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Feature Review

NAD+ in Aging: Molecular Mechanisms and Translational Implications

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The coenzyme NAD+ is critical in cellular bioenergetics and adaptive stress responses. Its depletion has emerged as a fundamental feature of aging that may predispose to a wide range of chronic diseases. Maintenance of NAD+ levels is important for cells with high energy demands and for proficient neuronal function. NAD+ depletion is detected in major neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, cardiovascular disease and muscle atrophy. Emerging evidence suggests that NAD+ decrements occur in various tissues during aging, and that physiological and pharmacological interventions bolstering cellular NAD+ levels might retard aspects of aging and forestall some age-related diseases. Here, we discuss aspects of NAD+ biosynthesis, together with putative mechanisms of NAD+ action against aging, including recent preclinical and clinical trials.

NAD+ Has a Key Role in Human Health

Nicotinamide adenine dinucleotide (NAD^+) is a cofactor for numerous enzymes involved in cellular energy metabolism, and for adaptive responses of cells to bioenergetic and oxidative stress. Over 110 years ago, NAD^+ was discovered as a 'cozymase' necessary for fermentation (Figure S1 in the supplemental information online) [1–5]. In his 1930 Nobel lecture, Dr Hans von Euler-Chelpin stated that 'cozymase (NAD^+) is one of the most widespread and biologically important activators within the plant and animal world' [6]. NAD^+ is a necessary cofactor for many metabolic pathways, such as glycolysis, fatty acid β-oxidation, and the tricarboxylic acid cycle, while the reduced form of NAD^+ (NADH) is a primary hydride donor in the production of ATP via anaerobic glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) [7]. Recently, the importance of NAD^+ has expanded from a key element in intermediate metabolism to a critical regulator of multiple cell signaling pathways, and is now a major player in aging and age-related diseases [8–11].

Mounting evidence links compromised NAD^+ status to the **hallmarks of aging** (see Glossary). Among the age-related cellular processes that may predispose to disease, impaired **autophagy** has emerged as an important component [12,13]. Indeed, recent studies in human cells and animal models have unveiled a novel role for NAD^+ in autophagy and **mitophagy**, in which impairments in lysosome-targeting and –recycling mechanisms result in the accumulation of damaged molecules and mitochondria, leading to cell dysfunction and/or death [14]. Here, we discuss the interconnected roles of autophagy (especially mitochondrial autophagy or 'mitophagy'), mitochondrial maintenance, DNA repair, and cell death relative to NAD^+ metabolism. We summarize new insights into pathways of NAD^+ biosynthesis and consumption, and highlight

Trends

Recent discoveries have demonstrated an age-dependent decrease in cellular and/or tissue NAD^+ levels in laboratory animal models. Moreover, NAD^+ depletion has been linked to multiple hallmarks of aging.

In premature aging animal models, NAD^+ levels are decreased, while NAD^+ replenishment can improve lifespan and healthspan through DNA repair and mitochondrial maintenance.

Mitochondrial autophagy (mitophagy) has a major role in clearance of damaged and/or dysfunctional mitochondria, and compromised mitophagy has been linked to metabolic disorders, neurodegeneration [including Alzheimer's disease (AD) and Parkinson's disease (PD)] in addition to aging, and other age-related diseases.

New evidence suggests that NAD^+ precursors, such as nicotinamide and nicotinamide riboside, forestall pathology and cognitive decline in mouse models of AD.

NAD^+ supplementation can inhibit multiple aging features in animal models. This highlights essential roles for NAD^+ in maintaining healthy aging, and suggests that NAD^+ repletion may have broad benefits in humans.

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how NAD^+ depletion might contribute to mammalian aging. We also examine how supplementation of NAD^+ precursors might constitute a promising therapeutic strategy to counter aging-associated pathologies and/or accelerated aging.

NAD^+ Biosynthesis and Consumption

Cellular NAD^+ Levels

NAD^+ is produced in all eukaryotic cells. The basal intracellular NAD^+ concentration can be up to 800 μM in yeast [15], 100-400 μM in human HEK293 cells [16,17], and approximately 0.2 mmol/kg in mouse tibialis anterior muscle [18]. New methods have been developed to enable the detection of subcellular NAD^+ levels (Box 1). NAD^+ is consumed in many catabolic pathways: in the cytosol, NAD^+ is reduced to NADH by lactate dehydrogenase (LDH) during anaerobic glycolysis [7]. In mitochondria, the three tricarboxylic acid cycle enzymes, isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase (α-KGDH), and malate dehydrogenase (MDH), reduce NAD^+ to NADH. NADH serves as the primary source of reducing equivalents for complex I (NADH dehydrogenase) of the electron transport chain (ETC) to fuel OXPHOS, generating NAD^+, ultimately reducing oxygen to H_2O and producing ATP [15]. Exercise and diet can affect NAD^+ concentration in various tissues. For example, 6 weeks of exercise was shown to improve glucose tolerance and increase muscle NAD^+ in a mouse model of high-fat diet (HFD)-induced obesity [19,20]. Studies in both mice and humans have shown that exercise can change circulating NAD^+ in a biphasic manner with moderate intensity exercise increasing, and strenuous exercise reducing, NAD^+ [21]. In addition, while a high-fat diet decreases muscle NAD^+ in HFD-induced obese mice, exercise and **caloric restriction** can increase NAD^+ in the muscle and liver of obese or aged mice, respectively [19,20]. Thus, intracellular NAD^+ is not only regulated by many cellular activities, including OXPHOS, mitochondrial metabolism, transcription, and signaling, but can also be significantly influenced by diet, exercise, and other health conditions.

NAD^+ Biosynthesis

In mammals, cellular NAD^+ is synthesized from a variety of dietary sources, including NAD^+ itself (it is metabolized in the gut, then synthesized again in cells), and from one or more of its major precursors: tryptophan (Trp), nicotinic acid (NA), nicotinamide riboside (NR), nicotinamide mononucleotide (NMN), and nicotinamide (NAM) [22]. Food sources of NAD^+ and its precursors are summarized in Box 1. Depending on the bioavailability of the precursors, there are three

Box 1. NAD^+ Measurement, Subcellular Concentrations, and Molecular Precursors

Different assays are used to detect NAD^+, including high-performance liquid chromatography (HPLC)-based methods (e.g., HPLC/MALDI/MS) [16] and NAD^+/NADH enzymatic cycling assays [28] (including commercial kits). The recent development of genetically encoded fluorescent biosensors, such as SoNar and a biosensor with a bipartite NAD^+-binding domain, allows the imaging of relative levels of free NAD^+ in subcellular compartments [17,120]. For example, in HEK293T cells, the concentration of NAD^+ is similar in the cytoplasm and nucleus (approximately 100 μM), and higher in the mitochondria (230 μM) [17]. These levels are consistent with other reports demonstrating that, in highly metabolically active, postmitotic cells, such as neurons, mitochondria have higher NAD^+ levels compared with other subcellular compartments [9,121]. Moreover, the nuclear and cytoplasmic NAD^+ pools are interchangeable, while the mitochondrial pool is relatively isolated, although a substantial decrease in the cytosolic pool may influence the mitochondrial pool [17].

NAD^+ precursors include nicotinamide (NAM), nicotinic acid (NA), tryptophan (Trp), nicotinamide riboside (NR), and nicotinamide mononucleotide (NMN). Niacin (vitamin B3; NA and NAM) is abundant in eggs, fish, meat, diary, some vegetables, and whole grains. Milk is a source of NR [122], and NMN is present in various types of food, including broccoli (0.25–1.12 mg/100 g), avocado (0.36–1.60 mg/100 g), and beef (0.06–0.42 mg/100 g) [44]. NAD^+, NADH, NADPH, and NADP are also present in many foods, and ingested NAD^+ can be metabolized to precursors, including NR (mild digestion), NMN (by intestinal brush border cells [22]), NAM, and NA (deaminated from NAM by gut bacterial nicotinamidase), followed by absorption in the intestinal epithelial cells and transfer to the blood [123] (see Figure 1 in the main text). The gut can also directly absorb these NAD^+ precursors from foods and dietary supplements.

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pathways for the synthesis of NAD^+ in cells: (i) from Trp by the ***de novo* biosynthesis pathway** or **kynurenine pathway**; (ii) from NA in the **Preiss–Handler pathway**; and (iii) from NAM, NR, and NMN in the **salvage pathway** (Figure 1).

The *de novo* biosynthesis pathway and the Preiss–Handler pathway are well characterized (recently reviewed elsewhere [8,9]). Here, we focus on the salvage pathway, which is important from a translational research perspective because it produces NAD^+ from the precursors NAM, NMN, and NR; these have shown potential as dietary supplements to increase intracellular NAD^+ levels (see below). The major steps and key enzymes in this pathway are discussed in Box 2.

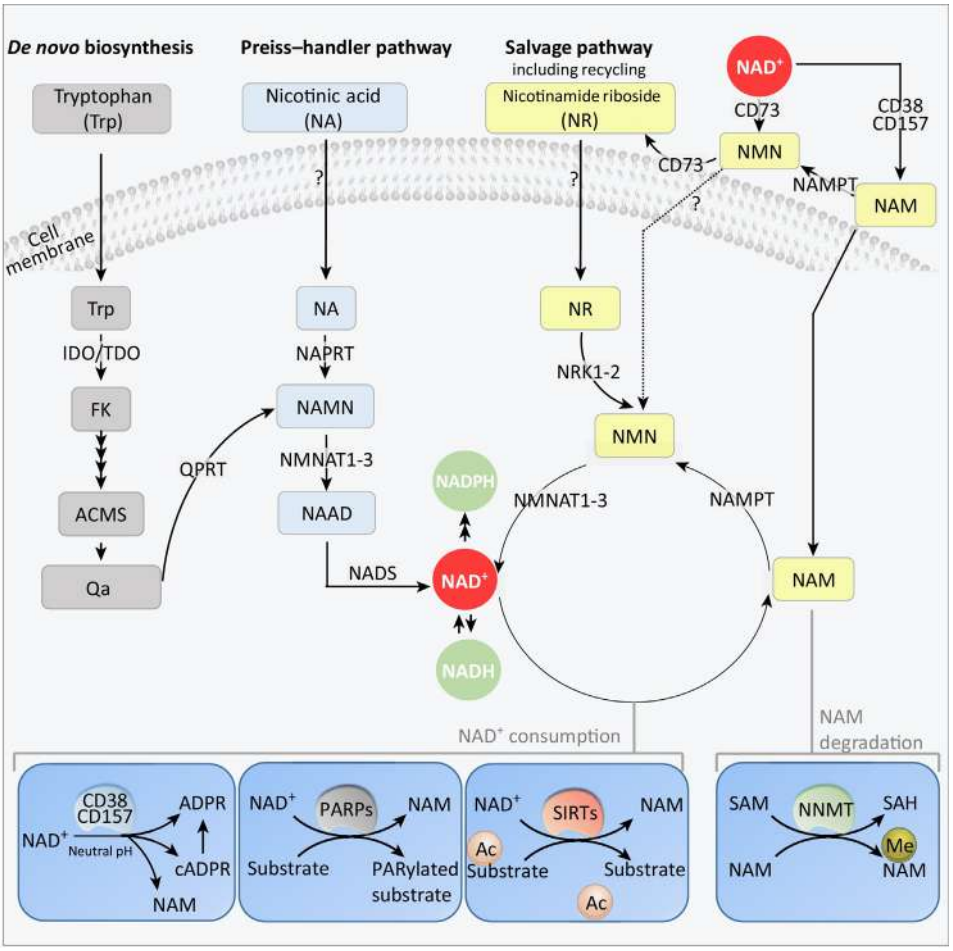
While there is inherent redundancy among the three NAD^+ synthetic pathways, distinct functions can be ascribed to the importance of specific metabolites and/or the tissue-specific expression of some of the enzymes in each pathway. The NA mononucleotide transferases (NMNATs), which are necessary for both the Preiss–Handler pathway and the salvage pathway (Figure 1), have pivotal roles in embryonic development in mice [23] and in neuroprotection across species [23–25]. *Nmnat1*$^{-/-}$ mice exhibit embryonic lethality [23] and studies in *Drosophila* suggest a general neuroprotective function for NMNAT. Specifically, overexpression of NMNAT in a *Drosophila* model of spinocerebellar ataxia 1 (SCA1)-induced neurodegeneration indicated that NMNAT could inhibit activity-induced neurodegeneration, injury-induced axonal degeneration, as well as SCA1-induced neurodegeneration [24]. In addition, mice lacking nicotinamide phosphoribosyltransferase (Nampt), a key enzyme in the salvage pathway, also exhibit embryonic lethality [26]. Furthermore, murine neurons and pancreatic β cells express low levels of Nampt protein compared with other cells and pancreatic β cells have been reported to be particularly vulnerable to NAMPT inhibition [26,27]. The importance of NAMPT has also been noted in mice with muscle-specific Nampt depletion; these mice experience fiber degeneration and loss of strength and endurance, whereas lifelong Nampt overexpression increases NAD^+ and improves the physical function of aged mice [28]. Of note, the three major NAD^+-synthesizing pathways use different substrates for the generation of NAD^+; thus, the production of NAD^+ may be dependent on both substrate availability and the local levels of synthesizing proteins (e.g., NAMPT, NMNATs, etc.).

NAD^+-Consuming Enzymes

NAD^+ is a coenzyme for three groups of enzymes: (i) deacetylases in the sirtuin family (SIRTs); (ii) ADP-ribosyltransferases, including poly(ADP-ribose) polymerases (PARPs); and (iii) cyclic ADP-ribose synthases (cADPRSs) (Figure 1). SIRTs are a group of NAD^+-dependent deacetylases and ADP-ribosyltransferases that promote mitochondrial homeostasis, neuronal survival, stem cell rejuvenation, and prevent certain aspects of the aging process, such as neurodegeneration, loss of stem cells, and mitochondrial dysfunction [29–31]. ADP ribosylation is an important protein post-translational modification that affects DNA repair, epigenetic regulation, neurodegeneration, and aging [14]. ADP ribosylation of proteins is executed by ADP-ribosyltransferases through transfer of the ADP-ribose moiety from NAD^+ to target substrates. PARPs are prominent members of the ADP-ribosyltransferase family, comprising 17 different enzymes in mammals [32]. PARPs transfer the first ADP-ribose unit from NAD^+ to target proteins, followed by the sequential addition of ADP-ribose units to the preceding ones to form poly(ADP-ribose) polymers (PARs) [32]. The cADPRSs include CD38 and its homolog CD157, in mammals and birds: CD38 and CD157 are transmembrane proteins, localized to the plasma membrane and to membranes of intracellular organelles, including the mitochondria, nucleus, and endoplasmic reticulum [33]. CD38 is expressed in immune cells, liver, testis, kidney, and brain [33]. It has important roles in several physiological processes, such as nuclear Ca^{2+} homeostasis, immunity, inflammation, as well as glucose and lipid homeostasis [34–36]. cADPRSs can function as glycohydrolases or NADases, hydrolyzing NAD^+ to NAM and ADP-ribose [9]. These NAD^+-

Glossary

3xTg AD mice: a model of AD. The mice express the human genes encoding Amyloid precursor protein (APP) and Presenilin 1 (*PS1*), similar to the APP/PS1 mouse, but also express human Tau. The mice show AD-like phenotypes, similar to APP/PS1 mice, including tau tangles and fibrils in the brain.
Amyloid beta (Aβ) plaques: formed from cleaved APP; Aβ aggregates to form large rafts of proteins that are toxic to neurons.
APP/PS1 mice: a model of AD. Mice express the human genes encoding *APP* and *PS1*. The mice show severe Aβ plaque formation and neurodegeneration and/or cognitive impairments.
Ataxia telangiectasia (A-T): a rare, untreatable, recessive inherited human disease characterized by severe neuromotor dysfunction, telangiectasia, sterility, cancer, and hypersensitivity to ionizing radiation. It is caused by mutations in the *ATM* gene, encoding ATM kinase, a regulator of the DNA damage response that is critical for genomic stability, telomere maintenance, and DNA double-strand break repair.
Autophagy: evolutionarily conserved process where cytoplasmic substrates are engulfed in an autophagic vesicle, fused to lysosomes, followed by degradation and recycling. Autophagy is necessary for cellular homeostasis through a balance with apoptosis and inflammation. Compromised autophagy occurs in many age-related diseases.
Caloric restriction: diet where the organism (e.g., mice) are not fed *ad libitum*. This restriction of food intake has been shown to increase lifespan.
Cockayne syndrome (CS): a rare premature aging disease with progressive neurodegeneration caused predominantly by mutations in genes encoding two DNA repair proteins: Cockayne syndrome group A (CSA) or Cockayne syndrome group B (CSB).
***De novo* biosynthesis pathway/ kynurenine pathway:** pathway from which NAD^+ is produced from tryptophan.
DNA damage response: the response of a cell to DNA damage. DNA damage activates a range of cellular processes, enabling the cell



Trends in Molecular Medicine

Figure 1. Nicotinamide Adenine Dinucleotide (NAD^+) Biosynthetic Pathways. NAD^+ is synthesized via three major pathways in mammals. The first is the *de novo* biosynthesis from tryptophan (Trp) (to the left) in a total of eight steps. Four steps are shown in this figure, including the conversion of Trp to formylkinurenine (FK), and a spontaneous reaction conversion of 2-amino-3-carboxymuconate semialdehyde (ACMS) to quinolinic acid (Qa). Qa is then converted to nicotinic acid mononucleotide (NAMN) by quinolinate phosphoribosyltransferase (QPRT). The second pathway is the Preiss–Handler pathway, initiated by the conversion of NA to NAMN by NA phosphoribosyl-transferase (NAPRT). NAMN, an intermediate in both *de novo* biosynthesis and the Preiss–Handler pathway, is then converted to form NA adenine dinucleotide (NAAD) by NA mononucleotide transferases (NMNATs). The link between Trp and NA shown here is the human pathway. The last step of these pathways is the conversion of NAAD to NAD^+ by NAD^+ synthase (NADS). The third pathway is the Salvage pathway, generating NAD^+ from nicotinamide riboside (NR), which also includes the recycling of nicotinamide (NAM) back to NAD^+ via NAM mononucleotide (NMN). Extracellularly, NAD^+ or NAM can be converted to NMN, which is in turn dephosphorylated to NR, possibly by CD73. NR is transported into the cell via an as yet unknown mechanism (possibly nucleoside transporters), where it is phosphorylated by nicotinamide riboside kinase (NRK)1 or NRK2 forming NMN. NMN is then converted to NAD^+ by NMNATs. The broken lines indicate that the mechanism and involved proteins are not yet known. The lower part of the figure shows the major NAD^+-consuming enzymes. From left: the cyclic ADP-ribose synthases (cADPRSs) CD38 and CD157 hydrolyze NAD^+ to NAM; in addition, CD38 can degrade NMN to NAM, removing NMN from NAD^+ synthesis. Poly(ADP-ribose) polymerases (PARPs), especially PARP1 and PARP2, use NAD^+ as a co-substrate to PARylate target proteins, generating NAM as a by-product. The deacetylation activity of Sirtuin (SIRT)1, SIRT3, and SIRT6 depend on NAD^+, generating NAM as a by-product, which can inhibit the activity of SIRTs. The enzyme NAM *N*-methyltransferase (NNMT) methylates NAM, using *S*-adenosyl methionine (SAM) as a methyl donor. This removes NAM from recycling, and indirectly affects NAD^+ levels. Abbreviations: IDO, indoleamine 2,3-dioxygenase; Me-NAM, methylated nicotinic acid mononucleotide; NAMPT, nicotinamide phosphoribosyl transferase (including extracellular and intracellular ones); TDO, tryptophan 2,3-dioxygenase.

to survive and maintain genome integrity.

Duchenne's muscular dystrophy: a genetic disorder caused by the lack of dystrophin, which results in muscle weakness and degeneration.

Hallmarks of aging: concepts or cellular and/or organismal processes all influencing and contributing to the process of aging.

Homologous recombination (HR)-based double-strand break repair (DSBR): repair process of DSBs by the repair pathway HR. HR is dependent on the cell cycle.

KK/H1J mouse model: a model often used to study metabolic syndromes because it presents inherited glucose intolerance and insulin resistance, which result in hyperglycemia. KK/HIJ mice have a strong tendency to develop type 2 diabetes mellitus in response to certain dietary regimens (e.g., high-fat diet) and aging.

Mdx mouse model: a model of Duchenne's muscle dystrophy with a phenotype resembling that of human patients, including muscle weakness and degeneration.

Metabolic syndrome: a group of risk factors and pathologies, including heart disease, diabetes, obesity, stroke, among others.

Mitophagy: a specialized form of autophagy that regulates the turnover of damaged and dysfunctional mitochondria.

***Nampt*$^{-/-}$ mice:** mice that do not express NAMPT, a key protein in the NAD^+ salvage pathway. They show embryonic lethality.

Nicotinamide adenine dinucleotide (NAD): a major coenzyme and/or compound in all human cells, exists in oxidized (NAD^+) and reduced (NADH) forms. NAD^+ has major roles in cellular energy metabolism, adaptive responses of cells to bioenergetic and oxidative stress, and aging.

Nonhomologous end joining (NHEJ)-based DSBR: the only DSBR pathway in postreplicative cells, including neurons.

Nucleotide excision repair (NER): a DNA repair pathway repairing bulky DNA adducts introduced in DNA by UV irradiation, environmental toxins, and certain antitumor agents.

Parkin: a E3 ubiquitin ligase; mutations in PARKIN, together with PINK1 (a serine/threonine kinase), are a leading cause of PD. PARKIN

Box 2. The NAD+ Salvage Pathway and Its Major Enzymes

The salvage pathway describes the formation of NAD+ from precursors including NR, NMN, and NAM. NR can be transported into cells via nucleoside transporters, although a NR-specific transporter has not yet been identified in mammals. In the cytoplasm, NR is phosphorylated by nicotinamide riboside kinases (NRK1-2) to form NMN [122]. NRKs are not expressed in mitochondria, suggesting that the phosphorylation step occurs in the cytosolic compartment [124]. In addition, extracellular interconversion of NAD^+ and its precursors may occur (see Figure 1 in the main text). Extracellular NMN can be converted from NAD^+ and NAM, with the latter in a nicotinamide phosphoribosyl-transferase (NAMPT) (extracellular)-dependent manner. NMN can either enter the cells directly, although a mechanism that is still unclear, or be converted to NR before transport into cells [124].

(Intracellular) NAMPT first converts NAM to NMN, which is then adenylated to NAD^+ by NMNATs. NMNATs are the final step in NAD^+ synthesis and are also involved in the Preiss–Handler pathway. In humans, there are three NMNAT isoforms with different subcellular localizations. NMNAT1 is localized in the nucleus, and shows the highest expression in skeletal muscle, heart, kidney, liver, and pancreas [125]. NMNAT2 is primarily found in the cytosol, tethered to the outer membrane of the Golgi apparatus by palmitoylation. NMNAT3 is localized to mitochondria and expressed at high levels in skeletal muscle and heart tissue [125]. Thus, intracellular NMN can be converted to NAD^+ in the nucleus, cytoplasm, or the mitochondria by one of the three NMNATs. It is still unclear how cytoplasmic NMN is imported into mitochondria.

NAM is not only an ingested NAD^+ precursor, but is also a byproduct of the degradation of cellular NAD^+ by NAD^+-consuming enzymes (detailed below). In mammalian cells, nicotinamide *N*-methyltransferase (NNMT) removes NAM from the NAD^+ biosynthesis pathway through methylation of NAM, which is further degraded into *N*-methyl-2-pyridone-5-carboxamide (Me2PY) and *N*-methyl-4-pyridone-5-carboxamide (Me4PY) [77].

consuming enzymes regulate a spectrum of cellular activities, including mitochondrial maintenance, DNA repair, and stem cell rejuvenation, processes that are critical for cellular health (see also recent reviews [9,11,35]).

Crosstalk among NAD^+-Consuming Enzymes: SIRTs, PARPs, and cADPRSs

The different classes of NAD^+-consuming enzymes compete for bioavailable NAD^+, which affects their cellular functions in human health. Thus, hyperactivity of one protein might limit the activities of the others and, conversely, inhibition of one protein may increase the NAD^+ pool for the others [35]. For example, SIRT1 activity can be substantially decreased when there is excessive PARP1 activation, and PARP1 or PARP2 deletion in human kidney cells and in *in vivo* models (*Parp2*$^{-/-}$ mice) have been reported to increase SIRT1 activity [37,38]. Persistent activation of PARP1, caused by DNA damage, has resulted in a greater than 50% decrease in cellular NAD^+ in DNA repair-deficient primary rat neurons and human neuroblastoma cells [14,39]. Moreover, in DNA repair-deficient human neuroblastoma cells, as well as in mouse and *Caenorhabditis elegans* models, cellular NAD^+ and Sirt1 activity have been reported to increase after treatment with Parp1 inhibitors {3-aminobenzamide, 3,4-dihydro-5-[4-(1-piperidinyl) butoxyl]-1(2H)-isoquinolinone} or after supplementation with NAD^+ precursors (NR or NMN) [14,39]. These studies suggest that PARP1 inhibition constitutes a potential therapeutic target to sustain cellular NAD^+ and to maintain SIRT activity.

CD38 is another major NADase in tissues. In **APP/PS1** mice, CD38 depletion and increased NAD^+ led to neuroprotection, as evidenced from a reduction in amyloid beta (Aβ) aggregates in the brain, which was associated with improved learning [40]. In addition, CD38-deficient mice showed protection against HFD-induced obesity and **metabolic syndrome**, exhibiting higher metabolic rates compared with wild-type mice [41]. This resistance to diet-induced obesity has been attributed, at least in part, to NAD^+-dependent activation of Sirt1 and the mitochondrial regulator peroxisome proliferator-activated receptor γ coactivator (PGC-1α) [41]. Of note, CD38-deficient mice show significant neuroprotection in the brain despite high levels of **PARylation** [42], indicating that the available NAD^+ appears to be sufficient in enabling the activity of Parp1, Sirt1, and other NAD^+-dependent enzymes. A recent study showed an age-dependent increase of CD38 in murine tissues, and documented (using CD38-knockout mice)

and PINK1 are also involved in mitophagy.

PARylation: post-translational modification performed by PARPs, mainly PARP1 in mammalian cells, via the use of NAD^+. PARylation is also known as poly(ADP-ribosylation).

Preiss–Handler pathway: pathway from which NAD^+ is produced from nicotinic acid.

Premature aging syndromes (accelerated aging/progeria): rare diseases in which patients show aspects of aging at a very early age.

Salvage pathway: primary pathway from which NAD^+ is produced from NR, NMN, or NAM.

Tau tangles: rafts or aggregates of Tau proteins, often hyperphosphorylated, causing dysfunction and cell death of affected neurons.

Xeroderma pigmentosum (XP): a rare autosomal-recessive disorder characterized by severe sun sensitivity and skin cancer. The etiology of XP is caused by mutation of genes encoding a group of DNA repair proteins, *XP* genes.

γ-H2AX foci: foci that occur when the histone H2A variant H2AX is phosphorylated rapidly after a DSB induction in DNA. Given that the phosphorylation of H2AX occurs rapidly after the induction of DSBs and correlates well with DSBs, it is often used as a DSB marker.

that CD38 was required for the observed age-related NAD^+ decline [43]. This is interesting because CD38 not only consumes NAD^+, but can also degrade the NAD^+ precursor NMN [43]. Thus, there appears to be a delicate balance between NAD^+ consumption and bioavailability, which is important for cellular function and survival. However, further studies of the functional significance of the interconnected networks between NAD^+-consuming enzymes is warranted.

NAD^+ in Aging and Age-Related Diseases

Age-Dependent Decrease of NAD^+ in Animals and Humans

Mice display an age-dependent decrease of NAD^+ in multiple organs, including brain, liver, muscle, pancreas, adipose tissue, and skin [14,44–46]. In *C. elegans*, an age-dependent reduction of NAD^+ has also been reported [14,39]. There is also evidence of decreased NAD^+ in aged human tissues. Specifically, *in vivo* NAD^+ assays have been used to demonstrate that intracellular NAD^+ declines with age in the human brain [47]. Also, NAD^+ in post-pubescent males and females negatively correlates with age [48]. Together, these data suggest that there is a universal age-dependent decrease of cellular NAD^+ across species. However, it is not clear whether this is due to increased NAD^+ consumption and/or decreased production.

NAD^+ Replenishment Can Improve Lifespan and Healthspan

The effects of different NAD^+ precursors, including NR and NMN, on the lifespan and healthspan of yeast, *C. elegans*, *Drosophila*, and mice have been investigated. One study showed that 10 μM NR could extend the replicative lifespan of wild-type yeast by more than ten generations [15]. In this yeast model, two NAD^+ synthetic pathways appeared to be necessary for the NR-induced lifespan extension, the tNrk1 and the Urh1/Pnp1/Meu1 pathways [15]. In *C. elegans*, 500 μM NR extended the average lifespan of wild-type worms (N2) via the SIR-2.1 (ortholog to mammalian SIRT1) pathway [39]. For *Drosophila*, no information is available regarding a direct effect of NAD^+ precursors on lifespan, but genetic overexpression of an NAD^+ synthetic enzyme nicotinamidase (D-NAAM) has been reported to extend lifespan [49]. D-NAAM, an ortholog of yeast PNC1, functions in the NAD^+ salvage pathway and converts NAM to NA [49]. In *Drosophila*, overexpression of D-NAAM can increase the NAD^+:NADH ratio, as well as the mean and maximal lifespan by up to 30% in a Sir2-dependent manner [49]. Notably, NR has been shown to improve mouse lifespan, even when administered late in life. At approximately 2 years of age, C57BL/6J mice were given NR, resulting in a significant increase in lifespan (5%) [46]. Supplementation with NAD^+ precursors not only extends lifespan, but also improves healthspan in yeast, flies, worms, and mice, as shown by various features, including improved mitochondrial health, muscle strength, and motor function [15,39,46,49–51]. In summary, these data suggest that NAD^+ replenishment delays normal aging in laboratory animal models.

NAD^+ may also delay the onset of aging in some premature aging diseases. These are a group of rare diseases in which patients exhibit aging features at a younger age. DNA repair impairment is a cause of many of these diseases, and some patients exhibit severe neurodegeneration, as in the case of **Xeroderma pigmentosum group A** (XPA), **Cockayne syndrome** (CS), and **Ataxia-telangiectasia (A-T)** [14]. XPA is caused by mutations in the *XPA* gene, which participates in **nucleotide excision DNA repair** (NER) [52,53]. The etiology of CS has been related to mutations in two proteins, CS group A (CSA) and CS group B (CSB) [54,55]. A-T is a multifaceted disease caused by mutations in A-T mutated protein (ATM), a master regulator of the **DNA damage response.** ATM has a key role in DNA double-strand break repair (DSBR). Interestingly, all three premature aging diseases show mitochondrial dysfunction and NAD^+ depletion (demonstrated in *C. elegans*, mice, and human cells) [14]. Furthermore, NAD^+ replenishment, using NR and/or NMN, improved the lifespan and healthspan of *C. elegans* in relevant models of XPA, A-T, and CS, relative to controls [14,39,56]. Remarkably, NR extended the lifespan in $Atm^{-/-}$ mice (B6;129S4-Atm^{tm1Bal}/J), which typically

perish at 3∼5 months of age. Specifically, NR supplementation was given at 12 mM in drinking water after weaning, resulting in 80% survival at 10 months of age [39]. In *C. elegans*, NAD^+ supplementation has been shown to improve neuronal DNA repair through deacetylation of the DNA repair protein Ku70, and to restore mitochondrial homeostasis via the mitophagy regulator NIX (DCT-1 in *C. elegans*) [39]. The DCT-1 mechanism of mitochondrial homeostasis was implicated based on evidence that DCT-1 co-localized with mitochondria in *Atm-1* worms [39]. Of note, NR and NMN exhibited similar beneficial effects in A-T models of *C. elegans* and mice [39]. In another example, mice hypomorphic for BubR1 (a mitotic checkpoint kinase) also presented with signs of premature aging, and mice overexpressing BubR1 exhibit an extended lifespan [57]. This is pertinent because loss of BubR1 during aging can result from NAD^+ depletion and decreased Sirt2 activity, rendering Sirt2 unable to deacetylate BubR1 (normally Sirt2 deacetylates BubR1, targeting it for ubiquitination and degradation) [57]. Both overexpression of Sirt2 or NMN treatment increased the lifespan of BubR1 mice, suggesting that BubR1 stabilization is important for achieving an increased lifespan [57]. In summary, restoration of intracellular NAD^+ has been shown to improve lifespan and healthspan in normal and prematurely aged laboratory organisms, but whether these findings can be translated to humans remains unproven.

NAD^+ and Neurodegeneration

Age is the greatest risk factor for neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), and hearing loss [58]. Recent studies in AD animal models suggest that **Aβ plaques**, **tau tangles**, and mitochondrial dysfunction (due to compromised mitophagy) are among the key features of AD [59]. Strategies to increase intracellular NAD^+ are considered as novel potential therapeutic interventions in AD. For example, 3 months of NR treatment has been documented to attenuate cognitive deterioration through Aβ reduction in the cortex and hippocampus in a mouse model (AD mouse crossed with a PCG-1α KO mouse) [60]. The study suggests that NR treatment promotes PGC-1α activity and induces the ubiquitin proteasome system, leading to degradation of Aβ aggregates [60]. Similarly to NR, NMN has also been found to ameliorate mitochondrial dysfunction and neuronal death in APP/PS1 mice, as evidenced from restored oxygen consumption rates, increased levels of Sirt1 and PGC-1α, and normalization of morphology of brain mitochondria from NMN-treated APP/PS1 mice [61]. NMN was also found to protect against Aβ oligomer-induced cognitive impairment, neuronal death, and cognitive dysfunction in a rat model of AD [62]. In another study, treatment with a different NAD^+ precursor, NAM, delayed pathology and cognitive decline in **3xTg AD mice,** through upregulation of neuronal bioenergetics, including neuroplasticity-involved kinases and transcription factors, as well as by improving autophagy processing (reduced autophagosome accumulation mediated by enhanced lysosome and/or autolysosome acidification) [63]. At the molecular level, NAD^+-dependent reduction of AD phenotypes may be attributed to the upregulation of autophagy and/or mitophagy because NAD^+/SIRT1 are able to upregulate autophagy through deacetylation of the major autophagy proteins Atg5, Atg7, and Atg8 in human cells, murine neurons, and *C. elegans* [39]. Furthermore, NAD^+/SIRT1 has been shown to upregulate mitophagy through the forkhead box-O3 (FOXO3)-NIX (BNIP3L/DCT-1) axis in *C. elegans* and in calorically restricted mice, or indirectly through an interaction between PGC-1α and **Parkin**, as evidenced from co-expression of PGC-1α and Parkin in murine cortical neurons that led to improved mitochondrial biogenesis and mitophagic activity [39,64–66]. Accordingly, upregulation of autophagy and/or mitophagy has been reported to clear Aβ plaques, tau tangles, and damaged mitochondria in mice, leading to improved mitochondrial function and neuronal survival [59,63,67].

PD appears to involve impairment of mitochondrial complex I, as well as compromised mitophagy in vulnerable neurons of the brain, including midbrain dopaminergic neurons [68]. There are several promising studies that suggest that NAD^+ precursors have therapeutic

potential in PD. Early clinical studies showed that supplementation (intravenous infusion or oral capsules) with the reduced form of NAD^+ (NADH) improved motor disability in patients with PD [69]. Recently, in models of PD *in vitro* (rotenone-treated PC12 cells) and *in vivo*, in *Drosophila* bearing *pink1* mutations, studies have shown that NR and NAM administration could ameliorate PD phenotypes [70,71]. For example, dietary NAM (5 mM) rescued thoracic defects and inhibited the loss of dopaminergic neurons in *pink1* mutant Drosophila, possibly through maintenance of a healthy mitochondrial pool, as evidenced from improved mitochondrial morphology and respiration [71]. However, further studies of NAD^+ precursor treatments in PD mice are necessary to elucidate the underlying molecular mechanisms and functional roles of NAD^+ in PD pathology.

Hearing loss is a common feature associated with advanced age, and noise exposure is a major cause of such loss [72]. Intense noise exposure can result in direct mechanical damage (acoustic trauma) to cochlear hair cells, and can trigger the delayed death of these auditory sensory neurons. NR administration (intraperitoneal injection) has been found to protect mice from transient and permanent noise-induced hearing loss and spiral ganglia neurite degeneration, as shown by the retraction of neurite ends from inner hair cells in the cochlea [73]. These effects appear to be mediated by NAD^+-dependent mitochondrial Sirt3, since the effect of NR was reduced in Sirt3-deficient mice [73]. This role of Sirt3 is supported by studies showing that caloric restriction prevents hearing loss in wild-type mice, but not in Sirt3-deficient mice [74]. Moreover, ototoxicity, caused by chemical damage to the inner ear, has been reported to be prevalent in patients with cancer receiving cisplatin chemotherapy [75,76]. Cisplatin causes DNA damage by crosslinking the two DNA strands. Increased NAD^+ has been reported to prevent cisplatin-induced cochlear damage through suppression of oxidative stress, DNA damage, and inflammatory responses in mice, as shown by a decrease in γ-H2AX signals, increased Sirt1 activity, and activation of p53 and NF-κB, proteins involved in inflammation in cochlear tissue [75,76]. These studies support the hypothesis that NAD^+ supplementation could provide a putative treatment option to preserve hearing during normal aging, or in individuals undergoing chemotherapeutic treatment. However, rigorous testing will be required to determine whether NAD^+ supplementation might modify the effectiveness of the chemotherapeutic treatment.

NAD^+ Can Mitigate Age-Associated Muscle Atrophy and Metabolic Disorders in Laboratory Animal Models

Studies in *C. elegans* and mice suggest that NAD^+ supplementation delays the onset of muscle atrophy, vision loss, as well as certain age-related diseases that might include metabolic disease, heart dysfunction, and cancer [39,44,50,73,77–82]. In Table 1 (Key Table), we summarize the known organismal benefits of NAD^+ supplementation for specific animal models, humans, NAD^+ precursor doses, and putative molecular mechanisms underlying these improvements.

Skeletal muscle mass and strength is reduced with aging as a result of muscle atrophy, leading to significant susceptibility to injury and reduced quality of life [83]. In the *mdx* mouse model of **Duchenne's muscular dystrophy**, which exhibits low muscle NAD^+, NR protects against disease progression through maintenance of muscle stem cell function and regeneration, where Sirt1-dependent enhancement of mitochondrial function and energetics as well as a reduction of PARylation have been implicated [46,77]. In another study, muscle-specific depletion of $Nampt^{-/-}$ in mice (***mNampt*$^{-/-}$ mice**) caused myocyte necrosis and progressive loss of muscle function, as well as inducing proinflammatory and regenerative transcriptional programs coincident with alterations in glucose metabolism [28]. In this model, oral NR supplementation reversed deficits in muscle mass, strength, and exercise capacity of $mNampt^{-/-}$ mice to wild-type levels [28]. In combination, these studies suggest that NAD^+

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Key Table

Table 1. Known Benefits and Mechanisms of Action of NAD^+ Precursors in Humans and Animal Models

Tissue	Supplement dose	Benefits	Pathways affected	Refs
Human				
Neurons 	NR 500 μM	Decreased AT pathology and restored mitochondrial function in ATM KD cells	↑ NAD^+, SIRT1 activation, BDNF levels and CREB activation, ↓ DNA damage	[38]
Blood 	Acipimox 250 mg/3×/d	↓ triglycerides and glucose in plasma of patients with T2DM; restored levels of NEFA	2-fold ↑ NAD^+	[11]
	NR 1000 mg/d/7 d		45-fold ↑ NAAD	[7]
Muscle 	Acipimox 250 mg/3×/d	Improved mt function in patients with T2DM; ↑ lipid content in patient skeletal muscle, due to ↑ NEFA	Gene sets affected were similar to those affected by NR and NMN in animal models	[6,11]
Worm				
Neurons 	NR 500 μM	Improved long- and short-term memory in Atm worms	↑ NAD^+, activation of sir-2.1, ↑ CREB, HSP-6	[38]
Muscle 	NR 500 μM	Improved mt network in Atm worms.	↑ NAD^+, sir-2.1 activation, CREB, HSP-6	[38,45,76]
Lifespan	NR 500 μM; NAM 200 μM; NR 500 μM	↑ Lifespan and improved fitness with age in wild-type N2 worms; ↑ lifespan of Atm worms	↑ NAD^+, sir-2.1, CREB. HSP-6, mt content and ATP, improved metabolism	[38,44]
Mouse				
Brain 	NR 400 mg/kg/d	↑ Neurogenesis; ↓ cognitive deterioration and Aβ production; ↑ synaptic plasticity	↑ NAD^+, activation of Sirt1 and PGC-1α and degradation of Bace1; ↑ LC3-II and altered fission/fusion balance	[45,59]
	NR 250 mg/kg/d			
	NR or NMN 12 mM	NR abolished metabolic profile of cerebellum from Atm KO mice; NR and NMN improved mt morphology and health, leading to neuron protection		
Ear/cochlear 	NR 1000 mg/kg/2×/d	Prevented transient and permanent hearing loss (noise-induced) by preventing degeneration of spiral ganglia neurites	↑ NAD^+ and Sirt3 activation	[38,72]
Eye 	NMN, 100 or 300 mg/kg/d	Prevented age-associated decline of rod and cone cell function; increased tear production.	Likely due to ↑ NAD^+ level and sirtuin activity	[43]
Muscle 	NR 400 mg/kg/d	↑ NAD^+ and mt content, prevented mt myopathy	Sirt1 and Sirt3 activation, and their targets Foxo1, Sod2, PGC1α, UPR^{mt}, Fgf21; improved mt function and mitophagy mediated via Sirt1 and EglN; ↑ mtDNA encoded proteins and/or nuclear encoded proteins	[43,45,49,76,80,84]
	NR 750 mg/kg/d	↑ Lifespan of $Trf1^{hrt/hrt}$ mutants and improved cardiac function		
	NMN 500 mg/kg/d	Reversed age-associated muscle atrophy and inflammation, impaired insulin signaling, and insulin-stimulated glucose uptake		
	NMN 300 mg/kg/d	Improved mitonuclear signaling and mt function		
Stem cells 	NR 400 mg/kg/d	Rejuvenated muscle stem cells; attenuated senescence in neuronal and melanocyte stem cells	Activation of UPR^{mt}, prohibitin pathways	[45,76]

Table 1. (continued)

Tissue	Supplement dose	Benefits	Pathways affected	Refs
Fat 	BAT: NR 400 mg/kg/d	↑ Mt content and mt respiratory capacity, reduced fat mass	↑ NAD^+ levels, Sirt1 and Sirt3 activation	[36,37, 50,84]
	WAT: IP NMN 500 mg/kg/d	Restored NAD^+ levels in diabetic mice and normalized glucose tolerance		
Liver 	NR 400 mg/kg/d, 500 mg/kg/d	↑ Oxidative profile, biogenesis, content and activity of mt; decreased tumorigenesis and DNA damage; prevented fatty liver and inflammation induced by high-fat high-sucrose diet	Sirt1 and Sirt3 activation, and Sirt1 target genes including Akt, prevention of NAFLD, decreased DNA damage	[43,50, 84,85]
	NMN 500 mg/kg/d, 300 mg/kg/d	Improved hepatic insulin sensitivity, decreased oxidative stress, and improved inflammatory response, immune response, and lipid metabolism		
Pancreas 	NMN 500 mg/kg/d	Improved glucose-stimulated insulin secretion	↑ NAD^+ and Sirt1 activation	[50]
Lifespan	NR 400 mg/kg/d	↑ Lifespan of mice	↑ NAD^+, Sirt1 and prohibition activity; improved mt function; mitophagy, DNA repair, and anticancer potential	[38,45]
	12 mM NR throughout life	↑ Lifespan of Atm KO mice		

Abbreviations: BAT, brown adipose tissue; KO knockout; mt, mitochondrial; T2DM, type 2 diabetes mellitus; UPR, unfolded protein response; WAT, white adipose tissue; ↑ increase; ↓ decrease.

supplementation inhibits muscular dystrophy in mice, which may have interesting implications for aged individuals.

NAD^+ replenishment has also been shown to have beneficial affects against obesity and/or other metabolic diseases. NR can enhance oxidative metabolism and protect mice against HFD-induced obesity and nonalcoholic fatty liver disease (NAFLD) by activating Sirt1 and Sirt3, and enhancing the energy expenditure and oxygen consumption rate of mitochondria [84,85]. NR induced a Sirt1- and Sirt3-dependent mitochondrial unfolded protein response, thereby improving mitochondrial metabolism (increasing mitochondrial complex formation and activity) [85]. NR administration (via an osmotic pump) lowered serum insulin levels and serum cholesterol concentrations in the **KK/H1J mouse model** of type 2 diabetes mellitus [86]. Other NAD^+ precursors, such as NMN (delivered intraperitoneally), have been reported to improve diet- and age-induced diabetes in 2-year-old mice by enhancing hepatic insulin sensitivity and antioxidative responses, including the production of glutathione *S*-transferase, among others [51]. NAD^+ precursors can also act partly through Sirt1 activation, as demonstrated by the abrogated effect of NMN via Sirt1 inhibition with EX527 (a Sirt1-specific inhibitor) [51]. NMN administration also ameliorated the impairment in glucose tolerance and glucose-stimulated insulin secretion in *Nampt*$^{+/-}$ mice [26].

The aforementioned data strongly suggest that NAD^+ replenishment has systemic benefits in aged laboratory animals and disease models in mice, although further testing is required. Importantly, translational human intervention studies with NAD^+ supplementation are already in progress[i].

Potential NAD^+-Mediated Mechanisms to Counter Aging

While phenotypic studies of NAD^+ replenishment on aging have been extensively explored in laboratory animals, the exact molecular mechanisms responsible for their beneficial effects are not yet understood. To dissect the interconnected mechanisms of the multifaceted functions of NAD^+ to aging, we can associate NAD^+ with most of the nine 'hallmarks of aging' [58] (Table 2). We also consider autophagy impairment as a NAD^+-related hallmark of aging.

Effects of NAD^+ Supplementation on DNA Repair

Cumulative evidence indicates that impaired genomic maintenance may causally contribute to aging. An age-dependent accumulation of DNA damage occurs in humans, possibly due to impaired DNA repair [87]. This suggests that maintenance of efficient DNA repair may delay the onset of aging and age-related diseases [88,89]. NAD^+ replenishment can improve DNA repair in cells, *C. elegans*, and mice. An early study reported that suitable doses of NAM (~3 mM) significantly enhanced DNA repair in gamma-irradiated XP cells *in vitro* [90]. In line with this finding, studies in human aortas suggest that the NAM-consuming enzyme NAMPT has a significant role in DNA repair to maintain genome integrity, as demonstrated from an increase in DNA oxidative DNA lesions and DNA DSBs in murine *Nampt*-deficient murine smooth muscle cells [91]. In addition to NAM, NA and NR also improve DNA repair *in vitro.* Indeed, studies revealed increased DNA repair *in vitro* in peripheral blood mononuclear cells, where NA treatment increased DNA repair efficiency and decreased micronuclei numbers following X-ray irradiation, and in murine Atm-deficient neurons, following NR administration [39,92]. NAD^+ supplementation can also increase DNA repair *in vivo*, because wild-type mice treated with NR showed decreased DNA damage, as evidenced from lower global PARylation and **γ-H2AX foci** relative to controls [46,77]. As previously mentioned, NAD^+ replenishment can also improve aging features of some DNA repair-deficient premature aging disorders (XPA, CS, and A-T), such as neurodegeneration, possibly through the upregulation and/or activation of DNA repair and mitophagy [14,39,93]. For example, due to the dysfunction of the DSBR protein ATM, accumulation of DNA damage occurs in both nuclear and mitochondrial genomes, as revealed from human patient fibroblasts and brain-tissue from Atm-deficient mice [94,95]. NR supplementation improved genomic stability in murine Atm-deficient neurons and *C. elegans* models of A-T, at least partially through Sirt1/Sirt6-dependent DSBR, upon protection against ionizing irradiation [39]. In neurons, there is no **homologous recombination (HR)-based DSBR**, only **nonhomologous end joining (NHEJ)-based DSBR** [96]. In murine Atm-deficient neurons, NR increases DNA-PKC-associated NHEJ through deacetylation of Ku70 [39]. These findings suggest that enhancing NAD^+ bioavailability can also target and increase DNA repair.

NAD^+ Maintains Mitochondrial Health

Mitochondrial abundance and quality are pivotal for health, and mitochondrial dysfunction is a hallmark of aging, detected in a broad spectrum of age-associated diseases [97]. NAD^+ replenishment can inhibit age-dependent mitochondrial decline or production in both *C. elegans* and mice [45,46]. For example, dietary NR treatment can compensate for a respiratory chain defect and reverse exercise intolerance in the mitochondrial disease mouse model $Sco2^{KOKI}$ [98]. NAD^+ can also bolster mitochondrial function by enabling mitochondrial biogenesis and mitophagy; mitochondrial biogenesis and respiration are induced by PGC-1α through the transcriptional upregulation of *Nrf1* and *Nrf2* in mouse myoblasts [99]. Thus, NAD^+ replenishment has been documented to induce mitochondrial biogenesis through the NAD^+/Sirt1-PGC1α pathway in aged mice, murine muscle stem cells, and *C. elegans* [45,46]. Indeed, NAD^+ can promote the removal of damaged and/or dysfunctional mitochondria via mitophagy, as detailed below.

NAD^+ in Autophagy Induction

Autophagy has multifaceted roles in health and aging, including the maintenance of cellular homeostasis, cellular energy (especially during nutrient starvation), neuroprotection, and anti-inflammation [13,67,100]. Compromised autophagy is a common signature of aging and contributes to age-related diseases, such as AD and PD [13,35,67]. Upregulation of autophagy can inhibit disease progression in animal models of AD and PD (3xTgAD models) [59,60,63,67]. Moreover, mutations of proteins in the autophagy pathway, including Atg1 (unc-1 in *C. elegans*) and other proteins encoded by the *Atg* genes, can lead to premature aging in yeast,

Table 2. NAD^+ and the Hallmarks of Aging

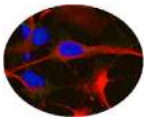
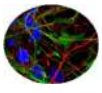
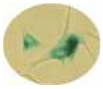
Hallmarks of aging	Pathways and conditions affected by changes in NAD^+ levels	Refs
Genomic instability 	↑ NAD^+ levels lead to ↑ DNA repair capacity; ↓ NAD^+ leads to ↑ ROS accumulation	[11,14,38,55]
	Mutations in DNA repair genes associated with premature aging disorders (e.g., XPA and CS), lead to ↓ NAD^+, PARP1 hyperactivation, neurodegeneration, and mitochondrial dysfunction	
	SIRT1 interacts with DNA repair enzymes Ku70, PARP1, and WRN. PARP1 is involved in both BER and NER; SIRT3 enhances NHEJ and HR via interaction with PARP1	
Telomere attrition 	Data not available	
Epigenetic alterations 	Treatment with NAD^+ precursors NR and NMN activates SIRTs, which deacetylate and activate transcription factors, including PGC-1α, FOXOs, and others, all related to aging	[8,9,44,49,59]
	PARP1 may be involved in chromatin structure modulation and insulation promotion, associated with changes in gene expression. Also, PARP-1 may function as a transcriptional co-factor. PARP-2 transcriptionally regulates SIRT1, indirectly connecting NAD^+ levels to epigenetic alterations	
Stem cell exhaustion 	NR treatment rejuvenated muscle, neuronal, and melanocyte SC pools through induction of UPR^{mt} and synthesis of prohibitin proteins; leads to ↑ oxidative respiration and/or ATP levels and higher mitochondrial membrane potential	[45,76]
	NR treatment prevented senescence of muscle SC in mouse model of muscular dystrophy	
	SIRT1 maintains naïve state of pluripotent SC by deacetylating Oct4. Restoration of NAD^+ in aged somatic cells (overexpression of NNT or NMNAT3) enhanced reprogramming efficiency and prolonged lifespan of mesenchymal SC by delaying senescence	
Loss of proteostasis 	NAD^+ precursor NR, or PARP1 inhibitors, activate UPR^{mt}, causing translocation of FOXO transcription factors, triggering ↑ antioxidant defenses in mice and worms, prolonging lifespan and health. UPR^{mt} activation also observed in yeast	[26,38,39,44,59]
	NR treatment of an AD mouse model (Tg2576) ↑ NAD^+ levels and PGC-1α-mediated degradation of Bace1 leading to ↓ Aβ production. AD mice crossed with CD38 KO mice showed attenuated AD pathology, suggesting that ↑ NAD^+ leads to ↓ aggregated dysfunctional proteins, such as Aβ	
Mitochondrial dysfunction 	Mouse and worm models of XPA, CS, and A-T show impaired mitophagy and mitochondrial biogenesis, likely due to ↓ activity of NAD^+-SIRT1-PGC-1α axis. These defects can be restored by ↑ NAD^+ with NAD^+ precursors	[14,40,49,50,84]
	↑ NAD^+ in aged mice restores mitochondrial function to that of young mice in a Sirt1-dependent manner either via PGC-1α/β or AMPK	
	↓ NAD^+ leads to ↓ TFAM signaling, likely via HIF-1α stabilization, resulting in ↓ mitochondrial biogenesis and loss of mitochondrial homeostasis. Short-term treatment with NMN ↑ NAD^+ and restored mitochondrial homeostasis in mice via Sirt1-PGC-1α activation	
Deregulated nutrient sensing 	NAD^+ levels affected in DIO. NR treatment can help to prevent high-fat DIO by ↑ NAD^+ and stimulating SIRT1 activity	[9,40,49,50,84]
	NNMT deficiency protects against DIO by ↑ SAM and NAD^+ in adipose and liver. Beneficial effects of calorie restriction are lost when SIRT1 and SIRT3 are inhibited	
	NMN treatment of a diabetes mouse model ameliorated glucose intolerance and ↑ hepatic insulin sensitivity or secretion by restoring NAD^+ levels (likely due to ↑ SIRT1 activity)	
	NR/NMN treatment of mice fed a HFD ↑ use of lipids as substrates, ↑ energy expenditure, and improved insulin sensitivity	
Altered cellular communication 	NR treatment prevented noise-induced hearing loss and led to regeneration of neurite ganglia mediated by NAD^+-dependent SIRT3 activity. ↑ NAD^+ directed SIRT1 activity delays axon degeneration	[25,40,50,72]
	NMN treatment reversed age-related changes in expression of genes related to inflammation, partly by increasing SIRT1 activity. Parp1 KO mice, CD38 KO mice, and NNMT KO mice exhibit ↑ NAD^+ levels and SIRT1 activation, correlating with ↓ risk of high-fat DIO	
Cellular senescence 	NAD^+ concentrations ↓ during senescence	[8,45,47]
	NAD^+ levels ↓ in aged human tissues, resulting in changes in oxidative stress and cellular metabolism, suggesting link between NAD^+ metabolism and senescence	

Table 2. (continued)

Hallmarks of aging	Pathways and conditions affected by changes in NAD^+ levels	Refs
	NR ↓ senescence in both neuronal and melanocyte SC by improving mitochondrial function, dependent on SIRT1 function	
	↑ NAD^+ (overexpression of NNT and NMNAT3) delays senescence in mesenchymal SC	
Comprised autophagy 	Exogenous NAD^+ administration ↑ autophagy in retinal pigment epithelium	[105]
	↑ NAD^+ synthesis, caused by AMPK activation through SIRT1/mTOR activation, leads to induction of autophagy in senescent cells	

Abbreviations: BER, base excision repair; DIO, diet-induced obesity; KO, knockout; mt, mitochondrial; ROS, reactive oxygen species; SAM, *S*-adenosyl methionine; SC, stem cell; UPR, unfolded protein response; ↑ increase; ↓ decrease.

nematodes, and *Drosophila*, and treatment with autophagy inhibitors can reduce their lifespan [101–103].

Autophagy induction may delay the onset of aging and potentially slow the initiation or progression of age-related diseases [13]. For example, upregulation of autophagy inhibits major markers of aging, including inflammation and senescence. Indeed, autophagy can inhibit inflammation through the regulation of immune mediators and their interaction with innate immune signaling pathways by removing endogenous inflammasome agonists [104]. In senescent cells, AMP-activated protein kinase (AMPK) can be inactivated; however, pharmacological activation of AMPK has been found to inhibit cellular senescence through NAD^+/SIRT1 induction and autophagy upregulation [105]. At the organismal level, pharmacological or genetic upregulation of autophagy has been reported to extend healthspan and lifespan in laboratory animal models [13]. Impaired autophagy might occur both upstream and downstream of other aging hallmarks, including loss of proteostasis and stem cell exhaustion. Moreover, mitophagy, which has a major role in mitochondrial maintenance, increases in an age-dependent manner [66].

NAD^+ has a fundamental role in the initiation of autophagy and is likely a key regulator in the molecular mechanism of autophagy induction. For instance, the NAD^+/SIRT1 signaling pathway stimulates autophagy, and several findings support a crucial role for SIRT1 in autophagy: (i) SIRT1 is required for autophagy induction and SIRT1 overexpression increases autophagic flux; (ii) the SIRT1 activator resveratrol induces autophagy; and (iii) autophagy is required for the lifespan prolonging effect of SIRT1 [13,106,107]. Several mechanisms have been implicated in NAD^+/SIRT1-mediated autophagy. For instance, the NAD^+/SIRT1 pathway may upregulate macroautophagy through the deacetylation of autophagy proteins, including Atg5, Atg7, and Atg8/LC3 [108]. NAD^+/SIRT1 may also stimulate autophagy and/or mitophagy through activation of the AMPK pathway: activated AMPK can upregulate autophagy and/or mitophagy through the phosphorylation of ULK1, the human autophagy protein 1 (hATG1) [109]. Given the strong association between NAD^+ and AMPK [18,105,110], it is likely that NAD^+ may also induce autophagy and/or mitophagy through activation of AMPK. In agreement, the upregulation of the rate-limiting enzyme in NAD^+ synthesis, Nampt, induces autophagy through the NAD^+/Sirt1 pathway in primary rat cortical neurons [111]. Specifically, NAD^+/Sirt1 can activate AMPK in primary rat cortical neurons, which then phosphorylates Tuberous Sclerosis Complex 2 (Tsc2) at Ser1387, leading to inhibition of the autophagy inhibitor mTOR [111]. In addition, Sirt1 can deacetylate Foxo1, leading to upregulation of the autophagic protein Rab7, which mediates late autophagosome–lysosome fusion in murine cardiomyocytes [112]. In addition to NR, another NAD^+ precursor, NAM, can also induce mitophagy and/or autophagy in human fibroblasts, murine cortical neurons, and 3xTg AD mice [63,113].

Recent studies suggest that NR induces mitophagy in both wild-type mice and *C. elegans*, and in some DNA repair-deficient premature aging diseases, including XPA, A-T, and CS. Specifically, nuclear DNA damage can induce mitochondrial dysfunction, which could be a common cause of the neurodegenerative phenotypes seen in XPA, A-T, and CS [14,39,93]. Cross-species studies using *C. elegans*, mice, and human patient cells reported increased mitochondrial reactive oxygen species (ROS) and accumulation of damaged mitochondria in models of XPA, A-T, and CS [14,39,93]. More specifically, in *atm-1 C. elegans*, NR treatment was shown to ameliorate mitochondrial dysfunction through mitophagy in a SIR2.1-DAF16-DCT-1-dependent manner [39]. The mammalian orthologs of DAF16 and DCT-1 are FOXO3 and NIX (BNIP3L), and this pathway is conserved in mammalian cells, because SIRT1-FOXO3-BNIP3L-dependent mitophagy is necessary to protect against hypoxia-induced mitochondrial damage, (revealed in mice) [64]. As mentioned above, NAD^+ replenishment can also improve DNA repair and, thus, we suggest that NAD^+ serves to link nuclear DNA repair and mitochondrial maintenance [35,108]. Also, NAD^+ can stimulate autophagy and/or mitophagy, which can help delay aging and extend longevity in certain models and across species.

Clinical Translation

NAD^+ precursors can delay aging and counteract a broad spectrum of age-related disease; however, the most important question is whether their beneficial effects will translate to humans. Preclinical and clinical safety assessments of some NAD^+ precursors in mice, rats, and humans have or are being conducted. In mice, there is no detectable toxicity of short-term (500 mg/kg body weight/day for 14 days) [14] or long-term NR treatment (~400 mg/kg/day NR treatment in drinking water for 6 weeks, or 570–590 mg/kg/day for over 10 months) [28,39]. The dose of 570–590 mg/kg/day in mice is equivalent to 3.19–3.30 g in humans based on weight [114]. Studies in rats reported no observed adverse effects of 300 mg/kg/day, and the lowest dose of NR that induced observed adverse effects was 1000 mg/kg/day, with target organs for toxicity assessment being the liver, kidney, ovaries, and testes [115]. Similarly, there is no detectable toxicity of short-term (500 mg/kg/day, intraperitoneal injection for 7 consecutive days) [50] or 12-month-long NMN treatment in wild-type C57BL/6N mice (100 mg/kg/day or 300 mg/kg/day) [44]. Consequently, the low toxicity of NAD^+ precursors in mammals may render these good candidates for clinical intervention.

Acipimox is a NA analog, and a 2015 report showed that it improved skeletal muscle mitochondrial function in patients with type 2 diabetes mellitus [116]. In the first controlled clinical trial of NR, researchers demonstrated that the compound was safe for humans, and increased blood levels of NAD^+ were detected relative to control subjects [117]. This trial involved six healthy men and six healthy women. Each participant received a single oral dose of 100 mg, 300 mg, or 1000 mg of NR with a 7-day gap between doses. The data indicated that NR administration increased NAD^+ in a dose-dependent manner with no serious side effects at any tested dose [117,118]. In addition, 1000 mg/day of NR taken orally resulted in a 2.7-fold increase of NAD^+ and 45-fold increase of the NAD^+ intermediate, NAAD [118]. Studies in mice also suggest that NR is more effective at increasing intracellular NAD^+ than other NAD^+ precursors, such as NAM and NA [118]. In 2016, a follow-up clinical trial was initiated with 140 healthy adults (aged 40–60 years), examining the benefits of 8 weeks of NR treatment [118]. However, the results are not yet available. Some recent clinical trials[i] have been designed to evaluate the efficacy of NAD^+ supplementation for the treatment of metabolic and age-related diseases. Over ten clinical studies have assessed or are currently assessing the safety and efficacy of NR (Box 3). It will be exciting to see whether NAD^+ supplementation has any effects on some of these human conditions going forward.

Box 3. Clinician's Corner

NAD^+ depletion may contribute to a wide spectrum of age-predisposed diseases, including neurodegenerative diseases, muscle atrophy, and progeria. Recent progress in animal studies support the hypothesis that NAD^+ replenishment may inhibit metabolic diseases, AD, hearing loss, and muscle atrophy, among others. However, further research elucidating the molecular mechanisms of the functions of NAD^+ precursor in delaying aging is warranted.

Over ten clinical studies have or are currently assessing the safety and efficacy of NR in humans. In addition to the focus on safety (ClinicalTrials.gov identifier number: NCT02678611), pharmacokinetics (NCT02300740 and NCT02191462), and bioavailability (NCT02712593), there are trials focusing on metabolic disturbance (NCT02689882), aging (NCT02921659 and NCT02950441), obesity, diabetes, or coronary artery disease (NCT02835664, NCT02812238, and NCT02303483), concussion (NCT02721537), and mild cognitive impairment (NCT02942888). Some of these clinical trials have been completed but have not yet been published. The first NMN clinical trial was launched in Japan in 2016, with a focus on safely and bioavailability of NMN in ten healthy humans [126]. In addition to NR and NMN, there are some clinical studies on NAM. Based on the benefits of NAM on AD mice [63], a safety study of the use of NAM to treat human AD, which involved 50 participants aged from 50 to 95 years, has just ended (NCT00580931). Even though some NAD^+ precursors (NR, NMN, and niacin/vitamin B3) are available in the market as dietary supplements, results from these clinical studies will determine the broad applications of NAD^+ in the aging population.

However, many questions remain. What are the therapeutic doses of NR/NMN needed for different diseases in clinical trials? Does long-term supplementation with NR/NMN have any side effects in humans? If NR and/or NMN show clinical benefit, what other clinical studies or combinational drugs should be pursued?

Outstanding Questions

What are the precise molecular mechanisms by which NAD^+ acts on mitochondrial homeostasis?

What molecular mechanisms underlie the autophagy and/or mitophagy-inducing activity of NAD^+?

In addition to deacetylation of major autophagic proteins (such as Atg-5, Atg-7, and Atg-8) and upregulation of certain autophagic proteins (e.g., DCT-1), are there other molecular mechanisms whereby NAD^+ modulates autophagy and/or mitophagy?

How are NA and NR transported into the cytosol from the extracellular milieu?

What cell membrane transport does NR use to enter the cells?

Which are the intracellular transporters for different NAD^+ precursors, especially for NR and NMN?

Concluding Remarks

The increasing population of older individuals presents a serious socioeconomic burden for families, societies, and the healthcare system. Lifestyle interventions, such as a healthy diet, fasting, and exercise, are ways to improve health and the quality of life [35,59,119]. However, not every individual at risk of developing an age-associated ailment may be willing or able to follow these lifestyle interventions. Therefore, the beneficial effects of NAD^+ precursors discussed in this review may promote healthy aging and delay various age-related diseases. Especially encouraging is the evidence demonstrating that NAD^+ replenishment is beneficial in multiple organs with varying disease conditions. The observations that NAD^+ replenishment delays or prevents muscle atrophy, hearing loss, and cognitive decline are remarkable. Moreover, preclinical data suggest that NAD^+ precursor treatment is a promising therapeutic strategy to improve clinical characteristics of the AD phenotype. Moreover, NAD^+ precursors, such as NR and NMN, are relatively safe and orally bioavailable. Thus, NAD^+ precursors may serve as promising candidates to combat normal aging and age-related disease. However, extensive research is warranted to validate their potential, particularly in humans.

While many studies on NAD^+ precursors are ongoing, major questions remain (see Outstanding Questions and Box 3). What predictive value does the alteration in NAD^+ levels have in normal aging and age-related disorders? Further studies evaluating alterations in cellular NAD^+ as a hallmark of aging are necessary. This task requires the development of new technologies to simultaneously detect NAD^+ and its metabolites in humans. Moreover, the pleotropic role of NAD^+ in human physiology is complex and requires further mechanistic insight. For example, the tissue and subcellular specificity of NAD^+ precursors need to be carefully evaluated. It is also possible that some unforeseen side effects may present in certain human populations. Thus, highly stringent and carefully designed clinical trials are necessary to ensure safety. Indeed, a decline in NAD^+ concentrations may have numerous roles in human physiology, some of which we are only beginning to understand. Some of these roles appear to be important in the aging process and are likely to be important drivers of aging and age-related dysfunction. With careful scientific evaluation, NAD^+ replenishment strategies might serve as a promising multifunctional approach to improve the quality of life for an increasingly aged population.

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Resources

[i]https://clinicaltrials.gov/ct2/results?term=nad%2B&Search=Search

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