Mitochondrial respiratory function is strongly associated with progressive glaucomatous vision loss

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Abstract

Intraocular pressure (IOP) has been the only modifiable risk factor for glaucoma. However, glaucoma develops with high IOP (high tension glaucoma; HTG) and normal IOP (normal tension glaucoma; NTG) and many lose vision despite IOP-lowering treatment, suggesting other factors confer susceptibility. We demonstrate that lymphocyte/monocyte mitochondrial oxygen consumption rate (OCR) is lower in glaucoma patients than in controls ($P < 0.001$), is lower in NTG than HTG ($P < 0.01$) and lower OCR is strongly associated with faster visual field (VF) progression in patients treated by lowering IOP ($P < 0.001$), explaining 13% of variance in the rate of progression. In a reference cohort of untreated glaucoma patents, IOP explained 16% of VF progression variance. Lymphocyte/monocyte nicotinamide adenine dinucleotide (NAD) levels are lower in glaucoma patients ($P < 0.001$) and strongly associated with OCR ($P < 0.001$). Our results support mitochondrial OCR and NAD levels as new biomarkers for glaucoma progression susceptibility and new targets for treatment.

Main

Affecting ~ 80 million people by the end of this decade, glaucoma is a chronic, progressive optic neuropathy in which retinal ganglion cells (RGCs) die, leading to vision loss. It is the leading cause of irreversible blindness worldwide [1]. High intraocular pressure (IOP) and older age have been established as the most important risk factors for glaucoma and its progression, with IOP being the only modifiable risk factor [2,3]. Whilst lowering IOP slows the rate of visual field (VF) progression [3–5], up to 60% of glaucoma patients with European ancestry [6] and up to 90% with Asian ancestry [7] lose vision despite IOP being within the normal range (normal tension glaucoma [NTG]). Many patients still progress despite IOP lowering or meeting target IOP [8,9] with 38.1% blind in one eye and 13.5% blind in both eyes 20 years from initial diagnosis [10]. This suggests that other factors confer susceptibility to glaucomatous neurodegeneration [11] and underlines the importance of identifying new, potentially treatable, risk factors. Several studies have indicated that altered mitochondrial bioenergetics is associated with both susceptibility and resistance to developing glaucoma [12–22]. However, to date, no study has shown the extent to which mitochondrial function influences glaucoma progression and its importance relative to other risk factors, and whether it can be used as a biomarker for assessing susceptibility to disease progression. In trying to understand potential causes and consequences of mitochondrial dysfunction for neurodegeneration, several research groups have investigated nicotinamide adenine dinucleotide (NAD) - an essential cofactor central to mitochondrial function, ATP synthesis, cellular metabolism, and a key regulator of axonal health [23,24]. NAD levels decline in the retina of glaucomatous DBA/2J mice, in an age- and IOP-dependent manner, rendering retinal ganglion cells (RGCs) susceptible to IOP-related stress and accelerating glaucomatous neurodegeneration [15]. Glaucoma patients have been reported to have low levels of nicotinamide (NAM; the amide form of vitamin B_3, an NAD precursor via the NAD-salvage pathway) in sera [25]. Furthermore, population-based studies have found that lower niacin (vitamin B_3) intake was associated with NTG [26], and greater niacin intake may be associated with a lower chance of

developing glaucoma [27,28]. The role of NAD in mitochondrial respiratory function suggests that bolstering cellular NAD + levels may improve cellular energetics and stress responses in neurons. The potential for modifiability of NAD levels, is evidenced by results of preclinical glaucoma models [15,29], and short term clinical trials showing electrophysiological and visual function evidence of recovery of RGC function with high-dose NAM supplementation [18,30]. Several clinical trials have now been initiated to evaluate the potential of high dose NAM to slow VF loss in glaucoma (e.g., the Nicotinamide in Glaucoma (NAMinG) trial - EME NIHR132758, NCT05405868).

Mitochondrial function and NAD levels may decline as a result of generalized changes, such as aging, or may be a consequence of local IOP-related stress in the optic nerve head (ONH). As the tissues of the ONH are not accessible *in vivo*, we assessed the generalized aspect, using peripheral blood monocular cells (PBMCs) as the model system for bioenergetic analyses.

We provide evidence that systemic mitochondrial function is lower in primary open-angle glaucoma (POAG) than in age-similar non-glaucomatous controls and is even lower in NTG compared to high-pressure POAG (HTG). Furthermore, we demonstrate a strong association between systemic mitochondrial function and the rate of visual field progression. We also demonstrate that total NAD levels are lower in POAG PBMC, and that NAD levels correlate positively with mitochondrial function providing new biomarkers for glaucoma susceptibility.

Results

Mitochondrial respiration is lower in glaucoma patients than in non-glaucomatous controls

Mitochondrial respiration was measured as oxygen consumption rate (OCR) using the XFe24 Analyzer in PBMC of 218 participants (99 NTG, 69 HTG, 50 age similar controls; **Table 1**; participant details). NTG patients had the lowest basal OCR (mean [SD] pmol/min/100,000 cells): 19.2 [4.1], followed by HTG 21.6 [3.9] and controls 26.8 [5.5] (Fig. 1A). The differences HTG and NTG versus control ($P < 0.001$) and NTG versus HTG ($P < 0.01$) were all statistically significant. Maximal OCR, ATP-linked OCR, and Reserve Capacity followed the same trend (**Supplementary Fig. 1, 2**). The intra-assay Coefficient of Variation (CV%) between technical repeats for basal OCR was 10%. Repeatability was measured in 31 NTG and 12 HTG participants on two occasions. Median (IQR) number of days between test and retest was 217 (126–746). Mean (SD) basal OCR was 20.8 (6.6) pmol/min/100,000 cells. 95% of values were within − 6.5 and + 6.2 pmol/min/100,000 cells of the test-retest mean difference (-0.1 pmol/min/100,000 cells) (**Supplementary Fig. 1**).

Extensive phenotypic information was collected for each participant. We next investigated the association of OCR with diagnostic category, demographic and clinical characteristics with univariable and multivariable linear regression models. We further analyzed those variables with a frequency of more than 15% and those detailed in the literature to have an impact on mitochondrial function. Covariates to

include in the final multivariable models were selected with the least absolute shrinkage and selection operator (LASSO) regression [31]. Analyses were repeated on control subjects alone for comparison.

Results of the multivariable model for factors associated with basal OCR in all participants and in the subset of control subjects are illustrated in **Fig. 1B** and **Supplementary Table 1**. Older age was associated with higher basal OCR in the whole cohort (POAG and controls), but not in the subset of control subjects alone (Fig. 1B). There was no association between age and OCR in the univariable models for the whole cohort or in the subset of control subjects. Type II diabetes was associated with lower maximal OCR (estimate [SE]: -46.7 [20.8] pmol/min/100,000 cells, $P = 0.030$) and reserve capacity (estimate [SE]: -44.0 [19.1] pmol/min/100,000 cells, $P = 0.026$), in the subset of control subjects (**Supplementary Fig. 3 and Supplementary Tables 2–4**). None of the other covariates in any model was associated with mitochondrial function in control subjects.

Mitochondrial respiratory function is strongly associated with the rate of visual field progression

VF progression rate and its association with mitochondrial respiration was analyzed in 229 eyes (NTG: 144 eyes, HTG: 85 eyes) of 139 patients. **Table 2** shows the demographic and clinical characteristics of the cohort. The summary value for visual field loss is the Mean Deviation (MD). The relationship between the rate of MD change and OCR was evaluated with linear mixed models with random slopes and random intercepts. In all models, the dependent variable was the MD value at each visit. Fixed effects were the follow-up time, covariates of interest, and their interaction. The random slope term was the follow-up time to account for the fact that different eyes may have different rates of progression over time; and the random intercept had a nested design with eye and patient IDs being the inner and outer level to account for within-eye (multiple tests from the same eye) and within-subject (both eyes of the same patient) correlations. Interactions between covariates and follow-up time modeled the variables' effect on the progression rate. In addition to mitochondrial OCR, we included the following potential variables of interest: baseline age, central corneal thickness (CCT), and mean IOP over the VF observation period. Other OCR parameters (basal, ATP-linked and maximal OCR and reserve capacity) and IOP metrics (mean, peak, fluctuation and relative reduction) over the VF observation period were included in separate models as sensitivity analyses (**Supplementary Figs. 4–7 and Supplementary Tables 5–12**). All analyses were repeated on the HTG and NTG cohorts separately. All independent variables were standardized (*i.e.* zero mean and unit standard deviation) to facilitate the comparison of the effect magnitude of different covariates by putting them on the same scale.

Overall, the mean (± SD) MD rate was − 0.59 (0.33) dB/year. Lower basal OCR was significantly associated with faster rates of VF progression (Fig. 2A **and Supplementary Table 5**); estimate for 1 SD (4.3 pmol/min/100,000 cells) difference [SE]: 0.19 [0.04] dB/year, $P < 0.001$. The coefficients for the NTG and HTG cohorts were similar when analyzed separately (Fig. 2A **and Supplementary Tables 6 and 7**). The proportion of variance in the rate of progression explained by basal OCR was 13% (partial R^2). Older age was associated with faster rates of progression in the whole cohort (for a 1 SD increase (10 years),

estimate [SE]: -0.09 [0.03] dB/year, $P = 0.002$) and in the HTG subset (for a 10-year increase, estimate [SE]: -0.20 [0.05] dB/year, $P < 0.001$) but not in the NTG subset (for a 10-year increase, estimate [SE]: -0.03 [0.04] dB/year, $P = 0.40$). Higher mean IOP was associated with faster rates of progression in the whole cohort (for 1 SD (2.8 mmHg) increase, estimate [SE]: -0.07 [0.03] dB/year, $P = 0.04$), but not in the NTG and HTG groups separately. This equates to -0.03 (95% CI -0.050 to -0.001) dB/year per 1 mmHg higher mean IOP. The proportion of variance explained by IOP was 1% (partial R^2). In this cohort, a 1 SD difference in basal OCR was equivalent to a 7.6 mmHg IOP difference and a 21-year age difference.

To test the hypothesis that the association between OCR and VF progression rates may change as a function of IOP values, we ran additional models with a triple interaction term among follow-up time, basal OCR and mean IOP. The interaction term in the whole cohort was not significant ($P = 0.97$), suggesting that the impact of mitochondrial dysfunction on disease progression is constant across the IOP spectrum (**Supplementary Table 8**). Similar associations between OCR parameters and VF rates were found in models for other IOP metrics (**Supplementary Figs. 4–7 and Supplementary Tables 9–11**). The interval from the end of the VF observation window and OCR measurement was a median (IQR) of 5.9 (1.7–9.5) years in patients who had undergone glaucoma surgery (50.7% of eyes) and 4.0 (2–18) days in patients who had not undergone glaucoma surgery. To test whether the association of OCR and VF progression rates was affected by whether patients had had glaucoma surgery or not (and the consequent time interval), we ran the analysis in two separate groups (surgery / no-surgery). The association between basal OCR and MD rate and the coefficients in both groups (surgery / no-surgery) were similar to those of the whole POAG cohort (**Supplementary Tables 12–13**). Furthermore, the interaction term among OCR and surgery status was not significant ($P = 0.97$), indicating that having had surgery was not associated with basal OCR differences (**Supplementary Table 14**). Taken together, these data support a hypothesis in which non-IOP factors (*e.g.* genetic or nutritional) alter mitochondrial respiration, and subsequently, confer a risk visual function deterioration in glaucoma patients.

The association of intraocular pressure with the rate of visual field progression in an untreated cohort provides a reference effect size

Glaucoma participants in our cohort were treated with IOP lowering medication or surgery and this may alter the association between IOP and the rate of VF progression. Therefore, we looked at an untreated glaucoma cohort as a reference dataset – the placebo arms of the UK Glaucoma Treatment Study (UKGTS).[3,32]. We included eyes from the placebo group with ≥ 5 reliable (< 15% false positive responses) VFs. Demographic and clinical characteristics of this cohort are detailed in **Supplementary Table 15**. The median (IQR) MD progression rate was − 0.23 (-0.73 to 0.11) dB/year. Only one eye per patient (worst eye), was included in the analysis as per the UKGTS protocol. In all models, the dependent variable was the MD value at each visit; fixed effects were the follow-up time, covariates of interest, and their interaction and the random slope term was the follow-up time. Results of the UKGTS multivariable model with standardized (by the SD of the clinic cohort) variables are illustrated in **Fig. 2B**. Higher mean IOP was significantly associated with faster VF progression: 1 SD (2.8 mmHg) increase in IOP was associated with (estimate [SE]) -0.25 [0.05] dB/year ($P < 0.001$) faster progression. Thicker central cornea was

associated with slower VF progression (for 1 SD increase, estimate [SE]: 0.18 [0.09] dB/year, $P = 0.046$). Baseline age was not associated with the rate of progression in this cohort (for 1 SD increase, estimate [SE]: 0.017 [0.08], $P = 0.83$). The proportion of variance in the rate of VF progressionexplained by IOP in the placebo arm of the UKGTS was 16% (partial R^2). In the clinic study, a reduction in basal OCR by 1 SD was associated with − 0.19 dB/year rate difference, an effect size equivalent to 2.1 mmHg in the UKGTS placebo arm.

Total NAD levels are lower in glaucoma patient PBMCs compared to controls

It has previously been reported that the NAD precursor, nicotinamide, is low in sera of a cohort of glaucoma patients [25]. To test the hypothesis that NAD levels themselves are lower in POAG (HTG and NTG), we measured total cellular NAD in the PBMC of 54 subjects (25 Controls, 10 HTGs and 19 NTGs). The intra-assay coefficient of variation (CV) between replicates was 9%. Cellular total NAD was statistically significantly different between the three groups ($P < 0.001$), with both NTG ($P < 0.001$) and HTG ($P = 0.01$) patients having significantly lower NAD levels compared to controls (Fig. 3A).

To test the hypothesis that NAD levels were associated with mitochondrial OCR, we ran multivariable linear regression models. Considering the smaller number of participants in this part of the study, covariates to include in the model were included based on the results of the multivariable models in the larger cohort. We chose to select covariates that were significantly associated with any of the OCR parameters (age, statin usage, number of systemic diseases, alcohol consumption and diabetes). Results of the multivariable model for basal OCR in the whole cohort of participants, and the subset of control subjects only, are illustrated in **Fig. 3B** and **Supplementary Table 16**. In the whole cohort, there was a significant association between higher cellular total NAD levels and higher basal OCR (estimate [SE]: 11.6 [2.1] pmol/min/100,000 cells per 1 pg NAD / mg of protein increase, $P < 0.001$). The proportion of variance in basal OCR in the multivariable model explained by total NAD levels was 16% (partial R^2). In control samples, the association of higher NAD levels with higher basal OCR values did not achieve statistical significance at the nominal 5% level, (estimate [SE]: 6.5 [3.4] pmol/min/100,000 cells per 1 unit of NAD increase, $P = 0.07$). There were similar associations between total NAD and other OCR parameters, whilst other covariates were not associated with OCR in any of the models (**Supplementary Fig. 8 and Supplementary Tables 17–19**). There was no significant association between NAD levels and rates of VF MD progression (estimate [SE]: 0.44 [0.37] dB/year per 1 SD (0.36 pg NAD / mg of protein) NAD increase, $P = 0.25$), possibly due to the small sample size (the association between basal OCR and rates of VF MD progression is also not significant (estimate [SE]: 0.14 [0.09] dB/year per 1 SD basal OCR increase (4.3 pmol/min/100,000 cells), $P = 0.12$) in this smaller subset of patients).

Discussion

Elevated IOP and greater age have been regarded as the major risk factors for glaucoma development and progression, with IOP being the only modifiable risk factor. The data presented here demonstrate

significantly lower mitochondrial OCR in both HTG and NTG patients compared to age-similar controls, with the difference being more marked in the NTG cohort. This implicates mitochondrial function as a susceptibility factor for glaucoma development across all IOP levels and especially in those with low IOP. These data also provide evidence of a strong, clinically relevant association between lower systemic mitochondrial function and faster glaucomatous VF progression in patients already treated by lowering IOP. Amongst various metabolites and pathways that may be implicated in mitochondrial function, lower NAD levels have been reported in various glaucoma models [15,33]. Our findings support this and demonstrate that total cellular NAD levels are lower in POAG patients and are strongly associated with lower mitochondrial respiratory function.

Initial reports of mitochondrial abnormalities in lymphocytes of glaucoma patients [12] have since been replicated by others [17], including in our own work [34]. The role of mitochondria in glaucoma susceptibility is further supported by *ex vivo* and animal model studies [15,35–40] and by genetic studies [41]. To understand the effect of mitochondrial function in glaucoma, we ran multivariable models including various factors that may have an influence on mitochondrial function. Type 2 diabetes was the only covariate associated with reduced (Maximal) OCR in the control group, in agreement with previous studies [42]. NTG patients had the lowest mitochondrial function, compared to HTG and controls. IOP is a well-established risk factor in glaucoma, however, NTG patients develop glaucomatous optic neuropathy with IOP in the statistical normal range, indicating that IOP is only one factor for developing glaucoma [43]. The phenotype of the NTG neuropathy may exhibit subtle differences to that of HTG. Caprioli and Spaeth [44] found VF defects in NTG to be significantly deeper and closer to fixation than in other types of glaucoma. Such VF defects bare similarities to those of mitochondrial optic neuropathies such as Leber's hereditary optic neuropathy (mitochondrial genome mutations) and dominant optic atrophy (mutations in a nuclear gene encoding inner mitochondrial membrane proteins) [45], supporting a mitochondrial contribution to the glaucomatous neuropathy. In fact, polymorphisms in the optineurin and TBK1 genes (which mediate mitophagy) and OPA1 (essential for mitochondrial fusion) account for a small proportion of NTG cases [46–49]. Given that mitochondrial function is lower in HTG than controls and lower in NTG than HTG, it seems to be an important risk factor across the range of IOP and more important at normal than high IOP levels. The interaction term (IOP*OCR*time) in our statistical model was not significant, suggesting that the IOP risk and mitochondrial risk is simply additive.

We evaluated the association between mitochondrial function and VF progression. The rate of VF progression is the most important clinical measurement of glaucoma deterioration and helps predict the likelihood of visual disability and blindness [50]. The identification of biomarkers for faster deterioration, identifying patients most at risk of visual disability and blindness, would considerably improve glaucoma care efficiency and enable personalized medicine. Such biomarkers would enable risk stratification for more intensive monitoring, more appropriate setting of treatment goals (such as target IOP) and, should mitochondria-targeted therapies become available, selection of patients for targeted treatment. Our study identifies OCR as a novel factor strongly associated with the rate of VF progression. Various clinical trials have demonstrated that reducing IOP slows down glaucoma progression in both HTG and NTG [4,5,51,52].

For instance, the Early Manifest Glaucoma Trial found that the risk of progression was reduced 10% for each 1 mmHg IOP reduction [5]. In our study we found the effect size of the association of basal OCR with the rate of VF loss in the whole cohort to be two and a half times that of IOP, when OCR and IOP were standardized to their respective SDs in this cohort. The proportion of variance explained by IOP (partial R^2 0.01) was low. The explanation for the weak association of IOP with VF progression rate is that this is a clinical glaucoma cohort treated for IOP lowering. In clinical practice, IOP is lowered to a level considered sufficient to slow VF progression to prevent symptomatic vision loss in the patient's lifetime [53] and treatment is escalated in patients showing VF progression. Such clinical intervention alters the association between IOP and progression rate. Therefore, we examined the association between IOP and the rate of VF progression in an untreated glaucoma cohort (the placebo arms of the UK Glaucoma Treatment Study; UKGTS) as a reference dataset. The proportion of variance in the rate of VF progression rate explained by IOP in the placebo arm of the UKGTS was about 16%. This compares to the proportion explained by basal OCR (13%) in the current study, once the contribution of IOP had been almost removed. The association of lower basal OCR with a faster rate of VF loss is clinically relevant, with 1 SD basal OCR in this study being equivalent to between 2.1 mmHg (in the UKGTS cohort) and 7.6mmHg (in this study's clinic cohort) higher mean IOP. Similarly, one standard deviation lower basal OCR was equivalent to being nearly 21 years older, in terms of glaucoma progression risk in this dataset. This demonstrates that OCR is an important biomarker for progressive VF deterioration in both HTG and NTG

Retinal NAD depletion in animal models of glaucoma and reduced NAM (an NAD precursor) in plasma of POAG patients have been reported by others [15,25]. Our results demonstrate that total PBMC cellular NAD levels in the POAG cohort were significantly lower than in age-similar controls. NAD is at the centre of various metabolic reactions culminating in ATP production. Therefore, its depletion will inevitably impair mitochondrial respiration and ATP synthesis, resulting in energy crisis which may lead to RGC functional impairment and death. The finding of lower PBMC NAD levels in NTG compared to HTG patients, lower NAD levels in HTG patients compared to controls, and the association of NAD levels with basal OCR, suggest that NAD levels may be a mediator of reduced mitochondrial respiratory function and a susceptibility factor for glaucoma progression. The low NAD levels in PBMC demonstrated in our study indicate a generalized metabolic dysfunction outside the eye. Although the precise mechanisms leading to mitochondrial dysfunction are still unclear, such dysfunction observed in glaucoma could be the result of lower NAD levels or it may be the cause of reduced NAD levels. Given the high energy dependence of RGCs, their axons and supporting glia, the findings in preclinical models, and similarities that NTG has with primary mitochondrial optic neuropathies, it is very likely that the mitochondrial dysfunction observed in this study is more than an epiphenomenon.

There are some limitations in this study. Estimates of VF progression are, by the nature of VF testing, imprecise. This imprecision weakens the strength of the observed association between the rate of VF progression and OCR measurements and IOP, so that both OCR and IOP likely explain more of the variance in the true rates of progression than is apparent in the analysis. The OCR and NAD measurements were made after the VF observation window, by several years in those who had glaucoma

surgery. However, an analysis of patients with a longer interval between measurements showed very similar results compared to those with a short interval, suggesting that the association of OCR and NAD measurements with progression risk is probably quite stable over time. The POAG patients in our study were recruited from clinics seeing patients at high risk of, or demonstrating, progression. This may over-estimate the role mitochondrial function in an unselected glaucoma population. Nevertheless, it quantifies the association of mitochondrial function with VF progression in the subset of patients of greatest importance – those more likely to lose vision from glaucoma. Mitochondrial function will be measured prospectively in an unselected POAG population in the NAMinG trial, providing information on the generalizability of the findings of this study.

In conclusion, this study provides the first evidence of the strong association between mitochondrial respiratory activity and the rate of disease progression in glaucoma. Low cellular NAD levels were associated with lower mitochondrial respiratory activity. To date the only modifiable risk factor for glaucoma progression has been IOP and all licensed treatments are for IOP lowering; none addresses neuronal resilience to disease-related stresses. Mitochondrial respiratory activity is potentially modifiable and is a new target for treatment [15,18] which has been shown to be effective in preclinical glaucoma models [29]. Furthermore, our results highlight potential new biomarkers, easily accessed in clinic through a blood sample, to identify patients at high risk of fast progression and visual disability early in the disease course.

Material and Methods

Study Design

This study was conducted in adherence with the tenets of the Declaration of Helsinki and was approved by the London - Surrey Borders and Health and Care Research Wales ethics committees. Participants were recruited between October 2018 to December 2021 after informed consent was taken from all participants. All participants underwent blood drawing. The study consisted of three parts (Supplementary Fig. 9). Part 1 – Association of PBMC mitochondrial function with glaucoma diagnosis. Part 2 – Association of PBMC mitochondrial OCR and the rate of VF progression. Part 3 – Association of PBMC NAD levels with glaucoma diagnosis and OCR. The association between IOP and the rate of VF progression was evaluated in a reference cohort of patients without IOP-lowering treatment (to avoid the confounding effect of treatment escalation on the association) – the placebo arm of the United Kingdom Glaucoma Treatment Study (UKGTS) [3,32]. Supplementary table 15 shows the main baseline demographic characteristics of this group.

Participants

A convenience sample of POAG patients was recruited from the glaucoma clinics at Moorfields Eye Hospital (London, UK) during their routine clinic appointments. Eligibility required a consultant ophthalmologist confirmed diagnosis of HTG (IOP $\geq$ 21mmHg before treatment) or NTG (IOP $\leq$ 21mmHg

before treatment), open drainage angles on gonioscopy and absence of a secondary cause for glaucomatous optic neuropathy or non-glaucomatous cause for VF loss. POAG participants were under usual care of IOP-lowering eye drops (64% of eyes), laser trabeculoplasty (16% of eyes) and/or surgical IOP reduction (50.7% of eyes). Age-similar control participants were recruited from the cataract clinics at Moorfields Eye Hospital (London, UK): eligibility required an ophthalmologist confirmation of absence of glaucomatous optic neuropathy, no family history of glaucoma in a first degree relative and IOP ≤ 21mmHg.

Ninety-nine NTG, 69 HTG and 48 Controls were recruited to Part 1 of the study. To be eligible for Part 2, participants were required to have had a minimum of 6 reliable (< 15% false positive responses) visual fields over a minimum of 3 years prior to having had any form of glaucoma surgery (trabeculectomy/tube), if applicable. For eyes having had glaucoma surgery, follow-up was censored from the listing visit onward so that the observation window was immediately prior to glaucoma surgery. 50.7% of eyes had undergone glaucoma surgery and 93% of eyes were on at least one IOP lowering eye drop at the time of OCR measurement.

Exclusion criteria for all participants were: secondary (including pseudoexfoliation and pigmentary), angle closure and congenital glaucomas. In addition, participants were not eligible if they had any medical conditions, or were on any treatments, known to affect lymphocyte function (active haematological malignancy, infection at the time of the blood sampling and recent chemotherapy/radiotherapy) or mitochondrial function (amiodarone, tetracyclines, chloramphenicol, antiretroviral drugs).

Visual function was quantified by standard automated perimetry with the Humphrey Field Analyzer (Carl Zeiss Meditec, Inc, Dublin, California, USA). We recorded the VF mean deviation (MD) from age-matched normative values from 24 – 2 Swedish Interactive Thresholding Algorithm (SITA) Standard tests, from both eyes. We also recorded the IOPs (Goldmann applanation tonometry - GAT) of both eyes during the period for which VF data were collected. Extensive phenotypic data were collected for each participant, including information on general and ocular health (Table 1, Table 2).

An untreated reference cohort was taken from the UKGTS, a randomised, multicentre, triple-masked, parallel-group, placebo-controlled trial [3,32]. The trial consisted of 516 participants randomized (1:1) to either latanoprost 0·005% or placebo, scheduled to have 16 24 – 2 Humphrey visual fields over 24 months. We included in our analysis only those eyes that had a minimum of five reliable visual fields over the observation period. VFs were also SITA Standard and IOP was measured with GAT. Variables included in the model were mean IOP over the observation period, CCT and baseline age.

Isolation of PBMCs

Venous blood was sampled in 10.0 mL Becton Dickinson (BD) Vacutainer blood collection tubes containing EDTA (Ethylenediamin tetra-acetic acid) (BD Biosciences). PBMCs were isolated from freshly drawn blood within 3 hours following venipuncture and were used immediately for oxygen consumption

rate (OCR) and total NAD measurements. Whole blood was mixed with 10 ml 1x Phosphate-buffered saline (PBS) on a 1:1 ratio, and then slowly pipetted down a 50mL falcon over 15mL of Lymphoprep™ (Accurate Chemical & Scientifix Corp. Cat # 1001967). Tubes were centrifuged at 1,300 g for 26 minutes with no brake at room temperature. After centrifugation, 4 mL of the cloudy PBMC band were collected using a sterile transfer pipette and added to two 15 mL tubes (2mL in each) and topped up with 6 mL PBS. The capped 15 mL tubes were mixed by inversion and centrifuged at 800 x g for 10 min at room temperature. The supernatant was carefully aspirated, the cell pellets were resuspended in 8 mL of PBS and centrifuged for a subsequent 10 min at 800 x g at room temperature. Following this centrifugation step, the supernatant was again carefully aspirated without disturbing the cell pellet, and PBMCs were resuspended in 0.8 mL of complete Seahorse media warmed at 37°C.

Application of Seahorse XFe24 to measure oxygen consumption in PBMC

The Mito Stress assay provides estimation of different bioenergetic measures by monitoring the oxygen consumption rates (OCR) of living cells through the addition of various inhibitors of OXPHOS. OCR was quantified using the XFe24 Analyzer (Agilent Technologies) according to the manufacturer's instructions. This technique allows real-time measurements of oxygen consumption rate (OCR) in living cells. Cells were seeded at a density of to 8 x 10^5 cells per well. One day before the assay, XFe24 Sensor Cartridge (Agilent) was hydrated overnight at 37°C in a non-CO2 incubator to equilibrate. On the day of the assay, PBMCs were suspended in 0.8 mL of pre-warmed (37°C) Seahorse Base media (# 102353-100, Seahorse Bioscience, Agilent) supplemented with 10 mM glucose, 1 mM sodium pyruvate, 2 mM glutamine final, adjusted to pH 7.4. Cells were counted using the Orflo MoxiGo II flow cytometer and diluted to 8 x 106 / mL. 100 µL of cell suspension was transferred into each well of a Seahorse XFe24 plate, pre coated with Corning Cell-Tak Cell and Tissue Adhesive (#CLS354240-1EA, Sigma-Aldrich), and the plate was centrifuged for 2 minutes at 200g, brake off to ensure cells were fully attached. The plate was transferred in a 37°C non-CO2 incubator for 30 minutes, following which, each well was topped up to a final volume of 500 mL with complete Seahorse Base media and transferred in a 37°C non-CO2 incubator for a further 30 minutes. Oxygen consumption rates (OCR) were monitored through sequential injections of oligomycin (1.5 µM), carbonylcyanide-4-trifluoromethoxyphenylhydrazone (FCCP, 1.5 µM), and antimycin A / rotenone (1.6 µM / 16 µM). The various parameters of the mitochondrial respiration were calculated as described in Supplementary methods, and results were expressed as pmol/min/100,000 cells.

Cellular total NAD content assay

Cellular total (NAD + plus NADH) NAD was measured in a convenience sample of 52 participants from the cohort of 218 subjects (25 Control, 10 HTG and 17 NTG). Median (IQR) number of days between OCR and NAD assays was 0 (0–123) days. Total NAD was quantified by a luciferase assay provided in the NAD+/NADH Glo Assay kit (#G9071, Promega). Luminescence was recorded at 20 minutes after the addition of the NAD/NADH-Glo™ Detection Reagent using Cytation1 Imaging plate reader (Biotek Instruments Inc., Agilent), with the Gen5 v3.05.11 microplate reader and imaging software. Following several titrations, the minimum and maximum amount of total cellular protein required to be in the linear

range of the assay were 2.1 µg – 39 µg (corresponding roughly to 150,000–4,000,000 PBMC). PBMCs were washed in ice cold PBS and centrifuged at 13,000 g for 10 minutes at 4°C. Supernatant was discarded and the cell pellet was suspended in 200 µL ice cold PBS. 30 µL of cell suspension was transferred in a 96-well, white-walled plate, followed by 30µl of NAD/NADH-Glo™ Detection Reagent. There was a minimum of two technical repeats for each participant. To ensure recordings were in the linear range of the assay, serial dilutions were performed. Technical repeats with protein levels outside the linear range and those with fluctuating results over time were excluded from analysis. Cellular total NAD levels were normalized to protein content and expressed as: pg NAD / mg of Protein.

Statistical analysis

We reported mean (± Standard deviation [SD]) and median (interquartile range [IQR]) for continuous variables and frequencies and proportions for discrete variables. We compared demographic and clinical characteristics among HTG, NTG, and control groups with ANOVA and chi-squared tests for continuous and categorical variables, respectively.

We investigated associations between the various OCR metrics and demographic and clinical characteristics of enrolled subjects with univariable and multivariable linear regression models. We ran separate models for each OCR metric (dependent variable). Candidate covariates included: age at the time of blood sample taking, sex, diagnostic status (i.e., control, HTG and NTG), smoking status, alcohol consumption, diabetes, systemic hypertension, hypercholesterolemia, migraine, number of systemic illnesses, body mass index (BMI), vasospasm, vitamin D supplementation, thyroid disease, and use of the following: angiotensin converting-enzyme (ACE) inhibitors, angiotensin receptor blocker (ARB), systemic beta-blocker, calcium channel blocker and/or diuretics. Covariates to include in each multivariable model were selected with the Least absolute shrinkage and selection operator (LASSO) regression LASSO is a form of penalized linear regression, which reduces some coefficients' magnitude and shrinks other variables to zero; non-zero coefficients are kept in the model [31]. Analyses were also repeated on control subjects (after excluding glaucoma patients) for comparison.

We used a one-way ANOVA with post-hoc Tukey’s HSD Test for multiple comparisons, to investigate whether cellular total NAD levels were different between groups (Control, HTG and NTG). When assessing the relationship between total NAD levels and different parameters of OCR, we used a multivariable model. Considering the smaller number of participants in this part of the study, we selected covariates based on the results of the multivariable models in Part 1. These were: age, statin usage, number of systemic diseases, alcohol consumption and diabetes.

All tests were 2-tailed, and p-values < 0.05 were considered statistically significant. We reported point estimates along with standard errors (SEs) and p-values for regression models. All analyses were performed with the statistical software R (R Foundation for Statistical Computing, Vienna, Austria). The relationship between the rate of MD change and IOP was evaluated with linear mixed models with random slopes and random intercepts. To evaluate the contribution of OCR within the multivariable model to VF progression rates, we calculated the coefficient of partial determination (partial R^2) of the

interaction term between OCR and time. The partial R^2 measures the incremental contribution of one regression term when all other variables are included in the model. To calculate the partial R^2, we constructed two multivariable linear mixed models: a full model and a reduced model. Both models had the same specifications, except that the reduced model lacked the OCR term. We then computed the sum of squares error (SSE) for the random effect in both models and calculated the partial R^2 using the following formula:

$$\text{partial}\mathrm{R}^2 = \frac{SSE\left(reducedmodel\right) - SSE\left(fullmodel\right)}{SSE\left(reducedmodel\right)}$$

For the placebo arm of the UKGTS, we also calculated the partial R^2 for mean IOP. We ran a full multivariable linear mixed model with random intercept and random slope having the same covariates (i.e., baseline age, CCT, mean IOP) as this study cohort, and their interaction with follow-up time, with the exception of OCR, the measurement of which was not part of the UKGTS protocol. We then ran a reduced model which lacked the mean IOP term and calculate the partial R^2 using the formula detailed above.

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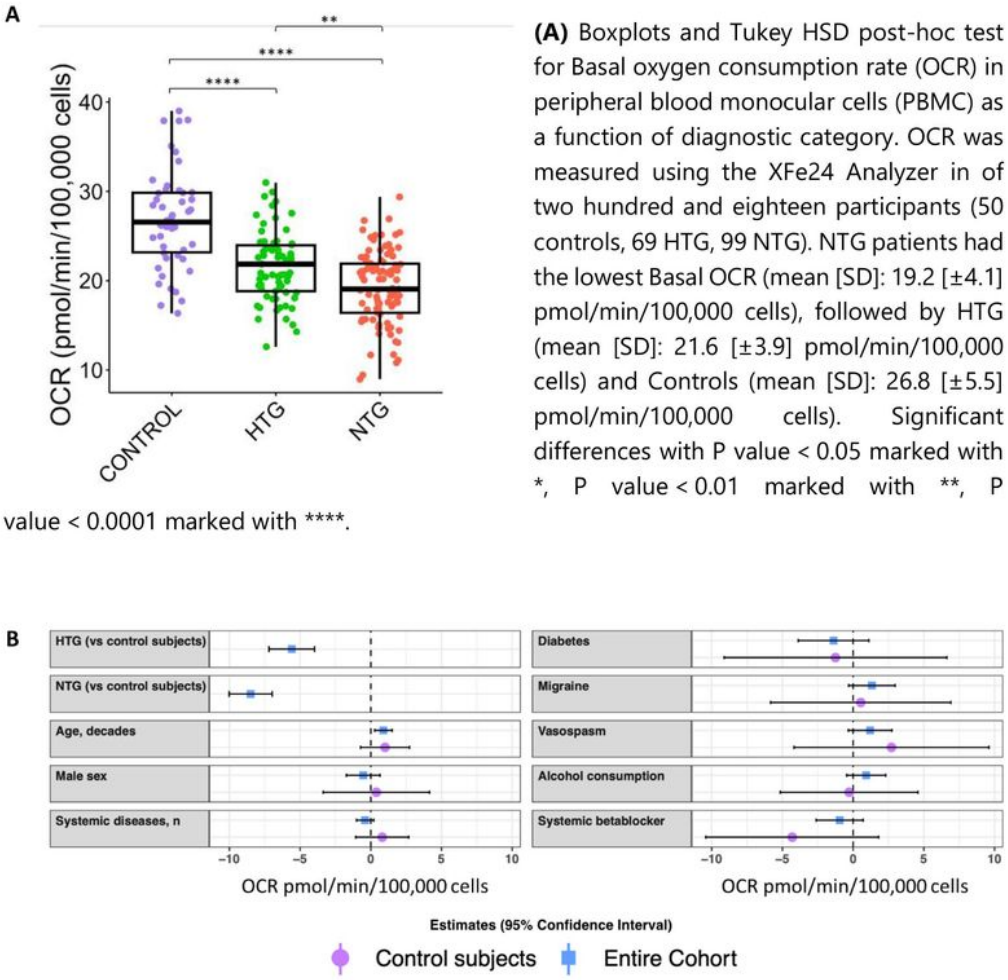
Tables

Tables 1 and 2 are available in the Supplementary Files section.

Figures

Figure 1. Mitochondrial respiration in peripheral blood mononuclear cells



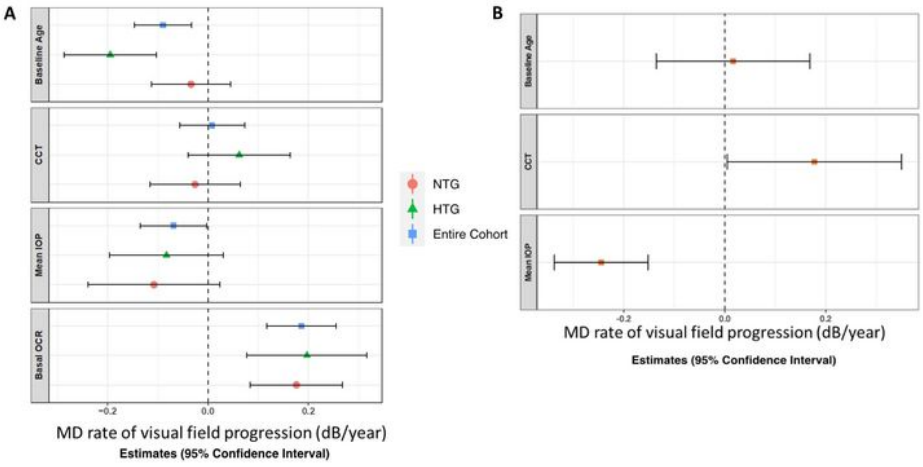
(A) Boxplots and Tukey HSD post-hoc test for Basal oxygen consumption rate (OCR) in peripheral blood monocular cells (PBMC) as a function of diagnostic category. OCR was measured using the XFe24 Analyzer in of two hundred and eighteen participants (50 controls, 69 HTG, 99 NTG). NTG patients had the lowest Basal OCR (mean [SD]: 19.2 [±4.1] pmol/min/100,000 cells), followed by HTG (mean [SD]: 21.6 [±3.9] pmol/min/100,000 cells) and Controls (mean [SD]: 26.8 [±5.5] pmol/min/100,000 cells). Significant differences with P value < 0.05 marked with *, P value < 0.01 marked with **, P value < 0.0001 marked with ****.

(B) Forest Plot showing the results of the multivariable model for factors associated with Basal OCR. Blue squares and purple circles represent central estimates and the horizontal bars represent the corresponding 95% confidence intervals. The model predicts that having NTG is associated with Basal OCR being 8.5 pmol/min/100,000 cells lower than Controls ($p < 0.001$) and having HTG is associated with Basal OCR being 5.6 pmol/min/100,000 cells lower than Controls ($p < 0.001$).

Figure 1

See image above for figure legend.

Figure 2. Association of mitochondrial respiration and intraocular pressure with visual field progression

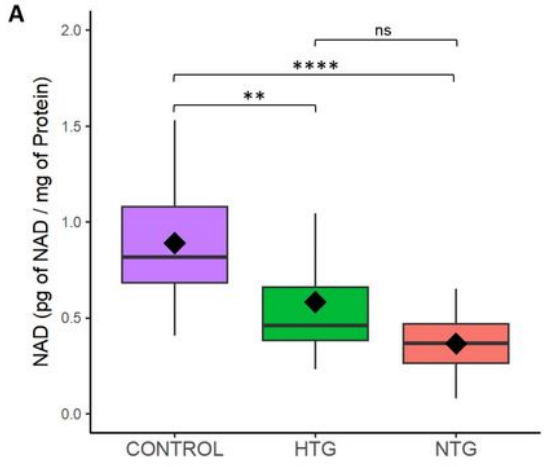


(A) Forest Plot showing the results of the Basal OCR mixed effect model for factors associated with the mean deviation (MD) visual field progression, expressed as dB/year. Blue squares, green triangles and red circles represent standardized Estimates, whereas horizontal bars represent their corresponding 95% confidence intervals. All independent variables were standardized (i.e., zero mean and unit standard deviation). A total of 229 eyes (NTG: 144 eyes, HTG: 85 eyes) of 139 patients were included in this analysis. Older age was associated with faster rates of progression in the entire cohort (for a 10-year increase, estimate [SE]: - 0.09 [0.03] dB/year, p=0.002). Higher mean IOP was associated with faster rates of progression in the entire cohort (for 1 SD (2.8 mmHg) increase, estimate [SE]: - 0.07 [0.03] dB/year, p=0.04). Lower Basal OCR was associated with faster rates of progression in the entire cohort (for 1 SD (4.3 pmol/min/100,000 cells) reduction, estimate [SE]: 0.19 [0.04] dB/year, p<0.001), NTG cohort (for 1 SD (4.1 pmol/min/100,000 cells) reduction, estimate [SE]: 0.18 [0.05] dB/year, p<0.001), and HTG cohort (for 1 SD (4.0 pmol/min/100,000 cells) reduction, estimate [SE]: 0.20 [0.06] dB/year, p=0.002). . One SD difference in basal OCR in the entire cohort was associated with a 0.19 dB/year difference in the rate of MD change and 1 SD (2.8mmHg) difference in mean IOP was associated with a 0.07 dB/year difference in the rate of MD change, the change in MD progression rate associated with 1 SD lower basal OCR was equivalent to 7.6 mmHg higher mean IOP. Similarly, 1 SD lower basal OCR was equivalent to being nearly 21 years older. **(B)** Forest Plot showing the results of the mean IOP mixed effect model for factor associated with the mean deviation (MD) visual field progression, in the placebo arm of the UKGTS cohort. A total of 213 eyes were included in the analysis. Red squares represent standardized Estimates, whereas horizontal bars represent their corresponding 95% confidence intervals. All independent variables were standardized (i.e., zero mean and unit standard deviation) using the SD and mean of the parameters from the main paper to facilitate comparison. Higher mean IOP was significantly associated with faster VF progression (for 1 SD increase, estimate [SE]: -0.25 [0.05] dB/year, *P*<0.001), while thicker CCT with slower VF progression (for 1 SD increase, estimate [SE]: 0.18 [0.09] dB/year, *P*=0.046).

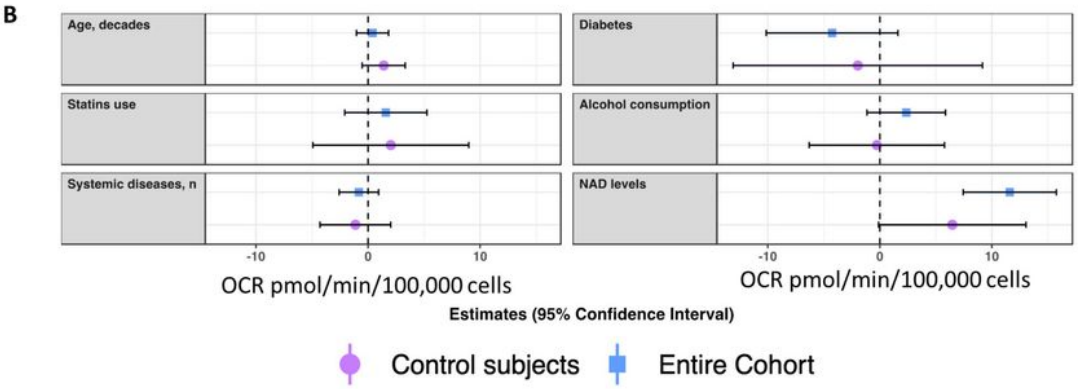
Figure 2

See image above for figure legend.

Figure 3. Nicotinamide adenine dinucleotide (NAD) levels in peripheral blood mononuclear cells and the association with basal oxygen consumption rate



(A) Boxplots and results of the Tukey HSD post-hoc test for total cellular NAD levels in PBMC as a function of diagnostic category. Total NAD levels were measured 54 (24.8%) subjects (25 controls, 10 HTG and 19 NTG). Total NAD levels followed the same pattern as Basal OCR, with NTG patients having the lowest NAD levels (mean [SD]: 0.4 [±0.2] pg NAD / mg of Protein), followed by HTG (mean [SD]: 0.6 [±0.3] pg NAD / mg of Protein) and Controls (mean [SD]: 0.9 [±0.3] pg NAD / mg of Protein). Significant change with P value < 0.05 marked with *, P value < 0.01 marked with **, P value < 0.0001 marked with ****.



(B) Forest Plot showing the results of the Basal OCR multivariable model for factors associated with total cellular NAD levels. Blue squares and purple circles represent central estimates and the horizontal bars represent their corresponding 95% confidence intervals. The model predicts that an increase in total NAD levels by 1 pg NAD / mg of Protein, would result in Basal OCR increasing by 11.6 pmol/min/100,000 cells in the entire cohort (p< 0.001).

Figure 3

See image above for figure legend.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.pdf
- SupplementaryTables.pdf
- Table1.pdf
- Table2.pdf