

Nampt/PBEF/visfatin serum levels: a new biomarker for retinal blood vessel occlusions

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Abstract: The main objective of the study was to quantify serum levels of nicotinamide phosphoribosyltransferase (Nampt/pre-B-Cell colony-enhancing factor 1/visfatin) in subjects with a history of retinal vascular occlusions (RVOs), disease conditions characterized by pronounced ischemia, and metabolic energy deficits. A case-control study of 18 subjects with a history of RVO as well as six healthy volunteers is presented. Serum Nampt levels were quantified using a commercially available enzyme-linked immunosorbent assay kit. Serum Nampt levels were 79% lower in patients with a history of RVO compared with that in healthy volunteers ($P < 0.05$). There was no statistically significant difference among the types of RVOs, specifically branch retinal vein occlusions ($n=7$), central retinal vein occlusions ($n=5$), hemiretinal vein occlusions ($n=3$), and central retinal artery occlusions ($n=3$; $P=0.69$). Further studies are needed to establish the temporal kinetics of Nampt expression and to determine whether Nampt may represent a novel biomarker to identify at-risk populations, or whether it is a druggable target with the potential to ameliorate the long-term complications associated with the condition, ie, macular edema, macular ischemia, neovascularization, and permanent loss of vision.

Keywords: Nampt, PBEF, visfatin, nicotinamide phosphoribosyltransferase, pre-B-cell colony-enhancing factor, retinal artery occlusion, retinal vein occlusion, biomarker, retina, vasculature

Introduction

Retinal vascular occlusions (RVOs) can present as retinal vein occlusions or retinal artery occlusions and are common causes of irreversible vision loss.¹ These occlusions can include either the central retinal vein or artery (CRVO or CRAO) or a branch vessel (BRVO or BRAO).¹ Of these, retinal vein occlusions are the most common and also are a common cause of retinal vascular disease, second only to diabetic retinopathy.² RVOs are prevalent in about 0.5% of the population, with an increased prevalence with increasing age, and some variance in terms of race and ethnicity.³ About half of these cases occur in patients older than 65 years of age, and more than half of these cases are found in patients with cardiovascular diseases such as hypertension, diabetes mellitus, and dyslipidemia. Compromised retinal venous outflow due to intraocular pressure, such as seen in open-angle glaucoma, can produce stasis, predisposing to RVOs.⁴

Vision loss in RVOs is most commonly caused by macular edema.⁵ Obstruction within blood vessels leads to serious exudation and, subsequently, macular edema. Permanent edema can lead to degeneration and changes such as subsequent macular edema and epiretinal membranes, ultimately resulting in vision loss.² Similarly, retinal ischemia increases the release of vascular endothelial growth factors (VEGFs), causing retinal neovascularization, which may in turn lead to retinal hemorrhage or glaucoma secondary to neovascularization.^{6,7}

Various treatment options have been explored; however, outcomes remain unsatisfactory. The current “gold standard” of treatment for BRVO is laser photocoagulation, which has been shown to reduce the risk of vision loss and to improve vision in up to two-thirds of patients with macular edema due to BRVO.⁸ Various newer treatment options include dexamethasone implants and anti-VEGF therapy, which help decrease central macular thickness, thereby promoting a clinically significant increase in visual acuity.⁹

However, currently there are no clinically or diagnostically relevant biomarkers that help predict an individual's risk for developing an RVO or even for disease and therapy outcome. In a first step to identifying novel predictive and diagnostic biomarkers and potential drug targets, we herein quantified the serum levels of Nampt in subjects with RVO and in healthy volunteers.

Nampt as nicotinamide phosphoribosyltransferase functions as an enzyme of critical importance for cellular energy metabolism. In addition, Nampt has been characterized as a proinflammatory cytokine that also possesses neuroprotective properties.¹⁰ Besides these physiological functions, Nampt has been correlated with a number of pathological states, including acute lung injury, rheumatoid arthritis, Leber congenital amaurosis,¹¹ and possibly diabetes mellitus (for review, see Sun et al¹⁰).

Nampt is the rate-limiting enzyme that catalyzes the first step in the biosynthesis of nicotinamide adenine dinucleotide from nicotinamide (for review, see Imai¹²). Given the fact that the retina is one of the tissues with the highest energy demand in the body, with most energy derived from oxidative metabolism,^{13,14} Nampt is a critical metabolic enzyme required for an adequate energy supply. Inhibiting Nampt activity results in detrimental consequences on retinal energy metabolism and in retinal toxicity,¹¹ analogously to mutations in nuclear nicotinamide mononucleotide adenylyltransferase (Nampt) in Leber congenital amaurosis.^{11,15,16}

Our results indicate significantly lower circulating Nampt levels in patients with fully resolved RVO compared with the same in healthy volunteers. Our data provide a strong rationale for performing future studies investigating the temporal kinetic profile of Nampt expression, from the acute phase of the RVO through the treatment and resolution phase, to determine the feasibility of using low serum Nampt levels as a novel biomarker for RVOs.

Methods

Subjects

The clinical research presented herein adhered to the tenets of the Declaration of Helsinki and was approved by

the Institutional Review Board. Subjects were identified in the Eye Clinic at Truman Medical Centers (Kansas City, MO, USA) based on the diagnosis of RVO. We enrolled 18 subjects with a previous diagnosis of RVO and six healthy, age-matched volunteers into our study. The patient demographics are shown in Table 1, and clinical parameters are presented in Table 2. All patients had fully resolved RVO; average time between RVO event and biomarker assessment was 6±2 months. None of the patients had a history of ophthalmological problems before the RVO.

Sample processing

For quantification of serum Nampt levels, 13 mL of blood was collected by venipuncture in a BD Vacutainer® venous blood collection tube with clot activator and gel for serum separation (BD, Franklin Lakes, NJ, USA). Serum was prepared according to the manufacturer's recommendations of inversion and centrifugation. Serum was aliquoted and stored at -80°C until use in experiments.

Table 1 Patient demographics (18 eyes of 18 patients)

Characteristic	N	%
Age (years)		
<40	0	0
40–49	2	11.1
50–59	8	44.4
60–69	6	33.3
≥70	2	11.1
Sex		
Male	7	38.9
Female	11	61.1
Study eye		
OD	11	61.1
OS	7	38.9
Clinical manifestation		
HRVO	3	16.7
BRVO	7	38.9
CRVO	5	27.8
CRAO	3	16.7
Treatment		
Anti-VEGF	11	61.1
Laser (focal or PRP)	11	61.1
Anti-VEGF + laser	7	38.9
PBEF gene polymorphisms		
T1001G	0	0
C1535T	0	0

Notes: Patient demographics are summarized. A total of 18 subjects (mean age: 59.1±2.1 years) with RVO were enrolled in the study. We tested for two PBEF1 gene polymorphisms, T1001G and C1535T, based on the study by Ye et al.¹⁹ None of the subjects carried a SNP allele.

Abbreviations: BRVO, branch retinal vein occlusion; CRAO, central retinal artery occlusion; CRVO, central retinal vein occlusion; HRVO, hemiretinal vein occlusion; Nampt, nicotinamide phosphoribosyltransferase; OD, right eye; OS, left eye; PBEF, pre-B-cell colony-enhancing factor; PRP, pan-retinal photocoagulation; RVO, retinal vascular occlusion; SNP, single-nucleotide polymorphism; VEGF, vascular endothelial growth factor.

Table 2 Clinical parameters of subjects with RVOs

ID	Age	Sex	Race	BMI	Event	Eye	Anti-VEGF y/n	Laser, y/n	BSCVA (initial)	BSCVA (final)	logMAR (initial)	logMAR (final)	Δ log MAR	Nampt (ng/mL)
1	57	Male	Black	34	HRVO	OS	Yes	Yes	20/CF	20/200	2	1	1	0.155
2	56	Male	White	39	BRVO	OD	No	Yes	20/200	20/200	1	1	0	0.54
3	62	Female	Black	38	CRVO	OD	No	Yes	20/CF	20/200	2	1	1	0.099
4	67	Female	Black	31	BRVO	OD	Yes	Yes	20/400	20/30	1.3	0.2	1.1	0.086
5	54	Male	Black	26	CRVO	OS	Yes	Yes	20/CF	20/100	2	0.7	1.3	0.291
6	46	Male	White	30	CRVO	OD	Yes	Yes	20/400	20/CF	1.3	2	−0.7	0.094
7	52	Male	White	28	BRVO	OS	Yes	No	20/50	20/50	0.4	0.4	0	0.104
8	62	Male	White	41	BRVO	OD	Yes	Yes	20/300	20/60	1.2	0.5	0.7	0.874
9	63	Female	Black	44	BRVO	OD	Yes	Yes	20/25	20/30	0.1	0.2	−0.1	0.064
10	50	Female	White	32	HRVO	OS	Yes	Yes	20/300	20/80	1.2	0.6	0.6	0.101
11	61	Female	Black	46	CRVO	OD	Yes	No	20/CF	20/300	2	1.2	0.8	0.079
12	49	Female	Black	22	HRVO	OD	Yes	No	20/CF	20/400	2	1.3	0.7	0.183
13	83	Female	Black	40	CRAO	OD	No	Yes	HM	NLP	3	^a	^a	0.252
14	59	Male	Black	29	CRVO	OD	No	No	HM	HM	3	3	0	0.103
15	66	Female	Black	51	CRAO	OD	No	No	20/25	20/20	0.1	0	0.1	0.224
16	54	Female	Black	41	BRVO	OS	No	No	20/30	20/20	0.2	0	0.2	0.178
17	53	Female	Black	62	CRVO	OD	Yes	No	20/400	20/200	1.3	1	0.3	0.145
18	70	Female	Black	45	BRVO	OS	No	Yes	20/200	20/40	1	0.3	0.7	0.099

Notes: Relevant clinical parameters describing manifestation, intervention, and visual acuity for subjects with RVO. The concentration of serum Nampt is provided for each subject. ^aLight perception is not an actual visual acuity measurement and can therefore not be converted to a numerical logMAR score.³¹

Abbreviations: BMI, body mass index; BRVO, branch retinal vein occlusion; BSCVA, best spectacle-corrected visual acuity; CF, counting fingers; CRAO, central retinal artery occlusion; CRVO, central retinal vein occlusion; HM, hand motion; HRVO, hemiretinal vein occlusion; logMAR, logarithm of the minimum angle of resolution; Nampt, nicotinamide phosphoribosyltransferase; NLP, no light perception; OD, right eye; OS, left eye; PRP, pan-retinal photocoagulation; RVO, retinal vascular occlusion; SNP, single-nucleotide polymorphism; VEGF, vascular endothelial growth factor; y/n, yes/no.

Enzyme-linked immunosorbent assay

Serum Nampt levels were quantified from subjects' sera using a commercially available and previously validated assay (Adipogen, San Diego, CA, USA),¹⁷ according to the manufacturer's instructions and using a Synergy H1 plate reader (Biotek, Winooski, VT, USA). Serum samples were tested in triplicates, and the results were validated by quantification using three separate enzyme-linked immunosorbent assay (ELISA) plates. The standard curve was established using a second-order polynomial (quadratic) fit in Prism software v5 (GraphPad Inc, La Jolla, CA, USA), according to the manufacturer's recommendations, which yielded a goodness of fit of $R^2=0.9996$ (Figure 1A).

Extraction of genomic DNA and single-nucleotide polymorphism analysis

Genomic DNA was extracted from the blood clots remaining after serum separation, essentially as described previously,¹⁸ in combination with a commercial DNA extraction kit (QIAamp DNA Blood Maxi Kit, Qiagen Inc, Germantown, MD, USA).

Purity and concentration of genomic DNA were assessed using absorbance at 260, 280, and 320 nm using a NanoVue™ spectrophotometer (GE Healthcare, Little Chalfont, Buckinghamshire, UK). We used molecular beacon technology for the custom designing of single-nucleotide polymorphism

(SNP) genotyping probes (sequences are listed in Table 3; Sigma-Aldrich Inc, St Louis, MO, USA) to determine the presence of T1001G and C1535T polymorphisms in the *PBEF1* gene. Molecular beacons were labeled with either 6-carboxyfluorescein (6FAM) or hexachloro-6-carboxyfluorescein (HEX) at the 5'-end and with the quencher Black Hole Quencher 1 (BHQ1®) at the 3'-end (Table 3). SNP genotyping was performed using Taqman Universal Mastermix and a StepOne Plus real-time polymerase chain reaction machine (both Applied Biosystems; Life Technologies Inc, Foster City, CA, USA), using 54.5°C as the annealing temperature. Analysis was performed using the SDS software v4 (Applied Biosystems). The probes were validated using a restriction digest approach, as described in Ye et al¹⁹ (data not shown). The polymorphisms T1001G and C1535T (originally described as C1543T¹⁹) had been identified as predisposing individuals to acute lung injury associated with sepsis.¹⁹

Statistics

Data are presented as individual data points on scatter plots; error bars represent the standard error of the mean. Nampt levels were compared between healthy volunteers and subjects with a history of RVO using the Student's *t*-test. To compare subtypes of RVOs, we performed one-way analysis of variance (ANOVA) with Tukey's post hoc test. For correlation analysis, we calculated a Pearson

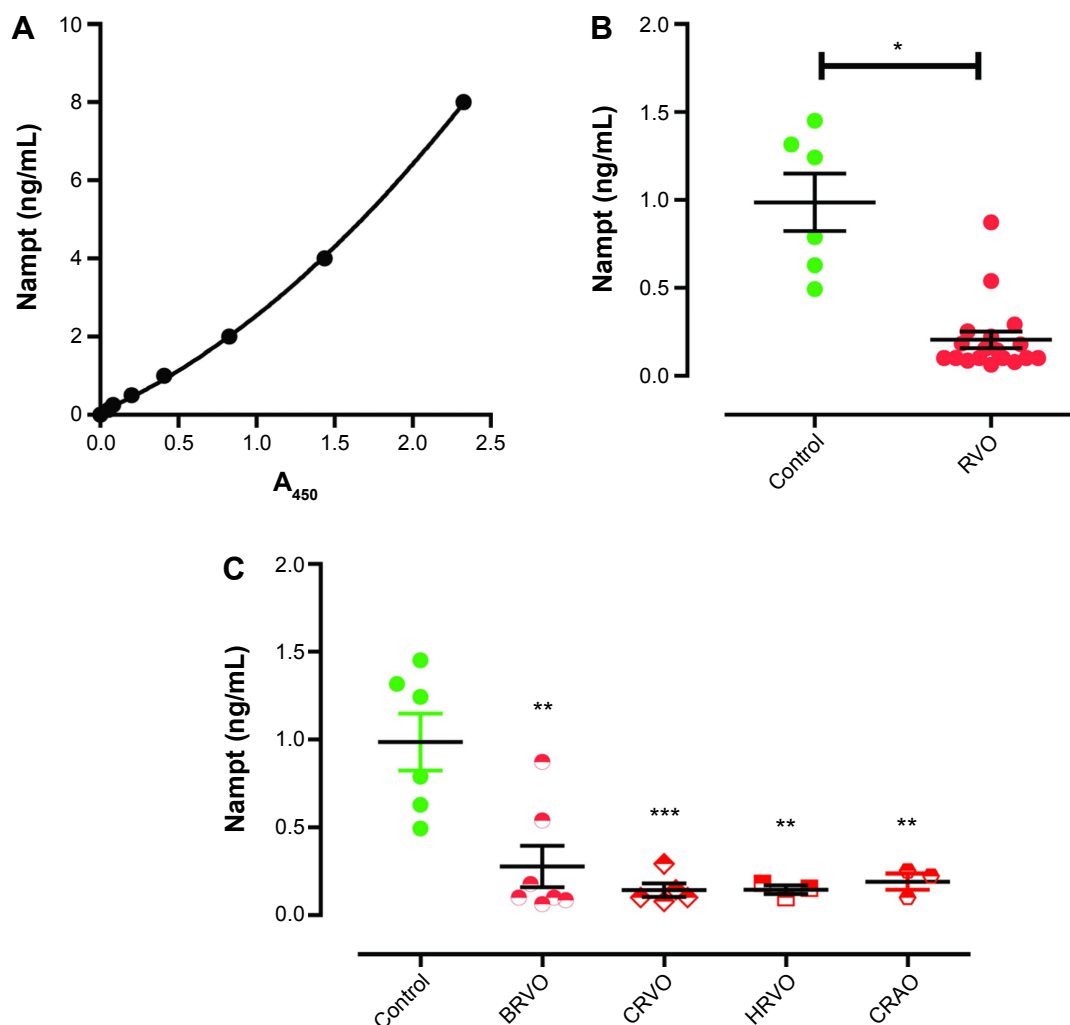


Figure 1 Serum Namp levels are lower in subjects with RVOs.

Notes: (A) Standard curve for serum Namp levels obtained using a commercially available ELISA kit. The R^2 value indicated the goodness of fit after fifth-parameter nonlinear fit regression. (B) Serum Namp levels are lower in subjects with RVOs compared with healthy volunteers ($n=6$ controls, $n=18$ subjects with RVO; unpaired t-test: $P < 0.05$). (C) There is no statistically significant difference in serum Namp levels among different types of RVOs. Bars represent mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Abbreviations: A_{450} , absorbance at 450 nm; BRVO, branch retinal vein occlusion; CRAO, central retinal artery occlusion; CRVO, central retinal vein occlusion; ELISA, enzyme-linked immunosorbent assay; HRVO, hemiretinal vein occlusion; Namp, nicotinamide phosphoribosyltransferase; RVO, retinal vascular occlusion; SEM, standard error of the mean.

Table 3 SNP determination

Type	Sequence (5'→3')	Label (5'/3')
SNP: T1001G		
Probe (T)	TTCTAACACATAATTGAGGTCTTTCT	6FAM/BHQ I
Probe (G)	TTCTAACACATAAGTGAGGTCTTTCT	HEX/BHQ I
Sense primer	AGGACATAAAGATCATAGC	None
Antisense primer	CACTGAGTTTGGGATATC	None
SNP: C1535T		
Probe (T)	TCCCTCATTTCTTCTGCTCTAGC	6FAM/BHQ I
Probe (G)	TCCCTCATTTCTTCTGCTCTAGC	HEX/BHQ I
Sense primer	CCCTGACCTCATCTTCTA	None
Antisense primer	TCACGTACTCCAGGATAG	None

Notes: Molecular beacons and primers used for SNP genotyping are listed. Molecular beacons were labeled with 6FAM or HEX to allow separation of the signal for each variant. Sequences were designed based on the PBEFI sequence in GenBank accession number AC007032.2, as described in Ye et al.¹⁹

Abbreviations: 6FAM, 6-carboxyfluorescein; BHQ, Black Hole Quencher I; HEX, hexachloro-6-carboxyfluorescein; SNP, single-nucleotide polymorphism.

product-moment correlation coefficient (r) to test the strength of the association. All statistical analysis was done in Prism software v5 (GraphPad Inc).

Results

Serum Namp levels are reduced in RVO

Namp levels in the sera of control subjects were 0.97 ± 0.16 ng/mL ($n=6$; Figure 1B, Table 2), in accordance with the published literature.¹⁷ In contrast, subjects with a history of RVO had statistically significant reduced Namp levels (0.20 ± 0.05 ng/mL, $n=18$, unpaired t -test: $P<0.05$; Figure 1B, Table 2).

We tested whether different types of RVOs were associated with different Namp levels. However, in our cohort, we did not identify any statistically significant differences in serum Namp levels among patients manifesting with hemiretinal vein occlusion (HRVO), BRVO, CRVO, or CRAO ($n=19$, $P=0.69$; Figure 1C). However, each type of RVO was significantly different from controls (ANOVA

$P<0.001$; HRVO: $n=3$, $P<0.01$; BRVO: $n=7$, $P<0.001$; CRVO: $n=6$, $P<0.01$; CRAO: $n=2$, $P<0.01$; Figure 1C).

When we compared the Namp levels between the sexes, we did not find any differences between females and males ($P=0.11$), in accordance with the published literature.²⁰

Furthermore, we correlated the serum Namp levels with the improvement of visual acuity after the RVO event (Figure 2A), as well as the clinical measurements of retinal thickness derived from spectral-domain optical coherence tomography data (Figure 2B–D) and did not identify any statistically significant associations. There was no statistically significant association between the time since the RVO event and serum Namp levels (data not shown).

Known confounding factors affecting serum Namp levels are absent in RVO patients

There are several disease conditions and biometric parameters, including stroke, hepatitis C, and hyperthyroidism,

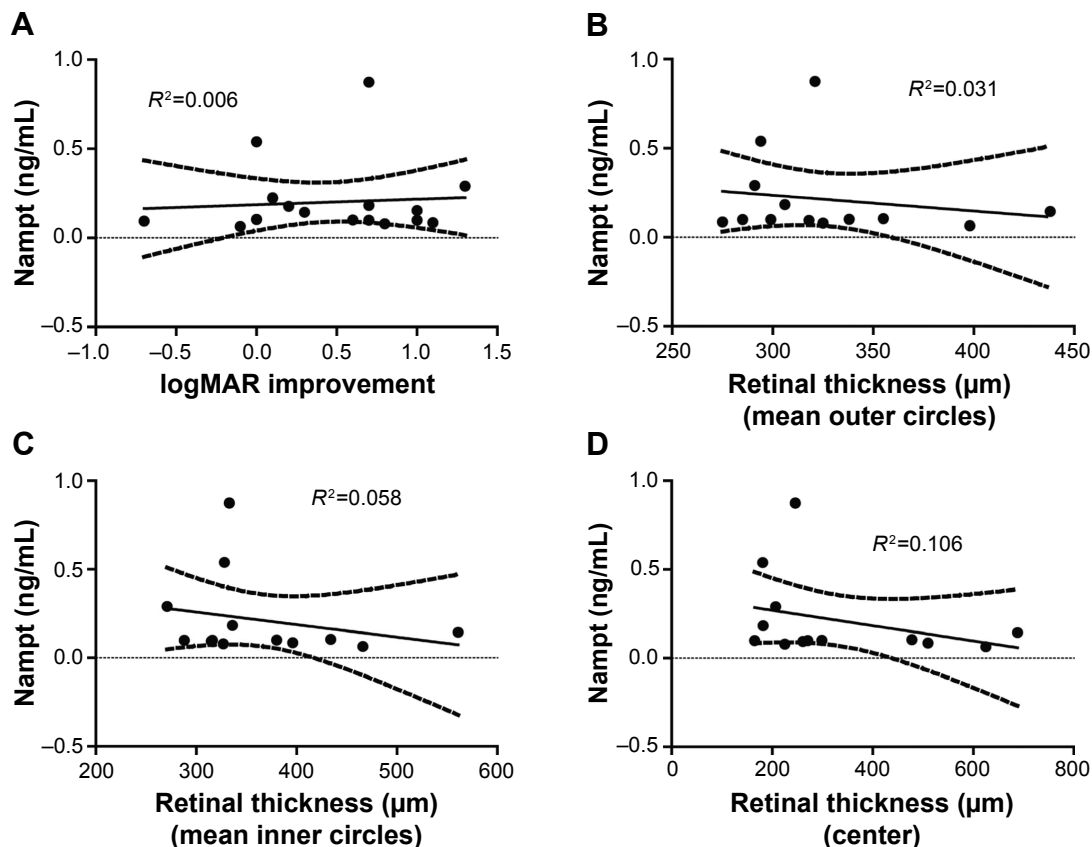


Figure 2 Serum Namp levels do not correlate with visual acuity or SD-OCT findings.

Notes: (A) Serum Namp levels in patients with a history of retinal blood vessel occlusions did not correlate with improvement of visual acuity after clinical intervention, expressed as the improvement in logMAR scores ($R^2=0.006$). (B–D) Furthermore, there was no statistically significant correlation between serum Namp levels and retinal thickness, as determined by SD-OCT. Correlations are shown for serum Namp levels with retinal thickness on the outer and inner circles, as well as the center retinal thickness measurement.

Abbreviations: logMAR, logarithm of the minimum angle of resolution; Namp, nicotinamide phosphoribosyltransferase; SD-OCT, spectral-domain optical coherence tomography.

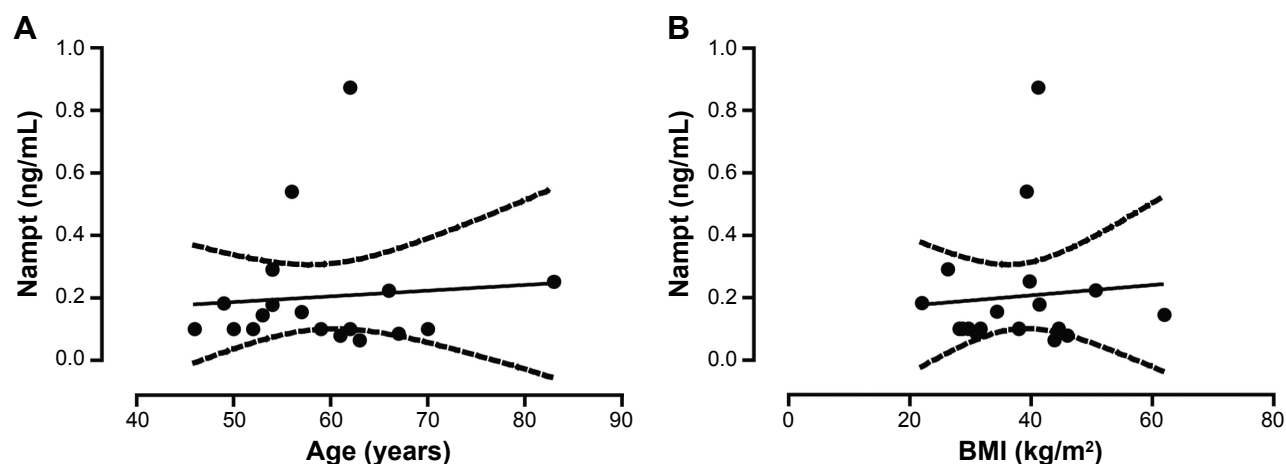


Figure 3 Serum Namp levels do not correlate with age or BMI.

Notes: (A) We tested for an association between serum Namp levels and age by calculating a Pearson product-moment correlation coefficient. The solid line represents the line of best fit ($R^2=0.006$), while the dotted lines represent the 95% confidence interval. There was no statistically significant association between serum Namp levels and age ($P=0.75$). (B) Similarly, we did not identify a statistically significant association between serum Namp levels and BMI ($R^2=0.007$; $P=0.75$).

Abbreviations: BMI, body mass index; Namp, nicotinamide phosphoribosyltransferase.

reported in the scientific and medical literature, which show potential correlations with Namp levels. We, therefore, systematically assessed them within our patient population as potential confounding factors. To determine whether Namp levels were correlated with age or weight, we calculated a Pearson product-moment correlation coefficient for age and body mass index (BMI). In our cohort, serum PBEF levels did not correlate with age ($P=0.75$, $R^2=0.006$; Figure 3A) or BMI ($P=0.75$, $R^2=0.007$; Figure 3B). Similarly, we did not detect any differences among ethnicities (data not shown). For four conditions, namely acute lung injury,¹⁹ chronic kidney disease,²¹ and rheumatoid arthritis²² as well as during chronic hemodialysis,²⁰ serum Namp levels are being discussed as a reflection of a patient's inflammation status rather than as a specifically disease-related change. None of the patients in our study suffered from these or related inflammatory disease conditions.

Additional studies have linked SNPs in the *PBEF1* gene with a predisposition for worse disease and therapy outcomes in acute lung injury and sepsis.^{19,23–25} Regarding the disease conditions and biometric parameters affecting serum Namp levels, we tested the patients' DNA samples for two known polymorphisms.¹⁹ We did not detect the presence of the T1001G or the C1535T polymorphisms in any of our patients (Table 1).

Discussion

We herein describe low serum Namp levels in subjects with a history of RVO. To our knowledge, this is the first report of Namp levels being associated with this disease condition and it represents the identification of the first potential biomarker for RVOs.

The level of serum Namp in healthy volunteers was in good agreement with the concentrations previously reported.¹⁷ Currently, there is significant discrepancy among the different methodologies used to detect Namp, as described in detail by Korner et al.¹⁷ Therefore, we chose a commercially available ELISA assay that has previously been shown to detect Namp accurately and reproducibly from human serum samples with respect to both epitope specificity and the ability to quantify Namp over a wide range of concentrations.¹⁷ Experimental parameters that we took into consideration were specificity of the antibody, the effect of diluted vs undiluted serum samples, and the fit of the standard curve. Specifically, we validated the Namp standard using Western blotting and the validated antibody used by Ye et al¹⁹ (data not shown). Furthermore, using an in-house-generated recombinant human Namp protein in the commercial ELISA yielded comparable serum concentrations that were not statistically significantly different from those reported herein using the provided protein standard (data not shown). Diluting the serum sample did not have any effect on quantitative analysis, further indicating the specificity of the antibody. For quantification, we used a second-order polynomial fit, as recommended by the manufacturer. In addition, we ensured that data points fell on the linear portion of the standard curve. Performing linear regression analysis yielded comparable serum Namp concentrations (data not shown). Overall, our data provide evidence that the ELISA used in the present study resulted in not only high reproducibility but also high specificity for accurately detecting Namp levels in human serum samples.

To exclude known confounding factors affecting serum Namp levels from our study's RVO patient population, we

assessed the patients' medical history, current health status, biometric measures, and genetically predisposing factors associated with the *PBEF1* gene. Our experiments did not reveal any differences in Nampt levels between females and males, in accordance with the published literature.²⁰ Furthermore, the role of Nampt in obesity remains unclear. Several studies suggest a direct correlation and role of the protein in obesity development, whereas other studies could not identify any correlation (Stastny et al²⁶ and references therein). The discrepancy among these studies may result from the chosen method for detecting visfatin,¹⁷ as well as differences in clinical trial design and statistical power.

Previous studies have identified increases in Nampt serum levels to be associated with acute lung injury,¹⁹ chronic kidney disease,²¹ and rheumatoid arthritis,²² as well as during chronic hemodialysis,²⁰ and it has been suggested that the serum Nampt level in these conditions may reflect the inflammation status rather than a disease-related change.²⁰ In Leber congenital amaurosis, mutations in the nicotinamide adenine dinucleotide (NAD) biosynthesis pathway result in effects reminiscent of decreased Nampt activity.¹¹ Furthermore, SNPs in the *PBEF1* gene have been identified as predisposing individuals to worse outcomes after acute lung injury and sepsis.^{19,23–25} We herein ruled out the two clinically significant polymorphisms, T1001G and C1535T,¹⁹ as contributing and/or confounding factors in our patient population.

In the present study, we identified a lower serum Nampt level in subjects with resolved RVOs compared with those in healthy volunteers and healthy control subjects reported in the literature.²⁰

Through its enzymatic activity, Nampt has been shown to exert potent protective effects both *in vitro*, in cellular models using neurons and cardiac myocytes,^{27,28} and *in vivo*, in a middle cerebral artery occlusion model for cerebral ischemia–reperfusion injury and stroke.^{27–29} Experimental downregulation of Nampt resulted in lower levels of autophagy in animals with cerebral ischemia–reperfusion injury and subsequent poorer outcomes had no effect, however, in the sham condition.²⁹ It is thus perceivable that circulating levels of Nampt correlate with its cellular concentration and thereby its Nampt enzymatic activity level in cells. Similarly, *in vitro* studies showed that Nampt protected neurons from energy deprivation in neurons using an oxygen–glucose deprivation model.²⁸ Accordingly, small interfering RNA-mediated knock-down of the *PBEF* gene resulted in increased levels of apoptosis of cardiac monocytes *in vitro* following glucose deprivation.²⁷

Thus, it can be speculated that subjects with lower systemic levels of Nampt would experience lower levels of cellular Nampt enzyme activity, resulting in reduced endogenous protection from ischemic events that could predispose to and increase the risk for RVOs. Alternatively, the RVO event may prevent normal release of Nampt into the blood stream. Given the different pathophysiology states of retinal vein vs retinal artery occlusions,¹ it is notable that patients with both types of occlusion had significantly lower serum Nampt levels than healthy volunteers.

Our results provide an important basis for future studies aimed at identifying whether serum Nampt levels represent a novel risk factor, and hence a potential diagnostic biomarker, for RVOs. Such studies will require a temporal kinetic analysis of serum Nampt levels. Furthermore, our data provide a rationale for devising neuroprotective pharmaceutical strategies targeting an increase in or induction of higher Nampt levels to increase endogenous protection of cells in general, and neurons in particular, in subjects with low circulating Nampt levels, and potentially other at-risk populations, to prevent subsequent deleterious effects on the retina and other metabolically active organs. Such strategies will probably need to consider multiple components of the NAD pathway, given the challenges faced by previous gene therapy approaches targeting NMNAT expression in Leber congenital amaurosis.³⁰

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Disclosure

The authors report no conflicts of interest in this work.

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