

# 1 NICOTINAMIDE DEFICIENCY IN PRIMARY OPEN-ANGLE 2 GLAUCOMA

3 Judith Kouassi Nzouget<sup>1</sup>, Juan Manuel Chao de la Barca<sup>1,2</sup>, Khadidja Guehlouz<sup>3</sup>, Stéphanie  
4 Leruez<sup>3</sup>, Laurent Coulbault<sup>4</sup>, Stéphane Allouche<sup>4</sup>, Cinzia Bocca<sup>1</sup>, Jeanne Muller<sup>3</sup>, Patrizia  
5 Amati-Bonneau<sup>1,2</sup>, Philippe Gohier<sup>3</sup>, Dominique Bonneau<sup>1,2</sup>, Gilles Simard<sup>2</sup>, Dan Milea<sup>5</sup>, Guy  
6 Lenaers<sup>1</sup>, Vincent Procaccio<sup>1,2</sup>, Pascal Reynier<sup>1,2</sup>

7 <sup>1</sup>Equipe Mitolab, Unité Mixte de Recherche MITOVASC, CNRS 6015, INSERM U1083,  
8 Université d'Angers, Angers, France

9 <sup>2</sup>Département de Biochimie et Génétique, Centre Hospitalier Universitaire, Angers, France

10 <sup>3</sup>Département d'Ophtalmologie, Centre Hospitalier Universitaire, Angers, France

11 <sup>4</sup>Service de Biochimie, EA4650, Centre Hospitalier Universitaire, Caen, France

12 <sup>5</sup>Singapore Eye Research Institute, Singapore National Eye Centre, Duke-NUS, Singapore

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14 **Correspondence to:** Judith Kouassi Nzouget, PhD, Equipe Mitolab, Institut MITOVASC,  
15 UMR CNRS 6015, INSERM U1083, 4 rue Larrey, 49933 Angers cedex 9, Angers, France; Tel:  
16 +33 (0) 244688412; Email: judith.kouassinzouget@univ-angers.fr

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18 Nicotinamide; Vitamin B3; Nicotinamide adenine dinucleotide; NAD; Optic nerve; Optic  
19 neuropathy.

20  
21 **Abbreviations and acronyms:** BMI: body mass index; CCT: central corneal thickness; CPP:  
22 comité de protection des personnes; HESI: heated electrospray ionization; HRMS: high  
23 resolution mass spectrometry; IOP: intraocular pressure; IS: internal standard; LC: liquid  
24 chromatography; MRM: Multiple Reaction Monitoring; NAD: Nicotinamide adenine  
25 dinucleotide; NM: Nicotinamide; NM-d<sub>4</sub> : nicotinamide-d<sub>4</sub>; OCT: optical coherence  
26 tomography; PFP: pentafluorophenyl; POAG: primary open-angle glaucoma; RGC: retinal  
27 ganglion cell; RNFL: retinal nerve fibre layer; VF-MD: visual field mean defect.

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34

35 **ABSTRACT**

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

38

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

45

46 **Results:** Using the semi-quantitative method, the plasma nicotinamide concentration was  
47 significantly lower in the initial cohort of POAG individuals compared to and further confirmed  
48 in the same cohort, using the targeted quantitative method, with mean concentrations of 0.14  $\mu\text{M}$   
49 (median: 0.12  $\mu\text{M}$ ; range: 0.06-0.28  $\mu\text{M}$ ) in the POAG group (-30 %;  $p = 0.022$ ), and 0.19  $\mu\text{M}$   
50 (median: 0.18  $\mu\text{M}$ ; range: 0.08-0.47  $\mu\text{M}$ ) in the control group. The quantitative dosage also  
51 disclosed a significantly lower plasma nicotinamide concentration (-33 %;  $p = 0.011$ ) in the  
52 replicative cohort with mean concentrations of 0.14  $\mu\text{M}$  (median: 0.14  $\mu\text{M}$ ; range: 0.09-0.25  
53  $\mu\text{M}$ ) in the POAG group, and 0.19  $\mu\text{M}$  (median: 0.21  $\mu\text{M}$ ; range: 0.09-0.26  $\mu\text{M}$ ) in the control  
54 group.

55

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

59 **INTRODUCTION**

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

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

75

76 Nicotinamide, also known as vitamin B3 or PP (pellagra-preventive) vitamin, is a water-soluble  
77 vitamin, the deficiency of which causes pellagra, a systemic condition associating diarrhoea,  
78 dermatitis and dementia, and ultimately leading to death. Despite its potential role in the  
79 pathogenesis of glaucoma, no study to our knowledge has yet established the involvement of  
80 nicotinamide in individuals with primary open-angle glaucoma (POAG)<sup>9</sup>.

81

82 To gain insight into the pathophysiology of POAG, we applied a non-targeted metabolomics  
83 approach, based on liquid chromatography coupled to high resolution mass spectrometry (LC-  
84 HRMS) <sup>10</sup>, to compare the plasma of individuals with POAG and controls. This study, showing  
85 that nicotinamide was the most discriminating metabolite of the signature, led us to investigate  
86 the plasma concentration of nicotinamide in individuals with POAG, as reported here.

87

## 88 **METHODS**

### 89 **Ethics Statement**

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

94

### 95 **Study participants**

96 Individuals were recruited from the Department of Ophthalmology of Angers University  
97 Hospital, France. The initial diagnosis of POAG was based on consensual criteria, i.e.  
98 glaucomatous optic nerve damage with progressive optic disc cupping, associated with an IOP  
99 >21 mmHg <sup>11</sup>. All the patients with POAG had an elevated IOP at the time of initial diagnosis,  
100 as well as open irido-corneal angles, as determined by gonioscopic examination. Individuals  
101 with isolated ocular hypertension, normal tension glaucoma, or any secondary form of  
102 glaucoma, were excluded from the study. Standard automated perimetry (Humphrey field  
103 analyser, Carl Zeiss, Dublin, CA, USA) with the 24-2 SITA-Fast algorithm was performed on  
104 all individuals with POAG, and values of the visual field mean defect (VF-MD) were used to  
105 grade the severity of POAG as “mild” with values lower than -6 dB, “moderate” with values

106 between -6 dB and -12 dB, and “severe” with values higher than -12 dB (perimetric Hoddapp-  
107 Parrish-Anderson criteria). The reliability indices retained were false positive or false negative  
108 rates under 15 %, and fixation losses under 20 %. The other tests performed on patients with  
109 POAG included evaluation of the thickness of the retinal nerve fibre layer (RNFL), using  
110 spectral domain optical coherence tomography (OCT), and measurement of the central corneal  
111 thickness (CCT) (Cirrus OCT, Carl Zeiss Meditec, Dublin, CA, USA). The best-corrected visual  
112 acuity was measured using the Monoyer decimal charts, with the results converted into logMAR  
113 units for statistical analysis. The IOP was measured using the Goldmann applanation tonometer.  
114 The history of glaucoma treatment was documented.

115  
116 Control subjects were selected among healthy individuals undergoing cataract surgery at the  
117 same Department of Ophthalmology. Their inclusion criteria were: visual acuity  $\geq$  20/50 and the  
118 absence of any other associated ocular condition, excepting cataract. The exclusion criteria  
119 were: a family history of glaucoma, ocular hypertension or any other intraocular pathology,  
120 including retinal disorders.

121  
122 Our study was carried out on two distinct cohorts recruited from the Department of  
123 Ophthalmology of Angers University Hospital. The first cohort, referred as the “initial cohort”,  
124 was composed of 34 individuals with POAG and 30 controls, and the second cohort, referred as  
125 the “replicative cohort”, was composed of 20 individuals with POAG and 15 controls. The  
126 initial cohort was subjected to a non-targeted metabolomics study, which led to the discovery of  
127 nicotinamide deficiency. This was followed by a quantitative analysis as developed in the  
128 Department of Biochemistry of Caen University Hospital, France. The replicative cohort was  
129 used only for the specific quantitative analysis of nicotinamide.

130

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

139

140 **Non-targeted semi-quantitative LC-HRMS nicotinamide analysis of plasma samples from**  
141 **the initial cohort**

142 The non-targeted LC-HRMS analysis was performed according to a method designed for the  
143 semi-quantitative measurement of 501 metabolites <sup>10</sup>. Briefly, metabolites were extracted from  
144 plasma samples using ice-cold methanol. The extracts were analysed by reverse phase (RP)  
145 ultra-high-performance liquid chromatography (UHPLC, Dionex<sup>TM</sup> UltiMate 3000) coupled to a  
146 high-resolution mass spectrometer (HRMS, Thermo Scientific<sup>TM</sup> Q Exactive<sup>TM</sup> platform).  
147 Acquisitions were performed in heated electrospray positive ionization (HESI+) mode. The  
148 semi-quantitative measurement of nicotinamide was based on an in-house library composed of  
149 501 endogenous metabolites, created using the Mass Spectrometry Metabolite Library of  
150 Standards (IROA Technology, Bolton, MA, USA). The method was validated over three days,  
151 and the extraction efficiency as well as the accuracy, precision, repeatability, and linearity of the  
152 method were assessed to ensure the quality of the results <sup>10</sup>.

153

154 The parameters of nicotinamide in the non-targeted method were the following: ionization:  
155 positive mode; RT: 1.66 min; Formula: C<sub>6</sub>H<sub>6</sub>N<sub>2</sub>O; M+H: 123.0553; Fragment ions: 80.0501 and  
156 96.0449. The repeatability (CV% performed on 6 duplicates) of the method for nicotinamide  
157 was as follow: 5.5% for peak area, 7.6% for peak intensity, 0.7% for retention time (RT) and 0%  
158 for m/z ratio. Mass spectrometry and chromatography accuracies were also satisfactory, with  
159 respectively 1 Δppm and 0.05 ΔRT; R<sup>2</sup> for dilutions linearity (1, 1/2, 1/4 dilutions) was equal to  
160 0.9.

161

162 **Quantitative LC-MS/MS nicotinamide analysis of plasma samples from the initial and**  
163 **replicative cohorts**

164 A blind independent external validation of nicotinamide dosage was performed on plasma  
165 samples from both the initial and replicative cohorts using a targeted LC-MS/MS method  
166 specifically designed for the quantification of nicotinamide. Nicotinamide (NM) and its isotope-  
167 labelled analogue, nicotinamide-d<sub>4</sub> (NM-d<sub>4</sub>), were purchased from LGC Standards GmbH  
168 (Wesel, Germany). Fifty microliters of plasma were mixed with 20 μL Internal Standard (IS)  
169 solution (NM-d<sub>4</sub>), and 130 μL of a cold methanol/acetonitrile solution (50/50; V/V) to  
170 precipitate proteins. Samples were incubated on ice for 5 min, and then centrifuged at 10 000 g  
171 for 5 min. Fifty μL of supernatant were mixed with 550 μL of water and filtered (0.45 μm)  
172 before injection into the chromatography and mass spectrometry system.

173

174 Liquid chromatography was conducted on a UFLC Prominence chromatographic system  
175 (Shimadzu, Kyoto, Japan) connected to a SCIEX QTRAP® 5500 mass spectrometer, equipped  
176 with a turbo V ion spray source (SCIEX, Toronto, Canada). Six μL of supernatant were injected,

177 and chromatographic separation was performed at +40 °C using a Pursuit pentafluorophenyl  
178 (PFP) column (150 x 2.1 mm, 3.5 µm; Agilent technologies, Santa Clara, CA, USA) connected  
179 to a guard column (Pursuit PFP). The flow rate was 0.4 ml·min<sup>-1</sup>. A gradient mobile phase was  
180 performed and started with 98 % mobile phase A (0.1% formic acid in water) and 2 % mobile  
181 phase B (methanol). After 1.5 min post-injection, the percentage of mobile phase B increased  
182 linearly from 2 % to 80 % in 1 min, and stayed at 80 % mobile phase B during 0.5 min. The  
183 return to baseline conditions (2 % B) was operated after 4 min and the system was allowed to  
184 stabilize for 2.3 min before the next injection. The total chromatographic run time was 6.3 min.

185

186 Mass spectrometry analysis was conducted using the electrospray ion (ESI) source in the  
187 positive mode. The parameters of the ion source were as follows: temperature 450 °C, ESI  
188 voltage 5500 V, Gas GS1 70 psi, Gas GS2 60 psi, CAD gas 8 psi, and Curtain gas 40 psi. For  
189 nicotinamide quantification, Multiple Reaction Monitoring (MRM) transitions were respectively  
190 m/z 123→80 and m/z 127→84 for nicotinamide and nicotinamide-d<sub>4</sub> respectively. For  
191 nicotinamide transition, the instrument parameters were 91 V, 27 V, and 12 V for DP, CE, and  
192 CXP, respectively. For nicotinamide-d<sub>4</sub> transition, the instrument parameters were 81 V, 27 V,  
193 and 38 V for DP, CE, and CXP, respectively.

194

195 Five standard calibration points were made in water at final concentrations of 0.082, 0.205,  
196 0.410, 0.819, and 1.639 µM for nicotinamide. A solution of nicotinamide-d<sub>4</sub> was prepared by  
197 dilution in water at a final concentration of 3.966 µM (IS solution).

198

199 Evaluation of the sensitivity and specificity of the protocol showed that the targeted LC-MS/MS  
200 method gave good results. The calibration curve was linear up to 200 µg/L (r>0.999), the limit

201 of quantification was 5 µg/L, and the recovery rate was 101±3 % in plasma samples spiked with  
202 nicotinamide. During the reproducibility assay, the coefficients of variation (CV) were lower  
203 than 5 % at three levels of concentration (CV = 4.8%, 20.4±1.0 µg/L for the low-level control).  
204 The retention times were 1.73 min and 1.71 min for nicotinamide and nicotinamide-d<sub>4</sub>,  
205 respectively. Typical chromatograms for nicotinamide and nicotinamide-d<sub>4</sub> in plasma samples  
206 are shown in the supplementary Figure.

207

## 208 **Statistical analyses**

209 The data matrix from non-targeted metabolomics contained one hundred and sixty metabolites;  
210 univariate analysis was performed using the non-parametric Wilcoxon rank sum test with  
211 Benjamini-Hochberg correction and keeping the False Discovery Rate (FDR) below 5%. These  
212 analyses were conducted using Metaboanalyst v4.0 <sup>12</sup>.

213

214 Univariate analyses of clinical data were carried out using two-tailed Student's *t*-test, with  
215 differences being considered significant at *p* < 0.05. A median test was used to compare the  
216 median concentrations of nicotinamide found in individuals with POAG *versus* controls, in both  
217 the initial and replicative cohorts. The level of significance for the two-tailed test was set at  $\alpha$  =  
218 0.05. This analysis was performed using SPSS Statistics v22 (IBM, Bois-Colombes, France).

219

220 The Chi-squared test was performed to assess the independence between POAG and control, in  
221 relation to the distribution of the blood collection hour (morning *vs.* afternoon).

222

## 223 **RESULTS**

224 This investigation was exclusively designed for a dedicated cohort of glaucoma patients and  
225 controls, and POAG was the only outcome under consideration.

226 As the literature does not report diurnal variations in vitamin B3 levels, we included patients  
227 who were selected in our ophthalmic clinics within the daily operating hours (from 8am to 4pm).  
228 In addition, subjects were included only if they had been fasting for at least 3 hours, before  
229 reaching the hospital. However, to exclude an eventual bias due to the collection time, we  
230 statistically compared the collection times of the patients and control cohorts, without finding  
231 significant heterogeneity (supplementary Table).

232

### 233 **Clinical characteristics of individuals with POAG and controls**

234 Comparisons between individuals with POAG (n=34) and controls (n=30) from the initial  
235 cohort, in terms of demographic and comorbidity data, medical conditions and general  
236 ophthalmological features, are presented in Table 1. There were no significant differences  
237 between the two groups in terms of mean age, sex ratio, systemic medications, or mean IOP.

238

239 Comparisons between individuals with POAG (n=20) and controls (n=15) from the replicative  
240 cohort, in terms of demographic and comorbidity data, medical conditions and general  
241 ophthalmological features are presented in Table 2. There was no significant differences  
242 between the two groups in terms of mean age, sex ratio, or systemic medications, except for  
243 anti-hypertensives ( $p<0.02$ ) and lipid-lowering medications ( $p<0.04$ ), which were significantly  
244 lower in individuals with POAG than in controls. In contrast to the initial cohort, the replicative  
245 cohort showed a difference between the two groups regarding the IOP, which was significantly  
246 higher in POAG individuals compared to controls ( $p<0.001$ ), the discrepancy with the initial

247 cohort being related to the presence in the replicative cohort of patients with an insufficiently  
248 efficacious treatment for IOP.

249

250 **Plasma nicotinamide concentrations**

251 The univariate analysis of the results obtained using the semi-quantitative LC-HRMS method on  
252 plasma samples from the initial cohort revealed significant differences between individuals with  
253 POAG and controls, with nicotinamide being the most discriminant metabolite (False Discovery  
254 Rate corrected  $p = 0.0027$ ), showing an average nicotinamide decrease of 36 % in individuals  
255 with POAG compared to controls (Figure A).

256

257 This observation, subsequently tested in both the initial and replicative cohorts, using an  
258 independent quantitative measurement of nicotinamide designed for a clinical laboratory setting,  
259 supported the results obtained with the metabolomics analysis (Figure B). The median  
260 concentrations of nicotinamide found in individuals with POAG and controls were 0.12  $\mu\text{M}$   
261 (0.06-0.28  $\mu\text{M}$ ) *vs.* 0.18  $\mu\text{M}$  (0.08-0.47  $\mu\text{M}$ ), and 0.14  $\mu\text{M}$  (0.09-0.25  $\mu\text{M}$ ) *vs.* 0.21  $\mu\text{M}$  (0.09-  
262 0.26  $\mu\text{M}$ ), respectively, in the initial and replicative cohorts, corresponding to a reduction of 30  
263 % ( $p = 0.022$ ) and of 33 % ( $p = 0.011$ ) of the nicotinamide concentration in the initial and  
264 replicative POAG *vs.* control cohorts, respectively. The mean concentrations of nicotinamide  
265 found in individuals with POAG and controls were 0.14  $\mu\text{M}$  *vs.* 0.19  $\mu\text{M}$ , and 0.14  $\mu\text{M}$  *vs.* 0.19  
266  $\mu\text{M}$ , respectively, in the initial and replicative cohorts.

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

271

272 **DISCUSSION**

273 Mitochondrial dysfunctions and decreased NAD content are hallmarks of aging in most organs  
274 <sup>13,14</sup> and many experimental studies, essentially performed on mouse models, have revealed that  
275 strategies based on NAD repletion effectively reverse age-related phenotypes and disorders <sup>15,16</sup>,  
276 such as those affecting the skeletal muscles <sup>17</sup>, the brain <sup>18</sup>, and the endothelium <sup>19</sup>. Recent  
277 studies on the DBA/2J mouse model of glaucoma, have further confirmed a dose-dependent  
278 protective effect of NAD repletion on the optic nerve, reaching a protection level of 93% at the  
279 highest nicotinamide dose tested (2000 mg/kg/day), despite a continuously elevated IOP <sup>6,7,20</sup>.  
280 More importantly, the age-dependent vulnerability of the RGCs in these mice was correlated  
281 with the decreased concentration of NAD in the retina. Thus, the nicotinamide deficiency we  
282 observed in the blood of POAG individuals parallels the NAD depletion observed in the DBA/2J  
283 mouse model. Interestingly, our study of plasma samples from individuals affected by dominant  
284 optic atrophy due to OPA1 mutations, another form of an age-dependent progressive optic  
285 neuropathy due to mitochondrial impairment, also revealed a 50 % reduction of nicotinamide  
286 whose chemical formula is C<sub>6</sub>H<sub>6</sub>N<sub>2</sub>O <sup>21</sup>.

287

288 The main function of NAD as a redox cofactor consists in providing electrons from oxidized  
289 nutrients to the mitochondrial respiratory chain complex I, thus sustaining ATP production. In  
290 parallel, NAD-consuming enzymes, such as those involved in DNA repair, e.g. poly (ADP-  
291 ribose) polymerase (PARP), may consume NAD stocks excessively during aging, in particular  
292 to prevent the accumulation of DNA mutations <sup>13</sup>. This excessive NAD consumption may  
293 compromise NAD-dependent complex I activity, the deficiency of which is frequently  
294 associated with inherited optic neuropathies, because of the particularly high energy required by

295 RGCs to transduce visual information from the retina to the brain. In this respect, lymphoblasts  
296 of patients with POAG showed a mitochondrial complex I deficiency reflecting a systemic  
297 mitochondrial impairment <sup>4,5</sup>. In addition, using targeted metabolomics on the plasma of POAG  
298 patients compared to controls, we have recently shown a metabolic profile combining the  
299 impaired utilization of energetic substrates and decreased levels of polyamines, attesting a  
300 mitochondrial dysfunction, and premature ageing <sup>22</sup>. Since nicotinamide is one of the main  
301 contributors to the regeneration of NAD through a salvage metabolic pathway, nicotinamide  
302 deficiency could reflect excessive age-related NAD consumption, which subsequently leads to  
303 complex I deficiency, and the energetic failure responsible for the degeneration of RGCs.

304 Despite extensive research in the literature, we were unable to find normative values for plasma  
305 nicotinamide levels in normal subjects. We believe that this can be explained by a technological  
306 gap, since the plasmatic nicotinamide levels are very low in humans. We assume that the recent  
307 technological advances in mass spectrometry have allowed us to perform these measures and we  
308 can only hope that further independent studies will explore this area.

309  
310 The main limitation of this study consists in the relatively small number of individuals in both  
311 the initial and replicative cohorts. However, we found a significant decrease in vitamin B3 levels  
312 in patients with POAG compared to controls using two different techniques, with highly similar  
313 results in the two independent cohorts. Further studies with larger cohorts are also required, as  
314 well as investigations in populations with various cultural dietary habits, to find out whether this  
315 deficiency is consistently associated with POAG and eventually with other forms of glaucoma.  
316 Finally, the convergence between recent studies showing that oral administration of  
317 nicotinamide prevents glaucoma in the DBA/2J mouse model <sup>6,7,20</sup> and our study on patients  
318 with POAG, opens promising therapeutic perspectives based on nicotinamide supplementation.

319

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390 **TABLE AND FIGURE LEGENDS**

391 **Table 1: Characteristics of individuals from the initial cohort.** Demographic data and  
392 comorbidity status, systemic medications, ophthalmological features and glaucoma medication  
393 of individuals with POAG compared to controls. BMI: body mass index (weight/height<sup>2</sup>). IOP:  
394 intraocular pressure; CCT: central corneal thickness; RNFL: retinal nerve fibre layer; VF-MD:  
395 visual field mean defect.

396

397 **Table 2: Characteristics of individuals from the replicative cohort.** Demographic data and  
398 comorbidity status, systemic medications, ophthalmological features and glaucoma medication  
399 of individuals with POAG compared to controls. BMI: Body mass index (weight/height<sup>2</sup>). IOP:  
400 intraocular pressure; CCT: central corneal thickness; RNFL: retinal nerve fibre layer; VF-MD:  
401 visual field mean defect.

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403 **Figure: Boxplots showing nicotinamide levels in the initial (34 POAG and 30 control  
404 individuals) and replicative (20 POAG and 15 control individuals) cohorts.** Error bars  
405 represent  $\pm$  SEM, and the black bars within the boxplots represent the median concentration for  
406 each group. (A) Peak area of nicotinamide found in the initial cohort following LC-HRMS  
407 analysis discloses a glaucoma/controls fold change of 0.65. (B) Concentrations of nicotinamide  
408 found in the initial and replicative cohorts following LC-MS/MS analysis. The  
409 glaucoma/controls fold changes were 0.70 and 0.67 for the initial and replicative cohorts,  
410 respectively. The *p*-values between groups for all conditions were \*: *p* < 0.05 and \*\*: *p* < 0.01.

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414 **Table 1: Characteristics of individuals from the initial cohort.** Demographic data and  
415 comorbidity status, systemic medications, ophthalmological features and glaucoma medication  
416 of individuals with POAG compared to controls. BMI: body mass index (weight/height<sup>2</sup>). IOP:  
417 intraocular pressure; CCT: central corneal thickness; RNFL: retinal nerve fibre layer; VF-MD:  
418 visual field mean defect.

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	<b>POAG</b> (N=34)	<b>Controls</b> (N=30)	<b>p-</b> <b>value</b>
<b>Demographic data and comorbidity</b>			
Average age (y)	73.06	73.77	0.65
Females (%)	50	50	1
Mean BMI (kg/m <sup>2</sup> )	26.22	26.99	0.59
Diabetes (%)	17.65	3.33	0.10
Hypertension (%)	50	63.33	0.29
Hyperlipidaemia (%)	26.47	43.33	0.165
Thyroid disease (%)	11.76	13.33	0.29
<b>Systemic medications</b>			
Anti-hypertensives (%)	47.06	63.33	0.19
Lipid-lowering medications (%)	23.53	43.33	0.09
Antiplatelet therapy (%)	26.47	36.67	0.39
Oral diabetes medications (%)	14.71	13.33	0.88
Insulin (%)	2.94	0	0.32
Corticosteroids (%)	2.94	3.33	0.93
Thyroid hormone (%)	17.65	13.33	0.64
Oestrogen (%)	0	0	1
Vitamin D (%)	11.76	20	0.38
<b>Ophthalmological features and glaucoma medication</b>			
Mean visual acuity (LogMar)	+0.12	+0.13	0.91
Mean IOP (mmHg)	13.42	14.10	0.27

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Mean CCT (μm)	529.95	-	-
Average RNFL thickness (μm)	66.91	-	-
Mean VF-MD (dB), (eye with worse MD)	-6.83	-	-
Glaucoma severity (%)			
Mild	82.35	-	-
Moderate	5.88	-	-
Severe	11.77	-	-
Glaucoma medications (%)			
Beta-blockers	55.88	-	-
Prostaglandin analogue	67.65	-	-
Alpha-2-agonists	11.76	-	-
Carbonic anhydrase inhibitor	26.47	-	-

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423 **Table 2: Characteristics of individuals from the replicative cohort.** Demographic data and  
424 comorbidity status, systemic medications, ophthalmological features and glaucoma medication  
425 of individuals with POAG compared to controls. BMI: Body mass index (weight/height<sup>2</sup>). IOP:  
426 intraocular pressure; CCT: central corneal thickness; RNFL: retinal nerve fibre layer; VF-MD:  
427 visual field mean defect.

	<b>POAG</b> (N=20)	<b>Controls</b> (N=15)	<b>p-value</b>
<b>Demographic data and comorbidity</b>			
Average age (y)	64.85	70.27	0.11
Females (%)	25	53.33	0.09
Mean BMI (kg/m <sup>2</sup> )	25.75	28.27	0.30
Diabetes (%)	25	13.33	0.39
Hypertension (%)	35	73.33	0.02
Hyperlipidaemia (%)	25	60	0.04
Thyroid disease (%)	5	0	0.33
<b>Systemic medications</b>			
Anti-hypertensives (%)	35	73.33	0.02
Lipid-lowering medications (%)	25	60	0.04
Antiplatelet therapy (%)	25	13.33	0.39
Oral diabetes medications (%)	25	13.33	0.39
Insulin (%)	0	0	-
Corticosteroids (%)	5	0	0.33
Thyroid hormone (%)	5	0	0.33
Oestrogen (%)	0	0	-
Vitamin D (%)	10	6.67	0.73
Others (%)	40	33.33	0.69
<b>Ophthalmological features and glaucoma medication</b>			
Mean visual acuity (LogMar)	+0.05	+0.03	0.37
Mean IOP (mmHg)	15.82	13.84	<0.001

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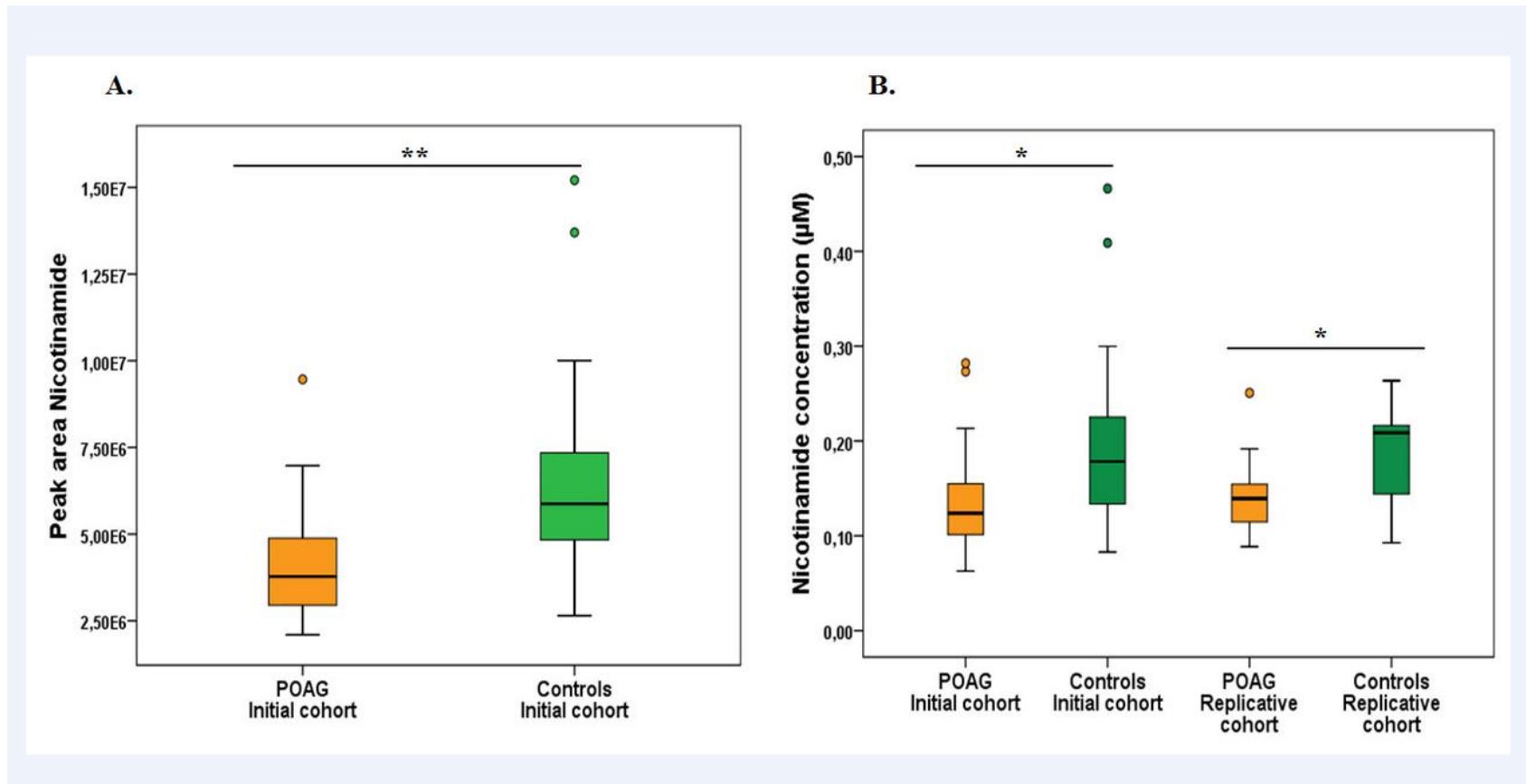
Mean CCT (μm)	544.44	-	-
Average RNFL thickness (μm)	68.7	-	-
Mean VF-MD (dB), (eye with worse MD)	-3.99	-	-
Glaucoma severity (%)			
Mild	80	-	-
Moderate	10	-	-
Severe	10	-	-
Glaucoma medications (%)			
Beta-blockers	60	-	-
Prostaglandin analogue	85	-	-
Alpha-2-agonists	0	-	-
Carbonic anhydrase inhibitor	15	-	-

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**Figure: Boxplots showing nicotinamide levels in the initial (34 POAG and 30 control individuals) and replicative (20 POAG and 15 control individuals) cohorts.** Error bars represent  $\pm$  SEM, and the black bars within the boxplots 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 glaucoma/controls fold changes were 0.70 and 0.67 for the initial and replicative cohorts, respectively. The *p*-values between groups for all conditions were \*:  $p < 0.05$  and \*\*:  $p < 0.01$ .

