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Review article

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Regulation of and challenges in targeting NAD$^+$ metabolism

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Abstract

Nicotinamide adenine dinucleotide, in its oxidized (NAD$^+$) and reduced (NADH) forms, is a reduction–oxidation (redox) co-factor and substrate for signalling enzymes that have essential roles in metabolism. The recognition that NAD$^+$ levels fall in response to stress and can be readily replenished through supplementation has fostered great interest in the potential benefits of increasing or restoring NAD$^+$ levels in humans to prevent or delay diseases and degenerative processes. However, much about the biology of NAD$^+$ and related molecules remains poorly understood. In this Review, we discuss the current knowledge of NAD$^+$ metabolism, including limitations of, assumptions about and unappreciated factors that might influence the success or contribute to risks of NAD$^+$ supplementation. We highlight several ongoing controversies in the field, and discuss the role of the microbiome in modulating the availability of NAD$^+$ precursors such as nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN), the presence of multiple cellular compartments that have distinct pools of NAD$^+$ and NADH, and non-canonical NAD$^+$ and NADH degradation pathways. We conclude that a substantial investment in understanding the fundamental biology of NAD$^+$, its detection and its metabolites in specific cells and cellular compartments is needed to support current translational efforts to safely boost NAD$^+$ levels in humans.

Sections

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Introduction

Nicotinamide adenine dinucleotide has a fundamental role in the biochemistry of all life. In oxidizing reactions, it serves as a hydride (H^-) acceptor through conversion from its oxidized form (NAD^+) to its reduced form (NADH) (Fig. 1). NADH, in turn, can provide a hydride to reduce other substrates, or, notably, to fuel the mitochondrial electron transport chain and thereby generate ATP. Glycolysis, β-oxidation and the tricarboxylic acid (TCA) cycle all require NAD^+. In fact, there is no sustainable path to produce ATP that does not require interconversion of NAD^+ and NADH, and the NAD^+/NADH ratio is a common point of control that links hundreds of reactions throughout the cell[1]. The phosphorylated form of NAD^+, $NADP^+$, has a similar role but works with a discrete set of enzymes, allowing the $NADP^+$/NAPDH ratio to be set independently from the NAD^+/NADH ratio (Box 1).

In addition to its roles in reduction–oxidation (redox) reactions, NAD^+ serves as a substrate for several classes of non-redox enzymes; these include mono-ADP and poly(ADP-ribosyl)transferases[2–4], sirtuins (the NAD^+-dependent deacylases sirtuin 1 (SIRT1)–SIRT7)[5], CD38 (also known as ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1) and its paralog CD157 (also known as BST1 or ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2)[6,7], and NAD^+ hydrolase SARM1 (ref. 8), all of which have important roles in cell signalling and fate decisions. The nicotinamide moiety of NAD^+ can be removed in enzymatic reactions and released as free nicotinamide (Nam). The remaining electrophilic ADP-ribosyl moiety may be covalently attached to nucleophilic side chains (predominantly of proteins in reactions catalysed by ADP-ribosyltransferases) or serve as an acceptor for acyl groups in reactions catalysed by sirtuins. Moreover, it can react with water or with itself to yield ADP-ribose or cyclic ADP-ribose (cADPR), respectively, catalysed by CD38, CD157 or SARM1. Finally, NAD^+ is used in RNA-capping events across all kingdoms. In eukaryotes, NAD^+ capping increases degradation of RNA, whereas in prokaryotes it generally stabilizes RNA[9]. Although $NADP^+$ has been considered mainly a redox co-factor, it can also serve as a substrate in a pyridinium exchange reaction catalysed by CD38 and SARM1, which replaces the nicotinamide moiety with a nicotinic acid moiety to generate the second messenger nicotinic acid adenine dinucleotide phosphate (NAADP)[10,11], which, similar to cADPR, is a potent calcium mobilizing agent[12,13]. These $NAD(P)^+$-consuming reactions, along with processes such as tissue growth or side reactions that chemically damage the structure of NAD^+, necessitate ongoing NAD^+ synthesis to maintain pools of the dinucleotides to serve as substrates and co-factors in redox reactions.

Although many of the key reactions involved in the synthesis and elimination of NAD^+ were described more than half a century ago, we are only just beginning to understand the physiological relevance of changes in NAD^+ and its metabolites in human tissues[14]. The observation that NAD^+ availability often decreases in aged tissues or disease states[15–21] has led to intense interest in the potential therapeutic value of supplemental NAD^+ precursors[22]. At the same time, evidence is mounting that our understanding of how to assess NAD^+ metabolism or the success of supplementation is woefully incomplete. Subcellular compartmentalization of discrete NAD^+ pools (spatially or functionally separated reservoirs; for example, in subcellular compartments such as mitochondria, cytosol and nucleus), along with the transport systems that establish and maintain them remain, poorly characterized[23]. Discrepancies between free and total NAD^+ concentrations within each pool are thought to reflect protein binding, but the identities of such proteins and the factors controlling binding levels are unknown[24]. Differences in NAD^+ metabolism and sharing of metabolites between cell types, tissues and species remain poorly understood in most cases. Although short-term studies have consistently supported the safety of boosting NAD^+ levels through supplementation, some potential risks remain to be fully addressed with direct measurements and longer-term studies.

In this Review, we discuss the complexities of NAD^+ synthesis and catabolism, focusing on how some of the less-appreciated issues, such as compartmentalization and non-canonical metabolites, may affect the success of NAD^+ boosting strategies. We do not comprehensively discuss the preclinical or clinical outcomes of NAD^+ boosting that have been obtained to date, as these are exhaustively reviewed elsewhere[22,25,26]. We selectively discuss NAD^+-dependent enzymes and their downstream products only when relevant to the biochemistry of the NAD^+ metabolome. We begin with a discussion of the key mechanisms of production and degradation of NAD^+, followed by its subcellular compartmentalization, transport and chemistry. We then discuss the benefits, risks and unknowns of strategies to increase NAD^+ levels.

Canonical pathways of NAD^+ synthesis and degradation

The discovery of NAD^+ synthesis pathways and identification of NAD^+ itself are inextricably linked with efforts to cure pellagra, which is a chronic and often fatal disorder that we now know is the result of severe NAD^+ deficiency[27]. The demonstration that Nam or nicotinic acid (NA) are NAD^+ precursors that could restore its level led to collective designation of the two precursors as vitamin B_3 and effectively eradicated pellagra in developed countries, although it remains a challenge in some developing regions. In subsequent studies it was shown that Nam and NA are converted to mononucleotides by distinct phosphoribosyltransferases, then to dinucleotides by nicotinamide mononucleotide adenylyltransferases (NMNATs) (Fig. 2). Nicotinic acid adenine dinucleotide (NAAD) then requires the action of NAD^+ synthase 1 (NADSYN1) to convert the nicotinic acid moiety to an amide[28]. Synthesis of NAD^+ from NA, known as the Preiss–Handler pathway, was first discovered in microorganisms but is also present in mammals, including in humans. However, in mammals, the largest portion of NAD^+ synthesis occurs through the Nam salvage pathway, which recycles Nam arising from NAD^+-dependent signalling reactions (Fig. 2). A third pathway, which begins from the amino acid tryptophan and is most active in the liver, has also been recognized and designated the de novo (or 'kynurenine') pathway[28]. With sufficient tryptophan availability, flux through this pathway can generate enough NAD^+ to support whole-body needs (through its conversion to circulating Nam) even in the absence of dietary vitamin B_3 in rodents and, at least for short periods, in humans[29,30]. Conversely, biallelic mutations in *NADSYN1* (which is required for both the Preiss–Handler and de novo pathways) lead to a severe congenital disease that may be improved by supplementation with amidated NAD^+ precursors[31].

The ribosylated forms of Nam and NA – nicotinamide riboside (NR; a niacin equivalent with a complete nicotinamide moiety) and nicotinic acid riboside (NAR), respectively – can enter synthesis pathways through direct conversion to the mononucleotides through nicotinamide riboside kinases (NRKs)[32] (Fig. 2). More recently, the reduced form of NR, NRH, was shown to form NADH independently of NRKs through adenosine kinase (AdK) and NMNATs[33–35], although the quantitative importance of this pathway in normal metabolism remains to be established. Unlike mammals, which directly salvage Nam, some evolutionary lineages, including yeast, worms, flies and most microbes, employ a nicotinamidase to deamidate Nam into NA for assimilation through the Preiss–Handler pathway[36] (Fig. 2). Many microbes also possess an alternative de novo pathway for NAD+ synthesis from aspartate[37].

The best studied and quantitatively most important pathway for elimination of NAD+ equivalents is through methylation of Nam by nicotinamide *N*-methyltransferase (NNMT)[38] (Fig. 2). Some methylated nicotinamide (me-Nam) is eliminated directly in the urine, but most undergoes further oxidation to *N*-methyl-6-pyridone 3-carboxamide (me-6PY; often referred to as *N*-methyl-2-pyridone 5-carboxamide and abbreviated as 2PY in the literature) or *N*-methyl-4-pyridone 3-carboxamide (me-4PY; referred to as 4PY in the literature)[39,40] (Fig. 2). In rodents, including mice, this reaction is catalysed by the action of multiple aldehyde oxidases (AOXs)[41]. However, other mechanisms are predicted for the oxidation of me-Nam in humans, as rodent AOX1 does not recognize me-Nam as a substrate and human AOX1 is the only isoform present in humans[42]. The methylated pyridones along with a small amount of unmethylated pyridones, nicotinamide *N*-oxide and nicotinuric acid (NUA) derived from excess NA are also cleared in the urine[43].

Subcellular NAD+ pools, their functions and maintenance

Many cellular processes, metabolites and pathways are compartmentalized to optimize the efficiency of biochemical activities. As NAD+ participates in as many as ~25% of all biochemical reactions[44],

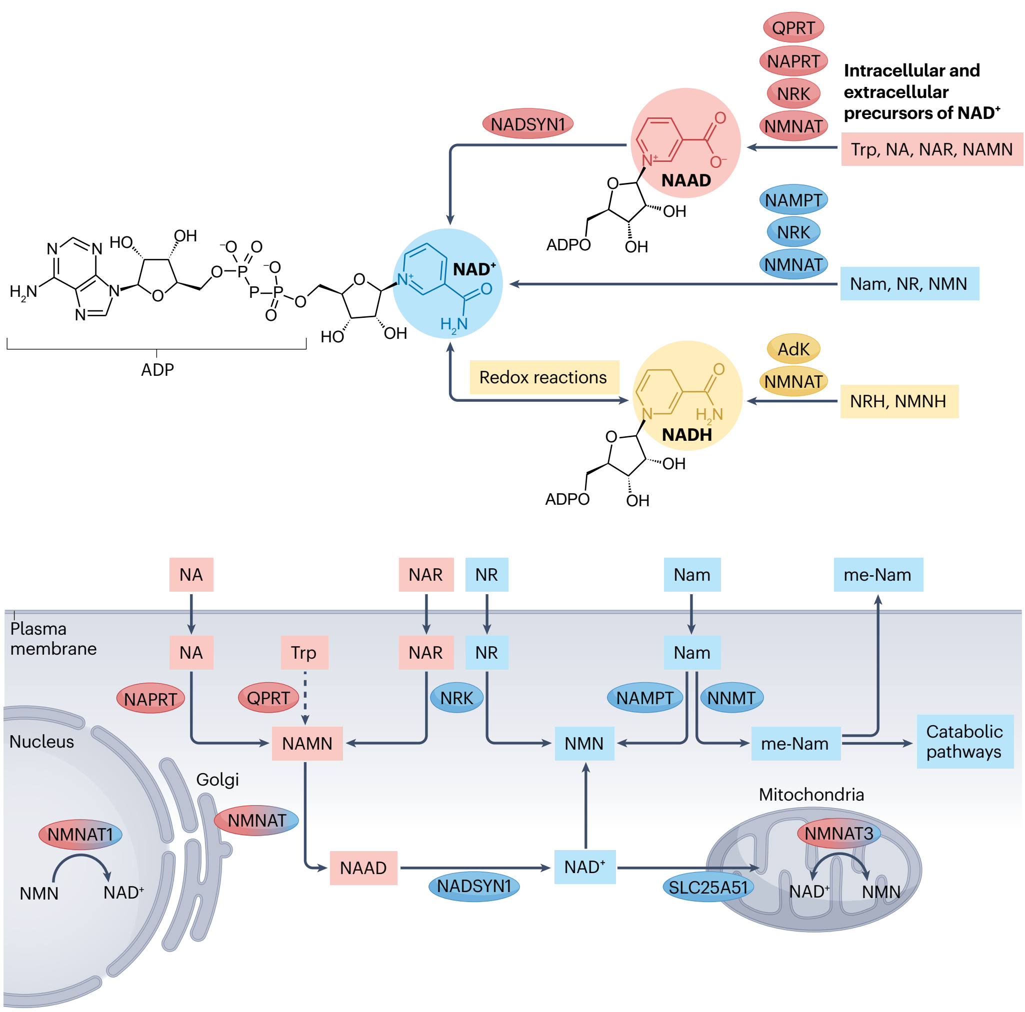

Fig. 1 | Structures and compartmentalization of pyridine adenine dinucleotides. The oxidized form of nicotinamide adenine dinucleotide (NAD+), the reduced form of nicotinamide adenine dinucleotide (NADH) and the acidic precursor nicotinic acid adenine dinucleotide (NAAD) are shown with their pyridine rings highlighted (top). NAD+ and NADH are reversibly interconverted in a multitude of redox reactions. Colour coding of the different pyridine moieties is maintained in subsequent figures. Key enzymes involved in the generation and maintenance of NAD+ pools are shown within their respective cellular compartments (bottom). The dashed downward arrow indicates intermediate steps not shown. AdK, adenosine kinase; me-Nam, methylated nicotinamide; NA, nicotinic acid; NADSYN1, NAD+ synthase 1; Nam, nicotinamide; NAMN, nicotinic acid mononucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NAPRT, nicotinic acid phosphoribosyltransferase; NAR, nicotinic acid riboside; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; NMNH, reduced form of nicotinamide mononucleotide; NNMT, nicotinamide *N*-methyltransferase; NR, nicotinamide riboside; NRH, reduced form of nicotinamide riboside; NRK, nicotinamide riboside kinase; QPRT, quinolinic acid phosphoribosyltransferase; SLC25A51, solute carrier family 25 member 51.

Box 1 | NADP(H) and NAD$^+$ kinase

Even though the redox properties of the oxidized form of nicotinamide adenine dinucleotide (NAD$^+$) and its phosphorylated form (NADP$^+$) are nearly identical, all organisms depend on the presence of both pyridine nucleotides. In contrast to NAD(H), the ratio of the phosphorylated adenine dinucleotides is maintained in favour of the reduced form, NADPH. Thus, a reservoir of reducing equivalents is maintained for key cellular functions, including detoxification, oxidative defence, reductive anabolic pathways and the generation of reactive oxygen species (ROS) by NADPH oxidase.

The only known reaction to generate NADP is the phosphorylation of NAD$^+$ to NADP$^+$, which is catalysed by NAD$^+$ kinase (NADK). Therefore, NADK is essential and ubiquitously present in all organisms. In humans, by far the predominant form of the enzyme is a cytosolic protein[252,253]. A mitochondrial counterpart (MNADK; encoded by *Nadk2*) has been reported[254,255], but its physiological function still needs to be established as its catalytic activity is two or three orders of magnitude slower compared with cytosolic NADK[254,255]. Additionally, *Nadk2*-null mice are viable, and their mitochondria still contain substantial amounts of NADP(H)[256]. Genetic modulation of *Nadk*, which encodes the cytosolic enzyme, by overexpression or depletion correlates with the total cellular NADPH concentration, supporting the predominant role of this enzyme form[253].

Cytosolic NADK activity is stimulated by a direct Ca^{2+}-dependent and calmodulin-dependent mechanism[257], similar to kinases from other animals and plants[258]. The human enzyme is also activated through phosphorylation by calmodulin-dependent kinase II[257] or AKT[259].

Recent research has demonstrated that the level of NADP(H) is tightly controlled by phosphatases, both in mitochondria (by nocturnin[260]) and the cytosol (by MESH1 (ref. 261)). These enzymes dephosphorylate NADP(H) back to NAD(H), indicating that the balance between the phosphorylated and unphosphorylated forms needs to be tightly controlled. Intriguingly, nocturnin expression undergoes circadian oscillations, and was proposed to establish an important link between metabolism and the circadian clock[260].

Given its activity, NADK is, in principle, also a NAD$^+$-consuming enzyme. However, this reaction probably does not substantially affect the NAD$^+$ pool because, relative to NAD(H), the concentration of NADP(H) is much lower[262], and there are many fewer reactions known that degrade NADP(H). By contrast, NAD$^+$ boosting could increase also the NADP(H) pool; however, this possibility has not been well explored. In principle, an increase in NADP(H) level could have favourable effects that might have been incorrectly ascribed to direct NAD$^+$-dependent mechanisms.

it needs to be available in many, if not all, subcellular compartments[23]. Moreover, the multitude of NAD$^+$-consuming signalling reactions continuously depletes the dinucleotide. In this section, we discuss mechanisms that enable the maintenance of compartmentalized NAD$^+$ pools, either through local NAD$^+$ synthesis or by mediating import of NAD$^+$ or NADH.

Subcellular differences in NAD(H) concentrations

Early studies addressing the subcellular distribution of NAD$^+$ established that mitochondria contain a large fraction of the cellular NAD$^+$ content, a fraction that varies between cell types and might exceed 50% in cardiomyocytes[45,46]. The need for a large mitochondrial pool has been attributed to its requirement in the TCA (Krebs) cycle and oxidative phosphorylation, the main NAD$^+$-dependent mechanism of ATP generation in most cells. Most of the remaining NAD$^+$ (typically >50% of cellular NAD$^+$ levels) has been detected in the nucleus and cytosol and ascribed to a single nuclear–cytosolic pool, assuming unrestricted exchange between the two compartments based on NAD$^+$ being small enough to pass through the nuclear pore[45,46]. Moreover, when cells were treated with an NAD$^+$ synthesis inhibitor, the rate of NAD$^+$ depletion was similar in the nucleus and cytosol, but significantly delayed in mitochondria[47]. Given that the total volume of mitochondria is very small compared with the nucleus and cytosol, it was clear early on that the NAD$^+$ concentration in mitochondria is substantially higher than its total cellular concentration. Indeed, the use of genetically encoded fluorescent NAD$^+$ sensors directed to the nucleus, cytosol or mitochondria confirmed the earlier observations[47]. In cultured human cells, the free NAD$^+$ concentration in mitochondria was estimated to be ~250 µM, whereas values of ~100 µM were measured in both the nucleus and cytosol[47]. Comparable but somewhat lower values for free NAD$^+$ were obtained using semi-synthetic Förster resonance energy transfer (FRET) sensors[48]. Notably, these values are lower than the total concentrations that are extractable from cells or tissues, and this difference has been attributed to lowering the free pool of NAD$^+$ through protein binding.

Dehydrogenase enzymes that use NAD(H) to catalyse oxidation and reduction reactions and have K_m values for NAD(H) that are similar to its free concentration range are kinetically regulated by fluctuations in NAD(H) concentration[44]. Many nuclear and cytosolic NAD$^+$-consuming enzymes, including sirtuins and ADP-ribosyltransferases[49,50], have K_m values for NAD$^+$ in the range of 50–200 µM. Consequently, their activity will also be modulated by physiologically relevant alterations in the concentration of NAD$^+$. In the nucleus, the transcription co-repressor carboxy-terminal binding protein (CtBP) acts as a NAD(H) sensor that modulates gene expression and reprogrammes metabolism in response to shifts in the redox ratio[51]. Although the capacity of the redox ratio per se to regulate sirtuins is controversial, there is good evidence for regulation of SIRT1 by circadian fluctuations in NADH levels[52,53]. In mitochondria, the K_m value for NAD$^+$ (~250 µM) of SIRT3 suggests it is a NAD$^+$ sensor that adjusts metabolic regulation within these organelles according to changes in NAD$^+$ levels[54]. Therefore, at least some of these proteins have a potential for metabolic sensing, as their activity could be regulated by fluctuations in NAD(H) concentration.

Despite the apparent equilibration between nuclear and cytosolic NAD$^+$ pools, it has been reported that by changing the abundance of nuclear or cytosolic NMNATs (the enzymes that catalyse the final step of NAD$^+$ synthesis), temporary NAD$^+$ gradients between these compartments could be established, and that they might be crucial for adipocyte differentiation[55]. Thus, a subset of NAD$^+$-dependent enzymes can likely facilitate immediate metabolic adaptation to changes in NAD(H) abundance or in the NAD$^+$/NADH ratio within each cellular compartment.

The assessment of NAD$^+$ content in other cellular compartments has been limited by the lack of adequate experimental tools (Table 1 and Box 2). Given that the NAD$^+$-dependent oxidation of very long-chain fatty acids takes place in peroxisomes, the presence of the dinucleotide in these organelles can be safely assumed. This assumption has been confirmed by targeted expression of the catalytic domain of poly(ADP-ribose) polymerase 1 (PARP1) in peroxisomes followed by immunocytochemical detection of ADP-ribose polymers[56], which are formed exclusively from NAD$^+$. Using this approach, the presence of NAD$^+$ was also demonstrated in the endoplasmic reticulum and the Golgi apparatus[56]. However, the roles of these subcellular pools are still unexplored.

Transport of NAD(H) across intracellular membranes

NAD$^+$ and its biosynthesis intermediates generally cannot passively diffuse across cellular membranes and require dedicated transporters to enter cells or membrane-bounded organelles. Nam may be

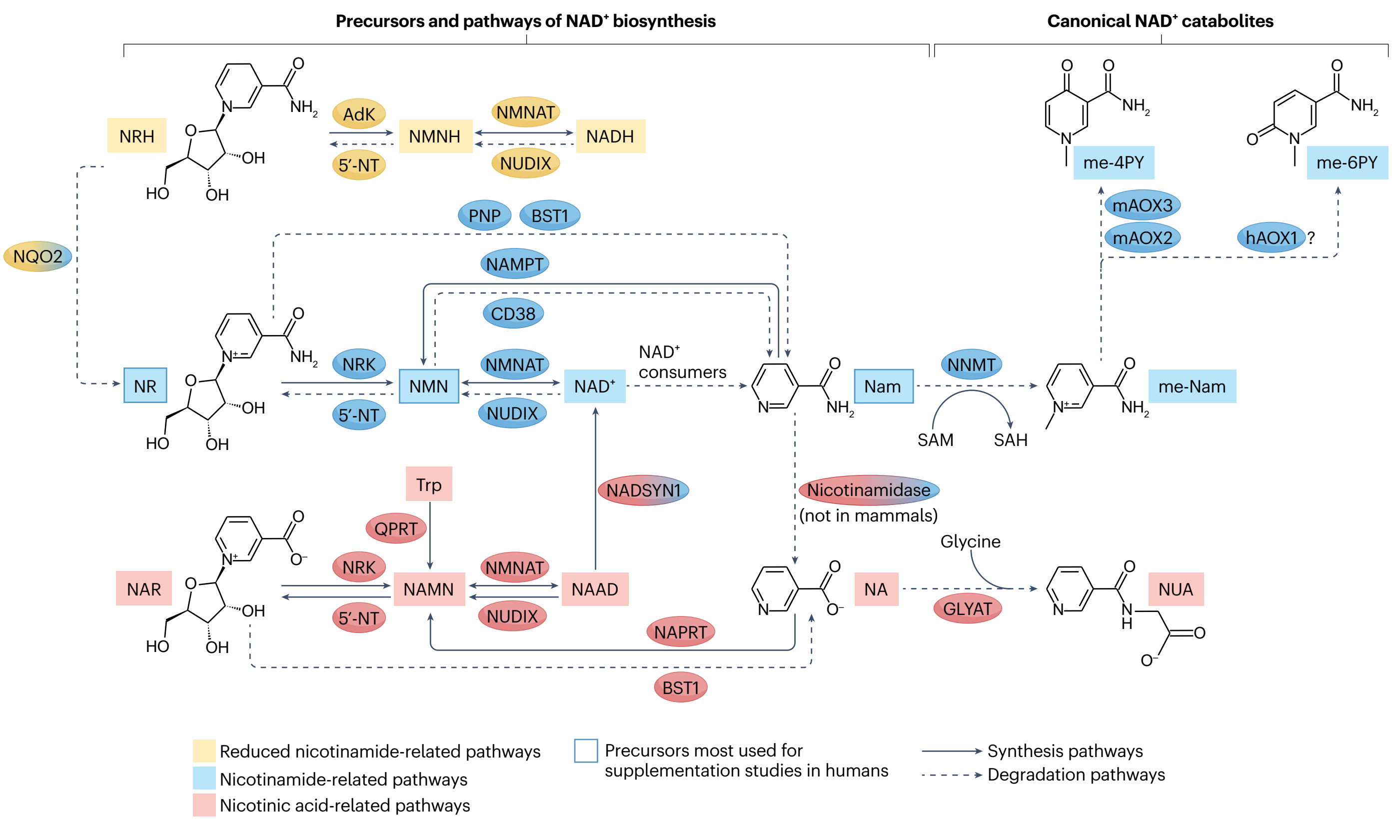

Fig. 2 | Biosynthesis and canonical degradation pathways of NAD$^+$. Nicotinamide (Nam) and nicotinic acid (NA) are the classical vitamin B_3 precursors; nicotinamide riboside (NR) and nicotinic acid riboside (NAR) are their respective derivatives; and nicotinamide mononucleotide (NMN) and nicotinic acid mononucleotide (NAMN) are their corresponding mononucleotides. Tryptophan is degraded in the de novo pathway to quinolinic acid (not shown), which is converted to NAMN by quinolinic acid phosphoribosyltransferase (QPRT). The reactions that transform Nam and NA into their mononucleotides are catalysed by nicotinamide phosphoribosyltransferase (NAMPT) and nicotinic acid phosphoribosyltransferase (NAPRT), respectively. NR and NAR are phosphorylated to NMN and NAMN, respectively, by nicotinamide riboside kinases (NRKs), whereas the kinase responsible for transforming reduced nicotinamide riboside (NRH) to reduced nicotinamide mononucleotide (NMNH) is adenosine kinase (AdK). NRH can also be oxidized to NR by *N*-ribosyldihydron icotinamide:quinone dehydrogenase 2 (NQO2). Formation of the dinucleotides in all pathways requires the activity of nicotinamide mononucleotide adenylyltransferases (NMNATs). As indicated, several intermediates can be degraded to their original substrates or to other precursors by the indicated enzymes 5′-nucleotidase (5′-NT), the hydrolase NUDIX (also known as NUD22), purine nucleoside phosphorylase (PNP), CD38 or BST1. The oxidized form of nicotinamide adenine dinucleotide (NAD$^+$) is degraded to Nam (and to ADP-ribose adducts) by 'NAD$^+$ consumers', including ADP-ribosyltransferases, sirtuins or NAD$^+$ glycohydrolases/ADP-ribosylcyclases. In lower organisms, Nam is deamidated to NA by nicotinamidase, which is absent in mammals. NA can be converted to nicotinuric acid (NUA) by glycine-*N*-acyltransferase (GLYAT). In mammals, when not recycled into NAD$^+$, Nam is methylated by Nam-*N*-methyltransferase (NNMT) to form *N*-methylnicotinamide (me-Nam). NNMT uses the universal methyl donor *S*-adenosylmethionine (SAM), which is concomitantly converted to *S*-adenosylhomocysteine (SAH). me-Nam is further converted to methyl pyridones (me-PY) with the oxo group added to the 4 position (*N*-methyl-4-pyridone 3-carboxamide (me-4PY)) or 6 position (*N*-methyl-6-pyridone 3-carboxamide (me-6PY)) of the ring. me-4PY generation is predominant in mice and catalysed by aldehyde oxidase 2 (mAOX2) and mAOX3. me-6PY is the major species produced in humans; although this reaction is often attributed to hAOX1, it is more likely non-enzymatic, as me-Nam is inherently susceptible to oxidation and mAOX1 cannot recognize me-Nam as a substrate. Note that several enzymes, including CD38 and BST1, can catalyse base-exchange reactions that are not represented here. NAAD, nicotinic acid adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADSYN1, NAD$^+$ synthase 1.

Table 1 | Analytical methods to measure NAD^+ and $NAD(P)^+$ + NAD(P)H

Method		Sensitivity	Biospecimen types	Metabolites measured	Limitations (L) and advantages (A)	Refs.
Direct detection methods						
MRS	^1H MRS	High micromolar to millimolar	Human tissues	NAD^+	L: expensive A: in situ measurements	107
	^{31}P MRS	High micromolar to millimolar	Human tissues	NAD(P)(H)	L: expensive; overlapping measurements; lack of internal standard for quantification A: in situ measurements	189
Intrinsic fluorescence	Two-photon spectrometry	Nanomolar	Whole cells	NADH	L: limited number of cells can be monitored, and only reduced forms are detectable A: spatial or redox redistribution can be monitored in real time	190
	FLIM	Nanomolar	Whole cells	NAD(P)H		191,192
Indirect detection methods						
Enzymatic assays		Nanomolar to millimolar	Cell, blood and tissue extracts	NAD(P)(H)[a]	L: requires specimen isolation and sample processing[a,b] A: easily implemented; numerous commercial kits are available	Reviewed[193,194]
NAD(P)(H) genetically encoded fluorescent sensors		Ratiometric	Whole cells, organelles	NAD(P)(H)-specific	L: limited dynamic range A: in situ measurements; minimal interference from NAD precursors	195–198
FRET-based semi-synthetic sensors		Ratiometric	Whole cells, organelles	NAD(P)(H)	L: requires several components (relatively large, overexpressed protein with two self-labelling tags and two tag-specific fluorophores) A: useable in a wide concentration range	48,199
Redox sensitive sensors		Ratiometric	Whole cells, organelles.	NAD(P)(H), but not limited to it	L: provides no information about absolute quantities A: immediate read-out of the redox status (and its changes)	200,201
^1H NMR spectrometry metabolomics		High micromolar and above	Extracts of cells, blood and tissues	Nam, NR, NMN, NAD(P)(H)	L: requires specimen isolation and sample processing[a,b] with low sensitivity; requires high-field NMR spectrometer A: analyses of ATP and of other key energetics metabolites can be performed concurrently	202–204
LC-UV		Mid micromolar to high millimolar	Extracts of cells, blood and tissues	All NAD metabolites possessing UV-absorbance properties	L: requires specimen isolation and sample processing[a,b] with relatively low sensitivity for which small sample sizes are not suitable; detection efficiency is dependent on the spectral properties of the metabolite being detected; quantification only of metabolites with known UV properties is possible[c] Liquid chromatography with detection by mass spectrometry, rather than by UV is preferable A: low cost and easy to implement	205,206
LC-MS^n metabolomics		Low nanomolar to high millimolar	Extracts of cells, blood and tissues	All NAD metabolites	L: requires specimen isolation and sample processing[a,b] A: ability to measure the full NAD^+ metabolome and discover new NAD^+-related entities; ability to trace how NAD^+ precursors are used and distributed using stable isotopologues	207–210
Poly(ADP-ribose) generation		Detection of relative changes	Organelles	NAD^+	L: no absolute quantification; suitable only for membrane-bounded organelles A: sensitive, robust, easy to implement	56

FLIM, fluorescence-lifetime imaging microscopy; FRET, Förster resonance energy transfer; LC-UV, liquid chromatography coupled with ultraviolet spectrometry; LC-MS^n, liquid chromatography coupled with mass spectrometry (n multidimensional); MRS, magnetic resonance spectroscopy; NAD^+, the oxidized form of nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, phosphorylated nicotinamide adenine dinucleotide; NADPH, reduced phosphorylated nicotinamide adenine dinucleotide; Nam, nicotinamide; NMN, nicotinamide mononucleotide; NMR, nuclear magnetic resonance; NR, nicotinamide riboside. [a]Low reproducibility due to variability in extraction and experimental protocols, detection of free versus protein-bound NAD(P)(H), and/or effects of extraction pH on NAD(P)(H) abundance. Results can be affected by the sample handling time. [b]Normalization to protein concentration, volume or tissue mass is required. [c]LC-UV has been supplanted by LC-MS^n, which provides increased sensitivity and ability to detect NAD metabolites regardless of their individual spectral properties.

an exception, especially at high concentrations, although evidence for carrier-mediated uptake also exists[57,58]. Therefore, such carriers should be key regulators of NAD^+ concentration in membrane-bounded organelles. However, knowledge about the exchange of NAD^+ or its biosynthesis intermediates across intracellular membranes has only begun to emerge. By contrast, the subcellular distribution of enzymes that can catalyse the final step of NAD^+ biosynthesis (NMNATs and NAD^+ synthase) is known and they are confined to the nucleus, cytosol and mitochondria[59]. Consequently, the presence of NAD$^+$ in other organelles requires dedicated NAD$^+$ or NADH transporters. Even the generation of the mitochondrial NAD$^+$ pool is largely independent of NMNAT3, the mitochondrial NMNAT[60].

Intense research led to the identification of solute carrier family 25 member 51 (SLC25A51; also referred to as MCART1) as a mitochondrial NAD$^+$ transporter in mammalian cells and established the major pathway responsible for the generation of this important pool[61–64]. Deletion of this carrier results in mitochondria that are almost devoid of NAD$^+$. The liver-specific carrier protein SLC25A47 was suggested to be another mitochondrial NAD$^+$ transporter[65]. However, a thorough experimental verification of this function is still pending, as hepatocytes clearly express SLC25A51. Likewise, the peroxisome membrane protein SLC25A17 has been reconstituted in phospholipid vesicles and shown to transport co-enzymes, including NAD$^+$ (ref. 66), yet whether this carrier has a physiological function in peroxisomal NAD$^+$ homeostasis remains to be firmly established. How the NAD$^+$ pools of other organelles, such as the endoplasmic reticulum and the Golgi complex, are generated and maintained remains unknown.

An expanded view of organismal NAD$^+$ metabolism

In recent years, there has been growing appreciation of the varied mixtures of NAD$^+$ precursors that we obtain from our diet, and how the gut microbiome interacts with host NAD$^+$ metabolism.

Dietary sources and uptake of NAD$^+$ precursors

A common misconception is that NAD$^+$ precursors arrive in the body exclusively as the conventional vitamin B_3 forms: Nam or NA. In fact, all living cells contain larger amounts of the active metabolites of NAD$^+$ (NAD$^+$, NADH, NADP$^+$, reduced phosphorylated nicotinamide adenine dinucleotide (NADPH)), which are the major forms of 'niacin equivalents' that are ingested from unprocessed plant-based or animal-based foods. 'Niacin' is a somewhat ambiguous term used inconsistently in the literature to refer to both Nam and NA, or to mean NA specifically,

Box 2 | Challenges in NAD$^+$ metabolome measurements

Even though the oxidized and reduced forms of nicotinamide adenine dinucleotide (NAD$^+$ and NADH, respectively) and phosphorylated NAD$^+$ (NADP$^+$ and NADPH, respectively) are comprehensively characterized molecules, their measurement in vivo and ex vivo remains challenging. Currently, direct NAD(P)(H) quantifications in humans can only be achieved by magnetic resonance spectrometry (MRS), and indirect measurements are achieved through invasive biopsy followed by extraction and quantification.

Although potentially very informative, measurements of NAD$^+$ by MRS are limited to only a few research sites and remain qualitative rather than quantitative, as internal standards are difficult to identify and characterize. Furthermore, both proton and phosphorus MRS require refinements to improve signal-to-noise ratios and, in the case of phosphorus MRS, other abundant biochemical entities possessing similar chemical properties can produce overlapping signals in the same analytical window (for example, NADH, NADP(H), ADP-ribose, co-enzyme A and uridine diphosphate sugars, although the α-phosphate of ATP can be the most obstructive signal[107,189,263,264]).

Isolation and processing of biospecimens are currently required to achieve quantitative measurements. These measurements can be carried out on cell, blood, tissue and fluid samples. Initial processing, which requires separating water-soluble materials that include all known NAD$^+$ metabolites from macromolecules and lipids, is often necessary and is usually followed by high performance liquid chromatography separation combined with detection by UV light or mass spectrometry[209]. This sequence, which is applicable to all biospecimens, has provided much impetus to understanding the biology of the NAD$^+$ metabolome. However, measurements have proven to be highly variable, depending on the operator, the protocol implemented and the separation and detection technique applied[265].

Crucial to measuring NAD$^+$, NADH, NADP and NADPH is the reactivity of NAD(P)H towards oxygen, particularly in the presence of riboflavin-containing proteins, which can result in their rapid conversion to NAD(P)$^+$ (ref. 161). This reaction renders an accurate and absolute quantification of all four co-factors challenging. Freeze–thaw is particularly detrimental to the reduced forms due to chemical degradation and hydrolysis[209]. Additional consideration should be given to the time between sample harvest and processing, which should be kept consistent across experiments. Moreover, as NAD(P)(H) are chemicals sensitive to pH (ref. 266) attention should be given to the extraction conditions and how these affect NAD(P)(H) distribution and abundance. If using liquid chromatography coupled with mass spectrometry (*n* multidimensional) (LC-MS^n) as an analytical method to measure the NAD$^+$ metabolome, use of isotopically labelled NAD(P)(H) as internal standards adequately resolves this issue and is strongly recommended to improve the consistency and accuracy of measurement.

Measurements of NAD(P)(H) in whole cells using protein reporters are gaining momentum and used to better understand the role of NAD(P)(H) pools. However, improvements are needed to expand the dynamic ranges of measurements, signal-to-noise ratios and the ability to provide quantitation in absolute terms for the individual NAD(P)(H) pools. Notably, the cells of interest have to be genetically modified and therefore this method is not applicable to humans. Even in mice, the use of protein reporters is only feasible in the context of dedicated models, which take time to generate.

Finally, some NAD$^+$ catabolites detected by LC-MS^n or liquid chromatography coupled with ultraviolet spectrometry (LC-UV) are misidentified, ignored or reported with conflicting nomenclature (for example, *N*-methyl-6-pyridone 3-carboxamide (me-6PY) and *N*-methyl-4-pyridone 3-carboxamide (me-4PY) are reported indiscriminately as PY, 2PY or even PYR, although each of these abbreviations corresponds to structurally different chemicals that are also NAD$^+$ catabolites[176]). NAD(P)(H) and its precursors are not only subject to enzymatic conversion but also readily chemically modified, and these chemical modifications can be predicted in order to help generate chemical standards to better explore the NAD(P)(H) metabolome and its functions. Although the complete characterization of the NAD(P)(H) metabolome lacks consistency, especially when considering species-specific NAD(P)$^+$ catabolism, a more systematic approach to characterizing the NAD$^+$ metabolome is now emerging[207,209].

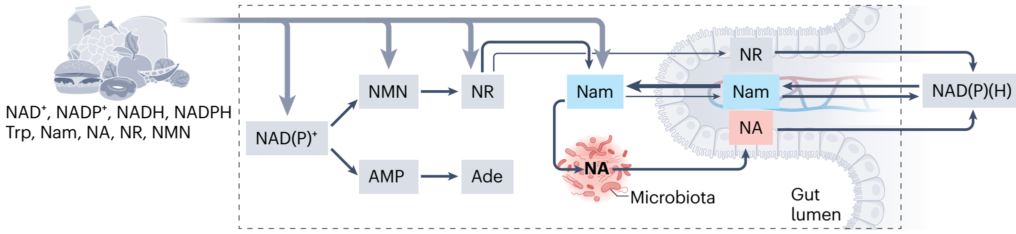

Fig. 3 | Fate of dietary precursors of NAD(P)(H). Unprocessed foods are rich in nicotinamide (Nam)-containing dinucleotides that are degraded by host metabolism, beginning in the acid environment of the stomach (dashed rectangle). Although some Nam can be absorbed directly, the net flux of this metabolite in the presence of a functional microbiome is from host circulation into the intestinal lumen. Microbial enzymes deamidate nicotinamide to nicotinic acid (NA), which can be used for microbial synthesis of the oxidized form of nicotinamide adenine dinucleotide (NAD^+) or absorbed by the host. Within host tissues, incoming NA, Nam and nicotinamide riboside (NR) generate NAD^+, which is further converted to reduced nicotinamide adenine dinucleotide (NADH) or the phosphorylated forms (NADP(H)) or is consumed to generate Nam that can then be salvaged to regenerate NAD^+ (not shown) or returned to the circulation. $NADP^+$, phosphorylated NAD^+; NADPH, reduced phosphorylated nicotinamide adenine dinucleotide; NMN, nicotinamide mononucleotide.

in which case Nam may be specified as 'niacinamide'. This terminology was adopted to avoid confusion with nicotine and hesitancy to consume 'acid'. Here we use the chemical names except when referring to 'niacin equivalents' as a collective term for molecules that contain or enable the formation of the nicotinamide moiety in NAD^+, and thus support the production of NAD^+.

As a general rule, phosphorylated nucleotides do not cross cell membranes[67], and instead NAD(P)(H) in the gut is broken down to NR (which can be taken up by equilibrative nucleoside transporters[68]) or further prior to uptake, with the majority absorbed as Nam or NA[69,70] (Fig. 3). A proposed exception is that nicotinamide mononucleotide (NMN) may be imported in the phosphorylated form through SLC12A8 (ref. 71), although the matter remains under debate[72,73]. Although some evidence has been presented for the association of labelled NMN with cells in an SLC12A8-dependent manner[74], its transformation into NAD^+ has required NRK activity (through NR as an intermediate) or degradation to Nam, in cell culture and in vivo studies to date[59,75]. Intact NAD^+ appears to be transported across the cell membrane through connexin 43, but the concentration gradient likely allows only export from cells through this pathway, potentially to support extracellular signalling enzymes such as CD38 (ref. 76). Intracellularly, connexin 43 allows movement of $NADP^+$ into endolysosomes[11].

The recommended daily intake for vitamin B_3 in the United States is 16 mg for men and 14 mg for women[77], and various organizations have set recommendations in this range based on urinary excretion of metabolites, with the intention of providing a safe margin to prevent pellgra[78]. However, this practice leaves open the question of what the 'optimal' level of vitamin B_3 consumption is. In addition, tryptophan contributes substantially to NAD^+ synthesis: a study conducted in Japanese women has estimated that every ~67 mg of tryptophan ingested supports NAD^+ synthesis equivalent to the amount produced from 1 mg of Nam[79]. In mice, tryptophan-dependent NAD^+ synthesis is sufficient to maintain the NAD^+ metabolome even in the absence of dietary vitamin B_3 or microbial contributions (that is, when the microbiome is removed by antibiotics or in germ-free mice); simultaneously inactivating the tryptophan pathway and withdrawing dietary vitamin B_3 causes a true deficiency[80,81]. Humans are generally considered to depend more on vitamin B_3 intake than mice, but notably they have also been shown to survive without vitamin B_3 when dietary tryptophan intake is sufficient[30]. Microbial metabolism can also provide some niacin equivalents from aspartate (or dietary precursors to aspartate), but the yield from these pathways is too low to prevent pellagra and, therefore, is of uncertain consequence. Notably, whereas many countries implement a mandatory food fortification programme that includes vitamin B_3 (for example, the United States, the United Kingdom, Australia and Canada), many industrialized countries of continental Europe (for example, France, Spain) and the Russian Federation do not. Thus, maintenance of the entire NAD^+ metabolome and the availability of its precursors, by means of diet or supplementation, depends not only on the physiological status of the host and its microbiome but also on the approach to nutrition in the general population.

The role of the microbiome

As noted above, the gut microbiome can contribute to de novo NAD^+ synthesis. In addition, microbes themselves require NAD^+, which creates the potential for 'net positive' or 'net negative' influences on total niacin equivalents available to the host. However, the role of the microbiome has proven more complex and fascinating than initially suspected, with Nam from the host circulation entering the gut lumen and returning largely as NA following transformation by microbes[81] (Fig. 3). This pathway accounts for ~50–80% of microbial NAD^+ synthesis and is sufficient to maintain circulating NA levels in the host in the absence of any dietary intake. The reasons for this sharing of niacin equivalents remain speculative – it may ensure the availability of NAD^+ precursors to microbes during times of fasting or low-quality diet, and provide metabolic flexibility to host tissues, which lack the ability to generate NA. For microbes, using deamidated NAD^+ intermediates is energetically less efficient but more chemically stable, and avoids inhibition of bacterial DNA ligase by NMN[82,83]. There is also potential for communication through G-protein-coupled receptor 109A (GPR109A; also known as HCAR2), which is present in the gut lumen[84] and responds to NA[85]. Furthermore, microbes have a much greater metabolic role during NAD^+ precursor supplementation than was initially appreciated. The majority of the increase of NAD^+ levels observed in the liver after a bolus of Nam, NR or NMN is generated through microbial production of NA, rather than from direct trafficking of the administered molecule to hepatocytes[70,81,86]. With higher doses, circulating concentrations of NA transiently reach levels sufficient to activate GPR109A throughout the body, rather than only in the gut lumen, and the potential importance of this mechanism remains unexplored[81]. Thus, the importance of microbiome composition or interventions such as antibiotics are areas that are ripe for exploration. Interestingly, providing reduced forms of NAD^+ precursors (that can be converted directly into NADH) might offer an opportunity to boost specifically host NAD(H) pools, as they appear to be much less affected by the gut microbiome[35].

The composition and structure of populations of microorganisms in the gastrointestinal tract are affected by many factors, predominantly genetics, host physiology, diet and environmental factors. However, boosting NAD$^+$ has also been shown to affect the microbial flora. For instance, NAD$^+$ precursors have been shown to restrict the colonization of pathogenic bacteria in the intestinal tract, while improving synthesis of hepatic bile acids, which are metabolites that possess inhibitory properties against microbial proliferation[87]. The consensus is that boosting microbial NAD$^+$ synthesis may help maintain normal microbial metabolism, and promoting host NAD$^+$ levels might be beneficial to intestinal bacteria and detrimental to harmful bacteria[37,88]. However, it will be important to test whether some of these benefits extend to humans as the change in microbiome composition following supplementation may be restricted to rodents[89].

Supplementing NAD$^+$ metabolism

Strategies to boost NAD$^+$ levels have received enormous attention in recent years, with many preclinical studies suggesting this has benefits (Table 2). However, in many cases there is little mechanistic understanding, and anecdotal claims continue to outnumber proven benefits in human trials.

Choosing when to supplement

Given that the intake of niacin equivalents in developed nations is well above the threshold required to prevent pellagra, it is prudent to examine the validity of evidence that further increasing NAD$^+$ synthesis capacity has any benefit. In fact, except for anecdotal reports, we are unaware of any such evidence in young healthy rodents or humans, and, by contrast, there are some hints of potentially detrimental effects on exercise performance (complicated by the potential influence of repeated gavages) and glucose tolerance with high doses in rodents[90–92]. However, there is abundant evidence that supplemental NAD$^+$ precursors are beneficial in rodents in conditions of metabolic stress, injury or disease, and can improve some aspects of ageing in mice[22]. In some, but not all, of these conditions, decreases in NAD$^+$ concentration have been measured in relevant tissues following supplementation, which is consistent with a model that NAD$^+$ levels typically found in healthy young individuals are near optimal and the goal of supplementation should be to restore the NAD$^+$ level only when it has fallen.

One of the biggest clinical successes of NAD$^+$ precursors to date has been the treatment of mitochondrial myopathy with NA[17]. In this case, the potential benefit of supplementation was detectable in the form of lower NAD$^+$ levels in muscle biopsies and whole blood of patients, with the degree of NAD$^+$ deficit predicting the degree of benefit. Importantly, it is not technically feasible at present to measure NAD$^+$ levels in rare cell types, or to assess the possibility that localized depletions occur within tissues. For this reason, even conditions in which no specific NAD$^+$ deficit is detected, but supplementation produces a benefit, may reflect restoration of NAD$^+$ levels. Moreover, the steady-state concentration of NAD$^+$ in a tissue is not a reliable indicator of turnover, as changes in NAD$^+$-consumer activity and synthesis rates that balance each other are invisible to steady-state measurements[93–95]. Excessive activation of one consumer could also limit NAD$^+$ availability to others, without a major change in total concentration, owing either to the kinetics or to the compartmentalization of the different enzymes. These uncertainties highlight the need for better tools and biomarkers to assess the need for and potential benefits of NAD$^+$ supplementation.

Choosing the most promising indications in which to invest clinical effort is a daunting task, given the broad range of rodent models that have shown benefits and the lack of readily available biomarkers or mechanistic understanding in most cases. Early trials in humans have had some successes but disproportionately more failures in attempting to recapitulate the results from rodents. Table 2 summarizes some of the indications that appear to warrant further testing, based on evidence of efficacy in rodents, an NAD$^+$-driven mechanism, clinical need and availability of biomarkers for assessing success. In addition to the mitochondrial myopathy study mentioned above, conditions that might benefit from NAD$^+$ supplementation include inflammation, heart failure, kidney disease, several premature ageing syndromes, including ataxia telangiectasia and Alzheimer disease, and aspects of healthy ageing. For both heart failure[18] and kidney disease[96], decreased NAD$^+$ levels have been measured in affected tissues in humans, and lower circulating Nam[97] and tryptophan[98] were recently noted in individuals with Alzheimer disease, although direct measurements of NAD$^+$ in brains of these individuals have not been reported. The longest human trial with NR to date (2 years) was performed in individuals with ataxia telangiectasia and recently reported improvements in motor coordination and eye movements[99]. Notably, heart failure and kidney disease have clear clinical markers that can be assessed over short time frames and NR supplementation has already been shown to suppress the inflammatory phenotypes of peripheral blood mononuclear cells isolated from individuals with heart failure[100,101].

Healthy ageing is perhaps the most difficult indication to assess, but also the one that is potentially the most impactful[14,22,102]. Decreases in NAD$^+$ levels are observed with age in multiple, but not all, tissues from rodents and in human skin[103], muscle[104], liver[105] and brain[106,107]. The decreases are mild (~30% between young and very old) and not entirely consistent between individuals, making it unclear whether they are sufficient to be functionally relevant and whether they are truly intrinsic to ageing or reflect specific disease processes in a subset of individuals[108]. Supporting these concerns, depletion of the NAD$^+$ synthesis factor nicotinamide phosphoribosyltransferase (NAMPT) in mouse skeletal muscle decreases NAD$^+$ levels far more than its effect of ageing, and is initially well tolerated[109]. In humans, the age-related decrease in muscle NAD$^+$ levels is correlated with muscle function, with aged trained athletes having levels that are no lower than those of young controls[104]. Although NR supplementation modestly extended the lifespan in one small study in mice[110], it failed to do so in a larger multicentre trial, albeit at a lower dose and with altered genetic background[111]. Nevertheless, both NR and NMN improve some age-related phenotypes in mice[110,112], and age is the greatest risk factor for most of the specific conditions that NAD$^+$ supplementation has shown promise for. Thus, it seems quite plausible that NAD$^+$ supplementation could extend the healthspan if not the lifespan. This possibility could be tested gradually, through approval of supplements for a specific indication in aged individuals and careful assessments of their overall health as secondary outcomes. Another possibility is performing a study more akin to the Targeting Aging with Metformin (TAME) trial, in which participants are enrolled prospectively and monitored for the occurrence and clinical course of multiple age-related conditions[113]. Studies testing specific mechanisms by which NAD$^+$ precursors have been proposed to exert anti-ageing effects[114] and a focus on biomarker development will greatly facilitate these translational efforts.

An overlooked concept is the need for other vitamin B-derived co-factors to support NAD$^+$ synthesis and functions. For instance, vitamin B_1 (thiamine), which is lower in aged individuals[115], forms

Table 2 | Evidence of benefits of supplemental NAD$^+$ precursors in humans

Parameter/disease	Improved by	Unchanged by	Comments
Physiological parameters			
Body weight	NMN[211]	NA[17] Nam[212,213] NR[214,215] NMN[216,217] (NMN[218])[a]	No clear trend in most studies
Adiposity	NA[17] NR[214] (NMN[219])	NR[215,220] NMN[216,221], NMN[211]* (NMN[218,222])	May be dose and/or duration dependent *Only abdominal fat is measured
Insulin sensitivity, glucose homeostasis	NMN[216]* Nam[212]	NMN[210,217,218] NR[214,220,223,224]	*Attributed specifically to muscles
Blood lipids (cholesterol, triglycerides)	NR[225] NMN[211] Nam[212]*	NR[220] NMN[216,219] (NMN[222])	*Beneficial for improving cholesterol, but not triglyceride, levels
Inflammation	NR[226], NR[100,227]* (NR[101]*) (NR[228])	NR[214]	*Assessments performed in peripheral blood mononuclear cells, monocytes or T cells isolated after treatment
Mitochondrial function	NA[17] NR[100]*, NR[215]**	Trp/NA/Nam[130] NR[214,226,229]	*Assessments performed in peripheral blood mononuclear cells isolated after treatment **Biogenesis, but not function, is measured in adipose tissue
Physical function	NA[17] Nam[230] NMN[217] (NMN[210,220,221,230]) (NR[231]*)	Trp/NA/Nam[130], NR[223,226], NMN[232] (NR[225])	*A single dose improved function in older individuals, but not in younger individuals
Hypertension and/or vascular dysfunction	NR[224]* NMN[211] (NMN[233])	NR[214] NMN[219,232]	*Nominally significant (before multiple comparison correction)
Muscle regeneration	None	NR/PT[234]	In individuals 55–80 years of age
Brown adipose thermogenesis	None	NR[235]	Positive results achieved in ex vivo treated adipocytes, but not in vivo
Sleep quality	(NMN[236])	None	Evening administration more promising than administration before midday
Cognitive function	None	Nam[237]*, NR[238]	*In individuals with previous skin cancer
Diseases/injuries			
Acute kidney injury	Nam[239]	None	Injury associated with cardiac surgery
Chronic kidney disease	None	NR[225]	Primary disease measures unchanged, but some evidence obtained of metabolic improvement
Mitochondrial myopathy	NA[17]	None	Contributions from NAD$^+$-independent effects on GPR109A or lipids cannot be excluded
Heart failure	None	NR[100]	Functional parameters unchanged; peripheral blood mononuclear cells had reduced inflammatory potential
Alzheimer disease	None	Nam[240]	No safety concerns noted
Parkinson disease	(NR[241]) (NR[242]*)	None	*Clinical improvement detected, but confounded by shorter interval since levodopa treatment in patients receiving NR
Amyotrophic lateral sclerosis	NR/PT[243,244]	None	NR-independent effects of PT have not been ruled out
Ataxia telangiectasia	NR[245] NR[99] (NR[246])	None	Improvements not associated with any change in neurofilament light chain (NfL)
Skin cancer, UV protection	Nam[122,247,248]	Nam[249]*	*12-month follow-up in transplant recipients
Osteoarthritis/joint immobility	Nam[230,250]	None	Multiple prior studies summarized in ref. 230

This table is not a complete listing of NAD$^+$-related studies and specifically excludes extensive literature on using NA to lower circulating lipids (through a mechanism not shared by nicotinamide and therefore likely NAD$^+$-independent), numerous promising but inconsistently controlled studies on intact NAD$^+$ and NADH supplementation[121], and early studies on Nam supplementation that often are based on case reports (for example, ref. 230). The table also excludes topical formulations, which have shown benefits in skin[251]. With the exception of a phase III trial for skin cancer[122], all studies in this table are early-stage investigational studies and, in some cases, may have failed to detect effects due to limited statistical power. We are not aware of human clinical studies that demonstrated detrimental outcomes for any of the listed parameters. Asterisked notes in the Comments column provide information about the asterisked items in the columns to the left in that row. GPR109A, G-protein-coupled receptor 109A; NA, nicotinic acid; NAD$^+$, the oxidized form of nicotinamide adenine dinucleotide; NADH, the reduced form of nicotinamide adenine dinucleotide; Nam, nicotinamide; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; NR/PT, nicotinamide riboside with pterostilbene. [a]Parentheses indicate studies with a duration of <4 weeks and n<10, that have mixed outcomes on related measures or that highlight non-significant trends.

an obligate co-factor for the pentose pathway, which produces the 5-phosphoriboside pyrophosphate that is needed for the glycosylation step of NAD^+ biosynthesis (catalysed by quinolinic acid phosphoribosyltransferase (QPRT), nicotinate phosphoribosyltransferase (NAPRT) or NAMPT)[116]. Similarly, thiamine-derived co-factors, as well as the vitamin B_2 (riboflavin)-derived co-factors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are necessary for the effective conversion of NADH reductive power into ATP production through the Krebs cycle and oxidative phosphorylation[116,117]. Sources and absorption of different B vitamins are similar, and unless genetic predispositions are responsible for poor absorption, populations deficient in niacin equivalents often display other B-vitamin deficiencies[118,119]. Thus, supplementation with only niacin equivalents might not be sufficient to meet the metabolic needs of an individual prone to B-vitamin deficiencies.

Current strategies for supplementing NAD^+

It is possible to simply supplement intact NAD(P)(H). In fact, intravenous NAD^+ has become fashionable although we are not aware of any scientific literature testing its effects, and a concern related to this type of administration is the potential for detrimental effects from a sustained increase in circulating adenosine[120]. In addition, there are scattered reports of supplementation with intact NAD^+ or NADH in neurological conditions that have generally not been well controlled or consistently reproduced (for example, studies reviewed in ref. 121). Most recent clinical efforts have focused on one of the following NAD^+ precursors.

Nicotinamide. Nam is the body's main currency for niacin equivalents and the most straightforward way to supplement NAD^+. In fact, phase III trials have already demonstrated safety and some evidence of efficacy against skin cancer[122]. Perceived weaknesses of this approach include the fact that high concentrations of Nam inhibit many NAD^+-dependent enzymes, and that it depends on the activity of NAMPT — a key enzyme that catalyses the phosphoribosyltransferase step in NAD^+ (re)generation from Nam (Fig. 2) — which is rate-limiting, energetically costly and downregulated in some conditions, including in models of heart failure[123]. NAMPT is also subject to feedback inhibition by NAD^+, thereby limiting the increase in NAD^+ level that can be achieved through routes depending on this enzyme[124]. In addition, Nam is the direct substrate of the catabolic pathway initiated by NNMT, making it theoretically easy to bypass NAD^+ synthesis altogether.

Nicotinic acid. NA has a long history of clinical use driven mainly by its lipid-lowering properties, which are not shared by Nam and, thus, appear independent of NAD^+ synthesis[125]. It also comes with the disadvantage of a flushing response (temporary reddening of the skin with an unpleasant sensation of heat and/or itching) triggered by its activation of GPR109A (ref. 85). Nevertheless, NA supplementation is an effective strategy to raise NAD^+ levels and has been successfully applied to the treatment of mitochondrial myopathy[17]. In terms of energy demand, synthesis of NAD^+ from NA is even more costly than from Nam as the NAD^+ synthetase step adds another ATP-dependent reaction (Fig. 1). Excess NA not used in NAD^+ production is converted to NUA (Fig. 2). NUA is the only known direct catabolite of NUA, but its putative physiological relevance has received little attention. Finally, NAD^+ synthesis relying on NAPRT rather than on NAMPT is associated with certain cancerous and precancerous lesions[126,127], which should be considered when using supplements that promote NA availability.

Tryptophan. Although the de novo pathway begins from tryptophan and total flux can be modulated by tryptophan intake (Fig. 2), this supplementation strategy is less attractive because tryptophan is also the precursor to kynurenines and serotonin, which have immunomodulatory and neurological effects, respectively[128]. Excess flux into the de novo pathway may also produce the neurotoxic intermediate quinolinic acid, which is associated with delirium and mortality. However, no adverse effects were noted in a study of up to 5 g per day supplemental tryptophan[129] and some studies continue to employ this strategy for NAD^+ boosting[130].

Nicotinamide riboside. in 2004, NR was recognized as an alternative entry point to the salvage pathway through NRKs[32] (Fig. 2). Its main advantage is bypassing NAMPT, thereby avoiding potential feedback inhibition, dependence on expression of that enzyme and some of the energetic cost of NAD^+ synthesis. Although these advantages clearly hold true in cell culture, much of an in vivo dose ends up as Nam and NA when administered orally[94,109]. Several studies have nevertheless shown advantages of NR over Nam in animals[109,123], which may be related to pharmacokinetics or the small proportion of NR that reaches tissues intact.

Nicotinamide mononucleotide. This intermediate of NAD^+ synthesis is positioned downstream of NAMPT and thus, similar to NR, also bypasses the need for NAMPT, thereby providing similar benefits to those of NR supplementation. Debate over whether there is direct uptake of NMN or whether it is first dephosphorylated to NR has fuelled a discussion regarding which of the two molecules is the most proximal to cellular NAD^+ (refs. 71–73). Few head-to-head comparisons testing NR and NMN in the same system at the same time have been performed in rodents, and none in humans, making it unclear whether there is a substantial advantage for either molecule. Notably, both performed similarly in a model of acute kidney injury[96,131]. Because NMN was tested as a drug prior to its submission as a dietary ingredient, the US Food and Drug Administration (FDA) has ruled that NMN is an investigational drug, ineligible for distribution as a dietary ingredient or supplement in the United States under the Dietary Supplement Health and Education Act (DSHEA). By contrast, NR was first granted status as a new dietary ingredient and then tested as a new investigational drug, exempting it from similar restrictions.

Other NAD^+ precursors. The redox properties of NAD^+ are preserved in NR and NMN; recently, their respective reduced forms NRH and NMNH have also been investigated with regard to having an effect on cellular NAD^+ levels. Both compounds profoundly elevate intracellular NAD^+ levels and, despite being acid labile in vitro, are more effective than their oxidized counterparts in rodents, which may be partly related to improved stability in the gastrointestinal tract and blood[34,35,132,133]. As NMNH needs to be dephosphorylated to NRH to enter cells, the intracellular conversion of the two molecules proceeds through the same route, namely through intracellular phosphorylation to NMNH by AdK, which is different from the NRK-dependent route taken by oxidized forms[33]. Whether this difference in enzymology or some other property accounts for the higher NAD^+ generation from reduced precursors remains to be fully elucidated. Ribosylated precursors entering NAD^+ synthesis through the deamidated pathway, such as NAR or nicotinic acid mononucleotide (NAMN), can maintain cellular NAD^+ levels[134]. However, their utility as NAD^+ boosters has not been well studied. Trigonelline (*N*-methylated nicotinic acid) occurs in plant-based foods such as coffee, and it remains uncertain whether the NA is liberated by microbial metabolism. Another consideration is whether supplementing ribose along with niacin equivalents will better support NAD+ synthesis[135]. This moiety is effectively built in with NR and NMN and could be an advantage of these molecules even if the ribose is largely separated from the Nam prior to absorption. Overall, it is very clear that many supplementation strategies are available and that making rational choices about which to apply will require direct comparisons that are not yet available.

Alternative strategies to restoring or increasing NAD+ levels

Apart from the well-studied and well-tested supplementation strategies using NR or NMN, two main options can be considered to enhance or stabilize NAD+ levels, namely, boosting NAD+ biosynthesis through stimulation of NAD+ synthesis enzymes and inhibition of excessive NAD+ consumption. Given the variety of entry points into NAD+ biosynthesis and the multitude of NAD+-degrading enzymes, there are many potential targets, some of which have already been addressed experimentally.

Supporting NAD+-synthesizing enzymes

NAMPT has a fundamental role in the recycling of Nam, which is generated by sirtuins, ARTs, CD38 and SARM1 in all of the NAD+-degrading signalling reactions (Fig. 2). As NAMPT is generally agreed to be rate-limiting in this pathway, acceleration of its activity is expected to contribute to higher NAD+ availability. Several synthetic NAMPT activators have been identified and undergone initial characterization, including demonstration of their NAD+ boosting capacity[136–138].

Acceleration of the conversion of tryptophan to NAD+ has also been proposed based on the inhibition of the de novo pathway enzyme ACMSD (also known as 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase)[80,139]. ACMSD decarboxylates aminocarboxymuconic semialdehyde (ACMS) to picolinic acid, which is further degraded to eventually form acetyl co-enzyme A. Crucially, the alternative pathway for ACMS metabolism – cyclization to quinolinic acid used to generate NAMN – is non-enzymatic[139]. Therefore, inhibition of ACMSD increases the flux towards NAD+ and reduces the need for other niacin equivalents. However, a concern is the potential to accumulate quinolinic acid, which is a neurotoxin[140], should downstream enzymes fail to metabolize the quinolinic acid as quickly as it is produced. Upregulation of NRK2 is observed in conditions of low NAD+ levels in the heart and skeletal muscle, suggesting that it may also provide a strategy to increase NAD+ production[123].

Inhibition of excessive NAD+ consumption

Inhibition of NAD+-consuming enzymes will contribute to the stabilization of NAD+ levels. However, it is not always clear which enzymes account for a substantial portion of NAD+ flux and many of the beneficial effects of NAD+-dependent processes are mediated by such enzymes, meaning their inhibition would be counterproductive. Therefore, it is important to identify those NAD+ consumers that exhibit excessive activity and to target them specifically, achieving a dual effect: the selective downregulation of an undesired or overactivated NAD+-dependent signalling process, along with the limitation of NAD+ consumption. The validity of limiting consumption to maintain high NAD+ levels was demonstrated by inhibition of PARP1 activity[141]. However, long-term PARP1 inhibition is considered undesirable owing to the important roles of this enzyme, particularly in DNA repair.

Age-related NAD+ decline has been proposed to depend on an increase in the activity of CD38, an ecto-enzyme found mainly on endothelial cells and in immune cells such as macrophages and B cells[142,143]. Moreover, it was recently shown that chronic inflammation, which is a hallmark of ageing, leads to increased expression of CD38 on macrophages owing to accumulation of senescent cells. Consequently, NAD+ cleavage is enhanced, lowering its levels in tissues[144,145]. Several inhibitors of CD38 have been developed that show promise in maintaining organismal NAD+ levels during ageing[146–148]. Another NADase of interest is SARM1. Following mechanical or chemical insult, SARM1 mediates the rapid NAD+ decline that ultimately drives axonal degeneration[149,150]. Although SARM1 is present in many mammalian tissues, its physiological role in non-neuronal cells has remained obscure. Inhibitors are under development[151–153] that might become of interest not only for protecting neurons but also to generally diminish potentially futile NAD+ consumption. Nevertheless, it needs to be borne in mind that due to the capacity of CD38 and SARM1 to synthesize (cyclic) ADP-ribose, their inhibition may have negative consequences on physiological calcium signalling. For example, oxytocin release and synaptic plasticity are impaired in mice lacking CD38 (ref. 154).

Potential risks of NAD+ overaccumulation

Excess intake of niacin equivalents beyond the level required to maintain NAD+ levels is a likely consequence of a healthy diet and certain for those using supplements. Although the risks of intermediate levels of precursor supplementation are being investigated, very high doses of Nam or NA can clearly be harmful[155]. For instance, according to recommendations made by the European Nicotinamide Diabetes Intervention Trial Group, Nam should be considered as a drug possessing toxic potential for adult doses in excess of 3 g per day, for which unsupervised use of Nam should be discouraged[156]. Although few adverse effects have been detected in preclinical studies to date, mild decreases in haemoglobin and haematocrit were noted following supplementation with NR[157] and NA[17], which may warrant monitoring in longer-term studies, and some theoretical concerns remain to be fully addressed.

Inside cells, NAD+ and NADH can be degraded to NMN and NMNH, which can be dephosphorylated to NR and NRH. NAAD, an NAD+ biosynthesis intermediate, was proposed to also be a substrate of pyrophosphatases such as NUDIX hydrolases and of phosphatases, and thereby a precursor of NAR[134]. These nucleosides can be released into the extracellular space[68]. In cell culture, the activity of equilibrative importers and exporters of NR and NAR appears to depend on the phosphorylation status of NR and NAR inside the cells[134], whereas the points of control of the import and export of NRH remain unknown. Circulating NAR and NR are readily hydrolysed to NA and Nam by glycohydrolases[158] and phosphorylases[159], whereas NRH remains intact. In cells, NRH is the redox co-enzyme of *N*-ribosyldihydronicotinamide:quinone dehydrogenase 2 (NQO2), a xenobiotic metabolizing enzyme[160,161]. Overall, it can be envisaged that excess of intracellular NAAD, NAD+ and NADH will undergo degradation through these innocuous pathways if these molecules are not needed in redox biology or signalling, and that excess of NA, Nam and their riboside forms enter circulation and are used in various tissues. However, overloading the system or driving chemical modification of NAD+ or its intermediates has the potential to create unintended metabolic consequences.

Methylation of nicotinamide

Administration of NAD+ precursors leads to a substantial increase of me-Nam levels[162,163]. Nam is methylated by NNMT (Fig. 2). Although methyl-nicotinic acid (trigonelline) is a known catabolite found in plants[164,165], NA is not a substrate of NNMT and production of methyl-nicotinic acid in mammalian cells has not been observed. Thus, NNMT appears to be specific for the removal of excess Nam[166]. me-Nam feeds back to inhibit NNMT[167], which could be important for limiting excessive Nam depletion.

Methylation of Nam consumes *S*-adenosylmethionine (SAM) and produces *S*-adenosylhomocysteine (SAH). Recycling of SAH to methionine is commonly referred to as the methyl cycle[168,169]. SAM is the second most used enzyme substrate after ATP[170–172] and its depletion is one mechanism proposed to explain the hepatotoxicity of high-dose Nam or NA[173]. Although it is tempting to speculate that supplementing SAM could therefore offset the effects of Nam, it was recently shown that SAM can be catabolized outside the methyl cycle into toxic catabolites, raising concerns over its safety as a freely available dietary supplement[174]. Conversely, increasing the levels of Nam might also affect the ability of SAM to contribute to the methylome, thereby reducing its effectiveness as a supplement. This effect should be of particular concern as co-supplementation of NAD^+ precursors with SAM-containing nutraceuticals is becoming more common[175]. Much remains to be elucidated in this area of NAD^+ supplement consumption in the context of co-administration.

Methylated pyridones

In mammals, me-Nam is readily oxidized to methylated pyridones: me-6PY (also known as 2PY) and me-4PY (also known as 4PY). The oxidation of me-Nam to me-PY allows the removal of excess Nam by NNMT, as it removes product inhibition. These methylated pyridones exist as three possible isomers, and can be generated enzymatically or form spontaneously without the need for enzyme catalysts[40]. Often, their detection and quantification in serum, plasma and urine is limited to only one of the isomers, providing only a partial estimate of overall Nam catabolism following administration of NAD^+ precursors[176]. These oxidized forms of me-Nam have been directly associated with the presence and progression of chronic kidney diseases and have been deemed uraemic toxins[177–179]. However, the toxic properties of me-PY and whether they cross cell membranes have yet to be ascertained both in eukaryotes and in prokaryotes. Very recently, circulating levels of me-PY were demonstrated to correlate with major adverse cardiovascular events in humans, and suggested to act through induction of vascular cell adhesion protein 1 (ref. 178).

Non-canonical metabolites

In addition to their catabolism through Nam, non-canonical products of NAD^+ and NADH metabolism can generate entities that are detrimental to cellular homeostasis and that counteract the benefits of increasing NAD^+ levels (Fig. 4). Excess of NAD^+ and excess of NADH are likely to affect cells and organelles differently in different circumstances. For instance, under oxidative stress, mitochondrial respiration and oxidative enzymes generate reactive oxygen species (ROS) that react with electrophiles to generate oxidized derivatives[180]. In the case of $NAD(P)^+$, NMN and NR, this oxidative chemistry occurs on the pyridinium ring of Nam (analogous to oxidation of me-Nam) (Fig. 4); it is promiscuous and takes place intracellularly as well as

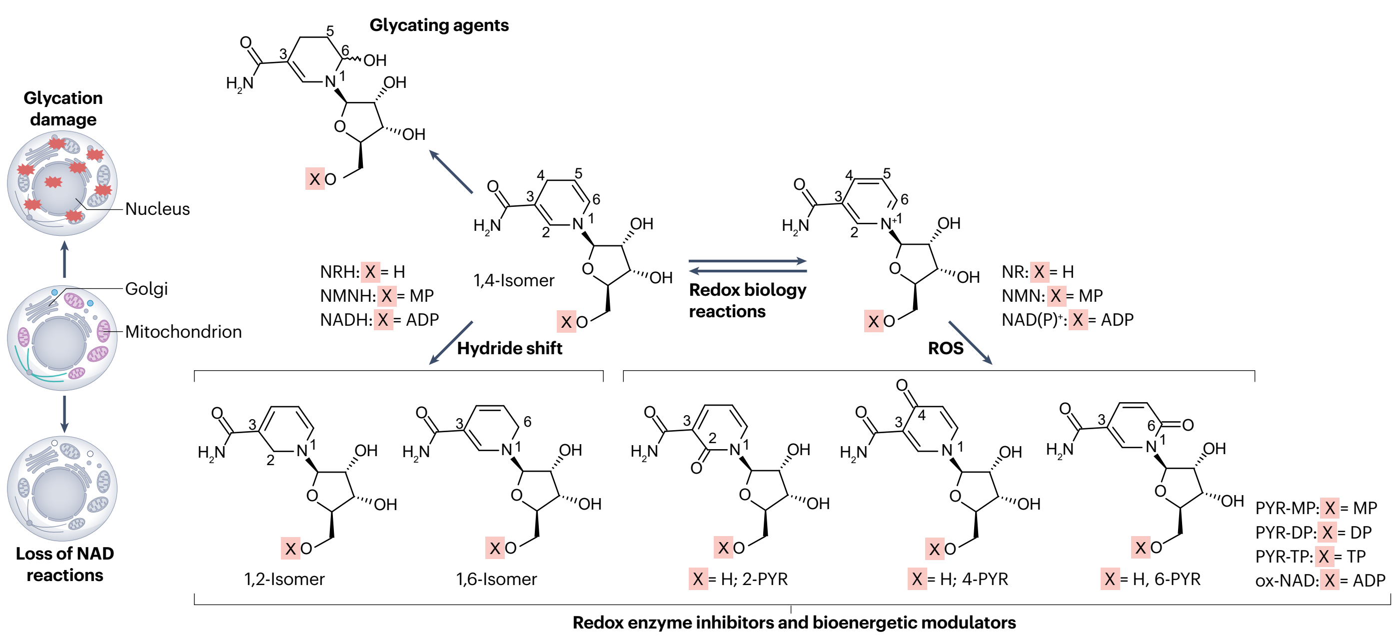

Fig. 4 | Non-canonical degradation of NAD^+ and NADH and of their ribosylated precursors. Reduced nicotinamide adenine dinucleotide (NADH) and its ribosylated precursors can undergo hydration, to generate glycating species, or isomerization, thereby yielding non-redox-active derivatives that interfere with redox biochemistry. The pyridinyl riboside of the oxidized form of nicotinamide adenine dinucleotide (NAD^+) and its ribosylated precursors also react with electron-rich reactive oxygen species (ROS) such as superoxide to generate stable ribosylated pyridones (PYRs). The nucleotides, such as pyridone riboside monophosphate (PYR-MP), and dinucleotides, such as over-oxidized NAD^+ (ox-NAD), are hydrolysed to pyridone ribosides (2-PYR, 4-PYR and 6-PYR) that are released into the extracellular environment, where they circulate and are taken up by other cells. Once inside cells, PYRs can be converted to their nucleotidic (monophosphate (MP), diphosphate (DP) or triphosphate (TP)) and dinucleotidic (ADP-linked) forms, with unknown consequences for nucleic acid synthesis and NAD^+-dependent enzymes. NMN, nicotinamide mononucleotide; NMNH, reduced nicotinamide mononucleotide; NR, nicotinamide riboside; NRH, reduced nicotinamide riboside.

Glossary

ADP-ribose

Generated by cleavage of the oxidized form of nicotinamide adenine dinucleotide (NAD^+) and the release of nicotinamide (Nam). It can be attached to proteins or nucleic acids in a process called ADP-ribosylation, which is catalysed by ADP-ribosyltransferases.

Nicotinamide

(Nam; also known as niacinamide). A major nutritional precursor of the oxidized form of nicotinamide adenine dinucleotide (NAD^+) and recyclable NAD^+ degradation product for NAD^+ (re)synthesis.

Vitamin B_3

Classically, refers to nicotinic acid (NA) and nicotinamide (Nam) for their capacity to cure pellagra; in a wider sense, natural compounds such as nicotinamide riboside (NR) and nicotinic acid riboside (NAR), which can be taken up in the gut and contain a pyridine moiety.

extracellularly[40]. Crucially, this oxidative process leads to the formation of non-canonical degradation products with as yet poorly defined properties. The three ribosylated pyridone (PYR) isomers – 2-PYR, 4-PYR and 6-PYR (Fig. 4) – generated through this chemistry can be found readily in cells, tissues, blood, serum and urine, although the phosphorylated forms, such as pyridone riboside triphosphate (PYR-TP), and the over-oxidized forms of NAD^+() are restricted to biospecimens containing cells, as they are intracellular entities[181,182]. Importantly, these over-oxidized forms of NAD^+ are structural mimics of NAD^+ and NADH, but are not redox active and do not possess a cleavable glycosidic bond, making them potentially potent inhibitors of NAD^+-dependent processes.

NADH and NADPH, similar to NRH and NMNH, can become hydrated[161,183]. This hydration leads to the formation of NADHX and NADPHX, which are NAD(P)H derivatives with properties that are poorly defined but detrimental to their functions as redox co-factors and substrates. When studying this process on NRH, it was discovered that NRH hydration led to the formation of conjugated protein adducts, which occurred preferentially on lysine residues[161]. Importantly, NAD(P)HX formation is reversed by a two-enzyme system that is essential to cell survival[184]. Therefore, excessive amounts of NAD(P)HX that would be susceptible to this chemistry present a risk that must be tightly controlled. Enzymes such as NAD(P)H quinone dehydrogenase 1 (NQO1) and NADPH oxidases are able to convert NAD(P)H to NAD(P), although in turn they may increase the production of ROS[185,186].

Considerably less often considered potentially detrimental is the ability of 1,4-NADH (the active form) to isomerize to 1,2-NADH or 1,6-NADH in the presence of riboflavin-based catalysts such as FMN and FAD, either free or protein-bound (Fig. 4). This isomerism is difficult to characterize in cells and tissues because it generates chemically unstable entities that can react readily with protein and generate irreversible protein conjugates, such as NAD(P)HX. Furthermore, these isomers are potent inhibitors of redox enzymes that are crucial to cellular metabolism and bioenergetics, and can potentially be detoxified through oxidation back to $NAD(P)^+$ by renalase[187,188]. As an increase in NADH can equate to an increase in the abundance of these isomers, an increase in total NAD(H) might present yet unanticipated drawbacks. In summary, an increase in NAD^+ levels through supplementation that allows for a decrease in ROS and avoids a sustained increase in NADH is most likely to offer the best physiological outcomes. It is still unknown how to best achieve this end point.

Conclusion and future perspective

Many open questions and controversies remain about the basic biology and metabolism of NAD^+-related molecules. Whole-tissue measurements lack the resolution to accurately determine the availability of NAD(P)(H) to enzymes in specific cells or subcellular compartments, either to diagnose deficiency or to judge the success of supplementation. We also lack tools to routinely assess NAD^+ turnover, which can vary independently from steady-state concentration. Determining these parameters is crucial for understanding which NAD^+-dependent processes are relevant in a given condition. To fully exploit the therapeutic potential of modulating NAD^+ metabolism, further research efforts are needed to understand the cellular mechanisms of NAD(H) distribution and how the different pools fuel NAD^+-dependent processes. Likewise, the interplay between different tissues in precursor generation and utilization is yet to be systematically studied. Moreover, the crucial roles of host–microbiome interactions and their interplay with other important co-factors, such as SAM, are receiving considerable interest, but will require further study to elucidate.

Rational design of translational experiments of NAD^+ boosting is going to require more sophisticated measurements and a focus on downstream mechanisms that can be tested. In humans, there is as yet no compelling reason to favour NR over NMN or vice versa, and although they both have a theoretical advantage over conventional B_3 vitamins, they lag behind in size and length of clinical trials. In future studies, it will be important to resolve controversies surrounding supplementation strategies to determine an optimal method for delivering niacin equivalents, whether there should be different strategies for different goals (for example, depending on the target tissue) and reliable parameters that can be evaluated as outcomes. It is also crucial to continue assessing potential risks of NAD^+ boosting.

In summation, NAD^+ and related molecules are essential to almost every facet of metabolism and the observations that their levels decrease and can be restored suggest the potential for exciting therapeutic opportunities. Fully achieving this potential will require embracing and continuing to explore the complexities of synthesis, transport and catabolism of the NAD^+ metabolome.

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

M.E.M. is supported, in part, by Elysium Health and holds a patent on manufacturing the oxidized and reduced forms of nicotinamide adenine dinucleotide (NAD^+ and NADH) precursors, their derivatives and applications thereof. M.Z. is scientific adviser for Blue Helix Health and declares no other competing interests. J.A.B. has received research funding and materials from Pfizer, Calico, Elysium Health and Metro International Biotech and consulting fees from Pfizer, Elysium Health, Cytokinetics and Altimmune, and holds a patent for using NAD^+ precursors in liver injury.

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