Systemic PTEN-Akt1-mTOR pathway activity in patients with normal tension glaucoma and ocular hypertension: A case series

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Abstract

Glaucoma is the most common optic neuropathy in humans and the leading cause of irreversible blindness worldwide. Its prevalence and incidence increase exponentially with ageing and raised intraocular pressure (IOP), while increasing evidence suggests that systemic mitochondrial

abnormalities may also be implicated in its pathogenesis. We have recently shown that patients who have not developed glaucoma despite being exposed for many years to high IOP (ocular hypertension - OHT) have more efficient mitochondria, measured in peripheral blood lymphocytes, when compared to age-similar controls and fast progressing normal tension glaucoma (NTG) patients. In this prospective case series we aimed to explore some of the molecular pathways involved in mitochondrial efficiency in glaucoma resistance by measuring the systemic activity (in peripheral blood) of key mitochondrial regulators: the mammalian target of rapamycin (mTOR) and its major upstream regulators and downstream effectors that form the PTEN-Akt1-mTOR signalling pathway. We found no statistically significant difference in the systemic mTOR activity between the three groups (control, NTG and OHT). In line with the mTOR results, there was no significant difference in the activity of both the two major upstream mTOR regulators (PTEN and Akt1) and its two main downstream effectors (S6K and 4EBP1). In a single NTG patient, with history of Raynaud's, significantly higher mTOR activity was noted. We conclude that the PTEN-Akt1-mTOR pathway does not appear to play a central role in mitochondrial efficiency in OHT.

Introduction

Worldwide, glaucoma is responsible for more blindness than any other eye condition, except cataract. Data from population-based surveys indicate that 1 in 40 adults older than 40 years has glaucoma with loss of visual function, which equates to 60 million people worldwide being affected; this number is constantly rising as the population ages (Quigley 2011).

Raised intraocular pressure (IOP) is the major risk factor for glaucoma, but patients with glaucoma deteriorate at all levels of IOP, strongly suggesting that this condition is multifactorial. The second most important established risk factor for the development and progression of glaucoma, consistently identified from large prospective clinical trials (Nouri-Mahdavi 2004, Leske 2007) and epidemiological studies (Mitchell 1996, Leske 2008), is increasing age. The increase in the prevalence of glaucoma with age is not accounted for by any increase in IOP with ageing (Klein 1992, Varma 2004), suggesting that ageing independently confers increased susceptibility to the disease.

Based on the well-established link between ageing and systemic mitochondrial functional decline, increasing evidence suggests that systemic mitochondrial abnormalities may be implicated in the pathogenesis of a range of neurodegenerative diseases (Lin 2006), including Parkinson's disease (Schapira 2012), Alzheimer's disease and glaucoma (Kong 2009, Lascaratos 2012). Indeed, a maternal family history of primary open angle glaucoma (POAG) is more likely than a paternal family history, suggesting a possible mitochondrial genetic influence (Nemesure 1996). The mean systemic mitochondrial respiratory activity, as measured in the peripheral blood, was significantly lower in 27 POAG patients compared to 64 control subjects (Abu-Amero 2006). Also, lymphoblasts from POAG patients have been shown to exhibit a defect in complex I of the oxidative phosporylation (oxphos) pathway, leading to decreased rates of respiration and ATP production (Lee 2012). In the presence of multiple cellular stressors, as seen in a multifactorial disease like glaucoma, it is possible that such generalised (systemic) mitochondrial abnormalities may accentuate any underlying elements of localised bioenergetic crisis at the level of the retinal ganglion cells (RGCs) and lead to increased susceptibility to cell death.

We have recently shown that patients 'resistant' to the major risk factor (raised IOP) for glaucoma have more efficient mitochondria, measured in peripheral blood lymphocytes, when compared to age-similar controls and fast progressing glaucoma patients (Lascaratos 2015). In line with these findings, efficient mitochondrial biogenesis has also been reported to drive incomplete penetrance in Leber's hereditary optic neuropathy carriers (Giordano 2014), while increased oxphos activity has been linked with vision preservation in autosomal dominant optic atrophy (ADOA) patients (Van Bergen 2011).

This study aims to explore the molecular pathways involved in mitochondrial efficiency in glaucoma resistance by measuring, to our knowledge for the first time, the systemic activity of key mitochondrial regulators, the mammalian target of rapamycin (mTOR) and its major upstream regulators and downstream effectors that form the PTEN-Akt1-mTOR signalling pathway, in the peripheral blood mononuclear cells (PBMCs) from a unique cohort of patients at the extremes of IOP susceptibility: normal tension glaucoma (NTG) patients with fast visual field (VF) progression (Mean Deviation change worse than -1.0 dB/yr) despite a low mean IOP (12.9mmHg) over a median follow-up of 6 years, and ocular hypertension (OHT) patients with no or minimal VF progression despite a relatively high mean IOP (24.5mmHg) over a median of 8 years. Age-similar subjects with normal IOP, healthy discs and no family history of glaucoma were also recruited as controls.

mTOR is a multi-domain, highly conserved serine/threonine kinase that controls cell growth and metabolism in response to nutrients, growth factors, cellular energy and stress (Khalil 2012). mTOR is a key regulator of mitochondrial function (Morita 2013) and co-localises with the outer mitochondrial membrane (Desai 2002). mTOR suppression has been found to result in increased autophagy, reduced mitochondrial function (Ramanathan 2009) and decreased mitochondrial respiration (Schieke 2006). This direct effect of mTOR on mitochondrial function is thought to be mediated to a certain extent by its ability to form complexes with the mitochondrial outer membrane proteins Bcl-xl (B-cell lymphoma-extra large) and VDAC1 (voltage-dependent anion-selective channel protein 1) (Ramanathan 2009). The potentially important role of mTOR in the eye has been demonstrated in adult mice, where mTOR activity is suppressed (Di Polo 2015) and protein synthesis is impaired following axotomy of the RGCs (Verma 2005). mTOR has also been shown to affect cellular metabolism by means of the transcriptional control of mitochondrial oxidative function through the YY1-PGC1α (yin-yang 1-peroxisome-proliferator-activated receptor co-activator 1α) transcriptional complex (Cunningham 2007).

Based on this link between mTOR activity and mitochondrial function, and to better understand the extent to which this kinase is involved in mitochondrial efficiency in OHT, we also measured the systemic activity of the main mTOR upstream regulators [PTEN (phosphatase and tensin homologue) and Akt1 (also known as PKBα, protein kinase B alpha)], as well as its major downstream effectors [S6K (p70 ribosomal protein S6 kinase) and 4EBP1 (eukaryotic translation-initiation factor 4E-binding protein 1)]. PTEN is a negative regulator of the mTOR pathway (Bossy-Wetzel 2004) by opposing phosphoinositide 3-kinase (PI3K) function and leading to inactivation of Akt1 and mTOR signalling (Figure 1). Akt1 can activate mTOR by mediating the inhibitory phosphorylation of its negative regulators TSC2 (tuberous sclerosis protein 2) and PRAS40 (proline-rich Akt/PKB substrate 40 kDa) (Song 2012). mTOR phosphorylates S6K and 4EBP1 to activate protein translation and cell survival (Gingras 2004).

Methods

Reagents

Reagents were supplied by Sigma–Aldrich (Poole, UK) and Merck (Nottingham, UK), unless otherwise stated.

Patient selection

Two cohorts of 7 subjects with ≥8 visual fields over ≥5 years of follow-up were recruited prospectively from Moorfields Eye Hospital: a) NTG group: rapidly progressing patients with Mean Deviation change > -1.0 dB/yr and mean IOP<16; b) OHT group: non- or slowly progressing patients with mean IOP>24. As mitochondrial function is known to decline with ageing, a third cohort of an equal number of age-similar subjects with normal IOP, healthy discs and no family history of glaucoma was recruited as controls. Since this was an exploratory study with carefully selected individuals at the very extremes of IOP susceptibility, no power calculation to determine the sample size was performed.

Schedule of investigations

Clinical phenotyping of all 21 participants included detailed medical history, fundus examination, IOP measurement using Goldmann applanation tonometry, measurement of central corneal thickness (CCT) with an ultrasound pachymeter (DGH Technology Inc, Exton, PA), and automated perimetry with the Swedish interactive threshold algorithm standard 24-2 program. 24-hour ambulatory blood pressure was measured with the Welch Allyn Ambulatory Blood Pressure Monitor 6100 (Aston Abbotts, Buckinghamshire, UK). Height and weight were also measured with participants wearing light clothing and no footwear. Peripheral blood was collected from each participant and processed for the isolation of unstimulated PBMCs and experimental analysis.

Isolation of human PBMCs

PBMCs were isolated from anticoagulated peripheral blood using Lymphoprep (Axis-Shield, Oslo, Norway), as described previously (Thorsby 1970) and resuspended in phenol red-free RPMI 1640 medium supplemented with 2mM glutamax, 12.5mM HEPES, 50U/mL penicillin and 50µg/mL streptomycin, to a density of 1×10^6/ml. 1ml was required for each of the 5 ELISA measurements described below.

Raji cell line

This well established immortalised human B- lymphocyte cell line was used as a reference (internal control) in the measurements of mitochondrial content by porin. Raji cells were cultured in RPMI 1640 medium, supplemented with 10% FCS, 2mM glutamax, 12.5mM HEPES, 50U/mL penicillin and 50µg/mL streptomycin in 75-cm^2 flasks at 37°C in an atmosphere of 5% CO_2. Doubling time was approximately 20 hours and cells were generally passaged at a 1:8 ratio and maintained at a density of 1×10^6/ml for use in experiments.

ELISA

mTOR is known to be phosphorylated at Ser2448 via the PI3 (phosphoinositide 3)-kinase/Akt1 signalling pathway. The PathScan Total mTOR and Phospho-mTOR (Ser2448) Sandwich enzyme-linked immunosorbent assay (ELISA) kits were used to measure total and phospho-mTOR (one measurement per participant), respectively. In order to maintain the phospho-activity, PBMCs immediately after isolation were resuspended in RPMI, washed in PBS and resuspended in 100µl of lysis buffer. The lysate was then microcentrifuged at 15,000 x g for 10 min at 4°C and 100µl of the supernatant added into each well of a 96-well plate pre-coated with mTOR mouse antibody, sealed with tape and incubated at 4°C overnight. After washing twice with wash buffer, 100µl of mTOR rabbit antibody were added to each well to detect the captured mTOR protein. After a second incubation at 37°C for 1hr and repeat wash, 100µl of anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody was added to each well to recognise the bound detection antibody. The plate was incubated at 37°C for 30min, washed as previously and 100µl of tetramethylbenzidine (TMB) substrate was added to each well, in order to develop the colour (blue). The reaction was terminated by the addition of an acidic STOP solution, changing the solution colour from blue to yellow. The absorbance was measured with a plate reader at 450nm, and mTOR activity was expressed as the ratio between Phospho-mTOR (Ser2448) and Total mTOR.

ADP Phosporylation

This assay provides a useful measure of mitochondrial function, and is designed to measure complex-specific ATP synthesis in live cells over a given period of incubation time, based on the exogenous supply of substrates. The rate of substrate-linked ADP phosphorylation for the different mitochondrial complexes was measured in digitonin permeabilised cells, as described previously by our group (Korlipara 2004). Live cells were harvested and resuspended in incubation medium containing 150mM KCl, 2mM K_2EDTA, 10mM K_2HPO4, 5mM Tris base, pH: 7.4 at a concentration of 1×10^6 cells/ ml. The reaction, performed in triplicate for each participant, was initiated at 37°C by mixing 250µl of cells with 250µl of incubation medium containing ADP (1mM), digitonin (20 µg/ml), and the following substrates: In the case of complex I, glutamate (10 mM) and malate (10 mM), and for complex II succinate (10 mM) and rotenone (10 µM). After 20 minutes of incubation in a rotating rack, the reaction was stopped with perchloric acid and this neutralised with 3M K_2CO_3/0.5M triethanolamine. ATP levels were measured using the ATP Bioluminesence Assay kit CLSII (Roche; Mannheim, Germany) and the emitted luminescence was measured with a Jade tube luminometer (Labtech; Uckfield, UK). Background levels of ATP, in the absence of substrate, were measured in duplicate for each participant and subtracted from the complex-specific ATP measurements. Protein

levels were estimated using a bicinchoninic acid (BCA) kit (Pierce Thermo Fisher; Basingstoke, UK) with reference to the protein standard supplied with the kit. Data were expressed as pmol ATP synthesised/min/mg protein and represent means of four analyses per sample.

Western blot analysis

Cells were harvested, washed with PBS and processed as described previously by our group (Cleeter 2013). 25–40 µg of whole cell lysates were electrophoresed on Novex gels (NuPage 4–12%; Invitrogen, Paisley, UK), transferred onto polyvinylidene fluoride membrane (Millipore; Watford, UK) and then probed with antibodies to porin (Abcam, Cambridge, UK; 1/1000 dilution). All were normalised to β-actin (Abcam, Cambridge, UK; 1/5000 dilution). Blots were developed using an enhanced chemiluminescence (ECL) kit (GE Healthcare; Little Chalfont, UK) and exposed to X-ray film (GE Healthcare). The film was developed and signal intensities in the linear range were quantified by the 'Alphadigidoc' software package (AlphaInnotech; San Leandro, CA). The densitometric reading of the protein of interest was expressed as a percentage of the control gene product (β -actin).

Statistical analysis

The Wilcoxon signed rank test was used for comparisons between pairs of groups. For comparisons between all three groups (control, NTG, OHT), the Kruskal Wallis test was used as the equivalent of one-way non-parametric ANOVA. Clinical data are presented as mean ± standard deviation (SD) for normally distributed measures and as median ± interquartile range (IQR) for data that are not normally distributed. In all experiments a P value of <0.05 was generally considered statistically significant.

In the box-and-whisker plots (IBM SPSS software, version 21.0), the middle (horizontal) line represents the median. The lower and upper horizontal lines of each box represent the lower and upper quartile values, respectively. The difference between the lower and upper quartiles is the interquartile range (IQR). Results are presented as median (IQR) for each parameter (n=7 from each group); NS: not significant. Outliers are highlighted with a circle or asterisk on the box-and-whisker plot.

Ethical approval

Consent was obtained from all the participants in this study and the relevant Research Ethics Committee approval (REC Ref: 11/H0715/10) was granted. This study adhered to the tenets of the Declaration of Helsinki.

Results

Baseline clinical data and past medical history (Tables 1 and 2)

The mean age (years) of the participants was almost identical between the NTG (70.7) and OHT (70.6) groups, and very similar to the controls (65.3). The median rate of VF progression for the whole field over the follow-up period, measured with the Moorfields Progressor software, was -1.07dB/year (IQR -1.05 to -1.09) in the NTG group (selected to represent a fast-progressing group), while the median rate only for the VF progressing points was even faster at -2.80dB/year (IQR -2.60 to -3.35). The OHT cohort showed minimal progression with a median rate of -0.15dB/year (IQR -0.14 to -0.21). All the OHT patients had full VFs. A significant number of VFs was performed in the NTG (median 9, range 8-15) and OHT (median 8, range 8-13) groups to provide sufficient information for representative progression measurements. Importantly, despite the fast disease progression, the mean IOP in the NTG group over 6 years of follow-up was only 12.9mmHg. In the OHT group, despite the minimal VF progression, the mean IOP over 8 years of follow-up was 24.5mmHg. The mean CCT was higher in the OHT group (574.9µm) and lower in the NTG group (525.8µm), as compared to the controls (550.4µm). The mean cup-to-disc ratio in the OHT subjects was 0.56 and in the controls 0.36, while the NTG group showed advanced cupping with a mean cup-to-disc ratio of 0.91. None of the 21 study participants reported a history of smoking or excessive alcohol intake, or suffered from active haematological malignancy or infection at the time of the blood sampling or had undergone recent chemotherapy, factors which if present might have negatively influenced mitochondrial function in the PBMC.

Higher systemic mitochondrial activity in ‘extreme’ OHTs as compared to NTGs

The rate of ADP phosphorylation by mitochondrial respiratory complex I and complex II was significantly higher in the lymphocytes of OHT patients, as compared to the NTG group (Figure 2). The median (IQR) rate of ADP phosphorylation by complex I in the control, NTG and OHT groups, was 4712 (3856-7732), 6063 (3016-8649) and 11237 (5716-12576), respectively (P=0.063 between control-OHT, P=0.018 between NTG-OHT and P=0.866 between control-NTG). The median (IQR) rate of ADP phosphorylation by complex II in the control, NTG and OHT groups, was 6712 (3962-8472), 7018 (3077-9897) and 11277 (6131-12285), respectively (P=0.128 between control-OHT, P=0.018 between NTG-OHT and P=0.612 between control-NTG). There was no significant difference in the porin levels, an established marker of mitochondrial content and the most abundant protein in the mitochondrial outer membrane, in the lymphocytes between the three groups, with the median (IQR) porin level in the control, NTG and OHT groups, being 28.5 (27.8-30.0), 27.7 (26.0-30.0) and 30.0 (28.3-31.1), respectively.

Systemic mTOR activity is similar between ‘extreme’ NTGs, ‘extreme’ OHTs and controls

In this small, but carefully selected cohort, there was no difference in the mTOR activity between the three groups (Figure 3). The median (IQR) systemic mTOR activity in the control, NTG and OHT groups, was 0.33 (0.17 to 0.42), 0.23 (0.16 to 0.28) and 0.19 (0.18 to 0.30), respectively. Activation of the mTOR pathway is known to mediate vasospastic phenomena (Zhang 2012) and of speculative

interest is that the only patient in our cohort with history of Raynaud's phenomenon (NTG no 6) had mTOR activity much higher (0.52) compared to the other patients in the cohort (outlier in Figure 3).

mTOR upstream regulator and downstream effector activity is similar between the three groups

In line with the mTOR results, there was no significant difference in the systemic activity of both the two major upstream mTOR regulators (PTEN and Akt1) forming the PTEN-Akt1-mTOR pathway (Figure 3) or the two main mTOR downstream effectors (S6K and 4EBP1) (Figure 4). In more detail, the median (IQR) PTEN activity in the NTG, OHT and control groups, was 0.45 (0.36 to 0.58), 0.48 (0.37 to 0.99) and 0.49 (0.36 to 0.70), respectively, while the median (IQR) Akt1 activity was 0.18 (0.14 to 0.28), 0.19 (0.11 to 0.31) and 0.21 (0.14 to 0.30), respectively. The median (IQR) S6K activity in the NTG, OHT and control groups, was 0.49 (0.45 to 0.64), 0.45 (0.32 to 0.52) and 0.51 (0.45 to 0.68), respectively, while the median (IQR) 4EBP1 activity was 1.26 (1.02 to 1.46), 1.15 (1.07 to 1.40) and 1.08 (0.70 to 1.23), respectively.

Conclusions

In view of the increasing evidence linking mitochondrial efficiency with glaucoma resistance and the central role of mTOR in regulating mitochondrial biogenesis, the purpose of this study was to determine whether differences in mitochondrial function between OHT and NTG patients could be explained by activity in the mTOR pathway. To gain useful insight into the mTOR-regulating cellular mechanisms, the most important upstream and downstream targets of mTOR were measured in this unique, prospectively selected cohort. In this series, despite the higher systemic mitochondrial activity in OHT patients compared to the NTG patients, there was no significant difference in the systemic PTEN-Akt1-mTOR-S6K-4EBP1 pathway activity between the three groups, suggesting that this pathway may not play a central role in mitochondrial efficiency in OHT. Nevertheless, a weak trend was observed for lower mTOR activity in OHT as compared to the other two groups, with an associated lower S6K and higher 4EBP1 activity in OHT. This is in line with a recent study showing that mTOR inhibition by rapamycin dramatically promotes RGC survival in a rat chronic ocular hypertension model, due to the inhibition of neurotoxic mediators' release and direct suppression of RGC apoptosis (Su 2014). This protective effect of rapamycin appears to contradict other studies that have linked mTOR suppression with lower mitochondrial function (Ramanathan 2009, Schieke 2006), although the use of different stressors and different animal and cell models in each study make direct comparisons difficult. mTOR is a versatile regulator that responds to a variety of cellular and extracellular stimuli, such as hypoxia, inflammation, low ATP levels, growth factors and neurotransmitters, and its role in health and disease and also during different stages of development is still poorly understood.

Although this study explores several major upstream and downstream mTOR targets, it is important to note the complexity of these mechanisms at a cellular level and the multiple interactions of these targets with other, still unexplored, pathways. Therefore, the absence of any significant difference in the PTEN-Akt1-mTOR-S6K-4EBP1 pathway between the three groups of participants, should not be interpreted as weakening the link between mitochondrial dysfunction and glaucoma or between

mitochondrial efficiency and glaucoma resistance, and should not minimise the potentially important role in the pathogenesis of glaucoma of other molecular targets interacting with this pathway. As an example, PTEN, a negative regulator of mTOR, and SOCS3 (suppressor of cytokine signalling 3), a negative regulator of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway, have been shown to regulate two independent pathways, which however enable robust and sustained long-distance axon regeneration in the adult central nervous system only when acting synergistically. While either PTEN or SOCS3 deletion in adult mouse RGCs individually promotes significant optic nerve regeneration following crush injury, such re-growth tapers off around two weeks after the insult, whereas simultaneous deletion of both PTEN and SOCS3 enables significant and prolonged axon regeneration (Sun 2011). Alternative pathways to be explored in future studies may include other important negative regulators of mTOR function, such as the tuberous sclerosis complex (TSC1/2). Hypoxia activates TSC1/2 through the regulated in development and DNA damage response (REDD) proteins leading to loss of mTOR function, while Akt and the extracellular-signal regulated kinases 1/2 (ERK1/2) inactivate TSC1/2 leading to mTOR stimulation (Di Polo 2015). Also, in view of the recent evidence of increased mitochondrial biogenesis in LHON carriers, it would be interesting for future studies to explore the role of PGC1α, a master regulator of mitochondrial biogenesis and downstream mTOR target within the mTOR-YY1-PGC1α pathway, in patients with OHT.

The utility of lymphoblasts to investigate mitochondrial dysfunction in optic neuropathies has been demonstrated previously in studies that determined oxphos defects in Leber's hereditary optic neuropathy (Brown 2001), autosomal dominant optic atrophy (Van Bergen 2011) and primary open angle glaucoma (Lee 2012). Unlike previous studies, a significant advantage of our approach was the use of unstimulated PBMCs (Lascaratos 2015), thus providing a more 'pure' human model that minimises any potential effect of viral transformation on measurements of mitochondrial function.

Another major strength of this study was its prospective and unique design, where patients at the very extremes of IOP susceptibility (fast progressing NTG with low IOP and non- or slow- progressing OHT with high IOP) were compared to age-similar controls. Although the small cohort size may limit the generalisability of our findings, the design employed here would be expected to maximise our chances of detecting mitochondria-related differences between groups. Also, instead of looking at the mTOR pathway at a single cellular level, we aimed to confirm our findings by measuring the activity of the most important upstream (PTEN, Akt1) and downstream (S6K, 4EBP1) mTOR targets.

With regards to co-existing systemic diseases, a reduction in the systemic mTOR activity has been associated with diabetes (Fraenkel 2008), with mTOR activation thought to promote the secretion of insulin and increase insulin sensitivity (Chong 2012). Yet, mTOR may also act via a negative feedback loop that can lead to poor insulin signalling and insulin resistance. Only one OHT patient (no 5) in our cohort suffered from diabetes (controlled on diet) and her mTOR activity was not particularly low at 0.19, coinciding with the median mTOR activity for this group. Also, it is unclear to what extent this condition would have affected mitochondrial function in the PBMCs. The latter may not be as critically involved in the pathophysiology of certain diseases as other tissues, and diabetes has been linked to mitochondrial dysfunction in insulin-sensitive tissues, including myocytes (Kelley 2002), hepatocytes (Sivitz 2010) and adipocytes (Choo 2006), but not in PBMCs. Also, the relatively short half-life of PBMCs (usually days to month) and their relatively high turnover would likely minimise the accumulating impact of chronic diseases on mTOR activity. Moreover, the role of mTOR as a

regulator of metabolism is well established and, while a relatively higher BMI was noted in the OHT group, the difference in the BMI between the three groups was not significant. A single OHT patient (no 1) showed a very high BMI (40.9) and even after excluding this individual from the analysis, no difference was found in the mTOR activity between the three groups.

Another systemic condition thought to be positively associated with mTOR activity is cancer, with increasing evidence that mTOR can drive tumourigenesis by activating Akt1, which promotes proliferation, survival and nutrient uptake in cancer cells (Zoncu 2011). Also, tumours can be driven by mutations in the tumour suppressor gene PTEN or by oncogenic mutations in PI3K, which promotes Akt1 signalling. However, these processes are likely to be more complex than we currently understand and perhaps apply only to the tissue of interest (and not PBMCs) or to specific types of cancers or specific stages in the disease process. The only participant in our study (control no 3) with active malignancy (breast cancer recently diagnosed at the time of blood sampling) showed a relatively low mTOR activity at 0.15, with the median in the control group being 0.33. Even after excluding the four patients with history of cancer (control no 3 and OHT no 1, 2 and 3) from the analysis, no significant difference in the mTOR activity was noted between the three groups. Perhaps it is more relevant to note than none of the participants suffered from active haematological malignancy or had undergone recent chemotherapy, factors which, if present, might have impacted more directly on PBMC function.

Unlike systemic diseases such as cancer and diabetes, glaucoma is a multifactorial disease of the optic nerve and it would perhaps not be surprising if systemic (peripheral blood) mTOR dysregulation was not a dominant risk factors in our NTG cohort. In some patients, other risk factors (such as vascular) may predominate and this could explain the lack of differences between the NTG and control groups. Also, while an increased vulnerability to widespread neurodegenerative disease has been reported in glaucoma, including a temporal processing defect in the auditory pathways (Rance 2012), none of our NTG patients had any co-existing neurodegenerative diseases, such as Parkinson's disease or dementia. The potential impact of topical medications on measurements was also considered, although there is no evidence to date to suggest that drugs applied topically would have a major impact on human circulating PBMCs. In terms of systemic medications, while aspirin has been linked to mTOR suppression (Spampatti M et al 2014), only two patients in our cohort (NTG no 5 and no 6) were on aspirin and after excluding these individuals from the analysis no significant difference was noted in the mTOR activity between the three groups.

The link between mTOR and vasospasm is new and interesting. The only patient in our cohort with a history of Raynaud's showed significantly higher mTOR activity, which is consistent with previous reports. mTOR inhibitors have been shown to decrease hypoxia inducible factor 1α (HIF1α) levels (Faivre 2006), while HIF1a inhibition is known to reduce the cerebral vasospasm following subarachnoid haemorrhage in rats and attenuate the expression of its downstream target vascular endothelial growth factor (VEGF) (Yan 2006). VEGF is a potent stimulant of angiogenesis and is involved in the pathogenesis of cerebral vasospasm (Borel 2003). Also, treatment with the mTOR inhibitors rapamycin and AZD8055 leads to the attenuation of angiographic vasospasm and improvement in clinical behavioural scores (Zhang 2012). As part of this study, a single case is presented that supports the above link between mTOR and vasospasm.

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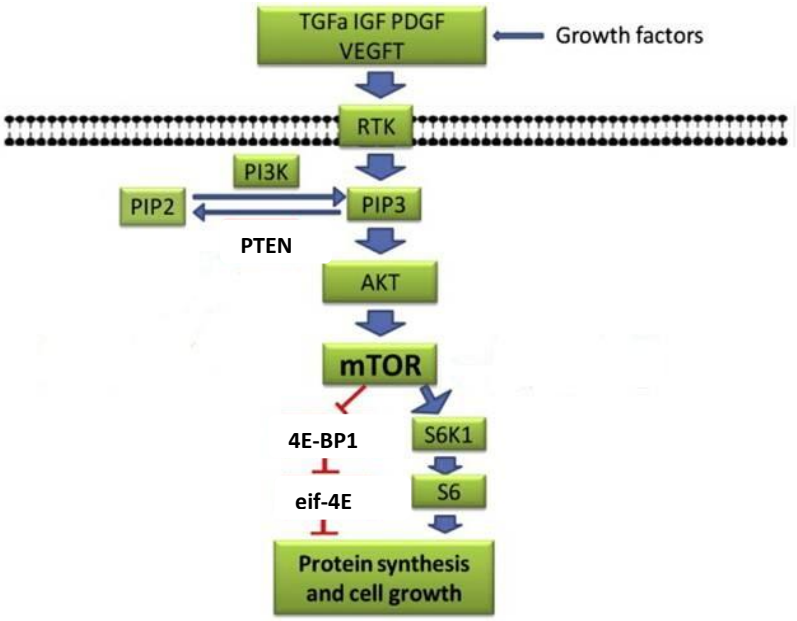

Figure 1: Schematic of the PTEN-Akt1-mTOR signalling pathway (modified from Liu et al 2012)

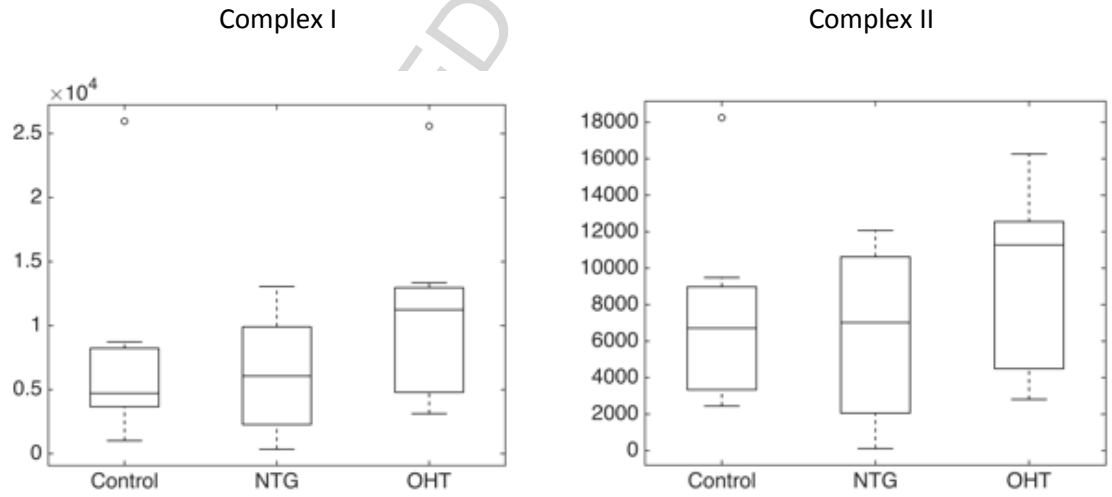

Figure 2: The rate of ADP phosphorylation (pmol/min/mg) by mitochondrial respiratory complex I and complex II in the PBMC from NTG and OHT patients, and age-similar controls (n= 7 from each group). The median (IQR) rate of ADP phosphorylation by complex I in the control, NTG and OHT groups, was 4712 (3856-7732), 6063 (3016-8649) and 11237 (5716-12576), respectively (P=0.063 between control-OHT, P=0.018 between NTG-OHT and P=0.866 between control-NTG). The median (IQR) rate of ADP phosphorylation by complex II in the control, NTG and OHT groups, was 6712 (3962-8472), 7018 (3077-9897) and 11277 (6131-12285), respectively (P=0.128 between control-OHT, P=0.018 between NTG-OHT and P=0.612 between control-NTG).

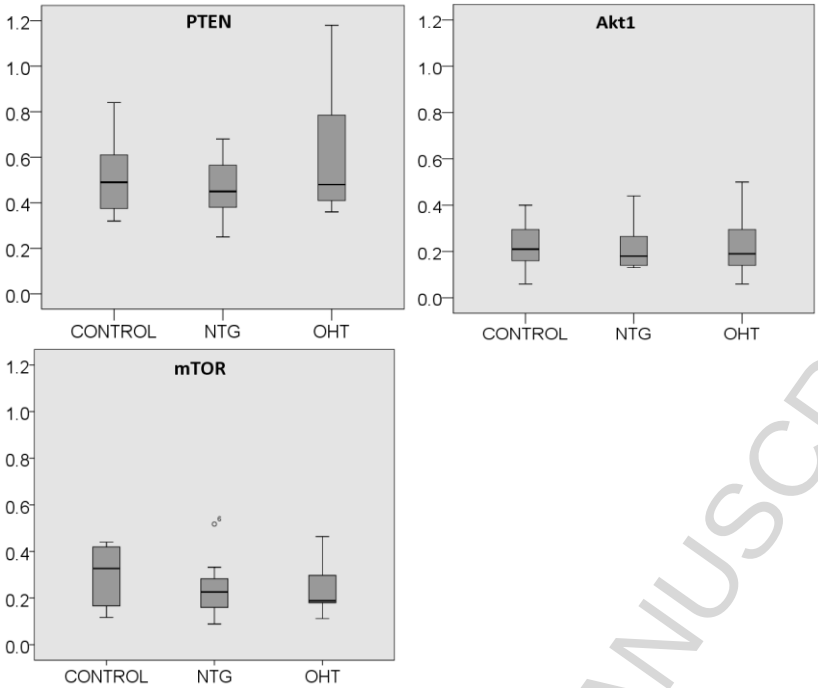

Figure 3: The PTEN-Akt1-mTOR pathway activity in the PBMC from NTG and OHT patients, and age-similar controls (n=7 from each group). All results are expressed as the ratio between the phosphorylated (PS380-PTEN, PS473-Akt1 and PS2448-mTOR) and total PTEN, Akt1 and mTOR, respectively.

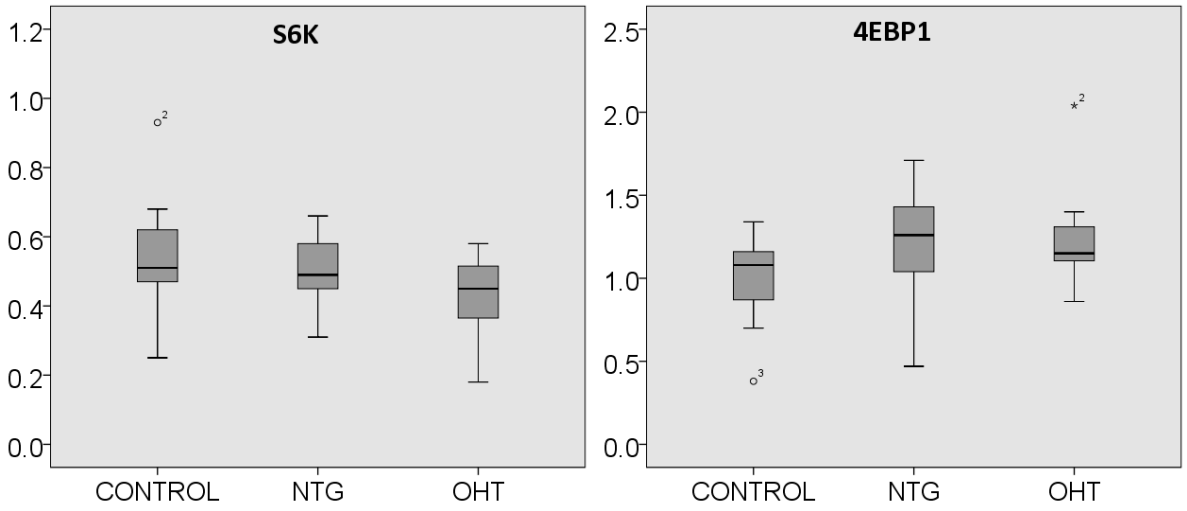

Figure 4: The activity of the two main mTOR downstream targets (4EBP1 and S6K) in the PBMC from NTG and OHT patients, and age-similar controls (n=7 from each group). All results are expressed as the ratio between the phosphorylated (PT37/46-4EBP1 and PT389-S6K) and total 4EBP1 and S6K, respectively.

Table 1: Demographic and other baseline clinical data for control, NTG and OHT subjects.

Variable	Control (n=7)	NTG (n=7)	OHT (n=7)
Mean age (±SD)	65.3 (±7.1)	70.7 (±20.0)	70.6 (±11.0)
Ethnicity (white)	7	5	7
Median (IQR) VF progression rate	n/a	-1.07 (-1.05 to -1.09)	-0.15 (-0.14 to -0.21)
Median number of VFs (IQR)	n/a	9 (8.5 to 11)	8 (8 to 11)
Median years of follow-up (IQR)	n/a	6 (5.8 to 6.5)	8 (6.5 to 11.1)
Mean GAT IOP during follow-up	n/a	12.9	24.5
Mean maximum reported GAT IOP	n/a	19.3	31.7
Mean cup-to-disc ratio	0.36	0.91	0.56

NTG: normal tension glaucoma; OHT: ocular hypertension; SD: standard deviation; IQR: interquartile range; GAT: Goldmann applanation tonometry; IOP: intraocular pressure; VF: visual field; n/a: not applicable. IOP is measured in mmHg and VF progression is expressed in decibels (dB) per year.

Table 2: Past medical history and vascular factors in the cohort.

	Control	NTG	OHT
Diabetes	0	0	1
Cancer	1	0	3*
Extreme dippers	0	0	1
BMI (mean ± SD)	22.8 ± 2.9	22.8 ± 3.7	27.1 ± 7.5
HT	0	3	4
NSAIDs	0	2	0
Statins	1	2	2
Other (CVA, MI)	1	0	0
Raynaud's or vasospasm	0	1	0
Migraine	1	0	0

HT: systemic hypertension; CVA: cerebrovascular accident; MI: myocardial infarction; BMI: body mass index; NSAIDs: non-steroidal anti-inflammatory drugs; extreme dippers defined as subjects whose mean night time systolic blood pressure was more than 20% lower compared to the mean day time ambulatory systolic blood pressure; *history of cancer many years prior to study with no evidence of recurrence