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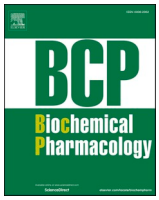

Review

A systems-approach to NAD+ restoration

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ABSTRACT

A decline in NAD+ is a feature of ageing and may play a causal role in the process. NAD+ plays a pivotal role in myriad processes important in cellular metabolism and is a cosubstrate for enzymes that play key roles in pathways that modify ageing. Thus, interventions that increase NAD+ may slow aspects of the ageing trajectory and there is great interest in pharmacological NAD+ restoration. Dietary supplementation with NAD+ precursors, particularly nicotinamide riboside, has increased NAD+ levels in several human intervention studies and arguably been the most robust approach to date. However, consistency and reliability of such approaches to increase NAD+, and also impact on markers of efficacy to slow or reverse features of ageing, has been inconsistent. We argue that a major element of this variability may arise from the use of single-target approaches that do not consider the underlying biological complexity leading to NAD+ decline. Thus, a systems approach – targeting multiple key nodes in the NAD+ interactome – is likely to be more efficacious and reliable.

1. NAD+ and ageing

Nicotinamide adenine dinucleotide (NAD+) is found in all living cells and plays a central role in many biological reactions. It was first discovered as a metabolic cofactor for critical redox reactions in pathways such as glycolysis, the tricarboxylic acid cycle and fatty acid β-oxidation. In these reactions, NAD+ functions as an oxidoreductase cofactor becoming reduced to form NADH, which subsequently acts as an electron donor in the production of ATP [1].

Aside from this key role as an intermediate in metabolism, NAD+ is also a critical regulator of a wide array of signaling pathways via its role as a co-substrate for more than three hundred enzymes [2]. Many of the enzymes that NAD+ interacts with are involved in making post-translational modifications to proteins that change their activity, generate cell-signalling molecules or modify histones to suppress or initiate transcription. This combination of metabolic and cell-signalling roles means that NAD+ acts as a metabolic messenger providing an important link between the energy status of the cell and downstream signaling for appropriate cellular adaptation to bioenergetic stress. Therefore, proper maintenance of NAD+ levels is required to maintain tissue homeostasis [3].

Despite its fundamental physiological role, an age-dependent decrease of cellular NAD+ is observed across species. In humans, age-related NAD+ decline has been observed in liver [4], skin [5], brain [6,7], plasma [8], skeletal muscle [9] and monocyte-derived macrophages [10]. NAD+ depletion is observed not only during normal ageing but also in accelerated ageing diseases including Ataxia Telangiectasia (AT), Xeroderma Pigmentosum group A (XPA), and Cockayne Syndrome (CS) [11]. A number of studies have also demonstrated a decline in NAD+ levels with aberrant nutritional status, such as obesity and metabolic disease [4,12,13]. An association between declining NAD+ availability and some neurodegenerative disorders is also evident, with NAD+ depletion reported in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and numerous accelerated ageing disorders that are associated with neurodegeneration [14,15]. Indeed, a initiating feature of many neurodegenerative diseases is axonal degeneration which is characterised by rapid NAD+ depletion [16]. The critical roles of NAD+ are known to be intimately linked with multiple key effectors of health and longevity and chronically low NAD+ has been found to correlate with multiple hallmarks of ageing and age-related disease states [17–19].

2. Preclinical benefits of NAD+ restoration

Restoration of NAD+ *in vivo* has been investigated extensively. Model organisms such as yeast, *C. elegans* and *Drosophila* all display an age-dependent reduction in NAD+ that when reversed promotes both lifespan and healthspan extension, with notable improvements to

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mitochondrial health, memory, strength and motor function [20–22].

NAD+ repletion has also been found to inhibit multiple ageing features in rodents. In mice, NAD+ levels have been found to decrease 2-fold by mid-age [4] resulting in a range of metabolic issues such as obesity, alcoholic steatohepatitis, non-alcoholic steatohepatitis (NASH), glucose intolerance and mitochondrial dysfunction, all of which were reversed upon restoration of NAD+ to levels found in younger mice [12,23–27].

NAD+ restoration in mice was also found to offer protection to multiple organs including renoprotection against acute kidney injury and protection of the liver against hepatoxicity [28–30]. An increased capacity for organ recovery after injury was also observed in mouse models of hepatoxicity and heart failure [31,32]. Cardiovascular improvements have also been observed with NAD+ restoration leading to improved endothelial function and reduced atrial stiffness [33].

Significant neurological benefits have also been demonstrated. In Alzheimer's disease models, treatments to boost NAD+ improved cognition in mice and were found to protect nerves after damage and promote regeneration [34]. Similar benefits were also observed in amyotrophic lateral sclerosis and Parkinson's Disease [35,36]. Furthermore, NAD+ restoration was found to rescue vision by reversing retinal degeneration [37] and offer protection to nerve cells in the inner ear from damage induced by loud noise [38].

Studies in rodents have also demonstrated significant improvements to sarcopenia and muscle weakness, another major feature of ageing. Boosting NAD+ levels in old mice dramatically improved muscle function and endurance [25]. At a cellular level enhanced NAD+ was found to increase mitochondrial function, ATP production [18] and to increase

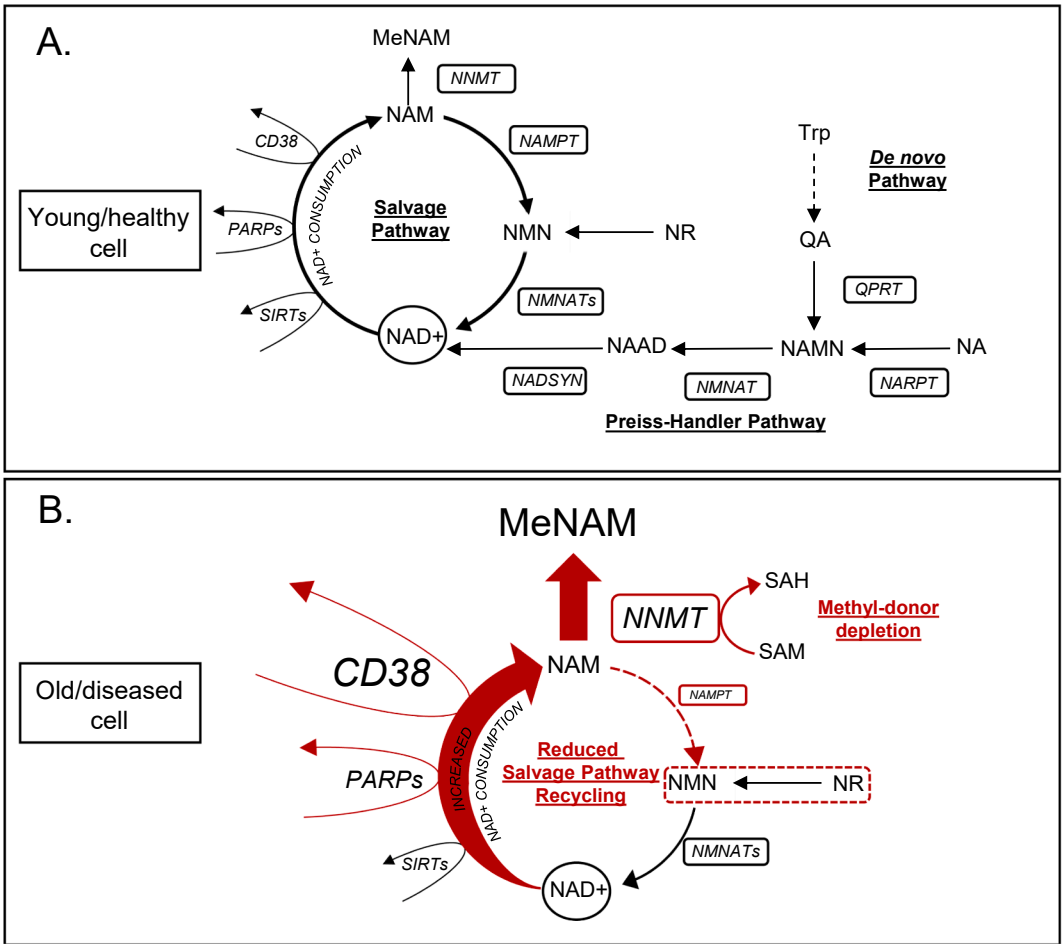

Fig. 1. A. NAD+ biosynthesis and consumption in healthy young mammalian cells. NAD+ can be produced in the body by three pathways. The de novo pathway, uses dietary tryptophan (Trp) which undergoes a five-step series of reactions to form quinolinic acid (QA). QA is then converted to nicotinic acid mononucleotide (NAMN) which enters the Preiss-Handler pathway to complete its conversion to NAD+. The Preiss-Handler pathway begins with the dietary precursor nicotinic acid (NA) which is converted to nicotinic acid mononucleotide (NAMN) by the enzyme nicotinic acid phosphoribosyltransferase (NAPRT). NAMN (and also NAMN from the de novo pathway) is then converted into nicotinic acid adenine dinucleotide (NAAD) by a family of enzymes called the NMN adenylyl transferases (NMNATs). Finally, NAAD is converted to NAD+ by NAD+ synthase (NADSYN). The Salvage pathway is a short pathway that recycles nicotinamide (NAM) to NAD+ via a two-step process. First, NAM is converted into nicotinamide mononucleotide (NMN) by the rate limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT), which is then converted directly into NAD+ via the NMNAT enzymes. Nicotinamide riboside (NR) can also use the Salvage pathway to make NAD+, but first it must be phosphorylated by NR kinase (NRK) to produce NMN which is then converted into NAD+. NAD+ is consumed within the cell as a cosubstrate by CD38, SARM1, CD157, the PARPs and the Sirtuins. During consumption NAD+ is broken down into NAM which is then recycled via the salvage pathway back into fresh NAD+. NAD+ recycling via the Salvage pathway is the primary source of NAD+ production within the cell. B. Multiple root causes of NAD+ decline in old or diseased cells. Old and diseased cells exhibit excessive NAD+ consumption due to increased expression and activity of CD38 and PARPs. Consumption of NAD+ produces NAM as a breakdown product. Reduced expression of NAMPT means the Salvage pathway is less efficient at recycling this NAM back into NAD+. As a result, NAM accumulates within the cell. To maintain cellular homeostasis cells upregulate NNMT to methylate and excrete excess NAM as MeNAM. This reaction utilises methyl groups from S-adenosylmethionine (SAM) which can ultimately result in methyl-donor depletion. Current strategies to increase NAD+ in older or diseased cells (such as supplementation with NR or NMN) do not address these underlying root causes of NAD+ decline and may serve to inadvertently increase NAD+ availability to CD38, whilst causing methyl donor depletion through forced upregulation of NAM methylation.

the number and quality of muscle stem cells [39]. Recently, studies have demonstrated that strategies to boost NAD+ levels improve oocyte quality and restore fertility in aged mice [40].

3. NAD+ biosynthesis

Given the promise of NAD+ restoration, an understanding of the cellular biosynthesis of NAD+ offers potential targets for intervention in the ageing process. Due to the critical nature of NAD+, it is perhaps unsurprising that the body has multiple pathways utilising several precursors as intermediates for NAD+ biosynthesis (Fig. 1A). Precursors include the amino acid tryptophan and the vitamin B3 derivatives nicotinic acid (NA), nicotinamide (NAM), nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN). These precursors are utilised by three NAD+ biosynthesis pathways: The Salvage pathway, de novo pathway, and Preiss-Handler pathway. During basal conditions, the Salvage pathway is thought to be the dominant source of NAD+ biosynthesis [41]. This is a short pathway that converts NAM released as a by-product of NAD+ degradation back into NAD+ via a two-step process. The first step is the conversion of NAM to NMN in a reaction catalysed by nicotinamide phosphoribosyltransferase (NAMPT), which is then converted directly into NAD+ via the nicotinamide mononucleotide adenylyl transferase (NMNAT1-3) enzymes. NAM is the primary substrate for the Salvage pathway but it can also use NR to make NAD+ if it is first phosphorylated by NR kinases 1 and 2 (NRK1 & 2) to produce NMN, which can then be converted into NAD+ [42,43]. An important distinction between these biosynthesis pathways is that the *de novo* and Preiss-Handler pathways rely on dietary derived-amino acids and vitamin B3 precursors whereas the Salvage pathway can utilise endogenous precursor from within the cell.

4. Current therapeutic approaches for NAD+ restoration

Given the clear role of NAD+ in age-related health decline there is great interest in modulating NAD+ pharmacologically to translate the benefits of NAD+ restoration into humans. The direct use of exogenous NAD+ is not practical due to its unstable nature and poor bioavailability to most cell types, so efforts have focused on supplementation with the NAD+ precursors NR and NMN, both of which have been extensively studied as NAD+ boosting therapies in preclinical testing.

To date, NR has been tested in ten clinical trials that have directly measured NAD+ levels in human blood and/or muscle samples (Table 1). Of these trials, seven demonstrated significant changes to NAD+ levels, with reported increases of between 40 and 168% when compared to baseline or placebo. Notably, many of these trials used NR at doses much higher than the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA) approved daily dose of 300 mg. Indeed, in studies using the approved dose of 300 mg NR, modest NAD+ increases of only 40–59% were observed, with larger increases in NAD+ only observed at much higher doses of up to 2000 mg NR. Additionally, three studies using 1000–2000 mg found no significant change to NAD+ levels. Compared to NR, NMN is less well studied in humans and although multiple trials are underway, there are currently no human clinical data demonstrating NAD+ enhancement.

To date, human clinical studies of precursor supplementation have primarily generated safety and tolerability data and only a small number of studies have started to look at the health benefits of elevated NAD+ to see if the beneficial results from preclinical studies translate to humans.

Some of the most notable effects of NR supplementation in preclinical studies have been improvements to mitochondrial function and muscle decline [44]. Oral NR was found to restore NAD+ in the muscle of both aged and muscular dystrophy (*mdx*) mice, resulting in positive effects on muscle function, mitochondrial function and stem cell pools [25,32,39,45]. In mice challenged with a high-fat diet (HFD), NR or NMN supplementation elevated skeletal muscle NAD+ resulting in increased sirtuin activity, restored glucose tolerance and an overall improvement of metabolic health [12,25]. Multiple clinical studies have sought to replicate these benefits in humans but, despite the promising results in rodents, augmentation of NAD+ in human skeletal muscle has proved challenging. Several clinical studies have been unable to replicate the NAD+ restoration observed in rodents in human muscle [46–49], even with long-term administration of high dose NR (1000 mg/day for 12 weeks) [49]. These studies also failed to replicate the beneficial health outcomes observed in rodents; no changes to resting metabolic function, exercise capacity or mitochondrial function were observed with NR supplementation in humans [46,48,49]. Interestingly, despite no detectable changes to NAD+ levels in these studies, it was recorded that concentrations of other NAD+ metabolites were altered, for example nicotinic acid riboside (NAR), NAM and the methylated nicotinamide breakdown products N1-methyl-2-pyridone-5-carboxamide (Me2PY) and N1-methyl-4-pyridone-3-carboxamide (Me4PY), demonstrating that NR was absorbed and metabolized in these studies [46–48]. This raises the idea that increased cellular NAD+ due to supplementation may have been rapidly metabolized by NAD+ consuming processes such that a measurable elevated steady state was not reached. However, were this the case, it would be reasonable to expect beneficial downstream effects of NAD+ or, at the very least, increased activity of downstream enzymes that consume NAD+, such as the sirtuins. However, in a study by Stocks *et al.* [47] there was no increase in SIRT activity, despite observed changes to NAD+ metabolites after supplementation with NR.

Animal studies have also repeatedly demonstrated a positive effect of NR on insulin sensitivity and hepatic steatosis in models of obesity and type 2 diabetes [12,25,27]. However, these beneficial effects also have not been replicated in human studies. There was no change in insulin sensitivity, glucose tolerance, or other metabolic parameters such as resting energy expenditure, lipolysis or oxidation of lipids in NR-supplemented obese study groups [46,50,51]. Mild changes to body composition were observed in one study of thirteen obese men and women with 1000 mg NR/day [46] but this was not observed in a similar study involving forty obese males with 2000 mg NR/day [50].

Despite the lack of robust, positive clinical outcomes with NR supplementation there were some notable observations. For example, a study by Martens *et al.* detected a trend towards an improvement in indicators of cardiovascular function including lower systolic blood pressure and aortic stiffness, which are major risk factors for negative cardiovascular events and age-related disease [52]. A promising reduction in the levels of circulating inflammatory cytokines was also observed in older males supplemented with 1000 mg NR for three weeks [48]. However, another study using the same dose of NR over six weeks in obese male and females failed to detect any change to plasma markers for inflammation [46].

Interestingly, a small study of four patients with heart failure demonstrated the most positive results, with NAD+ elevation, increased mitochondrial function and decreased pro-inflammatory factors all observed in these patients after NR supplementation [53]. As NAD+ decline has been found to be a contributing factor to myocardial failure [54] this indicates that NR may have a greater effect in conditions associated with severe NAD+ depletion.

There are a number of potential reasons why the results from these human *in vivo* studies do not fully corroborate findings obtained in preclinical studies. One is that the studies were not adequately powered to account for the inherent variability of human biology. For example, Airhart *et al.* found a wide response to supplementation with 2000 mg NR, with increases in NAD+ ranging from 35 to 168% amongst eight participants [55]. Other factors such as the age of participants, health status and NR dosage were also highly variable between studies making comparisons challenging (Table 1). Another consideration is the lack of standardisation between NAD+ measurement methodology; various techniques have been employed to measure NAD+ and its related metabolites. Commonly used approaches include enzymatic cycling assays [56], high-performance liquid chromatography with UV detection

Table 1
List of human clinical trials that have measured changes to NAD+ levels upon NR supplementation.

Intervention	Design	Participant characteristics	% NAD+ increase	Notable outcomes	Study reference
NR oral Single dose 100 mg, 300 mg, 1000 mg	Randomized, double-blind, crossover study	N = 12 Age = 33–55 BMI = 18.5–29.9 Healthy volunteers	100 mg = 48% 300 mg = 59% 1000 mg = 92% (AUC increase from baseline) Measured in PBMCs	NR safe and well tolerated	[60]
NR oral Dose-escalation 8 days 250 mg (Day 1, 2), 500 mg (Day 3, 4), 1000 mg (Day 5, 6), and 2000 mg (Day 7, 8)	Non-randomised, open-label, non-placebo controlled study	N = 8 Age = 21–50 (33 ± 8) BMI = unknown Healthy volunteers	35–168% (lowest vs. highest responder. Measurement taken on day 9 after final dose of 2000 mg NR) Measured in whole blood	NR safe and well tolerated	[55]
NR oral 6 weeks 1000 mg (2 × 500 mg/day) Crossover with placebo	Randomized, double-blind, placebo-controlled, crossover study	N = 24 Age = 65 ± 7 BMI = 24 ± 4 Healthy volunteers	60% (compared to baseline) Measured in PBMCs	Non-significant trend towards lower systolic blood pressure and reduced arterial stiffness amongst participants	[52]
NR oral + Pterostilbene (PT) 8 weeks Placebo or 250 mg NR + 50 mg PT or 500 mg NR + 100 mg PT.	Randomized, placebo-controlled, double-blinded study	N = 115 Age = 60–79 BMI = 18–35 Healthy volunteers	250 mg NR + 50 mg PT = 40% 500 mg NR + 100 mg PT = 90% (dropping to 55% at 60 days) (compared to placebo and baseline) Measured in whole blood	Total and LDL cholesterol levels were increased in NR + PT treated group	[61]
NR oral 8 weeks Placebo 100 mg or 300 mg or 1000 mg	Randomized, placebo-controlled, double-blinded study	N = 133 Age = 40–60 BMI = 28 ± 2 Overweight Healthy volunteers	100 mg = 22% 300 mg = 51% 1000 mg = 142 (compared to baseline measured after 2 weeks) Measured in whole blood	NR did not increase LDL cholesterol	[62]
NR oral 3 weeks 1000 mg (2x500mg/day) Crossover with placebo	Randomized, double-blind, placebo-controlled,crossover study	N = 12 Age = 70–80 (median 75) BMI = 21–30 (median 26.6) Healthy volunteers	Whole blood = 128% Skeletal muscle = no change detected (compared to placebo)	No change to mitochondrial bioenergetics Reduction in circulating inflammatory cytokines	[48]
NR oral 12 weeks Placebo or 2000 mg (2x1000mg/day)	Randomized, double-blind, placebo-controlled study	N = 40 Age = 60 ± 2 (placebo) 58 ± 2 (NR) BMI = 33.3 ± 0.6 (placebo) 32.4 ± 0.5 (NR) Obese and insulin resistant volunteers	Skeletal muscle = no change detected	No change to mitochondrial bioenergetics 14% decrease in NAMPT protein	[49]
NR oral 6 weeks 1000 mg/day Crossover with placebo	Randomized, double-blind, placebo-controlled, crossover study	N = 13 Age = 59 ± 5 BMI = 30.2 ± 2.6 Healthy obese volunteers	Skeletal muscle = no change detected	No improvement to insulin sensitivity Small but significant improvements to body composition, sleeping metabolic rate, skeletal muscle and acetylcarnitine concentrations	[46]
NR oral Dose-escalation 250 mg twice a day (day 1), 500 mg twice a day (day 2), 1000 mg twice a day (from day 3 for 5 to 9 days dependent on patient health)	Non-randomised, open-label, non-placebo controlled study	N = 4 Age = 51.8 ± 14.1 BMI = unknown Stage D heart failure patients	55–161% (lowest vs. highest responder, compared to baseline) Measured in whole blood	Improved PBMC respiration Reduced proinflammatory cytokine gene expression	[53]
NR oral 7 days Placebo or 1000 mg	Randomized, double-blind, placebo-controlled study	N = 8 Age = 28 ± 4 BMI = unknown Healthy volunteers	Skeletal muscle = no change detected	No change in mitochondrial adaptation to endurance exercise	[47]

(HPLC-UV–Vis) [57] and liquid chromatography coupled to mass spectrometry (LC-MS), which has been proposed recently to be the most reliable detection method [58]. Aside from determining a standardized detection method, there is also a clear need to establish the difference between transient NAD+ availability and absolute repletion when measuring NAD+ restoration. NAD+ is in a constant state of cellular flux so measurement of absolute levels may not fully reflect the cellular changes. Methods are now emerging using isotope tracer methods for NAD+ flux quantification [59]. Hence, measuring NAD+ turnover or utilisation should be considered in future studies.

Aside from study design considerations, failure to observe significant health outcomes from human *in vivo* studies, may also suggest that the underlying biological picture is more complex than first thought and simply using a precursor such as NR or NMN may be the wrong therapeutic approach to successfully address NAD+ decline. Since the first studies demonstrating the positive health benefits of NAD+ repletion, the scientific community has sought to understand the root causes of age-related cellular NAD+ decline and, despite clinical efforts focusing on the use of precursors, multiple studies now demonstrate that NAD+ decline goes far beyond a simple precursor supply issue. In fact, there is no evidence to suggest that this is a specific issue. Rather, findings suggest that NAD+ decline is much more complex with multiple root causes. So, whilst the research community remains distracted by which NAD+ precursor is best [63], it may be germane to consider in principle if supplementation with a precursor is likely to be an effective approach to NAD+ restoration at all [64].

5. The complexity of NAD+ decline

NAD+ is continually synthesized, catabolized and recycled, so it's biology is complex. Cellular NAD+ levels change during multiple physiological processes and are significantly affected by nutritional and environmental stimuli. The role of NAD+ in metabolic sensing requires a careful balance between production and utilisation. Thus, NAD+ metabolism comprises multiple precursors, production routes, recycling pathways, and a myriad of consuming enzymes, which in turn are often influenced by multiple complex factors themselves. Evidence now suggests that a major cause of NAD+ decline is a disruption of this finely-controlled network. Specifically, it has been found that the balance between catabolic and anabolic processes become dysregulated with age meaning that NAD+ consumption may start to outpace NAD+ production and salvage (Fig. 1B).

5.1. Increased NAD+ consumption

In its role as a coenzyme, NAD+ is effectively a substrate that is irreversibly degraded by three classes of NAD+ consuming enzymes: the protein deacetylase families of the sirtuins (SIRTs) and poly-ADP-ribose polymerases (PARPs) and the NAD+ glycohydrolases CD38, CD157 and SARM1. The expression and activity of some of these NAD+ consuming enzymes has been found to increase with age meaning that the demand for NAD+ also increases (Fig. 1B).

A range of factors contribute to these altered patterns of cellular NAD+ consumption. One influence is a tendency towards increased DNA damage with age, which activates NAD+ dependent repair processes [65,66]. The PARPs, a family of seventeen proteins, cleave NAD+ to produce NAM and ADP-ribose (ADPR). ADPR is then attached covalently to target proteins by a post translational modification process known as poly(ADP-ribosyl)ation (PARylation). PARPs 1–3 are known to play a key role in DNA damage repair by coordinating base excision repair of single strand breaks [67,68]. PARP1 is the best-characterized member and its activity in response to early DNA damage has been found to increase with age [21]. Upon activation, PARP1 PARylates itself along with histones and other key proteins at the point of DNA damage. These then that act as a scaffold to recruit and activate DNA repair enzymes to initiate DNA repair [69]. As each ADPR group is derived from a single NAD+ molecule, excessive PARylation represents a substantial drain on intracellular NAD+ levels and cells suffering acute DNA damage have been found to have faster PARP-mediated NAD+ consumption [59]. Although PARP1 is a critical DNA repair enzyme, its persistent activation may be harmful due to this contribution to NAD+ depletion. Indeed, persistent activation of PARP1 was found to result in a 50% decrease in cellular NAD+ in DNA repair-deficient primary rat neurons and, in response to excessive DNA damage, PARP1 can become hyperactivated, depleting the cell of mitochondrial NAD+ and inducing apoptosis [70–72]. Besides its crucial role in DNA repair, PARP1 also regulates other major cellular processes such as inflammation in an NAD+ dependent fashion. In LPS-stimulated macrophages PARP1 activity was found to be significantly upregulated and an associated increase in NAD+ consumption was observed [73]. Overactivation of PARPs with resulting severe depletion of NAD+ has also been recognised as a feature of pathogenic infection. This has been highlighted recently by severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), which is found to severely deplete the host NAD+ pool due principally to over-activation of PARPs [74]. PARP over-activation with age and in disease can, therefore, strongly compromise NAD+ availability in cells.

The NAD+ glycohydrolase CD38 also cleaves NAD+ as it acts to produce the downstream signalling factors ADPR and cADPR. CD38 is found throughout the body with high expression on the surface of immune cells [75]. Its expression is induced robustly during immune cell activation, playing an important role in multiple aspects of the inflammatory response including cell migration, activation, antigen presentation and cytokine release. It is now clear that CD38 becomes overexpressed during ageing due to chronic activation from persistent low level "inflammaging" [76,77]. Indeed, ADPR, a product of CD38 activity, was found to be significantly increased in the plasma of older people [8]. CD38 is currently recognised as the primary NAD+ consumer in mammalian tissues [78]. The profound effect of CD38 on NAD+ levels is due to CD38 being an inefficient cyclase; CD38 must degrade nearly one hundred molecules of NAD+ to generate just one molecule of cADPR [79]. The expression and activity of CD38 increases during ageing, coincident with the decrease in NAD+ levels [80–82]. Mice over-expressing CD38 had lower levels of NAD+, defective mitochondria, decreased oxygen consumption and increased lactate production [23,82]. CD38 can also cleave the NAD+ precursor nicotinamide mononucleotide (NMN) [81]. Thus, CD38 can potentially decrease NAD+ by direct consumption and by destruction of extracellular NMN that would otherwise serve as a precursor.

CD157 is a highly conserved homolog of CD38 that exhibits lower NAD^+-consuming activity [83]. Although less well studied than CD38, its expression is found to increase in M1 macrophages, which correlates with enhanced NAD+ degradation [84].

SARM1 has also emerged as an important NAD+ consumer found to be highly expressed in neuronal tissues. Its activation rapidly depletes NAD+ and it was shown to be an essential mediator of axonal degeneration after injury by initiating rapid breakdown of local NAD+ [85]. As a result, it is being investigated as a therapeutic target for neurodegeneration, but its role in age related NAD+ decline is yet to be investigated [86].

Given the above evidence, it is now well established that multiple NAD+ consuming pathways become overexpressed with age and subsequently can limit utilization of NAD+ by other NAD+ dependent enzymes, such as the sirtuins. Indeed, both PARP1 and CD38 have been demonstrated to exert dominance over SIRT1 in NAD+ consumption. In NAD+ depleted HeLa cells, PARP1 inhibitors were able to partially restore NAD+ while SIRT inhibitors could not [87]. Congruently, the endogenous activity of SIRT1 was found to be several times higher in CD38 knockout mice compared to aged wild-type [80].

5.2. Reduced NAD+ recycling

Despite their varying contributions to NAD+ decline, all of the abovementioned enzymes and processes that consume NAD+ generate NAM as a breakdown product. NAM can be recycled rapidly via the Salvage pathway to NAD+ . Thus, the Salvage pathway plays a major role in restoring NAD+ that has been degraded by NAD+ consuming enzymes.

Given the importance of NAD+, having a biosynthesis pathway that does not rely on NAD+ generation *de novo* from exogenous substrate but instead uses an endogenous precursor that becomes abundantly available when demand for NAD+ is high seems a good metabolic strategy. Theoretically, this means NAD+ levels within the cell should be self-sustaining regardless of demand without the need for additional exogenous precursor if proper recycling is maintained [42]. This recycling capability is likely to have evolved as a failsafe mechanism to protect such a vital cellular substrate. In addition, it has been found that plasma levels of most NAD+ precursors are probably insufficient to maintain high NAD + production rates, meaning mammals largely rely on NAD+ salvage from intracellular NAM [41], supporting the view that the Salvage pathway is the primary source of cellular NAD+ production [88]. Accordingly, the rate of NAD+ synthesis in mammals is largely determined by the first step in the Salvage pathway that converts NAM to NMN. In mammals, this is carried out by the rate limiting enzyme NAMPT [89]. Levels of NAMPT are highly dynamic, responding to changing cellular demands for NAD+ and cell stresses such as DNA damage and starvation [89]. Mice lacking NAMPT are not viable [90] and mutations on the NAMPT gene are correlated with diseases associated with low NAD+ [91]. It is now known that NAMPT levels decline with age in parallel with the decline in NAD+ as reported in aged tissues of rats [92,93], mice [4,94–97] and humans [4,98]. NAMPT has been found to be ~ 60 % lower in stem cells from aged compared to young rats with an associated significant decline in NAD+ (Ma et al. 2017). In a mouse model of Alzheimer's disease reduced NAD+ levels in the brain cortex were associated with decreased NAMPT levels [96], whilst an age-dependent decline in NAMPT was associated with a 50% decrease in NAD+ in retinal pigment epithelium in mice [95]. In human aortic endothelial cells, replicative aging was also associated with significant declines in NAMPT protein (53%) and activity (39%) [99]. In human hepatic tissue, NAD+ levels in older subjects (>60 years) were found to be only ~ 70% of that in middle-aged subjects (<45 years) and this was associated with a 50% reduction in NAMPT expression in samples from the older cohort [4].

This reduction in NAD+ biosynthesis via the Salvage pathway is a significant factor in older cells because as NAD+ consumption increases concurrently with age and demands for NAD+ replenishment and recycling increase, the resulting NAM is no longer efficiently recycled, exacerbating a situation of declining NAD+ levels (Fig. 1B) [4].

Obesity and high calorie diets, both associated with low NAD+ levels, have also been found to reduce NAMPT [100] and NAD+ levels in various tissues including liver, white adipose [12], muscle and brown adipose [25]. Disease conditions such as acute lung injury, atherosclerosis, cancer, diabetes, rheumatoid arthritis and sepsis have also been found to exacerbate NAMPT decline [91]. NAMPT is expressed ubiquitously in the body but there are large differences in the levels of expression between tissues [90] meaning some tissues, such as the brain and heart where the NAMPT-dependent pathway is known to be the preferred route of NAD+ synthesis [101,102], could be more severely affected by Salvage pathway decline than others.

The combined effect of increased NAD+ consumption and decreased NAM recycling has the potential to cause an accumulation of intracellular NAM in older cells. *In vitro* experiments have demonstrated that high concentrations of NAM can inhibit sirtuins [102,103]. Cells avoid this by further adjusting the NAD+ network by upregulating pathways that remove excess NAM from the cell, specifically nicotinamide-N-methyltransferase (NNMT) (Fig. 1B). NNMT is an enzyme that catalyses the methylation of NAM using S-adenosylmethionine (SAM) as a methyl donor to form methyl-NAM (MeNAM), which allows the urinary excretion of NAM. Indeed, older cells demonstrate increased expression of NNMT [84,104]. This further limits the cell's capacity to produce NAD+ by removing the critical endogenous supply of NAM by favoring its excretion from the cell rather than NAD+ regeneration via the Salvage pathway. Indeed, NNMT overactivation in mice was found to decrease the NAD+ content in the liver [105].

6. Limitations of precursor supplementation

As we begin to understand the complexity of NAD+ biology, it is becoming clear that supplementation with precursors alone does not address the underlying root causes of NAD+ decline. Whilst precursors may serve to temporarily increase cellular NAD+ at low levels, evidence now suggests that once this NAD+ has been broken down by hyperactivated NAD+ consumers, there is a high chance it will be effectively wasted due to the inefficiency of NAD+ salvage in older cells. This means that NAD+ generated by precursors may effectively get a "first pass" before it is irreversibly degraded and rendered useless, suggesting it is an unsustainable approach to NAD+ restoration (Fig. 1B).

In support of this, upon supplementation with NR, levels of the methylated excretory biproducts MeNAM and Me2YP are found to significantly increase in the plasma and urine of human subjects [48,60,62]. This suggests that NAD+ produced as a result of NR supplementation is metabolized to NAM by NAD+ consuming enzymes and then methylated and excreted via NNMT rather than being recycled back into NAD+ by the Salvage pathway. This supports an argument that precursor supplementation further dysregulates the NAD+ metabolome in older cells by NNMT upregulation to avoid NAM accumulation in cells with inefficient Salvage pathway recycling. A further consequence with likely negative impact is that chronic administration of precursor supplements at high doses depletes methyl groups via upregulation of NNMT (Fig. 1B), which may result in damaging secondary effects by reducing the cellular availability of methyl groups that are required elsewhere to maintain cellular homeostasis [105].

7. Alternative strategies to boost NAD+

Given these drawbacks of simple precursor supplementation, strategies that address the root causes of NAD+ decline by considering multiple key nodes of the NAD+ interactome seem more appealing. Furthermore, there is already a wealth of evidence to support these targets as interventions.

7.1. Targeting hyperactivated NAD+ consumption

7.1.1. CD38 inhibition

CD38 inhibition has emerged as a promising strategy to increase cellular NAD+ . Given the inefficient use of NAD+ by CD38, strategies to inhibit CD38 even at a low level may lead to substantial increases in cellular NAD+ levels. In support of this, CD38 inhibition by the flavonoid apigenin resulted in a 50% increase in NAD+ [106,107], which is comparable with reported NAD+ increases using 300 mg NR (Table 1). This intervention was also found to decrease global proteome acetylation and improve glucose and lipid homeostasis in obese mice by increasing the activity of downstream SIRT1 and SIRT3 [81,106]. The CD38 inhibitor 78c has also been found to reverse age-related NAD+ decline and improve several physiological and metabolic parameters of ageing, including glucose tolerance, muscle function, exercise capacity and cardiac function in mouse models of natural and accelerated ageing [82]. In addition, aged wild-type mice were shown to have around half the NAD+ levels of young mice, whilst CD38 knockout mice maintained their NAD+ levels and were resistant to the negative effects of a high fat diet, including liver steatosis and glucose intolerance, adding further support to CD38 inhibition being an attractive approach [23,82,106].

7.1.2. PARP inhibition

The detrimental action of PARP activity on NAD+ pools is evident in experiments where cells are treated with genotoxic agents to promote DNA damage. These agents lead to a sustained activation of PARP activity and a concomitant decrease in NAD+ levels to only 10–20% of their normal levels within 5–15 min [108,109]. Studies in DNA repair-deficient human neuroblastoma cells have shown that treatment with PARP inhibitors can reduce DNA-damage associated NAD+ loss [71]. In mouse models of early alcoholic steatohepatitis, a condition associated with NAD+ decline, pharmacological inhibition of PARP with Olaparib restored the hepatic NAD+ content and beneficially affected metabolic, inflammatory and oxidative stress parameters via increased SIRT1 activation [27,110]. Congruently, in PARP1 KO mice NAD+ levels were robustly increased in brown adipose tissue (BAT) and skeletal muscle by approximately 100% and 50% respectively, improving mitochondrial function [24,111]. PARP inhibitors have also proved promising in models of Cockayne Syndrome (CS) and Xeroderma Pigmentosum (XDP), accelerated ageing disorders characterised by persistent PARP activation and NAD+ decline. Notably, treatment of CS mice with PARP1 inhibitors promoted lifespan extension and ameliorated the severe phenotypes caused by PARP1 hyperactivation [11,70].

7.2. Targeting NAD+ salvage and recycling

7.2.1. NAMPT activation

Studies to reverse the age-related decline in the Salvage pathway by activating its rate-limiting enzyme NAMPT have also yielded promising results with regard to increasing NAD+ levels. Overexpression of NAMPT in mice was found to increase intracellular NAD+ levels in skeletal muscle by ~50%, an increase comparable with the effects of dietary NAD+ precursors (Table 1) [112]. The potential of NAMPT to restore NAD+ has also led to the development of small molecule activators. The aminopropyl carbazole P7C3 was able to rescue human cells *in vitro* from doxorubicin-induced NAD+ depletion [113], whilst *in vivo* P7C3 administration to mice increased brain NAD+ levels and offered protection from ischemic stroke [113,114]. SBI-797812, another small molecule activator of NAMPT, increased NAMPT activity and NAD+ levels in a dose dependent manner elevating NAD+ by ~40% in mouse liver [115]. The tripeptide Ile-Arg-Trp (IRW) has also recently been found to increase both NAMPT and NAD+ levels in muscle cells of obese mice [116]. Aside from the influence of pharmacological interventions, NAMPT levels fluctuate to match cellular NAD+ demand and as a result are influenced by activities that effect cellular energy stress such as fasting, calorie restriction and exercise [117–119]. Accordingly, in an exercise intervention study NAMPT protein increased by 127% in sedentary nonobese subjects after only three weeks of exercise training [119]. Another study demonstrated ten weeks of resistance training increased NAMPT levels in the muscle of middle-aged men by 15%, which was associated with a 127% increase in NAD+ levels [9]. This demonstrates that lifestyle interventions may also be a powerful strategy for NAD+ enhancement where clinically appropriate.

7.2.2. NNMT inhibition

NNMT was found to be overexpressed in the white adipose tissue (WAT) and liver of obese and diabetic mice, both of which are associated with decreased NAD+ [26,104]. NNMT knockdown was found to restore NAD+ and SAM indicating that NNMT inhibition promotes recycling of NAM to NAD+ via the Salvage pathway rather than methylation and excretion [26].

Treatment of mouse adipocytes with the NNMT inhibitor 5-amino-1MQ resulted in a concentration-dependent increase in NAD+ levels by ~1.2–1.6-fold relative to control adipocytes. Elevated SAM was also observed improving the availability of methyl groups to the cell [120]. Furthermore, treating mice with diet-induced obesity with a NNMT inhibitor caused significant loss of body weight and white adipose tissue mass, reduction in adipocyte size and decreased circulating cholesterol levels [120].

8. Multitarget strategies for NAD+ Restoration

Despite the focus on precursors to date, evidence now demonstrates that approaches to increase NAD+ are not limited to increasing precursor supply but can be pharmacological or lifestyle interventions that target pathways that synthesize, degrade or excrete NAD+ and/or its precursors. In addition, it is also evident that there is no single cause of NAD+ decline, but rather multiple points of dysregulation in the NAD+ interactome. Together, this highlights that a multitarget strategy for NAD+ restoration such as the use of a precursor in combination with inhibitors and/or activators acting at other key points in the NAD+ network may be the optimum approach to NAD+ restoration. The use of such multitarget interventions in areas of complex biology avoids the common pitfalls of molecular reductionism associated with focusing on single points of biology. This is because individual components of biological systems such as molecular pathways never work alone - they operate in highly structured and integrated biological networks, as exemplified by the NAD+ network. Reductionism, by its nature, cannot effectively address and intervene in the complexity of biological systems, the properties of which cannot be explained or predicted by studying their individual components [121]. Viewed from this perspective, NAD+ precursor supplementation provides a good example of molecular reductionism because the approach does not target the complexity of the underlying biology underpinning NAD+ decline. The use of NAD+ precursors in combination with CD38 inhibitors or NAMPT activators, for example, may provide synergistic increases in NAD+ levels by addressing the multiple root causes of NAD+ decline.

To add to this complex scenario, recent evidence has also emerged indicating that the local causes of cellular NAD+ decline, such as increased cellular consumption and decreased recycling, may be rooted within deeper, more systemic causes of ageing, leading us to question whether interventions acting on local NAD+ networks (even at multiple points) are also too simplistic. For example, it is now known that PARP1 and CD38 are major factors driving NAD+ decline but these are each in turn driven by the much greater influences of DNA damage and inflammation. Indeed, many scientists now agree that compromised NAD+ status is intricately linked to all nine hallmarks of ageing [84], but it remains to be determined whether NAD+ decline is a cause of the hallmark, a result of the hallmark or combination of both. This has been emphasised recently by the identification of a link between NAD+ decline and cellular senescence, another major manifestation of ageing that has often been studied separately from NAD+ decline [122].

Cellular senescence is a tumour suppressive response evolved to prevent the unrestricted division of damaged and potentially cancerous cells by inducing a state of growth arrest. Senescent cells have been found to contribute to numerous age-associated pathologies and their presence in tissues is accompanied by the chronic secretion of pro-inflammatory factors termed the ‘Senescence Associated Secretory Phenotype’ (SASP) [123]. Both restoration of NAD+ levels and the selective elimination of senescent cells independently promote healthspan in old or diseased animals [39,124] and strategies to develop therapies to mitigate each of these hallmarks of ageing have so far been approached individually. Recent studies, however, have demonstrated that falling NAD+ levels and senescent cell accumulation are linked. Furthermore, this link appears to be mediated via another key hallmark of ageing – chronic inflammation [122].

Multiple groups have now demonstrated that the accumulation of senescent cells with age leads to increased systemic secretion of the pro-inflammatory SASP, which in turn leads to spiralling levels of localised cellular CD38 expression and a concurrent decrease in NAD+ levels [84,122]. This highlights senescent cells as an important factor in NAD+ decline and raises the question as to whether localised strategies to restore NAD+ will ever be successful in the situation where the body carries a chronic burden of senescent cells. Interestingly, one study

demonstrated that high NAD+ in cultured senescent cells increased their secretion of pro-inflammatory SASP cytokines [125]. In this case, NAD+ itself would appear to be driving its own decline.

It is evident, therefore, that the relationship between NAD+ and other hallmarks of ageing is complex. On one hand, NAD+ may contribute to the pro-inflammatory phenotype of senescent cells, whilst on the other hand the pro-inflammatory SASP can contribute to a decline in NAD+ . The relationship between the two appears to be intertwined via chronic inflammation and CD38. These are all complex interactions that supplementation with an NAD+ precursor does not address.

Based on the above, circumstances in which higher NAD+ contributes to senescence-induced inflammation can be conceived. However, the balance of risk is more likely to favour restoring NAD+ levels as being protective against senescence and inflammation; if high NAD+ caused senescence-induced inflammation, then inflammation would reduce rather than increase with age, in contrast to the relationship actually observed [126], which in turn is congruent with the fact that NAD+ levels and senescence show a strong negative correlation through life [95,99,127]. Other observations also support the view that NAD+ protects against senescence. For example, in rat stem cells restoration of NAD+ by overexpression of NAMPT was found to attenuate senescence [93].

Nonetheless, the link between NAD+, senescence and inflammation merits further consideration. It throws light on the complexity of intervening to restore NAD+ as it declines with ageing and highlights the inappropriately simplistic, reductionist approach of current strategies. This point is exemplified by considering likely differential effects of simple precursor supplementation that depend on cell age and make older cells potentially less malleable in terms of achieving an increase in NAD+ using a single-agent approach. We now know that in older cells the enzymes in the metabolic networks regulating NAD+ have a reduced capacity to synthesise and recycle NAD+ via the Salvage pathway and have an increased capacity both to degrade NAD+ via CD38 and to methylate and excrete NAD+ breakdown products via NNMT. Thus, increasing NAD+ using a precursor without first inhibiting CD38 may 'fuel' CD38-mediated inflammation. Indeed, CD38 has a lower K_m for NAD+ than other (beneficial) enzymes with which it will compete for NAD+ as a co-substrate, notably the PARPS and sirtuins [41]. Evidence that supports this being a likely scenario includes an observation that levels of ADPR, a direct product of NAD+-consuming CD38 activity, increased significantly in humans after NR supplementation [60]. In further support, one of the most prominent phenotypes of CD38 knockout tissues is enhanced basal PARylation, which is consistent with the idea that CD38 out-competes PARPs for NAD+ [84]. Similarly, increased SIRT3 activity was reported in CD38 knockout mice [81]. Such unintended self-limiting interactions could explain why the downstream health benefits of NAD+ restoration in isolated cells or genetically modified organisms are not translating to human studies. It also presents an argument to support a multi-agent approach to counteracting age-related NAD+ decline. These could also include strategies targeting the emerging systemic causes of NAD+ decline such as senescence and inflammation.

Caloric Restriction (CR) is a complex intervention that speaks to an important interaction between NAD+, senescence and inflammation, and also the wider NAD+ interactome, in the context of ageing. It decreases DNA damage [128,129] and inflammation [130] with a consequent decrease in local NAD+ consumption via the PARPs and CD38. CR therefore presents as an existing strategy that appears to target multiple causes of NAD+ decline from a systems level. In support of this, CR has been found to increase NAD+ levels in multiple tissues of rodents [118,131], is correlated with upregulation of NAMPT [118], increases in Sirtuin activity [132–134] and has been found to be the most robust and reproducible intervention known to increase lifespan and healthspan across species [135,136]. Whilst CR may be an impractical solution for most, these observations hint that a pharmacological multitarget strategy of combined administration of an NAD+ precursor, a CD38 inhibitor, a NAMPT activator and a NNMT inhibitor, to simultaneously address multiple root causes of NAD+ decline, may hold potential for NAD+ restoration with greater measurable health benefits and reduce potential adverse effects associated with chronic high-dose administration of NAD+ precursors.

9. Conclusion and future considerations

As life expectancy rises globally and the proportion of older people in society continues to rise, age-related decline is predicted to bring major societal and economic problems. A wealth of evidence now demonstrates that NAD+ restoration has the potential to be effective in extending healthspan and preclinical studies suggest strong translational potential for future therapeutic targeting of NAD+ decline. However, it is clear that we need to rethink the therapeutic approach and the field must center its future efforts around strategies that address the root underlying causes of cellular NAD+ decline. Traditionally, science has taken a reductionist approach to understand biology and disease, dissecting biological systems into their constituent parts and studying them in isolation. Although scientists have made great progress using this method, it is now widely accepted that the reductionist approach vastly underestimates the complexity of biology and, as a result, efforts to treat many complex diseases have faced limited success. With this is mind, we now have a greater understanding of the mechanisms that lead to age-related NAD+ decline and the emerging roles of not only multiple local cellular alterations such as increased NAD+ consumption and reduced recycling, but also more global deep-rooted causes such as senescence and chronic inflammation. This bigger picture reveals that current strategies to enhance NAD+ using NAD+ precursors alone do not address the complexity of the underlying biology that we now know leads to age-related NAD+ decline. As a result, the use of multi-target strategies addressing several root causes of NAD+ decline in concert are emerging as a much more attractive strategy for NAD+ restoration and should be considered going forward to effectively develop NAD+ restoration therapies that may be used to target multiple age-related conditions.

CRediT authorship contribution statement

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