Review Paper:

Neuroprotection in Glaucoma:

Recent Advances and Clinical Translation

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Abstract: *(120 words)*

Intraocular pressure (IOP) reduction is currently the only evidence-based treatment strategy for glaucoma. However, IOP control in some individuals is challenging. Despite optimal treatment, a significant proportion of individuals will progress, with loss of visual field, loss of driving vision, and impaired quality of life. A new modality that could augment current treatment and reduce the rate of neurodegeneration to preserve vision throughout life would be a major breakthrough.

A vast number of studies have reported effective neuroprotection in animal models of glaucoma; however, translation to the clinic remains a major hurdle. Herein, we explore the therapeutic advancements in non-IOP dependent neuroprotection, research based upon potential pathogenic mechanisms, and propose strategies to improve the clinical translation of laboratory research in glaucoma.

Key Words: Glaucoma, Neuroprotection, Optic neuropathy. Retinal Ganglion Cell, Bioenergetics, Clinical Translation

Word Count: 5848 (excluding figures)

Contents

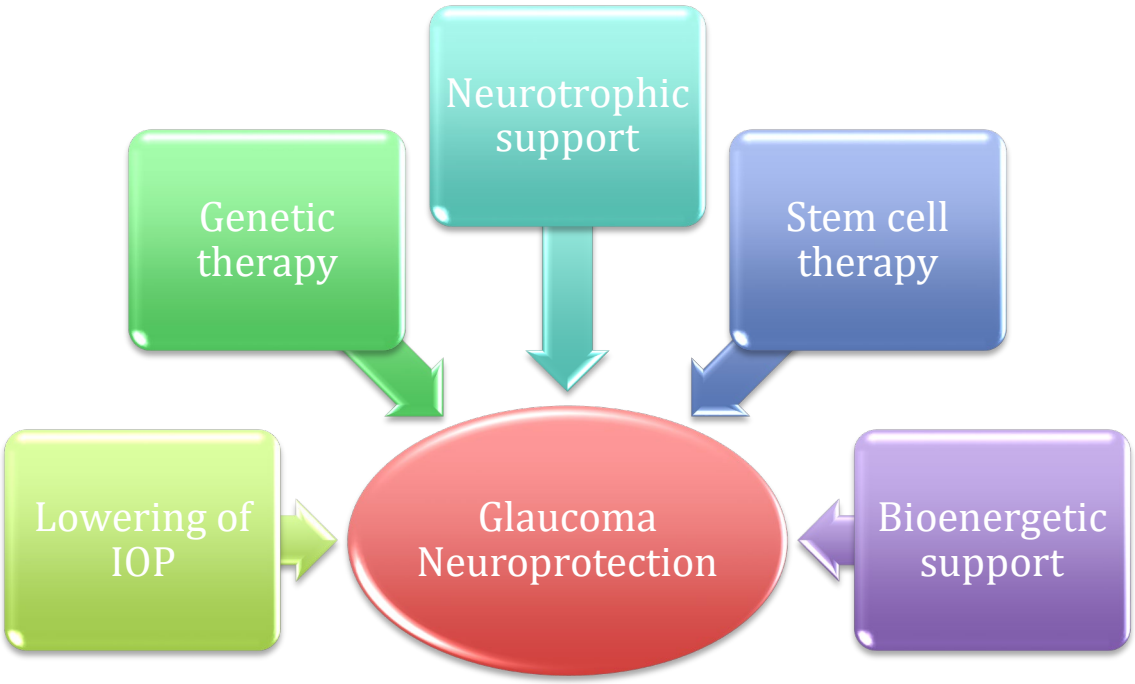
1. Introduction

Glaucoma refers to a group of ocular conditions united by a characteristic optic neuropathy and associated retinal ganglion cell (RGC) loss(1). The most common subtype is primary open-angle glaucoma (POAG). Approximately 1 in 6 individuals with POAG will progress to bilateral blindness within 20 years of being diagnosed (2). Clinical and histopathological evidence together indicate that the primary site of pathology in glaucoma is the optic nerve head (ONH)(3–5). Hence, glaucoma is primarily considered to be an axonopathy, with subsequent Wallerian degradation of the distal axon and loss of RGC somata and dendrites in the retinal ganglion cell layer(5–7).

In the broadest sense, neuroprotection refers to the relative preservation of neuronal structure and/or function(8). For a chronic neurodegenerative disease such as POAG, neuroprotection is conceptualized as a reduction in the rate of neurodegeneration. Intraocular pressure (IOP) reduction in glaucoma is arguably a form of neuroprotection. In fact, it is currently the only clinically proven strategy for successful neuroprotection. However, it is more common to consider IOP reduction as a distinct strategy with the notion of neuroprotection referring to a non-IOP related treatment modality that effectively reduces the rate of glaucomatous neurodegeneration independent of the IOP.

Whilst the pathogenesis of glaucoma remains incompletely understood, a spectrum of possible mechanisms to explain RGC pathology have been proposed, including genetic determinants, trophic factor withdrawal and loss of electrical activity, defective axon transport, chronic intermittent ischaemia, metabolic / bioenergetic failure, exposure to reactive oxygen species, and excitotoxicity(9–11). Laboratory studies continue to advance our understanding of these underlying pathogenic contributors in glaucoma, which ultimately pave the way to the development of improved neuroprotective strategies and subsequent clinical translation (Figure 1).

Figure 1: Neuroprotective Strategies in Glaucoma



2. POAG Genetics

POAG has a complex genetic basis with the ongoing discovery of new genetic loci that contribute to its pathogenesis. Mendelian gene variants account for only about 5% of all

POAG(9,12). Genetic linkage analyses have identified several key chromosomal loci shown to contribute to POAG risk (namely, MYOC, WDR36, OPTN, TBK1, NTF4, ASB10, EFEMP1, IL20RB). Genome-wide associations studies have identified additional gene loci (including CDKN2B-AS1, TMCO1, CAV1/CAV2, SIX1/SIX6, LRP12/ZFPM2, ABCA1, AFAP1, GMDS, GAS7, PMM2, ARH-GEF12, TGFBR3, TXNRD3, ATXN2, FOXC1, and C12ORF23) and many other additional loci have been found that affect IOP (12–14). Somatic mitochondrial DNA (mtDNA) mutations, which are not inherited but accumulated with increasing age, and mtDNA polymorphisms may also contribute to mitochondrial dysfunction in glaucomatous optic neuropathy(12,14). Furthermore, genes that are involved in cell cycle control and transforming growth factor-β (TGFβ) pathways have emerged as substantial risk loci for POAG(13).

Rather than mutations in single modifier genes it appears that it is the presence of polymorphisms in different genes which ultimately modulates the disease phenotype in glaucoma(12). Retinal gene replacement may prove a useful therapeutic avenue, which could conceivably, then, pave the way for the future development of targeted and individualised treatment strategies for glaucoma.

2.1. Genetic Therapy in POAG

Genetic therapies primarily aim to correct a fundamental molecular basis of the disease in question or prevent the transmission of pathogenic mutations across generations(12). In the context of glaucoma, most of these approaches are at the early experimental phase. Aside from ethical considerations, major practical limitations exist when

136 considering the development of genetic therapies for glaucoma. Whilst there is an
137 evolving range of tools available for retinal gene transfer, few to date have had a
138 significant impact on RGC neuroprotection. This appears to be reflective of the
139 challenges relating to gene transfer, penetration, precise localisation and binding to the
140 intended target site or, in the case of viral vectors, the associated safety risks with
141 insertional mutagenesis and potential immunogenic response(12,15).
142
143 The delivery of genes encoding therapeutic proteins such as neurotrophic factors (for
144 example, adeno-associated virus type 2 (AAV2) mediated delivery of brain derived
145 neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF)) have been
146 demonstrated to confer protection for RGCs in experimental models of retinal injury
147 (16,17). Several pre-clinical studies investigating intravitreal injection of viral vectors
148 carrying antiapoptotic genes have demonstrated protection against RGC and axonal loss
149 *in vivo*(17–20). The augmentation of tissue antioxidant status, via intravitreal injection
150 of viral vectors containing enzymes such as catalase or superoxide dismutase (SOD), for
151 example, has been investigated and has indicated improved RGC survival in animal
152 models of optic nerve crush and ischaemia/reperfusion damage(17,21–23). Furthermore,
153 a clinical trial initiated in 2003 utilised short-term adenoviral vector expression of the
154 cell cycle regulator p21 gene (encoding the CDKN1A protein) delivered before
155 trabeculectomy surgery, with the intent of modulating wound healing(17). Pre-clinical
156 studies in primates have already demonstrated that delivery of the virus prior to surgery
157 maintained the trabeculectomy outflow pathway with minimal biodistribution outside
158 the eye(17,24,25).
159

3. Neurotrophic Support

Neurotrophic factors generally function through tyrosine kinase signalling to support the growth, survival, and repair of neurons(26). Neurotrophic factors have been demonstrated to be strongly neuroprotective whilst promoting axon regeneration and enhancing neuronal cell function(9). Given their promising results in other neurodegenerative diseases of the central nervous system, neurotrophic factors offer an attractive therapeutic option to pursue in glaucoma.

Neurotrophic factors trialled for neurodegerative disease include: brain derived neurotrophic factor (BDNF) for amylotrophic lateral sclerosis (ALS), ciliary neurotrophic factor (CNTF) for ALS and for macular degeneration, glial derived neurotrophic factor (GDNF) for Parkinson's disease, and nerve growth factor (NGF) for Alzheimer's disease(17). However, none have succeeded in human trials(17). Oddone *et al.* (2017) found that both BDNF and NGF are reduced in the early and moderate stages of human glaucoma, and these could therefore act as potential biomarkers for early detection of glaucoma(27). NGF, CNTF, fibroblast growth factor 2 (bFGF) and BDNF have demonstrated efficacy in inhibiting RGC apoptosis in pre-clinical glaucoma models(28–30). Topical NGF therapy has recently been trialled in three patients with advanced glaucoma, improving several parameters of visual function(28).

Neurotrophic factor efficacy can be enhanced by stimulating RGCs with electrical activity or pharmacologically elevating cyclic adenosine monophosphate (cAMP), which greatly potentiates the pro-survival and growth effects of neurotrophic factor treatment(9,31,32). Evidence has shown that the combined administration of trophic

184 factors is more effective for the survival of RGCs than the administration of each factor
185 separately(33). Neurotech® has developed a polymeric device (NT-501) which can be
186 surgically implanted beneath the pars plana and which contains a genetically modified
187 human cell line which secretes CNTF(9). The CNTF is encapsulated into a semi-
188 permeable membrane, allowing it to diffuse out whilst evading potentially attacking
189 immune system cells(9). Phase I clinical trials for NT-501 in glaucoma have been
190 undertaken without serious adverse events (Clinical Trails USA NCT01408472) and
191 Phase II clinical trials were established in 2016 (Clinical Trails USA NCT02862938).
192
193 **4. Stem Cell Therapy**
194 Stem cell therapy is gaining increasing interest and has the aim of regenerating
195 endogenous cells *in vivo* to counteract damage caused by disease. Mesenchymal stem
196 cells (MSCs) have shown promising results in regard to their neuroprotective potential
197 in models of Parkinson's disease, multiple sclerosis, spinal cord injury, and Alzheimer's
198 disease among others (12,16,34–39). The optic nerve is a favourable target for MSC
199 therapy as it has the unique advantage of being somewhat contained and thereby
200 protected from direct systemic immunological reaction(13), as well as being open to
201 real-time monitoring *in vivo* via evolving technologies such as optical coherence
202 tomography (OCT).
203
204 MSCs are multipotent and therefore have the ability to differentiate into an array of
205 cells. They are easy to source from adult bone marrow or human umbilical cord blood,
206 and can be used without immune suppression(12,16,39,40). The neuroprotective and
207 regenerative effects of MSCs are achieved through their ability to differentiate into

208 neurons and glial cells, promote endogenous neuronal growth, promote
209 neuro/gliogenesis, encourage synaptic connection, reduce demyelination, induce
210 oligodendrogenesis, stimulate angiogenesis, decrease apoptosis, reduce oxidative stress
211 by lowering free radicals, modulate microglial activation, and suppress inflammatory
212 responses(39). Ultimately the goal of stem cell therapy in the context of optic
213 neuropathy is to recover vision via neuroregeneration of damaged or dead RGCs and
214 their axons.
215
216 **4.1. Mesenchymal Stem Cells in Animal Studies**
217 MSC transplantation with neuroprotective effects has been trialled in a variety of animal
218 models of optic nerve damage(12). Roubeix *et al.* (2015) used MSC transplantation in
219 the anterior chamber of hypertensive rat eyes to demonstrate a rapid and long-lasting *in*
220 *vivo* protective effect in peripheral RGC degeneration, achieved via improvement in
221 trabecular meshwork integrity and function resulting in IOP reduction(40). Likewise,
222 intravitreal MSC injection into hypertensive rat eyes demonstrated a neuroprotective
223 effect on RGC axon survival yet had no effect on optic nerve damage(41). Repair and
224 regeneration of axotomised RGC neurons was, however, demonstrated with MSC
225 grafting into the site of optic tract transection at the level of the lateral geniculate
226 nucleus in neonatal rats(42). Zhao *et al.* (2011) and Jiang *et al.* (2013) demonstrated the
227 protective effect of intravitreal delivery of human umbilical cord stem cells on optic
228 nerve crush injury in rats on RGC survival, optic nerve function, improved pathological
229 changes in the rat retina and up-regulation in the expression of trophic factors BDNF
230 and GDNF(43,44).
231

232 The attenuation of RGC death by MSC transplantation is largely attributed to the
233 extended release of trophic factors by the transplanted cells, such as CNTF, bFGF,
234 GDNF, HGFα and BDNF(33,43,45,46). Recently, platelet-derived growth factor
235 produced by human MSCs has been shown to have a strong neuroprotective action on
236 human RGCs. Ref (Osborne et al Neuroprotective Effects of Human Mesenchymal
237 Stem Cells and Platelet-Derived Growth Factor on Human Retinal Ganglion Cells.
238 CNTF is a potent RGC survival factor and bFGF is a stimulator of axonal growth(45).
239 Several studies have demonstrated that despite the significant RGC neuroprotection
240 afforded by intravitreal MSC injection, these latter cells did not migrate well and mostly
241 remained present in the vitreous cavity and inner limiting membrane with minimal
242 penetration through to the ganglion cell layer(33,41,45). Further, there was limited
243 differentiation of MSCs to retinal neuronal cells or RGC with functional axons and this
244 was reflective of poor integration of grafted MSCs into the retinal tissue or optic
245 tract(33,42,45). Therefore stem cells used as a vector for delivery and secretion of
246 neurotrophic factors to repair damaged RCGs could be more advantageous than their
247 seemingly limited ability to integrate and differentiate to repopulate retinal neuronal
248 tissue(46).
249
250 **4.2. Mesenchymal Stem Cells in Clinical Studies**
251 Intraocular injection of MSCs for the treatment of retinal and optic nerve disease are
252 currently being investigated in a large open-label non-randomised efficacy clinical trial,
253 the Stem Cell Ophthalmology Treatment Study (SCOTS; Clinical Trial USA Identifier
254 NCT01920867)(12,16,47). Whilst the study is currently in progress, preliminary reports
255 have detailed impressive results in isolated cases of optic neuritis and Leber's

Hereditary Optic Neuropathy(48,49). Although the level of evidence is not robust, these clinical results motivate ongoing research with MSCs as therapy for ophthalmologic mitochondrial disease with potential translation to glaucoma. Two registered phase 1 clinical trials specific to the treatment of glaucoma with MSCs are currently in progress: The Intravitreal Mesenchymal Stem Cell Transplantation in Advanced Glaucoma Study (Clinical Trials USA Identifier NCT02330978, estimated study completion date December 2016, no published results available), Effectiveness and Safety of Adipose-Derived Regenerative Cells for Treatment of Glaucomatous Neurodegeneration study (Clinical Trials USA Identifier NCT02144103, estimated study completion date January 2019).

4.3. Human Embryonic Stem Cells

The use of human embryonic stem cells (hESCs) is more ethically controversial despite the advantage of these pluripotent cells being able to differentiate into all derivatives of the three embryonic layers(12). Photoreceptor generation from hESCs has progressed more rapidly than has generation of RGCs. Sluch *et al.* (2015) described a cell culture protocol for the differentiation of hESCs to highly purified, functional RGCs using the CRISPR-engineered reporter cell line, including a comprehensive characterisation of the cells obtained(49). Pre-clinical studies have previously demonstrated the ability of retinal cells derived from hESCs to survive, integrate into the host retina, and mediate light responses(51,52). Whilst hESC technology is in essence still embryonic, with ethical and scientific challenges, it has the potential to revolutionalize the management of ocular neurodegenerative disease considered incurable through neuroregeneration of functioning retinal neurons.

5. Bioenergetic Protection

Bioenergetic neuroprotection refers to supporting the neuronal energy requirements at a cellular level which includes protecting cells against downstream metabolic failure to circumvent apoptosis which causes consequent neurodegeneration. This approach is gaining momentum in pre-clinical glaucoma research with promising therapeutic translation on the horizon.

5.1. Visual metabolic demands and RGC bioenergetics

Visual processing is metabolically expensive, which makes retinal cells exquisitely sensitive to bioenergetic disturbance. The metabolic cost for the processing of sensory information by photoreceptors has been estimated at 10^6-10^7 ATP molecules for graded signals(53). Thus the process of phototransduction places intense energy demands upon photoreceptors, which derive nutrients and oxygen from the choroidal circulation.

The optic nerve is formed by over one million RGC axons, which serve as a conduit between the retina and brain(10). The RGC axon has a tremendously long trajectory relative to the size of the cell body, upon which neuronal shuttling of cellular cargo occurs, which ultimately compounds the energy demands placed upon RGCs(10). Emerging evidence has strongly associated RGC mitochondrial dysfunction with retinal and optic nerve damage(54). Impaired axonal transport of mitochondria has been linked to RGC death and, given that the unmyelinated axons of RGCs in the pre-laminar retinal nerve fiber layer require a higher energy input, this may in part explain their vulnerability to metabolic injury(10,12). Ultimately RGC survival and function are

304 highly dependent on an intact energy supply and are therefore exquisitely sensitive to
305 homeostatic disruptions such as anoxia or metabolic substrate decline(10,12).
306
307 Light must pass through the entire thickness of the vertebrate retina to reach the
308 photoreceptors, hence, the retina needs to be as transparent as possible. This means that
309 within this tissue there must be a minimal level of blood vessel columns because these
310 structures are relatively opaque. This optical requirement for relatively limiting
311 vasculature, coupled with the large energy demands of the retina make it particularly
312 vulnerable to insults involving alterations in the available blood supply (55).
313 Furthermore, the blood supply of the optic nerve head (ONH) is primarily derived from
314 the choroid and is thus susceptible to pressure differentials within the tissue. These
315 unique vascular compromises inherent to the retina ultimately make the ONH more
316 vulnerable to ischaemia which in turn contributes to neurodegeneration in chronic
317 glaucoma(56).
318
319 The brain and retina are both dependent upon glucose metabolism to produce adenosine
320 triphosphate (ATP), but there are fundamental differences between retinal and cerebral
321 energy metabolism(57). Akin to the brain, much of the energy required for visual
322 functioning is derived from oxidative metabolism coupled to ATP synthesis(12,58). Yet
323 unlike the brain, the isolated mammalian retina also derives a considerable amount of
324 ATP from the conversion of glucose to lactate, even in the presence of oxygen(10,57).
325 This has the advantage that in the absence of oxygen, the mammalian retina has the
326 remarkable ability to maintain most of its ATP requirements via glycolysis(10,57).
327 However, the precise contribution of both glycolysis and oxidative phosphorylation to

328 energy metabolism in RGCs and their axons remains unclear and is likely to be both
329 species dependent and dependent upon the level of retinal vasculature(10).
330
331 **5.2. Consequences of Retinal Energy Failure**
332 **5.2.1. Reactive Oxygen Species**
333 As a downstream consequence of mitochondrial bioenergetic failure, oxidative stress
334 has received considerable attention for its contribution to RGC injury. Generation of
335 reactive oxygen species (ROS), a series of intracellular by-products derived from
336 mitochondrial respiration, is a process which is usually tightly regulated under normal
337 physiological conditions. The antioxidant response is typically carried out by enzymes
338 such as SOD, catalase, glutathione peroxidase, thioredoxin, peroxiredoxin as well as
339 non-enzymatic compounds such as retinol and carotenoids (together comprising vitamin
340 A), ascorbic acid (vitamin C), tocopherols (vitamin E) and melatonin(12). The increased
341 production of ROS from dysfunctional mitochondria in disease conditions, however,
342 leads to chronic oxidative damage which can contribute to cellular dysfunction and
343 consequent neurotoxicity(12). Apart from production via mitochondrial respiratory
344 chain reactions, there is also a simultaneous increase in extra-mitochondrial production
345 of ROS in the cytosol(59). This leads to oxidative deactivation of many enzymes
346 involved in, for example, regulation of glycolysis, in particular glyceraldehyde-3-
347 phosphate dehydrogenase (GAPDH) and pyruvate kinase(59). Rhodopsin and other
348 photosensitizers also augment the production of ROS in the retina during photopic
349 vision, which, by definition, involves exposure of the retina to light(59).
350

The retina is exquisitely sensitive to oxidative damage given its relatively high level of oxygen consumption(59,60). Consequently, oxidative stress is a pathogenic feature of many vision-impairing diseases, including glaucoma, age-related macular degeneration (ARMD), diabetic retinopathy, and uveoretinitis(12,59,60). However, dysfunctional, but not dead, RGCs may be amenable to neurorecovery with early intervention that targets potential mitochondrial dysfunction and elevated oxidative stress, via minimising the generation or accumulation of ROS(8,12).

5.2.2. Excitotoxicity

The pivotal role of excitotoxicity in neurodegenerative disease is gaining momentum and understanding its role in the treatment of optic neuropathies is receiving increasing attention. Excitotoxicity refers to cell death resulting from the toxic actions of excitatory amino acids(61,62). The stimulation of glutamate receptors, interleukin-1 receptors (IL-1Rs), JUN-linked receptors, and tumour necrosis factor receptors (TNFRs) triggers retinal neurons to undergo apoptosis through a cascade of cellular signalling events which in turn promote the release of cytochrome c and which activate the caspase pathways(13). Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and through prolonged exposure contributes substantially to the injury and death of neurons with the associated excessive influx of ions into the cell(61). The major ionotropic receptors activated by glutamate are N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainic acid (KA) receptors(61). Sustained activation ("overactivation") of glutamate receptors impairs cellular calcium homeostasis and activates nitric oxide synthesis, generation of free radicals and programmed cell death (Figure 2B)(61,62). Therapies targeting

375 excitotoxicity, such as memantine and brominidine, have thus been explored for their
376 therapeutic application in glaucoma.
377
378 **5.2.2.1. Memantine**
379 Memantine is a non-competitive NMDA receptor antagonist which blocks glutamate
380 excitotoxicity and is commonly used in the treatment of moderate to severe Alzheimer's
381 disease(9). Several animal glaucoma models have shown that memantine is protective
382 against RGC loss(9,13,16,63,64). However, large-scale multicentre, randomised double-
383 masked placebo-controlled Phase III clinical trials conducted to test the efficacy of oral
384 memantine for glaucoma failed to show any statistical benefit compared to placebo in
385 reducing visual field progression(13,16).
386
387 **5.2.2.2. Brimonidine**
388 Brimonidine, an α2-adrenergic receptor agonist, is commonly used to lower IOP in
389 glaucoma and is FDA approved for systemic administration. It has been demonstrated to
390 protect RGCs in animal models of optic nerve damage independent of its effect on IOP
391 by up-regulating anti-apoptotic factors and by blocking cellular toxicity induced by
392 mitochondrial oxidative stress(2,8,14,16,107,108). This is thought to be achieved
393 through the modulation of glutamate-induced excitotoxicity, vascular regulation via
394 inhibition of nitric oxide synthase or the endothelin pathway, oxidative stress, and
395 inhibition of glial activity(16). It has been demonstrated in clinical trials that
396 brimonidine monotherapy lowered the incidence of visual field progression compared
397 with timolol in treated patients (9 vs 30%) in the Low Pressure Glaucoma Study Group
398 over a period of 30 months despite similar IOP lowering effects(9,13,16,65). Yet this

study was limited by its small sample size and considerable drop out rate in the brimonidine group(13,65). Topical brimonidine 0.2% applied over 3 months has also been found to improve contrast sensitivity, in comparison to no improvement with timolol therapy, despite similar IOP lowering effects(66). A meta-analysis comparing timolol to brimonidine also confirmed no significant difference in IOP lowering effect(67). Tsai *et al.*(2005) described a statistically significant reduction in retinal nerve fibre layer damage following the use of brimonidine 0.2% compared with timolol 0.5% in ocular hypertensive patients over one year, independent of IOP reduction(68). These results suggest that brimonidine provides a non-IOP related neuroprotective effect.

5.3. Targeting Energy Metabolism

5.3.1. Glycolysis

Glycolysis is the oxygen independent metabolic pathway, which takes place in the cytosol of cells, to convert glucose into pyruvate to generate two ATP molecules per starting glucose. In the presence of oxygen, pyruvate is able to then enter the Kreb's cycle to generate 32-36 net ATP molecules within mitochondria, or in anaerobic conditions is instead converted to lactate in the presence of nicotinamide adenine dinucleotide (NAD+ or NADH)(Figure 2A). Whilst still in its infancy, experimental trials have demonstrated that through the manipulation of the glycoloytic pathway via substrate supplementation, RGC function and survival can be prolonged thereby affording a degree of neuroprotection in glaucoma(54,59,60,69).

5.3.1.1. Glucose

Using rat retinal explants Winkler *et al.* (1981) demonstrated that the majority of retinal ATP production can be maintained under conditions of oxygen deprivation provided that there is an abundance of glucose(70). *In vitro* the neuroprotective effect of glucose administration has been demonstrated to be predominantly due to glycolytic ATP production which suggests that RGCs can up-regulate glycolysis during ischaemia to generate ATP(71). Studies have demonstrated that elevated vitreous glucose levels provide robust neuroprotection of RGC somata and axons against experimental retinal ischaemic injury(57,72) and experimental glaucoma(69).

It was subsequently shown in clinical trials that contrast sensitivity was temporarily recovered in pseudophakic individuals with severe POAG after topical glucose application(73). Whilst glucose may provide a considerable degree of neuroprotection or recovery to damaged but not yet dead RGCs in the short term, long- term elevated vitreous glucose levels would possibly cause deleterious ocular complications such as cataract or diabetic-type retinal disease.

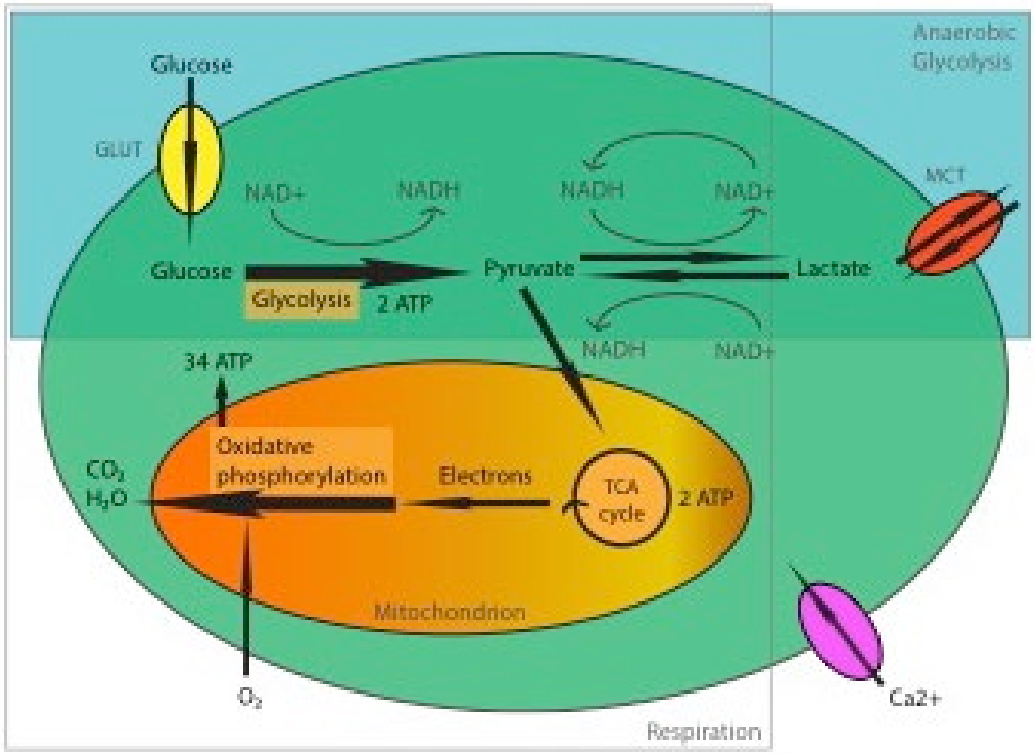
5.3.1.2. Pyruvate

Pyruvate is an endogenous alpha keto acid metabolite that protects against oxidative stress whilst simultaneously providing energy substrate support(59,60,74)(Figure 2C). Under normal circumstances, when oxygen is abundant, pyruvate is converted to acetyl CoA, which then enters the Kreb's cycle to contribute to the formation of substrates and electron donors for oxidative phosphorylation (OXPHOS) generating about 32 ATP molecules(71). In the face of oxygen deprivation, pyruvate is instead converted to lactate by lactate dehydrogenase (LDH) to regenerate nicotinamide adenine dinucleotide

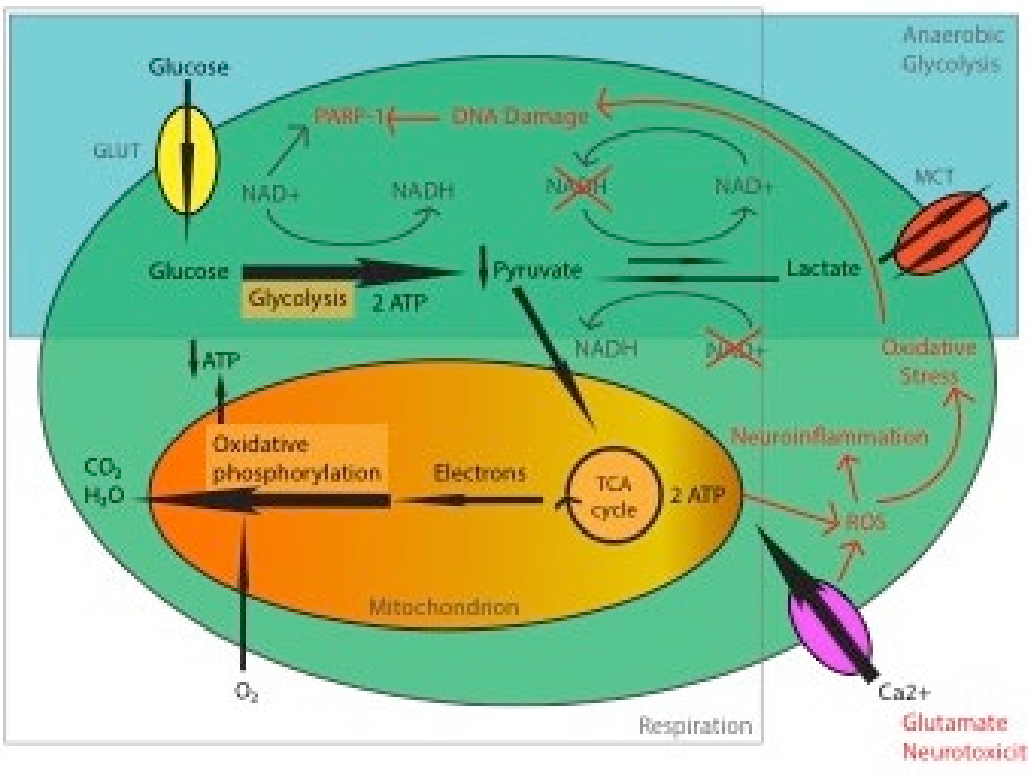
(NAD+)(71,74). It has been proposed that pyruvate could facilitate glycolysis by recycling of NAD+ required for the continued activity of the glycolytic pathway(59). By scavenging various ROS species, pyruvate is also able to inhibit oxidative stress. This prevents the toxic reactions of lipid peroxidation and loss of tissue thiols, thereby protecting the retina against further insult whilst supporting glycolysis(59,74). Pyruvate also reduces the blood glutamate level, thereby reducing glutamate-induced neurotoxicity, prevents neuronal network hyperexcitability, and exhibits potent anti-inflammatory action(74).

Evidence is accumulating to support the neuroprotective effect of pyruvate in experimental models of neurodegenerative disease, such as ischaemic brain injury, hypoglycaemic brain injury, Huntington's disease, neuroblastoma, closed head injury, and Parkinson's disease(75–83). Preliminary *in vivo* experiments by Hegde *et al.*(2008 and 2010) demonstrated that pyruvate substantially promotes glycolysis in the retina in the face of ROS-induced inhibition(59,60). This could be explained by the inhibition of oxidative inactivation of –SH containing enzymes such as GAPDH and pyruvate kinase, and the continued regeneration of NAD+ (produced during the reduction of pyruvate to lactate by LDH) with consequent stimulation of the oxidation of glyceraldehyde-3-phosphate to 1,3 diphosphoglycerate by GAPDH dependent on NAD+ availability(59).

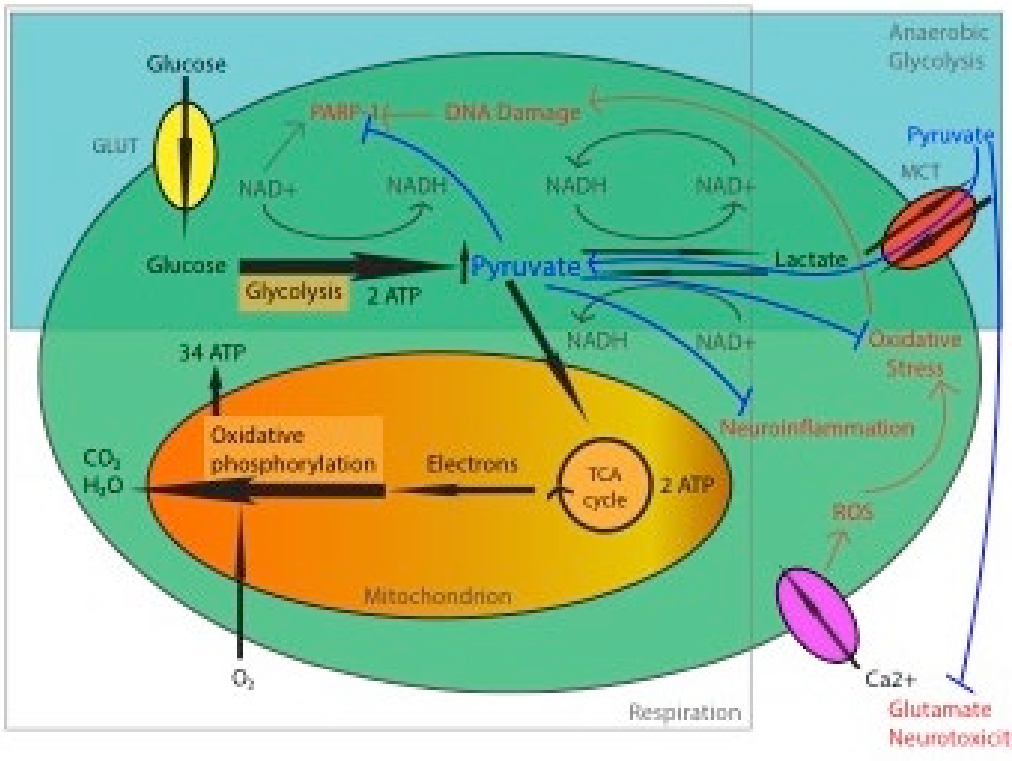
Figure 2: Cellular Mechanism of Neurodegeneration and Pyruvate Neuroprotection*


Anaerobic Glycolysis
Glucose
GLUT
NAD+
NADH
NADH
NAD+
MCT
Glucose
Glycolysis
2 ATP
Pyruvate
Lactate
NADH
NAD+
34 ATP
Oxidative phosphorylation
Electrons
TCA cycle
2 ATP
CO_2
H_2O
Mitochondrion
O_2
Ca2+
Respiration

469 **(A)**


Anaerobic Glycolysis
Glucose
GLUT
PARP-1
DNA Damage
NAD+
NADH
NADH
NAD+
MCT
Glucose
Glycolysis
2 ATP
Pyruvate
Lactate
NADH
NAD+
Oxidative Stress
ATP
Neuroinflammation
Oxidative phosphorylation
Electrons
TCA cycle
2 ATP
CO_2
H_2O
ROS
Mitochondrion
O_2
Ca2+
Glutamate Neurotoxicity
Respiration

470 **(B)**



(C)

(A) ***Normal cellular respiration:*** *Under normal conditions glucose enters the cell through the transmembrane glucose transporter (GLUT) and is metabolised to pyruvate via glycolysis. If oxygen is abundant, pyruvate enters the tricarboxylate acid (TCA or 'Kreb's') cycle where it is metabolised via oxidative phosphorylation to form an abundance of ATP. Under anaerobic conditions pyruvate is instead preferentially converted to lactate, by lactate dehydrogenase (LDH), in the presence of NADH. Conversely, lactate can also enter cells through the monocarboxylate transporter (MCT) and can be converted to pyruvate by LDH in the presence of NAD+.*

(B) ***Pathology of neurodegeneration:*** *Both oxidative stress and extracellular glutamate trigger excitotoxicity, which contributes to neuronal degeneration. Glutamate accumulation triggers an influx of excessive calcium into cells. Reactive oxygen species accumulate and contribute to neuroinflammation and oxidative stress. DNA damage is induced by ROS leading to the overactivation of poly-ADP ribose polymerase-1 (PARP-*

1) causing depletion of cytostolic NAD+. Reduced NAD+ inhibits glycolysis with subsequent decline in mitochondrial ATP production, as well as limiting the conversion of lactate to pyruvate.

(C) *Pyruvate supplementation*: *Pyruvate is able to counteract substrate decline and support mitochondrial ATP production in conditions of neuronal stress (only when O2 is present). It also serves as a potent scavenger of reactive oxygen species, reduces neuroinflammation and subsequent oxidative stress. Pyruvate directly acts within the blood stream to lower glutamate levels, thereby reducing neuronal calcium (Ca2+) overload. Pyruvate promotes glycolysis by inhibiting PARP-1 overactivation, which effectively restores NAD+ levels.*

Adapted from:* ***Zilberter, Y. et al.(2015) A unique array of neuroprotective effects of pyruvate in neuropathology. Front. Neurosci; 9:17.*

5.3.1.3. Nicotinamide

Nicotinamide adenine dinucleotide (NAD) is a key molecule for mitochondrial health and nicotinamide (NAM) is a major precursor in the formation of NAD in mammals *in vivo*(84,85). NAM is unique among NAD precursors because it is a physiological inhibitor of the major NAD catabolic enzymes, namely CD38, PARPs (Poly (ADP-ribose) polymerase), and SIRTs (sirtuin)(84,86,87). Its physiological efficacy in glaucoma is supported by its favourable effects on calcium channel and calcium signalling (important in axon degeneration) (88–90), its vasoactive properties (with vascular dysfunction implicated in glaucoma) (91,92) and its ability to improve endothelial function and stabilising blood flow by reversing endothelin-mediated

508 vasoconstriction (with endothelin receptor blockers shown to protect against glaucoma)
509 (84,93).
510
511 The retinal level of NAD has recently been discovered to decline in an age-dependent
512 manner(84,94), rendering RGC mitochondria vulnerable to IOP-dependent
513 stresses(54,95). In a mouse model of hereditary glaucoma (DBA/2J (D2) mice)
514 Williams *et al.*(2017) demonstrated that oral administration of nicotinamide and/or gene
515 therapy (driving expression of *Nmnat1*, a key NAD+ -producing enzyme) was
516 protective prophylactically and as an intervention, both histologically and functionally
517 on pattern electroretinogram(54). More pertinently, up to 93% of eyes did not develop
518 glaucoma with high dose nicotinamide supplementation, which also had an IOP
519 lowering effect(54).
520
521 The Wallerian degeneration slow allele, Wld^s, deceases the vulnerability of RGCs
522 subjected to elevated IOP by increasing retinal NAD levels(95). This extends upon the
523 finding that a mouse strain called Wallerian degeneration slow mice (Wld^s) contains a
524 spontaneous dominant mutation that protects against neuronal insults, such as
525 Parkinson's disease, hypoxic-ischemic injury, toxic neuropathy (taxol), and
526 others(94,96). Williams *et al.*(2017) demonstrated that when coupled with nicotinamide
527 administration, 94% of eyes were protected against glaucomatous neurodegeneration in
528 a mouse glaucoma model (DBA/2J (D2) mice)(95). Certainly this preliminary evidence
529 advocates for the use of nicotinamide and/or gene therapy in glaucoma and other
530 neurodegenerative disease, with further studies required exploring its safety and
531 efficacy in human disease.

5.3.1.4. Lactate and Other Glycolytic Intermediates

Before glucose can reach the retina from the choroidal circulation it must first traverse the retinal pigment epithelium (RPE), which functions as a blood-retina barrier. Most of the glucose that reaches the retina is consumed by glycolysis and converted to lactate, predominantly by photoreceptors. Kanow *et.al.* (2017) recently demonstrated in *in vivo* vertebrate retinas that the lactate converted by photoreceptors serves as a fuel source for neighbouring retinal cells, and that glycolysis can be suppressed by lactate in RPE cells to permit more glucose to reach the retina(97).

It widely accepted that intercellular lactate movement, via monocarboxylate transporters, performs an essential function in the metabolic interaction between neurons and glia via the astrocyte-neuronal lactate shuttle. In the CNS it has recently been demonstrated that lactate has neuroprotective effects in models of excitotoxicity and energy depletion(98). It appears to constitute an alternative energy substrate for neurons lacking standard nutrients. This has been demonstrated in both *in vitro* and *in vivo* models of cerebral ischaemia(98). It has also been proposed that both the L- and D-forms of lactate play a role in intercellular communication via interaction with the HCA1 receptor(99). Studies performed by Tekkök *et.al.* (2005) further support the hypothesis that L-lactate is released from astrocytes and taken up by axons as an energy source for sustaining their excitability(100). These findings support the theory that the metabolic astrocyte-neuronal lactate shuttle within the retina could hold promise for manipulation in future glaucoma neuroprotection studies.

5.3.2. OXPHOS

Mitochondrial aerobic respiration through oxidative phosphorylation (OXPHOS) generates the majority of ATP in neurons and their axons. Given that OXPHOS, by definition, is dependent on oxygen and knowing that ischaemia likely plays a role in the pathogenesis of glaucoma, erythropoietin (EPO) has been proposed as a possible neuroprotective candidate. EPO is a glycoprotein cytokine secreted by the kidney in response to hypoxia, which in turn stimulates red blood cell production to improve the blood stream's oxygen carrying capacity. EPO and its receptors are distributed within the human retinal tissue and RPE(101), and Szabo *et.al.* (2008) demonstrated that RGCs principally produce and secrete EPO(102). Exogenous EPO administration has been demonstrated in *in vivo* glaucoma and optic nerve transection models to improve RGC survival and restore mitochondrial structure(103–105). Yet a significant draw-back of EPO therapy is its promotion of angiogenesis causing pathological neovascularisation(101). Whilst promising pilot studies have been performed looking at neuroprotection in optic neuritis in humans(106–109), no trials to date have been undertaken to assess the clinical utility of EPO in glaucoma.

Neuroglobin (Ngb) is linked to oxidative metabolism and is hypothesised to have a myoglobin-like role in supplying oxygen to the respiratory chain of neurons whilst also protecting them from ROS(110). Both Ngb and Cytoglobin are present in distinct nerve cell populations, including human retinal neurons and RPE(111). In fact, the Ngb concentration in the retina is 100-fold higher than any other nervous tissues with this protein being especially abundant (~10-fold higher) in the RGC layer and optic nerves than in the other layers of the retina(112). Ngb has shown a promising neuroprotectant

property in murine cerebral and retinal ischaemia(110,113), and has also been found to prevent RGC damage induced by glutamate cytotoxicity in vitro and/or by chronic IOP elevation *in vivo*(114,115). Ngb has not only been demonstrated to improve the survival of RGCs after optic nerve injury but, in mouse retinas showing enhanced Ngb expression, was found to regenerate central optic axon outgrowth(116). Ngb may modulate RGC susceptibility to glaucomatous neural damage and may therefore represent a novel neuroprotective and neuroregenerative therapy for this disease.

The general aim of pharmacological therapy in targeting mitochondrial dysfunction in glaucoma is to improve energy production and protect cells from ROS toxicity(12). A review of experimental mitochondrial therapies in neurodegenerative disease with possible translation to optic neuropathies is comprehensively detailed by Lopez *et.al.*(2016)(12). Those compounds/therapies targeted against oxidative damage include mitoquinone mesylate (Mito-Q), co-enzyme Q10 (CoQ10), carotenoids, idebenone, exogenous glutathione, and methylene blue(12). However, to date there are no clinically trialled drugs with definitive therapeutic efficacy for the treatment of mitochondrial dysfunction in glaucoma.

5.3.3. Pentose Phosphate Pathway (PPP)

Parallel to the glycolytic pathway is the pentose phosphate pathway, taking place in the cytosol of cells. Using glucose as its primary substrate, the PPP generates NADPH and pentose sugars as well as ribose 5-phosphate (a precursor for the synthesis of nucleotides)(117). The production of cellular reducing equivalents can in turn be used in reductive biosynthesis reactions within cells. NADPH also functions to prevent

oxidative stress thereby preventing apoptotic cell death(118) and in photoreceptors, this compound is further involved in the recycling of photopigments(119,120). It has been hypothesised that glucose can directly provide cytoprotection through its oxidation via the PPP by maintaining cellular reducing power(117,118).

Using rat retinal cultures, Han *et.al.* (2013) demonstrated that administration of the 6-phosphogluconate dehydrogenase inhibitor, 6-AN, inhibited the PPP and reduced the protective effect of glucose against rotenone-induced retinal cell toxicity(121). Contrary to this, Winkler *et.al.* (1997) showed that the portion of total glucose metabolised via the PPP did not increase significantly in the isolated retina when glucose was elevated from 5mM to 30mM(122). This suggests that PPP-derived NADPH may only play a minor role in neuronal functioning. It further supports previous findings that ATP production from glycolysis constitutes the most important glucose-induced neuroprotective mechanism in retinal ischaemia(121).

6. Clinical Translation

Despite large numbers of laboratory-based reports of successful neuroprotection in animal glaucoma models, clinical translation has been extremely limited. Herein, we address both the potential reasons underlying this 'translational gap' and possible solutions to this.

6.1. Who needs neuroprotection?

Currently, the only treatment strategy for glaucoma is reduction of the intraocular pressure. The basic management algorithm comprises an iterative process of setting a

target pressure at which it is believed progression will not occur, monitoring the individual and lowering the target pressure if progression is noted. There is considerable evidence for the efficacy of this strategy even in individuals with normal-tension glaucoma(123). Furthermore, there is evidence that if IOP is well controlled at relatively normal levels then the majority of individuals do not progress(124). However, the cumulative incidence of bilateral blindness from glaucoma after 20 years is approximately 14%. Whether or not this incidence could be mitigated or even eliminated with earlier diagnosis and further reduction of IOP is unclear. Although robust evidence is not forthcoming, clinical experience suggests that there is a small but significant group of individuals in whom a target pressure is unreachable even in principle. In addition, there is a considerable group of individuals who would benefit from adjunctive treatment where attempting to reach target pressure with more aggressive treatment may not be desirable. Arguably, a safe, efficacious neuroprotective therapy as part of a "belt and braces" strategy could be added to the management of all individuals with glaucoma.

6.2. Strategies to Advance Clinical Neuroprotection Studies

Obstacles to clinical neuroprotection studies relating to glaucoma include: slow progression, heterogeneity of pathogenesis, and the fact that evidence of neuroprotection would need to be detectable beyond the therapeutic effect of routine IOP reduction. The chronicity of glaucoma and the general slow rate of progression make clinical neuroprotection studies challenging and potentially prohibitive in terms of the time and cost required to investigate a new therapy. However, there are strategies that could considerably reduce the time and sample size required to obtain a definitive

result. Traditionally, the gold standard primary outcome in clinical glaucoma studies has been automated perimetry. However, modern practice incorporates optical coherence tomography (OCT) as a routine clinical tool, with structural changes generally detectable at an earlier stage than visual field changes. Guided Progression Analysis (GPA) on the Cirrus HD-OCT (Carl Zeiss Meditec, Dublin, CA) provides a trend-based statistical analysis that could conceivably replace perimetry as a primary outcome in neuroprotection studies.

It may be judicious to initially target "lower hanging fruit" where outcomes could be assessed more rapidly. This could include a randomized controlled trial of neuroprotectant therapy versus placebo in acute glaucoma or situations where IOP may be temporarily poorly controlled. Similarly, it would be advantageous to select a sample of individuals with chronic glaucoma who were rapidly progressing. Identification of such individuals would be enhanced by large databases at national or international levels.

Thoughtful study design and sophisticated statistical analyses can considerably reduce sample sizes. When obtaining multiple measurements over time comparing rates of progression between two groups (treated and placebo) where individuals have variable starting points and rates of progression, the ideal statistical framework is a linear mixed model incorporating random intercepts and random slopes. This model accounts for the correlated nature of the data and neatly handles inevitable missing numbers. Estimates of intercepts, slopes, and correlations can be obtained relatively easily from existing data. Sample sizes are easily determined using open source software(125).

The numbers needed can be surprisingly small. As an example, consider a trial in which assessments are taken every three months for 24 months (9 visits) and we estimate a 30% reduction in the rate of progression in the group receiving a novel neuroprotectant compared to the control group. The participants are selected to have a relatively rapid rate of progression (1 dB per year). We estimate the random intercept to have a variance of 0.3, the random slope to have a variance of 0.7, and a residual variance of 0.1. The estimate the correlation between random slope term and random intercept term is 0.7. Using these estimates, for a power of 80% and alpha value of 0.05, only 46 individuals are required in each group(126,127).

7. Conclusion

Glaucoma has a multifactorial pathogenesis, broadly categorised into vascular and mechanical theories, which drives progressive optic neurodegeneration and consequent blindness. Thus treatment strategies have evolved based upon potential root causes. Early identification of those at risk through genetic screening and unveiling the underlying patient-specific aetiology may, in the future, offer customised therapy tailored to the individual patient.

Whilst IOP lowering therapies generally slow the progress of glaucoma progression they are limited by the extent of their effect despite optimal IOP control. Thus there is growing demand for, and investment in, neuroprotection research to provide an adjunct to IOP lowering therapies and prevent visual decline.

The holy grail of laboratory glaucoma research would be to develop animal models that more closely recapitulate human disease in order to validate new therapeutic agents prior to embarking on human trials. Whilst there is an ever-increasing abundance of preclinical research, clinical translation remains in early infancy and not without its inherent challenges. Refinement of clinical trial design and the use of validated home monitoring techniques may improve the cost burden and efficiency of clinical neuroprotective trials in glaucoma research.

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