhile therapeutic target in atherosclerosis
20 remains unclear. It may be that netrin-1 delivery would have to be targeted in order to exert a
21 useful therapeutic effect. Further work is needed to address this question and to further
22 elucidate the precise roles played by the different isoforms of netrin-1 in the pathophysiology
23 of atherosclerosis.

Funding

This work was supported by a King's British Heart Foundation Centre for Excellence Award [RE/18/2/34213]. V.C. is supported by British Heart Foundation PhD Studentship [FS/18/69/33884].

Conflicts of interest

None declared.

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Figure legends

Figure 1 – Schematic structure of the netrin family members.

Figure 2 – Predicted unbound netrin-1 tertiary structure generated by Phyre2 software, using the human netrin-1 UniProt sequence. It has a flower-shaped structure, formed by the N-terminus on the bottom right hand-side of the Figure (green and blue motifs), followed by three V-type epidermal growth factor domains and the C-terminus at the top (red motif).

Figure 3 – Netrin-1 dependence receptors. **(A)** Schematic structure of DCC and UNC5 cytoplasmic and extracellular domains. **(B)** Netrin-1 binding to DCC-DCC and DCC-UNC5 complexes, promoting neuronal attraction and repulsion respectively. **(C)** Apoptosis promoted by DCC receptor in the absence of netrin-1. **(D)** Apoptosis promoted by UNC5 receptor in the absence of netrin-1.

Figure 4 – Schematic structure of netrin-1 isoforms.

Figure 5 – Overview of netrin-1 effects in the context of normal physiology and atherosclerosis. **(A)** Endothelial-derived netrin-1 interacts with UNC5b receptors on the monocyte surface, causing chemorepulsion. **(B)** In atherosclerosis, pro-inflammatory cytokines act on endothelial cells to downregulate expression of endothelial-derived netrin-1, as well as upregulate endothelial cell adhesion molecules, the resultant effect of both being increased monocyte recruitment to the plaque.

Figure 6 – Proposed model for the protective role of netrin-1 in endothelium. In the absence of netrin-1, pro-inflammatory stimuli induce the TLR4 cascade leading to transmigration of NF- κ B to the nucleus, promoting expression of pro-inflammatory cytokines and adhesion molecules. The protective effects of netrin-1 are believed to be exerted through inhibition of IKK activation and I κ B phosphorylation and degradation, via the UNC5b receptor, thereby preventing nuclear translocation. Image adapted from⁴⁵.