Is Netrin-1 Deficiency Responsible for Inflammation and Systemic Diseases Related to Pseudoexfoliation?

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Precis: Serum netrin-1 levels are significantly lower in patients with pseudoexfoliation syndrome (PES) and pseudoexfoliative glaucoma (PEG) compared with the control group.

Purpose: To investigate serum netrin-1 levels in PES and PEG patients and to determine the relevance of this molecule in the etiopathogenesis of PES-related and PEG-related diseases.

Materials and Methods: This prospective study included 29 PES and 17 PEG patients in the study groups and age-sex matched 47 cataract patients without pseudoexfoliative accumulation as a control group. Serum netrin-1 levels were measured by enzyme-linked immunosorbent assay.

Results: Serum netrin-1 level was significantly lower in the PES and PEG groups compared with the control group (P = 0.007). Multinomial logistic regression analysis was performed in terms of netrin-1 levels ≤ 712.9 pg/mL, > 712.9 pg/mL and sex which may affect PES and PEG. It was found that netrin-1 was a significant negative predictor for PES (odds ratio, 3.45; 95% confidence interval, 1.230-9.716; \( P = 0.019 \)) and PEG (odds ratio, 3.57; 95% confidence interval, 1.008-12.669; \( P = 0.049 \)), respectively.

Conclusions: Decreased serum netrin-1 levels were detected in PES and PEG patients, similar to atherosclerosis and Alzheimer disease. Inflammation lays behind in the common pathogenesis of these diseases. Therefore, netrin-1 promises a potential anti-inflammatory role.

Key Words: pseudoexfoliation syndrome, pseudoexfoliative glaucoma, netrin-1, inflammation

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Pseudoexfoliation syndrome (PES) is an age-related disease with progressive abnormal fibrillar extracellular material accumulation in many ocular and extracocular tissues. Pseudoexfoliative material accumulates in the pupillary margin, iridocorneal angle, anterior lens capsule, zonules, and anterior vitreous in the eye. As a result of the accumulation, various complications such as pseudoexfoliative glaucoma (PEG), cataract formation, weak pupillary dilation, posterior synechiae, zonular weakness, and pseudouveitis may occur.1-3 Exfoliation material also accumulates in many extracocular tissues such as lung, heart, gall bladder, liver, kidney, blood vessels, extraocular muscles, and cerebral meninges.3-5

In recent years, the association between PES and several systemic diseases such as abdominal aortic aneurysm, atherosclerosis, coronary artery disease, systemic endothelial dysfunction, vein occlusions, renal artery stenosis, transient ischemic attacks, coronary artery ectasia, retinal artery and middle cerebral artery blood flow velocities, ischemic brain lesions and Alzheimer disease (AD) was reported.6-13 PES is accompanied by systemic low-grade inflammation and higher levels of inflammatory molecules such as plasma homocysteine, interleukin-6, interleukin-1β, and C-reactive protein were detected in PES patients.14-17

Netrins are a small family of highly conserved guide molecules in glycoprotein structure, which were discovered for the first time in a nematode, Caenorhabditis elegans. They direct cell and axon migration as extracellular proteins, during embryogenesis. Netrin family has 5 defined members: netrin-1, netrin-3, netrin-4, netrin-G1, and netrin-G2. Netrin-1 has substantial roles in physiological responses such as angiogenesis and inflammatory suppressive effects.18 Several studies investigated the anti-inflammatory effects19-21 and its relationship with atherosclerosis22-24 and AD.25,26

According to recent literature, none of the previous studies investigated serum netrin-1 levels in PES and PEG patients. This study investigated the serum netrin-1 levels in PES and PEG patients. Thus, it is aimed to assess the roles of this molecule in common pathogenesis of PES, PEG, and related diseases.
receiving medications such as HMG-CoA reductase inhibitors, corticosteroids, and non-steroidal anti-inflammatory drugs including acetylsalicylic acid were also excluded from the study. In contrast, patients receiving antihypertensive medications and whose blood pressure was <140/90 mm Hg were included in the study.

All patients underwent a routine ophthalmologic examination including best-corrected visual acuity, slit-lamp examination, intraocular pressure measurement, gonioscopy, pachymetry, dilated fundus examination, retinal nerve fiber layer (RNFL) thickness analysis, and 30/2 Humphrey visual field test. In patients who had dense cataracts that caused a misinterpretation of the results of RNFL thickness analysis and 30/2 Humphrey visual field test, the examinations were performed when the optic medium was transparent after phacoemulsification surgery. Participants with a normal optic disc appearance, visual field test, RNFL thickness analysis, intraocular pressure measurement ≤21 mm Hg, and a white fluffy pseudoexfoliation material accumulation and/or transillumination defect in the iris near the pupil in the ophthalmoscopic examination were diagnosed as PES. Those with a history of confirmed glaucoma diagnosis by optic disc and perimetry findings, and the characteristic white fluffy pseudoexfoliation material accumulation on the anterior lens capsule or the pupillary edge, and iris near the pupil in the ophthalmoscopic examination were diagnosed as PEG. The control group included age-sex–matched cataract patients without any pseudoexfoliative material accumulation.

Blood Sampling
Blood samples (5 mL) were collected from all participants through venous puncture to the serum separator tubes, just before patients were prepared for cataract surgery after overnight 8-hour fasting. After coagulation occurs the tubes were centrifuged at 3000g for 10 minutes to obtain serum samples. The serum samples were stored at −20°C until the analysis was done.

Netrin-1 Analyses
Serum netrin-1 levels were determined by ELISA (SunRed Bio, China) as suggested by the manufacturer. According to the manufacturer instructions, the sensitivity of the assay was 9.711 pg/mL, the assay range was 12 to 300 pg/mL, and intra-assay and interassay CVs were <12%.

Statistical Analyses
Statistical analyses were performed using version 23.0 of the SPSS software package for Windows (SPSS Inc., Chicago, IL). The results were expressed as mean ± SD, median, minimum, and maximum. Kolmogorov-Smirnov tests were used to determine whether variables were distributed normally. Sex was compared using the χ² test. The 1-way analysis of variance test and Kruskal-Wallis test were used to compare variables among groups. Bonferroni-corrected Mann-Whitney U test was used for comparisons between subgroups. The receiver operating characteristic (ROC) curve and the area under the ROC curve were used to discriminate the control group from PES and PEG groups. On the basis of Youden index, the best cutoff value for netrin-1 was determined. Multinominal logistic regression analysis was performed by creating a model including factors affecting the presence of PES and PEG, such as sex and netrin-1 levels. All analyses were performed with a power of 95% confidence interval (CI). An overall P-value of <0.05 was considered as statistically significant.

RESULTS
Table 1 shows the demographic and clinical data of the 93 individuals who participated in this study. There was no statistically significant difference between the groups in terms of age, sex, and hypertension.

The netrin-1 levels of groups are described in Table 2. The median (minimum to maximum) of netrin-1 levels in patients of control, PES, and PEG groups were 719.60 (208.70 to 2609.00) pg/mL, 615.00 (229.40 to 1019.00) pg/mL, and 556.60 (383.60 to 853.00) pg/mL, respectively (Table 2). The median (minimum to maximum) of netrin-1 levels in patients with PES and PEG were significantly less than those in the

| TABLE 1. Demographic and Clinical Data by Study Groups |
|-----------------------------------------------|
| Groups                        | Control Group | PES Group | PEG Group | P   |
| Age (mean ± SD)          | 72 ± 9        | 74 ± 7    | 72 ± 7    | 0.535* |
| Sex, n (%)                  |               |           |           |      |
| Female                       | 27 (58.7)     | 12 (26.1) | 7 (15.2)  | 0.298†  |
| Male                         | 20 (42.6)     | 17 (36.2) | 10 (21.3) |        |
| HT, n (%)                    |               |           |           |      |
| No                           | 28 (52.8)     | 16 (30.2) | 9 (17.0)  | 0.931†  |
| Yes                          | 19 (47.2)     | 13 (29.8) | 9 (17.0)  |        |

*One-way analysis of variance.  †Kruskal-Wallis test.

| TABLE 2. The Netrin-1 Level of the Patients in All Groups |
|-----------------------------------------------|
| Groups                        | Control Group | PES Group | PEG Group | P*   | P†   | P‡   | P§   |
| Netrin-1 levels (pg/mL)       |               |           |           |      |      |      |      |
| Median                       | 719.6         | 615.0     | 556.6     | 0.007| 0.012| 0.009| 0.690|
| Minimum                      | 208.7         | 229.4     | 383.6     |      |      |      |      |
| Maximum                      | 2609.0        | 1019.0    | 853.0     |      |      |      |      |

*Kruskal-Wallis test.  †Comparison of control versus PES.  ‡Comparison of control versus PEG.  §Comparison of PES versus PEG.  PEG indicates pseudoexfoliation glaucoma; PES, pseudoexfoliation syndrome.
control group ($P = 0.007$) (Fig. 1). The Bonferroni corrected Mann-Whitney $U$ test was performed to compare subgroups, and the significance level was set as $P < 0.017$. There were significant differences between the control group and both of the PES and PEG groups ($P = 0.012$ and 0.009, respectively). However, there was no significant difference between the PES and PEG groups in terms of netrin-1 levels (Fig. 1).

We calculated the best cutoff value on the basis of Youden index and the sensitivity and specificity in that criteria. In the ROC analysis, the precision of netrin-1 levels in predicting pseudoexfoliative groups (PES and PEG) compared with control group was moderate and the area under the ROC curve was 0.689 (95% CI, 0.584-0.782) with a sensitivity and specificity of 76.09% (95% CI, 61.2-87.4) and 55.56% (95% CI, 65.3-93.4), respectively. The best cutoff values for netrin-1 levels was <712.9 mg/mL and revealed a statistically significant difference ($P = 0.0007$) (Table 3; Fig. 2).

Multinominal logistic regression analysis was performed in terms of netrin-1 levels $\leq 712.9$ pg/mL; $> 712.9$ pg/mL and sex which may affect PES and PEG. It were found that netrin-1 was a significant negative predictor for PES [odds ratio (OR), 3.45; 95% CI, 1.230-9.716; $P = 0.019$] and PEG (OR, 3.57; 95% CI, 1.008-12.669; $P = 0.049$), respectively (Table 4).

**DISCUSSION**

In this study, we detected lower serum netrin-1 levels in PES and PEG patients compared with the control group. Moreover, serum netrin-1 seems to possess a wide spectrum of anti-inflammatory effects, on the basis of the current literature that emphasized the relationship between PES and inflammatory states or elevated proinflammatory agents. Netrin-1 is expressed and regulated by human coronary artery endothelial cells. Netrin-1 expression increases with atheroprotective laminar flow and decreases with inflammatory cytokines. Besides, it has anti-inflammatory, monocyte adhesion, and migration inhibitory effects on vascular endothelium. It was also demonstrated that netrin-1 protects endothelial barrier function. Previous studies reported the relationship between PES and several vascular pathologies such as ocular ischemia, iris hypoperfusion, anterior chamber hypoxia, decreased ocular, and retrobulbar microvascular and macrovascular blood flow, decreased blood flow in the neural rim and lamina cribrosa, and decreased blood flow in the peripapillary retina and optic nerve rim. Our results suggest that netrin-1 deficiency contributes to the ocular vascular pathologies related to PES.

Atalar and colleagues reported that patients with PES had arterial endothelial dysfunction. They attributed this dysfunction to the accumulation of pseudoexfoliative material in vascular tissue. Consistently, the results of this study demonstrated that netrin-1 deficiency contributes to the arterial endothelial dysfunction seen in PES.

Inflammation is one of the common pathogenesis of PES with atherosclerosis. It has a substantial role in all phases of atherosclerosis. An animal study revealed that

**TABLE 3. Receiver Operating Characteristic Curves and AUC for Netrin-1 Levels**

| Criterion | Sensitivity | 95% CI | Specificity | 95% CI | +LR | 95% CI | AUC | P   |
|-----------|-------------|--------|-------------|--------|-----|--------|-----|-----|
| $\leq 712.9$ | 76.09 | 61.2-87.4 | 55.56 | 40.0-70.4 | 1.71 | 1.2-2.5 | 63.6 | 0.0007 |

AUC indicates area under curve; CI, confidence interval; LR, likelihood ratio.
the level of netrin-1 in the vascular tissue of atherosclerotic mice was decreased.\textsuperscript{22} Moreover, Munoz et al\textsuperscript{24} found that the level of serum inflammatory mediators was elevated and the netrin-1 level was diminished in individuals with subclinical atherosclerosis compared with the healthy controls. In this aspect, the netrin-1 deficiency in atherosclerosis supports our study results and also, shows one another common pathogenesis of PES and atherosclerosis.

The relationship between AD and PES was previously investigated.\textsuperscript{11–13} Sun et al\textsuperscript{25} found that netrin-1 levels were low in both cerebrospinal fluids and serum of experimentally AD model compared with healthy mice. Lourenço et al\textsuperscript{26} reported decreased netrin-1 expression in the AD model transgenic mice. All these studies demonstrating the low netrin-1 levels in AD, were consistent with our study results. Thus, our study suggests new common pathogenesis for PES and AD coexistence.

The PES is associated with systemic inflammation and netrin-1, which is an anti-inflammatory molecule, seems to be insufficiently secreted because of several factors also leading to PES. This hypothesis is also supported by the multinominal logistic regression analysis which revealed that PES occurs 3.45 times and PEG occurs 3.57 times more in netrin-1 deficient patients. Therefore, the most

FIGURE 2. Receiver operating characteristics curve of netrin-1 levels. AUC indicates area under curve. Figure 2 can be viewed in color online at www.glaucomajournal.com.

| Groups* | B   | SE  | P     | OR   | Lower Bound | Upper Bound |
|---------|-----|-----|-------|------|-------------|-------------|
| PES group | Intercept | -0.964 | 0.494 | 0.051 | 0.211 | 1.470 |
| Sex = F | -0.585 | 0.495 | 0.237 | 0.557 | 0.211 | 1.470 |
| Sex = M | 0 | — | — | — | — | — |
| Netrin-1 ≤ 712.9 | 1.241 | 0.527 | 0.019 | 3.458 | 1.230 | 9.716 |
| Netrin-1 > 712.9 | 0 | — | — | — | — | — |
| PEG group | Intercept | -1.520 | 0.610 | 0.013 | 0.174 | 1.755 |
| Sex = F | -0.592 | 0.589 | 0.315 | 0.553 | 0.174 | 1.755 |
| Sex = M | 0 | — | — | — | — | — |
| Netrin-1 ≤ 712.9 | 1.274 | 0.646 | 0.049 | 3.574 | 1.008 | 12.669 |
| Netrin-1 > 712.9 | 0 | — | — | — | — | — |

\( R^2 = 0.10 \) (Cox-Snell), 0.12 (Nagelkerke). Model \( \chi^2 (4) = 10.37, \ P = 0.35 \).

*The reference category is control.

\(^{†}\) \( P < 0.05 \).

\( B \) indicates \( \beta \) coefficient; CI, confidence interval; F, female; M, male; OR, odds ratio; PEG, pseudoexfoliation glaucoma; PES, pseudoexfoliation syndrome.
remarkable result of this study is netrin-1 deficiency may also be one of the etiological factors causing PES.

Nevertheless, future studies may also shed light on the role of serum netrin-1 deficiency in cerebrovascular and cardiovascular diseases such as PES and/or PEG, as well as other vein occlusions, aortic aneurysm, and transient ischemic attacks. Besides, that would be interesting to investigate the level of aqueous humor netrin-1 to evaluate its role in ocular inflammation of PES/PEG and progression from PES to PEG.

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