Review

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Focus: Metabolism

Modulating NAD+ metabolism, from bench to bedside

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Abstract

Discovered in the beginning of the 20th century, nicotinamide adenine dinucleotide (NAD+) has evolved from a simple oxidoreductase cofactor to being an essential cosubstrate for a wide range of regulatory proteins that include the sirtuin family of NAD+-dependent protein deacylases, widely recognized regulators of metabolic function and longevity. Altered NAD+ metabolism is associated with aging and many pathological conditions, such as metabolic diseases and disorders of the muscular and neuronal systems. Conversely, increased NAD+ levels have shown to be beneficial in a broad spectrum of diseases. Here, we review the fundamental aspects of NAD+ biochemistry and metabolism and discuss how boosting NAD+ content can help ameliorate mitochondrial homeostasis and as such improve healthspan and lifespan.

Keywords aging; metabolic disorders; neurodegeneration; nicotinamide adenine dinucleotide; poly ADP-ribose polymerase

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Introduction

The first cofactor ever described, nicotinamide adenine dinucleotide (NAD^+), was discovered by the British biochemists Arthur Harden and William John Young in 1906 (Harden & Young, 1906). They observed that adding boiled yeast extracts to non-boiled yeast extracts significantly accelerated alcoholic fermentation, suggesting that the boiled yeast fraction contained something capable of promoting the fermentation reaction. They named this heat-stable, but yet unidentified factor, "cozymase". Almost 25 years later, Hans von Euler-Chelpin established the chemical composition of the cozymase as an adenine, a reducing sugar group and a phosphate (von Euler & Myrbäck, 1930). Finally, in 1936, Otto Heinrich Warburg discovered the capability of the cozymase to transfer hydride from one molecule to another and identified nicotinamide base as the site of redox reactions (Warburg & Christian, 1936). Together with its reduced counterpart, NADH, NAD^+ has since been known for being involved in reactions that required the transfer of electrons from one molecule to another. As such, the redox couple NAD^+/NADH has been reported to participate in numerous reactions requiring an electron exchange, such as glycolysis, pyruvate-to-lactate and pyruvate-to-acetyl-CoA interconversions, β-oxidation, citric acid cycle (TCA cycle), and oxidative phosphorylation. Moreover, addition of a phosphate to the adenosine ribose of NAD^+ by NAD^+ kinases (NADKs) leads to a formation of nicotinamide adenine dinucleotide phosphate ($NADP^+$). $NADP^+$ and its reduced form, NADPH, play a key role in cellular defense against oxidative stress, as well as in the synthesis of fatty acids, cholesterol, and DNA. Detailed description of the physiological roles of the $NADP^+$/NADPH redox couple is reviewed elsewhere (Ying, 2008). Although the role of NAD^+ in redox reactions is now rather well understood, NAD^+ biology underwent a renaissance when NAD^+ was reported to influence the activity of the sirtuins (Imai *et al*, 2000), a family of NAD^+-dependent deacylases, implicated in the regulation of metabolism and mitochondrial function (Haigis & Sinclair, 2010; Houtkooper *et al*, 2012). Besides sirtuins, other enzymes, such as the poly ADP-ribose polymerase (PARP) protein family and the cyclic ADP-ribose (cADPR) synthases, such as CD38 and CD157, are currently known to require NAD^+ as a cosubstrate to perform their function. The dependence of these important metabolic enzymes on NAD^+ levels provides an attractive possibility to modulate their activity and thereby achieve health benefits and has led to an increased interest in NAD^+ metabolism over the last decade. The therapeutic potential of NAD^+ boosting techniques to activate the sirtuins has now been explored in a large spectrum of preclinical disease models that mimic rare genetic disorders, such as the Cockayne syndrome, as well as pandemic-like contemporary diseases, such as obesity or non-alcoholic fatty liver disease (NAFLD). The near future will hopefully see these studies translate from the bench to the bedside.

Biosynthesis of NAD+

Intracellular NAD^+ can be produced through either *de novo* synthesis or via salvage pathways from precursor molecules, naturally occurring vitamins: nicotinamide (NAM), nicotinic acid (NA), and nicotinamide riboside (NR) (Bogan & Brenner, 2008; Houtkooper *et al*, 2010) (Fig 1). The NAD^+ *de novo* synthesis pathway starts from the amino acid tryptophan (Bender, 1983; Houtkooper *et al*,

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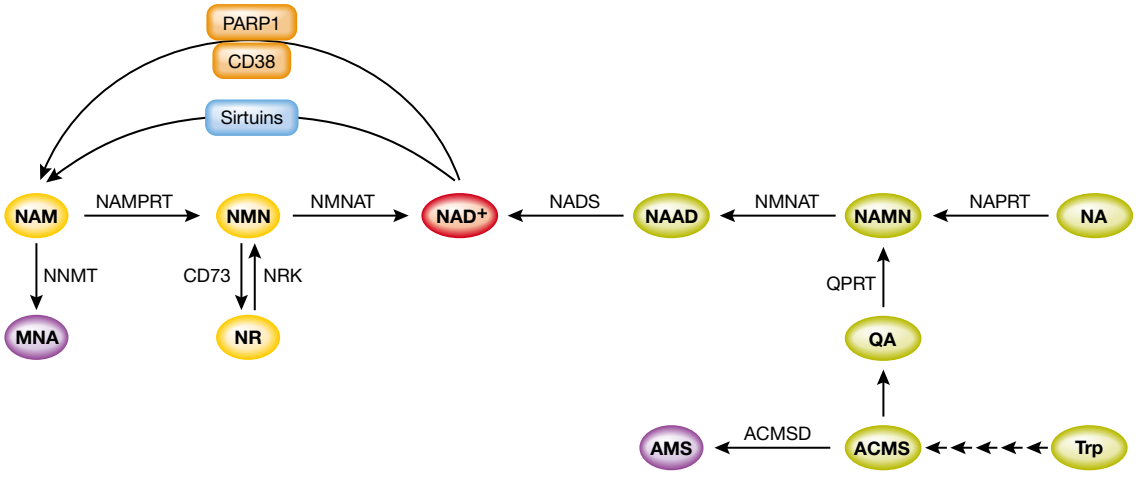

Figure 1. Pathways modulating NAD+ content in mammals.
Intermediates of the amidated and deamidated routes are depicted in yellow and green, respectively. NAD+-consuming enzymes competing with sirtuins for NAD+ availability are depicted in orange. Purple color indicates metabolites not recycled in the NAD+ synthesis pathway.

2010) and most likely takes place in the cytosol, since all the enzymes catalyzing the different steps of this process are localized there (Houtkooper *et al*, 2010).

NAD^+ synthesis from NAM requires only two steps: NAM gets first converted by nicotinamide phosphoribosyltransferase (NAMPRT) into NAM mononucleotide (NMN), which in its turn leads to the production of NAD^+ in a reaction catalyzed by nicotinamide mononucleotide adenylyltransferase (NMNAT) (Fig 1). Three different isoforms of NMNAT have been reported, each of them possessing a specific subcellular localization: NMNAT1 is a nuclear enzyme (Emanuelli *et al*, 2001; Yalowitz *et al*, 2004), NMNAT2 is located in the cytosol and Golgi apparatus (Yalowitz *et al*, 2004; Berger *et al*, 2005), while NMNAT3 was detected in the cytosol and mitochondria (Zhang *et al*, 2003; Berger *et al*, 2005; Yang *et al*, 2007). NR also gets converted into NMN by nicotinamide riboside kinase (NRK) (Bieganowski & Brenner, 2004). Mammals possess two isoforms of NRK: an ubiquitously expressed NRK1 and NRK2, whose expression was mainly detected in heart, skeletal muscle, brown adipose tissue (BAT), and liver (Bogan & Brenner, 2008). Interestingly, it has been recently reported that NRK1 is required for NAD^+ synthesis not only from the exogenously administered NR, but also NMN (Ratajczak *et al*, 2016). Both NAM and NR operate via the "amidated" route to produce NAD^+ (Fig 1).

Nicotinic acid, in its turn, initiates the "deamidated" route (Fig 1). Conversion of NA into NA mononucleotide (NAMN) constitutes the first step of this route, which most often is referred as the Preiss–Handler pathway (Preiss & Handler, 1958). The NMNATs recognize both NAMN and NMN as substrates; however, in the case of NAMN the conversion results in NA adenine dinucleotide (NAAD), and therefore, one additional step, catalyzed by NAD synthetase (NADS), is required to produce NAD^+ (Fig 1). Interestingly, it has been recently reported that NR leads to the production of NAAD via a yet-unknown mechanism (Trammell *et al*, 2016a).

The *de novo* NAD^+ synthesis pathway, which converts tryptophan into NAD^+, consists of eight steps. The first reaction of this pathway constitutes of a conversion of tryptophan into N-formylkynurenine, which in mammals can be catalyzed by two different enzymes: tryptophan-2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO). This conversion is considered to be the first rate-limiting step for the pathway. TDO is the major contributor to NAD^+ biosynthesis in liver, while IDO is ubiquitously expressed in extrahepatic tissues, with the highest activity detected in lung, intestine, and spleen (Yamazaki *et al*, 1985; Kudo & Boyd, 2000). TDO is induced by tryptophan and glucocorticoids (Comings *et al*, 1995), while IDO is induced by inflammatory stimuli (Yoshida & Hayaishi, 1978; Yoshida *et al*, 1979; Takikawa *et al*, 1986; Heyes *et al*, 1992; Reinhard, 1998; Sanni *et al*, 1998; Daubener & MacKenzie, 1999). N-formylkynurenine gets converted by formamidase (KFase) into kynurenine. Kynurenine in its turn leads to 3-OH kynurenine in a reaction catalyzed by kynurenine 3-hydroxylase (K3H). Kynureninase (Kyase) then forms 3-hydroxyanthranilate, which gets transformed into α-amino-β-carboxymuconate-ε-semialdehyde (ACMS) by 3-hydroxyanthranilate 3,4-dioxygenase (3HAO). The formation of this unstable ACMS constitutes a branching point of the *de novo* NAD^+ synthesis pathway (Bender, 1983; Houtkooper *et al*, 2010). ACMS can either undergo cyclization forming quinolinic acid (QA), which is then converted by quinolinate phosphoribosyltransferase (QPRT) into NAMN and from this point fuses with the Preiss–Handler pathway to produce NAD^+ (Fig 1). Otherwise, the carbon group of ACMS can be removed, which either leads to the production of picolinic acid or is directed to total oxidation to CO_2 and H_2O. While the cyclization of ACMS is a spontaneous reaction, the transformation of ACMS into α-amino-β-muconate-ε-semialdehyde (AMS) is catalyzed by the enzyme α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD) (Fig 1).

Preferential source for NAD+ production

The existence of different pathways leading to NAD^+ production raises questions on the relative importance of each pathway and which of them possess the highest potential to boost NAD^+ levels. The preferable precursor for NAD^+ production within the organism is hence still a matter of debate. There is evidence that NAM possesses a higher NAD^+ boosting capability when compared to NA in different organs in mice (Collins & Chaykin, 1971, 1972; Mori *et al*, 2014; Yang *et al*, 2014). Additionally, in human plasma, levels of NAM were reported to be fivefold higher than NA levels (Jacobson *et al*, 1995). However, several other studies claim the opposite: NA is a more effective NAD^+ precursor than NAM (Ijichi *et al*, 1966; Hagino *et al*, 1968; Lin & Henderson, 1972; Williams *et al*, 1985; Jackson *et al*, 1995; Hara *et al*, 2007). It is important to mention that in Mori *et al* (2014) the authors quantified the activity of NMNAT and NADS; therefore, the comparison was rather made between the "deamidated" (e.g., from NA) and "amidated" route, which includes both NAM and NR. And even if the authors of this study claim that NAM is the main precursor for NAD^+ synthesis, the possibility of a significant contribution of other precursors using the amidated NAD^+ biosynthesis route (e.g., NR) cannot be discounted. In support of this, a very recent study showed that NR has a greater capacity over NA and NAM to boost hepatic NAD^+ levels (Trammell *et al*, 2016a). It is also important to mention that both NA and NAM have reported side effects, whereas no adverse effects are currently reported for NR. NA activates the G protein-coupled receptor, GPR109A and causes flushing, characterized by vasodilation and a burning sensation (Benyo *et al*, 2006). While NAM raises health concerns for treatment of diabetic patients, as high doses of NAM can be hepatotoxic (Knip *et al*, 2000).

As for tryptophan, its administration to humans has been used as treatment for pain, sleep disorders, depression, hyperactivity, and bulimia (Richard *et al*, 2009). No severe adverse effects have been reported for tryptophan administration, even with doses going as high as 20 g/day in schizophrenic patients (Sidransky, 2001). A large number of reviews attribute a marginal role to the *de novo* NAD^+ synthesis pathway. However, a solid support for this claim is lacking. One of the studies frequently cited to sustain this point of view reports that tryptophan alone is not sufficient to maintain the physiological NAD^+ concentration of the cell (Nikiforov *et al*, 2011). However, this conclusion was exclusively based on the observation that supplementation with tryptophan is not sufficient to protect cells from the death induced by NAMPRT inhibitor FK866 and no NAD^+ quantification was performed in this study. In addition, some studies show that, at least in the liver, tryptophan constitutes the preferable substrate for NAD^+ production. Rat primary hepatocytes, treated with NA, NAM, or tryptophan, were reported to use exclusively tryptophan for their NAD^+ biosynthesis, even though they were still able to take up NA and NAM from the culture medium (Bender & Olufunwa, 1988). Administration of tryptophan, NA, or NAM to rats showed that tryptophan resulted in the highest hepatic NAD^+ concentrations (Bender *et al*, 1982). Moreover, it has been shown that in rat liver, NA and NAM have a very limited capacity for NAD^+ production, probably due to the saturation of the involved phosphoribosyltransferases, whereas no such limitations were detected for the NAD^+ synthesis from tryptophan (Williams *et al*, 1950; Bender *et al*, 1982; McCreanor & Bender, 1986).

NAD+ consuming enzymes

Sirtuin proteins require NAD^+ as a cosubstrate for their activity. A detailed description of their role in the regulation of metabolism and aging is beyond the scope of this review, but has been extensively covered elsewhere (Haigis & Sinclair, 2010; Satoh *et al*, 2011; Houtkooper *et al*, 2012; Chang & Guarente, 2014). Besides sirtuins, two different protein families are well known to use NAD^+ as a cofactor for their enzymatic activities. These include the PARPs and the cADPR synthases, CD38 and CD157. PARPs are involved in DNA repair, maintenance of genomic integrity, and cell death, with PARP1 accounting for more than 85% of NAD^+ consumption of this protein family (Bai & Canto, 2012). cADPR, which is generated by CD38 and CD157, is a signaling molecule that controls intracellular calcium fluxes. The catalytic efficiency of CD38 is significantly higher than that of CD157 (Quarona *et al*, 2013). While CD38 expression was initially considered to be limited to the immune system, it was later found to be ubiquitously distributed. CD38 is an important NAD^+ consumer, as its loss of function (LOF) in mice led up to a 30-fold increase in NAD^+ levels in different tissues (Barbosa *et al*, 2007). Combined increases in PARP1 (Braidy *et al*, 2011; Mouchiroud *et al*, 2013) and CD38 (Camacho-Pereira *et al*, 2016) activities upon aging were reported to cause age-associated reduction in NAD^+ content. For more extensive coverage of these NAD^+ consuming enzymes, we refer the readers to a few reviews on PARPs (Bai & Canto, 2012; Canto *et al*, 2013; Jubin *et al*, 2016) and cADPR synthases (Malavasi *et al*, 2008; Quarona *et al*, 2013).

Regulation of NAD+ content

Regulation of NAD+ content by diet and aging

An increase of NAD^+ levels followed by sirtuin activation is observed in situations of energy deficit, such as fasting (Rodgers *et al*, 2005; Chen *et al*, 2008; Cantó *et al*, 2010), calorie restriction (CR) (Qin *et al*, 2006; Chen *et al*, 2008; Cantó *et al*, 2010) or low glucose feeding (Fulco *et al*, 2008), and exercise (Canto *et al*, 2009; Cantó *et al*, 2010; Costford *et al*, 2010). On the contrary, multiple studies reported that high-fat (HF)/high-fat high-sucrose (HFHS) feeding diminishes NAD^+ content in liver (Yoshino *et al*, 2011; Gariani *et al*, 2016, 2017; Trammell *et al*, 2016b), skeletal muscle (Canto *et al*, 2012), BAT (Canto *et al*, 2012), and white adipose tissue (WAT) (Yoshino *et al*, 2011). Orotic acid administration (Fukuwatari *et al*, 2002) or feeding a methionine-/choline-deficient (MCD) diet (Gariani *et al*, 2017) also leads to the concomitant appearance of liver fat accumulation and a drop in hepatic NAD^+ levels. A recent study, however, reported that administration of a HF diet for 11 weeks led to an increase in NAD^+ content in mouse liver, accompanied by enhanced Sirt1 activity (Penke *et al*, 2015). The stimulation of the NAD^+ biosynthesis could represent an initial compensatory attempt to maintain energy homeostasis (Penke *et al*, 2015; Drew *et al*, 2016), whereas more prolonged exposure to HF diet reduces NAD^+ content, resulting in functional damage (Drew *et al*, 2016). Interestingly, in *Saccharomyces cerevisiae* NAD^+

content is affected by the carbon source used: Yeast grown on ethanol contain practically double the amount of NAD^+ compared to yeast grown on glucose (Agrimi *et al*, 2011).

Finally, a decrease in NAD^+ content was also reported to be associated with aging in *Caenorhabditis elegans*, mice, rats, and humans (Braidy *et al*, 2011; Yoshino *et al*, 2011; Massudi *et al*, 2012; Gomes *et al*, 2013; Mouchiroud *et al*, 2013; Camacho-Pereira *et al*, 2016; Guan *et al*, 2017).

Regulation by circadian rhythm

Circadian rhythm is another important regulator of NAD^+ content. The key regulators of the mammalian circadian clock machinery are the transcription factors CLOCK and BMAL1, which act together as a heterodimer. Their transcriptional targets, PER and CRY, form a negative feedback loop by repressing CLOCK-BMAL1 activity. Hepatic NAD^+ levels oscillate in a diurnal manner (Nakahata *et al*, 2009; Ramsey *et al*, 2009). Mice with LOF mutations in the circadian activator genes, *Clock* and *Bmal1*, show reduced NAD^+ content, while NAD^+ levels were elevated in mice with mutations in the clock repressor genes *Cry1* and *Cry2* (Ramsey *et al*, 2009). The circadian clock-controlled expression of *Namprt* is thought to be responsible for this fine-tuning of the NAD^+ availability (Nakahata *et al*, 2009; Ramsey *et al*, 2009). Importantly, clock-driven oscillations of NAD^+ were claimed to regulate the activity of both SIRT1 (Nakahata *et al*, 2009; Ramsey *et al*, 2009) and SIRT3 (Peek *et al*, 2013). It is possible that modulation of the redox state of NAD^+ can reciprocally impact on the circadian clock. *In vitro* NADH was shown to enhance binding of the CLOCK-BMAL1 heterodimer to DNA, whereas NAD^+ was inhibiting this process (Rutter *et al*, 2001). On their turn, the NAD^+-dependent enzymes SIRT1, SIRT6, and PARP1 were reported to control the circadian clock machinery via post-translational modifications of the core clock transcription factors and via regulation of their transcription (Asher *et al*, 2008, 2010; Chang & Guarente, 2013; Masri *et al*, 2014).

NAD^+ boosting strategies

NAD^+ levels can be increased either by promoting its synthesis—by enhancing the enzymes involved in NAD^+ biosynthesis or administration of NAD^+ precursor molecules—or by limiting its consumption. Supplementation with NA, NAM, NR, NMN, or tryptophan can increase NAD^+ content (Canto *et al*, 2015). Overexpressing or activating enzymes catalyzing the rate-limiting steps of NAD^+ biosynthesis also are efficient to boost NAD^+ levels (Araki *et al*, 2004; Sasaki *et al*, 2006; Hsu *et al*, 2009; Wang *et al*, 2014a; Williams *et al*, 2017). However, the translational potential of this approach may be lower compared to the other strategies.

Pharmacological or genetic inhibition of non-sirtuin NAD^+ consumers, such as PARP-1 or CD38 (Fig 1), can help to preserve NAD^+ levels for sirtuin activation (Aksoy *et al*, 2006a; Barbosa *et al*, 2007; Bai *et al*, 2011; Pirinen *et al*, 2014), especially in situations when non-sirtuin NAD^+ consumers are overactivated (Bai *et al*, 2011; Braidy *et al*, 2011; Mouchiroud *et al*, 2013; Fang *et al*, 2014; Mukhopadhyay *et al*, 2014; Ryu *et al*, 2016; Gariani *et al*, 2017). For instance, DNA damage is known to cause a dramatic decline in NAD^+ intracellular levels, which is due to PARP activation (Berger, 1985), and overexpression of CD38 in cells leads to a ~35% decrease in NAD^+ levels (Hu *et al*, 2014). On the contrary, *Cd38*$^{-/-}$ and *PARP-1*$^{-/-}$ mice have increased NAD^+ content in different organs (Aksoy *et al*, 2006a,b; Young *et al*, 2006; Bai *et al*, 2011).

Increased nicotinamide methyl transferase (*NNMT*) expression was reported in obesity and type 2 diabetes (Lee *et al*, 2005; Yaguchi *et al*, 2005; Salek *et al*, 2007; Kraus *et al*, 2014). NNMT is the enzyme catalyzing the transformation of NAM into methylnicotinamide (MNA) (Fig 1) and is highly expressed in liver and adipose tissue (Aksoy *et al*, 1994; Riederer *et al*, 2009). Inhibiting NNMT in these tissues should increase NAD^+ content, since NAM would not be degraded but exclusively reconverted into NAD^+ (Fig 1). In line, the knockdown of *NNMT* increased NAD^+ levels in adipose tissue, but not in the liver (Kraus *et al*, 2014).

Resveratrol, a natural polyphenol found in red wine, activates AMPK and thereby increases NAD^+ levels (Fulco *et al*, 2008; Canto *et al*, 2009; Price *et al*, 2012; Desquiret-Dumas *et al*, 2013), which promotes sirtuin activation.

Therapeutic potential of NAD^+

Pellagra

Pellagra, a disease that was epidemic in the XVII–XIX centuries in several rural areas of Europe and the United States (Bender, 1983), owes its name to the Italian "pelle" = skin and "agra" = sour or rough, which describes its most noticeable feature. Otherwise, it is also called the disease of "the three Ds": Diarrhea, dermatitis, and dementia, which, if untreated, can lead to the fourth "D", i.e. death. In 1915, Joseph Goldberger demonstrated that pellagra was not an infectious disease, as previously thought, but is due to poor nutrition and could be prevented by consumption of fresh meat and milk (Bender, 1983). Administration of both NAM and NA (Elvehjem *et al*, 1937), and later tryptophan (Krehl *et al*, 1945) were also reported to prevent pellagra.

Aging

Sirtuins are well-known longevity regulators, and their decreased function with age might at least be partially explained by a systemic decline in NAD^+ levels upon aging (Mouchiroud *et al*, 2013) [reviewed in (Imai & Guarente, 2014; Menzies *et al*, 2016)]. Rising NAD^+ content, followed by sirtuin activation, has been reported to increase lifespan in yeast (Lin *et al*, 2004; Belenky *et al*, 2007; Easlon *et al*, 2008), worms (Mouchiroud *et al*, 2013), and mice (Zhang *et al*, 2016). Administration of NR, NMN, or NAM recovered NAD^+ content and protected against aging-related complications, such as mitochondrial dysfunction (Gomes *et al*, 2013; Mouchiroud *et al*, 2013; Mills *et al*, 2016), decline in physical performance (Mills *et al*, 2016; Zhang *et al*, 2016) and muscle regeneration (Zhang *et al*, 2016), arterial dysfunction (de Picciotto *et al*, 2016), decline in vision (Lin *et al*, 2016; Mills *et al*, 2016), including glaucoma (Williams *et al*, 2017), and age-associated insulin resistance (Mills *et al*, 2016).

The most striking benefits of NAD^+ supplementation on aging were observed in several rare diseases linked to abnormal DNA repair that are typified by accelerated aging, such as the Cockayne syndrome group B (CSB), xeroderma pigmentosum group A (XPA), or ataxia-telangiectasia (A-T). In a mouse model of CSB, neurons

show mitochondrial defects, which have an impact on the cerebellum and inner ear. Administration of PARP inhibitors or the NAD^+ precursor, NR, to $csb^{-/-}$ animals attenuated many of the phenotypes of CSB and restored altered mitochondrial function in their neurons (Scheibye-Knudsen *et al*, 2014). Another DNA damage repair disorder is XPA, which is also characterized by mitochondrial alterations and reduced NAD^+-SIRT1 signaling due to the overactivation of PARP1 (Fang *et al*, 2014). Treatment with NAD^+ precursors, NR and NMN, or with the PARP inhibitor, Olaparib, rescued the XPA phenotype in cells and worms. Similar observations of increased PARylation, NAD^+ depletion, and mitochondrial dysfunction were made in mouse and worm models of another progressive neurodegenerative disease, A-T (Fang *et al*, 2016). Restoring the NAD^+/SIRT1 pathway, by NR and NMN administration to *C. elegans* and mice, improved A-T neuropathology (Fang *et al*, 2016).

Metabolic disorders

The importance of NAD^+ as a metabolic regulator has been demonstrated by its efficacy to attenuate many features of the metabolic syndrome, a cluster of pathologies including insulin resistance, fatty liver, dyslipidemia, and hypertension, with increased risk of developing type 2 diabetes and heart failure. Different approaches aiming to raise NAD^+ levels were shown to provide protection against obesity, such as (i) inhibition of NAD^+ consumers, PARPs (Bai *et al*, 2011; Gariani *et al*, 2017) and CD38 (Barbosa *et al*, 2007), (ii) administration of NAD^+ precursors, such as NR (Canto *et al*, 2012; Gariani *et al*, 2016; Trammell *et al*, 2016b) or NMN (Yoshino *et al*, 2011), (iii) or inhibition of NNMT (Kraus *et al*, 2014). NAD^+ boosting was also efficient to improve glucose homeostasis in obese, prediabetic, and T2DM animals (Barbosa *et al*, 2007; Bai *et al*, 2011; Yoshino *et al*, 2011; Canto *et al*, 2012; Kraus *et al*, 2014; Gariani *et al*, 2016, 2017; Trammell *et al*, 2016b). Likewise, re-establishing NAD^+ levels with NR or PARP inhibitors also protected from non-alcoholic steatohepatitis (NASH) (Gariani *et al*, 2016, 2017; Mukhopadhyay *et al*, 2017) as well as alcoholic steatohepatitis (ASH) (Mukhopadhyay *et al*, 2017).

Muscle function

Increase in muscle NAD^+ content, resulting from NR administration or PARP inhibition, improved muscle function and exercise capacity in mice (Canto *et al*, 2012; Pirinen *et al*, 2014), including in aged animals (Zhang *et al*, 2016). Interestingly, muscular dystrophy is characterized by a dramatic drop in NAD^+ in the muscle (Ryu *et al*, 2016). NR administration to the *mdx* mouse, a model for muscular dystrophy, improved muscle function by enhancing bioenergetics, attenuating inflammation and fibrosis (Ryu *et al*, 2016), as well as, by favoring regeneration and preventing the exhaustion and senescence of muscle stem cells, typical to the *mdx* mice (Zhang *et al*, 2016).

The beneficial effects of improving muscle bioenergetics are also illustrated in models of mitochondrial myopathies. Increasing muscle NAD^+ levels by the administration of NR or a PARP inhibitor preserved muscle function in two different models of mitochondrial myopathy (Cerutti *et al*, 2014; Khan *et al*, 2014). Similar benefits on mitochondrial myopathy were seen with the AMPK agonist, AICAR (Viscomi *et al*, 2011), which may at least in part be due to the recovery of NAD^+ content upon AMPK activation.

Cardiac function

Exposing the heart to different types of stresses was reported to result in a decline in cardiac NAD^+ content (Pillai *et al*, 2005, 2010; Karamanlidis *et al*, 2013; Yamamoto *et al*, 2014). For instance, cardiomyocyte hypertrophy is characterized by a drop in cellular NAD^+ levels. Supplementation with NAD^+ was hence protective against cardiac hypertrophy in mice, and these anti-hypertrophic effects were in part attributed to the activation of SIRT3 (Pillai *et al*, 2010).

Cardiac ischemia is another condition causing a steep decrease in NAD^+ levels. NMN administration protected the mice from ischemic injury via the recovery of cardiac NAD^+ content and subsequent SIRT1 activation (Yamamoto *et al*, 2014). Similarly, cardiac-specific overexpression of NAMPRT in mice increased NAD^+ content and reduced the extent of myocardial infarction and apoptosis in response to prolonged ischemia and ischemia/reperfusion (Hsu *et al*, 2009). Maintaining NAD^+ levels in pressure-overloaded hearts is crucial for myocardial adaptation and protection from heart failure, as demonstrated by NMN administration to mice treated with the NAMPRT inhibitor FK866 (Yano *et al*, 2015) and to cardiac-specific mitochondrial complex I-deficient mice (Lee *et al*, 2016). In a mouse model of heart failure caused by iron deficit, reconstituting NAD^+ content also improved mitochondrial quality, protected cardiac function, and increased lifespan (Xu *et al*, 2015). Similarly, NR administration improved cardiac function in aged *mdx* mice, which, like muscular dystrophy patients, display cardiomyopathy (Ryu *et al*, 2016).

Renal function

Multiple studies demonstrated the loss of SIRT1 and SIRT3 activity as a key feature of kidney dysfunction, including kidney abnormalities linked with aging (Koyama *et al*, 2011; Zhuo *et al*, 2011; Morigi *et al*, 2015; Ugur *et al*, 2015; Guan *et al*, 2017). Acute kidney injury (AKI) is characterized by a reduction in NAD^+ content and NAMPRT expression (Morigi *et al*, 2015; Ugur *et al*, 2015). Promoting NAD^+ synthesis via NAM or NMN supplementation was reported to mitigate AKI in ischemia/reperfusion- and cisplatin-induced mouse models of AKI (Tran *et al*, 2016; Guan *et al*, 2017). Furthermore, administration of the AMPK agonist, AICAR, which positively impacts on NAD^+ levels (Canto *et al*, 2009), was protective against cisplatin-induced AKI in SIRT3-dependent manner (Morigi *et al*, 2015). Although no NAD^+ quantification was performed in this particular study, the involvement of SIRT3, as well as the increase in *Namprt* expression detected upon AICAR administration, points toward a potential increase in NAD^+ levels (Morigi *et al*, 2015). Kidney mesangial cell hypertrophy is also characterized by a depletion of NAD^+ content (Zhuo *et al*, 2011) and restoring intracellular NAD^+ levels via supplementation with exogenous NAD^+ prevented its onset by activating SIRT1 and SIRT3 (Zhuo *et al*, 2011).

Neurodegeneration

NAD^+ boosting has also been shown to be neuroprotective. Raising NAD^+ levels protects against neuronal death induced by ischemic brain (Klaidman *et al*, 2003; Sadanaga-Akiyoshi *et al*, 2003; Kabra *et al*, 2004; Feng *et al*, 2006; Kaundal *et al*, 2006; Zheng *et al*, 2012) or spinal cord injuries (Xie *et al*, 2017). Axonal degeneration is considered as an early pathological mechanism in

this type of neurodegeneration. An accumulating amount of data indicates that axonal degeneration is not only limited to ischemic brain and spinal cord injuries, but constitutes a hallmark process, preceding neuronal death, in a much larger spectrum of disease states, including traumatic brain injury, inflammatory disorders, like multiple sclerosis, and degenerative disorders, such as Alzheimer's and Parkinson's diseases (Lingor *et al*, 2012; Johnson *et al*, 2013). Degenerating axons show a decrease in NAD^+ content (Wang *et al*, 2005; Gerdts *et al*, 2015), while replenishing NAD^+ by supplementing NAM (Wang *et al*, 2005), NR and NMN (Sasaki *et al*, 2006), and high doses of NAD^+ (Araki *et al*, 2004), or overexpressing enzymes involved in NAD^+ biosynthesis (Araki *et al*, 2004; Sasaki *et al*, 2006) delayed axonal degeneration. In line with this, supplementation with NAM, NMN, or NR was neuroprotective in rodent models of Alzheimer disease (Qin *et al*, 2006; Gong *et al*, 2013; Liu *et al*, 2013; Turunc Bayrakdar *et al*, 2014; Wang *et al*, 2016a), and supplementation with NAM or LOF of PARP were protective in *Drosophila* models of Parkinson's disease (Lehmann *et al*, 2017).

NAD^+ depletion is also involved in the neurodegeneration induced by highly toxic misfolded prion protein (Zhou *et al*, 2015). Replenishment of intracellular NAD^+ stocks, either by providing NAD^+ or NAM, rescued the neurotoxic effects of protein aggregates (Zhou *et al*, 2015). Importantly, restoring NAD^+ content is not exclusively protecting neurons, since it has also been reported to prevent the death of astrocytes (Alano *et al*, 2004).

P7C3, a compound that enhances neurogenesis (Pieper *et al*, 2010) and that was neuroprotective in mouse models of Parkinson's disease (De Jesus-Cortes *et al*, 2012), amyotrophic lateral sclerosis (Tesla *et al*, 2012) and brain injury (Yin *et al*, 2014), was subsequently identified as an NAMPRT activator (Wang *et al*, 2014a). Therefore, the beneficial effects of P7C3 on neuron preservation seem at least in part to be due to a NAMPRT-mediated increase in NAD^+ levels (Wang *et al*, 2014a).

Nicotinamide riboside supplementation recovered depressed sensory and motor neuron conduction velocities and thermal insensitivity in T2DM mice (Trammell *et al*, 2016b) and alleviated chemotherapy-induced peripheral neuropathy in rats (Hamity *et al*, 2017), indicating that NAD^+ also is beneficial in the peripheral neuronal system.

NAD^+ boosting was also able to protect mice from loss of vision and hearing (Shindler *et al*, 2007; Brown *et al*, 2014). Intravitreal injections of NR in mice attenuated optic neuritis in a dose-dependent manner (Shindler *et al*, 2007). Even if no NAD^+ quantification was performed in this study, SIRT1 activity was necessary for the neuroprotective effects of NR, since the protection was blunted in the presence of sirtinol, a SIRT1 inhibitor (Shindler *et al*, 2007). Furthermore, systemic administration of NAM and overexpression of Nmnat1 had spectacular effects on vision in DBA/2J mice, which are prone to glaucoma (Williams *et al*, 2017). Noise exposure results in degeneration of the neurons innervating the cochlear hair cells. Increase in NAD^+ levels induced by NR administration prevented against noise-induced hearing loss and neurite degeneration (Brown *et al*, 2014). In line with this, CR was shown to protect against cochlear cell death and aging-associated hearing loss in a Sirt3-dependent manner (Someya *et al*, 2010). It is therefore tempting to speculate that this improvement could also be associated with increased NAD^+ levels upon CR, though no direct measurements of NAD^+ levels were performed in this study.

Future challenges and perspectives

Although it was thought that the NAD^+ biosynthetic pathways were entirely understood, we still continue to discover new actors of NAD^+ metabolism. For instance, whereas the conversion of NR into NMN by NRK was established some time ago (Bieganowski & Brenner, 2004), the opposite reaction, that is, the transformation of NMN into NR by CD73, was only recently shown to occur in humans (Garavaglia *et al*, 2012). Another example is the recent description that NR not only induced NAAD concentrations by 45-fold, but that it is also a direct biosynthetic precursor of NAAD (Trammell *et al*, 2016a). The biochemical basis of this conversion cannot be explained with our current state of knowledge, as NAD^+ synthetase, which catalyzes the transformation of NAAD into NAD^+, works unidirectionally (Bieganowski *et al*, 2003; Wojcik *et al*, 2006) and can therefore not catalyze the reverse reaction from NAD^+ into NAAD.

Additionally, it is possible that we still ignore some functions that NAD^+ might accomplish within the cell. For instance, very recently NAD^+ was found to be linked to RNA in bacteria (Chen *et al*, 2009). By forming a cap at the 5′-terminus of bacterial RNA molecule, it is not only increasing its stability (Cahova *et al*, 2015), but also serves as non-canonical initiation nucleotides for *de novo* transcription initiation (Bird *et al*, 2016). Initially thought to be prokaryote-specific, this RNA modification appears to be also conserved in eukaryotic systems (Jiao *et al*, 2017; Walters *et al*, 2017). Similarly to bacteria, in eukaryotic cells NAD^+ addition seems to occur during transcription initiation (Bird *et al*, 2016; Walters *et al*, 2017). Intriguingly, a subset of eukaryotic non-coding RNAs have also been reported to possess a NAD^+-cap. Since these RNAs are formed exonucleolytically, NAD^+ cap addition in their case would occur post-transcriptionally (Jiao *et al*, 2017). Oppositely to prokaryotes, in mammalian cells the NAD^+ cap was reported to rather promote mRNA decay (Jiao *et al*, 2017). The full physiological significance of NAD^+-capping is yet to be discovered. It is, however, tempting to speculate that the proportion of cellular mRNA possessing NAD^+ cap might be influenced by intracellular NAD^+ content and thus by the energy state of the cell.

Devising better NAD^+ quantification methods is a critical challenge in the field. Measurements based on UV–Vis methods are less accurate and sensitive than mass spectrometry methods (Trammell & Brenner, 2013). Moreover, accurate NAD^+ quantification in different subcellular compartments is challenging due the complexity of subcellular fractionations and the NAD^+ isolation procedures. Over the last few years, a new generation of NAD^+ biosensors was developed, allowing NAD^+ quantification in intact cells as well as within specific subcellular compartments (Hung *et al*, 2011; Bilan *et al*, 2014; Cambronne *et al*, 2016). Further development and wider application of these biosensors combined with strategies to explore the kinetics of NAD^+ biosynthesis and metabolism, using flux studies, will hence be important for future research. Besides, according to a recent study NADP and NADPH were more significantly deregulated in T2DM and obesity than NAD^+ and less correctable by NR

supplementation (Trammell *et al*, 2016b). Monitoring of the entire NAD^+ metabolome could hence help our further understanding of its role in metabolism, which might extend far beyond NAD^+–sirtuin or NAD^+–PARP axis.

As reviewed here, manipulations of NAD^+ concentrations have demonstrated multiple beneficial effects in a large spectrum of diseases in animal models (Fig 2). Translating these effects into clinical benefits now becomes one of the main challenges. The fact that the long-term administration of the NAD^+ precursor molecules showed no deleterious effects in animals should be considered promising. As such, administration of NMN for 12 months demonstrated no toxicity in mice (Mills *et al*, 2016). Similarly, administration of NR to mice for a duration of 5–6 months (Gong *et al*, 2013), 10 months (Zhang *et al*, 2016), and 12 months (Tummala *et al*, 2014) showed no obvious adverse effects. Moreover, 2016 was marked by the first report on the effects of NR in humans, showing that the oral administration of NR led to a dose-dependent increase in NAD^+ levels in blood in healthy volunteers (Trammell *et al*, 2016a). Another NAD^+ precursor, NAM, has also been already tested in humans and protected β-cell function in type 1 diabetes patients (Olmos *et al*, 2006) and even though the clinical trials for NAM as a treatment for type 1 diabetes failed, no adverse effects of NAM were detected (Gale *et al*, 2004; Cabrera-Rode *et al*, 2006). Furthermore, a slow release form of NA (acipimox) was effective in inducing mitochondrial activity in skeletal muscle of type 2 diabetic patients (van de Weijer *et al*, 2015).

In theory, all this bodes well for the use of NAD^+ precursors in the clinic. The fact that many of these NAD^+ precursors are rightfully considered vitamins (which are generally regarded as safe (GRAS)), and that they are widely available to the public at large, however, also poses some issues. Despite the many health benefits that are inferred from their use in animal disease models, these NAD^+ precursors need still to undergo rigorous clinical testing in diseases setting, before one can recommend their widespread use. Therefore, some caution is required, so that the overuse or improper use in uncontrolled settings does not hamper their clinical development as nutra- or pharmaceutical agents.

It will also be important to identify which pharmacological strategies aiming to boost NAD^+ content would be the most appropriate in patients. Various monoclonal antibodies targeting CD38 have been developed as a treatment for hematological malignancies, some of them being in preclinical, and some even in late clinical, studies (van de Donk *et al*, 2016). Many flavonoids were reported to inhibit human CD38 at low micromolar concentrations

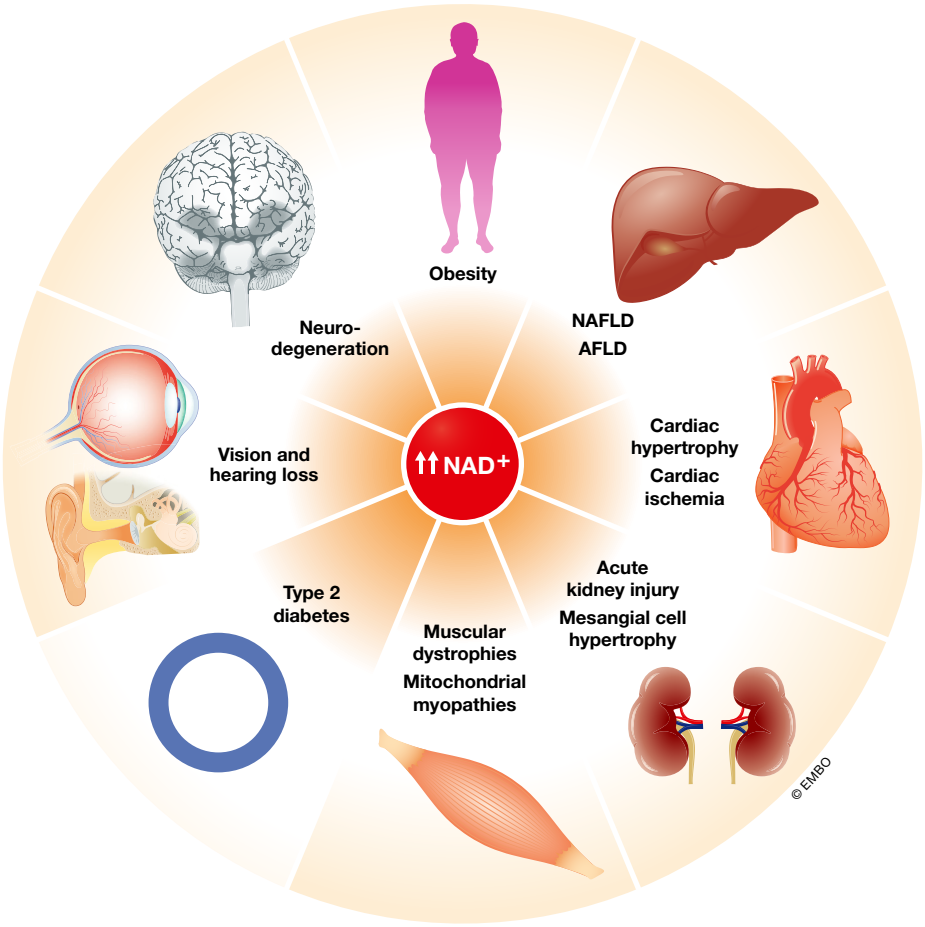

Figure 2. Therapeutic potential of NAD^+ boosting in humans based on findings in animal studies.
NAFLD, non-alcoholic fatty liver disease; AFLD, alcoholic fatty liver disease.

(Kellenberger *et al*, 2011; Escande *et al*, 2013), some of them showing promising therapeutic effects in mice (Escande *et al*, 2013; Boslett *et al*, 2017). Thiazoloquin(az)olinones have recently been described as potent CD38 inhibitors, able to elevate NAD^+ in plasma, liver, and muscle in mice (Haffner *et al*, 2015). Moreover, several reports have disclosed different small molecules blocking CD38 activity at low micromolar concentrations (Zhou *et al*, 2012; Moreau *et al*, 2013; Swarbrick *et al*, 2014; Wang *et al*, 2014b; Becherer *et al*, 2015). Testing and exploring their therapeutic potential in animal models is now just a matter of time.

Several PARP inhibitors are currently either marketed (Olaparib) or undergoing advanced clinical trials for the treatment of cancers in patients with BRCA mutations (Wang *et al*, 2016b). The drawback of these PARP inhibitors is that none of them is selective for a specific PARP family member (Wang *et al*, 2016b). For instance, the clinically approved compound, Olaparib, inhibits PARP-1, PARP-2, PARP-3, and PARP-4 (Wahlberg *et al*, 2012), increasing chances of adverse effects. As a case in point, the loss of both PARP-1 and PARP-2 in mice was reported to cause embryonic lethality (Menissier de Murcia *et al*, 2003), while a T cell-specific deficiency in both them leads to highly aggressive lymphomas (Navarro *et al*, 2017). Moreover, all current PARP inhibitors are genotoxic (Ito *et al*, 2016), which raises concerns about their use for non-oncologic indications. Indeed, some side effects that could be tolerated in case of the treatment of life-threatening diseases such as advanced cancers (Brown *et al*, 2016) may not be tolerated for non-oncologic indications. All these evidences indicate that the development of safer and more selective PARP inhibitors is necessary.

The growing literature on the beneficial effects of raising and maintaining NAD^+ levels in different disease models and the high evolutionary conservation of the NAD^+–sirtuin signaling axis suggests that strategies that increase cellular NAD^+ content may have a preventive and/or therapeutic potential in a large number of human diseases (Fig 2). With the first reports on human trials of various NAD^+ boosting techniques that start to appear, we are entering in the exciting era of NAD^+ therapeutics. While there is no certitude that NAD^+ boosting will be able to extend lifespan in humans, such strategies definitely possess the potential to delay age-associated physiological decline, and therefore, we predict that they will be useful to manage aging-related diseases and extend healthspan.

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Conflict of interest

JA is a founder and SAB member of Mitobridge, a company that develops NAD^+ boosting therapeutics.

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