




# Targeting Netrin-1 and -4 as a Novel Diagnostic Parameter and Treatment Option for Diabetic Retinopathy

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**Abstract:** Diabetic retinopathy (DR) is a retinal vascular disorder associated with both type 1 and type 2 diabetes mellitus (DM). It is characterized by specific loss of pericytes, which leads to an augmented blood vessel permeability, and development of new blood vessels (retinal neovascularization). Moreover, stiffening of eye membrane, inflammation, and apoptosis of endothelial cells also lead to damage of the blood–retinal barrier and blindness in most cases unless it's detected and managed early. Hence, this review was intended to assess the potential roles of Netrin-1 and -4 as new/alternative biomarkers and therapeutic options for DR. Netrin-1 and -4 have been the most known ligands and are well known for their role in neural guidance. DR has both neural and vascular components; therefore, biomarkers used for both neural and vascular retinal tissues are potentially important. According to different experimental and clinical studies, as compared to the normal groups, there was a significant increment in both retinal Netrin-1 and -4 mRNA and protein levels in the retinopathy groups. In addition, exogenous supplementation of these proteins is also used as a therapeutic agent for DR.

**Keywords:** diabetic retinopathy, Netrin-1, Netrin-4, biomarker, diabetes mellitus

## Introduction

Diabetic retinopathy (DR) is a retinal vascular disease<sup>1</sup> and the most prevalent microvascular complication of both type I and type II diabetes mellitus (DM).<sup>2</sup> The prevalence is strongly correlated to the duration of DM and level of glycemic control.<sup>3</sup> Globally, nearly 90 million people have DR, of which 21 million have diabetic macular edema (DME), 28 million have sight-threatening retinopathy, and 17 million have proliferative retinopathy.<sup>3,4</sup> In the United States, retinopathy is the major cause of loss of sight in productive age groups.<sup>1</sup>

In patients with type II DM, retinopathy may not be detected for years until significant damage occurs. Therefore, patients are diagnosed with DR after it has caused serious damage (visual problems). Even though type I DM is mostly diagnosed in early age, it still needs early detection for prevention of related complications, including retinopathy and other complications.<sup>5</sup>

Different biological mechanisms have been associated with the pathology of hyperglycemia-induced retinopathy. Among them, polyol and hexosamine pathway activity, activation of protein kinase C isoforms, oxidative stress, and formation of advanced glycation end-product are mostly reported.<sup>3,6</sup> DR is characterized by specific loss of pericytes, which leads to an augmented blood vessel permeability,

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and the development of new blood vessels (retinal neovascularization).<sup>7</sup> In addition, stiffening of the membrane in the eye and apoptosis of endothelial cells are detected during the pathogenesis, which collectively contributes to the damage of the blood–retinal barrier. Moreover, marked loss of endothelial cells and pericytes leads to upregulation of vascular endothelial growth factor (VEGF) by activating hypoxia-inducible factor 1 (HIF-1). Likewise, phospholipase A2's (PLA2) elevation due to hyperglycemia also prompts upregulation of VEGF.<sup>6</sup> DME can occur at any stage of DR and cause distortion of visual images and a decrease in visual acuity.<sup>8</sup> Individuals with DR has been a higher chance to develop other vascular complications, including diabetic nephropathy, diabetic neuropathy, and cardiovascular diseases.<sup>9</sup>

Different conventional and newer diagnostic tools including glycated hemoglobin (HbA1c), thioredoxin-interacting protein, fructosamine, and glycated albumin have been used for detection of retinopathy in diabetic patients. However, most of them are used after it causes significant damage to patients.<sup>9,10</sup> In addition, they have limited specificity, sensitivity, and are imprecise in certain clinical conditions.<sup>11</sup> Moreover, increased cost related to newer diagnostic tools were also a substantial challenge.<sup>12</sup> Hence, the present review was intended to assess the potential role of Netrin-1 and -4 as a novel biomarker and therapeutic option for DR.

## Netrin-1 and -4 and Their Receptors

Netrin is among axon guidance molecules with Semaphorins and Ephrins. It has been known to regulate axonal growth in the development of the nervous system, regulation of immune and inflammatory responses (inflammation of the nervous system).<sup>13,14</sup> Netrin-1 and -4 are the most known

ligands of the family and critical axonal guidance protein during morphogenesis, embryonic development, and angiogenic processes.<sup>15,16</sup> Netrin-1 related axonal functions have been linked to two classes of receptors: the deleted in colorectal cancer (DCC) family, including DCC and its orthologue Neogenin-1, and the Unc5s family, including Unc5A<sup>17,18</sup> while Netrin-4 performs its activity majorly through binding to the laminin  $\gamma$ 1 chain and disrupts laminin networks and basement membranes<sup>15,16,19</sup> (Figure 1).

## The Role of Netrin in Retina Development

Netrin receptors have been expressed in developing retina and Netrin-neogenin signaling, which might stimulate retinal ganglion cell axon growth into and along the optic nerve.<sup>20,21</sup> Netrin-1/UNC5B may activate retinal vessel development and they can stimulate pro-angiogenesis.<sup>22</sup> The guidance of retinal ganglion cell axons through the optic disc is dependent on the DCC/Netrin-1 axonal guidance system.<sup>21</sup> Furthermore, netrin-1 contributes to steering axons out of the retina.<sup>23</sup> Under hypoxic conditions, the Netrin-1 level increases and it might be a key factor in inducing retinal neovascularization.<sup>25,26</sup> Retinal neovascularization is the major pathological condition of several diseases including diabetic retinopathy, retinal vein occlusion, and age-related macular degeneration.<sup>8,24,27</sup> Netrin-1 promotes retinal angiogenesis in oxygen-induced retinopathy.<sup>28,29</sup>

Netrin-4 is highly expressed in the retina and its role is as an angiogenesis modulating factor in oxygen-dependent vascular homeostasis.<sup>24</sup> In an *in vivo* study, Netrin-4 is found to be a negative regulator of corneal epithelial cell proliferation and retinal vascular branching. It is also expressed in the retinal vascular basement membrane.<sup>16</sup>

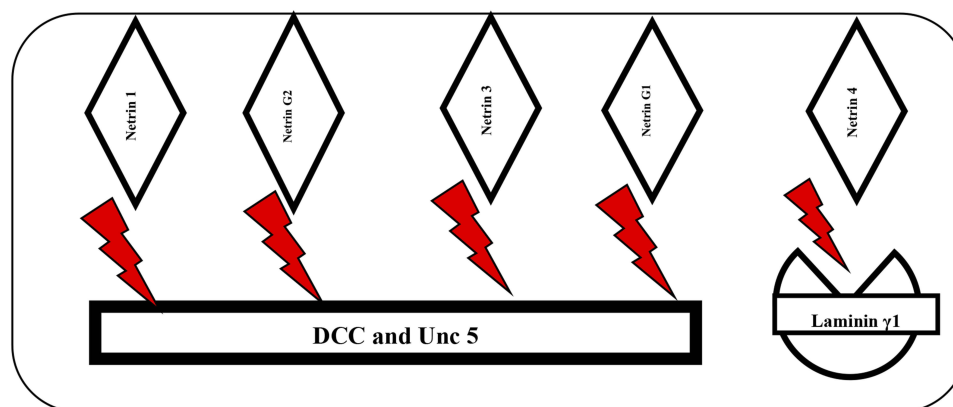


Figure 1 Netrin and its Receptors.

**Table 1** Role of Netrin-1 and -4 in Diabetic Retinopathy as a Biomarker and Treatment Modality for DR

Study Design	Method and Intervention	Treatment Outcome	References
Clinical and animal study	A total of 18 eyes from 18 patients were included in the study and 10 of them were collected from DR patients and 8 from non-DR patients. OIR mice models were established.	The levels of Netrin-1 in the vitreous of DR patients were significantly higher than those in the controls Level Netrin-1 was up-regulated in OIR mice.	34
Experimental animal model	C57BL/6J mice were exposed to 75 ± 5% oxygen for 5 days (OIR).	Compared to the normoxic group, there were significant increases in both retinal Netrin-1 mRNA and protein levels in the hypoxic group.	26
Experimental animal model	STZ induced DM Retinal tissues were harvested and the expression of Netrin-1 mRNA was examined by RT-PCR.	Both Netrin-1 mRNA and protein levels retina were dramatically increased in the DM rats compared to the NC group.	35
Experimental animal model	Retinal neovascularization was induced by OIR in C57BL/6J mice. RT-PCR and Western blot were used to examine the expression of Netrin-1 receptor subtypes in the mouse retinas.	Netrin-1 receptor subtypes were significantly expressed in the retina of OIR mice. Western blots showed that UNC5B expression was significantly increased. Treatment of OIR mice with the UNC5B shRNA plasmid dramatically reduced neovascular tufts and neovascular outgrowth into the inner limiting membrane.	25
In vitro and in vivo experimental study	Effects of Netrin-4 on vascular smooth muscle cell (VSMC) activity, wound migration assays, proliferation cell assay, cell adhesion assay, HUAEC and VSMC co-cultures on Matrigel, Small interfering RNA and transfection assays, RT-PCR and transfection of PC3 prostate carcinoma cells and xenograft in nude mice analysis were performed.	VSMC express Netrin-4 and three of the six known Netrin receptors. Netrin-4 promotes mural cells migration. Netrin-4 promotes VSMC adhesion. Netrin-4 induces VSMC adhesion via DCC, Neogenin, and Unc5B receptors. Netrin-4 enhances VSMC recruitment in an in vitro model of endothelial cell-pericyte interaction. Overexpression of Netrin-4 by PC3 cells increases mural cell coverage in tumor vasculature.	36
In vitro and in vivo experimental study	In vitro angiogenesis assay in human umbilical vein endothelial cell (HUVEC) Netrin-4 was topically applied in vivo on alkali-burned rat corneas on day 0 and/or day 10 post-injury.	The levels of Netrin-4 in endothelial cells (HUVEC) and cornea was increased. Netrin-4 decreases HUVECs viability in a dose-dependent manner by regulating HUVEC proliferation and apoptosis Netrin-4 inhibited HUVECs tube formation. Netrin-4 prevents corneal neovascularization in alkali-burned rat corneas. Netrin-4 promotes the regression of corneal neovascularization and inhibits apoptosis in alkali-burned rat corneas.	19
Experimental animal study	STZ-induced rats 0.1 µg/mL or 5 µg/mL Netrin-1 was injected intravitreally to assess retinal neovascularization.	HUVECs formation, viability and proliferation, migration, and invasion were upregulated by Netrin-1 at a concentration of 0.1 µg/mL, while 5 µg/mL Netrin-1 had an opposite effect. Netrin-1 was found to increase HUVEC viability by comparing the samples with the CTR at every time point. Netrin-1 was found to suppress and reverse retinal neovascularization at a concentration of 5 µg/mL, while 0.1 µg/mL Netrin-1 led to an increase in the number of new retinal blood vessels, after 6 weeks' injection.	37

(Continued)

Table 1 (Continued).

Study Design	Method and Intervention	Treatment Outcome	References
Experimental animal model	STZ induced DM to 6-week-old C57BL/6J mice over 5 consecutive days.	Truncated Netrin-1 is highly expressed in retina during Streptozotocin-induced diabetes. The fragments of Netrin-1 compromise endothelial barrier function.	32
Experimental animal model	<i>Ntn-4</i> <sup>-/-</sup> mice with oxygen-induced retinopathy (OIR) and laser-induced choroidal neovascularization (CNV), mimicking hypoxia-mediated neovascularization and inflammatory mediated angiogenesis.	Netrin-4, but not Netrin-1 mRNA expression, increased in response to relative hypoxia and recovered to normal levels at the end of blood vessel formation. No changes in the retina were found in normoxic <i>Ntn-4</i> <sup>-/-</sup> mice. In OIR, <i>Ntn-4</i> <sup>-/-</sup> mice initially displayed larger avascular areas which recovered faster to revascularization. Ganzfeld electroretinography showed faster recovery of retinal function in <i>Ntn-4</i> <sup>-/-</sup> mice. Expression of Netrin receptors, Unc5H2 (Unc-5 homolog B, <i>C. elegans</i> ) and DCC (deleted in colorectal carcinoma), was found in Müller cells and astrocytes.	24
In vitro experimental model	Isolation of human mesenchymal stem cells from Wharton's jelly of the umbilical cord (WJ-MSC) HUVEC isolation and culture. Conditioned media precipitation and Netrin-1 determination. Histological analysis and immunohistochemistry.	Pharmacological blockage of Netrin-1 in WJ-MSC resulted in diminished angiogenesis on HUVEC. HUVEC were stimulated with exogenous Netrin-1 assayed at physiological concentrations, endothelial vascular migration occurred in a concentration-dependent manner. WJ-MSC-secreted Netrin-1 promotes increased angiogenesis in vivo. Netrin-1, secreted by WJ-MSC, might mediate its angiogenic effect through specific cell surface receptors on the endothelium, such as UNC5b and/or integrin $\alpha 6 \beta 1$ , expressed in HUVEC.	38
Clinical and animal study	50 proliferative DR patients with tractional detachment and 30 patients with idiopathic macular epiretinal membrane (IMEP) served as the control group. Eighty adult male SD rats were randomly divided into the experimental group and control group.	RT-PCR detected Netrin-1, Netrin-4, and VEGF mRNA expression in patient fibro vascular membranes, the epiretinal membrane, and SD rat retinas, respectively. Netrin-1 and Netrin-4 are weakly expressed in the fibro vascular membranes of PDR patients from the immunofluorescence staining and RT-PCR analysis. Exogenous injection of Netrin-1 and Netrin-4 have efficiently protective effects on reducing vascular permeability of DR. Aggrandize concentrations of Netrin-1 and Netrin-4 can reduce VEGF levels in DR.	39
Experimental animal study	Vessel segmentation and analysis were performed using MATLAB. Retina flat-mount and Fundus autofluorescence Scotopic Ganzfeld ERG was performed in <i>Ntn4</i> <sup>-/-</sup> (C57Bl/6-129SvJ-Netrin4 <sup>tm</sup> ) mice.	NTN4 KO has increased retinal vascular tortuosity and hyaloid artery persistence. NTN4 KO has an increased amount of amoeboid mononuclear phagocytes in the retina. NTN4KO has decreased rod function.	40

**Abbreviations:** NTN4KO, *Ntn4*<sup>-/-</sup>; VEGF, vascular endothelial growth factor; OIR, oxygen-induced retinopathy; VSMC, vascular smooth muscle cell; HUVEC, human umbilical vein endothelial cells; AMD, age-related macular degeneration.

## Preclinical and Clinical Studies on the Level of Netrin in Diabetic Retinopathy

DR has both neural and vascular components; proteins used for both neural and vascular retinal tissues are a potentially important role. Through time the early diagnosis of retinopathy is increasing by different biomarkers related with the neural component.<sup>30,31</sup> Among them, Netrin was studied by various experimental and clinical models.<sup>7,19,24,32,33</sup> The level of Netrin protein in the plasma has been positively correlated with DR. As compared with the normal controls, the level of Netrin was markedly increased in the early phase of DR. As shown in Table 1, different experimental studies revealed that the level of Netrin in the cornea increased substantially at the early phase of DR (Table 1).

## Association of Netrin-1 and -4 with Diabetic Retinopathy

DR is one of the major complications of DM and known to cause visual loss in many cases.<sup>5</sup> It involved a neurovascular lesion that can damage blood vessels and nerves of the retina.<sup>2</sup> Due to that protein mostly found in nerve endings were used as indicators for changes which occurred in the system.<sup>7</sup> Among them, Netrin has been recently considered as a novel biomarker and therapeutic agent for DR. Netrin-1

and -4 has been well studied regarding their role in DR.<sup>39</sup> Alteration of the body's Netrin-1 and -4 level may be reflected as upcoming biological protein to identify retinopathy promptly and to decide its severity.

Angiogenesis secondary to DR causes pericytes cell injury, which is due to infiltration of neutrophils and monocytes like IFN- $\gamma$  and IL-17.<sup>6</sup> Generally, Netrin-1 regulates hypoxia-induced inflammation through COX-2/PGE2 and NF $\kappa$ B pathways during inflammation secondary to hypoxia. It has a protective role in DR by conversing new blood vessel formation and overwhelming inflammation in the cornea<sup>32,38</sup> (Figure 2).

Different studies also proved that the level of Netrin in the cornea significantly increased at the early stage of DR. So considering this protein as a biomarker is imperative to detect the disease as timely before it progresses and causes severe damage.<sup>26,32,34</sup> According to Miloudi et al, Netrin-1 level has been increased in the inflamed retina. In addition, degradation of Netrin-1 by collagenases and MMPs generated fragments, such as the VI-V peptide, that exacerbate retinal edema. Moreover, these data further suggest that cells of the ganglion layer undergo diabetes-induced retinopathy.<sup>32</sup>

Netrin-4 also has a role in vascular remodeling such as an increased retinal blood vessel tortuosity, persistence of the hyaloid artery in adult mice and spontaneous focal retinal

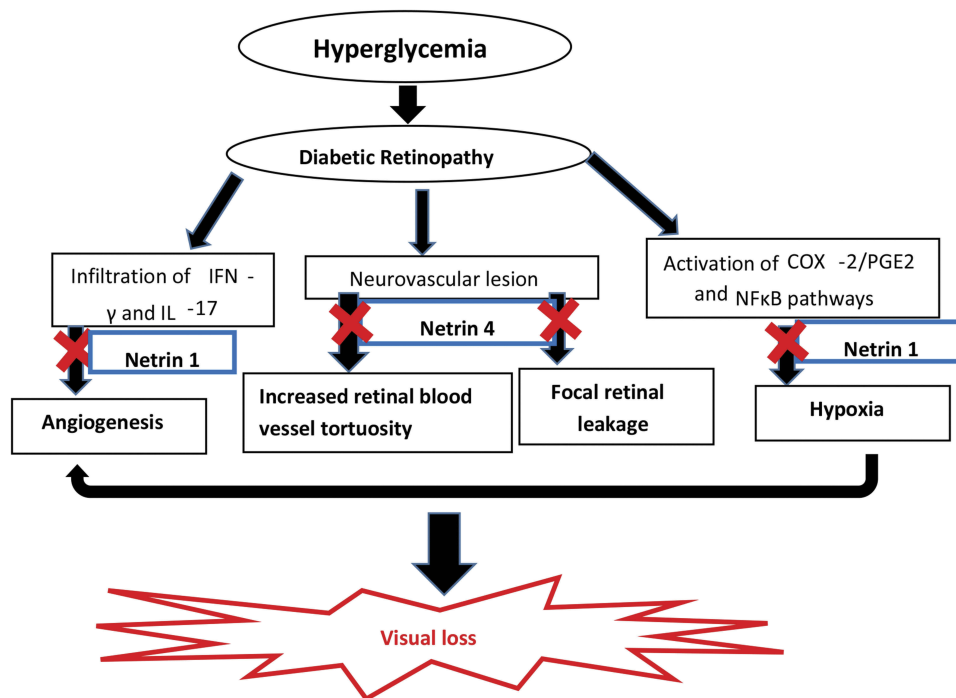


Figure 2 Relation of Netrin-1 and -4 with Diabetic Retinopathy.

leakage.<sup>36,40</sup> Studies done on knockout mice (*Ntn-4*<sup>-/-</sup>) confirmed that lack of Netrin-4 has a major role in the pathophysiology of neovascularization due to ischemia but it does not relate to inflammatory neovascularization<sup>24</sup> (Figure 2).

Unlike other biomarkers, various preclinical/clinical studies proved that administration of exogenous Netrin (Netrin-1 and -4) has a therapeutic effect (Table 1).<sup>19,25,36,39</sup> According to Crespo-Garcia et al, exogenous administration of Netrin-1 and -4 into the vitreous of diabetic rats reduced retinal vascular permeability. These effects may relate to the potential role of Netrin-1 in vascular repair, inhibition of angiogenesis, and preferentially preserves the remaining normal vasculature to supply ischemic tissue during retinopathy. In addition, Netrin-4 acts as an antiangiogenic factor through the regulation of both endothelial and perivascular cells in the retina.<sup>40</sup> Netrin-4 decreased human umbilical vein endothelial cells (HUVECs) viability in a dose dependent manner by regulating HUVEC proliferation and apoptosis. It also prevented corneal neovascularization, enhanced the regression of corneal neovascularization, and inhibited apoptosis in alkali-burned rat corneas. Another study done by Prieto et al<sup>38</sup> revealed that Netrin aroused endothelial cell migration in vitro at minimal concentrations while at higher concentrations it tended to promote endothelial cell migration to a smaller degree.

## Conclusion and Future Perspectives

The review revealed that Netrin-1 and -4 are a potential to be an alternative biomarker for DR, and exogenous supplementation of these proteins can be used as a therapeutic agent. Change of the retinal/corneal Netrin-1 and -4 levels may be reflected as upcoming biological protein to identify DR promptly, which in turn becomes eye-opening for novel drug discovery for this upsetting disease condition. Further detailed animal and human studies are required to advance the role of Netrin as a novel biomarker for DR. Additionally, further investigations are needed to determine the mechanisms by which Netrin is used as a therapeutic agent for the treatment of DR.

## Abbreviations

DCC, deleted in colorectal cancer receptors; DM, diabetes mellitus; DME, diabetic macular edema; DR, diabetic retinopathy; HIF-1, hypoxia-inducible factor 1; HUVECs, human umbilical vein endothelial cells; KO, knockout; OIR, oxygen-induced retinopathy; UNC5, uncoordinated 5 receptors; VEGF, vascular endothelial growth factor.

## Author Contributions

All authors contributed to data analysis, drafting, or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare that there is no conflict of interest.

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