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Therapeutic potential of nicotinamide adenine dinucleotide (NAD)

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ABSTRACT

Nicotinamide adenine nucleotide (NAD) is a small ubiquitous hydrophilic cofactor that participates in several aspects of cellular metabolism. As a coenzyme it has an essential role in the regulation of energetic metabolism, but it is also a cosubstrate for enzymes that regulate fundamental biological processes such as transcriptional regulation, signaling and DNA repairing among others. The fluctuation and oxidative state of NAD levels regulate the activity of these enzymes, which is translated into marked effects on cellular function. While alterations in NAD homeostasis are a common feature of different conditions and age-associated diseases, in general, increased NAD levels have been associated with beneficial health effects. Due to its therapeutic potential, the interest in this molecule has been renewed, and the regulation of NAD metabolism has become an attractive target for drug discovery. In fact, different approaches to replenish or increase NAD levels have been tested, including enhancement of biosynthesis and inhibition of NAD breakdown. Despite further research is needed, this review provides an overview and update on NAD metabolism, including the therapeutic potential of its regulation, as well as pharmacokinetics, safety, precautions and formulation challenges of NAD supplementation.

1. Introduction

Nicotinamide adenine nucleotide (NAD) is a small ubiquitous hydrophilic cofactor that is involved in multiple aspects of cellular metabolism (Nelson and Cox, 2017). As shown in Fig. 1., it consists of adenine and nicotinamide nucleotides linked through their 5′-phosphate groups (PubChem, 2019). It has two forms, oxidized and reduced, abbreviated as NAD^+ and NADH, respectively (Belenky et al., 2007). Although NADH is a powerful reducing agent, the redox reaction can be easily reversed, and the coenzyme can constantly cycle between NAD^+ and NADH without being consumed (see Fig. 2.) (Pollak et al., 2007). As a consequence, it is able to accept and donate electrons, acting as a shuttle for the transfer of electrons and protons in enzymatic reactions. This redox cycling is an essential process for biological reactions that occur across a wide range of metabolic pathways, such as glycolysis, citric acid cycle, and mitochondrial oxidative phosphorylation. Thus, it is an indispensable molecule for catabolism and ATP synthesis and storage (Lin and Guarente, 2003). NAD is very compartmentalized, and it is mainly found in the mitochondria. Since heart and muscle have a greater energetic demand than other tissues, mitochondria and NAD are more abundant in these tissues (Houtkooper et al., 2010).

It has to be noted that besides its typical role as a cofactor in reductive biosynthesis and oxidative breakdown, non-redox roles of NAD have been recently untangled, and have renewed the interest on this molecule. So, not only it participates in the regulation of energetic metabolism, but it is also a cosubstrate for several enzymes that regulate crucial biological processes (Pehar et al., 2018). Therefore, regulation of NAD metabolism can severely impact different biological processes, such as transcriptional regulation, DNA repairing, signaling, longevity and cellular viability (Chiarugi et al., 2012). In fact, around 500 enzymes, which are indispensable for maintaining homeostasis, use NAD as a cofactor or a cosubstrate, and NAD-dependent enzymes cannot function without it (Pankiewicz et al., 2015). NAD can be endogenously synthesized, and while redox reactions do not alter the overall levels of coenzyme, some reactions require continuous NAD^+ expenditure. Fluctuations in NAD^+ levels and/or NAD^+:NADH ratio have pronounced effects on cellular function, therefore NAD^+-synthesizing, -dependent and -consuming enzymes are interesting and potential therapeutic targets for drug discovery (Khan et al., 2007). Since high NAD^+ tissue concentrations have been associated to metabolic benefits, different strategies to raise tissue NAD^+ have been used, including enhancement of biosynthesis and inhibition of NAD^+ breakdown (Sultani et al., 2017). While the therapeutic importance of supplementation with NAD^+ and its precursors has been long recognized

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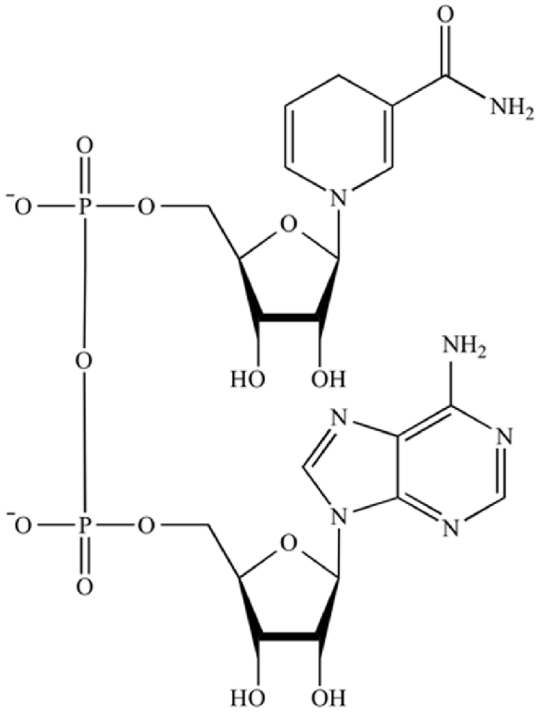

Fig. 1. NADH chemical structure (PubChem, 2019).

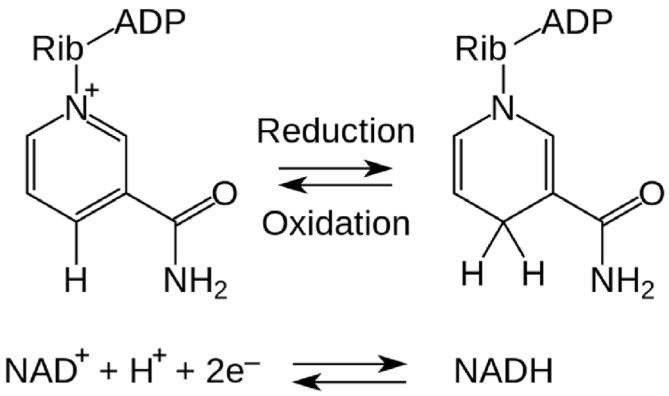

Fig. 2. NAD redox reaction (Nelson and Cox, 2017).

for the prevention and treatment of pellagra, considering the aforementioned key functions of NAD^+, the regulation of its levels and/or oxidized:reduced ratio is also an attractive target for the prevention and treatment of different conditions and pathologies including aging and age-related diseases like obesity, type 2 diabetes, neurodegenerative diseases and cancer, among others (Houtkooper et al., 2010). Since, NAD^+-consuming enzymes are sensitive to variations in NAD^+ concentrations, it is able to trigger adaptative changes in bioenergetics and survival. In fact, part of the positive effects of calorie restriction (CR) and exercise are believed to be due to sirtuin activation by the upregulation of NAD^+ production. This has raised interest in elevating NAD^+ levels as a potential approach to achieve beneficial health effects in humans (Yang and Sauve, 2016).

Considering that exogenous administration may enhance its biosynthesis, NAD and its precursors are interesting compounds for the formulation of food supplements and medicinal products. Besides its role in aging and age-associated diseases, and although efficacy has not been demonstrated for the following indications, some healthy individuals take oral NAD supplements to improve memory and concentration, as an antioxidant, and to enhance athletic performance through a presumed increase in ATP synthesis (Mero et al., 2008). These potential health benefits, and the increase in antioxidant consumption due to increasing healthy-living trend (Arenas-Jal et al., 2019), have led to an increased consumer demand for NAD. However, the extent of absorption, action and effectiveness of its supplementation is unclear. Despite it can be regarded as safe, and that there have been no reports of overdose or toxicity, high doses of NADH (> 10 mg/day) may cause agitation, anxiety and sleeplessness (DrugBank, 2019). When it comes to its formulation in food supplements and medicinal products, the lack of stability under different environmental conditions such as heat, pH, light and oxygen, and its high hygroscopicity, hinder its use (Rover et al., 1998; Wu et al., 1986). However, despite there are limited works regarding NAD formulation, different approaches have already been tested to overcome these issues.

The aim of this paper is to review and summarize the existing evidence for the therapeutic potential of NAD metabolism regulation. For this reason, its metabolism, synthesis and food sources, the therapeutic potential in different indications, as well as the pharmacokinetics, safety, precautions and formulation challenges of NAD supplementation are reviewed in more detail below.

2. NAD metabolism and NAD^+-consuming enzymes

As previously mentioned, not only NAD as a coenzyme has an essential role in catabolism and ATP synthesis and storage, but it is also a cosubstrate for several enzymes that regulate crucial biological processes. These non-redox roles of NAD have been recently untangled and have renewed the interest on this molecule. Since certain reactions need continuous NAD^+ consumption, its oxidative state and tissue concentration, which is regulated by the relative balance between consumption and biosynthesis, can affect the activity of these enzymes, and therefore have an effect on different processes such as transcriptional regulation, DNA repairing, signaling, longevity and cellular viability (Johnson and Imai, 2018). Since alterations in NAD^+ homeostasis have raised as a common characteristic of a variety of diseases, NAD^+-synthesizing, -dependent and -consuming enzymes have become interesting and potential therapeutic targets for drug discovery (Lin and Guarente, 2003). Considering the wide range of enzymes regulated by its levels, NAD^+ mimics lack selectivity leading to side effects. Despite several powerful and selective inhibitors of NAD^+-dependent enzymes have been described, being potentially effective as therapeutic agents, further research is needed to confirm its therapeutic potential and absence of severe side effects (Pankiewicz et al., 2015).

When it comes to the main families of NAD^+-consuming enzymes, sirtuins, poly(ADP-ribose)polymerases (PARPs) and ADP-ribosyl cyclases (ADPR-cyclases) stand out. Since they consume NAD^+ as a cosubstrate, not only NAD^+ availability can regulate the activity of these enzymes, but at the same time, their enzymatic activity along with NAD^+ biosynthetic rate can impact on NAD^+ tissue concentration (Katsyuba and Auwerx, 2017).

2.1. Sirtuins

Sirtuins are NAD^+-dependent deacetylases that play a key role in transcriptional regulation, DNA repair, metabolism, oxidative stress resistance, longevity and circadian rhythm regulation (Houtkooper et al., 2012; Nakagawa and Guarente, 2011). They have deacetylation activity so that NAD^+ hydrolysis is coupled to the removal of the acetyl group from lysine residues present in several substrate proteins, such as histones. Acetylation is a major regulatory mechanism of protein function and can affect several protein features like subcellular location, enzymatic kinetics and interactions with other proteins among others (Drazic et al., 2016; Narita et al., 2019). Thousands of acetylated proteins from diverse subcellular compartments have been identified. By way of example, 63% of mitochondrial proteins are subject to reverse acetylation, which is a crucial regulatory mechanism for optimal mitochondrial function. Since lysine acetylation negatively affects the

activity of most metabolic enzymes, sirtuin-mediated NAD^+-dependent deacetylation is usually associated with activation of enzymatic processes (Baeza et al., 2016; Pehar et al., 2018). The activity of sirtuins has been linked with the regulation of a wide spectrum of important cellular functions that will be discussed below. However, since sirtuins have less affinity to NAD^+ than other NAD^+-consuming enzymes, higher NAD^+ levels are required to enhance their activity. For this reason, either overexpression or knockdown of nicotinamide phosphoribosyltransferase (NAMPT), which is the rate-limiting enzyme in NAD^+ salvage pathway, has been associated with increased or reduced sirtuin activity, respectively (Cantó et al., 2013).

2.2. PARPs

PARPs are NAD^+-consuming enzymes involved in DNA repair, epigenetic modifications, tumorigenesis, cell differentiation, metabolism and many other cellular processes (Bai, 2015). Despite their specific role in the regulation of DNA damage has been highly characterized, PARPs also regulate adaptive responses to inflammatory, oxidative, proteotoxic, and genotoxic stresses (Mouchiroud et al., 2013). PARPs are widely distributed in all tissues and hydrolyze NAD^+ to transfer an ADP-ribose moiety to a receptor amino acid. While PARP activation is an integral part of the cellular response to oxidative stress-induced DNA damage, excessive activation impairs mitochondrial function and has been linked to cell death. This detrimental effect resultant of PARP overactivation is likely to be due to an excessive NAD^+ consumption that leads to a catastrophic decline in cytosolic NAD^+, which is translated into glycolytic inhibition and cell death. Thus, PARP overactivation is a major component in oxidant-induced mitochondrial dysfunction (Ying et al., 2005).

2.3. ADPR-cyclases

ADPR-cyclases, also known as cyclic ADP-ribose synthases, which include CD38 and CD157, are NAD^+-consuming enzymes with multiple cellular functions such as calcium signaling and immune function regulation (Czura and Czura, 2006). Despite ADPR-cyclases have receptor functions in immune and myeloid cells, they are also multifunctional enzymes that not only catalyze the cyclization of NAD^+ to cyclic ADP ribose, but also the production of several second messengers that act as powerful intracellular calcium-mobilizing agents to control chemotaxis of dendritic cells and activation of microglia. In fact, CD38 is highly expressed in neurons and astrocytes, where it consumes 100 NAD^+ molecules to generate 1 molecule of cyclic ADP-ribose (cADPR), a second messenger (Hogan et al., 2019; Pehar et al., 2018). For this reason, since the main enzymatic activity of CD38 is NAD^+ hydrolysis, it is regarded as an important regulator of intracellular NAD^+ pools, and therefore of metabolic pathways (Camacho-Pereira et al., 2016; Chini, 2009). CD157 instead, is involved in immune development and has been recently associated with the development of Parkinson's disease in specific populations. Besides, although it also produces cADPR, its catalytic efficiency is significantly lower than CD38 (Quarona et al., 2013).

Finally, when it comes to the interaction between these NAD^+-consuming enzymes, the Michaelis constant (Km), which is an inverse measure of affinity, is higher in sirtuins than in PARPs and CD38. So, since sirtuins require elevated NAD^+ levels to boost their activity, the activation of other NAD^+-consumers may reduce NAD^+ availability and therefore, limit sirtuin activity (Verdin, 2015). In contrast, a reduction in PARP and CD38 activity is translated into an increase in total NAD^+ levels leading to sirtuin activation. This is markedly interesting considering that the aforementioned NAD^+-consumers have opposed functions compared to sirtuins (Cantó et al., 2013).

3. Synthesis, food sources and deficiency

Despite redox reactions do not affect the overall levels of NAD, certain reactions need continuous NAD^+ consumption. Therefore, since cells need it to maintain viability, NAD is constantly produced (Dölle et al., 2013). It is biosynthesized via two major pathways: *de novo* synthesis from the essential amino acid tryptophan, or via salvage pathways that recycle byproducts of intracellular NAD catabolism such as nicotinic acid, nicotinamide and nicotinamide riboside, which are altogether referred to as niacin or vitamin B3, and can also be acquired from the diet (Braidy et al., 2019). In salvage pathways, nicotinamide, which is the principal dietary precursor in mammals and also the main by-product of NAD^+-consuming enzymes in eukaryotic cells, is converted back to nicotinamide mononucleotide by NAMPT. Then, the latter is converted back to NAD^+ by nicotinamide mononucleotide adenylyltransferase (NMNAT) enzymes. Despite all the biosynthetic pathways meet at dinucleotide formation step, which is catalyzed by NMNAT, NAMPT is the rate-liming enzyme in the salvage pathway (Dölle et al., 2013). For this reason, overexpression of NAMPT, and not NMNAT, elevates NAD^+ cellular levels (Revollo et al., 2004).

While the components for the *de novo* biosynthesis of NAD from tryptophan are circumscribed to the cytosol, NAD might be carried to other compartments. However, considering the high NAD^+ consumption rates, it seems possible that different compartments have independent biosynthesis. Indeed, the nucleus and mitochondria, both of which require NAD^+ replenishment due to sirtuin- and/or PARP-mediated consumption, are able to synthetize NAD^+ from nicotinamide (Cantó et al., 2015). Finally, although NAD can be synthesized from *de novo* and salvage pathways, the circulating levels of most NAD precursors are generally lower than required to keep high intracellular NAD synthetic rates (Cantó et al., 2015). For this reason, and because salvage pathways, which need the uptake of NAD precursors from the diet, are the main source of NAD (Denu, 2007), its synthesis needs to be reinforced with nutrition. Otherwise, a persistent lack of dietary niacin or tryptophan causes pellagra, which is a vitamin deficiency disease manifested with diarrhea, dermatitis, dementia and even death. However, it can easily be cured with tryptophan or niacin supplementation (Caballero et al., 2003).

4. Therapeutic potential of NAD metabolism regulation

NAD relevance as a therapeutic agent has long been recognized in pellagra. However, the current incidence of this disease is low due to improved nutritional status of populations (Caballero et al., 2003). For this reason, and mainly due to the recent progress in untangling NAD key roles in many cellular processes, regulation of NAD metabolism has become an attractive therapeutic target for other indications. While NAD abundance in cells is regulated by breakdown and by the genetic and transcriptional factors that control the expression of biosynthetic enzymes, variations in NAD levels can have a significant effect on metabolic efficiency and cellular function (Cantó et al., 2015). NAD^+ levels oscillate in a circadian fashion, allowing proper diurnal coupling of transcription with metabolism (Sultani et al., 2017). However, not only NAD^+ levels but also NAD^+:NADH ratio, play a key role in the regulation of metabolic enzymes and the intracellular redox state. In fact, since this ratio varies in response to changes in metabolism, it is used as an indicator of the metabolic state (Bilan et al., 2014).

NAD homeostasis is susceptible to aging and other conditions that lead to NAD^+ levels decline, which is translated into defects in nuclear and mitochondrial functions, resulting in many age-associated diseases (Camacho-Pereira et al., 2016). In fact, several age-related diseases have been associated with a change in NAD^+ levels or NAD^+/NADH redox state (Braidy et al., 2011). In addition, models of obesity and metabolic dysfunction have also been linked to NAD^+ depletion, whereas high NAD^+ levels have been reported in response to CR and exercise, which are associated to metabolic benefits (Poljsak and

Milisav, 2016). These findings have encouraged research in the potential therapeutic effects that NAD^+ levels replenishment may have on aging or age-associated diseases. Preliminary evidence shows that restoring or elevating NAD^+ concentrations can drastically ameliorate age-associated functional defects and even counteract age-associated diseases (Aman et al., 2018). Thus, enzymes involved in NAD^+ metabolism have emerged as attractive therapeutic targets for drug discovery against several human diseases. Despite the development of potent and selective inhibitors of NAD^+-consuming enzymes could be efficacious in the treatment of different human diseases, research is in its early stages (Khan et al., 2007). Besides, administration of NAD or its precursors has been shown to be able of effectively increasing NAD^+ levels in different cell types (Okabe et al., 2019; Srivastava, 2016). The current scientific evidence regarding different potential therapeutic indications of NAD metabolism regulation is reviewed below.

4.1. Aging

Aging is a generalized physiological progressive decline characterized by a lower ability of the organism to maintain cellular homeostasis over time due to the accumulated molecular, cellular and organ damage. Despite that all the causal factors of aging are not fully understood, loss of regenerative potential, defects in DNA repair and mitochondrial decline appear to be common aspects of aging in mammals. If this decline is not counterbalanced, it can lead to the development of several age-associated diseases (Cui et al., 2012; Malavolta and Mocchegiani, 2016). Considering the aging human population and the increase of age-associated mortality and morbidity, improving healthspan is an attractive target for pharmacological intervention (Figueira et al., 2016; Khan et al., 2017).

CR increases the lifespan, reduces the incidence and retards the onset of several age-associated diseases in different models, and it is the only intervention capable of extending mammalian lifespan (Omodei and Fontana, 2011). Despite more studies are needed to confirm if CR is capable of extending human lifespan, studying its molecular basis is crucial to develop effective strategies to retard the onset of aging outcomes (Balasubramanian et al., 2017). The positive effects of CR seem to require sirtuins, which can only function in the presence of enough NAD^+, which suggests that the metabolic state of cells controls their activity (Nakagawa and Guarente, 2011; Zullo et al., 2018). In fact, studies in yeast suggest that CR might shift the metabolism towards tricarboxylic acid cycle, leading to increased respiration and therefore, increased NAD^+ levels and NAD^+:NADH ratio, that activate sirtuins (Lin and Guarente, 2003). It has to be noted that not only CR, but also different situations of energy stress such as exercise or low glucose bioavailability, lead to an increase of NAD^+ levels and boost sirtuins activity (Houtkooper et al., 2012). The activation of sirtuins stimulates transcriptional programs that promote metabolic efficiency as well as the upregulation of oxidative metabolism, antioxidant pathways and DNA repair. Therefore, as it has been reported in yeast, worms, flies and murine models, their activation results in a lower incidence of aging diseases and promotes longevity (Imai and Guarente, 2014). Despite aging in humans is considerably more complex, and that further studies are required to understand its fundamental mechanisms, deacetylation of p53 by human sirtuins is known to promote cell survival under stress, suggesting an crucial role of NAD^+ and sirtuins in human aging and lifespan (Khan et al., 2017; Luo et al., 2001).

Besides, the characteristic accumulation of DNA damage and inflammation in aging leads to excessive PARP activation. This results in NAD^+ depletion, which is translated into a decline in mitochondrial function (Maynard et al., 2015), and loss of sirtuin function (Mendelsohn and Larrick, 2017). Considering that sirtuins control the circadian clock, which is responsible for NAD synthesis regulation, there is also a decline in NAD biosynthesis in aging (Masri, 2015). This feedback circuit promotes cell senescence, impaired capacity for tissue maintenance and regeneration, health deterioration, disease and premature aging. Since conservation of appropriate NAD^+ pools is essential to maintain metabolic health in older age, there is an increasing interest in replenishing NAD^+ levels. In fact, while a reduction of NAMPT expression results in NAD decline, NAMPT overexpression leads to delayed senescence and enhanced oxidative stress defense (Revollo et al., 2004; van der Veer et al., 2007). Inhibition of PARP and CD38 has also been suggested as a potential therapeutic approach to maintain NAD^+ levels during aging, which would allow the activation of other NAD^+-consuming enzymes that compete for the same pool, such as sirtuins (Mouchiroud et al., 2013). In fact, while aging in mice is associated with an increase in CD38 activity, leading to NAD^+ levels and mitochondrial activity depletion, CD38 knockout mice seem to be protected from this decline due to an increase in NAD^+ availability (Camacho-Pereira et al., 2016). However, PAPR and CD38 have complex roles, and considering that their activation contributes to maintain genomic integrity, its inhibition could cause severe side effects. Thus, further studies are required to evaluate whether this strategy may have a therapeutic value (Houtkooper et al., 2010). For this reason, supplementation with NAD^+ or its precursors seems the most reasonable approach at this stage. In animal studies, NAD^+ precursors supplementation restored NAD^+ levels, improved mitochondrial function and reversed sirtuin inactivation triggered by aging (Imai and Guarente, 2014). Therefore, enhancing NAD^+ levels might also be useful to improve lifespan and healthspan in conditions sharing common pathological mechanisms with aging (Zhang et al., 2016). For instance, long-term administration of NAD^+ precursors in different age-associated disease provided improved energy and lipid metabolism, insulin sensitivity, eye function and bone density among others (Aman et al., 2018).

4.2. Neurodegenerative diseases

Aging is the main risk factor for the development of neurodegenerative diseases (Kritsilis et al., 2018). As previously mentioned, disrupted NAD homeostasis is involved in stem cell senescence and impaired capacity for tissue maintenance and regeneration, which leads to several age-associated diseases. In contrast, regulation of NAD metabolism has emerged as a key therapeutic target for neurodegenerative diseases. Restoring NAD^+ levels might delay senescence, enhance oxidative stress defense and prevent, delay or treat several neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease as well as amyotrophic lateral sclerosis (Verdin, 2015). Besides modulating mitochondrial function and oxidative stress defense, NAD has also a more specific role in neurodegeneration (Elfawy and Das, 2019). A critical event in neurodegenerative conditions is Wallerian degeneration or axonopathy, which is the damage to nerve fibers that occurs in response to different factors, including aging, and is usually accompanied by ATP and NAD^+ depletion (Wang and He, 2009). The expression of the genetic alteration Wallerian degeneration slow (Wld^s) preserves neuronal NAD^+ levels leading to sirtuin activation, which confers resilience to nerve damage. Thus, modulation of NAD^+ metabolism might be capable of delaying and protecting against axonal degeneration (Araki et al., 2004). In fact, while reduction of NMNAT expression leads to degeneration even in uninjured axons, NMNAT activity is crucial in the neuroprotective effect of Wld^s mutation, probably due to NAD^+ levels replenishment that is translated into sirtuin activation and improved energetic function (Pehar et al., 2018; Verdin, 2015). In addition, vincristine-induced axonopathy was characterized by NAD^+ depletion, whereas bathing neurons in NAD^+ had a protective effect. Therefore, therapies that preserve, restore or elevate neuronal NAD^+ levels may be neuroprotectant (Belenky et al., 2007).

Besides, there is a PARP upregulation in the central nervous system in neurodegenerative diseases. Excessive PARP and CD38 activation depletes NAD^+, compromising mitochondrial function and leading to cell death. Thus, their inhibition could be beneficial in the treatment of neurodegenerative diseases. In fact, PARP inhibition improves axonal

regeneration and prevents neuronal death in certain models of neurodegeneration (Martire et al., 2015). At the same time, CD38 inhibition increases NAD^+ levels in the brain and has a neuroprotective effect, which is likely to be due to its role in the regulation of inflammatory mediators such as amyloid beta (Blacher et al., 2015; Tarragó et al., 2018). However, since PARP and CD38 are involved in DNA repair, oxidative stress defense and inflammation, which are prevalent in neurodegenerative processes, it is hard to predict whether the inhibition will have a positive effect.

Finally, NAD^+-mediated neuroprotection depends on sirtuins. Transgenic mice overexpressing sirtuins had similar phenotypes to CR and were capable to prevent the detrimental effects of energy-dense diet and aging (Bordone et al., 2007). In addition, these were protected from Alzheimer's, Parkinson's and Huntington's disease. Overexpression of brain sirtuins in murine models of Alzheimer's disease inhibited the production of amyloidogenic peptides and reduced the resulting neuropathology (Donmez and Outeiro, 2013; Herskovits and Guarente, 2013). Also, sirtuins in oligodendrocytes may contribute to myelinization processes (Li et al., 2007). For this reason and considering that sirtuins have a NAD^+-dependent activity, NAD^+-boosting molecules might replace CR in treating specific diseases. In fact, nicotinamide mononucleotide and nicotinamide ribose were able to penetrate to the brain and effectively restore NAD^+ and ATP levels, increase sirtuin activity, improve mitochondrial function, memory, cognition and behavior, and to reduce reactive oxygen species (ROS), toxicity and amyloid β production (Wang et al., 2016). Thus, the administration of NAD^+ precursor compounds is promising for the prevention and treatment of neurodegenerative diseases. However, further research is needed to determine whether the supplementation of NAD^+ intermediates in humans is safe and effective.

4.3. Cancer

The role of NAD^+ metabolism in the regulation of cancer is particularly complex. Several NAD^+-dependent enzymes affect genomic stability, mutagenesis and metabolic reprogramming, which are the main factors that promote cancer initiation and progression. Therefore, oscillations in NAD^+ levels and redox state could have a great influence on tumorigenesis (Poljsak, 2016). In fact, increased ATP production and biomass generation are characteristic attributes of cancer. For this reason, restricting NAD^+ availability could prevent cells growth, and sensitize cancer cells to oxidative damage caused by chemo- and radiotherapy (Chiarugi et al., 2012). This would cause apoptosis leading to reduced tumorigenesis, metastasis and angiogenesis. However, this could also disrupt other NAD^+-dependent processes, leading to toxicity for normal cells and severe adverse effects in patients. Thus, highly selective inhibitors should be designed to target only cancer cells (Khan et al., 2007).

Since tumors consume large amounts of NAD^+, the expression of NAD^+ biosynthetic enzymes is upregulated in certain cancers. By way of example, NAMPT, NMNAT and nicotinic acid mononucleotide adenylyl transferases are overexpressed in a variety of tumors, and it seems that they contribute to tumorigenesis by several NAD^+-dependent mechanisms. In fact, high NAMPT levels correlate with poor patient survival (Yaku et al., 2018). In addition, NAD^+-dependent enzymes are also often overexpressed in cancer cells. Therefore, its selective inhibition has been considered a potential anticancer strategy. Indeed, inosine monophosphate dehydrogenase inhibitors limit DNA replication and nucleotide biosynthesis, and could also be useful as antibacterial and antiparasitic agents, since the amino acid sequences of these enzymes vary significantly in bacteria, parasites and mammals (Pankiewicz et al., 2015). When it comes to NAD^+-consuming enzymes, PARP activity adjusts NAD+ levels according to cell damage, leading to either DNA repair or apoptosis. Acute or severe DNA damage following ionizing radiation or exposition to genotoxic agents results in PARP overactivation, which triggers a sudden depletion of NAD^+ that lowers ATP generation rate leading to apoptosis of damaged cells (Yaku et al., 2018). However, its activity is enhanced in tumors, promoting DNA repair and survival of those cancer cells under genomic stress. For this reason, PARP inhibitors could sensitize tumor cells to apoptotic killing by chemo- and radiotherapy (Curtin and Szabo, 2013). In addition, PARP inhibition leads to elevated NAD^+ levels allowing enhanced sirtuin activity (Poljsak, 2016). Despite sirtuins play contradictory roles in cancer, they have been linked to genomic stability as well as reduced replication of cancer cells and tumor progression (Bosch-Presegué and Vaquero, 2011).

Whereas as previously mentioned, restricting NAD^+ availability could prevent tumorigenesis, metastasis and angiogenesis, CR, which is known to increase NAD^+ levels, is associated with reduced susceptibility to many cancers (Meynet and Ricci, 2014). Therefore, increasing NAD^+ levels might also have protective effects. In fact, while niacin deficiency is prevalent in patients with neuroendocrine cancers and has been linked with increased cancer risk and complications, niacin supplementation prevents the development of skin and liver cancer (Sultani et al., 2017), and might prevent secondary cancer after chemotherapy (Boyonoski et al., 2002). Taking all these into consideration, despite there is no doubt that alterations in NAD^+ levels and regulation of NAD^+-synthesizing, -dependent and -consuming enzymes can influence cancer development, its relationship during the malignant process is very complex. Thus, further research is needed to elucidate the role of NAD^+ metabolism in cancer prevention, initiation and treatment.

4.4. Diabetes

Glucose homeostasis and release of insulin and insulin-like growth factor (IGF) are regulated by sirtuins (Kuang et al., 2018). Since these are NAD^+-dependent enzymes, NAD^+ metabolism also plays an important role in diabetes. During glucose deprivation, NAD^+ levels and sirtuin activity increase, which finally leads to gluconeogenesis promotion (Rodgers et al., 2005). In turn, insulin and IGF increase glycolysis in cells, which reduces NAD^+/NADH ratio and inhibits sirtuin activity. Thus, insulin and IFG are negatively regulated by sirtuins (Houtkooper et al., 2010; Nogueiras et al., 2012). Besides, other NAD^+-consuming enzymes have also been associated to insulin production. Indeed, in type 1 diabetes, PARP overactivation leads to depletion of NAD^+ levels, resulting in death of insulin-producing β cells (Charron and Bonner-Weir, 1999). In addition, hyperglycemia in type 1 and type 2 diabetes also decreases NAD^+ and NAD^+:NADH ratio, ultimately resulting in diabetic complications (Wu et al., 2016). In this context, selective inhibition of these enzymes could restore NAD+ levels and prevent diet- and age-induced type 2 diabetes (Mouchiroud et al., 2013). In fact, PARP and CD38 knockout mice had increased NAD + levels, mitochondrial function and sirtuin activity, as well as improved lipid and glucose homeostasis. Thus, they were protected from metabolic dysfunction, obesity and diabetic symptoms induced by high-fat feeding (Bai et al., 2011; Barbosa et al., 2007). However, at this stage, this is not a feasible therapeutic approach for human diabetes due to potential severe side effects. For this reason, the administration of NAD^+ precursors has been suggested as an alternative to replenish NAD^+ levels. In fact, following the administration of NAD^+-precursors in prediabetic and diabetic mice, glycemic control biomarkers were improved, weight gain was reduced and protection against neuropathy development was increased (Sultani et al., 2017; Yang and Sauve, 2016). In addition, NAD^+ tissue levels that were declined due to a high-fat diet were replenished, and protection against the development of diet-induced obesity and insulin resistance in wild type mice was conferred. These positive effects were probably due to enhanced oxidative metabolism that led to increased lipid utilization (Imai and Guarente, 2014). Last but not least, axonopathy is a critical event in diabetes-induced peripheral neuropathy. Since it is usually linked with ATP and NAD^+ depletion, the restoration of NAD^+ levels could be translated

into neuroprotective effects (Araki et al., 2004; Wang and He, 2009). Thus, therapies that stimulate NAD^+ biosynthesis, such as activation of NAD^+-synthesizing enzymes or administration of NAD^+ precursors, might have potential for the prevention of diabetes-induced peripheral neuropathy.

4.5. Other indications

4.5.1. Antioxidant and anti-inflammatory properties

ROS are produced as a result of normal metabolic processes but may come from exogenous sources as well. While the accumulation of oxidative damage is associated with age-related decline and diseases, there is a complex system of endogenous antioxidants and damage repair mechanisms that counteract the deleterious effects generated by oxidants (Massudi et al., 2012). Sirtuins have a direct influence on mitochondrial function and antioxidant defenses by means of mitochondrial-superoxide-2 and isocitrate-dehydrogenase-2, among others. In addition, while changes in protein acetylation affect the redox status of cells, changes in redox status can also regulate several metabolic and antioxidant pathways (Singh et al., 2018). While molecular damage as well as different pathogens, diseases, and conditions such as obesity led to overactivation of the inflammasome complex, which is a component of innate immune surveillance (Vandanmagsar et al., 2011), fasting and administration of NAD^+ precursors decreased inflammasome activation in a sirtuin-dependent manner in human-derived macrophages. This suggested that regulation of NAD^+ signaling could be a potential therapeutic approach to inhibit excessive inflammation (Li et al., 2017, 2016).

4.5.2. Mitochondrial disorders

Since elevated NAD^+ levels enhance mitochondrial function and cell survival, increased NAD^+ production could be beneficial in mitochondrial disorders. In fact, studies in animal models of mitochondrial disease showed that enhanced NAD^+ production by either administration of NAD^+ precursors, PARP inhibition or PARP genetic knockout, improved exercise tolerance and mitochondrial function (Yang and Sauve, 2016). In addition, sirtuins, which depend on NAD^+ levels, were able to protect mitochondria from oxidative stress, promoting mitochondrial integrity (Singh et al., 2018). Despite further research is needed, these results have stimulated interest in the potential benefits of boosting NAD^+ in mitochondrial diseases.

When it comes to chronic fatigue syndrome (CFS), despite its etiology is unknown, inflammation, oxidative stress and mitochondrial dysfunction have been linked to this extremely debilitating illness. Since NAD^+ plays a role in these factors, it could confer potential therapeutic benefits for CFS patients. In fact, Castro-Marrero et al. (2015) carried out a clinical trial in patients with CFS and reported that coenzyme Q10 plus NADH supplementation resulted in a significant improvement of fatigue. In addition, biochemical parameters such as NAD^+:NADH ratio and ATP were restored.

4.5.3. Hearing loss

Hearing loss results from cochlear degeneration following damage produced by different factors such as high-intensity sound, ototoxic agents, aging and intrinsic disorders like systemic diseases (Liu and Yan, 2007). It is remarkable that either Wld^s mouse, which leads to sirtuin activation, or sirtuin overexpression, prevents noise-induced hearing loss (Brown et al., 2014). As mentioned in Section 4.2., by means of NMNAT activity, Wld^s confers protection of neuronal NAD^+ levels, leading to sirtuin activation and improved energetic function, which results in resistance to nerve damage. Besides, while the prevalence of hearing loss increases with age, CR reduces the progression of age-related hearing loss in a sirtuin-dependent manner. Sirtuins increase oxidative stress resistance and prevent the mitochondrial decay associated with aging and age-related hearing loss (Someya et al., 2010). Since sirtuins are NAD^+-dependent enzymes, to confirm whether increased NAD^+ could promote sirtuin activation and prevent hearing loss, nicotinamide ribose was administered before and after, or only after noise exposure. Despite all treatments fully protected hearing in all frequencies, and therefore, increasing NAD^+ levels in neurons and ear tissues might provide protection from trauma-induced and progressive hearing loss, further research is needed (Brown et al., 2014).

Moreover, several drugs are known to induce ototoxicity, such as the chemotherapeutic agent cisplatin. Different mechanisms like oxidative stress, DNA damage and inflammation are involved in cisplatin-induced cochlear damage (Rybak et al., 2009; Sheth et al., 2017). In addition, impairment in intracellular NAD^+ levels and NAD^+:NADH ratio is also critical. Despite further research is needed to elucidate the precise mechanisms underlying cisplatin ototoxicity, it seems that its administration leads to the production of ROS, causing oxidative DNA damage which hyperactivates PARP that consume NAD^+. This is translated into a significant decrease in sirtuin activity, which has a protective effect against ototoxicity (Kim et al., 2014). Besides, the active immune response following cisplatin administration is also implicated in cochlear damage and hearing loss. In contrast, an increase in NAD^+ levels and NAD^+:NADH ratio results in sirtuin activation, which suppresses the adverse effects of cisplatin by down-regulating oxidative stress factors and inflammatory responses (Kim et al., 2016, 2015). Thus, despite further research is needed, modulation of NAD^+ metabolism could be a promising and new therapeutic approach for the treatment of cisplatin-induced ototoxicity.

4.5.4. Retinal degenerative diseases

Retinal degenerative diseases are a main cause of morbidity, as vision disability significantly affects the quality of life. While different factors are involved, death of light-sensitive photoreceptors that leads to blindness is a common outcome of retinal degenerative diseases (Lin and Apte, 2018). Sirtuins counteract oxidative stress and retinal degeneration, contributing to survival and function of retinal photoreceptors (Balaiya et al., 2017; Luo et al., 2017). However, retinal NAD^+ deficiency, and resultant low sirtuin activity has been reported in multiple retinal degenerative disorders, including age-associated dysfunction, diabetic retinopathy and light-induced degeneration. NAD^+ seems to play a central role in retinal degeneration, since alterations of its homeostasis may contribute to neurodegeneration and photoreceptor death (Lin and Apte, 2018). Furthermore, mutations in NAD^+ biosynthetic enzymes have been associated to childhood blinding disease and retinal degeneration (Lin et al., 2016). Therefore, sirtuin activation via supplementation with NAD^+-precursors could be a feasible therapeutic strategy for the treatment of retinal degenerative diseases. In fact, administration of nicotinamide nucleotide in mice was able to restore NAD^+ levels and retinal function (Mills et al., 2016).

4.5.5. Cardiovascular diseases

DNA damage in myocytes activates PARP (Zhang et al., 2019), which adjusts NAD+ levels according to cell damage. Since PARP and sirtuins compete for the same limiting NAD^+ pool, PARP might act as regulators of sirtuin activity. Sirtuins have antiapoptotic effects in cardiomyocytes, exerting a remarkable protective effect against heart failure (Chung and Joe, 2014; Pillai et al., 2005). For this reason, replenishment of NAD^+ levels by administration of precursors or increased activity of NAD^+ biosynthetic enzymes could recover cell viability (Hsu et al., 2009; Yamamoto et al., 2014). In fact, the administration of NAD^+ precursors improved survival in mice with transferrin-receptor-1 deletion, which caused iron deficit and mitochondrial defects in the heart leading to premature lethality. This protective effect of NAD^+ could be attributed to the improvement in mitochondrial function (Xu et al., 2015). Besides, sirtuins enhance vascular function by suppressing the expression of inflammatory factors, and improve free fatty acid, triglyceride, total cholesterol and blood glucose levels. Therefore, they act as anti-atherosclerosis agents (Johnson and Imai, 2018). Considering that NAD^+ regulates sirtuin

activity, nicotinamide, which is a NAD^+ precursor, has been used to lower cholesterol and triglyceride levels and to improve HDL/LDL ratio (Rajman et al., 2018). The improvement in lipid metabolism was attributed to NAD^+ levels increase that resulted in sirtuin activation (Ye et al., 2017). Considering the wide impact of cardiovascular diseases in human population, further studies should be carried out to clarify the potential cardioprotective effect of increasing NAD^+ levels.

4.5.6. Jet lag

Jet lag is a syndrome characterized by psychological and physiological effects such as fatigue, irritability, disrupted sleep, gastrointestinal distress or memory loss, that occur after a long flight through several time zones. It probably results from disruption of circadian rhythms in the human body (Choy and Salbu, 2011; Weingarten and Collop, 2013). Since the impairment of circadian rhythm leads to a range of metabolic defects, and current remedies for jet lag are limited in efficacy and practicality, there is increased interest in finding a substance capable of counteracting jet lag (Birkmayer et al., 2002).

The circadian rhythm is regulated by a transcriptional feedback system that coordinates metabolism and behavior to recurring daily changes such as light/dark cycles and food availability (Eckel-Mahan and Sassone-Corsi, 2013; Reinke and Asher, 2019). NAMPT expression displays circadian rhythmicity and leads to circadian oscillation of NAD^+ levels, which regulate the activity of sirtuins. In turn, in an NAD^+-dependent manner, sirtuins not only regulate key enzymes in mitochondrial processes and antioxidant defenses, but also the circadian core clock, which is responsible for NAD synthesis regulation (Masri, 2015). Since NAD increases cellular production of ATP and facilitates dopamine synthesis, it may counteract the effects of jet lag on cognitive function and sleepiness. In fact, Birkmayer et al. (2002) reported that NADH reduced jet lag-induced disruption of cognitive function, with no adverse effects.

4.5.7. Depression

Sirtuins are involved in the development of depression and other mood disorders such as anxiety. While NAD^+ regulates the activity of sirtuins, these regulate the expression of monoamine oxidase A, which lowers serotonin and drives anxiety-like behaviors (Libert et al., 2011). Genetic or pharmacological inhibition of sirtuins, as well as long-term stress, which has been linked with a decrease in sirtuin activity, lead to depressive-like behaviors (Johnson and Imai, 2018). Since NAD metabolism regulates the activity of sirtuins, and it is involved in the production of energy, it could have beneficial effects on diseases affecting the central nervous system. Rex et al. (2004) studied the potential antidepressant role of NADH and nicotinamide in a forced swimming test in Wilstar rats, and found that NADH but not nicotinamide, increased swimming behavior. This behavioral profile was similar to fluoxetine, and since NADH did not produce hyperlocomotion, the antidepressant-like effect was not attributed to an increase in motor activity but to the antidepressant potential of NADH.

4.5.8. Fatty liver disease

Since NAD^+ homeostasis is impaired in fatty liver disease, regulation of NAD^+ metabolism is potentially useful for the treatment of this disease, which is associated with several metabolic disorders (Zhou et al., 2016). While hepatic NAD^+ levels are depleted in fatty liver disease, supplementation with NAD^+ precursors or inhibition of PARP enzymes prevents its development in several models on a high-fat diet. In addition, NAD^+ replenishment reverses the existing pathology (Gariani et al., 2016; Komatsu et al., 2018). However, further studies are needed to understand how NAD^+ increase exerts its protective effects, and to determine whether NAD supplementation could be an effective therapeutic approach for fatty liver disease and other metabolic disorders (Okabe et al., 2019).

4.5.9. Candidiasis

Candida glabrata is an opportunistic pathogen yeast, and it is the second leading cause of candidiasis. Since it lacks genes encoding the enzymes responsible for the *de novo* biosynthesis of NAD^+, it is a nicotinic acid, nicotinamide and nicotinamide riboside auxotroph (Gazzaniga et al., 2009). Low levels of these precursors of the NAD^+ salvage synthesis leads to NAD^+ depletion, limiting the function of sirtuins, which repress the transcription of genes encoding adhesins that promote urinary-tract infections. Therefore, increased dietary nicotinic acid provides protection against urinary tract infections (Domergue et al., 2005). However, since high doses of nicotinic acid may cause flushing, nicotinamide riboside instead should be tested in humans for the treatment against *C. glabrata* (Belenky et al., 2007). In addition, *C. glabrata* utilizes mainly nicotinamide riboside, and to a lesser extent nicotinic acid as NAD^+ sources during disseminated infection (Ma et al., 2007).

4.5.10. Enhanced endurance capacity

Skeletal muscle has a high energy demand, and it is therefore a major consumer of glucose and fatty acids. Since NAD^+ plays a crucial role in energy production and post-translational modifications, its levels influence a wide range of cellular processes that impact muscle function, regeneration, aging, and disease (Goody and Henry, 2018). While an accelerated decline in muscle mass and function is produced when NAD^+ levels are dramatically depleted due to NAMPT deletion, these defects were overcome when NAD^+ precursors were administered to mice. In line with these findings, NAMPT overexpression in skeletal muscle is associated with preservation of NAD^+ levels and enhanced endurance capacity in animals (Frederick et al., 2016; Wang et al., 2017).

5. NAD and NAD-precursors supplementation

5.1. Pharmacokinetics

When it comes to its pharmacokinetics, the majority of studies have focused on the administration of NAD precursors such as nicotinic acid, nicotinamide and nicotinamide riboside (Rajman et al., 2018; Trammell et al., 2016). However, few studies have been carried out using NAD^+ or NADH. In addition, there are no pharmacokinetic studies of NAD supplementation in humans.

Rex et al. (2002) studied NADH pharmacokinetics in rats and concluded that although NADH absorption after oral administration could be assumed, the slow absorption could lead to a relatively small bioavailability. In a subsequent study, Rex and Fink (2008) assessed NADH absorption in vitro and concluded that NADH was absorbed mainly in the small intestine. Absorption increased with concentration, but the rate was found to be independent from concentration. The absorption was relatively quick, reaching a plateau after 20–30 min. However, previous studies found that oral NADH was degraded in the acidic environment of the stomach (Kimura et al., 2006). For this reason, in order to assure NADH absorption and bioavailability, enteric-coated dosage forms should be developed, or other administration routes such as intranasal or sublingual, which would bypass the stomach, should be investigated. In fact, some food supplements already in the market use these approaches to overcome NADH degradation in acidic conditions during gastric passage.

To sum up, despite it can be concluded that NAD is absorbed in the small intestine, further studies regarding its pharmacokinetics in humans should be carried out.

5.2. Safety and precautions

Niacin is a water-soluble vitamin (vitamin B3) and is the main NAD^+ precursor. Despite niacin from foods is safe, adverse effects have been reported with medicinal products containing higher doses. Typical

side effects, with a frequency of ≤1.5%, include skin blushing and tingling, as well as gastrointestinal disruption including nausea and vomiting (Knip et al., 2000). These effects might be more prevalent in patients with active peptic ulcer, abnormal liver function or liver disease, diabetes, inflammatory bowel disease, migraine, alcoholism, gout and cardiac arrythmias than in general population. Despite as an over-the-counter medicinal product, niacin can be found at doses up to 5 g per day (Mills et al., 2003), the European Safety Authority (EFSA) and the US Federal Drug Administration (FDA) have set tolerable upper intake levels for niacin as a vitamin found in food products and dietary supplements. While EFSA stablished a tolerable upper intake level for adults of 10 mg/day for nicotinic acid and 900 mg/day for nicotinamide, the FDA limit for adults was set at 35 mg/day for all forms of vitamin B3.

When it comes to the safety of NADH supplementation, in a study evaluating the long-term administration of oral NADH in rats, no chronic toxicity was observed in terms of hematology, clinical chemistry and histology, and there was no apparent effect on urine analysis parameters. It was well tolerated at doses equivalent to 875 mg for a 70 kg human subject (Birkmayer and Nadlinger, 2002). In addition, Nadlinger and Hallström (2004) reported that NADH intravenous administration to beagle dogs for 15 days did not result in toxicity, and that the maximum tolerated intravenous dose was 500 mg/kg/day. As for the safety in humans, in several clinical trials assessing NADH protective role against chronic fatigue syndrome, Alegre et al. (2010), Castro-Marrero et al. (2015) and Forsyth et al. (1999) declared that no adverse effects were observed or reported by patients. In addition, consumers in a long-term treatment with ENADA®, which is a commercial product containing 5 mg of NADH, have not reported any side effects, which provides additional proof of the safety of NADH supplementation (Birkmayer et al., 2002). Besides, NADH has also been used intravenously since the 1960s to alleviate withdrawal from a variety of drugs and alcohol, at doses between 800 and 1800 mg per day, and no side effects have been reported. Taking all these into consideration, NADH supplementation can be considered safe. However, although no overdose or toxicity has been reported, high doses of NADH (> 10 mg/day) could induce agitation, anxiety and sleeplessness (DrugBank, 2019).

5.3. Formulation challenges

The main NAD precursor, niacin, is stable in dry form and in solution, and has been widely used in food supplements and medicinal products. However, although the majority of interventions described above to replenish NAD levels in different indications have used NAD precursors such as niacin, the direct administration of NAD could also be interesting. Since the physicochemical properties of NAD precursors are well described, and its activity is not affected by heat, light, acid, alkali or oxidation, this section would focus on the physicochemical, galenic and stability properties of NAD, which pose several formulation challenges.

Thanks to its physiological function and antioxidant activity, NAD is an approved and interesting compound for the formulation of food supplements and medicinal products (DrugBank, 2019). In line with the healthy living trend, consumption of antioxidants is on the rise, with an expanding consumer base (Arenas-Jal et al., 2019). However, the lack of NAD stability under different environmental conditions, and its poor flow properties and high hygroscopicity, limit its applications in food supplement and medicinal products. When it comes to NAD physicochemical characteristics, it is a white to yellowish amorphous powder, hygroscopic and highly water-soluble. Solids are stable if stored desiccated and protected from light, but storage at −20 °C or colder is recommended. In general, due to its oxidation state, NADH tends to be oxidized to NAD, but overall it is more stable than NAD, which decomposes easily during storage and manipulation. For this reason, despite the higher price of NADH, it is more frequently used in formulations than NAD. In general, solutions are stable for about a week at 4 °C and neutral pH, but NADH and NAD^+, respectively, decompose rapidly in acids or alkalis. For this reason, water alone should not be used to prepare NADH solutions, as it tends to be acidic. Instead, solutions with a pH of 9–11 should be used to dissolve NADH (Rover et al., 1998; Wu et al., 1986). Phosphate buffers should also be avoided, as they could accelerate NADH degradation. NADH solutions are also susceptible to oxidation, especially if light and heavy metals are present. For this reason, concentrated solutions should be stored in a low temperature freezer, at least between −20 and −40 °C. In contrast, NAD^+ solutions are stable at neutral or slightly acidic pH for at least 2 weeks at 0 °C; and for at least 6 months at −70 °C. NAD^+ solutions are sensitive to light, rapidly degraded upon heating, and very labile in alkaline pH, especially in the presence of phosphate, maleate or carbonate. Ideal storage conditions for NAD^+ solutions would be at −70 °C and pH of 2–6 (Sigma-Aldrich, 2019).

So, NAD is not only vulnerable to heat, pH, light and oxygen (Rover et al., 1998; Wu et al., 1986), but also has poor flow properties and high hygroscopicity, which limits its applications in food supplements and medicinal products. Many research efforts have been made to clarify NAD mechanism of action, efficacy and safety, but few works available are addressing NAD stability and hygroscopicity issue. Microencapsulation, a rising technology both in the pharmaceutical and food industry, could be effective to solve these issues. The encapsulation of biomolecules in a suitable delivery system has received great attention over the past few decades because of its increasing potential in therapeutic applications. Biomolecules are typically susceptible to degradation, and thus require protection to ensure their stability and bioavailability (Machado et al., 2013). In addition, when it comes to NADH, either microcapsules or the final pharmaceutical dosage form should be enteric, otherwise it would be degraded before reaching the intestine since it is unstable in acidic media.

As for different microencapsulation techniques, granulation followed by fluid bed coating with an enteric polymer would be able to protect NADH from different environmental conditions during storage, as well as from the acidic gastric fluids during administration. Despite this technique has been widely applied for the microencapsulation of many food and pharmaceutical active ingredients such as vitamins and probiotics (Desai and Jin Park, 2005; Schell and Beermann, 2014), it requires large quantities of product for each trial, at least 300 g of powder for a pilot plant equipment (Glatt, 2019). Thus, considering NADH's high cost (900 €/100 g) (Oriental Yeast Co, 2019), trials to microencapsulate NADH by this technique might be limited. For this reason, ionotropic gelation, which is a common approach for the encapsulation of biomolecules, could be more cost-effective for testing NADH microencapsulation. It is based on the use of hydrogels, and although gel particles can be obtained from different hydrocolloids, sodium alginate (SA) is one of the more commonly used as it is biocompatible, nontoxic, biodegradable and cheap (Giri et al., 2012). SA is a natural polysaccharide extracted from brown algae, and it is widely used for the encapsulation of cells, DNA, microorganisms and nutrients (Sánchez et al., 2013). SA gel particles prepared by ionic crosslinking with cations, create a protective gel matrix, which is independent of temperature. The preparation method is simple and mild, being suitable for thermolabile active ingredients. In addition, SA gel locks the core materials inside conferring protective benefits that minimize sensitive encapsulated compounds' degradation, and it is also capable of modifying their release (Gaonkar et al., 2014; Lee and Mooney, 2012). When it comes to the divalent cations used to induce ionotropic gelation, despite SA has a higher affinity to Pb, Cu and Cd, Ca is the most commonly used, thanks to its nontoxicity (Ching et al., 2017; Orive et al., 2006). Gel particles' size can range from < 0.2 mm (nano) to > 1 mm (macro) depending on the preparation method (Lee et al., 2013). The simplest method is dripping, which involves the drop-wise extrusion of SA into a Ca gelling bath and produces large gel particle size. Scale-up difficulties limit this method to a lab scale setup only, and

many of the potential applications involving biomolecules require the preparation of vehicles that can be efficiently taken up by cells and mucosal tissue, and thus, require sizes within the nanometer or micrometer range (Machado et al., 2013). For this reason, several modified extrusion techniques were developed. One of them is atomization, which produces fine droplets by pumping air and polymer solution concurrently into a nozzle. When these polymer fine droplets come in contact with calcium gelling bath, fine gel particles are produced (Ching et al., 2017).

Finally, probably due to NADH's vulnerability to heat, pH, light and oxygen, as well as its poor flow properties and high hygroscopicity, few available formulations contain NADH as active ingredient. In addition, stability issues have been reported for some of the formulas that are already in the market, especially in those containing non-encapsulated NADH. These might be related to NADH's high cost, that limits the number of trials and suitability of available techniques hindering the development of stable formulas. While few patents describe NAD formulations, and contain broad descriptions that are difficult to disclose, there are no research articles detailing the procedure and formula for its obtention. Thus, further research is needed to obtain stable NAD formulas that will allow to test the administration of NAD in the indications described above.

6. Conclusion

Thanks to the recent progress in understanding non-redox roles of NAD, its therapeutic potential has been recognized and the interest on this molecule has been renewed. NAD can be synthesized *de novo* from tryptophan or from salvage pathways that require the uptake of NAD precursors from the diet, and its synthesis is required since it is not only a cofactor in redox reactions but is also a cosubstrate for a series of NAD^+-consuming enzymes like sirtuins, PARP and ADPR-cyclases. While these regulate essential biological processes such as transcriptional regulation, signaling and DNA repairing among others, their activity is regulated by oscillations in NAD^+ oxidative state and concentration, which is determined by the relative balance between consumption and biosynthesis. Disruption of NAD^+ homoeostasis has emerged as a common characteristic of several diseases, whereas increasing NAD^+ levels has been associated with positive health effects. Therefore, enzymes implicated in NAD^+ metabolism have become interesting targets for drug discovery, with an extensive therapeutic potential in different indications. Different strategies to increase NAD^+ levels have been used, including enhancement of biosynthesis and inhibition of NAD^+ breakdown. Despite restoring or increasing NAD^+ concentrations could ameliorate age-associated functional defects and diseases, as well as potentially extend lifespan in humans, further research is needed to clarify the targets and mechanisms of NAD^+ function, and to determine the safety and efficacy of nutritional, pharmacological and genetic interventions to regulate NAD metabolism in humans. At this stage, since knocking out, blocking or inhibiting NAD^+-consuming enzymes could cause potential severe side effects, supplementation with NAD precursors seems the most reasonable approach, until safe and effective selective inhibitors are developed. The administration of NAD or its precursors was capable of effectively increasing NAD^+ levels, leading to beneficial physiological effects. However, further research is needed to translate these findings into clinical trials that analyze the impact of NAD^+ treatment in different human pathologies, mainly in age-related diseases.

This review provided an overview and update of NAD metabolism, including the regulation of several enzymatic families such as PARP, sirtuins and ADPR-cyclases, which play an important role in the pathogenic and pathologic mechanisms of different conditions and diseases. In addition, the therapeutic potential of NAD metabolism regulation in several indications, as well as the safety and precautions, and formulation challenges of NAD administration were also reviewed.

Author contributions

Conception, literature research and writing were performed by M. Arenas-Jal. Field experience and critical review of the manuscript were performed by J.M. Suñé-Negre and E. García-Montoya.

Author disclosures

As a part of an industrial PhD, M. Arenas-Jal works as R&D manager for Vitae Health Innovation, S.L.

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