Nicotinamide Deficiency in Primary Open-Angle Glaucoma

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Purpose. To investigate the plasma concentration of nicotinamide in primary open-angle glaucoma (POAG).

Methods. Plasma of 34 POAG individuals was compared to that of 30 age- and sex-matched controls using a semiquantitative method based on liquid chromatography coupled to high-resolution mass spectrometry. Subsequently, an independent quantitative method, based on liquid chromatography coupled to mass spectrometry, was used to assess nicotinamide concentration in the plasma from the same initial cohort and from a replicative cohort of 20 POAG individuals and 15 controls.

Results. Using the semiquantitative method, the plasma nicotinamide concentration was significantly lower in the initial cohort of POAG individuals compared to controls and further confirmed in the same cohort, using the targeted quantitative method, with mean concentrations of 0.14 μM (median: 0.12 μM; range, 0.06–0.28 μM) in the POAG group (−30%; $P = 0.022$) and 0.19 μM (median: 0.18 μM; range, 0.08–0.47 μM) in the control group. The quantitative dosage also disclosed a significantly lower plasma nicotinamide concentration (−33%; $P = 0.011$) in the replicative cohort with mean concentrations of 0.14 μM (median: 0.14 μM; range, 0.09–0.25 μM) in the POAG group, and 0.19 μM (median: 0.21 μM; range, 0.09–0.26 μM) in the control group.

Conclusions. Glaucoma is associated with lower plasmatic nicotinamide levels, compared to controls, suggesting that nicotinamide supplementation might become a future therapeutic strategy. Further studies are needed, in larger cohorts, to confirm these preliminary findings.

Keywords: primary open-angle glaucoma, metabolomics, mitochondrial dysfunction, nicotinamide, vitamin B3, nicotinamide adenine dinucleotide, NAD, optic nerve, optic neuropathy

Glaucoma, the leading cause of irreversible blindness worldwide, is due to a progressive optic neuropathy involving the loss of retinal ganglion cells (RGCs).[1] Although age and increased intraocular pressure (IOP) are the main risk factors of the disease, other factors may contribute to the occurrence and progression of glaucoma, such as genetic variants, which account for approximately 5% of the cases, together with vascular impairment and metabolic disturbances.[2]

Since the local absence of myelinated axons in the intraocular portion of the optic nerve leads to high energy requirements, the question of mitochondrial dysfunction has been raised in glaucoma similarly to what is observed in hereditary optic neuropathies.[3] Indeed, several studies have revealed a true respiratory chain deficiency in glaucoma.[4,5] The central role of mitochondrial dysfunction was recently demonstrated in a DBA/2J mouse model of glaucoma with high IOP.[6,7] These authors highlighted decreased retinal levels of nicotinamide adenine dinucleotide (NAD), an essential oxidation-reduction cofactor, and showed that the oral administration of high doses of nicotinamide, a precursor of NAD, structurally and functionally prevented the loss of RGCs, posing the rationale for a translational application in humans.[8]

Nicotinamide, also known as vitamin B_3 or PP (pellagra-preventive) vitamin, is a water-soluble vitamin, the deficiency of which causes pellagra, a systemic condition associated with diarrhea, dermatitis, and dementia, and ultimately leading to death. Despite its potential role in the pathogenesis of glaucoma, no study to our knowledge has yet established the involvement of nicotinamide in individuals with primary open-angle glaucoma (POAG).[9]

To gain insight into the pathophysiology of POAG, we applied a nontargeted metabolomics approach, based on liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS),[10] to compare the plasma of individuals with POAG and controls. This study, showing that nicotinamide was the most discriminating metabolite of the signature, led us to investigate the plasma concentration of nicotinamide in individuals with POAG, as reported here.

Methods

Ethics Statement

Participants were included in the study after having given their informed written consent for the research. The study was conducted according to the ethical standards of the Declaration of Helsinki and its later amendments, and with the approval of the University of Angers ethical committee (Comité de Protection des Personnes [CPP] OUEST 2), agreement number: CB 2013-04.

Study Participants

This investigation was exclusively designed for a dedicated cohort of glaucoma patients and controls, and POAG was the only outcome under consideration. Individuals were recruited from the Department of Ophthalmology of Angers University Hospital, France. The initial diagnosis of POAG was based on consensual criteria, that is, glaucomatous optic nerve damage with progressive optic disc cupping, associated with an IOP >21 mm Hg.[11] All the patients with POAG had an elevated IOP at the time of initial diagnosis, as well as open iridocorneal angles as determined by gonioscopic examination. Individuals with isolated ocular hypertension, normal-tension glaucoma, or any secondary form of glaucoma were excluded from the study. Standard automated perimetry (Humphrey field analyzer; Carl Zeiss Meditec, Dublin, CA, USA) with the 24-2 SITA-Fast algorithm was performed on all individuals with POAG, and values of the visual field mean defect (VF-MD) were used to grade the severity of POAG as "mild" with values lower than –6 decibels (dB), "moderate" with values between –6 and –12 dB, and "severe" with values higher than –12 dB (perimetric Hoddap-Parrish-Anderson criteria). The reliability indices retained were false-positive or false-negative rates under 15% and fixation losses under 20%. The other tests performed on patients with POAG included evaluation of the thickness of the retinal nerve fiber layer (RNFL), using spectral-domain optical coherence tomography (OCT), and measurement of the central corneal thickness (CCT) (Cirrus OCT; Carl Zeiss Meditec). The best-corrected visual acuity was measured using the Monoyer decimal charts, with the results converted into logMAR units for statistical analysis. The IOP was measured using the Goldmann applanation tonometer. The history of glaucoma treatment was documented.

Control subjects were selected from among healthy individuals undergoing cataract surgery at the same Department of Ophthalmology. Their inclusion criteria were visual acuity ≥20/50 and the absence of any other associated ocular condition, excepting cataract. The exclusion criteria were a family history of glaucoma, ocular hypertension, or any other intraocular pathology, including retinal disorders.

Our study was carried out on two distinct cohorts recruited from the Department of Ophthalmology of Angers University Hospital. The first cohort, referred to as the initial cohort, was composed of 34 individuals with POAG and 30 controls, and the second cohort, referred to as the replicative cohort, was composed of 20 individuals with POAG and 15 controls. The initial cohort was subjected to a nontargeted metabolomics study, which led to the discovery of nicotinamide deficiency. This was followed by a quantitative analysis as developed in the Department of Biochemistry of Caen University Hospital, France. The replicative cohort was used only for the specific quantitative analysis of nicotinamide.

Blood samples from each participant were collected in heparin tubes at least 3 hours after the last meal. The transfer of the blood tubes was carried out according to a very strict protocol, securing the fastest possible storage at –80°C. Thus, after blood sampling, the tubes were immediately transported on ice to the certified Biological Resource Center (Hospital of Angers), where they were immediately processed for centrifugation (10 minutes at 3000*g* at +4°C) to recover the supernatant (plasma), which was divided into 500-µL aliquots and immediately stored at –80°C until further analysis. The delay between sampling and storage was less than 1 hour for every included subject.

Nontargeted Semiquantitative LC-HRMS Nicotinamide Analysis of Plasma Samples From the Initial Cohort

The nontargeted LC-HRMS analysis was performed according to a method designed for the semiquantitative measurement of 501 metabolites.[10] Briefly, metabolites were extracted from plasma samples using ice-cold methanol. The extracts were analyzed by reverse phase (RP) ultra-high performance liquid chromatography (UHPLC; Dionex UltiMate 3000) coupled to a high-resolution mass spectrometer (HRMS, Thermo Scientific Q Exactive platform; Bremen, Germany). Acquisitions were performed in heated electrospray positive ionization (HESI+) mode. The semiquantitative measurement of nicotinamide was based on an in-house library composed of 501 endogenous metabolites, created using the Mass Spectrometry Metabolite Library of Standards (IROA Technology, Bolton, MA, USA). The method was validated over 3 days, and the extraction efficiency as well as the accuracy, precision, repeatability, and linearity of the method was assessed to ensure the quality of the results.[10]

The parameters of nicotinamide in the nontargeted method were the following: ionization: positive mode; RT: 1.66 min; Formula: $C_6H_6N_2O$; M+H: 123.0553; Fragment ions: 80.0501 and 96.0449. The repeatability (coefficient of variation [CV]% performed on six duplicates) of the method for nicotinamide was as follows: 5.5% for peak area, 7.6% for peak intensity, 0.7% for retention time (RT), and 0% for m/z ratio. Mass spectrometry and chromatography accuracies were also satisfactory, with, respectively, 1 Δppm and 0.05 ΔRT; R^2 for dilutions linearity (1, 1/2, 1/4 dilutions) was equal to 0.9.

During the semiquantitative LC-HRMS several metabolites related to nicotinamide were assessed: 1-methylnicotinamide, 6-hydroxy-nicotinic acid, nicotinic acid, nicotinamide mononucleotide, and NAD. Only 1-methylnicotinamide was accurately detected, but this metabolite was not discriminant between POAG and controls.

Quantitative LC-MS/MS Nicotinamide Analysis of Plasma Samples From the Initial and Replicative Cohorts

A blind independent external validation of nicotinamide dosage was performed on plasma samples from both the initial and replicative cohorts using a targeted LC-MS/MS method specifically designed for the quantification of nicotinamide. Nicotinamide (NM) and its isotope-labeled analogue, nicotinamide-d_4 (NM-d_4), were purchased from LGC Standards GmbH (Wesel, Germany). Fifty microliters of plasma were mixed with 20 µL Internal Standard (IS) solution (NM-d_4) and 130 µL of a cold methanol/acetonitrile solution (50/50, vol/vol) to precipitate proteins. Samples were incubated on ice for 5 minutes and then centrifuged at 10,000*g* for 5 minutes. Supernatant (50 µL) was mixed with 550 µL water and filtered (0.45 µm) before injection into the chromatography and mass spectrometry system.

Liquid chromatography was conducted on a UFLC Prominence chromatographic system (Shimadzu, Kyoto, Japan)

Table 1. Distribution of the Blood Collection Hour Between Patients and Controls

Time of Sample Collection	8–12 AM	1–4 PM	*P* Value
Controls, initial cohort	16	14	
POAG, initial cohort	18	16	0.97
Controls, replicative cohort	9	6	
POAG, replicative cohort	12	8	1

connected to a SCIEX QTRAP 5500 mass spectrometer, equipped with a turbo V ion spray source (SCIEX, Toronto, ON, Canada). Supernatant (6 µL) was injected, and chromatographic separation was performed at +40°C using a Pursuit pentafluorophenyl (PFP) column (150 × 2.1 mm, 3.5 µm; Agilent Technologies, Santa Clara, CA, USA) connected to a guard column (Pursuit PFP). The flow rate was 0.4 mL·min^{-1}. A gradient mobile phase was performed and started with 98% mobile phase A (0.1% formic acid in water) and 2% mobile phase B (methanol). After 1.5 minutes post injection, the percentage of mobile phase B increased linearly from 2% to 80% in 1 minute, and stayed at 80% mobile phase B during 0.5 minute. The return to baseline conditions (2% B) was operated after 4 minutes and the system was allowed to stabilize for 2.3 minutes before the next injection. The total chromatographic run time was 6.3 minutes.

Mass spectrometry analysis was conducted using the electrospray ion (ESI) source in the positive mode. The parameters of the ion source were as follows: temperature 450°C, ESI voltage 5500 V, Gas GS1 70 psi, Gas GS2 60 psi, CAD gas 8 psi, and Curtain gas 40 psi. For nicotinamide quantification, Multiple Reaction Monitoring (MRM) transitions were, respectively, m/z 123→80 and m/z 127→84 for nicotinamide and NM-d_4. For nicotinamide transition, the instrument parameters were 91 V, 27 V, and 12 V for DP, CE, and CXP, respectively. For NM-d_4 transition, the instrument parameters were 81 V, 27 V, and 38 V for DP, CE, and CXP, respectively.

Five standard calibration points were made in water at final concentrations of 0.082, 0.205, 0.410, 0.819, and 1.639 µM for nicotinamide. A solution of NM-d_4 was prepared by dilution in water at a final concentration of 3.966 µM (IS solution).

Evaluation of the sensitivity and specificity of the protocol showed that the targeted LC-MS/MS method gave good results. The calibration curve was linear up to 1.639 µM ($r > 0.999$), the limit of quantification was 0.041 µM, and the recovery rate was 101 ± 3% in plasma samples spiked with nicotinamide. During the reproducibility assay, the coefficients of variation (CV) were lower than 5% at three levels of concentration (CV = 4.8%, 0.17 ± 0.01 µM for the low-level control). The retention times were 1.73 and 1.71 minutes for nicotinamide and NM-d_4, respectively. Typical chromatograms for nicotinamide and NM-d_4 in plasma samples are shown in the Figure, panel C.

As the literature does not report diurnal variations in vitamin B_3 levels, we included patients who were selected in our ophthalmic clinics within the daily operating hours (from 8 AM to 4 PM). In addition, subjects were included only if they had been fasting for at least 3 hours before reaching the hospital. However, to exclude an eventual bias due to the collection time, we statistically compared the collection times of the patients and control cohorts, without finding significant heterogeneity (Table 1).

Statistical Analyses

The data matrix from nontargeted metabolomics contained 160 metabolites; univariate analysis was performed using the nonparametric Wilcoxon rank sum test with Benjamini-Hochberg correction and keeping the false discovery rate (FDR) below 5%. These analyses were conducted using Metaboanalyst v4.0.[12]

Univariate analyses of clinical data were carried out using 2-tailed Student's *t*-test, with differences being considered significant at $P < 0.05$. A median test was used to compare the median concentrations of nicotinamide found in individuals with POAG versus controls, in both the initial and replicative cohorts. The level of significance for the 2-tailed test was set at $\alpha = 0.05$. This analysis was performed using SPSS Statistics v22 (IBM, Bois-Colombes, France).

The χ^2 test was performed to assess the independence between POAG and control, in relation to the distribution of the blood collection hour (morning versus afternoon), as shown in Table 1.

Results

The characteristics of the study cohorts are given in Table 2. Regarding plasma nicotinamide concentrations, the univariate analysis of the results obtained using the semiquantitative LC-HRMS method on plasma samples from the initial cohort revealed significant differences between individuals with POAG and controls, with nicotinamide being the most discriminant metabolite (FDR *Q* value = 0.0027), showing an average nicotinamide decrease of 36% in individuals with POAG compared to controls (Fig., panel A).

This observation, subsequently tested in both the initial and replicative cohorts, using an independent quantitative measurement of nicotinamide designed for a clinical laboratory setting, supported the results obtained with the metabolomics analysis (Fig., panel B). The median concentrations of nicotinamide found in individuals with POAG and controls were 0.12 µM (0.06–0.28 µM) versus 0.18 µM (0.08–0.47 µM), and 0.14 µM (0.09–0.25 µM) versus 0.21 µM (0.09–0.26 µM), respectively, in the initial and replicative cohorts, corresponding to a reduction of 30% ($P = 0.022$) and of 33% ($P = 0.011$) of the nicotinamide concentration in the initial and replicative POAG versus control cohorts, respectively. The mean concentrations of nicotinamide found in individuals with POAG and controls were 0.14 vs. 0.19 µM and 0.14 vs. 0.19 µM, respectively, in the initial and replicative cohorts. We did not find any correlation between the nicotinamide levels and the age of POAG patients and controls, suggesting that the nicotinamide deficiency observed in POAG is rather due to the disease.

Discussion

Mitochondrial dysfunctions and decreased NAD content are hallmarks of aging in most organs,[13,14] and many experimental studies, essentially performed on mouse models, have revealed that strategies based on NAD repletion effectively reverse age-related phenotypes and disorders,[15,16] such as those affecting the skeletal muscles,[17] the brain,[18] and the endothelium.[19] Recent studies on the DBA/2J mouse model of glaucoma have further confirmed a dose-dependent protective effect of NAD repletion on the optic nerve, reaching a protection level of 93% at the highest nicotinamide dose tested (2000 mg/kg/day), despite a continuously elevated IOP.[6,7,20] More importantly, the age-dependent vulnerability of the RGCs in these mice was correlated with the decreased concentration of NAD in the retina. Thus, the nicotinamide deficiency we observed in the blood of POAG individuals parallels the NAD depletion observed in the DBA/2J mouse model. Interestingly, our study of plasma samples from individuals affected by dominant optic atrophy due to *OPA1* mutations, another form of an age-

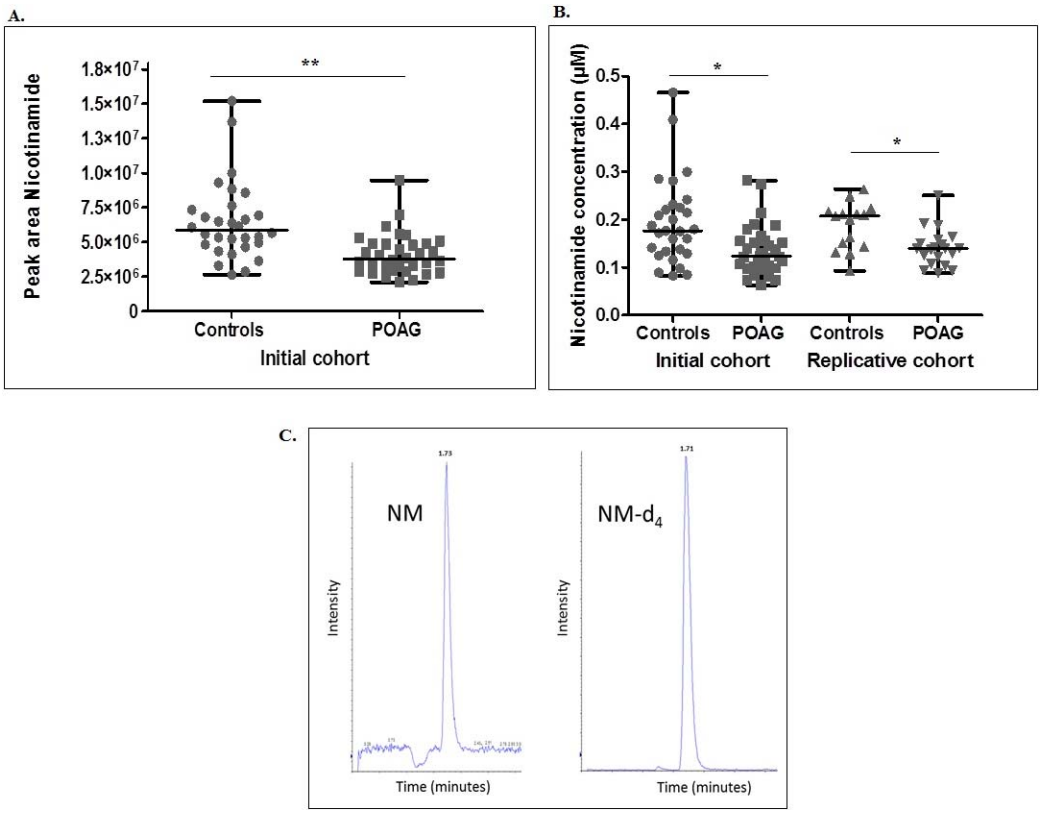

FIGURE. Dot plots showing nicotinamide levels in the initial (30 control and 34 POAG individuals) and replicative (15 control and 20 POAG individuals) cohorts. The *central black bars* within the dot plots represent the median concentration for each group. (**A**) Peak area of nicotinamide found in the initial cohort following LC-HRMS analysis discloses a glaucoma/controls fold change of 0.65. (**B**) Concentrations of nicotinamide found in the initial and replicative cohorts following LC-MS/MS analysis. The controls/glaucoma fold changes were 1.43 and 1.49 for the initial and replicative cohorts, respectively. The *P* values between groups for all conditions were $^{*}P < 0.05$ and $^{**}P < 0.01$. (**C**) Chromatograms obtained for nicotinamide (NM; *left*) and its isotope-labeled analogue nicotinamide-d_4 (NM-d_4; *right*) in a plasma sample. Deuterated nicotinamide, NM-d_4, was used as internal standard for the absolute quantification of nicotinamide concentration in the plasma from the cohorts. For quantitative analysis, the use of a stable isotope-labeled analogue of the analyte, as internal standard, is recommended to correct for possible loss during sample preparation steps and matrix effects during mass spectrometry acquisitions. The ideal internal standard is a substance not contained in the sample, structurally related to the analyte (NM in our case), and having a retention time close to that of the analyte (retention times of NM and NM-d_4 are 1.73 and 1.71 minutes, respectively). NM-d_4 perfectly fulfils these criteria.

dependent progressive optic neuropathy due to mitochondrial impairment, also revealed a 50% reduction of nicotinamide.[21]

The main function of NAD as a redox cofactor consists in providing electrons from oxidized nutrients to the mitochondrial respiratory chain complex I, thus sustaining adenosine triphosphate (ATP) production. In parallel, NAD-consuming enzymes, such as those involved in DNA repair, for example, poly (ADP-ribose) polymerase (PARP), may consume NAD stocks excessively during aging, in particular to prevent the accumulation of DNA mutations.[13] This excessive NAD consumption may compromise NAD-dependent complex I activity, the deficiency of which is frequently associated with inherited optic neuropathies, because of the particularly high energy required by RGCs to transduce visual information from the retina to the brain. In this respect, lymphoblasts of patients with POAG showed a mitochondrial complex I deficiency reflecting a systemic mitochondrial impairment.[4,5] In addition, using targeted metabolomics on the plasma of POAG patients compared to controls, we have recently shown a metabolic profile combining the impaired utilization of energetic substrates and decreased levels of polyamines, attesting to a mitochondrial dysfunction, and premature aging.[22] Since nicotinamide is one of the main contributors to the regeneration of NAD through a salvage metabolic pathway, nicotinamide deficiency could reflect excessive age-related NAD consumption, which subsequently leads to complex I deficiency, and the energetic failure responsible for the degeneration of RGCs. Alternatively, nicotinamide may be protective in glaucoma through other routes such as those involving the sirtuins that are NAD-dependent enzymes regulating the metabolism. Indeed, a marked reduction of SIRT1 expression was evidenced in aged retinal neurons in several species[23] and the upregulation of SIRT1 has been shown to have an important protective effect against various ocular diseases, including retinal degeneration.[24] Nicotinamide may also be protective in glaucoma through protection of vascular injury as has been shown in a rat model subjected to ischemia/reperfusion.[25]

Despite extensive research in the literature, we were unable to find normative values for plasma nicotinamide levels in normal subjects. We believe that this can be explained by a technological gap, since the plasmatic nicotinamide levels are very low in humans. We assume that the recent technological advances in mass spectrometry have allowed us to perform these measures and we can only hope that further independent studies will explore this area. Since vitamin B_3 has been proposed as a treatment for hyperlipidemias,[26] we carefully searched for its presence in the medications taken by the participants to the study, and failed to find any presence of this vitamin.

The main limitation of this study consists in the relatively small number of individuals in both the initial and replicative

Table 2. Characteristics of Individuals From the Initial and the Replicative Cohorts. Demographic Data and Comorbidity Status, Systemic Medications, Ophthalmologic Features, and Glaucoma Medication of Individuals With POAG Compared to Controls

Characteristics	Initial Cohort			Replicative Cohort		
	Controls, $N = 30$	POAG, $N = 34$	*P* Value	Controls, $N = 15$	POAG, $N = 20$	*P* Value
Demographic data and comorbidity						
Average age, y [range]	73.77 [57–83]	73.06 [61–86]	0.65	70.27 [55–80]	64.85 [42–95]	0.11
Females, %	50	50	1	53.33	25	0.09
Mean BMI, kg/m^2	26.99	26.22	0.59	28.27	25.75	0.3
Diabetes, %	3.33	17.65	0.1	13.33	25	0.39
Hypertension, %	63.33	50	0.29	73.33	35	0.02
Hyperlipidemia, %	43.33	26.47	0.165	60	25	0.04
Thyroid disease, %	13.33	11.76	0.29	0	5	0.33
Systemic medications						
Antihypertensives, %	63.33	47.06	0.19	73.33	35	0.02
Lipid-lowering medications, %	43.33	23.53	0.09	60	25	0.04
Antiplatelet therapy, %	36.67	26.47	0.39	13.33	25	0.39
Oral diabetes medications, %	13.33	14.71	0.88	13.33	25	0.39
Insulin, %	0	2.94	0.32	0	0	–
Corticosteroids, %	3.33	2.94	0.93	0	5	0.33
Thyroid hormone, %	13.33	17.65	0.64	0	5	0.33
Estrogen, %	0	0	1	0	0	–
Vitamin D, %	20	11.76	0.38	6.67	10	0.73
Ophthalmologic features and glaucoma medication						
Mean visual acuity, logMAR	+0.13	+0.12	0.91	+0.03	0.05	0.37
Mean IOP, mm Hg	14.1	13.42	0.27	13.84	15.82	<0.001
Mean CCT, µm	–	529.95	–	–	544.44	–
Average RNFL thickness, µm	–	66.91	–	–	68.7	–
Mean VF-MD, dB, eye with worse MD	–	−6.83	–	–	−3.99	–
Glaucoma severity, %						
Mild	–	82.35	–	–	80	–
Moderate	–	5.88	–	–	10	–
Severe	–	11.77	–	–	10	–
Glaucoma medications, %						
Beta-blockers	–	55.88	–	–	60	–
Prostaglandin analogue	–	67.65	–	–	85	–
Alpha-2-agonists	–	11.76	–	–	0	–
Carbonic anhydrase inhibitor	–	26.47	–	–	15	–

BMI, body mass index (weight/height^2).

cohorts. However, we found a significant decrease in vitamin B_3 levels in patients with POAG compared to controls using two different techniques, with highly similar results in the two independent cohorts.

Further studies with larger cohorts are also required, as well as investigations in populations with various cultural dietary habits, to find out whether this deficiency is consistently associated with POAG and eventually with other forms of glaucoma. Finally, the convergence between recent studies showing that oral administration of nicotinamide prevents glaucoma in the DBA/2J mouse model[6,7,20] and our study on patients with POAG opens promising therapeutic perspectives based on nicotinamide supplementation.

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