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Role of Nicotinamide Adenine Dinucleotide and Related Precursors as Therapeutic Targets for Age-Related Degenerative Diseases: Rationale, Biochemistry, Pharmacokinetics, and Outcomes

Nady Braidy
University of New South Wales

Jade Berg
Australasian Research Institute

James Clement
BetterHumans Inc.

Fatemeh Khorshidi
University of New South Wales

Anne Poljak
University of New South Wales

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Nady Braidy [1], Jade Berg [2], James Clement [3], Fatemeh Khorshidi [1], Anne Poljak [4,5], Tharusha Jayasena [1], Ross Grant [2,5,6] Perminder Sachdev [1,7],

[1] *Centre for Healthy Brain Ageing, School of Psychiatry, University of New South Wales, Sydney, Australia*

[2] *Australasian Research Institute, Sydney Adventist Hospital, Sydney, Australia*

[3] *BetterHumans Inc, USA*

[4] *Mark Wainwright Analytical Centre, University of New South Wales, Sydney, Australia*

[5] *School of Medical Sciences, University of New South Wales, Sydney, Australia*

[6] *Sydney Medical School, University of Sydney*

[7] *Neuropsychiatric Institute, Euroa Centre, Prince of Wales Hospital, Sydney, Australia*

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Abstract

Significance: Nicotinamide adenine dinucleotide (NAD+) is an essential pyridine nucleotide that serves as an essential cofactor and substrate for a number of critical cellular processes involved in oxidative phosphorylation and ATP production, DNA repair, epigenetically modulated gene expression, intracellular calcium signalling and immunological functions. NAD+ depletion may occur in response to either excessive DNA damage due to free radical or UV attack, resulting in significant PARP activation and a high turnover and subsequent depletion of NAD+, and/or chronic immune activation and inflammatory cytokine production resulting in accelerated CD38 activity and decline in NAD+ levels. Recent studies have shown that enhancing NAD+ levels can profoundly reduce oxidative cell damage in catabolic tissue, including the brain. Therefore, promotion of intracellular NAD+ anabolism represents a promising therapeutic strategy for age-associated degenerative diseases in general, and is essential to the effective realisation of multiple benefits of healthy sirtuin activity. The kynurenine pathway represents the de novo NAD+ synthesis pathway in mammalian cells. NAD+ can also be produced by the NAD+ salvage pathway. ***Recent Advances:*** In this review, we describe and discuss recent insights regarding the efficacy and benefits of the NAD+ precursors, nicotinamide (NAM), nicotinic acid (NA), nicotinamide riboside (NR), and nicotinamide mononucleotide (NMN), in attenuating NAD+ decline in degenerative disease states and physiological ageing. ***Critical Issues:*** Results obtained in recent years have shown that NAD+ precursors can play important protective roles in several diseases. However, in some cases, these precursors may vary in their ability to enhance NAD+ synthesis via their location in the NAD+ anabolic pathway. Increased synthesis of NAD+ promotes protective cell responses, further demonstrating that NAD+ is a regulatory molecule associated with several biochemical pathways. ***Future Directions:*** In the next few years, the refinement of personalised therapy for the use of NAD+ precursors, and improved detection methodologies allowing the administration of specific NAD+ precursors in the context of patients' NAD+ levels will lead to a better understanding of the therapeutic role of NAD+ precursors in human diseases.

Introduction

Pellagra is a syndrome cause by a diet seriously deficient in synthetic precursors for the essential pyridine nucleotide nicotinamide adenine dinucleotide (NAD+), namely niacin (vitamin B3), and tryptophan (75,117,255). This lethal disorder can develop within 60 days

of maintaining a deficient diet due to the absence of free stores of nicotinic acid (NA) or nicotinamide (NAM) (298). Pellagra is pathologically characterised by a distinct dark pigmented skin rash and the three D's of dermatitis, diarrhoea, and dementia (5). Interestingly the shares AIDS Dementia Complex (ADC) shares some neurological similarities with pellagra in its clinical presentation (55).

In the last century, pellagra was a common disease in rural areas in the poorer southern United States, and was attributed to an unknown infectious pathogen (299). However, it was Dr Joseph Goldberger, and his associates, of the U.S. Public Health Service, who in 1914, examined the hypothesis that pellagra was due to a dietary deficiency. Subsequently, pellagra was prevented using a diet rich in maize, fresh milk, eggs and cured meat in these populations (1,121). Despite these advances, it was not until 1937 that Conrad Elvehjem, a biochemistry Professor, first demonstrated the anti-pellagra genic effect of NAM and NA on the related black tongue disease in malnourished dogs (99,100).

Individuals diagnosed with pellagra-induced dementia can be successfully treated in the early stages of the disease. However, untreated pellagra results in irreversible neurological damage and eventually death (148). This is due to primarily to reduced NAD+ production and availability as NAD+ and its phosphorylated form NADP+ are both essential cofactors and substrates for numerous biological processes (365). A focal reduction in NAD+ availability due to increased turnover or reduced synthesis may also be foundational to the pathology seen in other conditions. It seems to the fit the observation of an apparently reversible dementia before frank pathology in patients with ADC. At present, pellagra is a rare condition that has been reported in severe cases of alcoholism and anorexia, or malnourishment in the underdeveloped world (21,332).

Several biochemical studies have shown that an inefficient production of NAD+, where catabolism exceeds anabolism, may produce cellular dysfunction simply due to dietary lack of niacin (27,325). It may also be due to the rate-limiting action of co-substrate dependent quinolinate phosphoribosyltransferase (QPRT) (267,304). Excess of the amino acid leucine, , inhibits QPRT, which prevents the formation of niacin or NA to nicotinic acid mononucleotide (NAMN) (189). Reduced tryptophan availability, particularly after chronic immune activation, or in the absence of a tryptophan -rich diet (i.e. soy, meat, fish, eggs and peanuts), may also be associated with the development of pellagra (217). Essential

differences however, may be observed between ADC and pellagra, as the latter develops as a result of a global bodily deficiency of tryptophan and niacin, while ADC develops as a result of increased tryptophan and NAD+ catabolism at specific, although possibly numerous sites (Figure 1). Activation of the tryptophan catabolism may be both positive and negative in ADC. Immune activated oxidative tryptophan catabolism can positively increase cell viability through increased NAD+ metabolism in brain cells. However, chronic activation of tryptophan catabolism may occur in response to increased NAD+ catabolism. Increased astrocyte and mononuclear phagocyte activation stimulates tryptophan catabolism to maintain NAD+ levels in the early stages of immune activation. However, prolonged immune activation leads to excess macrophage recruitment and activation which reduces the astrocyte-to-neuronal NAD+ supply, resulting in pellagra-like neuronal dysfunction which may be reversible in the short term (Figure 1). The characteristic mood disorders and depression of end stage HIV may be due to increased tryptophan catabolism leading to reduced availability of tryptophan for catabolism via serotonergic pathways (Figure 1).

It is well known that NAD+ concentrations increase under conditions associated with reduced energy loads. These include activities such as fasting, glucose deprivation, caloric restriction, and exercise (68). However, apart from pellagra, NAD+ levels decline in animals on high fat diets, and during ageing and cellular senescence (293). Given that NAD+ levels are elevated under conditions of increased lifespan or health span and decline under conditions of accelerated ageing and/or reduced health span, suggests that reduced NAD+ levels may represent a major contributor to the ageing process (102). Therefore, supplementation with NAD+ and its precursors may represent a potential therapeutic strategy to mediate protection against accumulation of inflammation and highly volatile reactive oxygen species during the ageing process.

NAD+ biosynthesis pathways

Several NAD+ precursors have been identified in our natural diet. These include the amino acid tryptophan, and three forms of vitamin B3 - NA, NAM and nicotinamide riboside (NR) (Figure 2). Tryptophan catabolism via the kynurenine pathway can lead to *de novo* NAD^+ synthesis (128). When dietary tryptophan is limited, the efficiency of the conversion of tryptophan to NAD+ declines below the well-established conversion ratio of 60:1 (107,164). NA and NR are precursors that are found in the basic food chain. NA is produced by plants

and algae, while NR is present in milk (338). NAM is formed as a by-product of enzymatic degradation of pyridine nucleotides, and is the main form of vitamin B3 that can be absorbed from animal-based food. The provision of these vitamins to NAD+ is aided by several factors, including the gut microbiome (212,213). Biosynthetic genes are also regulated by circadian rhythms (243). Additionally, the expression levels of a number of enzymes involved in NAD+ anabolism decline with age (236).

Tryptophan catabolism via the Kynurenine Pathway

Tryptophan is the least abundant amino acid of animal and plant proteins, making up only 1-1.5% of the protein amino acid content (261). Tryptophan was first isolated in 1901 by Sir Frederick Gowland Hopkins and his student S. W. Cole (154), and by 1906 was reported as the first amino acid necessary for growth (261). The kynurenine pathway was first described as a principle route for tryptophan catabolism in 1947 (122). Two major routes for tryptophan catabolism have been identified in mammals that are active independent of protein anabolism. In the periphery, the kynurenine pathway accounts for up to 95% of tryptophan metabolism, while only about 1% of TRYP content is converted via the indoleamine pathway to form the neuroactive metabolites, serotonin and melatonin (261).

IDO-1/2 and TDO

The kynurenine pathway proceeds with the oxidative cleavage of tryptophan by either indoleamine 2,3-dioxygenase-1 (IDO-1) (**EC** 1.13.11.52) and its isoform indoleamine 2,3-dioxygenase-2 (IDO-2), or tryptophan 2,3-dioxygenase also called tryptophan pyrolase (TDO) (**EC** 1.13.11.11) to produce formylkynurenine (23,103,313) (Figure 2 Step a). Both IDO and TDO are haem-requiring enzymes. IDO is mainly found in extrahepatic tissue including the brain, placenta, spleen, lung, kidney, alimentary tract, and epididymis. It does not contain activating site for tryptophan analogues and is primarily activated by proinflammatory cytokines, such as interferon-gamma (IFN-γ) (109). Concomitant induction of IDO and free radical production of interferon-γ (IFN-γ) may at first increase NAD+ biosynthesis to contribute to the regeneration of intracellular NAD+ levels in an environment of increased NAD+ turnover and demand. This suggests a protective role for increased tryptophan catabolism in activated macrophages (Figure 3). However, TDO is predominantly located in the mammalian liver and can be activated by numerous factors, including fasting, glucocorticoids, hydrocortisone, NA, and L-tryptophan (369).

The metabolic product of IDO-1/2 and TDO activity is the unstable intermediate metabolite, N-formyl kynurenine (N-f-YN) (140) which is rapidly hydrolysed by kynurenine formylase (EC 3.5.1.9) to form kynurenine (Figure 2 Step b), the first appreciably stable product of the kynurenine pathway. Kynurenine can cross the blood brain barrier (BBB) (240) and represents a significant branch point from which three products can be synthesised with the use of three different enzymes, Kynureninase (**EC** 3.7.1.3), Kynurenine aminotransferase (KATs) (**EC** 2.6.1.7), and Kynurenine 3-hydroxylase (**EC** 1.14.13.9) (22).

Kynureninase

Kynureninase is a cytosolic enzyme which produces anthranilic acid (AA) by the cleavage of the alanine side chain from kynurenine (Figure 2 Step e). AA can undergo hydroxylation to 5- or 3- hydroxyanthranilic acid (5- or 3-HAA) via non-specific microsomal hydroxylating enzymes (184,257,262). AA can also cross the BBB via passive diffusion. Kynureninase also plays a role in the production of 3-HAA from 3-hydroxykynurenine (3-HK) (Figure 2 Step e).

The formation of NAD+ from tryptophan is inhibited by inadequate levels of vitamin B6 as kynureninase is dependent on pyridoxyl-5'-phosphate (PLP) (vitamin B6) as a coenzyme for the conversion of kynurenine to AA, or 3-HK to 3-HAA (238). Low levels of vitamin B6 has been shown to correlate with higher levels of psychological distress (173,306). The mechanism of B6 involvement in depression is most likely due to the fact that B6 is a cofactor for 5-hydroxytryptophan decarboxylase, the enzyme which catalyses the last step in serotonin biosynthesis (126,233).

However, pyridoxine is also a cofactor for several reactions in the brain neurotransmitter pathway, including glutamate decarboxylase and gamma-aminobutyric acid (GABA)-transaminase, the two enzymes required for the synthesis for GABA from glutamate (124,125,368). In pyridoxine dependent epileptic children, inefficient B6 levels resulted in markedly elevated levels of glutamate in the brain (141). Moreover, a decrease in vitamin B6 levels has been associated with a deficiency in both humoral and cell mediated immune responses including lymphocyte differentiation and maturation (134).

It has been noted that a decrease in kynureninase activity will reduce further flux through the kynurenine pathway thereby decreasing production of the NMDA receptor agonist and

excitotoxin, quinolinic acid (QUIN) (111). However, QUIN levels are increased during inflammation, suggesting that kynureninase activity may not be significantly reduced. Vitamin B6 may therefore be used preferentially by the cell for kynureninase activity (e.g. QUIN/NAD production over GABA transaminase (EC 2.6.1.19; GABA production), 5-hydroxytryptophan decarboxylase (EC 4.1.1.28; Serotonin synthesis), and glutamate decarboxylase activity (EC 4.1.1.15), indicating a cell priority for *de novo* NAD+ biosynthesis under these conditions. The increase in QUIN secretion by activated mononuclear phagocytes during neuroinflammation may indicate an increased demand for NAD+ in these cells, the production of which may be limited under certain conditions by a saturated QUIN ribosylation system (252,253).

Kynurenine aminotransferases

Kynurenine aminotransferases (KATs) produce kynurenic acid by the transmission of kynurenine (Figure 2 Step c). Kynurenic acid is a stable compound with non-specific antagonist action in the brain at the glutamate subtype, N-methyl-D-aspartate (NMDA) receptor. Both KATs and kynureninase are vitamin B6 dependent enzymes (245).

Kynurenine 3-hydroxylase

Kynurenine 3-hydroxylase is a mitochondrial enzyme that also converts kynurenine to 3-HK by the hydroxylation of the aromatic ring (Figure 2 Step d). 3-HK is an NADPH-dependent enzyme whose activity appears to be reduced with oestrogen and in conditions of hyperthyroidism (28). 3-HK can also cross the BBB, stimulate free radical production and mediate vasodilation (104).

3-hydroxyanthranilic acid oxygenase

The catabolism of 3-HAA is mediated by 3-hydroxyanthranilic acid oxygenase (3-HAAO) (EC 1.13.11.6), an enzyme that is found in both cytosol and synaptosomal fractions to produce the intermediate 2-amino-3-carboxymuconic semialdehyde (307) (Figure 2 Step f).

Picolinic acid carboxylase

The enzyme picolinic acid carboxylase (PICAC) (EC 4.1.1.45) preferentially converts 2-amino-3-carboxymuconic semialdehyde to 2-aminomuconic semialdehyde with subsequent non-enzymatic conversion to picolinic acid (PIC) (Figure 2 Step g) (188,230), a metal chelator (106,270) and NMDA-receptor antagonist or enzymatic rearrangement leading

finally to acetyl CoA (30,79,230). The non-enzymatic rearrangement of 2-amino-3-carboxymuconic semialdehyde occurs when PICAC is saturated with substrate to produce QUIN (Figure 2 Step h). The activity of PICAC has been shown to be inversely proportional to the amount of NAD+ synthesised from tryptophan (305).

Quinolinic acid phosphoribosyl transferase

QUIN is converted to NAMN by the enzyme quinolinic acid phosphoribosyl transferase (QPRT) (EC 2.4.2.19) (Figure 2 Step i). QPRT catalyses the reaction between 5-phosphoribosyl-1-pyrophosphate (PRPP) and QUIN in the presence of Mg^{2+} to produce NAMN. The maximal enzymatic rate for QPRT is apparently the lowest of all kynurenine pathway enzymes, and is 80 times lower than the preceding enzyme, 3-HAAO. However, the Michaelis-Menton constant (Km) for both 3-HAO and QPRT has been calculated to be the same, and this is likely due to the fact that 3-HAA provides substrate for the production of PIC as well as QUIN. The relative amount of QUIN formed from 3-HAA will therefore by determined by the rate of PICAC activity (168,176). The behaviour of PICAC under inflammatory conditions in the human brain or elsewhere does not appear to have been investigated. However, as IFN-γ appears to only induce IDO, it may cautiously be speculated that PICAC activity is not increased during an inflammatory response. Thus, increased flux through the kynurenine pathway will proportionately increase QUIN production.

QPRT is widely distributed in several tissues including the liver and brain, and may play an important role in mediating neuroprotection against QUIN-induced toxicity, associated with neurodegenerative diseases, including epilepsy and Huntington's disease (58,132,175,229,235,250,333,364). The physiological levels of QUIN are thought to be in the low nanomolar range, and QPRT activity increases with increased levels of QUIN. However, at high levels of QUIN (> 500 nM), neuronal QPRT activity is saturated (267). This leads to the production of QUIN at a greater rate than the production of NAD+, leading to the accumulation of QUIN and NMDA-mediated excitotoxicity (254).

PRPP is important in the regulation of QPRT activity (Figure 4) (33,161,162,168). The rate at which PRPP is synthesised and used determines its steady state concentration within the cell, which then determines the metabolic progress of pathways competing for PRPP. PRPP is synthesised in the cell in the reaction catalysed by 5-phosphoribose pyrophosphokinase or PRPP synthetase (EC 2.7.6.1) utilising a ribose-5-phosphate and ATP. PRPP synthetase has

an absolute requirement for inorganic phosphate (Pi) and is elevated in cells undergoing rapid cell division. The activity of PRPP synthetase is competitively inhibited by increased levels of ADP and ATP. The ribose 5-phosphate used in this reaction is generated from glucose 6-phosphate metabolism via the hexose monophosphate shunt or from ribose-1-phosphate (generated by the phosphorolysis of nucleotides) via a phosphoribomutase reaction (73).

Disorders in PRPP-synthetase activity and NAD+ metabolism have been implicated in the development of neurological disorders. PRPP concentrations have been positively correlated with cytosolic NAD+ and ATP levels in whole animals, and the availability of PRPP for NAD+ synthesis may be reduced in the presence of high turnover and *de novo* synthesis of purine and pyrimidine nucleotides (96). This may occur in ADC, and neurodegenerative diseases as a result of free-radical induced DNA damage and astrogliosis. The increase of QUIN seen in some neuroinflammatory conditions may therefore be a combination of increased flux through the kynurenine pathway coupled with decreased enzyme activity possibly due to the use of PRPP for purine and pyrimidine synthesis in DNA damaged or mitotic cells.

NAD pyrophosphorylase (nicotinamide mononucleotide adenylyltransferase)

Further transformations leading to the synthesis of the parent molecule of the pyridine nucleotides, NAD occurs in the nucleus and possibly the mitochondria. NAMN is catalysed by NAD pyrophosphorylase or nicotinamide mononucleotide adenylyltransferase (NMNAT) (EC 2.7.7.1) in the presence of ATP to produce desamido NAD (193,296) (Figure 2 Step u). In the presence of glutamine, desamido-NAD is amidated to the parent pyridine nucleotide, NAD+ (Figure 2 Step m), the final product of the kynurenine pathway (367). Three isoforms have been identified in humans in several different organelles, namely NMNAT-1 (nucleus), NMNAT-2 (golgi complex), and NMNAT-3 (mitochondria) (31). The differential localisation of these enzymes suggests an organelle-specific function for these proteins, and independent nuclear, mitochondrial, and Golgi-specific NAD+ biosynthetic pathways. Unlike NMNAT-1, which is the preferred enzyme for NAD+ synthesis (157), NMNAT-2 and -3 can also form NADH directly from reduced nicotinamide mononucleotide (NMN) (165). NMNAT activity (and predominantly NMNAT-1) is high and non-rate-limiting in catabolic tissue, but not in blood (236).

Apart from NAD+ biosynthesis, some studies have demonstrated that NMNAT isoforms can protect against axonal degeneration both *in vitro* and *in vivo* (80,183,211). NMNAT has been shown to serve as a stress response protein necessary for thermotolerance and attenuation of oxidative-stress induced shortened lifespan (11). The same study further showed that NMNAT is transcriptionally regulated by the heat shock factor (HSF) and hypoxia-inducible factor 1α (HIF1α) *in vivo*. During conditions of heat shock, HSF can bind to the NMNAT promoter, thus inducing NMNAT expression. However, under hypoxic conditions, HIF1α enhances NMNAT levels indirectly via induction of HSF (11). Additionally, NMNAT isoforms may exhibit protein chaperone function, exerting neuroprotection in several drosophila and mouse models of neurodegeneration (54,382). Overexpression of NMNAT-1 has also been shown to partially maintain neuronal function and reduce the levels of biochemical insoluble tau in a mouse model of chronic tauopathy with no significant effect on tau phosphorylation, tau aggregation, or tau-induced inflammation and hippocampal atrophy (12). Furthermore, overexpression of NMNAT-3 mediated axonal protection against tumor necrosis factor-induced and intraocular pressure (IOP) elevation-induced optic nerve degeneration by reducing the expression of p62 and increasing autophagic flux in a retinal ganglion cell line (182). Taken together, these studies suggest the possibility for new mechanisms of protection for NMNAT enzyme activity in addition to its role as an enzyme for NAD+ biosynthesis.

NAD+ production from the vitamin niacin

In addition to its *de novo* synthesis from tryptophan, NAD+ can also be synthesised from either the acid, amide or riboside form of the vitamin niacin (vitamin B3).

Nicotinic acid phosphoribosyl transferase

NA is converted to NAMN by the enzyme nicotinic acid phosphoribosyl transferase (NAPRT) (Figure 2 Step l) (EC 6.3.4.21) using PRPP as a co-substrate, in an ATP-dependent manner. As QUIN is converted to NAMN by the enzyme QPRT, the sequence of events leading to NAD+ production is identical after NAMN formation from either substrate (112). NAPRT appears to be expressed in several catabolic tissues including the colon, heart, kidney, and liver (95). The non-deamidated route of NAD+ synthesis displayed a higher relative proportions in blood and small intestine, and higher absolute values in liver and small intestines compared to the amidated (NAMPT) route, suggesting the significance of NA as a

precursor for NAD+ synthesis in these tissues (236). This has been reaffirmed by several feeding studies that have shown that NA is a more favourable precursor for NAD+ synthesis than NAM in the liver, intestine and kidney (81). As well, NA has been shown to increase intracellular NAD+ levels in a kidney cell line. Additionally, overexpression of NAPRT1 has been shown to mediate protection against oxidative stress-mediated NAD+ depletion (142).

Although tryptophan can be converted to NAM, it cannot be used to produce NA in vertebrate cells expressing the *de novo* synthesis pathway and NAD+ consuming enzymes, such as poly(ADP-ribose) polymerases (PARPs). NAM can be converted to NA in the intestinal lumen by bacterial nicotinamidase (EC 3.5.1.19) (Figure 2 Step o). However, one study suggested that sufficient levels of pyrophosphate and NAMN in cells can induce NAPRT to yield NA, thus allowing for the production of NA from tryptophan (212,214). Further studies are required (and are planned) to test this hypothesis. Bacterial and fungal degradation of NAD+, and direct NA supplementation can also increase NA levels in the alimentary canal for distribution to the rest of the body via vascular blood flow (212).

Nicotinamide phosphoribosyl transferase

The enzyme nicotinamide phosphoribosyl transferase (NAMPT) (**EC**:2.4.2.12) using PRPP as a co-substrate converts NAM to NMN (Figure 2 Step p), and then to NAD+ by the action of NAD pyrophosphorylases in the presence of ATP (Figure 2 Step u) (351). This amidated route of NAD+ synthesis predominantly displayed the highest rates in liver and kidney, and lowest in blood (161). The expression of NAMPT is encoded by the pre B-cell colony enhancing factor (PBEF1) gene. NAMPT also known as PBEF or visfatin has been identified as a cytokine that promotes the maturation of B cells when other cytokines, such as IL-7, and stem cell factors are available. It also exhibits insulin mimetic effects (260,374). The intracellular domain has been shown to activate lymphocytes and function as an NAD+ biosynthetic enzyme (281). However, both the extracellular and intracellular domains exhibit favourable phosphoribosyl activity.

In a cisplatin-induced acute kidney injury model, pharmacological manipulation of NAMPT expression via AICAR significantly improved renal function and reduced tubular injury. This effect has been associated with increased mRNA expression of SIRT3 – a mitochondrial sirtuin – and reduced protein hyperacetylation (237). Inhibition of the NAMPT pathway can impair glucose tolerance and insulin secretion in mice, an effect which can be ameliorated by

subsequent supplementation with NMN (275). Despite these findings, inhibition of NAMPT, which anabolises the substrate for NMNAT in mammalian cells had no significant effect on NMNAT-1 mediated axonal protection in another study (289).

Nicotinamide N-methyltransferase

The ability of a cell to salvage NAM into the generation of NAD+ via NAMPT versus methylation of NAM by the enzyme nicotinamide N-methyltransferase (NNMT) (**EC**:2.1.1.1) (Figure 2 Step q) to N-methylnicotinamide (MeNAM) modulates the efficiency of biological processes dependent on NAD+ (6,263). N-methylation also regulates the biotransformation and detoxification of certain drugs and other xenobiotic compounds by the liver. The enzymatic activity of NNMT uses S-adenosyl methionine as the methyl donor to form pyridinium ions such as S-adenosyl-L-homocysteine (287). This enzyme is predominantly expressed in the liver. A lower expression has been reported in the kidney, lung, skeletal muscle, placenta, heart and adipose tissue, although it was not detected in the brain or pancreas (287). Increased activity of NNMT has been shown to facilitate the production of toxic N-methylpiridinium compounds, which have demonstrated neurotoxic properties, and which may be involved in the nigrostriatal degeneration (366).

Nicotinamide riboside kinases

NR or nicotinic acid riboside (NAR) represent newly identified precursors that can be converted to NAD+ via the NR kinase (NRK) (EC 2.7.1.173) pathway (Figure 2 Step j), or by the action of nucleoside phosphorylase and the nicotinamide salvage pathway (38). NRKs are highly conserved in eukaryotic cells, and are encoded by the Nmrk genes. Two NRK enzymes have been identified, NRK1 and NRK2, however, their exact physiological roles remain unclear. While NRK1 is ubiquitously expressed in mammalian tissue, NRK2 is not expressed in the kidney, liver, lung, pancreas and placenta (272). Using the Nmrk1-deficient mouse model (NRK1KO), it has recently been shown that NRKs are rate-limiting for NR/NMN-mediated NAD+ synthesis (272).

Purine nucleoside phosphorylase

The second NR salvage pathway is NRK-independent, whereby NR is broken into a ribosyl product and NAM (Figure 2 Step k), the latter of which yields NAD+ by nicotinamide salvage. Purine nucleoside phosphorylase (PNP) (EC 2.4.2.1) has been shown to convert NAR to NA (Figure 2 Step k), which is then converted to NAMN by the catalytic action of

NAPRT (331). PNP deficiency has been shown to increase the levels of deoxyGTP (dGTP) levels. This in turn inhibits ribonucleotide reductase, which is required for the formation of deoxynucleotides (20,285,327). The enzyme deficiency leads to the accumulation of metabolites that can induce toxicity in lymphoid lineage cells (291).

Cytosolic 5'-nucleotidases

A recent study showed that NAR can be produced by human cells and forms a critical role in intracellular NAD+ anabolism (190). The study showed that cytosolic 5'-nucleotidases (5'-NT) can dephosphorylate NAMN, and to a lesser extent, NMN, to form NAR. The amount of NAR formed appears sufficient to promote NAD+ synthesis in neighbouring cells that are missing the machinery required to utilise non-riboside NAD+ precursors (190).

Biological roles of NAD+

NAD+ is an essential pyridine nucleotide that plays major roles in a number of critical biological processes, including oxidative phosphorylation and ATP production, and synthesis of cholesterol, fatty acids, and steroids (224). The primary function of NAD+ was identified by Warburg and Christian in 1936 (357). NAD+ serves as a hydrogen acceptor allowing the transfer of electrons for oxidation-reduction (i.e. redox) reactions leading to ATP production in the mitochondria. ATP represents the cellular 'energy currency', and a decline in intracellular NAD+ levels leads to reduced levels of ATP, culminating in cell death via energy restriction (373).

Apart from NAD+, its closely related phosphate NADP (Figure 2 Step r), serves as a cofactor in several anabolic processes, such as fatty acid and cholesterol synthesis (315). The reduced form of NAD+ and NADP are NADH (Figure 2 Step t) and NADPH (Figure 2 Step s) respectively. These nucleotides serve as hydride donors, in over 400 enzymatic reactions throughout the body involving dehydrogenases, hydroxylases and reductases (219). These reduced and phosphorylated forms can interconvert, but do not alter the levels of NAD+.

Importantly, NADPH is an essential coenzyme required for the reduction of reactive oxygen species (ROS) (29). Thioredoxin (TXN) is an antioxidant protein that is reduced by thioredoxin reductase (TXNRD) in an NADPH-dependent process (61). Glutathione disulphide (GSSG) is also a substrate for glutathione reductase (GSR) for reduction back to glutathione (GSH) using NADPH. The generation of GSH and TXN is pivotal for the

elimination of ROS such as hydrogen peroxide (H_2O_2) (123). Reduced NADPH production due to decreased NAD+ anabolism (or increased catabolism) can lead to impairments in the cells redox balance leading to perturbations in mitochondrial function and genomic signalling and stability subsequently leading to increased vulnerability to ~~oxidative stress~~ necrotic and apoptotic pathways.

Apart from its roles in redox reactions, a large body of evidence has shown that NAD+ is more than a regulator of metabolism, but rather can also participate as the required substrate for several important enzymatic reactions, including DNA repair, epigenetically modulated gene expression, maintenance of intracellular calcium homeostasis and immunological roles (52,118,120) (Figure 5).

Poly(ADP)-ribosylation and DNA repair

DNA strand breaks are known to occur in response to free radicals, ultraviolet light or alkylating chemicals which activate the enzyme poly(ADP-ribose) polymerase (PARP) (Figure 2 Step n) (320). Neuronal and astroglial cells exposed to cytotoxic levels of glutamate and QUIN show both an increase in intracellular oxidative stress and PARP activity (46). PARP-1 (the dominant member of a super family of 18 PARP proteins) efficiently detects the presence of DNA breaks by its N-terminal zinc-finger domain (312). The ADP-ribosylation of PARP triggers the recruitment of key proteins that stimulates the repair of the DNA damage in less than 15 s (85). Importantly, in order for PARP to carry out its ADP-ribosylating function it uses the ADP ribose moiety of NAD+ for its supply. Thus, PARP breaks down NAD+ to NAM and an ADP-ribosyl product (Figure 6A) (145). Possibly as a consequence of DNA strand breaks, recent evidence suggests that the poly(ADP)ribosylation of histones or transcription factors may also be involved in nuclear receptor signalling. Poly(ADP-ribose) metabolism is a dynamic process in which the degradation of ADP-ribose polymers occurs relatively rapidly through the action of poly(ADP-ribose) glycohydrolase (295) (Figure 6A).

A significant decrease in intracellular NAD+ has been reported in the brain and a variety of other cell types as a result of DNA strand breaks and PARP activation following exposure to hydrogen peroxide, nitric oxide, HIV infection, or during inflammation (7,326,330). Increased PARP activity resulting in decreased NAD+ has been shown to decrease ATP and neurotransmitter levels in the brain as well as cause cell lysis and death (45,203) (Figure 6B).

Inhibition of PARP activity, following oxidant injury has been shown to preserve NAD+ and ATP levels preventing cell lysis (14), although damage to the DNA is probably not prevented. In a pancreatic islet cell population lacking expression of PARP, NAD+ depletion does not occur after oxidant injury despite DNA strand breaks occurring to the same degree (68). This demonstrates that activation of PARP is the major cause of NAD+ depletion in these oxidant injury cells. Elevated levels of free radicals, oxidants, and excitotoxins have been reported in inflammatory mediated diseases of the brain, and in some cases, DNA damage has been demonstrated (2,220,221,341,380). This suggests that NAD+ depletion through PARP activation may play a role in CNS dysfunction and pathology under these conditions (Figure 6).

More recently, it has been suggested that PARP activation, rather than NAD+ decline may be responsible for cell death following exposure to genotoxic insult. For example, poly(ADP)ribosylation has been shown to directly inhibit the glycolytic enzyme hexokinase leading to a significant reduction in glycolysis prior to NAD+ depletion, mitochondrial dysfunction and neuronal cell death (17). Moreover, direct poly(ADP)ribosylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is the primary cause of cell death in kidney tubules following ischaemic injury (94). These studies suggest that the beneficial effects of PARP inhibition may be due to altered metabolic effects independent of maintenance of NAD+ levels during pathological conditions.

PARP also appears to play a positive role in the up regulation of the tumor suppressor protein, p53. For example, PARP-deficient cell lines derived from Chinese hamster V79 cells failed to undergo poly(ADP)ribosylation and activate p53 following treatment with etoposide (363). PARP can also activate DNA dependent protein kinases which regulate p53 activity through phosphorylation (318). Therefore, on the contrary to reported benefits of PARP inhibitors, pharmacological inhibition of PARP activity may contribute to genomic instability with resulting risk of cancer formation.

CD38/CD39/CD73/CD157 and secondary messenger signalling

The immune associated ectoenzymes CD38, CD39, CD73 and CD157 represent another class of NAD^+-consuming enzymes (155) (Figure 2 Step n). These enzymes require NAD+ to produce ADP ribose (ADPR) and hydrolyse the secondary messenger signalling molecule, cyclic-ADP-ribose (cADPR) which helps mediate intracellular calcium transients (Figure 7).

CD38 has also demonstrated an immunomodulatory role (135). For instance, the presence of CD38 on T-lymphocytes influences the ability of antigen presenting cells to stimulate antigen specific T-cells (256). Upregulation of CD38 expression also signals maturation of dendritic cells during inflammatory cytokine activation and acts as a modulating adhesion and signalling molecule between dendritic cells and lymphocytes (105). In cardiomyocytes, exogenous stimulants may stimulate an increase in intracellular calcium, which leads to activation of CD38 (147). CD38 expression has also been shown to increase with age (65), and this is most likely attributed to an age-related increase in circulating inflammatory cytokines, and reduced CD38 function has been associated with poor immune responses.

Given that 100 molecules of NAD^+ must be hydrolysed to generate one molecular of cADPR, it is highly likely that CD38 is a major regulator of intracellular NAD^+ levels (77). Accordingly, we found a 5 fold increase in NAD^+ levels in CD38 knockout neuronal cells compared to controls (52). Therefore, CD38 may not only represent an inefficient secondary messenger enzyme, but also as a NADase which primarily regulates intracellular levels of NAD+ and its physiological processes (Figure 8).

CD38 has also been shown to use β-NAD+ as a substrate, but no α-NAD+ or NADH. CD38 can also catalyse a base exchange between NADP and NA, leading to the formation of nicotinic acid adenine dinucleotide phosphate (NAADP), which is also used as a hydrolytic substrate (90). It can also metabolize analogues of NAD+, including nicotinamide guanine dinucleotide (NGD+) and nicotinamide hypoxanthine dinucleotide (NHD+), yielding cyclic compounds (cGDPR and cIDPR, respectively). These compounds exhibit fluorescent properties, but not calcium-releasing (383). They represent useful biochemical agents for examining ADP-ribosyl cyclase activity.

Prolonged activation of CD38 following cardiac stress has been shown to induce a sustained Ca2+ release leading to cardiac hypertrophy and arrhythmias (130). Supporting evidence comes from male CD38-knockout mice which reported improved cardiac function, while treatment with ADPR cyclase inhibitors led to anti-arrhythmic affects in multiple in vitro models and cardiac Ca2+ overload studies (131). Similarly, inhibition of CD73 has been shown to mediate protection against renal stressors, and CD39 activity mediated protection against renal ischaemic injury (268).

CD38 can also regulate the activity of PARP and other NAD+-dependent enzymes SIRT1 activities by potentially reducing the accessibility of NAD^+ to its preferred enzymatic targets (348). NAM, which is generated by the catalytic activity of CD38, also represents an endogenous metabolite of SIRT1 enzyme. Therefore, it has been postulated that CD38 may in fact be an important regulator on intracellular NAD+ levels and SIRT1 activity, thus influencing SIRT1 functions, including maintenance of cellular bioenergetics, obesity, and senescence. Interestingly, one study reported no significant effects on NAD+ levels in CD38 knock-out mice compared to wild-type animals (377). Therefore, the amount of benefit due to CD38 inhibition or ablation warrants further investigation.

Novel CD38 inhibitors may also be useful for the treatment degenerative disorders where optimal NAD^+ and NADPH anabolism remains crucial to attenuate oxidative stress insult, the latter of which serves as the ultimate electron donor supporting glutathione peroxidases, peroxiredoxins, and glutaredoxins. However, inhibition of CD38 may also result in a deleterious impact on immunological function. CD38/cADPR also signals oxytocin release, which regulates many social behaviours, and inhibiting this process may induce several forms of mental impairment. Moreover, niacin deficiency which has been observed in patients with pellagra often progresses to a dementia similar to schizophrenia (98,264), and this could be due to impaired cADPR formation.

Sirtuin activity

Another important NAD+-dependent function is the activity of the silent information regulators of gene transcription, or sirtuin family of enzymes (Figure 2 Step n). Sirtuins are a family of class III NAD+ dependent histone deacetylases exhibit protein lysine deacetylase, and partial ADP-ribose transferase activities. In the reaction mediated by sirtuins, an acetyl-modified lysine is bound to a target protein and NAD+ in specific pockets (346). Deacetylation occurs when the modified lysine side chain is coupled to the cleavage of the glycosidic bonds in NAD+, leading to the generation of the deacetylated lysine, acetylated ADP-ribose, and NAM as byproducts (101) (Figure 9).

At, present seven classes of sirtuins (SIRT1-7) have been identified in mammalian cells, each of which are localised in various cellular organelles, and mediate a diverse range of important biological functions (53) (Figure 10). SIRT1 and SIRT6 are nuclear proteins associated with the maintenance of chromatin structure, DNA repair, and gene expression. It has been

suggested that SIRT1 may play a pivotal role in promoting cellular longevity and may hold the key to slowing development of the aging phenotype (290). SIRT1 has been shown to influence the acetylation status of several important transcription factors, including the metabolic regulator, peroxisome proliferator-activated receptor-γ (PPARγ), tumour suppressor protein (p53), and the cell growth linked FOXO forkhead family of transcription factors (192). However, some evidence suggests that SIRT6 may also contribute to an age-resistant phenotype (300). SIRT2 is predominantly a cytoplasmic protein where it regulates gene expression by deacetylating transcription factors which shuttle from the cytoplasm to the nucleus (282). SIRT3, SIRT4 and SIRT5 are found in the mitochondrion where they respond to changes in mitochondrial redox status by altering the enzymatic activity of specific downstream targets, including manganese superoxide dismutase (MnSOD) (249). SIRT7, is localised in the nucleolus of mammalian cells and has been associated with cellular growth and metabolism (347). The biological relevance of sirtuins in redox processes will be discussed further in another section of this review. Importantly, the beneficial effects of sirtuin activity are only achieved if NAD+ levels are optimal.

Principle causes of NAD+ decline

Apart from deficiency within the NAD+ biosynthesis process, there are principally two conditions under which NAD+ depletion may occur: (1) Excessive DNA damage due to free radical or UV attack, leading to hyperactivation of PARP. This ultimate leads to a high turnover and subsequent depletion of NAD+. The resulting energy crisis and reduced ATP production can lead to cell death via either an apoptotic or necrotic pathway (2). A chronic increase in immune activation and inflammatory cytokine production can accelerate CD38 activity and contributing to NAD+ decline. While several clinical disorders and degenerative disorders can meet these criteria, chronic accumulation of oxidative stress and inflammation during advanced age represents a major driver of NAD+ decline (49). Promotion of NAD+ anabolism using NAD+ precursors may represent a clinically relevant therapeutic strategy to ameliorate the age-related decline in cellular energy.

Redox roles of sirtuins and transcriptional regulation

Since the term of their discovery, sirtuins have been associated with lifespan extension. However, while the longevity enhancing capacity of sirtuins have been established in several small model systems, the modes of action of sirtuins underlying these beneficial effects

remain unclear. Chronic accumulation of damage over time represents the main phenotype associated with the ageing process. In particular, chronic oxidative stress can induce damage to diverse macromolecules, and perturb mechanisms with which they are repaired. Recent evidence suggests that the beneficial effects of sirtuins may be mediated by their ability to regulate redox processes. In this section, we will investigate the association between sirtuins and their redox environment, and review how sirtuin-mediated deacetylation affects target enzymes and transcription factors.

SIRT1

As previously mentioned, tumor suppressor p53 represents the first deacetylation substrate of SIRT1. The transcription factor p53 has been shown to activate numerous pro- and antioxidant genes including sestrins, manganese superoxide dismutase (MnSOD) and glutathione peroxidase 1 (GSHPXD1) (284). The binding and deacetylation of p53 by SIRT1 at Lys382 mediates its transcriptional activity (208). SIRT1 deacetylation of p53 has been shown to influence the cellular localisation of p53 in response to oxidative stress, and may serve as a metabolic switch between antioxidant protection and apoptotic cell death. For instance, in murine embryonic stem cells, the absence of antioxidants in cell culture media induced mitochondrial translocation of p53, whilst in SIRT1 knockout cells; increased oxidative stress induced nuclear translocation of p53leading to an antioxidant response (139). Similarly, up-regulation of SIRT1in mesangial cells attenuated the induction of p53-mediated apoptotic pathway following exposure to pathological concentrations of H_2O_2. However, at lower concentrations of H_2O_2, the SIRT1-p53interaction led to an induction of antioxidant processes (191).

While the adaptive role of SIRT1 against ROS stress has been well established *in vitro*, studies using live animal have been less convincing. This is due to the high levels of embryonic lethality following the production of $SIRT1^{-/-}$ mice. However, one study using heterozygous SIRT1 knockout mice reported increased vulnerability to renal oxidative stress, and combined $SIRT1^{+/-}$ $p53^{+/-}$ showed greater susceptibility to tumor development compared to p53 haploinsufficiency alone (146).

SIRT1 has also been shown to deacetylate and activate FOXO3a following exposure to oxidative stress (57). FOXO3a appears to be an important transcriptional activator of the SOD2 gene which encodes for the production of the endogenous antioxidant protein MnSOD.

The catalase enzyme, which acts directly on free radicals is predominantly localised in peroxisomes, and represents another target of FOXO3a (185). As per the relationship between SIRT1 and p53, low levels of H_2O_2 can mediate FOXO3a-mediated induction of catalase, ,whilst cytotoxic levels of H_2O_2 can induce FOXO3a-mediated apoptosis (144). In cardiovascular disease, increased oxidative stress can upregulate SIRT1expression and stimulation of catalase and MnSOD expression. However, higher levels of SIRT1 can lead to cardiac hypertrophy and cell death via apoptotic pathways (9). Taken together, these studies collectively suggest that SIRT1 serves as an ROS sensor, capable of inducing protection at low-level stress, whilst inducing apoptosis at severe stress levels.

Recently, it has also been shown that mechanisms responsible for the regulation of the intracellular NAD^+:NADH ratio can also affect SIRT1 function via AMP-activated kinase (AMPK), an essential regulator of cellular energy homeostasis. Several studies have shown that reduced glucose available in myoblasts induced activation of AMPK and upregulation of NAMPT, leading to increased levels of intracellular NAD^+ and activated SIRT1, and culminating in the activation of several transcriptional mediators, including FOXO proteins and PGC-1α, thus enhancing catabolism and mitochondrial biogenesis (110). Furthermore, activation of AMPK stimulated transcriptional activity downstream of SIRT1 in another study (66). SIRT1 can also activate AMPK through positive feedback mechanism. For instance, Liver kinase B1 (LBK1), which phosphorylates and activates AMPK under low nutrient levels can be deacetylated by stimulation or overexpression of SIRT1 (either directly or indirectly). This promotes translocation of LBK1 from the nucleus to the cytosol, which further phosphorylates AMPK (192). As such, there seem to be multiple levels of metabolic regulation occurring through the AMPK–SIRT1 axis, and many of these steps require further elucidation.

Apart from the AMPK-SIRT1 axis, SIRT1 can also interact and deacetylate PGC-1α. PGC-1α is an important transcriptional coactivator that stimulates mitochondrial biogenesis and indirectly also mitochondrial dynamics in a tissue-dependent manner. For instance, increased hepatic SIRT1 due to fasting can deacetylation PGC-1α leading to both inhibition of glycolytic genes, and increased expression of genes associated with gluconeogenesis (276). Another study showed that SIRT1 could directly interact with and deacetylate PGC-1α in adrenal PC12 cells leading to reduced PGC-1α transcriptional activity and related mitochondrial oxidative metabolism (246). However, in skeletal muscle, increased SIRT1

activity and PGC-1α deacetylation led to an increase in mitochondrial fatty-acid oxidation (116). Reduced PGC-1α activity has associated with reduced expression of the mitochondrial antioxidant protein, MnSOD, providing additional support for the role of SIRT1 on control of redox stressors (207).

Recent studies have shown that calorie restriction, and the phytochemical resveratrol, which are known another to activate SIRT1, can enhance endothelial nitric oxide synthase (NOS) expression and promote mitochondrial biogenesis by upregulating transcription factors such as PGC-1α (78). Similarly, SIRT1 has been shown to deacetylated eNOS *in vivo*, leading to increased eNOS activity and intracellular NO production (225). Therefore, SIRT1 represents a key regulator of vascular tone dependent on eNOS.

Moreover, it is well established that the transcriptional response to hypoxia is regulated mainly by the HIF family of proteins, of which HIF1α and HIF2α are well characterised (reviewed in (216)). It has been demonstrated that both HIF1α and HIF2α can be deacetylated by SIRT1 by two separate and distinct mechanisms. Under normal physiological conditions, SIRT1 can bind to, and deacetylate, HIF1α, preventing HIF1α from interacting with the transcriptional coactivator p300, inhibiting its transcriptional activity (200). However, under hypoxic conditions, the decline in the NAD^+:NADH ratio, and available NAD+ for optimal SIRT1 activity due to reduced oxygen levels allows HIF1α to remain acetylated, thus preventing its hypoxic transcriptional activity (200). On the contrary to its effect on HIF1α, SIRT1 can also form a complex with SIRT1 under hypoxic conditions and is deacetylated at three lysine residues (K385, K685, and K741) in the carboxy terminus, leading to increased transcriptional activity of HIF2α and related proteins and erythropoietin in particular (92).

SIRT2

The expression of SIRT2 have also been shown to be upregulated at both the mRNA and protein level in response to cellular stressors such as oxidative stress. Numerous studies have demonstrated that Increased SIRT2 expression following oxidative insult can lead to cellular apoptosis via induction of the proapoptotic protein Bim (350). Overexpression of SIRT2 has also been shown to promote neurodegeneration, although the exact mechanism remains unclear (322). In the absence of the SIRT2 gene, upregulation of the cytosolic chaperone 14-3-3ζ, sequesters the proapoptotic mitochondrial protein BAD in the cytosol and mediates protection against anoxia–reoxygenation-induced cell death (210). SIRT2 inhibitors have been shown to ameliorate α-synuclein-mediated toxicity in a cellular model of Parkinson's

disease (210). However, under low-stress conditions, SIRT2 upregulates mitochondrial MnSOD via FOXO3a deacetylation, leading to a reduction in the levels of ROS.

SIRT3

Isocitrate dehydrogenase 2 (IDH2) represents another major target of SIRT3, a mitochondrial sirtuin. IDH2 uses NADPH to generate reduced GSH to mediate an antioxidant affect. Schlicker et al., showed SIRT3, but not SIRT5, could deacetylated IDH2 at K211 and K212 residues to promote its activity (294). It has been shown that the GSH:GSSG ratio and the level of NADPH are increased in the liver, brain, and the inner ear following CR in a SIRT3-dependent manner (311). As well, SIRT3 directly deacetylates and inhibits the activity of IDH2, and SIRT3 overexpression increased NADPH levels and reduced oxidative stress-mediated cell death (311). Taken together, these studies suggest that CR, SIRT3, and IDH2 represent important targets for the management and treatment of age-related hearing loss, and that maintenance of intracellular NAD+ levels modulate the cellular response to degeneration.

Like IDH2, SIRT3 has been shown to mediate SOD2 activity by regulating mitochondrial FOXO3a activity, although the exact mechanism remains unclear. One study using overexpression of SIRT3 in mouse embryonic fibroblasts shows that the levels of ROS were dramatically reduced in a SOD2-dependent manner (266). Similarly, hyperacetylation of SOD2 in SIRT3-deficient mice led to reduced SOD2 activity and upregulation of ROS production (329). However, differences in the site-specific regulation of SOD2 by SIRT3 have been reported, and this is likely due to differences in cell type, species, or stress conditions

Additional mitochondrial targets of SIRT3 and SIRT5 also been recently identified which can regulate oxidative stress. SIRT3 has been shown to deacetylate complexes I, II, III, and IV and glutamate dehydrogenase (GDH), which regulates glutamate oxidative stress, yielding NADPH, is deacetylated by SIRT3 (and antagonised by SIRT4-mediated ADP-ribosylation) (205). SIRT5 can also deacetylate cytochrome *c* (294). As well, SIRT3 and SIRT5 can both regulate mitochondrial-localized reactions of the urea cycle. To be more specific, SIRT3 can deacetylate ornithine transcarbamoylase while SIRT5 acts on carbamoyl-phosphate synthase 1, to enhance urea cycle function, and promote the clearance of oxidative stress-promoting ammonium (137).

SIRT3 has been recently shown to be important for the regulation of normal cardiac function and protection against cardiac pathologies. Knockout of SIRT3 has been shown to increase the hyperacetylation of mitochondrial protein, leading to spontaneous cardiac hypertrophy with age and greater than 50% reduction in ATP levels (319). Reduced SIRT3 expression and hyperacetylation of cardiac mitochondrial enzymes have also been reported in mouse models for cardiac disorders, and poor human hearts (156). As well, increased activity of acyl-CoA dehydrogenase and other enzymes involved in fatty acid oxidation (FAO) have also been reported in SIRT3 knockout mice (13). However, another study reported reduced rates of FAO in the hearts of fasted animals (151). These differences may be attributed to variation in the type of stressors which can influence the activity of protein acetylation.

Renal stress has been shown to reduce the expression of SIRT3. For instance, SIRT3 mRNA expression was shown to be decreased in a model of free fatty acid (FFA) associated tubulointerstitial inflammation, and this occurred parallel to increased levels of ROS and markers of inflammation compared to age-matched control animals (187). Interestingly, retroviral overexpression of SIRT3 attenuated these changes, suggesting that optimal SIRT3 function is necessary for renal function (370). Similarly, high glucose levels were shown to decrease the mRNA and protein expression of SIRT3, and supplementation with NAD+ ameliorated high-glucose induced mesangial hypertrophy and SIRT3 expression at both the genomic and protein level (390). Taken together, these findings suggest that SIRT3 can protect against renal degeneration in diabetic nephropathy.

SIRT4

Like SIRT3, SIRT4 appears to be highly expressed in catabolic tissue such as the brain, heart, liver and kidney (136). SIRT4 has been shown to protect against hypoxia-induced apoptosis in cardiomyoblast cells (202). However, knockout of SIRT4 protected against angiotensin-II induced cardiac hypertrophy and fibrosis in mice, suggesting that SIRT4 may be directly involved in the pathogenesis of cardiovascular disease (209). While both studies suggest a discrepancy for the exact role of SIRT4 in cardiac function, it appears likely that these effects are due to the modulatory role of SIRT4 on cellular oxidative stress levels.

There also exists a strong correlation between kidney function, SIRT4 levels and the NAD+ metabolome. For instance, co-treatment with cisplatin and the phytochemical curcumin

restored NAD+ levels and attenuated the decline in NAMPT, SIRT1, SIRT3 and SIRT4 expression due to cisplatin-induced nephrotoxicity (342). However, it is unlikely that these effects are directly in response to SIRT4, as the levels of NAMPT, SIRT1 and SIRT3 were also affected. Additional work is necessary to evaluate the role and modes of action of SIRT4 in degenerative disorders of the brain, heart and kidney, and other age-related conditions associated with NAD+ depletion.

SIRT5

The exact roles of SIRT5 in maintaining normal cellular homeostasis is not well understood. One study found no significant differences between the heart weight and rate, and systolic blood pressure in SIRT5 knockout mice exposed to a high fat diet (379). However, another study showed that protein succinylation is uniquely elevated in SIRT5 knockout mice (248). These proteins include those that are involved in fatty acid metabolism, amino acid catabolism, the TCA cycle, oxidative phosphorylation, ketone and pyruvate metabolism (41). In mice exposed to cardiac ischemia, a larger infarct volume and elevated oxidative stress were reported in SIRT5 knockout hearts compared to wild-type controls (41). These changes were accompanied by increased fibrosis, and reduced shortening fraction and ejection fraction compared. Increased activity of succinate dehydrogenase (SDH) was also reported in SIRT5 knockout mice, and SDH inhibitors reduced infarct size to 'normal' levels (41). This suggests that the protective effects of SIRT5 may be mediated by desuccinylation of SDH.

Similarly, knockout of SIRT5 also resulted in hypersuccinylation of mitochondrial protein, and post translational modification of malonylation and glutarylation in the kidney (198). Additionally, SIRT5 has been shown to deacetylate carbamoyl-phosphate synthetase 1 (CPS1), leading to increased activity of CPS1 and reduced plasma urea levels (242). Increased blood ammonia levels were reported in SIRT5 knockout mice compared to age-matched wild-type controls. These findings provide a key role for the role of SIRT5 in the regulation of ammonia.

SIRT6

While the effect of redox stressors on SIRT6 function remains nascent in current literature, one study has shown that knockdown of SIRT6 can induce accelerated senescence as evidenced by the development of degenerative features, shortened telomere length, and reduced life span (239). Interestingly, HIF1α has been shown to be upregulated in cells

lacking SIRT6, leading to an increased glucose uptake and improved glycolysis (384). In normal mice embryonic fibroblast cells, SIRT6 serves as an H3K9 histone deacetylase, inhibiting HIF1α-dependent transcription of multiple glycolytic genes, thus acting as a co-repressing of HIF1α.

SIRT7

Of the family of sirtuins, SIRT7 remains the least investigated. One study showed that knockdown of SIRT7 enhances the acetylation of p53, leading to increased vulnerability to genotoxic insult (241). SIRT7 has also been shown to inhibit cell proliferation following exposure to high oxidative stress levels (344).

Activation by NAD+ precursors

A growing body of evidence suggests that upregulation of NAD+ anabolism can influence processes regulated by sirtuins. These pathways may therefore be upregulated with NAD+ or NAD+ precursors, or other means of manipulating NAD+ biosynthesis pathways. It has been shown that the Km for SIRT3 and SIRT5 is significantly lower than the levels of mitochondrial NAD+, suggesting that the activity of these sirtuins is rate-limited by the availability of mitochondrial NAD+ levels (150). Current evidence suggests the importance of SIRT1 and SIRT3 in regulating the beneficial effects of NAD+, and the effects of NAD+ supplementation on other sirtuins remains unclear. Examining whether the activity of other sirtuins are affected by NAD+ therapy represents an emerging area of research. It is likely that NAD+ supplementation may activate multiple members of the sirtuin family leading to diverse effects on multiple biological processes, and thus improving brain, cardiac and renal function under different stressors.

Distribution of the NAD+ metabolome

It is well established that NAD+ (in particular the NAD+/NADH ratio) is a master regulator of cellular bioenergetics. The total intracellular NAD+ content is estimated to be in the range of 0.2-0.5 mM (388). This concentration is within the estimated NAD+ Km value of PARPs (0.02-0.08 mM) (15) and SIRT1 (0.56 mM) for NAD+ (278). This means that the availability of the essential substrate NAD+, is rate-limiting for PARPs and SIRT1. For instance, low NAD+ levels due to increased PARP activity leads to reduced SIRT1 activity, whereas higher NAD+ levels enhances PARP and SIRT1 activities . Research from our group has

demonstrated that reduced levels of NAD+ due to chronic oxidative stress and hyperactivation of PARPs are associated with significantly reduced sirtuin activity (51,223).

Metabolomic profiling of the NAD+ metabolome in peripheral blood mononuclear cells (PBMCs), plasma and urine in an overnight fasting human subject has recently been published (337). The study showed that the phosphorylated NAD+ metabolites – NAMN, NAAD, NADP+, NMN and ADPR – are found exclusively in blood cells, but not in plasma or urine. The levels of NA, NAM and NR are considerably low in normal fasting blood (337). Very few studies have examined the levels of these NAD+ metabolites due to limitations in accurately measuring them. Using GC-MS, one study reported that the concentration of NAM is fasting blood was about 300 nM, and the level of NA was 30 nM (72). This provides evidence for the physiological importance of NAM as the preferred form of niacin to extrahepatic tissue. The blood levels of both NA and NAM can be significantly increased following supplementation with vitamin B3. These pharmacological doses range between 1-3 grams of NA or NAM. In comparison, a niacin-rich meal contains about 10 grams of vitamin B3 composed of a mixture of NA and NAM, the concentrations of which vary with their content in plant and animal foods (231,232).

It has been previously shown that small amounts of NA can be converted to NAD+ in the intestine and liver, and NA may not be detected in systemic blood. Moreover, the catalytic activity of NAD+ glycohydrolases or ADP-ribosylation in the small intestine or liver can induce the release of NAM into the blood stream (274). NAM from the diet may also be used to form NAD+ in the small intestine and liver, and may also be released into the blood stream. Expression of hepatic NNMT leads to the formation of MeNAM from NAM, thus maintaining SIRT1 activity in the liver (152).

It remains unclear whether NAM can accumulate in the blood stream following a NAM-rich meal, or stored in several tissue for generation of NAD+ as required, and later released into the blood stream to maintain threshold levels in the blood stream. However, one study previously showed that up to 60% of the total NAD+ levels are depleted in red blood cells in a rat model of niacin deficiency. The remaining 40% appeared resistant to depletion (274). On the contrary, the levels of NAD+ in the liver were considerably higher and depleted at a slower rate during deficiency (274). Short- or long-term storage of NAD+ may take place in the liver and red blood cells, where it regulates blood NAM levels during periods of niacin

deficiency eg. during fasting. Under normal physiological conditions, high affinity transporters are required to facilitate the transfer of NA and NAM into extrahepatic tissues, which are present in the blood stream at low to mid nanomolar concentrations. Understanding the interactions between these precursors can help us to elucidate appropriate pharmacological doses of NA and NAM.

Recently NR has been identified as an NAD+ precursor vitamin that is uniquely and orally bioavailable in mice and humans (337). Blood NAD+ levels have been shown to increase by 2.7 fold following a single daily dose of NR (1000 mg) for 7 days, with a concurrent increase in NAAD by up to 45 fold in PBMCs. While it is unclear how an oral dose of NR can raise NAAD levels, it has been suggested that NR may be partially converted to NAM via the NAD+ salvage pathway (337). Such conversion may stimulate bacterial hydrolysis of NAM to NA, culminating in the production of NAD+ using an NAAD intermediate. Another study showed that NMN is metabolised extracellularly to yield NR, which is then converted to NAD+ intracellularly (272). Therefore, NR and NMN represent convergent supplementation strategies to enhance NAD+ anabolism.

Subcellular compartmentalisation of NAD+

Traditionally, it was thought that NAD+ was distributed in the nucleus, as only one form of NMNAT was identified as nuclear in origin (296). Nuclear NAD+ was therefore available to catalyse poly(ADP-ribose) formation, but could also equilibrate in the cytosol via nuclear pores (32). Until recently, the significance of mitochondrial NAD+ was unclear, and it was thought that NAD+ could be transported in its intact form into the mitochondria (138). However, it is now understood that there are three intracellular NAD+ compartments – the nucleus, cytosol and mitochondria (31). Subcellular compartmentalisation of NAD+ is thought to play a critical role following niacin depletion. As total intracellular NAD+ levels decline, distinct subcellular stores of NAD+ may influence the outcome of competition between biochemical processes dependent on NAD+ consumption, leading to significant alterations in metabolic pathways that are involved in tissue pathologies.

Recently, three distinct NMNAT enzymes have been discovered, localised to the nucleus (NMNAT-1), mitochondria (NMNAT-2), and the Golgi apparatus (NMNAT-3) (31). While the levels of NMN required for the catalytic activity of NMNAT-1 and NMNAT-2 are very

close, a higher amount of NMN is required for NMNAT-2 activity (121). The differential expression of NMNAT enzymes in different intracellular compartments suggests multiple roles for promoting optimal metabolic function in a variety of cells, or an additional mechanism for adaptive response to stress. For example, one study showed that niacin deficiency with normoxia reduced lung NAD+ levels in Fisher-344 rats by 40 % (273). Interestingly, exposure to chronic hypoxic conditions induced poly(ADP-ribose) formation in lung tissue, but did not reduce lung NAD+ content, rather NAD+ levels remained at near control non-treated levels in niacin-deficient lung tissue.

NMNAT-1 plays an important role in mediating NAD+ synthesis to close proximity of the main enzyme responsible for ADP-ribosylation, PARP-1, but also including PARP-2 and 3, tankrases, and sirtuins. While it is likely that nuclear NAD+ may enter the cytosol via specific nuclear pores, there are also additional benefits for the formation of NAD+ in the nucleus. Overexpression of NMNAT-1 has been shown to rescue neurons from axonal degeneration, known as Wallerian degeneration (381). Similarly, inactive mutant forms of NMNAT-1 also demonstrated beneficial effects against neural loss, possibly due to a chaperone effect (54,382). NMNAT-1 can direct NAD+ synthesis towards the active site of automodified PARP-1 via non-covalent interactions between NMNAT-1 and poly(ADP-ribose) (32).

Moreover, the mitochondrion represents the main site for important redox reactions, including the TCA cycle and oxidative phosphorylation for ATP production. As well, it is also home to mitochondrial poly(ADP-ribose) metabolism and SIRT3-5 activities (247). These fundamental processes need to be maintained if possible even in the presence of NAD+ decline due to increased cellular ADP-ribosylation and niacin deficiency. NAD+ can be released from the mitochondria and into the cytosol and nucleus through specific permeability transition pores during conditions of apoptosis or necrosis (89,149). Therefore, high starting mitochondrial levels of NAD+, which are an order of a magnitude greater than cytosolic levels, are necessary to maintain optimal redox function.

On the other hand, the Golgi apparatus is involved in packaging and transfer of macronutrients to other organelles, and for clearance from the cell. It is likely that the Golgi apparatus may regulate NAD+ levels in other organelles, although this remains uncertain. NAD+ may be excreted from the Golgi apparatus and into the cytosol, or it may be released

in the extracellular space to act as a substrate for important ecto-mono(ADP-ribosyl)transferases and/or ADP-ribosylcyclases, which do not normally have access to significant amounts of NAD+ (31).

The effect of NAD+ precursors in the subcellular distribution remain uncertain and several questions remain unanswered. Will nuclear NAD+ be made more available following treatment with high levels of vitamin B3, since it has the greatest capacity to modulate poly(ADP)ribosylation and repair of DNA damage? Will there also be an increase in cytosolic NAD+, given that brain cyclic ADP-ribose levels can increase? What are the effects of high levels of vitamin B3 on the mitochondrial NAD+ pool? Interestingly, NAPRT, the enzyme responsible for the conversion of NA to NAD+ is found in the cytoplasm (142). Therefore, supplementation with high levels of NA may alter the subcellular contents of NAD+.

Modulation of NAD+ metabolism by caloric restriction

It is well established that caloric restriction (CR) represents the most efficacious intervention to promote longevity in several short-lived species including mice and rats, and maintain a healthy and average lifespan in primates. CR is defined as a 20% reduction in calorie intake compared to ad libitum feeding without incurring malnutrition or reduction in important vitamins and nutrients (222). Although the molecular basis of CR remains unclear, it is thought that CR regulates fat and carbohydrate metabolism, ameliorates oxidative stress and inflammation, activates a stress-induced hormetic response that down-regulates insulin and insulin-like signalling (ILS), amino signalling target of rapamycin (TOR)-S6 kinase pathway, and the glucose signalling Ras-protein kinase A (PKA) pathway (36). It is believed that regulation of macromolecule consumption is a direct response to reduced diet, while hormesis and down-regulation of TOR and PKA is most likely the molecular aspect of CR.

Several studies have examined the effect of CR in a variety of model organisms. In yeast, exposure to sublethal stress conditions increases expression of nicotinamidases, thus altering NAD+ metabolism and enhancing the activity of Sir2, a yeast homolog of mammalian SIRT2 (16). This is evidenced by repression of age-associated extrachromosomal ribosomal DNA circles (309). Down-regulation of TOR and PKA also mediates the beneficial effects of CR on lifespan as reported in cell survival studies (361). On the contrary, longevity in worms is

mediated by inactivation of ILS or Forkhead FoxO transcription factor daf-16 (25). While additional mechanisms may be attributed to CR in mammals, alterations in the NAD+ metabolome and increased sirtuin activity may play a prominent role in mediating health benefits reported in the brain and liver following a CR diet.

In rodents, brain total NAD levels were reportedly increased in CR-treated mice, while NAM levels decreased concurrently (265). These observations occurred in parallel to increased neuronal SIRT1 activity which lowered Alzheimer's associated-neuropathology. In another study, hepatic total NAD levels increased in fasted mice, and these changes were accompanied by increased SIRT1 activation, PGC1α deacetylation, and increased mitochondrial biogenesis (277) (Figure 11). Three mechanisms have been developed to explain these changes in the NAD+ metabolome following CR: (1) Increased systemic mobilisation of NAD+ precursors, NAM and NR, since increased L-tryptophan and NA availability is dependent on dietary availability; (2) Reduced NAD+ catabolism if major NAD-consuming enzymes such as PARPs and CD38 are negatively modulated by CR; and (3) CR-mediated negative regulation of the NR and/or NAR pathways may increase brain and hepatic NAD+ levels.

Information obtained from experimental small model organisms has provided insight into the molecular basis of CR and ageing. However, there are still discrepancies in studies using larger animals. CR has been investigated in rhesus monkeys, which are the closest experimental model organism to human in a controlled environment (226). One study conducted by the National Institute of Aging (NIA) reported no significant improvement in lifespan. However, a positive trend to slow-down the onset of age-related degenerative diseases was observed (227). In contrast, another study by the Wisconsin National Primate Research Center (WNPRC) showed significant improvements in both lifespan and health span (82,83). These discrepancies have been attributed to differences in dietary composition and heterogenic genetic backgrounds of the subjects (Figure 11).

Nevertheless, the beneficial effects of CR have been documented in the Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE) study conducted by the National Institute of Health (NIH). The study showed that a two-year 25% CR regimen provided significant health benefits in non-obese humans, including reduced inflammatory markers and cardiometabolic risk factors (280). However, given the results observed in

rhesus monkeys, longer studies with a larger sample size need to be conducted to validate the potential effects of CR on human lifespan and normal physiological function.

Beneficial effects of NAD+ precursors

NAD+ anabolism in mammalian cells is known to occur through two major pathways; the *de novo* and the salvage pathways. To determine nutritional and therapeutic benefits due to maintenance of NAD+ levels in tissues, organs and cells, supplementation with either NAD+ and its reduced form NADH, or its precursors represents a potential therapeutic strategy to slow down the ageing process and/or improve the management of age-related degenerative disease. Oral supplementation with NAD+ and NADH have not shown any significant elevation in plasma or tissue levels of NAD+, potentially due to inefficient metabolism of NAD+ through the gut, thus leading to poor bioavailability (177). Additionally, oral NADH may not be oxidised to NAD+ in the body, may not be efficiently absorbed by the gastrointestinal system, or may be converted to a product prior to absorption that cannot yield NAM (34,35). At present, intravenous infusion of NAD+ is the only recognised effective means of clinically increasing systemic NAD+ levels. However, it is anticipated that some of the alternative NAD+ precursors, including NA, NAM, NMN, NR and NAR (Figure 12) are likely to provide some benefits.

Nicotinic acid

NA represents the acid form of niacin. It is commonly prescribed clinically for the treatment of hyperlipidemia. It has been reported that daily intake of 1-3 grams reduces blood triglyceride levels and low-density lipoproteins (LDL), whilst increasing the level of high-density lipoprotein (HDL), thus favourably regulating the LDL:HDL ratio (133,343). Our research group was the first to show that exogenous NA efficiently increased intracellular NAD+ levels in brain cells (127). However, NA therapy induces significant skin flushing in a majority of individuals, thus limiting its clinical uses. A mild skin flush has been reported in patients exposed to 50 mg oral NA, and the upper tolerable limit for NA has been set to 35 mg per day for adults in USA and Canada (314). The lipid lowering effects of NA are thought to be mediated by binding of NA to the cell surface of a G-protein coupled receptor known as HM74A or GPR109A (314). This association in adipocytes suppresses triglyceride lipolysis, culminating in the reduction of circulating fatty acids, and reduced liver VLDL formation and circulating LDL-cholesterol (314) (Figure 13).

The uncomfortable side effect occurs because of an NA mediated stimulation of HM74A in some skin immune cells which results in the conversion of the omega-6 metabolite arachidonic acid (AA) into prostaglandin E2, stimulating vasodilation of skin capillaries, causing skin flush (314) (Figure 14). RUP25, a receptor that differs from HM74A by only one amino acid, has been identified. RUP25 has been shown to exhibit greater affinity to NA than HM74A, and has been associated with extreme skin flush reactions in some people (314). This often dramatic and unwelcome side effect has therefore restricted NA applications to essentially a treatment-resistant lipid lowering therapy (170).

In addition, a ketone body, beta-hydroxybutyrate is the natural ligand for HM74A, which is produced during fasting (314). While NA demonstrated a greater affinity to HM74A (100 nM required for half maximal) compared to beta-hydroxybutyrate (700 nM required for half maximal), endogenous NA levels do not reach the concentrations required to activate this receptor, whilst ketone bodies circulate at the required levels (314). However, other mechanisms have been prescribed to account for the effects of NA on dyslipidemia. These include but are not limited to inhibition of liver diacylglycerol acetyltransferase, inhibition of pathways associated with the clearance of HDL in the liver (74,345), and activation of PPAR-mediated cholesterol transport from extrahepatic tissue (84,169,171). More recently, SIRT1 has been shown to be a positive regulator of the liver X receptor (LXR). SIRT1-mediated deacetylation of LXR at conserved lysine residues can lead to activation of LXR, which regulates cholesterol levels, HDL biogenesis, and lipid homeostasis (199).

Likewise, elevated levels of NA have been shown to improve genomic integrity by reducing micronucleus frequency, and NA deficiency results in chromosomal instability (178-181). Treatment with NA has been reported to delay carcinogenesis, enhance repair efficiency following γ- and X-irradiation in mouse melanoma cells and human PBMCs, and improved neuronal function following hypoxic insult (244,362). NA has also been shown to enhance endothelial protection by increasing endothelial levels of NADP+ and glutathione (GSH) (114). However, these studies were performed using concentrations ranging between 250-1000 μM in the culture medium, which is beyond the physiological concentrations in humans.

Nicotinamide

NAM, the amide form of vitamin B3 is also generated as a by-product of SIRT-mediated deacetylation, PARP-mediated ADP-ribosylation and CD38 NADase and ADP-ribosylation activities which can be converted back to NAD+ via the salvage pathway. High levels of NAM have been used to enhance radiotherapy or chemosensitise solid tumours by promoting microvascular flow inside the tumour (3,4). The clinical regimen describes oral doses (3-6 g) aimed at increasing systemic blood levels to 700 μM or higher, combined with inhalation of 95 % oxygen/5 % carbon dioxide (358,359). This leads to improved tumour blood flow and oxygen generation, thus enhancing the effect of radiation by inhibiting myosin light chain kinase (MLCK). Decreased phosphorylation of MLCK disrupts vascular smooth muscle contraction, promoting vasodilation (283). However, the concentrations used *in vitro* are an order of magnitude higher than clinical systemic levels, and the macrovascular effects appear to be independent of its effects on NAD+ production.

NAM has also been shown to prevent or slow down the progression of several types of diabetes in animal models, although this effect was not reproducible using gram amounts of NAM in a randomised control trial (10,113,215,323,371,386,387). NAM has also been used as a potential therapeutic strategy to limit vascular injury and ischemia to the brain and other tissue in response hypoxic and/or chemotoxic insult in several animal models with some success (91,301,302,334,352).

Topical NAM formulations have also been successfully used for the treatment of inflammatory skin conditions, including rosacea, autoimmune bullous dermatoses, and acne (251). NAM has also been previously used for the maintenance of skin integrity, lowering sebum levels, and reducing hyperpigmentation spots and redness (328). NAM has also been shown to reduce acute and chronic effects of UV-induced skin damage by preventing the expression the inflammatory mediators IL-6 and TNFα, and the DNA damage markers cyclobutane pyrimidine dimers and 8-oxo-7,8-dihydro-2-deoxyguanosine (234). As well, NAM has been shown to improve UV-induced immunosuppression and photocarcinogenesis in rodent models and human studies (115). Similarly, human clinical studies have shown that oral NAM can significantly reduce actinic keratosis compared to a placebo, and may likely to also be useful for the prevention of non-melanoma skin cancer (321).

However, it is well established that as a by-product of NAD+ catabolism, NAM also serves as a natural feedback inhibitor for NAD-dependent enzymes (Figure 15). For example, PARP, sirtuin and CD38 activities are proportionately inhibited as NAM concentrations increase, and this has been postulated as the mechanism for the anti-diabetic effects of NAM in humans. While NAD+ levels are still elevated, the important NAD-dependent functions (e.g. SIRT1 activity) are inhibited. Moreover, NAM supplementation worsened liver fat accumulation in a choline deficient rat model, and this effect was attributed to the accumulation of poly(ADP-ribose), and a reduction in epigenetic methylation due to the use of methyl groups in NAM excretion (19,172). Therefore, though exogenous NAM can be converted to NAD+ it is again not considered an ideal supplement, particularly in the medium to longer term due to its enzyme inhibiting and methyl depleting potential.

Nicotinamide mononucleotide

NMN is an important precursor for NAD+ synthesis from NAM. Supplementation with NMN has been shown to have a positive effect on insulin levels most likely through action on pancreatic β cells (316). NMN supplementation has also been shown to reduce obesity and vascular damage in several *in vitro* and *in vivo* models (87,355). NMN has also been reported to improve CNS function by increasing brain mitochondrial respiratory deficits, protecting against amyloid-beta (Aβ) oligomer-induced toxicity and cognitive impairment, and ameliorate reactive glial-induced motor neuron loss, and maintenance of neural stem and progenitor cells (356). It has also been shown that NMN can protect against cerebral ischemia-induced apoptosis and enhances neurogenesis following cerebral injury (360). NMN treatment also up-regulated Nrf2 and HP-1 protein expression and promoted Nrf2 nuclear translocation for its transactivation following post-ischemic neuroinflammation, to attenuate secondary neurological injury (360). Similarly, overexpression of NAMPT, the enzyme required for NMN anabolism, appears neuroprotective in stroke (353,354).

A recent study showed that NMN treatment significantly improved major pathological hallmarks of Alzheimer's disease (AD) in APPswe/PS1dE9 AD transgenic (tg) mice, including cognitive impairment, neuroinflammation, Aβ deposition, and synaptic loss (372). Another study also found that NMN treatment inhibited JNK activation and APP-mediated amyloidogenic processing by APP-cleavage secretase in AD-tg mice (349,356). Accordingly,

it is likely that NMN may represent a new therapeutic target for the treatment and management of AD and other age-related degenerative diseases.

In NDUSF4KO mice – a mouse model for cardiac-specific complex I deficiency – mice exhibited a reduced NAD+/NADH ratio, hyperacetylation of mitochondrial protein including the mitochondrial permeability transition pore (mPTP), impaired oxidative phosphorylation and increased vulnerability to cardiac stress (174). Treatment with NMN was able to partially restore the intracellular levels of NAD+ and attenuated the hyperphosphorylation of mPTP and improved heart failure following exposure to chronic stress. Moreover, these mice also exhibited increased activation of SIRT3, and its deacetylation of key protein including mPTP due to enhanced activity following increased NAD+ levels following NMN treatment may also explain the beneficial effects of NAD+ in NDUFS4KO (174).

Despite these reported benefits of NMN treatment, evidence also suggests that as NMN is effectively contained within the cells membranes and is not subject to high diffusion gradients. This has raised the question of whether NMN is able to effectively traffic across most cells. Interestingly, extracellular NMN may be actively produced from the direct metabolism of exogenous NAD+ (388). However, further work is required to establish the range of conditions for which NMN may prove beneficial in humans.

Nicotinamide riboside

NR is a naturally occurring precursor of NAD+ originally isolated from fresh milk (338). Exogenous treatment with NR has been shown to increase intracellular NAD+ levels in a variety of cell lines. Supplementation with NR protected murine dorsal root ganglion neurons from axonopathy via a mechanism involving the transcriptional induction of NRK2 gene (288). As this effect is not reproducible by NA or NAM, NR represents a major precursor in the CNS when the *de novo* synthesis of NAD+ by the kynurenine pathway is impaired. NR has been shown to efficiently increase NAD+ levels without causing any adverse skin flushing in contrast to NA, or liver damage in contrast NAM (337). NR has also been shown to serve as a cholesterol-lowering agent in obese mice (67). Recent studies have shown that NR is the mitochondrially favoured NAD+ precursor , and the beneficial *in vivo* effects of NR have been attributed to modulation of mitochondrial sirtuin activities, as well as nucleocytosolic targets including PARPs, sirtuins, CD38, NAD-dependent oxidoreductases, and NADPH-dependent ROS detoxification enzymes . Supplementation with NR has also been shown to reduce the acetylation state of several protein targets of SIRT3, including

SOD2 and NADH ubiquinone oxidoreductase subunit A9, suggesting that NR may be used to pharmacologically activate SIRT3 (67). Moreover, administration of NR slowed down neurite degeneration after noise exposure by the NAD+-SIRT3 pathway (56).

As yet, the effect on metabolic health of NR as an exclusive source of niacin remains unclear. Supplementation of dietary NR in mice overexpressing the putative human oncogene, unconventional prefoldin RBP5 interactor (URI) reduced dysplastic lesions and prevented tumour development, thus providing evidence for NAD+ supplementation as a novel approach for the treatment and management of hepatocellular carcinoma (340). Overexpression of human URI drives the development of dysplasia in hepatocytes via mechanisms involving the aryl hydrocarbon receptor (AhR) and oestrogen receptor (OR), and impaired kynurenine pathway metabolism (340). End-stage tumours in hURI-overexpressing mice regressed with increased apoptosis in mice supplemented with NR (320,340). Despite the beneficial effects of NR supplementation, the doses often used to produce beneficial effects are remarkably high (400 mg NR/kg body weight/day) compared to current commercially available supplements (6-500 mg/ kg body weight/day). One study showed that NR (300 mg/kg body weight/day) reduced exercise performance in rats, and previously reported ergogenic effects of NR could not be confirmed (186). Two hypotheses have been postulated to explain this observation: (1) Based on similar effects of NA and NAM, it is likely that NR may also reduce fatty acid oxidation during exercise, leading to earlier fatigue; and/or (2) NR may also alter the redox properties of NAD+ and NADP+, leading to a non-optimal reductive state (186). Additional studies are warranted to examine the effects of NR on exercise performance.

Recently, the effect of a wide range of dietary NR concentrations on metabolic flexibility and gene expression in epididymal white adipose tissue was examined in mice exposed to a mildly obesogenic (40 % fat) diet (303). The study showed that 30 mg NR per kg diet was most beneficial for improving metabolic health, with regards to metabolic flexibility and increased expression of PPAR-γ, a master regulator of adipogenesis, and SOD2 and PRDX3, two antioxidant genes (303). The study concluded that 30 mg NR/kg diet represented the optimal concentration to potentiate metabolic health.

Nicotinic acid riboside

The least examined of the NAD+ precursors, NAR has been shown to be produced in human cells through NMN and NAMN dephosphorylation by cytosolic 5'-NTs (38,190). It is anticipated that this metabolite will represent an important precursor for NAD+ generation. Low micromolar concentrations of NAR have already been demonstrated to produce sufficient amounts of NAD+ to maintain cell viability. One study showed that NAR can be produced and delivered by cells at physiologically sufficient levels (190). It is likely that other cell types may use NAR and transport it between each other.

Pharmacokinetics of NAD+ precursors

Boosting the NAD+ pool by utilising precursor molecules may have multiple health benefits and a diverse range of therapeutic implications. NA, NAM, NMN and NR have been publicised as potent NAD+ boosters. NMN and NR may also be used as a general supplement in patients who have adverse responses to NA and NAM. The pharmacokinetics of NA and NAM has been extensively investigated, while the pharmacokinetic properties of NR have only recently been determined in mice and a middle-aged human subject (337). However, pharmacokinetics effects of NMN and more so, NAR have not yet been fully investigated in either the human or murine models.

Nicotinic acid

The pharmacokinetics of NA have been previously examined using pharmacological doses of NA and several extended release formulations. An open-label, dose-rate escalation, crossover study administered 12 human subjects with 2000 mg NA in solution form in slow (25 mg niacin aqueous solution administered every 10 minutes for 80 doses), intermediate (50 mg niacin aqueous solution administered every 10 minutes for 40 doses), or fast (100 mg niacin aqueous solution administered every 10 minutes for 20 doses) (232). Peak NA levels varied between 10 μM (slow release) to 240 μM (fast release). Interestingly, the area under the curve (AUC) for the slow release formulation was 25-fold lower than the fast release counterpart. NA in the slow release preparation is taken up by the intestine and liver, forming NAD+, and the released into the circulation as NAM (232). Importantly, a concentration of 10 μM is estimated to be about 30-fold higher than physiological levels of NAM in the blood. Fast release formulation of NA not only yields higher peak levels and AUC, it also elevates peak NAM levels to 16 μM. NA is preferentially removed from circulation at high levels with a half-life of 1 h, compared to a half-life of 4 h for NAM (231,232). Therefore, high doses of

NA also elevated the levels of NAM to supra-physiological levels. Therefore, the effects of NA on lipids may also be due to the protective effects of NAM and increased NAD+ anabolism.

In a recently study comparing the efficiency of NAD+ precursors to generate NAD+ in mice following oral gavage, NA produced the lowest levels of NAD+ (337). However, the kinetics of hepatic NAD+ accumulation was 4-6 h faster than either NAM or NR. Oral administration of NA doubled hepatic NAD+ (from 1 to 2 mM) by increasing the level of NAAD (an intermediate), and enhanced NAD+ catabolism as reported by increased levels of MeNAM (337). The liver promoted NAD+ anabolism as long as enough NA is available, while increasing the activity of NAD-dependent processes, some of which generate NAM as a by-product (337). Increased NNMT expression due to increased levels of MeNAM stabilises hepatic SIRT1 protein and regulates lipid levels in mice and humans (152,194,336).

Another study reported significant changes in the levels of NAD+ following oral supplementation over a 2-3 week feeding interval (26). It was previously thought that NAD+ levels will continue to increase in response to time before reaching a plateau. In rats supplemented with NA (30 and 4000 mg/kg), bone marrow NAD+ significantly increased in animal fed with 4000 mg/kg (26). However, NAD+ levels were down-regulated as consumption became chronic, and it was unclear whether this effect was due to either NA uptake alone, associated with the conversion of NA to NAM, or altered NAD+ catabolism in bone marrow. Therefore, it has been postulated that pharmacological responses to long-term supplementation with NAD+ precursors may change over time (26). This also raises the important question of whether higher NAD+ level have the potential to induce a deleterious impact on cellular function, thus stimulating an adaptive response.

Nicotinamide

The pharmacokinetics of 3-6 g of oral NAM in humans has been previously investigated (93). Higher doses are prone to produce adverse reactions including nausea and vomiting. The peak blood levels of NAM were between 1-2 mM. This is estimated to be more than 3000-fold higher than circulating levels (93). This figure is also well above the minimum concentration associated with radiation sensitivity. The half-life of NAM is 4-5 h, which provides sufficient time to facilitate carbogen breathing and radiation therapy. It has been suggested that if MLCK represents the likely target of NAM, then it is required in order to

accumulate inhibitory concentrations inside a cell. While intracellular transporters for NAM have been previously identified, mechanisms for cellular responses to high concentrations of NAM remain unclear. One study showed that radiation sensitivity remained after a decline in circulating levels of NAM, suggesting that the beneficial effects of NAM may be related to additional downstream effects, including increased NAD+ generation (279), and/or inhibition of poly(ADP-ribose) and DNA damage-induced apoptosis (153).

While high doses of NA have been shown to increase NAM levels, the effect of increased NAM on NA levels remains unclear. At present, enzymatic conversion of NAM to NA has not been identified. However, the deamidation of NAM via oral and intestinal microflora is possible (212). One study showed that significant amounts of salivary NAM were converted to NA, although circulating levels of NA following large oral dosing of NAM remained undetected (317). It is likely that the quantification methods used in this study were not sensitive enough to clearly delineate changes in the NAD+ metabolome. Skin flushing, a common adverse effect following NA treatment, has also been reported with NAM, suggesting that NA may be increased following administration of high doses of NAM (167).

A recent study comparing three NAD+ precursor vitamins provided in bolus at equivalent oral doses also demonstrated increased hepatic NMN, NAAD, NAD+ and NADP+ levels, and NAD+ catabolic activity as evidenced by elevations in MeNAM and ADP-ribose (337). However, while the AUC of increased NAD+ due to NAM showed a 50% benefit compared to NA, the study demonstrated a 50% deficit in NAM-mediated accumulation of ADP-ribose compared to NA (337). These studies also suggest that NAAD may represent a biomarker for increased NAD+ synthesis, and is independent of traditional NAAD precursors such as L-tryptophan and NA (337). Unlike NA, NAM is not a potent cholesterol-lowering agent, and high levels of NAM may inhibit PARP and sirtuin activities.

Evidence for potential adaptive responses following high doses of NAM has been previously reported. Rats supplemented with high doses of NAM (4g/kg) exhibited elevated levels of NAD+ and ADP-ribose in the brain. Interestingly, these changes were accompanied by impaired cognition as reported by impaired performance in a hippocampal-dependent spatial learning test (197). Increased NAD+ can ultimately lead to increased activity of a diverse range of enzymes including PARPs, sirtuins and CD38/CD157. This in turn may have dynamic effects in cellular function.

Nicotinamide mononucleotide

The detection of NMN in blood remains challenging. While the concentration of NMN has been reported to be around 50 μM is plasma (275), NMN levels were undetectable in another study (272). These differences can be attributed to different detection techniques for NMN. For instance, using an HPLC-based method, intracellular concentrations of NMN and NAD+ were reported to increase up to 500 pmol/mg and 50 pmol/mg of white adipose and pancreatic tissue 15 min after intraperitoneal injection of 500 mg/kg of NMN (376). However, hepatic NMN and NAD+ levels were reported to reach 10 and 4000 pmol/mg respectively, 6 h after oral gavage of 185 mg/kg (337). Similarly, the level of NMN has been reported to be around 1.5 pmol/mg tissue in tumours, and 80 nM in ascite fluid (310).

NMN appears to be stable in plasma and cell media supplemented with 10 % foetal bovine serum (FBS), and no increases in NAM levels were reported after 1 h incubation (272). However, NMN injections led to significant increases in NAM levels in plasma, which suggests that NMN may be partially converted to NAM following intraperitoneal injection. The presence of NAM in mice plasma following NMN injection suggests that NMN may be initially converted to NR (272).

A recent study demonstrated that dephosphorylation of NMN into NR, which is required to produce NAD+ in yeast, represents a major step as an exogenous NAD+ precursor in mammalian cells (272). It is thought that the extracellular receptor CD73 may act as an NR-releasing enzyme. CD73 has both pyrophosphatase and 5'NT activity which facilitates the conversion of extracellular NAD+ and NMN to NR, which in turn can be used to stimulate further NAD+ synthesis. This is supported by another study which showed that gene silencing of CD73 inhibits the use of NMN as a potential NAD+ precursor (310). Additionally, NRK1 has recently been identified as an important rate-limiting enzyme for the conversion of NMN to NR for NAD+ synthesis (272), and provides a reliable explanation to account for overlapping effects reported for NMN and NR.

Nicotinamide riboside

Evaluating the pharmacokinetics of NR in mammalian tissue has been limited by poor sensitivity and detection of NR in biological samples. As well, results of a clinical trial aimed at investigating the pharmacokinetics of NR in healthy human subjects are not yet available.

However, NR was degraded rapidly after incubation in murine plasma (~10% NR was degraded after 10 min, and 66% was degraded after 1 h), leading to comparable increases in NAM. These results hint at the presence of plasma factors that can degrade NR to NAM (272). NR degradation followed by detection of NAM has also been observed in cell media containing 10 % FBS (272). Importantly, NR is stable in protein factions in milk with a potential lifetime of one week (338). NR may also be circulated in a cell-bound form for several hours.

One study showed that NR is safe and orally bioavailable in mice and humans with no adverse effects reported (337). However, a future study will need to incorporate a validated flushing symptom questionnaire to assess whether NR may be associated with any flushing episodes. Higher levels of NMN, NAMN, NAM, NAAD, NAD+ and NADP+ were produced following oral gavage of NR compared to oral NAM (337). In addition, ADP-ribose levels were more significantly elevated compared to NA and NAM, suggesting that NR can enhance the activity of NAD+ consuming enzymes more than mole-equivalent doses of NAM and NA (337). More recently, a randomised double-blinded placebo-controlled study showed that NR in combination with pterostilbene, a naturally occurring phytochemical found in blueberries, can increase NAD+ in a dose-dependent manner in whole blood lysates throughout the entire 8 week trial (88). Taken together, this suggests that NR is a more potent precursor of NAD+ synthesis and NAD-dependent activities than amidated and acidic forms of niacin.

Nicotinic acid riboside

To our knowledge, the pharmacokinetic properties of NAR have not been reported in the literature. Understanding the pharmacology of NAR has remained difficult due to its low physiological sub-micromolar concentration. The ^{1}H-NMR method has shown some success in detecting NAR in cell culture medium because of the observed chemical shifts (190). Also, low sensitivity of detection methodologies requires acquisition of spectra over extensive periods of time. Overexpression of NAPRT in HEK293 cells led to the detection of NAR in the cell culture medium, and this affect is due to increased NA catabolism (190). In HeLa cells, NAR but not NR, is released in amounts that are sufficient to maintain NAD+ biosynthesis and cell survival (190).

Nicotinic acid adenine dinucleotide – a biomarker of elevated NAD+ metabolism

As mentioned above, NA, NAM and NR have been shown to increase the levels of the intermediate, NAAD. However, the increase in the levels of NAAD was lowest following ingestion of NA (337). This finding suggests that increased NAD+ anabolism by supplementation with NAD+ precursors not only increases the accumulation of by-products of NAD+ catabolism (such as ADP-ribose and MeNAM), but also stimulates retrograde synthesis of NAAD and NAMN. As the rate of NAD+ anabolism increases, NAD+ is deamidated to form NAAD by a yet unknown mechanism. Similarly, it is also possible that deamidation of NMN can lead to increased levels of NAMN and NAAD. While the deamidation reaction is yet to be verified, it is possible that NAAD may also be formed from NAADP, although the mechanism responsible for this elusive biochemical reaction is yet to be identified.

Effects of NAD+ precursors on NAD-dependent processes

Apart from the beneficial effects of NAD+ precursors on normal cellular function, including NAD+ and NADP-dependent reactions and ADP-ribosylation, these substrates for NAD+ anabolism share a common effect of increasing intracellular NAD+ levels in multiple cellular compartments. It has been considered that normal metabolic processes may be fulfilled following recommended daily intake of vitamin B3, and that higher doses may induce alternate mechanisms. However, it is now clear that physiological roles of NAD+ precursors may be different than the doses present naturally in the diet.

Nicotinamide and PARPs

Whether NAM can inhibit PARP-1 activity remains controversial. The Ki value for NAM-mediated inhibition of PARP-1 ranges between 30-200 μM in a cell-free system. The Ki value in cultured cells was 3-fold greater than in a cell-free system (271). This suggests that the uptake and/or conversion of NAM to NAD+ may be limited in cell cultures. In mammals, oral NAM is metabolised by the small intestine and liver before it enters the blood stream. NAM is taken up by extrahepatic tissue in small amounts where it is immediately converted to NAD+. Moreover, blood volumes are significantly lower than tissue volumes. Therefore, it is less likely that tissue NAM levels will reach the concentrations needed to inhibit PARP-1 activity.

On the other hand, the conversion of NAM to NAD+ due to increased substrate has been shown to promote poly(ADP-ribose) levels. For instance, supplementation with NAM (1 g/kg) increased hepatic NAD+ levels by 50%, while basal poly(ADP-ribose) levels increased by 2 fold (163). This suggests that NAM was more effective at enhancing substrate pools than mediating PARP-1 inhibition. However, poly(ADP-ribose) content was the same when the same animals were exposed to a hepato-carcinogen to enhance PARP-1 activity. NA also promoted higher levels of poly(ADP-ribose) formation (163). Taken together, it is likely that basal PARP-1 activity may be regulated differently than DNA-damage induced PARP-1 activity, and NAM may be more effective at inhibiting the latter form of PARP-1 activity.

In another study, increased PARP-1 activity was reported in extrahepatic tissue in response to oral dosing of NAM (4 g/kg). In that study, bone marrow NAD+ levels increased by 2.5 fold, basal poly(ADP-ribose) levels increased by 5-fold, while DNA-damage-induced poly(ADP-ribose) increased by 2-fold (42,43). Similarly, studies in radiation sensitisation models showed that radiation sensitivity due to NAM was due to mechanism(s) independent of inhibition of PARP activity and DNA repair processes (279).

Nicotinamide and sirtuins

Mammalian sirtuins have developed low NAD+ binding affinities which ensured that their deacetylase activities can be efficiently regulated by minor changes in the intracellular concentrations of NAD+, thus serving as potent NAD+ sensors. Reduced intracellular levels of NAD+ during ageing can downregulate sirtuin activity and SIRT1-mediated deacetylation of p53 (51). On the other hand, increased intracellular NAD^+ levels. either due to CR or NAD^+ supplementation. can upregulate sirtuin activity. While resveratrol, a plant-derived stilbene putatively allosterically activates SIRT1 only, NAD^+ supplementation can activate almost all seven forms of mammalian sirtuins. For example, regulation of SIRT3 by intracellular NAD+ levels has been demonstrated to be the major determinant of cellular resilience against apoptosis (143).

If the increase in NAM in biological systems is capable of inhibiting PARP-1 activity, then it may also inhibit other NAD+ dependent processes such as sirtuins. High levels of NAM may inhibit NAM cleavage reactions or mediate competitive inhibition at NAD+-binding sites leading to altered function or sirtuin enzymes, to ultimately enhance the levels of NAD+.

Physiological levels of NAM are within the same range as the IC50 of several sirtuins (159), therefore suggesting that sirtuins may act as NAM sensors as well as NAD+ sensors .

NAM has been shown to bind to a specific conserved region in the catalytic site of sirtuins, inducing a reverse base-exchange reaction with an intermediate, rather than deacetylation, thus inhibiting sirtuin deacetylase activity (129). The base-exchange equilibrium constant has been estimated to be about 20 for SIRT1(37). This means that the maximum possible activation of SIRT1 by full inhibition of the base exchange reaction at any NAM concentration is greater than for Sir2 in yeast. Recently, isonicotinamide (isoNAM), a synthetic analogue of NAM has been shown to compete with NAM for binding at the catalytic site (228). However, unlike NAM, isoNAM does not substantially react with the intermediate, leading to increased Sir2 activity.

NAM represents a physiological inhibitor of sirtuins. The IC_{50} values for inhibition of bacterial Sir2, yeast Sir2, mouse Sir2, SIRT1, SIRT2, SIRT3, and SIRT5 were measured to be 26, 120, 160, 50, 100, 36.7 μM, and 1.6 mM respectively (129). The concentration of NAM in yeast nuclei has been estimated to be 10–150 μM, which suggests that NAM is a regulator of Sir2 activity *in vivo (292)*. Yeast and bacterial sirtuins have lower Km's and Kd's for NAD+ compared to mammalian sirtuins, and therefore may be less sensitive to changes in intracellular NAD+ concentrations than their mammalian counterpart (68). Therefore, increased NAD+ levels are more likely to result in activation of mammalian sirtuins. In mammalian cells, low levels of NAM have been reported in several rat tissues (142). This is likely due to rapid catabolism of NAM for the production of NAD^+ and related pyridine nucleotides. However, high concentrations of NAM (up to 300 μM) have been reported in the brain of Tg2576 mice (265), providing additional evidence for NAM as a regulator of sirtuin activity in mammalian tissue. Moreover, the ratio of NAD^+ to NAM in subcellular sub-compartments can decline with age, therefore lowering sirtuin activities (223). This effect may be due to increased utilisation of NAD^+ by PARPs and reduced NAD+ anabolism from NAM by NAMPT-mediated salvage pathway (160).

Depending on their physiological roles, several other mechanisms have been attributed to account for the regulation of sirtuins by NAM. For instance, NAM can only partially inhibit Sir2AF2 (SIRT2 homolog from Archaeon Archaeglobus fulgidus), whereas it is a full inhibitor of SIRT1 (129). As well, NAM is a competitive inhibitor of SIRT3, in contrast to

non-competitive inhibition reported for other sirtuins (129). Mammalian SIRT3 is a mitochondrial sirtuin that has demonstrated tumour suppressive effects and regulates glycolytic metabolism (18). Inhibition of SIRT3 by NAM may increase glycolytic metabolism and inhibit aerobic glycolysis and thus reducing cancer cell biomass, and improving chronic tissue damage.

Apart from base exchange, which is known to increase the rate of forward reaction for sirtuin activity, direct competition has been postulated as another mechanism to explain the greater degree of competition between NAM and NAD^+ in the inhibition kinetics of SIRT3 compared to that of SIRT1 (129). This involves NAD+ binding to the catalytic site in the presence of NAM (129). Elucidating the mechanisms of action of NAM against SIRT3 and other sirtuins can help to develop more efficient inhibitors and activators of sirtuins that can be translated to the clinic.

CD38-mediated processes

It has been proposed by our group and others that CD38 can regulate SIRT1 activity by modulating the availability of the essential substrate NAD+, and NAM to the SIRT1 enzyme (52). This can have a profound effect on modulating obesity, metabolic disorders, cellular energy homeostasis and cellular senescence, and ageing. By promoting intracellular NAD+ anabolism whilst reducing NAM levels, inhibition of CD38 can increase SIRT1 activity.

Several CD38 inhibitors have been identified. These include NAM and NA, NAD+ analogs such as arabiono-NAD, and reducing agents including dithiortheitol (76). CD38, due to its effect on calcium generation, also serves as an important mediator of smooth muscle contraction, cell death, and apoptosis, neural and hormonal signalling, and egg fertilisation (76). Therefore, CD38 inhibition may be useful under pathological conditions where calcium homeostasis is impaired, including hypertension, cardiac ischemia, asthma and dysfunctional labour.

However, CD38 is also involved in the release of hormones such as oxytocin and ACTH which regulate maternal and social behaviour (166). Inhibition of CD38 in these conditions may have significant negative effects on psychological function. In addition, CD38 plays an important role in the immune system, and knock-out of CD38 has been shown to increase susceptibility to bacterial infection (201). Therefore, the effect of NAD+ precursors and

changes to the NAD+ metabolome may have previously unknown effects of CD38 activity and NAD-dependent processes, and may serve as important therapeutic strategies for the treatment of metabolic and inflammatory conditions if appropriate dosage regimens are devised and adapted to meet individual patient requirements.

Redox reactions

In eukaryotic cells, the generation of ATP is achieved predominantly by mitochondrial oxidative phosphorylation. In this process, free energy released following the break-down of carbon substrates is captured by exchanges between electron donors and electron acceptors via the electron transport chain (ETC) leading to ATP production (308). NAD+ serves as an electron acceptor, and its reduction leads to the generation of NADH, which can be subsequently oxidised by complex I of the ETC to produce NAD+. The NAD+/NADH ratio serves as an important indicator of several oxidoreductase enzymes. Elevated levels of NADH can inhibit NAD-dependent processes. A metabolic imbalance in oxidative phosphorylation has been associated with several cardiac, neurological and renal pathologies (206). Alterations to the ETC can lead to a significant decline in ATP production, increased intracellular Ca^{2+} influx and free radical production, and lowered NAD+/NADH ratio. A switch between oxidative to anaerobic metabolism in response to several cardiac stressors has been shown to reduce oxidative damage and maintain ATP levels. However, this compensatory mechanism impairs oxidative phosphorylation whilst limiting the mitochondrial NAD+ pool (97).

Similarly, the NAD+/NADH ratio appears to play a crucial role in the heart and kidney and supplementation with NAD+ precursors has been shown to protect against impairments in oxidative phosphorylation due to cardiac stressors and AKI-induced renal damage (150). In PGC1α-deficient mice, treatment with NAM increased fatty acid oxidation, ATP generation and the NAD+/NADH ratio to protect against AKI toxicity (339). Therefore, under degenerative conditions associated with impaired oxidative phosphorylation, or other abnormality leading to a decline in NAD+, upregulation of NAD+ anabolism through NAD+ precursors may improve redox function.

While human *in vivo* studies regarding the effect of NAD+/NADH ratio remain nascent. However, using two-photon microscopy for the quantification of NADH and NADPH in epidermal skin layers, one study reported a significant increase in NADH fluorescence

following arterial occlusion, suggesting that there is a reduction in oxidative phosphorylation due to a decline in the need for electron donation for the oxidation of NADH to NAD+ (24). Similarly, reduced NADPH fluorescence emission has been reported in the facial skin of older aged females compared to younger subjects (286). Taken together, these studies provide supportive evidence for the role of NAD+ in regulating cellular bioenergetics.

Impaired poly(ADP)ribosylation has been associated with increased sensitivity to DNA damage underlying skin lesions reported in the human disease of niacin deficiency better known as pellagra (274). Additionally, impairment in the formation of cyclic ADP-ribose, which regulates intracellular calcium levels, may contribute to neuronal loss observed in pellagrous dementia (378). However, redox reactions corresponding to the ratio of NAD+/NADH are less prone to be affected by altered NAD+ levels as ADP-ribosylation reactions. NAD+/NADP+ serve as soluble cofactors in a multitude of oxidation/reduction reactions. The catalytic enzymes utilise riboflavin-based nucleotides as a source of prosthetic groups. Others contain iron to facilitate electron transfer. Unlike poly- and mono(ADP-ribosyl)ation reactions, iron and riboflavin deficiencies are not known to induce sun sensitivity of the skin, and dementia, which are two main characteristics of pellagra .

One study investigated the effect of NADH, the reduced form of NAD+, on proliferation, cytokine release, and cell redox status of lymphocytes collected from healthy aged subjects (40). Cells exposed to NADH (500 µM/L) showed increased levels of glutathione (GSH), and catalase activities, while malondialdehyde and carbonyl proteins are markedly decreased (40), suggesting a decline in oxidative stress. Recently, it has been shown that treatment with 1 mM NADH increased the expression of nuclear Nrf2, catalase activity, and total GSH by increasing SIRT2 function (69).

As well, the effect of niacin deficiency on endogenous antioxidant defence mechanisms, NADPH:NADP+ and GSH:GSSG redox couples remains unclear. Two studies showed that niacin deficiency increased markers of oxidative stress, but did not induce either NADPH or GSH decline (27,325). This suggests that niacin deficiency impaired poly(ADP-ribose) accumulation but did not stimulate further tissue damage, whilst maintaining GSH defences. Several mechanisms have been postulated to account for the maintenance of redox reactions during periods of niacin deficiency. These include variations in substrate affinity for NAD+,

subcellular localisation of enzymes and cofactors, and direct modulation of enzyme activity/expression levels.

Does NAD+ and related precursors display hormesis

There is a growing body of evidence which suggests that NAD+ decline is a major contributor to the ageing process and may be involved in the pathogenesis of several age-related degenerative diseases affecting the heart, brain, liver, kidney and skin. These results collectively highlight the potential for NAD+ supplementation, whether using NAD+ alone or NAD+ precursors to protect against ageing and associated pathologies. While such prospects are of major clinical significance, the role of NAD+ and its modulation in human ageing remains only partially understood. In particular, little is understood regarding the impact of having 'very high' NAD+ levels. We suggest that modulation of NAD+ levels may induce a hormetic dose response that may confound numerous clinical outcomes.

The term hormesis was first incorporated into the biomedical context by Southam and Ehrlich in 1943 to account for the effects of Red cedar tree extracts on wood-rotting fungi (62). The study showed that various species of fungi exhibited low-dose stimulation and a high dose inhibitory effect on cellular metabolism. By the 21st century, hormesis is now used to define the biphasic dose response that occurs following exposure to a chemical or physical agent, or as an over-compensatory response to cytotoxic insult (63). Resveratrol, an activator of sirtuins has recently been shown to induce a hormetic dose response in a variety of biological models including breast, prostate, colon, lung, uterine and leukemia tumor cell lines (64). In these studies, lower concentrations of resveratrol enhanced tumor cell proliferation. However, at higher concentrations, resveratrol induced an inhibitory effect. For instance, resveratrol increased the activity of the vitamin D receptor and promoted proliferation of T47D breast cancer cells up to 4 μM, above which led to reduced proliferation of the tumor cell line (64).

Other studies have shown that resveratrol can protect cultured hippocampal neurons against oxidative stress at concentrations between 5-25 μM (108). Resveratrol could also ameliorate inflammation and oxidative stress in cultured tumor cells by inhibition of COX-2. (391) However, when these cells are under conditions of reduced oxygen and glucose availability, resveratrol can induce apoptotic cell death (64).

In light of these findings, it is likely that upregulation of NAD+ anabolism may also conform to a hormesis biphasic dose response. For example, where neuronal cells are exposed to cellular stress, as may occur due to ischemic insult, or cytotoxins such as glutamate, and Aβ aggregates, increasing NAD+ levels may provide both beneficial and deleterious effects which may be dependent on the dosage and duration of administration relative to the cytotoxic stimulant. For instance, *in vitro* incubation of naive T cells with NAD+ induced apoptosis, while activated T cells incubated with NAD+ showed no signs of apoptosis (204). It was suggested that ecto-NAD, as substrate of ADP ribosylation, acts on naive, but not on activated T cells (297). This indicates that many effects of NAD+ are dependent on environmental factors that would seem to produce a favourable response.

Competition between NAD+-consuming enzymes also displays hormesis. For example, as previously mentioned, PARP1 activity increases with age due to accumulation of oxidative DNA damage, and in response to high energy intake. Since the Km for NAD+ for PARP1 and SIRT1 is relatively similar, the decline in NAD+ levels following PARP1 activation can also induce a decline in SIRT1 activity. Therefore, while low levels of PARP1 activity can repair DNA damage following exposure to mild oxidative stress levels, increased PARP1 activity can lead to cell death via reduced SIRT1 activity and energy restriction, therefore exacerbating disease progression (269). Therefore, rigorous double blind and placebo controlled clinical trials are needed to assess the nature of the dose-response effect of NAD+ in humans. Further work will be required to gain further understanding of the role of NAD+ anabolism against ageing and age-related diseases.

Limitation of using *in vitro* and *in vivo* studies

Despite the importance of NAD+ metabolism to human health and diseases, determining the levels of NAD+ remains a challenge. As well, while there is clinical significance for supplementation with NAD+ precursor in the clinic, evidence showing increased NAD+ levels upon such supplementation is limited. While cell culture and animal models are commonly used in research studies, they are not a true representation of human physiology. Moreover, biochemical assays or analytical methods that are currently used to analyse tissue samples or cell homogenates are vulnerable to changes in pH, temperature, light, and chemical agent or buffer solution. Therefore, more accurate and reliable quantification and extrapolation to *in vivo* conditions are warranted.

Cell-culture systems

Accumulating evidence suggests the involvement of oxidative stress, inflammation and increased L-tryptophan catabolism in several degenerative disorders. This has paved the way for investigation of basic mechanisms of free-radical damage and modulation of the NAD+ metabolome as a therapeutic strategy to protect against it. For example, primary murine and human brain cell cultures, and immortalised cell lines remain highly useful as models for examining the effect of oxidative damage and adaptive cellular responses. The most common approach to modelling CNS oxidative stress and altered kynurenine pathway metabolism is through exposure of primary glial and neuronal cells to deleterious conditions, and addition of exogenous prooxidants and neuroprotective agents (44,46,48,50,71,195,333). Cell culture models have also been used to examine the effects of niacin deficiency, and inhibition of NAD-dependent processes (such as PARP, sirtuin and CD38 inhibition) on cellular function in several *in vitro* disease models (46,47,52,119,267). However, most cell culture components, which are fundamental to these studies, are aimed at maximising cell growth and survival in culture, and do not fully, recapitulate natural *in vivo* biological processes. There is also strong evidence that the beneficial effects of NAD+ precursors in culture systems may be incurred via non-physiological mechanisms.

Vitamin B3 is present in cell culture in both its amide and acidic form. However, NAM is present at highest concentrations, and this reflects the significance of NAM as the main form of niacin in the blood stream. Commonly used cell culture media (e.g. MEM, Williams, RPMI, BME, L-15, Dulbecco's) contain between 1-4 mg/L of NAM, although more specialised media (MCDB 131, BGjB) may contain between 6-20 mg/L. The equivalent molar concentration for 4 mg/L is approximately 33 μM. This amount is about 300-fold greater than the average levels of NAM in plasma. It is likely that these concentrations may have a profound effect on intracellular NAD+ storage, cyclic-ADP- and monoribosylation, and inhibitor studies. Other cell culture media contain equal contents of NAM and NA at concentrations of up to 4 μM. Similarly, the concentration is well above the physiological concentration of NA in systemic circulation. Moreover, these levels are significantly greater than the amount required to activate HM74A receptors if these are expressed in cells in culture.

In vivo models

The human life-span is much longer than smaller mammalian species, making it difficult to fully characterise the influence of NAD+ metabolism during normal human aging. Therefore, traditional *in vivo* studies have been performed using animals with phenotypically accelerated ageing or prolonged longevity, transgenic, mutant, and knockout models that focus on a single gene's role, to generate reproducible results. Due to their short lifespan, inbred laboratory rodents, particularly rats and mice (e.g. senescence accelerated mice – SAM), are used as models to investigate the effects of intrinsic and extrinsic factors on lifespan (324). However, this is quite limited since these inbred models do not provide significant genetic diversity to be comparable to humans and correlate poorly with human conditions. To date, more than 150 clinical trial candidates to attenuate inflammation in critically ill patients have failed due to over-reliance on inadequate animal models.

We have addressed the conceptual translation of biochemical data collected from aging female Wistar rats to further enlighten our understanding of the role of NAD+ metabolism and other molecular changes occurring as part of 'normal' human ageing (51-53). Our physiologically aged Wistar rats were an outbred model which displays significant genetic diversity within a small number of individuals. This diversity-outcrossed rat model is more representative of a natural population and is therefore a powerful tool in identifying the genetic basis for assessing the efficacy of these pharmacological strategies, and identify adverse effects in first-line therapeutic tests which are otherwise nascent in previously inbred animals. Additional effects of aging previously demonstrated in this animal model include a marked decrease in the astrocyte-neuronal ratio; altered pericyte-endothelial relations affecting 'vessel stability'; marked inflammatory cascade, CNS neovascularization and breakdown of the blood-retinal barrier, and a decline in defence-related Fos expression (158,218).

Methods of Detection

NAD+ and its related metabolites have been previously measured using a variety of methods. For instance, enzymatic and colorimetric assay which provide indirect measurements, have inherent difficulties that can affect reliability and are susceptible to significant variation in metabolite levels due to minor differences in temperature and pH, and cannot detect low picomolar levels. Moreover, reverse phase HPLC, which relies on mobile phases containing

buffer salts and ion pairing agents have been used to increase sensitivity, but are still limited to low micromolar detection levels (70).

In contrast, tandem LC-MS/MS allows more robust quantification of trace levels of NAD+ metabolites in different biological samples with high specificity and sensitivity. It represents the gold standard in NAD+ metabolomics. However, unlike Nuclear Magnetic Resonance (NMR), complex sampling processes are required (335). The diversity of NAD^+ metabolites (i.e. free bases, mono and di-nucleotides) makes their simultaneous differential analysis a major challenge (335). We recently developed an improved method to quantify the NAD+ metabolome and adenosine phosphates across biological samples, including brain and reproductive cells. Its principle features are enhanced resolution and simultaneous quantification of 17 analytes on an amino phase column, avoiding the need for two separate (i.e alkaline and acidic chromatographic gradients) (60).

Development of non-destructive detection and quantification of the NAD+ metabolome is desirable to elucidate intracellular NAD+ levels and redox state in the intact human and animal body. Recently, a novel magnetic resonance imaging (MRI)-based has been developed to determine the endogenous ^{31}P MR signals of the NAD+ molecules in live animal brains (389). This technique can resolve the MRI signal of NADH from that of NAD^+ by utilising specific spectroscopic characteristics at a given magnetic field strength. This approach requires ultrahigh fields of 9.4 and 16.4 T. This non-invasive technique has been further used to measure intracellular NAD^+ and NADH contents and NAD^+/NADH redox state in healthy human brains using a 7-T human MR scanner (389). We were the first to show that intracellular NAD^+ levels, which is the essential substrate for sirtuin activity, decline with age in humans and physiologically aged rats (51,52,223). MRI was used to reaffirm these age-dependent increase of intracellular NADH and age-dependent reductions in NAD+, total NAD contents, and NAD+/NADH redox potential of the healthy human brains.

It is anticipated that improvement in methods to quantify the NAD+ metabolome will be developed to help standardise NAD+ research across different laboratories, and to overcome challenges associated with translation of preclinical studies towards clinical practice.

Prospects of using NAD+ precursors in the clinic

Pellagra, a syndrome caused by a diet deficient in either NA or L-tryptophan can lead to

psychotic symptoms leading to pre-senile dementia likely due to upregulation of IDO, which can deplete neurons of the essential amino acid, L-tryptophan causing neurodegeneration. Administration of the NAD+ precursors, NA or NAM previously improved the neurological state of dementia patients in the 1930's. Pharmacological doses of either NA or NAM have also provided dramatic therapeutic benefits for other diseases, including, lipid dyshomeostasis, rheumatoid arthritis, type I diabetes, colitis, multiple sclerosis (MS), and schizophrenia in both animal models and in the clinical setting. Among these precursors, NA appears to specifically activate the G-protein coupled receptor, GPR109, leading to the release of the prostaglandins, PGE2 and PGD2 (314). These prostaglandins exert potent anti-inflammatory effects through endogenous signalling mechanisms. While NAM can prevent MS in animal models, it is also an inhibitor of sirtuins, and may therefore prove detrimental on long-term cell survival and longevity (258,259).

There is growing evidence suggesting that NAD+ administration may also reduce cellular injury in multiple oxidative stress induced degenerative diseases. NAD+ treatment has been shown to reduce PARP1-induced astrocyte death (7). PARP1 has been implicated in the pathogenesis of several diseases including diabetes, Alzheimer's disease (AD) and Parkinson's disease (PD) (196,221). Since supplementation with NAD+ can protect against PARP1 mediated cell death, NAD+ administration may improve cell viability in these diseases by at least partially ameliorating PARP1 toxicity. *In vitro* studies have shown that NAD+ remains protective even when administered at 3-4 hours following PARP1 activation, suggesting that NAD+ administration has a long window period for reducing cellular injury (8). In addition, NAD+ may also improve cell viability by enhancing sirtuin activities and/or improving energy metabolism.

While the potential involvement of NAD+ metabolic pathways in energy metabolism and mitochondrial function have been known for quite some time, suggestions of the involvement of NAD+ in DNA repair and longevity have grown at a rapid rate in the last decade. Characterisation of the NAD+ synthetic pathways has not only made these advancements possible, but also contributed extensively to the understanding of the diverse roles of pyridine nucleotides in cellular biology. Despite this, information regarding the fundamental roles of NAD+ in neurodegeneration and ageing remains limited. Further investigations are necessary in this increasingly relevant field.

While the current review herein focussed on PARP1 in cellular degeneration, the role of other PARPs such as tankyrases in cellular function remains largely unknown. Since NAD-dependent tankyrases are primary mediators of telomerase activity it is highly likely that NAD+ may also affect the aging process through regulation of tankyrase activity (385). It would therefore be intriguing to study the effects of NAD+ precursors on tankyrases and telomerases on certain biological functions, including neurogenesis, which might be relevant in the aging brain.

Additionally, NAD+ regulates diverse pathways which may control lifespan. The importance of NAD^+ is further underscored by recent work providing genetic evidence for the existence of several pathways necessary for NAD^+ synthesis. For example, the newly identified NAD^+ precursor, NR has been shown to contribute to NAD^+ synthesis by at least two unique pathways in the yeast *Saccharomyces cerevisiae*, and can upregulate intracellular NAD+ levels in mice and humans (338). Both pathways require the NAM ring for entry into the previously established pathways for NAD^+ synthesis. Future studies are required to address the importance of NR in human health and disease, and whether it can be effectively used to replenish lowered NAD^+ levels in age-related diseases, such as AD. Given the adverse effects associated with high-dose use of NA and NAM, NR may represent an alternative precursor to enhance NAD+ levels.

As well, changes in the NADH level, NAD+ redox potential and NAD+ levels are likely to be present in other pathological conditions and may be associated with disease progression. In particular, increased oxidative stress and immune activation in AD, PD, ADC and Amyotrophic lateral sclerosis (ALS) may influence the available concentration of these molecules. Future investigation into the metabolism and biological function of NAD+ in these and other degenerative diseases may expose fundamental properties that may involve the use of NAD+ precursors as adjunct therapy for treatment in these diseases, and perhaps may help in slowing down the age-related disease process.

Resveratrol is a polyphenol with major health benefits that is thought to operate through direct activation of the ‘anti-aging’ enzyme SIRT1. However, recent reports have challenged this ‘direct-activation’ hypothesis, suggesting that the mechanism by which resveratrol increases SIRT1 function is still unknown (39,86). Previous work from our group has shown for the first time that resveratrol induces a dose dependent increase in NMNAT-1 activity. As SIRT1 requires NAD+ as a substrate to perform its gene silencing function, higher NAD+

levels will enhance SIRT1 activity. This finding suggests that resveratrol may promote SIRT1 function by enhancing NAD+ synthesis in whole cell systems without requiring direct activation. Our observation that resveratrol increases NAD+ levels in primary human brain cells by acting on NMNAT, together with the neuroprotective effects of green tea polyphenols against QUIN-mediated excitotoxicity (47), supports the view that polyphenols have considerable therapeutic potential, particularly for the treatment of neurodegenerative diseases. As NMNAT can accelerate NAD+ synthesis from all six substrates, QUIN, NR, NMN, NA, NAR and NAM, NMNAT activation by resveratrol may represent an ideal natural therapeutic to replenish NAD+ levels. Maintenance of higher cellular NAD+ will enhance SIRT1 activity and other NAD+-dependent pathways, impacting positively on cell viability and longevity.

Finally, increased NAMPT has been reported in a mice model of collagen-induced arthritis both in the serum and in the arthritic paw (59). NAMPT inhibition reduced arthritic severity comparable to etanercept, and significantly lowered the levels of cytokine release in affected joints, and reduced intracellular NAD+ concentrations in inflammatory cells (59). Therefore, NAMPT may play an important role during inflammatory diseases associated by cytokine secretion from leukocytes. Therefore, increasing NAD+ levels may be deleterious in inflammatory conditions and may exacerbate the disease due to increased NAMPT activity and NAD+ use in immune cells. This represents an additional potential negative to 'one-size-fits-all' use of NAD+.

While most of the evidence reviewed in this paper strongly supports the current enthusiasm to investigate and develop strategies for increasing NAD+ levels in conditions where NAD+ turnover is high and/or concentrations are reduced, the use of NAD+ enhancing therapeutics in circumstances where cellular NAD+ levels are already adequate may be unwise. Given the complexity of the biochemical systems affected by NAD+ and its associated metabolites a simplistic, one size fits all, approach to NAD+ therapeutics will likely limit the true potential of NAD+ treatment and may in fact cause harm under some circumstances. As well, while several NAD+ precursors have been recently identified and examined in several models, a side-by-side comparison of these precursors is nascent in current literature. It is anticipated that these precursors may exhibit important differences in their effect in various pathological disorders (375).

To circumvent this, in addition to the many studies focussed on identifying efficient ways of increasing NAD+, additional effort must be applied to the development of cost-effective methods of measuring and correlating NAD+ levels in both tissue and extracellular fluids to cellular and organ health in an effort to establish a clear understanding of what a 'healthy' NAD+ level actually is. Armed with this knowledge the clinician may confidently apply NAD+ therapy after an appropriate assessment of NAD+ levels to determine whether the treatment is likely to be effective in each client's case.

Concluding remarks

NAD+ research has generated multiple discoveries in the last two decades. Identification of the important role of NAD+ as a cofactor in cellular respiration and energy production was followed by discoveries of numerous NAD+ biosynthesis pathways. In recent years, NAD+ has been shown to play a unique role in DNA repair and epigenetic control through protein deacetylation. Elucidation of the pivotal roles played by NAD+ in linking the key biochemical and cellular processes of oxidative stress and immune activation, energy metabolism, epigenetic control and cell viability in degenerative disorders and ageing will likely prove seminal to the advancement of effective therapeutics in degenerative diseases. NAD+ remains the central molecule in the metabolism and functions of NAD+, NADH, NADP+ and NADPH. Of these four molecules, only NAD+ can be synthesised *de novo* via the kynurenine pathway, while the generation of NADH, NADP+ and NADPH requires NAD+ as the original precursor. Maintenance of intracellular NAD+ levels is pivotal for the regulation of DNA repair, stress resistance, and cell death, suggesting that NAD+ synthesis through the kynurenine pathway and/or salvage pathway is an attractive target for therapeutic intervention in age associated degenerative disorders. Agents such as NR, and to a lesser degree, NA and NAM, have been shown to protect severed axons from degeneration in animal models for Wallerian degeneration, and extend lifespan in small organisms. However, further studies are necessary to clarify the conditions under which specific NAD+ precursors should be used to efficiently promote intracellular NAD+ anabolism. This involves evaluating the pharmacokinetics, safety, and efficacy in healthy and disease models to develop targeted therapies that ameliorate degenerative processes and help maintain and improve health span and longevity.

While it will almost certainly be proved true that NAD+ therapy alone is not the mythical 'elixir of life', its foundational role in cellular energetics, nuclear signalling and viability suggest it just may be a key ingredient.

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List of Figures

Figure 1. Putative relationship between changes in tryptophan catabolism and de novo NAD+ synthesis in ADC neuropathology. Immune activated oxidative L-tryptophan catabolism can contribute positively to the maintenance of cell viability through increased metabolism of NAD+ in astrocytes and mononuclear phagocytes. However, chronic activation of this pathway may also enhance neuronal excitotoxicity through the production of QUIN and possibly 3-HK.

Figure 2. The NAD+ metabolome. L-Tryptophan (L-TRYP), nicotinic acid (NA), nicotinamide (NAM), nicotinamide mononucleotide (NMN), nicotinamide riboside (NR) and nicotinic acid riboside (NAR) can be used as precursors for NAD+ synthesis. L-TRYP is catabolised to N-formylkynurenine (N-f-KYN) by indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO) **(a)**. N-f-KYN is catabolised by arylformidase to form kynurenine (KYN) **(b)**. Kynurenine aminotransferases (KATs) catabolise KYN to form kynurenic acid (KA) **(c)**. Kynurenine 3-hydroxylase uses KYN as a substrate to form 3-hydroxykynurenine (3-HK) **(d)**. Kynureninase then forms 3-hydroxyanthranilic acid (3-HAA) **(e)** which is converted to 2-amino-3-carboxymuconate semialdehyde (not shown) by 3-hydroxyanthranilic acid oxygenase (3-HAAO) **(f)**. This product is then converted to picolinic acid by picolinic acid carboxylase **(g)**. Alternatively, the semialdehyde undergoes spontaneous condensation and rearrangement to form quinolinic acid (QUIN) **(h)**, which

forms nicotinic acid mononucleotide (NAMN) by quinolinic acid phosphoribosyltransferase (QPRT) **(i)**. NAMN undergoes adenylylation by nicotinamide mononucleotide adenyltransferases (NMNAT1-3) to form nicotinic acid adenine dinucleotide (NAAD) **(u)**, which forms NAD+ by glutamine-dependent NAD+ synthetases **(m)**. NA is used by the Preiss-Handler pathway. NAMN is formed by nicotinic acid phosphoribosyltransferase (NAPRT) following addition of 5-phosphoribose group from 5-phosphoribosyl-1-pyrophosphate to NA **(l)**. Nicotinamide phosphoribosyltransferase (NAMPT) forms NMN by addition of phosphoribose moiety to NAM **(p)**. NMN is then converted to NAD+ via the catalytic activity of NMNAT1-3 **(u)**. NAM is also produced as a by-product of NAD-dependent enzymes eg. PARPs, sirtuins, and CD38 **(n)**. NAM can also be converted to NA by bacterial nicotinamidases **(o)**. NR is phosphorylated to form NMN by nicotinamide riboside kinases (NRK1/NRK2) **(j)** which is then subsequently converted to NAD+ by NMNAT1-3. NAR can also be used to form NAMN by NRK1/NRK2 **(j)** or NA by purine nucleoside phosphorylase **(k)**. NAM is methylated nicotinamide N-methyltransferase (NNMT) to N-methylnicotinamide (MeNAM) and modulates the efficiency of NAD-dependent biological processes **(q)**. NAD+ can be reduced to form NADH **(t)**. NAD+ can also undergo phosphorylation to NADP+ **(r)** and then further reduction to NADPH **(s)**.

Figure 3. Concomitant induction of IDO and free radical generation by IFN-γ. Chronic immune activation of macrophages and astrocytes will result in increased reactive oxygen and nitrogen species and elevates glutamate levels (in the absence of efficient uptake into astrocytes). A possible relationship exists between IFN-γ stimulated free radical production and IDO induction, leading to increased *de novo* synthesis of NAD+.

Figure 4. Cofactors required for QPRT activity and NAD+ synthesis. PRPP is important for the regulation of QPRT activity.

Figure 5. Cellular roles of NAD+. The mechanisms of degradation of NAD+ including CD38, PARPs, and sirtuins. NAD+ can be phosphorylated to NADP+. There are also oxireduction reactions of NAD+ to NADH and NADP+ to NADH. CD38 is an NAD-dependent enzyme which leads to the production of cADPR from NAD+ and NADP+

respectively. Cytosolic cADPR target to ryanodine receptors on endoplasmic reticulum, and transient receptor potential mucolipin 1 on lysosomes, regulating intracellular calcium signalling from the endoplasmic reticulum and lysosome-mediated intracellular calcium signalling.

Figure 6 (A). PARP and PARG enzymatic activity. PARP breaks down NAD+ to NAM and an ADP-ribosyl product degradation of ADP-ribose polymers occurs relatively rapidly through the action of poly(ADP-ribose) glycohydrolase. **(B)** Relationship between DNA damage, PARP activation, and NAD+ depletion. Under normal physiological conditions, PARP activation leads to repair of damaged DNA. However, increased PARP activity resulting in decreased NAD+ has been shown to decrease ATP as well as cause cell lysis and death (45,203) (Figure 6B).

Figure 7. Stoichemistry of CD38-mediated Ca^{2+} mobilising, and NADase activities. CD38 require NAD+ to produce ADP ribose (ADPR) and hydrolyse the secondary messenger signalling molecule, cyclic-ADP-ribose (cADPR) which helps mediate intracellular calcium transients.

Figure 8. Schematic representation of CD38-mediated intracellular Ca^{2+} secondary messenger signalling. , CD38 is also an NADase which primarily regulates intracellular levels of NAD+ and its physiological processes.CD38 also catalyses a base exchange between NADP and NA, leading to the formation of nicotinic acid adenine dinucleotide phosphate (NAADP), which is also used as a hydrolytic substrate.

Figure 9. Sirtuin enzymatic activity. NAM is rendered as a by-product of sirtuin-mediated deactylation. Deacetylation occurs when the modified lysine side chain is coupled to the cleavage of the glycosidic bonds in NAD+, leading to the generation of the deacetylated lysine, acetylated ADP-ribose, and NAM as by-products.

Figure 10. Functions of NAD-dependent sirtuins and relevant transcription factors. Sirtuin-mediated deacetylation affects numerous target enzymes and transcription factors relevant to

ageing and disease. Importantly, sirtuin activities stimulate oxidative phosphorylation (OXPHOS) while yet unknown acetylation mechanisms serve to inhibit oxidative phosphorylation (anti-OXPHOS).

Figure 11. Modulation of NAD+ and NAD-dependent pathways by caloric restriction in mice and humans. Caloric restriction has been shown to increase neuronal SIRT1 activity in humans. In mice, hepatic total NAD+ levels increased in fasted mice, and these changes were accompanied by increased SIRT1 activation, PGC1α deacetylation, and increased mitochondrial biogenesis.

Figure 12. Chemical structure of NAD+ precursors.

Figure 13. Mechanisms of action of nicotinic acid (NA) in dyslipidemia. The lipid lowering effects of NA are thought to be mediated by binding of NA to the cell surface of a G-protein coupled receptor known as HM74A or GPR109A. This association in adipocytes suppresses triglyceride lipolysis, culminating in the reduction of circulating fatty acids, and reduced liver VLDL formation and circulating LDL-cholesterol.

Figure 14. Schematic representation of the molecular mechanism of skin flushing following treatment with nicotinic acid (NA). NA mediated stimulation of HM74A in some skin immune cells results in the conversion of the omega-6 metabolite arachidonic acid (AA) into prostaglandin E2, stimulating vasodilation of skin capillaries, causing skin flush.

Figure 15. Mechanisms of action of nicotinamide (NAM) and its effect on the NAD+ metabolome. NAM also serves as a natural feedback inhibitor for NAD-dependent enzymes. For example, PARP, sirtuin and CD38 activities are proportionately inhibited as NAM concentrations increase, and this has been postulated as the mechanism for the anti-diabetic effects of NAM in humans. While NAD+ levels are still elevated, the important NAD-dependent functions (e.g. SIRT1 activity) are inhibited.

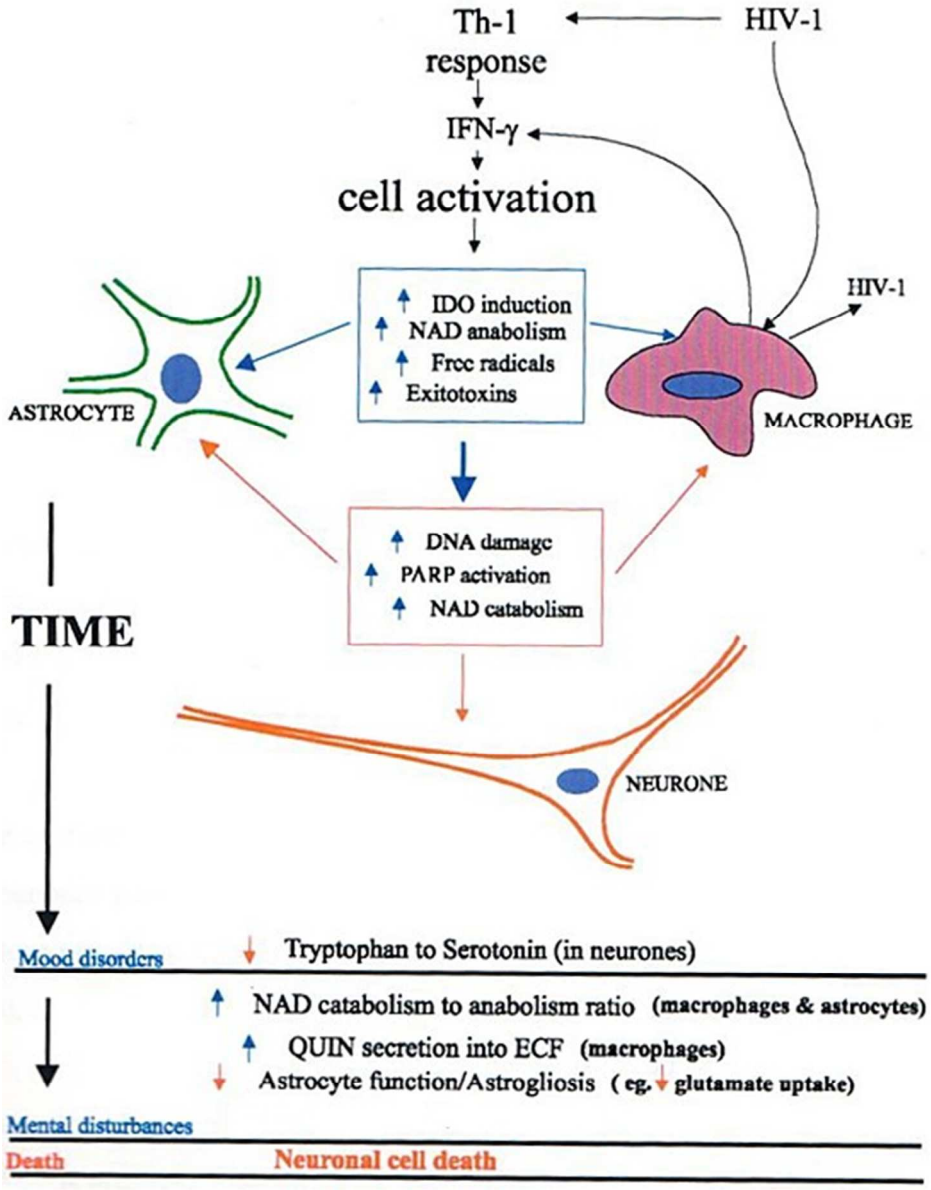

Figure 1. Putative relationship between changes in tryptophan catabolism and de novo NAD+ synthesis in ADC neuropathology.

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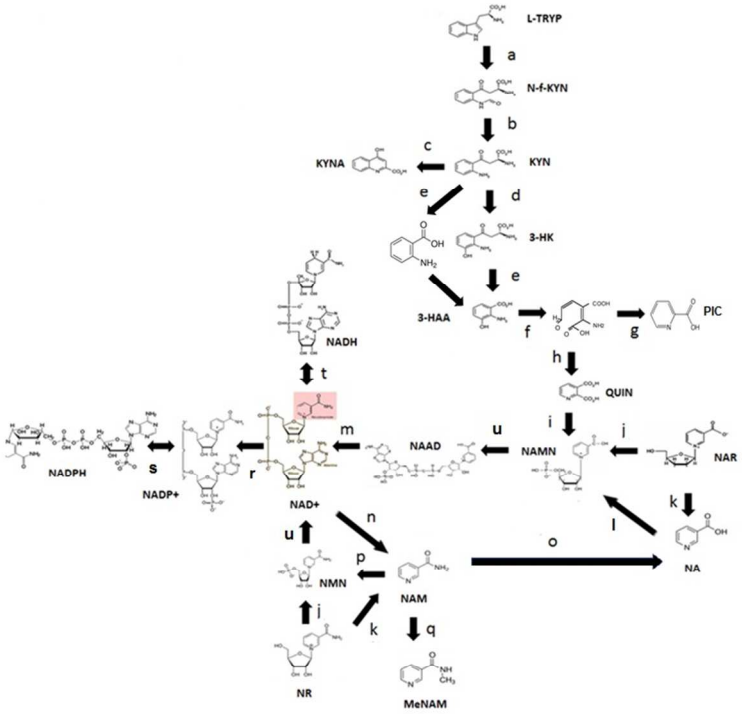

Figure 2. The NAD+ metabolome.

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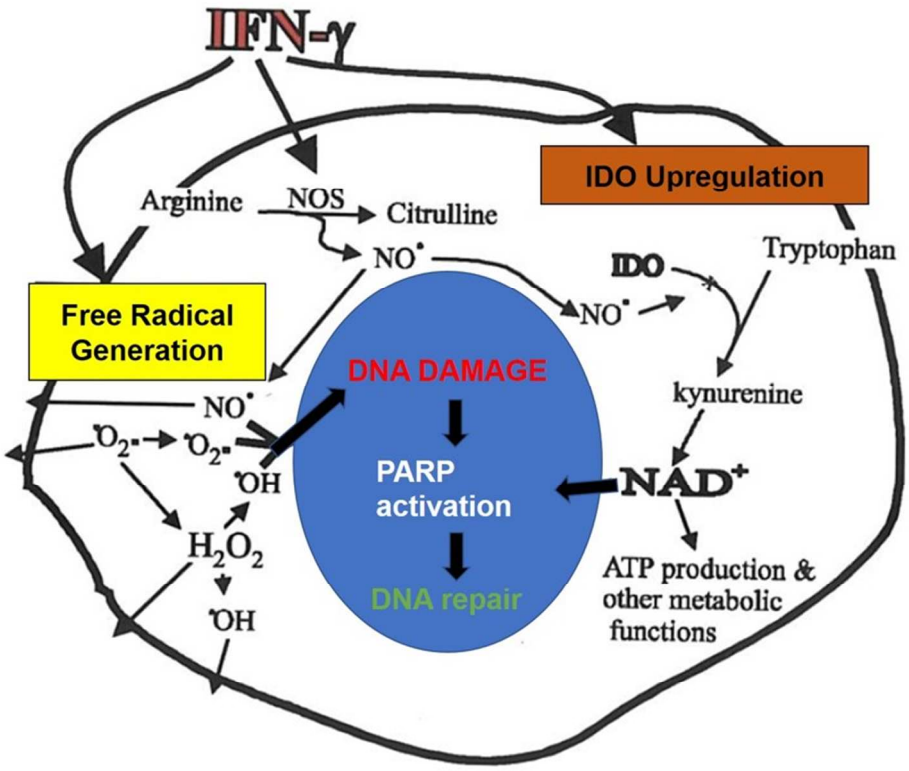

Figure 3. Concomitant induction of IDO and free radical generation by IFN-γ.

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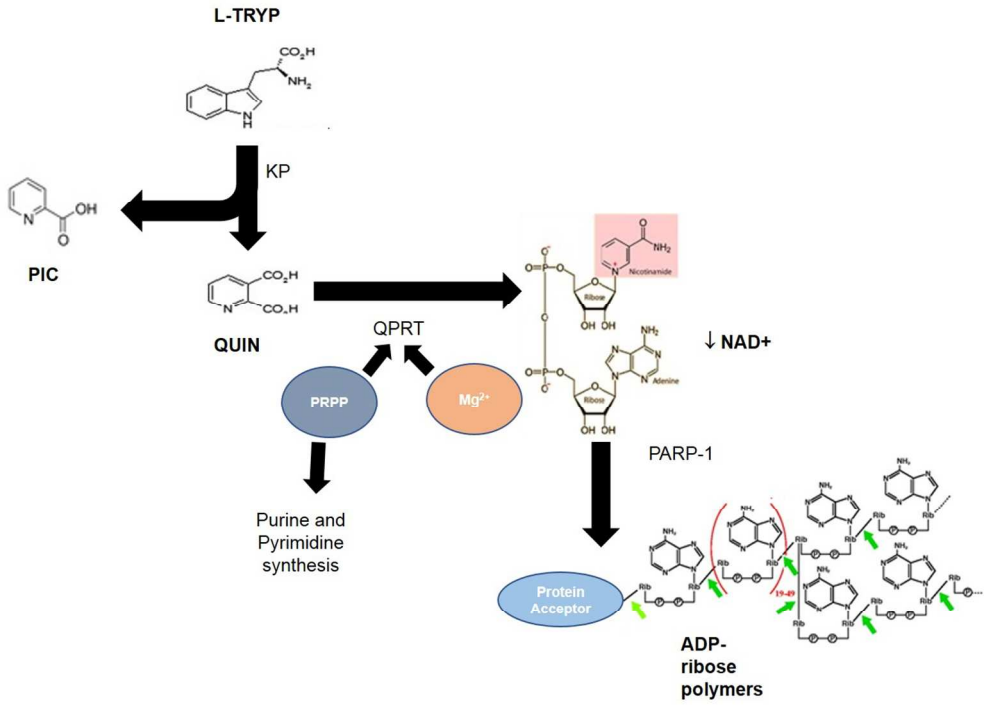

Figure 4. Cofactors required for QPRT activity and NAD+ synthesis.

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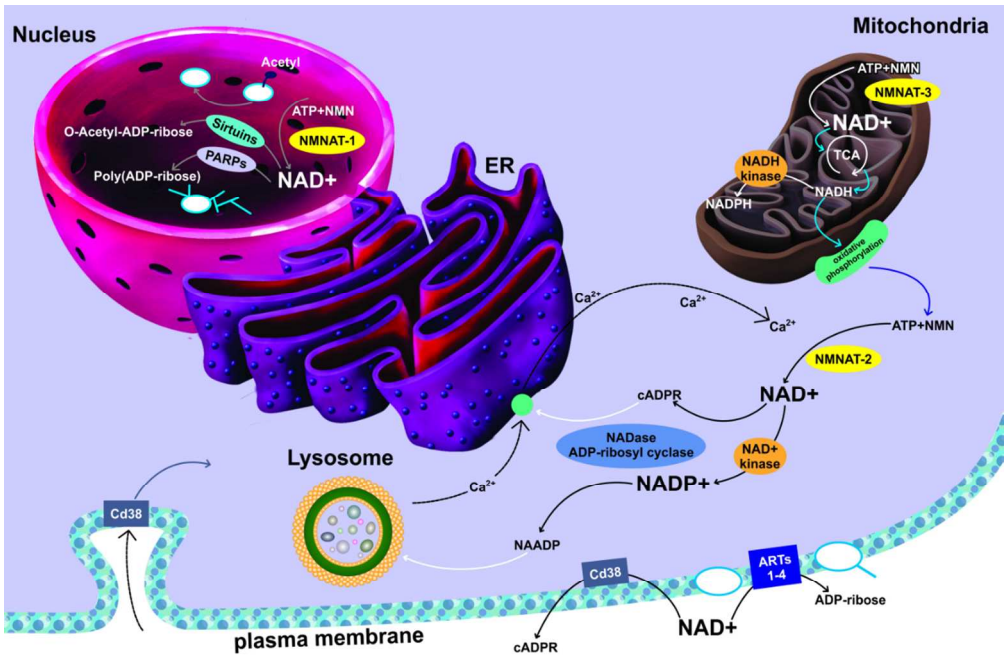

Figure 5. Cellular roles of NAD+.

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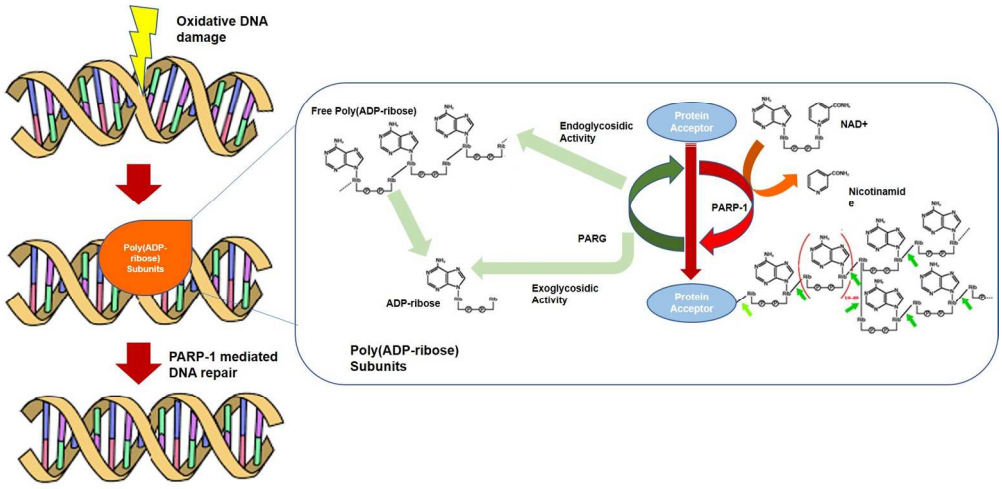

Figure 6 (A). PARP and PARG enzymatic activity.

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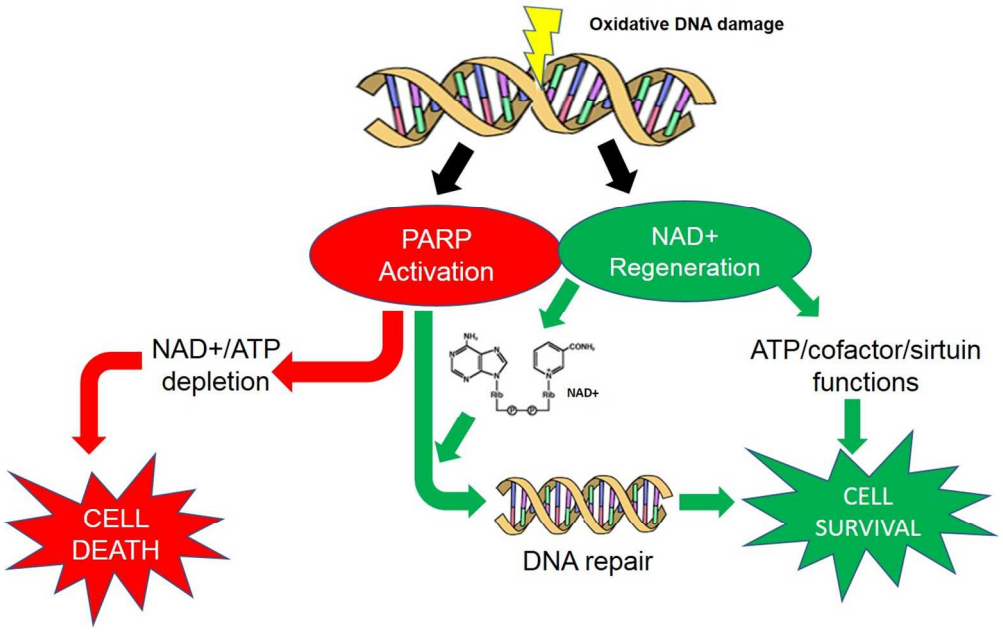

Figure 6 (B) Relationship between DNA damage, PARP activation, and NAD+ depletion.

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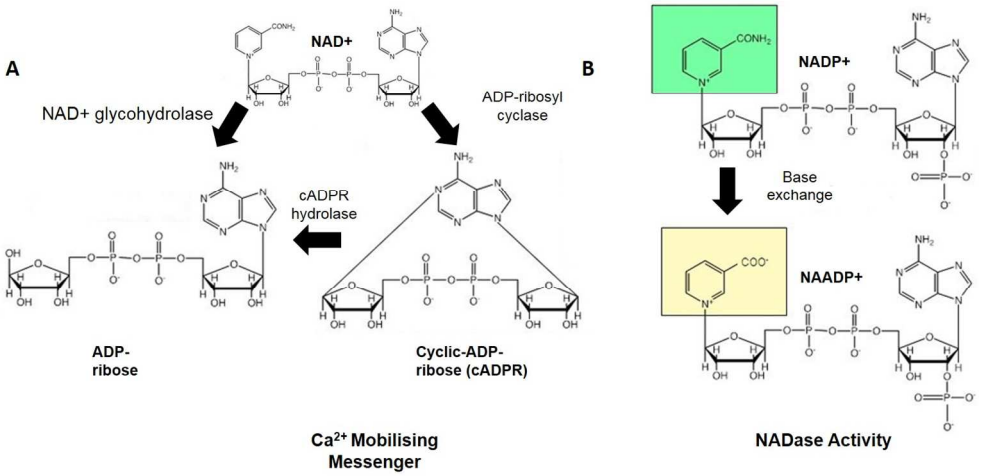

Figure 7. Stoichemistry of CD38-mediated Ca2+ mobilising, and NADase activities.

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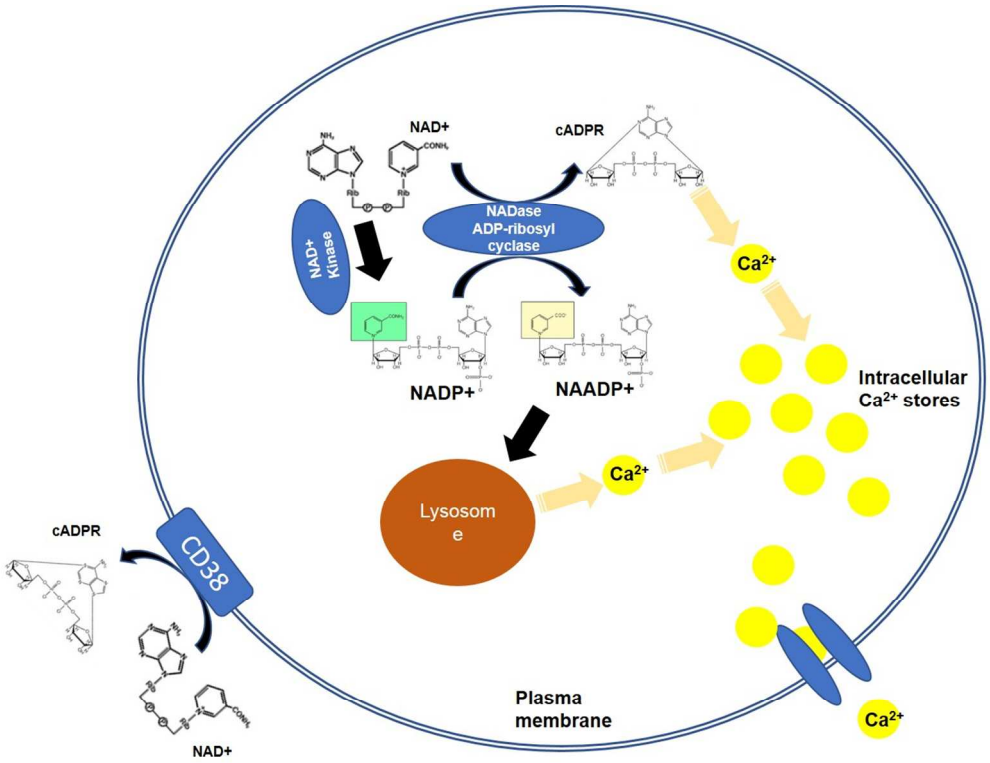

Figure 8. Schematic representation of CD38-mediated intracellular Ca2+ secondary messenger signalling

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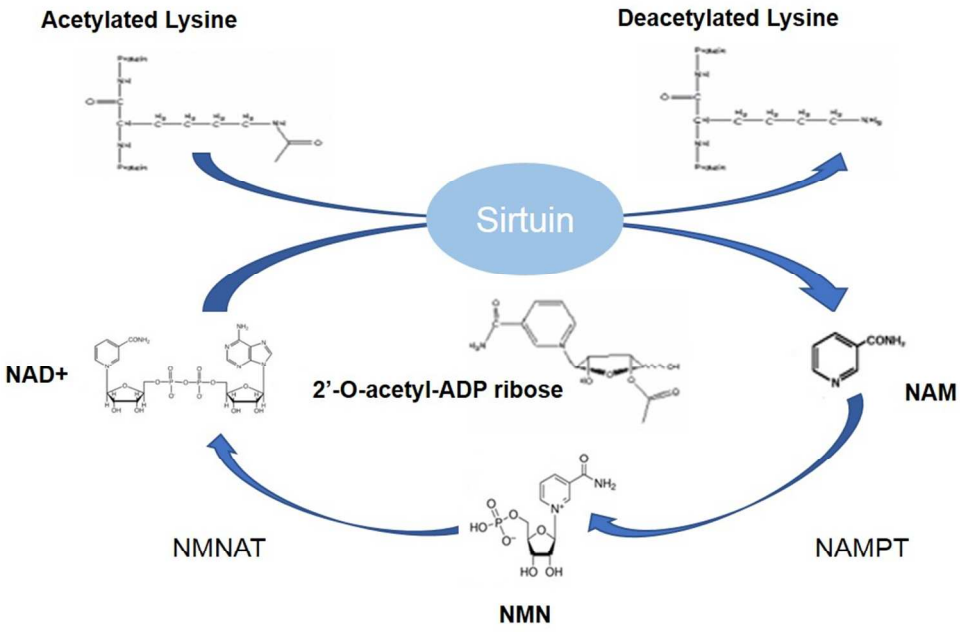

Figure 9. Sirtuin enzymatic activity.

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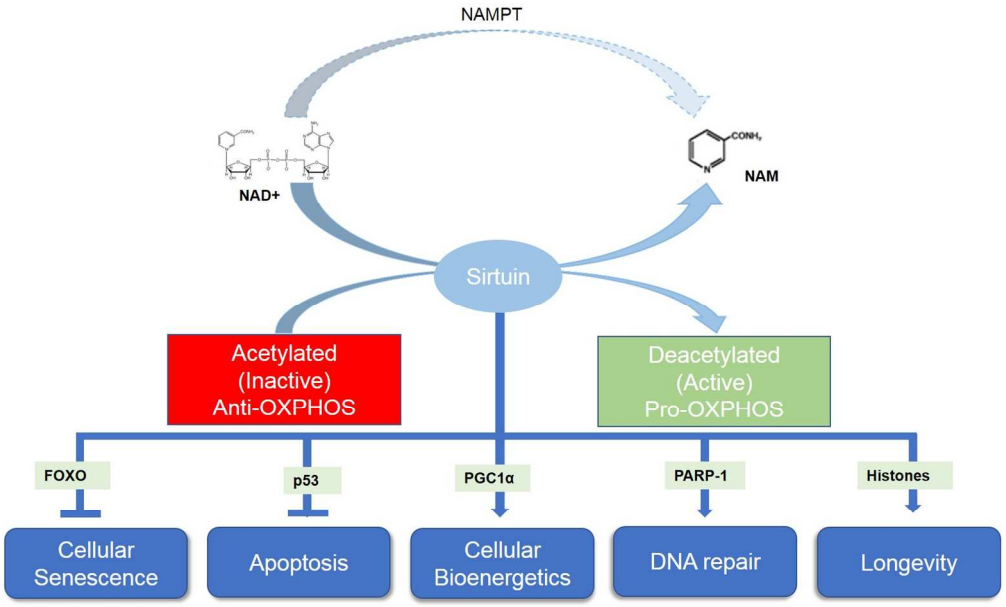

Figure 10. Functions of NAD-dependent sirtuins and relevant transcription factors.

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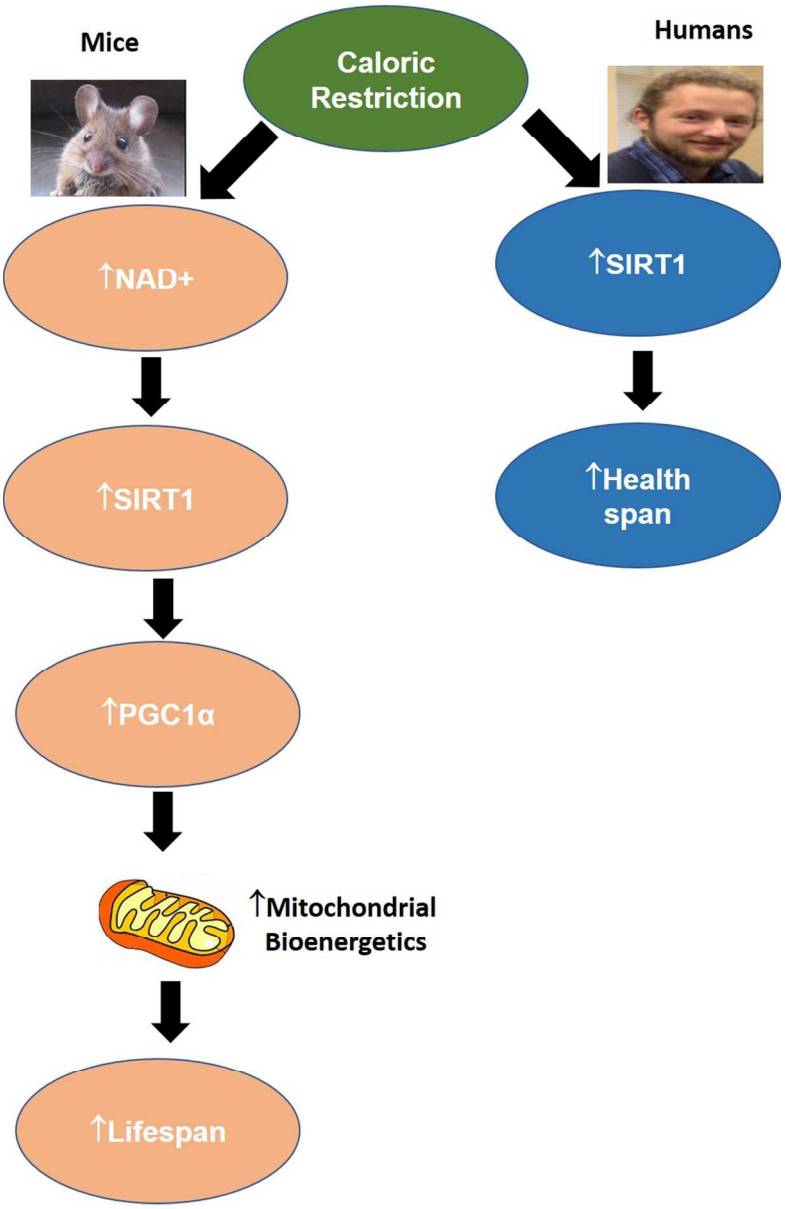

Figure 11. Modulation of NAD+ and NAD-dependent pathways by caloric restriction in mice and humans.

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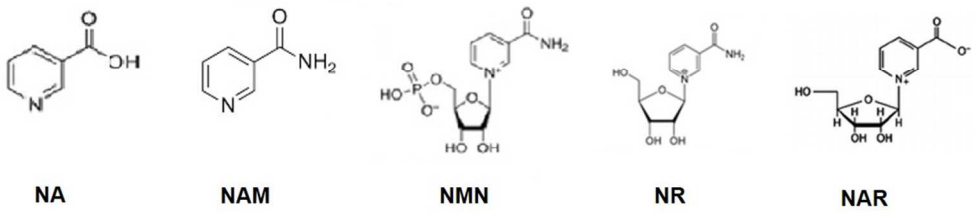

Figure 12. Chemical structure of NAD+ precursors.

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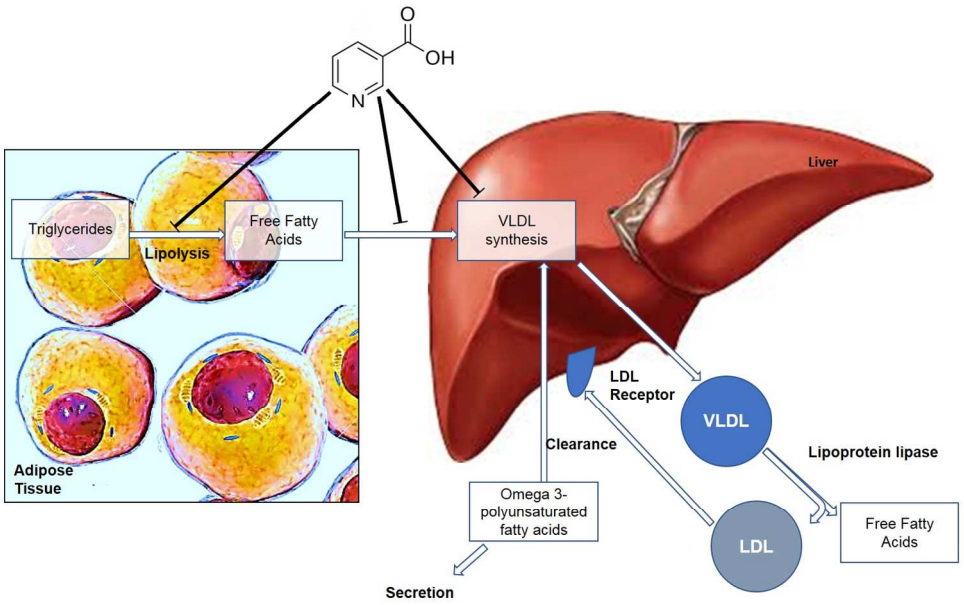

Figure 13. Mechanisms of action of nicotinic acid (NA) in dyslipidemia.

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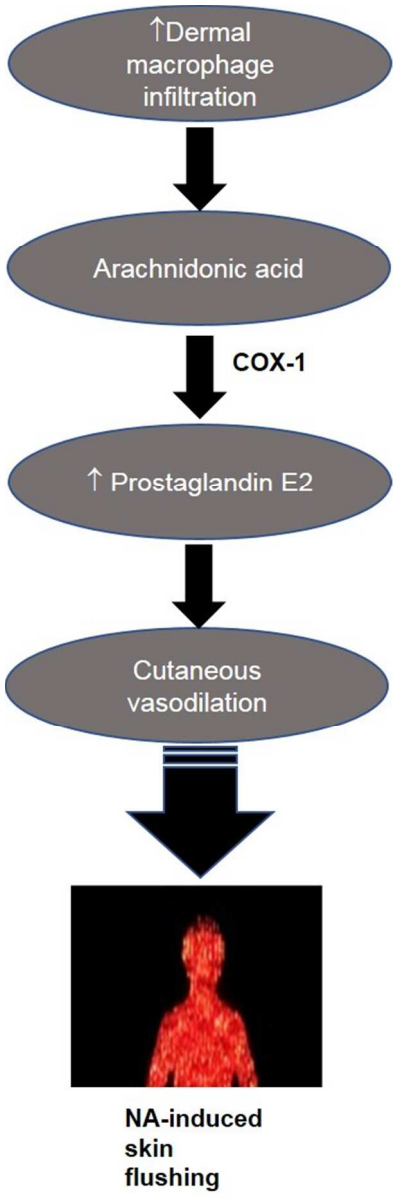

Figure 14. Schematic representation of the molecular mechanism of skin flushing following treatment with nicotinic acid (NA).

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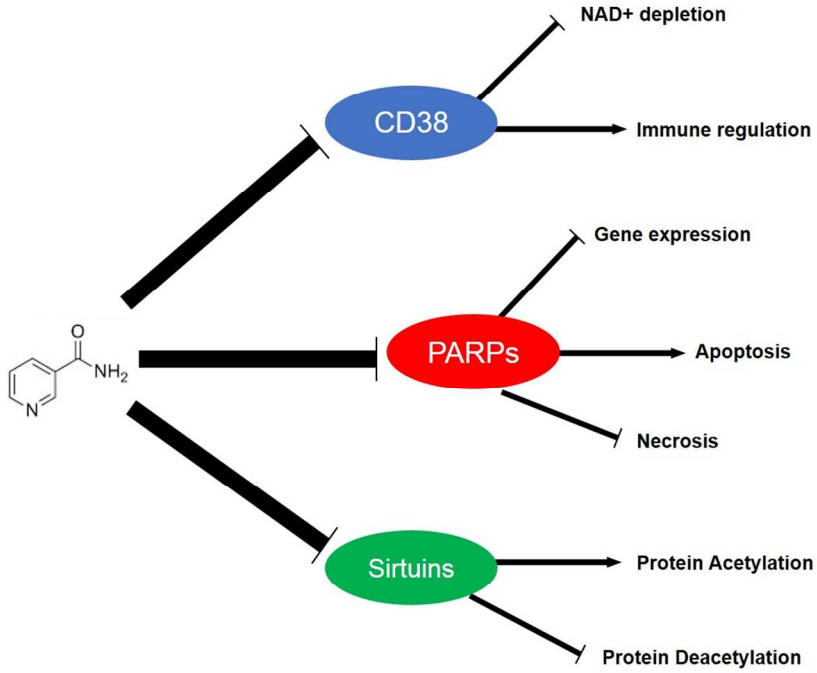

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339x232mm (96 x 96 DPI)