Role of endothelial NAD^+ deficiency in age-related vascular dysfunction

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Running head: NAD boosters improve vascular function in aging

Abstract

Age-related alterations in endothelium and the resulting vascular dysfunction critically contribute to a range of pathological conditions associated with old age. To rationally develop therapies that improve vascular health and thereby increase health span and lifespan in older adults, it will be essential to understand the cellular and molecular mechanisms contributing to vascular aging. Pre-clinical studies in model organisms demonstrate that NAD^+ availability decreases with age in multiple tissues and that supplemental NAD^+ precursors can ameliorate many age-related cellular impairments. Here we provide a comprehensive overview of NAD^+ dependent pathways (including the NAD^+ utilizing sirtuins and poly (ADP-ribose) polymerase enzymes) and the potential consequences of endothelial NAD^+ deficiency in vascular aging. The multifaceted vasoprotective effects of treatments that reverse the age-related decline in cellular NAD^+ levels as well as their potential limitations are discussed. The preventive and therapeutic potential of NAD^+ intermediates as effective, clinically relevant interventions in older adults at risk for ischemic heart disease, vascular cognitive impairment and other common geriatric conditions and diseases that involve vascular pathologies (e.g. sarcopenia, frailty) is critically discussed. We propose that NAD^+ precursors (e.g., nicotinamide riboside, nicotinamide mononucleotide, niacin) should be considered as a critical component of combination therapies to slow the vascular aging process and increase cardiovascular health span.

Key words: geroscience, senescence, oxidative stress, endothelial dysfunction, microcirculation

Successful vascular aging determines lifespan and health span

Over the coming decades the average age of the population of the Western world will continue to grow. Due to the significant increase in the average life expectancy combined with unfavorable trends in fertility those aged ≥65 will become a much larger share of the population (e.g., in the European Union rising from 19% to 29%(2)). The share of those aged ≥80 will increase from 5% to 13% of the population of European Union by 2070. Similar trends will be manifested both in Japan and the United States. The increasing fiscal strain linked to pensions, health care and long-term care combined with the increases in the old-age dependency ratio (people aged 65 and above relative to those aged 15 to 64; in the European Union: 29.6% in 2016, 51.2% in 2070) are expected to be a significant challenge to the societies of each industrialized nation(64).

While aging affects physiology and pathophysiology throughout the body, the consequences of age-related alterations of the cardiovascular system are especially relevant to the lifespans and health spans of the populations of the developed countries. Cardiovascular and cerebrovascular diseases are the most common cause of death among older people in these nations(1) accounting for approximately 1/3 of all deaths at the age of 65 and nearly 2/3 at an age of 85(164). In addition, aging-induced functional and structural alterations of the vasculature contribute to the pathogenesis of a wide range of age-related diseases that limit health span, contributing to decreased workforce participation, increased dependency and institutionalization in older adults. These age-related diseases include coronary heart disease (CHD), myocardial infarction, vascular contributions to cognitive impairment and dementia (including stroke), Alzheimer's disease, hypertension, peripheral artery disease, sarcopenia, kidney and eye diseases(164). Aging promotes endothelial apoptosis, impairs endothelial angiogenic capacity and promotes capillary regression(13, 36, 40, 45). A decline in capillary density ("microvascular rarefaction"(13, 142, 149, 157, 168, 169)) contributes to decreased tissue perfusion with age, which is a major contributor to mortality and morbidity. Vascular pathologies also contribute to gait and balance disorders(57, 145, 151, 165) promoting falls. Age-related pro-inflammatory changes in the vasculature contribute to the pathogenesis of chronic inflammatory diseases associated with old age, including atherosclerotic diseases (including CHD, stroke, peripheral artery disease, renal artery stenosis), osteoarthritis(6), metabolic disease and diseases of the gastrointestinal tract. Age-related endothelial changes promote increased coagulation and impair stem cell biology (e.g. by altering the local microenvironment in vascular stem cell niches(81, 129)). Aging-induced dysfunction of microvascular barrier and transport function (e.g. promoting the leakage of microbial breakdown products to the systemic circulation) likely promotes chronic systemic low-grade sterile inflammation and distant organ damage(135). Age-related alterations in the endothelial phenotype alter the secretion of growth factors, chemokines and enzymes that can degrade the extracellular matrix, likely promoting tumor progression, intravasation and cancer metastases(173). Finally, impaired release of gaseotransmitters (including NO) from the microvessels negatively impacts mitochondrial function and cellular bioenergetics in the skeletal muscle, the heart and the central nervous system(105, 106).

Therefore, it is critical to understand mechanisms underlying vascular aging(83) to better predict and prevent vascular contributions to the pathogenesis of multiple diseases associated with old age. A better mechanistic understanding of macro- and microvascular aging processes is also critical to develop and evaluate dietary, lifestyle and pharmacological countermeasures to address this growing health issue.

Role of oxidative stress and endothelial dysfunction in vascular aging

Impairment of endothelium-dependent nitric oxide (NO)-mediated vasodilation ("endothelial dysfunction") is a frequently used indicator of vascular health(29, 35, 60, 120, 132). Endothelial dysfunction associates with cardiovascular events (reviewed in(86)), is an early feature of atherosclerotic vascular diseases, and significantly contributes to impaired microvascular perfusion(149, 164, 167). Importantly, clinical and preclinical studies demonstrate that aging is a major cause for endothelial dysfunction(9, 44, 51) and that beneficial effects of anti-aging interventions are predicted by their ability to restore endothelial NO mediation in aging(36, 37, 40, 42, 50, 114, 152). In many cases, the loss of NO signaling with age or disease is a direct reflection of oxidative stress, since superoxide readily reacts with NO to generate peroxynitrite, a free radical-containing molecule that lacks NO's signaling ability and damages other molecules. The sources of superoxide include mitochondrial production and NAD(P)H oxidase activation(36, 37, 44, 136, 143, 151). NO released from the vascular endothelium is a potent vasodilator, which regulates vascular resistance and thereby tissue perfusion. In addition, endothelium-derived NO also confers important vasoprotective, cardioprotective, anti-inflammatory and anti-aging effects. For instance, NO was demonstrated to regulate cell division and survival, inhibit platelet aggregation and inflammatory cell adhesion to endothelial cells, promote angiogenesis, disrupt pro-inflammatory signaling pathways, and regulate mitochondrial function and cellular energy metabolism(149, 164, 167). Endothelial dysfunction contributes to the pathogenesis of cardiovascular disease, stroke and hypertension, vascular cognitive impairment and dementia, and a range of pathological conditions from erectile dysfunction to impaired exercise tolerance in older adults(164, 167). The critical role of endothelium-derived NO in aging is underscored by the findings that mice genetically deficient for endothelial nitric oxide synthase (eNOS) exhibit premature vascular, metabolic, brain and cardiac aging phenotypes associated with early mortality(89, 150), many of which can be reversed by supplying NO through exogenous nitrite(147). The mechanisms underlying age-related endothelial dysfunction prominently involve increased oxidative stress(5, 44, 53, 140, 164, 167). Previous preclinical and clinical studies have tested various experimental interventions designed to attenuate oxidative stress and interfere with oxidative stress-mediated pathways to improve endothelial function in animal models of aging(40, 61, 87, 88, 92, 110, 113, 114, 143, 148, 152, 164, 166). Despite these exciting studies, the molecular mechanisms that lie upstream of age-associated increased oxidative stress remain elusive.

Key objectives of geroscience research are to understand the biology of aging and to translate scientific insight obtained in models of aging into translationally relevant interventions that improve late-life health, including cardiovascular health. The prevailing view in the field of geroscience is that fundamental aging processes are causally upstream of, and the cause of, all age-related pathologies, including cardiovascular diseases. Intervening in these fundamental cellular and molecular processes of aging thus should provide protection against a wide range of age-related diseases and conditions, including endothelial dysfunction. What is currently identifiable about organismal and tissue aging is that it is a very complex process, involving diverse biological mechanisms. However, the exact roles of fundamental cellular and molecular processes of aging in the genesis of increased oxidative stress and consequential endothelial dysfunction in the aging vasculature remain to be elucidated.

Role of NAD^+ deficiency and cellular energetic impairment in aging-induced endothelial dysfunction

There is strong evidence that with advanced age there is decreased availability of cellular NAD^+ (62, 95, 177), which may be a common contributor to aging processes across tissues and in

evolutionarily distant organisms. In support of this theory it was demonstrated that enhancing NAD^+ biosynthesis extends lifespan in yeast, worms and flies(7, 8, 12, 102, 103) and improves both general health and longevity in mice(100, 181). Here we review the evidence supporting the concept that age-related decline in [NAD^+] plays a critical role in vascular aging.

Biological functions of NAD^+

Nicotinamide adenine dinucleotide (NAD) and its phosphorylated form nicotinamide adenine dinucleotide phosphate (NADP) have central roles in cellular metabolism, energy production and survival(15). Over 400 enzymes require the NAD^+ and $NADP^+$, predominantly to accept or donate electrons for redox reactions. NADP is synthesized by NAD^+ kinase, which phosphorylates NAD^+. Although both NAD and NADP participate as electron carriers in a multitude of redox reactions, they support distinct functions. NAD^+ participates primarily in energy-producing reactions requiring an electron exchange, including the catabolism of carbohydrates, fatty acids, proteins, and alcohol (e.g. glycolysis, pyruvate-to-lactate and pyruvate-to-acetyl-CoA interconversions, β-oxidation, citric acid cycle, and oxidative phosphorylation). NADP predominantly participates in anabolic pathways, including the synthesis of fatty acids, cholesterol and DNA. NADP is also critical for the regeneration of components of antioxidant systems. To support these distinct functions, mammalian cells maintain NAD predominantly in the oxidized state to serve as oxidizing agent for catabolic reactions, whereas NADP exists predominantly in a reduced state (NADPH) to be able to readily donate electrons for reductive cellular biochemical reactions. The cycling of NAD and NADP between oxidized and reduced forms in redox reactions is easily reversible, since when NAD(P)H reduces another molecule it is re-oxidized to $NAD(P)^+$. Thus, these coenzymes can continuously cycle between the reduced and oxidized forms without being consumed. Altering the availability of these coenzymes, either through a shift in the redox ratio or via changes in cellular synthesis and/or degradation of NAD(H) and NADP(H) will likely affect the function of hundreds of NADH-dependent and NADPH-dependent enzymes.

NAD^+ is also the substrate for at least four classes of enzymes important for cellular survival, aging and normal physiological functioning. These include enzymes with mono adenosine diphosphate (ADP)-ribosyltransferase and poly (ADP-ribose) polymerase (PARP) activities, which catalyze ADP-ribosyl transfer reactions. NAD^+ is a rate-limiting co-substrate for Silent information regulator-2 (Sir2)-like enzymes (sirtuins), which are key regulators both of pro-survival pathways and mitochondrial function and catalyze the removal of acyl groups from acylated proteins, utilizing ADP-ribose from NAD as an acceptor. Importantly, both NAD^+-dependent PARP enzymes and sirtuins are involved in DNA repair pathways. Finally, ADP-ribosylcyclases such as CD38, which have relevance for calcium signaling and endothelial NO mediated vasodilation(180), also require NAD^+.

Biosynthesis of NAD^+

In mammals, NAD^+ can be synthesized *de novo* in the cytosol from the amino acid tryptophan, from nicotinic acid, or salvaged from nicotinamide or intermediates containing this moiety (Fig. 1). In the first step of the *de novo* pathway, tryptophan is converted into N-formylkynurenine by either of two different enzymes: tryptophan-2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO). TDO is critical for NAD^+ biosynthesis in liver, whereas IDO is expressed in many extrahepatic tissues, including endothelial cells(19) and is known to be upregulated in response to inflammatory cytokines. N-formylkynurenine is converted into kynurenine by formamidase. Kynurenine is metabolized in one of two ways: one pathway yields kynurenic acid, whereas the other yields 3-hydroxykynurenine and quinolinic acid, precursors of NAD^+.

The Preiss-Handler and NAD^+ salvage pathways recycle components of NAD^+ that are taken up from food or released by biochemical reactions that break down NAD^+. Three vitamin precursors containing a pyridine base that are used in these pathways are nicotinic acid (NA), nicotinamide (Nam) and nicotinamide riboside (NR) (Fig. 1). These compounds are termed vitamin B3 or niacin (although niacin may also refer to nicotinic acid specifically). NAD^+ synthesis from nicotinamide requires two steps: nicotinamide is first converted into nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT)(69), then the production of NAD^+ from NMN and ATP is catalyzed by nicotinamide mononucleotide adenylyltransferases (NMNATs). NMNAT1 is a nuclear enzyme, NMNAT2 is located in the cytosol and Golgi apparatus, while NMNAT3 is located in the mitochondria in most cell types(76). NAMPT is considered the rate-limiting component in this NAD^+ biosynthesis pathway(123). In the Preiss–Handler pathway, NA is converted into NA mononucleotide (NaMN) by the addition of ribose-phosphate (from phosphoribosyl pyrophosphate by nicotinic acid phosphoribosyltransferase [NAPRT]). NaMN is then converted into NA adenine dinucleotide (NaAD) by NMNATs, and lastly into NAD^+ the presence of ATP and ammonia by NAD synthase. In mammals, which lack nicotinamidase, NA seems to be derived primarily from extracellular sources. Exogenously administered NA has been demonstrated to be a good precursor of NAD biosynthesis, significantly increasing tissue NAD^+ levels(34, 71, 90) in addition to its better-known effect a lipid lowering agent via direct inhibition of triglyceride synthesis and decreasing secretion of VLDL and LDL particles from hepatocytes(74). Important for the present review is that treatment with niacin is associated with improved endothelial function(126). NR and nicotinic acid riboside are converted to NMN and nicotinic acid mononucleotide (NaMN), respectively, by nicotinamide riboside kinase 1 (NRK1) and NRK2(15, 16, 121).

Despite the presence of the *de novo* pathway, the NAD^+ salvage pathway is essential in mammals: a lack of niacin in the diet results in significant decline in tissue NAD^+(122) and mice lacking NAMPT constitutively are not viable(124). Even with an intact salvage pathway, the lack of niacin in the diet causes the severe vitamin deficiency disease pellagra(84), which is characterized by dermatitis, diarrhea, dementia and ultimately death. Data derived from the 1995 Continuing Survey of Food Intakes by Individuals indicate that in the United States the greatest contribution to the niacin intake of the adult population comes from mixed dishes high in meat, fish, or poultry, enriched and wholegrain breads and fortified cereals(70). Fish, such as tuna (niacin content: 18.4 mg/100 g), sardines ((3)) and salmon (niacin content: 7.8 mg/100 g), as well as chicken meat (niacin content: 13.9 mg/100 g) and liver (niacin content: 11 mg/100 g) are relatively rich in NAD^+ precursors. One of the best food sources of niacin is yeast (niacin content: 40.2 mg/100 g)(4). Milk and milk products also contain NAD^+ precursors (60% as nicotinamide, 40% as NR)(156), although the niacin content in them is significantly lower relative to aforementioned food items (niacin content in milk: 0.089 mg/100 g). Several food items contain particularly high concentrations of NMN, including edamame, avocado and broccoli(100).

It should be noted that niacin intake in the adult population in the United States is generous in comparison with the Estimated Average Requirement (EAR)(70). For instance, the median intake by adult women is 17 to 20 mg of niacin, which exceeds the Estimated Average Requirement of 11 mg of niacin equivalents needed to prevent pellagra. The Boston Nutritional Status Survey reported that people over age 60 in this cohort has a median niacin intake of 21 mg/day for men and 17 mg/day for women(70). Niacin intake from supplements is also significant. Over one third of adults participating in the National Health and Nutrition Examination Survey (1999–2000) reported taking a multivitamin dietary supplement containing niacin in the previous month(119). Data from the Boston Nutritional Status Survey indicates that in elderly individuals taking supplements, the fiftieth percentile of

supplemental niacin intake was 20 mg for men and 30 mg for women(70). Of note, supplements containing up to about 400 mg of niacin are available without a prescription. It should also be noted that nicotinic acid has been also used as a lipid lowering agent since the 1970s, based on its inhibitory effect of triglyceride synthesis, accelerated intracellular hepatic apo B degradation and the decreased secretion of VLDL and LDL particles.

Endothelial cells abundantly express the enzymes required to metabolize NAD^+ precursors (Csiszar and Ungvari, unpublished observation 2018), suggesting that endothelial NAD^+ levels are likely to be responsive to exogenously administration or dietary intake of NAD^+ precursors. For a more extensive review on the biosynthesis of NAD^+, the reader is directed to references(15, 76).

Mechanisms of age-related decline in cellular NAD^+ levels

NAD^+ concentration decreases in multiple tissues over the course of normal aging. Although the dispersion of endothelial cells within a given tissue makes it difficult to measure their NAD^+ pools directly in situ, studies on endothelial cells isolated from the brains of young and aged animals provide evidence that [NAD^+] also falls in the endothelial compartment (Tarantini, Csiszar and Ungvari, submitted, 2019).

The mechanisms underlying the age-related decline in [NAD^+] are likely multifaceted(127) and may include decreased expression of nicotinamide phosphorybosyltransferase (NAMPT; which catalyzes the rate limiting step in the biosynthesis of NAD^+)(178), increased utilization of NAD^+ by activated poly (ADP-ribose) polymerase (PARP-1)(110), and increased activity and expression of the NADase CD38 (23, 146) (Fig. 2). The functional relevance of these pathways is shown by the findings that genetic depletion of NAMPT and/or pharmacological inhibition of NAMPT (by the inhibitor FK866) decreases cellular NAD^+ levels and mimic aspects of the aging phenotype in endothelial cells(171), skeletal muscle(131) and neuronal cells(138, 139). PARP-1 is a constitutive factor of the DNA damage surveillance network. In aged cells PARP-1 is activated in response to DNA damage induced by increased oxidative/nitrative stress. PARP-1 cleaves NAD^+ and transfers the resulting ADP-ribose moiety onto target nuclear proteins and onto subsequent polymers of ADP-ribose, depleting cellular NAD^+ pools in the process. There is evidence that in human tissues (skin samples) advanced aging results in increased DNA damage, which correlates with increased PARP activity and decreased NAD^+ levels(95). Importantly, genetic depletion(11) and/or pharmacological inhibition of PARP-1 were shown to increase tissue NAD^+ levels in rodent models of accelerated aging. Pharmacological inhibition of PARP-1 was also shown to improve endothelial function in aged rodents(110-112). Two recent studies demonstrated that the expression and activity of the NADase CD38 increase with age, and that blocking CD38 activity is sufficient to increase [NAD^+] and prevent the age-related decline in multiple tissues including skeletal muscle, liver and adipose tissue(23, 146). Endothelial cells are known to express CD38 and CD38-mediated NAD^+ depletion in this cell type has been linked to loss of eNOS mediated NO generation(22, 125).

In addition to the intrinsic effects of age, cardiovascular risk factors that promote accelerated vascular aging result in cellular NAD^+ depletion. Accordingly, there is evidence linking high fat diet-induced obesity(27, 59), high homocysteine levels(20), diabetes(133, 134) to a decline in cellular NAD^+ levels, which would likely contribute to endothelial dysfunction.

Anti-aging effects of treatment with NAD^+ boosters

Cellular NAD^+ levels can be increased by up-regulating the enzymes involved in NAD^+ biosynthesis, by inhibition of NAD^+ consumers(76), or by treatment with NAD^+ precursors(26), including niacin, nicotinamide mononucleotide (NMN)(48, 107, 159), nicotinamide riboside (NR).

While overexpression of enzymes catalyzing NAD^+ biosynthesis (NAMPT or NMNATs) effectively boosts NAD^+ levels (54, 76), the translational potential of this approach is limited. Significant data are available to support the efficacy and translational relevance of NMN and NR treatment(177). NMN is considered an especially promising candidate as an anti-aging therapeutic approach due to its multi-targeted effect(80).

Administration of NMN or NR to aged mice increases tissue NAD^+ levels(100, 177, 181). The rise in NAD was detected within minutes in some studies, indicating that NMN is quickly absorbed in the gut and is either efficiently transported in the circulation and readily converted by the cells to NAD^+, or, alternatively is converted to another NAD^+ precursor in the liver, which then circulates to peripheral tissues, increasing cellular NAD^+ levels. Recent findings support the latter view, showing that there is a significant first-pass effect and orally administered NMN and NR are readily metabolized to nicotinamide in the liver, which then can get into the circulation, increasing NAD^+ levels in other organs (91). There are strong data to show that human blood NAD^+ can rise as much as 2.7-fold with a single oral dose of NR and that oral NR elevates tissue NAD^+ in the mouse liver with superior pharmacokinetics to those of nicotinic acid and nicotinamide(154). Additionally, single doses of 100, 300 and 1,000 mg of NR were demonstrated to result in dose-dependent increases in the blood NAD^+ metabolome in humans(154). Note that the doses of NAD^+ precursors used in preclinical and clinical studies to reverse the adverse effects of aging are significantly higher than the Estimated Average Requirement (EAR)(70) of ~11 mg of niacin equivalents needed to prevent pellagra in humans even if allometric scaling is used.

There is increasing evidence that restoration of cellular NAD^+ levels by treatment with NAD^+ precursors in aged mice exerts multifaceted anti-aging effects, reversing age-related dysfunction in multiple organs, including the eye(100), the skeletal muscle(62) and the brain(73). Even short-term administration of NMN or NR has been demonstrated to exert significant protecting effects in a wide range of age-related pathophysiological conditions, improving skeletal muscle energetics and function(62), protecting neuronal stem cells and increasing mouse lifespan(181). The NAD^+ booster acipimox, a niacin derivative used for treatment of hyperlipidemia in type 2 diabetic patients, was also shown to improve mitochondrial function in the skeletal muscle(170). NR was also shown to exert protective effects against high-fat diet-induced metabolic abnormalities(27, 155).

Importantly, chronic treatment of aged mice with NAD^+ boosters was shown to improve endothelial function in the aorta (Ungvari and Tarantini, unpublished observation, 2015)(50) and in the cerebral circulation (Ungvari and Tarantini, unpublished observation, 2015). Studies are currently underway to determine whether chronic treatment with NR improves cerebral blood flow (ClinicalTrials.gov Identifier: NCT03482167) in older adults with mild cognitive impairment. More recently, treatment of aged mice with NMN was shown to reverse age-related capillary rarefaction and increase blood flow in the skeletal muscle(48), likely by increasing the angiogenic capacity of endothelial cells(21, 48). There is also evidence suggesting that in old mice NMN treatment restores fenestration of liver sinusoidal endothelial cells(66). Fenestration of liver sinusoidal endothelial cells enables the bidirectional exchange of substrates (including insulin, lipoproteins and pharmacological agents) between the blood and hepatocytes and thereby importantly contributes to metabolic homeostasis. With increasing age the frequency and diameter of fenestrations significantly decrease, likely due to age-related disruption of VEGF and NO dependent signaling pathways, which promote pathologic remodeling of the actin cytoskeleton and cell membrane lipid rafts(32, 72, 108). It is likely that NMN treatment exerts its protective effects on the liver sinusoidal endothelial cells by restoring endothelial NO mediation. The available evidence suggest that higher dietary niacin intake is also associated with improved vascular endothelial function in older

adults(75). Yet, niacin as add-on treatment to high dose statins in patients with established coronary artery disease does not appear to improve endothelial function(116). Consistent with the protective effects of diverse NAD^+ boosters treatment of aged rodents with PARP-1 inhibitors, which should spare NAD^+ (25, 28), was also shown to improve endothelial function(110-112).

Mitochondrial dysfunction and elevated mitochondrial oxidative stress play a critical role in aging-induced cardiovascular dysfunction(47, 136, 161) and vascular impairment(61, 143). The mechanisms contributing to mitochondrial oxidative stress in the aged endothelium are likely multifaceted and involve a dysfunctional electron transport chain. Reduced electron flow through the electron transport chain, in particular due to aging-induced dysregulation of complex I and complex III(82), likely promotes electron leak and favors increased mtROS production. A key mechanism underlying the anti-aging action of NMN treatment is improving cellular energetics by rescuing mitochondrial function(62), at least in part, by activating sirtuin deacylases (SIRT1-SIRT7; Fig. 2). Sirtuins are known to mediate beneficial anti-aging(33, 102, 174) and vasoprotective effects(36, 37, 42) of caloric restriction as well. In support of this concept, knock-down of SIRT1 in aged cerebromicrovascular endothelial cells was shown to abolish the anti-oxidative and mitochondrial protective effects of NMN treatment (Ungvari and Csiszar, 2018, unpublished observation). There is direct evidence that activation of SIRT1 underlies NMN-induced restoration of endothelial angiogenic capacity and increased capillarization in aged mice(141). Previous studies suggest that the age-related decline in oxidative phosphorylation (OXPHOS) and/or increased mitochondrial oxidative stress may be due, at least in part, to the specific loss of mitochondrially encoded transcripts(62). In that regard it is important that NMN treatment was shown to restore expression of mitochondrial encoded OXPHOS subunits in aged mice in a SIRT1 dependent manner(62). Treatment with NR was also shown to up-regulate mitochondrial gene expression and promote mitochondrial biogenesis in the mouse skeletal muscle(27). Moreover, recent studies show that pharmacological inhibition of alpha-amino-beta-carboxymuconate-epsilon-semialdehyde decarboxylase (ACMSD)(115), the enzyme that limits spontaneous cyclization of alpha-amino-beta-carboxymuconate-epsilon-semialdehyde in the de novo NAD^+ synthesis pathway, can also boosts de novo NAD^+ synthesis and sirtuin 1 activity, ultimately enhancing mitochondrial function in kidney and liver(77). We posit that rescue of vascular mitochondrial function by restoring the expression of mitochondrial encoded OXPHOS subunits contributes to the vasoprotective effects of treatment with NAD boosters. These observations accord with findings from earlier studies demonstrating that many of the health benefits of SIRT1 activation are linked to improved mitochondrial function(14). Further, SIRT1-activating compounds (STACs) such as resveratrol and SRT1720 have been demonstrated to exert significant vasoprotective effects in aging and models of accelerated vascular aging(30, 39, 56, 101, 114, 161-163, 179) similar to NAD^+ boosters, including up-regulating mitochondrial biogenesis(38), attenuating mitochondrial oxidative stress(43, 160), activating antioxidant defense mechanisms(41) and inhibiting apoptosis(114) in endothelial and vascular smooth muscle cells. STACs were also shown to increase capillary density(109), improve endothelial function and blood flow regulation(152) and prevent microvascular fragility(151) in the aged mouse brain and to exert similar vasoprotective effects in non-human primate models as well(18, 96). Future studies should determine whether NAD^+ boosters also confer similar vascular health benefits. In addition to sirtuin-mediated effects, because mitochondrial ATP production and membrane potential require NAD as an essential coenzyme, restoring an optimal NAD/NADH ratio itself should also promote efficient mitochondrial function in vascular cells.

Perspectives

Taken together, progress in geroscience research investigating the role of fundamental aging processes in the development of age-related chronic diseases(55, 79, 94, 130), including cardiovascular pathologies has been rapid in recent years(10, 46, 52, 55, 85, 98, 104, 117, 164), from both the basic science and the clinical perspectives. The field of vascular aging research matured and expanded when researchers started to apply breakthrough discoveries in biogerontology to the development of new therapeutic strategies to prevent/reverse age-related pathologic functional and phenotypic alterations of blood vessels. In particular, NAD^+ boosting strategies were shown to confer multifaceted health benefits in aging, including potential translationally relevant vasoprotective effects. However, understanding the cellular and molecular mechanisms by which age-related NAD^+ deficiency contribute to age-related vascular pathologies, elucidating the exact mechanisms by which NAD^+ boosting strategies exert their anti-aging vascular effects and translating the preclinical findings to the clinics remain a substantial challenge and an active area of research with numerous open questions.

It remains unclear what downstream mechanisms mediate the beneficial vascular effects of NAD^+ boosters. In addition to the role of established NAD^+ biosynthetic pathways new research may reveal new aspects of NAD^+ metabolism, including novel pathways that utilize NAD^+ (e.g. NAD^+ addition to RNAs(76)) that contribute to the biological effects of NAD^+ boosters in the aged vasculature.

Although NMN and NR have been tested in diverse disease models, no side-by-side comparisons have been conducted between NMN and NR in the context of macrovascular and microvascular aging. Future pharmacological and nutraceutical strategies to rescue vascular NAD^+ levels in aging will also need to take into account the limited oral bioavailability of NR and NMN as well as the tissue-specificity of important pathways in NAD+ metabolism(91). Further, a recent meta-analysis of all randomized studies that compared niacin with placebo, either alone or in combination with statin treatment or other treatments that lower low-density lipoprotein cholesterol levels also showed that niacin does not affect significantly all-cause mortality rates and does not lower the risk of cardiovascular mortality, nonfatal myocardial infarction, stroke, or the need for revascularization(58). With that regard, studies aimed at understanding the differential biological effects of treatment with niacin, NMN and NR will be highly informative.

Compartmentalization of NAD^+ biosynthesis is also not well understood. Subcellular compartments (e.g. the nucleus, cytosol, and mitochondria) appear to express distinct pathways to synthesize NAD^+(176). However, it is not clear what the relevance of this spatial organization is, given that individual enzymes appear to be dispensable in most cases(24, 175) and tracer studies suggest that intact NAD^+ can move between the cytosol and mitochondria(49). It is presently unclear how NAD^+ intermediates are transported across cell membranes and shared among different subcellular compartments in endothelial cells. Novel isotope-tracer methods to analyze NAD synthesis-breakdown fluxes have been developed(91), which could be adapted to study endothelial cell-specific NAD^+ metabolism.

In 2009 Imai and coworkers proposed an interesting concept, named the "NAD World," which implicated NAD^+ metabolism and SIRT1 in systemic regulation of mammalian aging and longevity(67). Since then the concept has evolved and now NMN is hypothesized to function as a systemic signaling molecule that participates in inter-tissue communications among three key tissues, namely, the hypothalamus, adipose tissue, and skeletal muscle, for regulation of aging processes and longevity control(68). The concept implies that the hypothalamus is a high-order control center of systemic aging processes and that inter-tissue communication between the adipose

tissue, skeletal muscle and the hypothalamus, mediated by circulating factors (including myokines and adipokines), comprises a critical feedback loop. Importantly, transport and uptake of circulating NMN as well as inter-tissue communication via circulating factors depends on the function of the (micro)vasculature. Endothelial cells also express key components of pathways involved in NAD^+ biosynthesis and degradation (including PARP-1 and CD38). Additionally, SIRT-1 is known to regulate several aspects of endothelial function, including angiogenesis, vasodilatory function. Further, NMN appears to significantly impact the function and phenotype of endothelial cells in aging. Thus, it would be interesting to incorporate in the model the function and age-related changes of the microvascular endothelial cells and consider the role endothelial cells (which represent the largest endocrine organ) in systemic regulation of aging within the framework of the NAD World.

When translating the protective effects of NAD^+ boosting strategies into clinical benefits several challenges should be considered, including the side effect profiles of such treatments. Treatment with L-tryptophan is known to cause a range of unwanted side effects (belching and gas, blurred vision, diarrhea, dizziness, drowsiness, dry mouth, headache, heartburn), including the potentially severe eosinophilia-myalgia syndrome (for which it was recalled from the market in 1990). Niacin treatment can cause a flushing reaction(17) as well as gastrointestinal side effects, and liver problems and may promote impaired glucose tolerance(99, 128) at high doses (e.g. ~3 g/day nicotinic acid). Adverse effects (nausea, vomiting, and signs of liver toxicity) have been reported at nicotinamide intakes of 3 g/day (118) and intakes of nicotinic acid of 1.5 g/day(97). The niacin derivative lipid lowering agent acipimox (Olbetam) also causes flushing and gastrointestinal side effects in 10% of the patients. Individuals with liver disease, diabetes mellitus and alcoholism are more susceptible to the adverse effects of excess niacin intake. Unlike other NAD^+ boosters, Nam has the capacity to exert end-product inhibition on SIRT1 deacetylase activity, which may result in unwanted side effects as well. Importantly, chronic administration of NMN resulted in no apparent toxicity in mice(100). Similarly, chronic treatment of laboratory mice with NR for 5–6 months(63), 10 months(181) or 12 months(158) was not associated with any obvious toxic adverse effects. It is promising that small-scale clinical studies with NR treatment have not reported adverse effects in humans(154). A small randomized, placebo-controlled, crossover clinical trial of NR supplementation (2x500 mg/day for 2x6 weeks) in older adults(93) also reported no major adverse effects. Nevertheless, subsequent clinical trials on larger cohorts should carefully monitor adverse events associated with NMN and NR treatment. It is expected that soon reliable information will be available on the pharmacokinetics, dosing and side effect profiles of NMN and NR treatments in older adults. Multiple clinical studies are ongoing, investigating the effects of treatment with NAD^+ boosters in humans, including the effects of NMN on metabolic health in women (ClinicalTrials.gov Identifier: NCT03151239). Ongoing clinical trials with NR treatment include studies to investigate the effects of NR on mitochondrial biogenesis and mitochondrial function (ClinicalTrials.gov Identifier: NCT03432871 and NCT02835664). Importantly, many of the NAD^+ precursors are considered vitamins and are widely available to the public as dietary supplements. New studies should also determine which pharmacological strategies aiming to boost cellular NAD^+ levels by inhibiting degradation of NAD^+ would be the most appropriate for vasoprotection in older adults. Several PARP inhibitors are currently available or are undergoing clinical trials for oncologic indications. One important consideration is that PARP inhibitors are potentially genotoxic, which may limit their use in patients with non-oncologic diseases.

The effects of an initial study using longer treatment with NR (2x500 mg/day, for 6 weeks) on endothelium-dependent dilation and arterial stiffness (ClinicalTrials.gov Identifier:

NCT02921659) was recently reported (93). However, the results on the effects of NR on endothelial function and vascular health were inconclusive. While NR was found to elicit small decreases in blood pressure and somewhat reduce aortic stiffness, it did not improve endothelium-dependent, flow-mediated dilation of brachial arteries(93). However, this initial clinical trial had important limitations, which necessitates targeted follow-up studies with fewer outcomes based on two-sided statistical inference to confirm the effects of NR treatment on vascular health. It is becoming evident that in addition to testing the effects of NAD^+ boosters in healthy adults exhibiting near-normal vascular function, future investigations should also include older patients with cardiovascular and metabolic diseases characterized by significantly impaired endothelial function. Additional research is also needed to develop sensitive NAD^+ quantification methods, preferably assessing the entire NAD^+ metabolome in relevant tissues, that could be used in the clinical setting to evaluate treatment efficiency(31).

Research over the past two decades has broadened our view of the multi-factorial nature and heterogeneity of cellular aging processes(78) that contribute to age-related cardiovascular pathologies(164). Furthermore, there is considerable cross talk between signaling pathways involved in the vascular aging process. With age multiple regulatory and homeostatic mechanisms become dysfunctional and impairment of these compensatory mechanisms significantly decrease cellular resilience to other stressors as well. Due to the complexity of age-related physiological dysfunction there is a strong scientific rationale for pursuing multiple targets to delay cardiovascular aging. To rationally develop 'anti-aging' interventions that target multiple steps in the vascular aging process will likely require a combination therapy approach. Future studies should explore how NAD boosting strategies can be combined with selective inhibitors of other cellular pathways involved in the aging process (e.g., mTOR) and determine the dose-limiting toxicities of such combination targeted therapies.

Finally, understanding of NAD^+ depletion in smooth muscle cell pathophysiology is also a promising area for research. There is evidence that NAD+ levels affect vascular smooth muscle cells contractility and impact structural integrity of the vascular wall(65). For example, vascular smooth muscle-specific Nampt-deficient mice exhibit an ~40% reduction in aortic NAD+ , which appears to promote pathogenesis of aortic aneurysms(172). It will be interesting to determine whether treatment with NAD+ boosters can reverse/prevent alterations in vascular structure and function, which are secondary to aging-induced phenotypic changes in smooth muscle cells(136, 137, 144, 151, 153, 165).

Collectively, we are entering a new era of vascular aging research and it will change the way we approach prevention and treatment of age-related cardiovascular pathologies. Pharmaceutical companies that prepare for this paradigm shift will realize tremendous benefits for years to come. NAD^+ boosting therapeutic strategies have the potential to delay/reverse age-associated physiological decline in the cardiovascular system and therefore, we predict that they will be useful components in future anti-aging treatment protocols for prevention of aging-related diseases and extension of cardiovascular health span.

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Figure legends

Figure 1. Schematic representation of *de novo* and salvage pathways for NAD^+ biosynthesis. The figure summarizes the key features of both the de novo pathway whereby L-tryptophan is metabolized to NAD+ and the salvage pathway whereby NAD+ is synthesized from the NAD+ precursors nicotinic acid (NA), nicotinamide riboside (NR) and nicotinamide (Nam). The *de novo* biosynthesis of NAD+ starts from L-tryptophan (Trp) which is enzymatically converted in a series of reactions to quinolinic acid (QA). QA is converted by quinolinate phosphoribosyltransferase (QPRT) to nicotinic acid mononucleotide (NaMN), which is then converted to nicotinic acid adenine dinucleotide (NAAD) by nicotinamide mononucleotide adenylyltransferase (NMNAT) enzymes. NAD synthase (NADS) generates NAD+ by the amidation of NAAD. In the salvage pathway nicotinamide mononucleotide (NMN) is synthesized from Nam by the rate-limiting enzyme, nicotinamide phosphoribosyltransferase (NAMPT). NMN is also synthesized from nicotinamide riboside (NR) via phosphorylation by NR kinase (NRK). NMN is converted into NAD^+ by NMNATs. NA, the other substrate of the NAD^+ salvage pathway, is converted by nicotininc acid phosphoribosyltransferase (NAPRT) to nicotinic acid mononucleotide (NaMN), which is then converted into nicotinic acid adenine dinucleotide (NaAD) by NMNATs, and lastly into NAD by NADS. Multiple enzymes break-down NAD+ to produce NAM and ADP-ribosyl moiety, including sirtuins and Poly (ADP-ribose) polymerase-1 and -2 (PARP-1/2). NMN is a substrate of ectoenzyme CD73, with generation of NR. IDO: indoleamine 2,3-deoxygenase; KAT: Kynurenine aminotransferase; KMO: kynurenine 3-monooxygenase; 3-OHKyn: 3-hydroxyl kynurenine; 3-HAA: 3-Hydroxyanthanillic acid; 3-HAO: 3-hydroxyanthranilate-3,4-dioxygenase; QPRT: Quinolinate phosphoribosyltransferase;

Figure 2. Role of NAD^+ deficiency in aging-induced endothelial dysfunction. Aging-induced mechanisms contributing to an age-related decline in NAD^+ content may include up-regulation of pathways consuming NAD^+ (PARP1 activation, CD38) and decreased biosynthesis of NAD^+ (e.g. due to down-regulation of nicotinamide phosphoribosyltransferase [NAMPT]). PARP-1 is a key NAD^+-consuming enzyme competing with sirtuins for NAD^+ availability. In aging increased DNA damage results in nuclear PARP-1 activation, lowering NAD^+ availability. The consequences of age-related NAD^+ depletion in endothelial cells include decreased activation of sirtuins (SIRT1,2,6 and 7 in the nucleus, SIRT3,4 and 5 in mitochondria and SIRT1 and 2 in the cytosol), which contribute to dysregulation of mitochondrial biogenesis, impaired mitochondrial energetics, increased mitochondrial production of reactive oxygen species (mtROS), up-regulation of NOX oxidases, decreased eNOS activity and impaired bioavailability of NO, increased activity of NfKB-driven pro-inflammatory pathways, down-regulation of pro-survival and stress resilience pathways and pathways involved in angiogenesis. Decreased NAD^+ supply also alter NADH levels and synthesis of NADP/NADPH, contributing to age-related changes in a wide range of NADH and NADPH dependent catabolic and anabolic pathways as well as impairment of NADP(H) dependent regeneration of antioxidant systems (e.g. GSH). These changes impair endothelium-dependent vasodilation, promote inflammation, decrease capillarization and tissue blood flow and impair transport and barrier function of the endothelial cells. The multifaceted impairment of microvascular endothelial function contributes significantly to the age-related dysfunction of multiple organs. Yellow arrows highlight potential targets for intervention to rescue the function of the NAD^+/SIRT-1 axis in aged endothelial cells. These anti-aging interventions include rescuing NAD^+ levels by treatment with NAD^+ precursors (NR, NMN), pharmacological inhibition of NAD^+ utilizing PARP-1 activation or treatment with sirtuin activating molecules (STACS).