

# Glaucoma as a Metabolic Optic Neuropathy: Making the Case for Nicotinamide Treatment in Glaucoma

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**Abstract:** Mitochondrial dysfunction may be an important, if not essential, component of human glaucoma. Using transcriptomics followed by molecular and neurobiological techniques, we have recently demonstrated that mitochondrial dysfunction within retinal ganglion cells is an early feature in the DBA/2J mouse model of inherited glaucoma. Guided by these findings, we discovered that the retinal level of nicotinamide adenine dinucleotide (NAD, a key molecule for mitochondrial health) declines in an age-dependent manner. We hypothesized that this decline in NAD renders retinal ganglion cells susceptible to damage during periods of elevated intraocular pressure. To replete NAD levels in this glaucoma, we administered nicotinamide (the amide of vitamin B<sub>3</sub>). At the lowest dose tested, nicotinamide robustly protected from glaucoma (~70% of eyes had no detectable glaucomatous neurodegeneration). At this dose, nicotinamide had no influence on intraocular pressure and so its effect was neuroprotective. At the highest dose tested, 93% of eyes had no detectable glaucoma. This represents a ~10-fold decrease in the risk of developing glaucoma. At this dose, intraocular pressure still became elevated but there was a reduction in the degree of elevation showing an additional benefit. Thus, nicotinamide is unexpectedly potent at preventing this glaucoma and is an attractive option for glaucoma therapeutics. Our findings demonstrate the promise for both preventing and treating glaucoma by interventions that bolster metabolism during increasing age and during periods of elevated intraocular pressure. Nicotinamide prevents age-related declines in NAD (a decline that occurs in different genetic contexts and species). NAD precursors are reported to protect from a variety of neurodegenerative conditions. Thus, nicotinamide may provide a much needed neuroprotective treatment against human glaucoma. This manuscript summarizes human data implicating mitochondria in glaucoma, and argues for studies to further assess the safety and efficacy of nicotinamide in human glaucoma care.

**Key Words:** glaucoma, NAD<sup>+</sup>, nicotinamide, axon degeneration, retinal ganglion cell, optic nerve head cupping

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## GLAUCOMA, MITOCHONDRIAL DYSFUNCTION, AND NICOTINAMIDE TREATMENT

Affecting ~80 million people by the end of this decade, glaucoma is a leading cause of blindness worldwide.<sup>1</sup> It represents a significant economic and health burden. Glaucoma is characterized by the progressive dysfunction and loss of retinal ganglion cells (RGCs) and their axons, which

make up the neural tissue of the optic nerve. Major risk factors for glaucoma include increased intraocular pressure (IOP) and age. The DBA/2J (D2) mouse is an age-dependent, inherited model of high IOP and glaucoma.<sup>2–11</sup> Ourselves and others have established that the key features of glaucomatous neurodegeneration in DBA/2J mice match those in human patients. This includes the progressive nature and specificity of RGC demise,<sup>12,13</sup> the location of a critical insult to RGC axons within the optic nerve head,<sup>14</sup> the topographic pattern of cell loss<sup>15</sup> (fan-shaped from the optic disk<sup>14</sup>), and the lessening of neurodegeneration by lowering IOP.<sup>7,16–18</sup> The similarities extend to molecular changes, with changes in specific pathways being demonstrated in both DBA/2J mice and human patients (including the endothelin pathway and its receptors and various complement pathway molecules<sup>2,12,19–22</sup>).

To elucidate the earliest molecular changes that occur in glaucoma we have used RNA-sequencing (RNA-seq) to analyze D2 RGCs at different ages and stages of disease.<sup>23</sup> We discovered that metabolic dysfunction and mitochondrial abnormalities occur before neurodegeneration, at a time-point that corresponds with early decreases in electrical activity as assessed by pattern electroretinogram (PERG). Guided by these results, we performed metabolic profiling, which identified an age-dependent decrease in the levels of retinal NAD. Targeting this metabolic decline, by repleting levels of NAD by administering its precursor nicotinamide, robustly protected eyes from glaucoma<sup>23</sup> (Fig. 1). This treatment protected all assessed RGC compartments including the axon, soma, and synapses.<sup>23,24</sup> In addition, it protected from declines in the very early and sensitive measures of RGC dysfunction; PERG and axoplasmic transport. Some of the very earliest detectable changes in RGCs following periods of elevated IOP were also prevented (mitochondrial dysfunction and transcriptomic changes) (Fig. 2). To develop a “one-shot” treatment for glaucoma, we tested a gene therapy (over expressing *Nmnat1*, coding a key NAD producing enzyme). Gene therapy has the advantage of overcoming compliance issues by being long-lasting (possibly life-long). This *Nmnat1* gene therapy was sufficient to protect the majority of eyes from glaucoma. Combining this gene therapy with nicotinamide treatment was even more effective, protecting significantly more eyes.<sup>23</sup>

Further evidence of the robust protection from glaucoma in mice with increased NAD levels is provided by our findings using D2 mice carrying the Wallerian degeneration slow allele (*Wld<sup>S</sup>*, which encodes additional NMNAT1 activity).<sup>14,26–29</sup> D2 mice with the *Wld<sup>S</sup>* allele were protected from glaucoma to a similar degree as mice with nicotinamide treatment alone (~70% of eyes not developing glaucoma).<sup>24</sup> We also tested the effect of combining both enzyme and precursor (ie, *WLD<sup>S</sup>* plus nicotinamide). This robustly prevented development of glaucoma more than either treatment alone (94% of eyes not developing glaucoma).<sup>24</sup> Importantly, these treatments protected from RGC synapse loss and prevented dendrite pruning out to the

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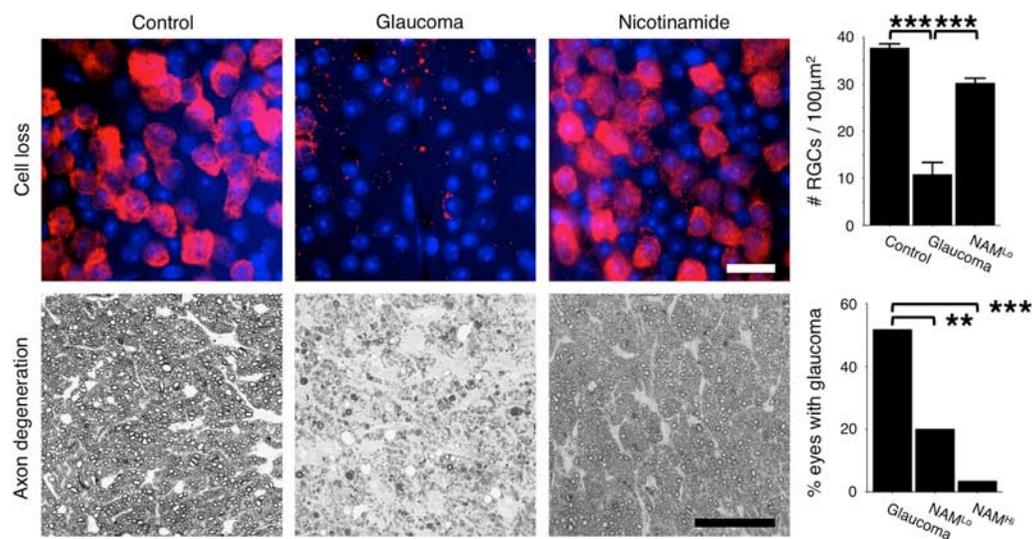
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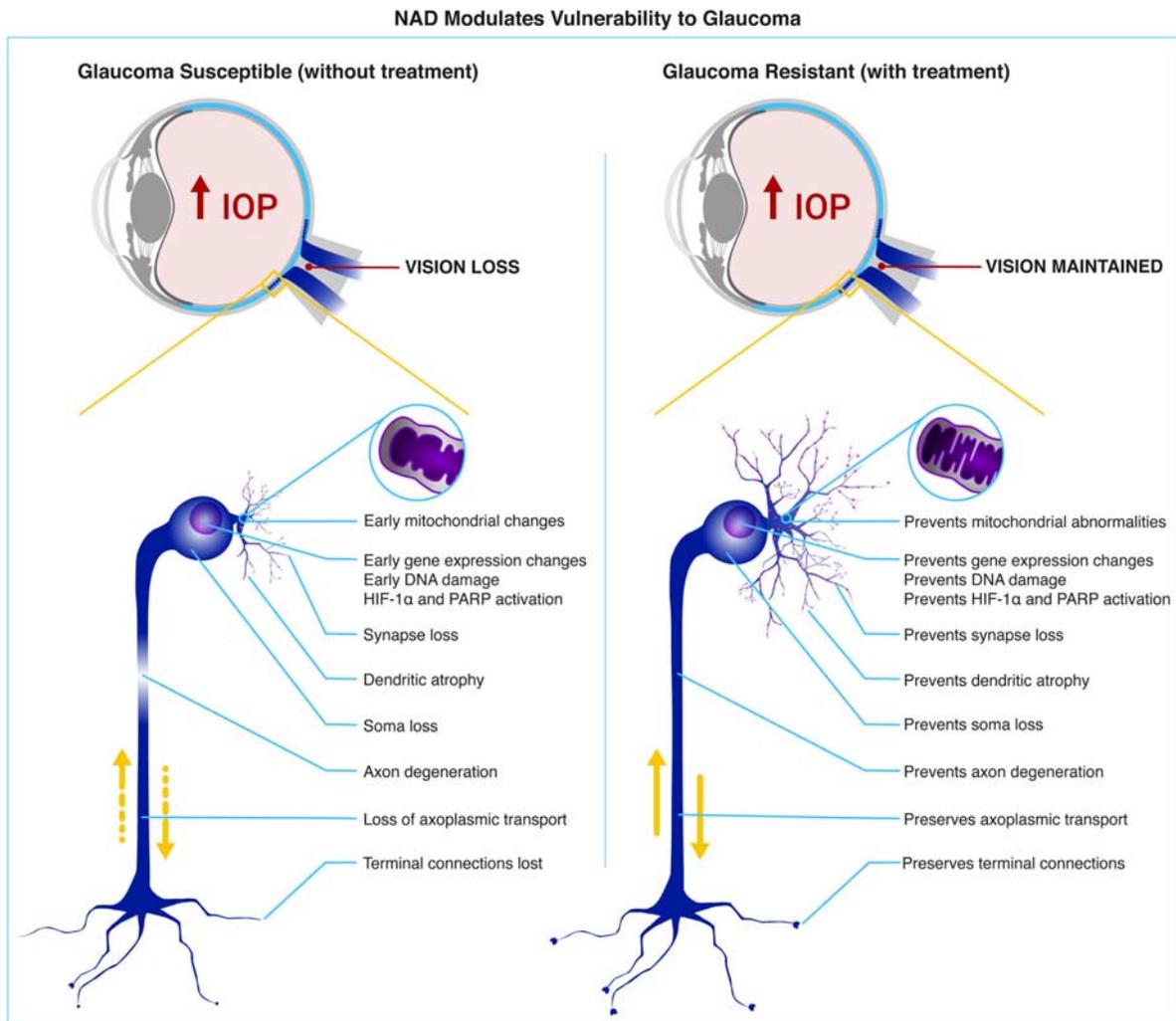
**FIGURE 1.** Retinal ganglion cell protection following nicotinamide treatment in the DBA/2J mouse model of inherited glaucoma. Nicotinamide profoundly protects retinal ganglion cells and prevents optic nerve degeneration in a dose-dependent manner. Top row shows flat-mounted retinas stained with an anti-RBPMS antibody that specifically labels retinal ganglion cells (red) and counterstained with DAPI that stains nuclei (blue). There is a significant loss of retinal ganglion cells following periods of elevated IOP (top row, middle panel), which is prevented by nicotinamide treatment (top row, right panel). Bottom row shows cross sections of the optic nerve stained with PPD. Following periods of elevated IOP retinal ganglion cell axons in the optic nerve degenerate and glial scars are formed (bottom row, middle panel). Nicotinamide treatment (NAM<sup>Lo</sup>) robustly protected the axons, and the number of optic nerves with glaucoma was significantly decreased. At a higher dose (NAM<sup>Hi</sup>) 93% of optic nerves did not develop glaucoma (bottom row, chart). Scale bars = 20 μm (top row), 50 μm (bottom row). \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , Student  $t$  test (top), Fisher exact test (bottom). Glaucoma = untreated, DBA/2J mice. In the charts NAM<sup>Lo</sup> and NAM<sup>Hi</sup> refer to DBA/2J mice treated with the indicated doses of nicotinamide. All images are for mice that were 12 months of age. See references<sup>23,24</sup> for more details.

oldest ages tested.<sup>24,25</sup> Although further experiments with a variety of animal models are needed to assess how widespread and robust such NAD-based protection may be in glaucoma, we also demonstrated protection against two experimentally induced models of RGC death and protection has been shown in other models by other groups.<sup>23</sup> Expression of a cytoplasmically localized variant of NMNAT1 (in mice) or NMNAT3 (in rats) protected from experimentally induced models of RGC death and glaucoma.<sup>30,31</sup> Together, these data suggest that interventions that target NAD levels protect both RGC morphology and function, and may transform patient care for glaucoma.

## MITOCHONDRIA AND HUMAN GLAUCOMA

When assessing the translational relevance of our findings, it is important to consider the potential importance of mitochondrial dysfunction in human glaucoma. Mitochondria have been considered relevant to glaucoma for some time.<sup>32–37</sup> Our studies extend this by demonstrating that mitochondrial dysfunction occurs as one of the earliest detectable events within RGCs following IOP elevation in vivo in a chronic mouse model of glaucoma and that nicotinamide treatment is remarkably protective.<sup>23</sup> As further discussed below, a growing literature suggests that mitochondrial functions are relevant to the human disease and that they may underlie both susceptibility and resistance to developing glaucoma. Together with our findings, this suggests that nicotinamide treatment may prove beneficial in human glaucoma and studies to directly evaluate it are of the utmost importance.

Although further studies are needed, emerging research suggests that a systemic vulnerability to mitochondrial abnormalities and metabolic demise exists in open-angle glaucoma patients compared with controls (in mitochondrial complexes I, III, IV, and V).<sup>38</sup> DNA analysis has demonstrated increased mitochondrial DNA content<sup>38</sup> as well as a spectrum of mtDNA mutations and mutations in nuclear-encoded mitochondrial protein-coding genes in both open-angle and normal tension glaucoma patients.<sup>39–45</sup> Such mitochondrial abnormalities were present in peripheral blood leukocytes suggesting a systemic susceptibility to metabolic abnormalities (as opposed to mitochondrial changes in the eye as a consequence of high IOP).<sup>46</sup> Increased mitochondrial DNA content provides evidence of imbalance between mitochondrial and nuclear genomes that predisposes to mitochondrial dysfunction. Decreased plasma citrate levels have been suggested as a biomarker for human glaucoma.<sup>47</sup> Citrate is an important substrate in energy production within mitochondria. It is produced within mitochondria and so reduced mitochondrial activity may underlie the lower citrate levels. In another study, lymphoblasts from open-angle glaucoma patients had decreased mitochondrial complex I-mediated oxidative phosphorylation, again supporting systemic susceptibility of mitochondria.<sup>48</sup> Such systemic susceptibility is expected to contribute to mitochondrial damage and increasing vulnerability to glaucoma with increasing age. Taken together with our data, this susceptibility would be predicted to increase the likelihood of an energetic crisis and RGC dysfunction when RGCs are subject to stresses induced by high IOP in human glaucoma. In contrast, having more reliable or efficient mitochondria may protect from glaucoma when IOP is high. In fact, glaucoma resistant individuals who have not developed glaucomatous neuropathy despite years of



**FIGURE 2.** NAD protects from retinal ganglion cell degeneration and glaucoma. NAD levels decrease with age rendering retinal ganglion cells vulnerable to IOP-induced damage. Preventing this age-dependent change by increasing NAD availability robustly protects from all assessed signs of glaucoma. Increasing NAD using nicotinamide,<sup>23</sup> *Nmnat1* gene therapy,<sup>23</sup> the *Wld<sup>s</sup>* allele,<sup>24,25</sup> or combinations of these robustly protect retinal ganglion cells from degeneration. This protection is neuroprotective, as neither the low dose that we used (NAM<sup>LO</sup>) or the other treatments changed the IOP. Higher doses of nicotinamide may have an additional benefit by also limiting the degree of IOP elevation.<sup>23</sup> The inhibitory affect of nicotinamide on various enzymes may enhance its potency against glaucoma through different mechanisms (see text). Left shows a retinal ganglion cell undergoing glaucomatous changes because of high IOP. Right shows a retinal ganglion cell that is protected from the detrimental effects of IOP because of increased NAD levels presumably within the RGC. The affects of NAD on other cell types may also be important. See references<sup>23–25</sup> for more details.

high IOP are reported to have systemic mitochondrial efficiency,<sup>49</sup> including increased rates of ADP phosphorylation by mitochondrial complexes I, II, and IV as compared with both unaffected controls and glaucoma patients. Other lines of inquiry have demonstrated that OPA1 expression (which promotes mitochondrial stability) was decreased in open-angle glaucoma patients<sup>50</sup> and genome wide expression studies (GWAS) have linked a key mitochondrial gene (*TXNRD2*) to glaucoma susceptibility.<sup>51</sup> A recent study has identified certain African (and African American) mtDNA haplogroups as risk factors for primary open-angle glaucoma. These haplogroups contain ancestral variants for mitochondrial genes *MT-RNR2* and *MT-CO1* that have known roles in other degenerative diseases.<sup>52,53</sup> Another study found that groups of common variants related by shared membership in mitochondrial and metabolic pathways had associations with

primary open-angle and normal tension glaucoma.<sup>54</sup> Given all of these observations, our findings of mitochondrial damage as an early and key driver in D2 glaucoma, may well generalize to human patients. Taken as a whole, these studies support including glaucoma in the spectrum of mitochondrial optic neuropathies.<sup>55–57</sup>

**CHOICE OF NAD PRECURSOR AND SAFETY**

Therapies to increase NAD levels and improve metabolic reliability under stress may be effective against glaucoma.<sup>23–25,58</sup> Thus, the administration of NAD precursors offers promise for improving glaucoma prevention and care. As decreasing NAD levels seem to be a common feature of aging in different tissues and species,<sup>59–63</sup> the use of NAD precursors may be effective in a wide variety of

glaucoma cases. The profound nicotinamide-mediated protection that we have demonstrated supports testing the use of nicotinamide in human glaucoma. Nicotinamide has a good safety profile especially when used at 3 g/d or below.<sup>64</sup> Despite the extensive use of nicotinamide, human safety studies in aged glaucoma populations are necessary. In our studies, the lowest dose used (nicotinamide low dose; NAM<sup>L</sup>) is equivalent to ~2.7 g/d for a 60 kg human (the human dose equivalent is based on a mouse dose of 550 mg/kg/d<sup>65</sup>). At this dose structural and functional changes were prevented and the overall neural protection was robust, despite no impact on IOP elevation. The highest dose that we tested (nicotinamide high dose; NAM<sup>H</sup>, 2000 mg/kg/d) is equivalent to ~9.8 g/d for a 60 kg human and was extremely protective against glaucoma. At this dosage, IOP became elevated but to a lesser magnitude than in untreated eyes, suggesting that NAM has effects on a variety of cell types and may have dual benefit in glaucoma.

Other NAD precursors must also be considered. The most appropriate or most effective NAD-precursor may depend on tissue and disease context. Compliance issues, including ease of dosing, are important when considering specific precursors, with more frequent dosing being more problematic, especially in the elderly. Of the commonly used precursors, nicotinic acid has the most unpleasant side effects and nicotinic acid (NA) is more noxious than nicotinamide (NAM) and nicotinamide riboside (NR).<sup>64,66</sup> These side effects of NA include flushing and gut irritation that significantly impact compliance. Nevertheless, both NA and NAM have a long history of human use that demonstrates good safety with minimal adverse effects. Safety data are derived from studies evaluating high doses ~3 to 9 g/d (or more in some cases) for long periods of time (up to 5 y<sup>64</sup>). In one study, there were only 3 cases of hepatotoxicity among 6000 patients on megadoses of NA and/or NAM. One of these cases normalized without withdrawing NA, whereas another normalized when concomitant phenothiazine was withdrawn but the patient was still on niacin. The other patient was on 9 g/d niacin and likely had individual susceptibility to this hepatotoxicity.<sup>64</sup> It is important to note that the term niacin originally referred to NA, and this patient was likely on NA. The term niacin is confusing, however, as it is also used to refer to a mixture of NA and NAM or rarely NAM alone. Thus, caution must be taken when referring to niacin, nicotinic acid, or vitamin B<sub>3</sub> in older literature. A case of hepatotoxicity caused by 9 g/d NAM has been reported; however, emphasizing the need to monitor for individual susceptibility when high doses are consumed.<sup>67</sup>

Regarding ocular side effects, there is 1 case in which NA raised IOP.<sup>68</sup> Despite the common long-term use of high doses of niacin (often 2 to 3 g/d) to lower cholesterol or treat other conditions, we have not found any other reports of this effect on IOP. In fact, niacin was reported to lower IOP in 12 AMD patients and our highest dose of NAM lessened the degree of IOP elevation in DBA/2J mice.<sup>23,69</sup> Macular edema without fluorescein leakage is a rare complication of NA (0.67% of patients treated for hyperlipidemia) that can be easily detected and reversed by stopping NA supplementation.<sup>70</sup> It is not clear if NAM or NR ever induces this phenotype, however, and there are limited safety data for NR. Given its documented safety, tolerability and other potential advantages discussed below, we chose to work with NAM over NA or other NAD precursors.

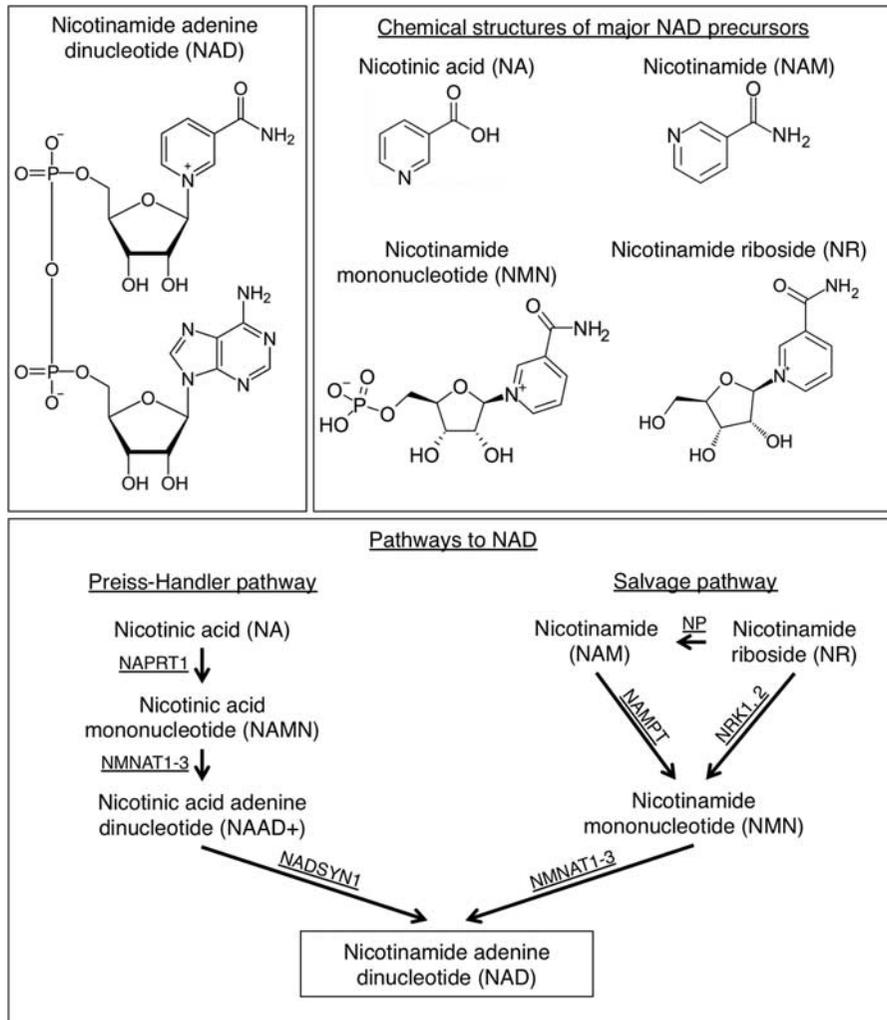
In recent years, various studies have promoted the use of NR over NAM. NR can be converted to NAD independently of NAM (through NRKs) and is claimed to be more bioavailable and more effective at increasing NAD levels for a given dose. NR is also claimed to be superior to NAM because it does not inhibit the activity of sirtuins (SIRT6).<sup>71</sup> NAM is a physiological regulator that inhibits SIRT6. SIRT6 are important NAD consuming enzymes that deacetylate lysines on proteins. They are key regulators of metabolism and mitochondrial reprogramming with aging. Their activities are known to be important for NAD-mediated protection in various settings.<sup>62,71–73</sup> Although there is merit to these arguments, we do not necessarily agree that NR is superior for treating glaucoma (discussed in more detail below) and comparative tests are required to definitively test this.

A recent mouse and human study comparing NR and NAM claimed superior bioavailability of NR.<sup>66</sup> NR was effective at raising NAD in liver, adipose tissue, and muscle shortly after administration, and NR was reported to be more bioavailable and more effective at raising NAD than NAM. From the reported data, it is clear that NR raised NAD levels more rapidly and to a greater degree than NAM in liver. However, when NAD totals are calculated as the area under the curve over a 12-14 hour period there is no clear difference (in liver<sup>66</sup>). The area under the curve for both NR and NAM are almost identical with NAM providing a more sustained NAD increase (see fig. 5b of Trammell et al<sup>66</sup>). Thus, NR may be better if frequent or continuous dosing is possible. However, because of the preference for a simpler less frequent administration protocol to enhance compliance, it could be argued that the more sustained NAD altering kinetics of NAM are preferable. Ultimately, the kinetics of NAD increase and duration in the retina and optic nerve (and possibly brain) are important for glaucoma. In mammalian cells, NRKs are necessary for the conversion of NR to NAD (and rate limiting), whereas NRKs are not required for conversion of NAM to NAD.<sup>74</sup> Initial studies suggest that NRK protein levels are low in brain,<sup>74</sup> but NR successfully increased NAD levels in whole brain by ~1.5-fold.<sup>66</sup> In our previously published and publically available RNA-seq data sets, the transcript abundance of NRK genes (*Nmrk1*, *Nmrk2*) indicates that they are only lowly expressed in RGCs.<sup>23</sup> Thus it is not clear that NR would be more effective against glaucoma than NAM, although whole retina effects and systemic effects cannot be discounted. Thus, although NR should not be discounted, NAM warrants serious consideration and may prove equally or even more effective against glaucoma. An overview of the major NAD precursors and the pathways involved are shown in Figure 3.

The current literature would suggest that NAM makes it into RGCs intact, thus there are several potential advantages of NAM treatment for glaucoma over other NAD precursors as discussed in the following paragraphs.

Firstly, NAM administration is already shown to rapidly increase retinal NAD levels (an ~3-fold increase that is sustained using our NAM<sup>L</sup> dose—550 mg/kg/d) and to robustly prevent all assessed signs of glaucoma including RGC and optic nerve degeneration.<sup>23</sup> If these changes hold true in glaucoma patients, then NAM will be an attractive treatment option.

Secondly, NAM is unique in that it is a major natural precursor for NAD in mammals *in vivo*.<sup>75,76</sup> It is also unique among NAD precursors because it is a physiological inhibitor of the major NAD catabolic enzymes: (1) CD38 is



**FIGURE 3.** NAD synthesis. The chemical structure of nicotinamide adenine dinucleotide (NAD) (top left) and 4 major NAD precursors; nicotinic acid (NA), nicotinamide (NAM), nicotinamide mononucleotide (NMN), and nicotinamide riboside (NR) (top right). NAD can be produced de novo from dietary tryptophan. Alternatively, NAD can be produced through 2 other core pathways; the Preiss-Handler pathway from NA, or through the salvage pathway from NAM (bottom panel). NAM is available in diet and readily absorbed as a major NAD precursor through the salvage pathway in vivo. Enzymes: NAPRT, nicotinamide phosphoribosyltransferase; NADSYN1, NAD synthetase 1; NAMPT, nicotinamide phosphoribosyltransferase; NMNAT1, -2, and -3 nicotinamide nucleotide adenylyltransferases 1, 2 and 3; NRK1, -2, nicotinamide riboside kinases (mouse gene names *Nmrk1*, -2); NP, purine nucleoside phosphorylase.

a major NAD consumer, and CD38 is inhibited by NAM<sup>77</sup>; (2) PARPs are upregulated in the retina during glaucoma and are major NAD consumers that deplete ATP under stress conditions, PARPs are inhibited by NAM<sup>78</sup>; (3) SIRT6 are major NAD consumers and SIRT6 are inhibited by NAM.<sup>79,80</sup> The unique inhibitory effects of NAM may have multiple complex effects that are not easily predictable and some of these may be advantageous in a glaucoma setting. For example and by inhibiting PARP, NAM may protect from further ATP depletion until metabolism is normalized. In addition, inhibition of NAD catabolism may be advantageous as it would be expected to allow more rapid or greater local increases in NAD. SIRT6 are class III histone deacetylases (HDACs) and HDAC inhibition is at least partially protective in various models of neurodegenerative disease, including RGC death and DBA/2J glaucoma.<sup>81-83</sup> In contrast, NAMs inhibitory functions have been suggested to be detrimental as SIRT6 activity is required

for NAD-mediated protection in some settings.<sup>62,71,73,84,85</sup> Further experiments are needed to determine whether or not inhibition of some HDACs or activation of specific SIRT6 is required for the protection against glaucoma. It is also possible that the inhibition of potentially protective SIRT6 by NAM is relieved at higher NAD concentrations. NAM-based inhibition of SIRT6 was initially thought to be a traditional noncompetitive base-exchange inhibition. However, more recent kinetic data demonstrate different inhibition characteristics between various SIRT6 that include apparent competition between NAM and NAD<sup>+</sup>.<sup>86</sup> Differences in inhibition kinetics can be explained by differences in NAD<sup>+</sup> binding affinity between specific enzymes.

Thirdly, NAM has documented effects on calcium channels and calcium signaling at least in part through inhibition of ADPRC and its target the ryanodine receptor.<sup>87</sup> Calcium signaling/mobilization is important in axon degeneration and may impact glaucoma.<sup>88-90</sup>

Fourthly, NAM has vasoactive and vasoprotective properties. Vasculature dysfunction is implicated in glaucoma.<sup>91,92</sup> NAM can improve endothelial function and stabilize blood flow by preventing transient flow interruptions.<sup>93</sup> Endothelins are very potent vasoconstrictors that are implicated in glaucoma in humans and animal models, with endothelin receptor blockers protecting from glaucoma.<sup>19,94–97</sup> NAM can reverse endothelin-mediated vasoconstriction though its inhibitory actions on ADPRC.<sup>98–100</sup>

Lastly, as NAMPT is the rate-limiting enzyme in the conversion of NAM to NAD,<sup>101</sup> the NAD precursor NMN, which is an intermediate in this process, should not accumulate with NAM administration, as it should be rapidly converted to NAD (Fig. 3). This is also true for NR, as NRKs are rate limiting in its conversion to NMN. Although administration of NMN protects from retinal photoreceptor degenerations induced by NAMPT deficiency or light exposure,<sup>102</sup> potential accumulation of NMN may be detrimental for glaucoma as NMN can participate in axon degeneration<sup>103</sup> (axon injury and activation of axon intrinsic degeneration pathways are central in glaucoma). Thus NAM may have better efficacy than NMN in glaucoma. On the basis of the above considerations of NAM's properties as well as existing data demonstrating a very robust protection in mice, we propose testing NAM as a neuroprotective agent to combat human glaucoma.

## CONCLUSIONS

In conclusion, nicotinamide (NAM; the amide of vitamin B<sub>3</sub>) has promise to be a safe and potent neuroprotective agent in human glaucoma. Although further animal studies and human clinical trials are needed, the growing literature implicating mitochondrial dysfunction and systemic mitochondrial susceptibility as determinants of vulnerability to glaucoma support this. Nicotinamide may offer an attractive combinational therapeutic with agents that lower IOP, and may have additional benefit in normal tension glaucoma patients or glaucoma patients refractory to IOP lowering medications. The possibility that nicotinamide can prevent glaucomatous neurodegeneration is an exciting prospect, with potentially important implications for other age-related or ophthalmic diseases.

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