REVIEW ARTICLE

# Impaired nicotinamide adenine dinucleotide (NAD+) metabolism in diabetes and diabetic tissues: Implications for nicotinamide-related compound treatment

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**ABSTRACT**

One of the biochemical abnormalities found in diabetic tissues is a decrease in the cytosolic oxidized to reduced forms of the nicotinamide adenine dinucleotide ratio (NAD+/NADH also known as pseudohypoxia) caused by oxidation of excessive substrates (glucose through the polyol pathway, free fatty acids and lactate). Subsequently, a decline in NAD+ levels as a result of the activation of poly adenine nucleotide diphosphate-ribose polymerase (mainly in type 1 diabetes) or the inhibition of adenine nucleotide monophosphate-activated protein kinase (in type 2 diabetes). Thus, replenishment of NAD+ levels by nicotinamide-related compounds could be beneficial. However, these compounds also increase nicotinamide catabolites that cause oxidative stress. This is particularly troublesome for patients with diabetes, because they have impaired nicotinamide salvage pathway reactions at the level of nicotinamide phosphoribosyl transferase and phosphoribosyl pyrophosphate, which occurs by the following mechanisms. First, phosphoribosyl pyrophosphate synthesis from pentose phosphate pathway is compromised by a decrease in plasma thiamine and transketolase activity. Second, nicotinamide phosphoribosyl transferase expression is decreased because of reduced adenosine monophosphate-activated protein kinase activity, which occurs in type 2 diabetes. The adenosine monophosphate-activated protein kinase inhibition is caused by an activation of protein kinase C and D1 as a result of enhanced diacylglycerol synthesis caused by pseudohypoxia and increased fatty acids levels. In this regard, nicotinamide-related compounds should be given with caution to treat diabetes. To minimize the risk and maximize the benefit, nicotinamide-related compounds should be taken with insulin sensitizers (for type 2 diabetes), polyphenols, benfotiamine, acetyl-L-carnitine and aldose reductase inhibitors. The efficacy of these regimens can be monitored by measuring serum NAD+ and urinary nicotinamide catabolites.

## INTRODUCTION

Both nicotinic acid and nicotinamide are known as vitamin B3 (niacin). Its deficiency causes pellagra, with symptoms including inflamed skin, diarrhea and dementia. In developed countries, it is mainly used as a supplement for various conditions[1–3] although its efficacy in humans is still not clear. Both forms of niacin are utilized to synthesize nicotinamide adenine

dinucleotide (NAD+)[1,4,5]. However, as nicotinic acid causes flush and other symptoms, nicotinamide is mainly used at a high dose[5]. NAD+ metabolism is currently one of the hottest research topics in the field of aging following the recent increase in resveratrol research. It has been shown that human skin NAD+ content sharply declines as people age[6]. Similar findings were observed in plasma NAD+ levels[7]. Whether this decline relates to the aging process, and whether replenishment of NAD+ slows aging are hot issues. Nevertheless, nicotinamide

and related compounds have been and are currently being tested to treat conditions, such as cardiovascular disease[8] and diabetes, in humans.

Our research focuses on diabetic complications[9], NAD+ redox abnormalities in diabetes (pseudohypoxia)[9–13], sirtuin-1 (SIRT1)[14–16], resveratrol[15,17,18] and adenosine monophosphate (AMP)-activated protein kinase (AMPK)[16,18–21], all of which control and at the same time are controlled by nicotinamide and NAD+. In both types of diabetes (type 1 and 2), the metabolism of NAD+ appears to be dysregulated. This is evident in animal models of diabetes and is highly suggested in human diabetes. The etiology of metabolic dysregulation in NAD+ is likely to coincide with the etiology of diabetic vascular and neural complications, which has been elucidated in the past five decades of research. However, our research interest has been more than diabetic complications studied in various tissues. As explained in this article, the main causative abnormality – a decrease in cytosolic NAD+/NADH – has been observed not only in the kidney, retina and nerves, typical tissues in the field of diabetic complications, but also found in the liver, heart, skeletal muscle and even in pancreatic β-cells of individuals with diabetes, prediabetes and obesity[9]. In these tissues, the effects of this abnormality are still not well explored. For example, this redox change is the main force that increases diacylglycerol (DAG) and triacylglyerol synthesis. Consistent with this idea, expression of aldose reductase in the mouse liver causes the same redox change in the absence of diabetes, which leads to fatty liver ,as shown recently[22]. Thus, the data and research we present in this article are intended for researchers studying diabetes in general. We review the relationship between NAD+, diabetes and its complications. After an introduction to nicotinamide, AMPK, SIRT1 and resveratrol, we discuss how NAD+ dysregulation might be corrected, and how nicotinamide related compounds, such as nicotinamide riboside

(NR) and nicotinamide mononucleotide (NMN), might be used as a treatment for diabetes, but with several caveats.

## PSEUDOHYPOXIA AND NAD+ METABOLISM ABNORMALITIES IN DIABETIC TISSUES

In this section, we start to define the concept of pseudohypoxia. This term was first used in a paper by Professor Williamson[10], who was a mentor of one of the authors of the present article (YI). Together we wrote a number of papers explaining the concept[10,11,13,21,23–28]. Pseudohypoxia is a state of lower than normal cytosolic NAD+/NADH ratio (the normal free ratio is ~600). It is known from studies carried out in the 1970s that various tissues (first observed in the liver) from diabetic animals have abnormal values in total NAD, and its ratio in reduced (NADH) and oxidized (NAD+) forms[29–31]. For example, the lens from rats post 6 weeks of streptozotocin-induced diabetes has a three- to fourfold lower NAD+/NADH ratio compared with non-diabetic rats due to increased NADH and decreased NAD+. Total NAD was decreased by 15%. Such a high decline was also observed with NADPH[29]. By using metabolite indicator methods[30] in the retina and sciatic nerve to calculate the free ratios of NAD+ and NADH in the cytosolic and mitochondrial compartments (i.e., by measuring lactate, pyruvate for cytosolic, and 2-oxoglutarate, ammonium and glutamate for mitochondria), we and others found that a low NAD+/NADH ratio is only observed in the cytosol and not in mitochondria[10,11,13,23,26], suggesting that the diabetic effect is, at least in early stages, solely in the cytosol[10]. Similar changes can be observed by incubating rat retina with high glucose for 2 h[12,32]. In this case, change occurred only in the ratio, not in total NAD. Clear involvement of the polyol pathway (oxidation of sorbitol to fructose) to cause a low NAD+/NADH ratio was observed in early diabetic rats[9,13], and by incubating human red blood cells with high glucose, in which aldose reductase
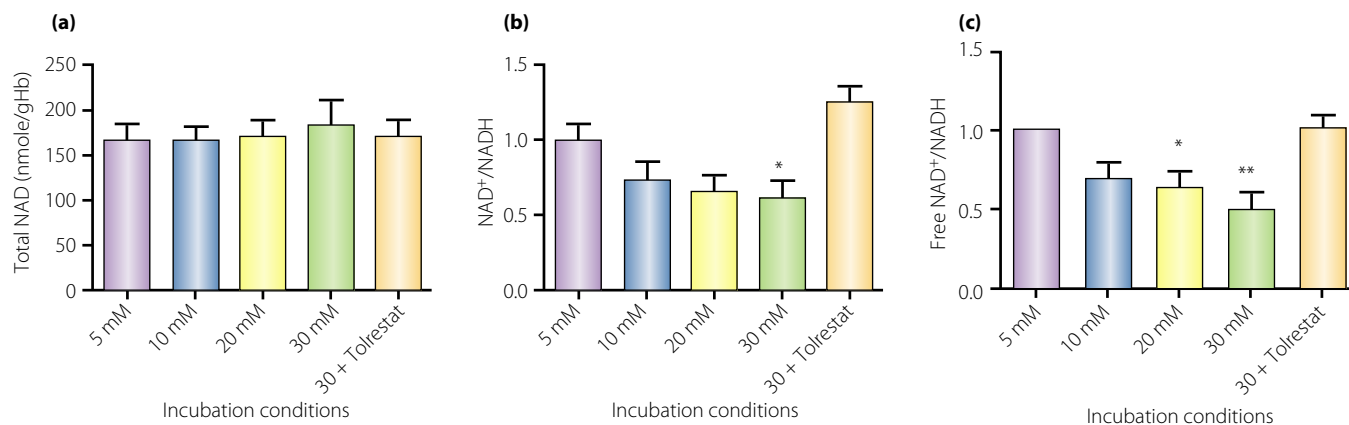


**Figure 1** | Total and free nicotinamide adenine dinucleotide (NAD+/NADH) ratio changes in human red blood cells incubated in the indicated conditions for 1 h. One hour incubation of red blood cells with high glucose decreased the ratios of oxidized to the reduced form of NAD+/NADH, which was completely prevented by 70 μmol/L aldose reductase inhibitor, tolrestat. See Data S1 for details of the experiment. * $P < 0.05$ vs 5 mM, ** $P < 0.01$ vs 5 mM

inhibitors prevented NAD$^+$/NADH ratio change (Figure 1). Thus, acute hyperglycemia, such as post-prandial hyperglycemia, likely changes the cellular NAD$^+$/NADH ratio without decreasing the total NAD amount. However, this acute change of NAD$^+$/NADH is enough to increase triose phosphate levels by glyceraldehyde phosphate dehydrogenase (GAPDH), the synthesis of diacylglycerol and production of superoxide[9,33], as follows. Increased NADH produces glycerol-3-phospate (a precursor for DAG), which fuels electrons at mitochondria complex II through the glycerol phosphate shuttle by electron transfer from mitochondrial glycerol-3-phosphate dehydrogenase[34,35]. This leads to reverse electron flow to complex I, leading to superoxide production[36]. In addition, cytosolic NADH might directly produce oxidative stress (Figure 2).

A decline of NAD$^+$ levels is clearly observed in chronic type 1 insulinopenic diabetic animal models, such as streptozotocin diabetic rats. 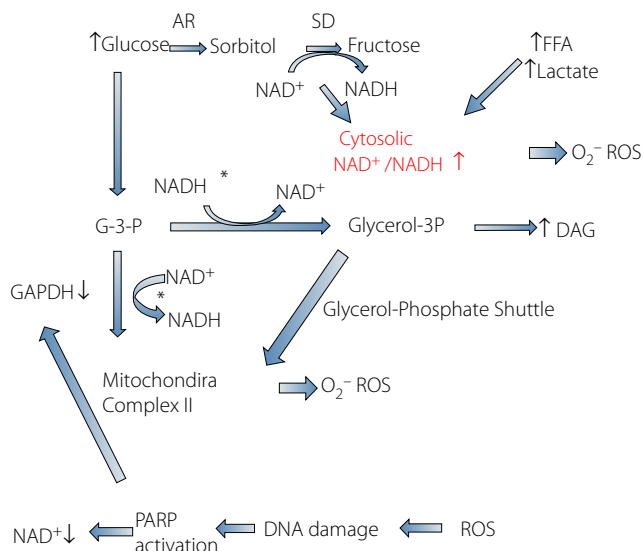A measurable decrease might take weeks to months to occur, depending on the tissues. The aorta from 1-month-old diabetic mice showed 60% decline in NAD$^+$ levels[37]. This decrease is apparently caused by activation of poly adenosine diphosphate (ADP)-D-ribosyl polymerase (PARP), likely caused by oxidative stress-mediated deoxyribonucleic acid (DNA) damage, as its inhibitor, BJ-34, prevented it. Declining NAD$^+$ levels is a signature of an imbalance in energy homeostasis, which is manifested as decreased adenosine triphosphate (ATP) levels[37] or a decreased ratio of ATP/(ADP or AMP). Declining ATP levels are reported in type 1 diabetic animal models[37], as well as in the liver of human type 1 diabetic patients[38].

Compared with type 1 diabetes, the declining NAD$^+$ levels in type 2 diabetes (particularly in the hyperinsulinemic stage) and its animal models seem to be mild. A high-fat diet in mice causes insulin resistance and impaired glucose tolerance similar to early type 2 diabetes. In this model, a decline of NAD$^+$ was reported in the liver and white adipose tissue[39]. In addition, despite a modest increase of plasma glucose, activation of the polyol pathway and PARP are observed in the sciatic nerve[40]. Energy levels are also compromised, albeit slightly. Skeletal muscle ATP and high energy intermediate phosphocreatine levels in humans with type 2 diabetes were decreased by 12%, but ADP was not changed significantly. In the liver, ATP synthesis was decreased[41]. The activation of PARP in both types of diabetes, which appears to be a consequence of oxidative damage to DNA, accelerates the decreasing NAD$^+$/NADH ratio further by inactivating GAPDH (Figure 2)[42].

In addition to high glucose, non-esterified fatty acids are increased in type 1 diabetes by insulinopenia, and type 2 diabetes by increased ectopic fat deposition. Fatty acid toxicity in the development of diabetes was firstly proposed by McGarry[43,44]. This theory was preceded by the seminal work of Randle[45], who described fatty acid-induced insulin resistance observed by tissue incubation. Recent findings of selective insulin resistance in the liver observed in type 2 diabetes concurs with these fatty acid toxicity theories[46]. In the development of diabetic complications and insulin resistance, free fatty acids (FFA) are utilized by the mitochondria, resulting in increased NADH that slows shuttling activities to mitochondria, which causes pseudohypoxia more easily. The second effect of increased FFA and FFA-CoA in cytosol is to increase the synthesis of acyl-glycerols including DAG (Figure 2).

A decreasing cytosolic NAD$^+$/NADH ratio affects the cytosolic lactate/pyruvate ratio through lactate dehydrogenase, which induces higher lactate production. Thus, plasma lactate levels are increased in obesity[47] and diabetes[48]. This high level of plasma lactate, in turn, causes the NAD$^+$/NADH ratio change in other cells through the lactate dehydrogenase reaction[9]. Thus, the uniqueness of lactate is that it can transmit the redox change from one organ to another. It was shown that a lactate infusion causes insulin resistance in rats[49]. Similarly, high plasma lactate was associated with a high risk of developing diabetes[50].



**Figure 2** | Biochemical cascades postulated to cause diabetic complications. Intracellular hyperglycemia increases in polyol pathway flux, free fatty acids (FFA) and lactate that cause a cytosolic nicotinamide adenine dinucleotide (NAD$^+$/NADH) ratio increase (pseudohypoxia). This redox change induces: (i) production of reactive oxygen species (ROS) from cytosol and mitochondria; and (ii) increased flux to glycerol-3-phosphate and diacylglycerol (DAG). An excess of long-chain acetyl coenzyme A also augments ROS and production of DAG. Increased NADH produces glycerol-3-phosphate, which fuels electrons at mitochondria complex II through the glycerol phosphate shuttle by electron transfer from mitochondrial glycerol-3-phosphate dehydrogenase. Chronically increased ROS production causes deoxyribonucleic acid damage and activates poly-adenosine diphosphate ribosyl-polymerase (PARP), which utilizes NAD$^+$ as a substrate. PARP activation might cause post-translational modification of glyceraldehyde phosphate dehydrogenase (GAPDH), resulting in inhibition and translocation. AR, aldose reductase; G-3-P, glyceraldehyde-3-phosphate; SD, sorbitol dehydrogenase.

Taken together, the redox change characterized by pseudohypoxia, which is caused by excessive substrate oxidation, leads to oxidative stress and DNA damage, occurs in various tissues, and is found in obesity, prediabetes and diabetes.

## SIRT1 AND AMPK

SIRT1 belongs to a family of sirtuins that use $NAD^+$ as a substrate catalyzing the reaction of deacetylation or mono-ADP-ribosylation of proteins[19]. AMPK is known as a master regulator of energy metabolism, as it is activated by lowering the ratio of ATP to AMP or ADP[19]. Both SIRT1 and AMPK are known to activate genes involved in longevity, and work together as the master regulators of various metabolic pathways[14,18,19,51,52]. Well-known targets of both are peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), which activates mitochondrial biogenesis[53–56], and forkhead box O3, which determines the fate of the cells (life or death signaling pathways)[14,57,58]. They also suppress pro-inflammatory nuclear factor-kappa B signaling[59–61] and upregulate autophagy by suppressing mammalian target of rapamycin signaling (Figure 3)[62–64].

There is a positive reciprocal relationship between AMPK and SIRT1. Activation of AMPK has been shown to increase $NAD^+$ levels, which results in SIRT1 activation[65]. This effect is
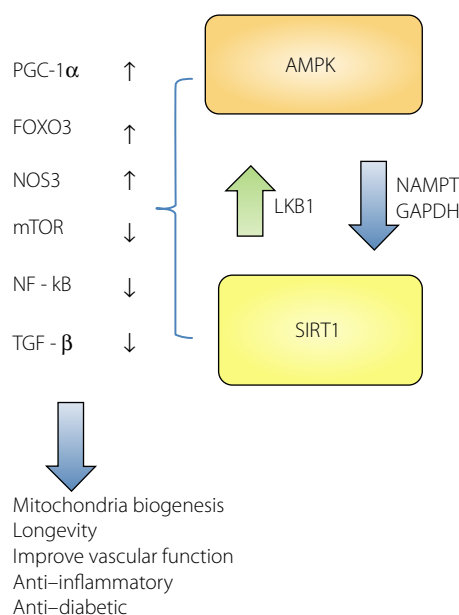


**Figure 3** | Adenosine monophosphate (AMP)-activated protein kinase (AMPK) and sirtuin-1 (SIRT1) interaction. AMPK and SIRT1 activate each other through liver kinas B1 (LKB1) and nicotinamide phosphoribosyltransferase (NAMPT), and regulate target molecules, such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), forkhead box O3 (FOXO3), mammalian target of rapamycin (mTOR) and so on. Polyphenol, such as resveratrol, activates both AMPK and SIRT1 in multiple ways. NF-κB, nuclear factor-κB; TGF-β, transforming growth factor-β.

mediated by upregulation of nicotinamide phosphoribosyltransferase (NAMPT) messenger ribonucleic acid and protein expression. Our results showed an absolute requirement of AMPK for the upregulation of $NAD^+$. The enzyme is thought of as a rate-limiting step to convert nicotinamide to $NAD^{+66}$. This increase in $NAD^+$ level might cause SIRT1 (and other sirtuins) activation by two closely related mechanisms. One mechanism is to increase $NAD^+$ levels and another is to decrease nicotinamide levels. As nicotinamide is a competitive inhibitor of sirtuin family enzymes[67,68], the second effect affects activities of SIRT1 and other members of the family. For example, an increase in nuclear and cytoplasmic $NAD^+$, and a decrease in nicotinamide might activate SIRT1 and 6.

We showed that SIRT1 deacetylates liver kinase B1 (LKB1), which is an upstream kinase of AMPK and causes its activation, thus controlling AMPK activity indirectly (Figure 3)[16,19]. LKB1 activity appears to play crucial roles in the context of diabetic complications. The Schwann cell-specific LKB1 knockout mouse showed biochemical and morphological changes predominantly in peripheral nerves characterized by energy depletion, decreased NAD, $NAD^+/NADH$ ratio and axonal atrophy morphologically similar to diabetic neuropathy[69].

Recently, there is an additional mechanism to activate SIRT1 by AMPK involving GAPDH phosphorylation[64]. In this mechanism, GAPDH Ser122 phosphorylation by AMPK causes GAPDH nuclear translocation where it binds SIRT1 directly and activates it. Thus, there is reciprocal or positive feedback relationship between SIRT1 and AMPK (Figure 3)[14,19].

## RELATIONSHIP BETWEEN CELLULAR $NAD^+$ AND ATP

The main function of $NAD^+$ is as the electron acceptor for catabolic pathways[70]. Under strong reduction of $NAD^+$ (production of NADH), even glycolysis is inhibited at the step of GAPDH. $NAD^+$ synthesis using nicotinamide requires at least three ATPs (Figure 4)[70]. In addition, the last step of $NAD^+$ synthesis catalyzed by NMN adenylyltransferase (NMNAT) is completely reversible[71], meaning that there might be equilibration between cytosolic $NAD^+$ and ATP levels (Figure 4). Such a relationship was reported in hepatocytes. A titration experiment that changed cellular ATP levels clearly showed that cytosolic $NAD^+$ positively correlates with ATP levels[72]. This indicates that if the $NAD^+$ level becomes low enough, it affects ATP levels.

This could be the case with excess DNA damage. In cells undergoing DNA damage, PARP is activated, which produces high levels of nicotinamide at the expense of $NAD^+$. Although PARP enzyme activity is necessary to maintain genetic stability in the long term[73], there are numerous reports showing that the inhibition of PARP is beneficial at least temporarily[73,74]. This is likely due to maintaining $NAD^+$ and ATP levels. Streptozotocin, used to render rats diabetic, causes β-cell DNA damage. This activates PARP, resulting in lowering NAD and ATP, and apoptosis. A high-dose injection of nicotinamide (250 mg/kg) has been used to prevent streptozotocin-treated rats from
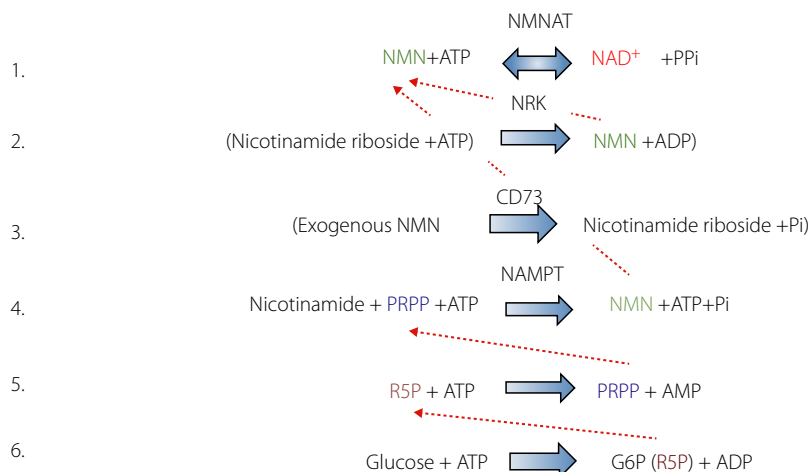
**Figure 4** | Nicotinamide adenine dinucleotide (NAD$^+$) synthesis cascade from nicotinamide (salvage pathway). NAD$^+$ catabolite, nicotinamide, can be salvaged to re-synthesize NAD$^+$ by the reaction of nicotinamide mononucleotide adenylyltransferase (NMNAT) from nicotinamide mononucleotide (NMN) (1). NMN is produced by nicotinamide phosphoribosyltransferase (NAMPT) (4) from phosphoribosyl diphosphate (PRPP), which originates from pentose phosphate pathway product, ribose-5-phosphate (5, 6). In addition to this endogenous cascade, nicotinamide riboside (NR), a milk ingredient, can produce NMN (2). Recently, NMN has been shown to boost NAD$^+$. The majority of exogenously administered NMN is believed to be decomposed to nicotinamide riboside (3). One of the peculiarities of the salvage pathway is its dependency of adenosine triphosphates (ATPs). In addition, because of the reversibility of NMNAT reaction (1) there is a positive correlation between cytosolic NAD$^+$ and ATP. NRK, nicotinamide riboside kinase (ribosylnicotineamide kinase); RSP, ribose-5-phosphate.

developing full diabetes. Similarly, nicotinamide prevents autoimmune diabetes in animal models, possibly through inhibition of PARP and prevention of β-cell NAD$^+$ depletion[75]. This was a main rationale for the European Nicotinamide Diabetes Trial (ENDIT), a clinical trial to prevent type 1 diabetes by nicotinamide. Nicotinamide can inhibit PARP activity by 90% at 1 mmol/L. Although nicotinamide levels were not measured in pancreatic β-cells, such levels of nicotinamide have been shown to prevent PARP activation and also promote NAD$^+$ synthesis[76].

## AMPK ACTIVITY IN TYPE 1 AND 2 DIABETES

Energy homeostasis is believed to be regulated by AMP-activated protein kinase, which is activated by lower ATP/(ADP or AMP) ratios[19,77]. AMPK activation upregulates catabolic processes and downregulates anabolic processes to restore ATP levels[19,77]. Because of the energy imbalance reported in type 1 diabetes, AMPK could be increased. We observed this in the retina from streptozotocin-induced diabetic rats (Figure 5).

In contrast to type 1 diabetes, AMPK activity is thought to be downregulated, not upregulated in the tissues of type 2 diabetes. The clinically approved drug for type 2 diabetes, metformin, is known to activate AMPK[78], and its action is believed to promote beneficial effects in preventing fatty liver and cardiovascular events. Thus, although type 2 diabetes might have an energy production problem likely by mitochondrial dysfunction[79–81], the energy levels are not low enough to activate AMPK. Rather, AMPK is downregulated. We observed this

phenomenon in the retinas from *db/db* mice (Figure 6). High-fat diet and type 2 diabetes are associated with insulin resistance and lipid metabolism abnormalities resulting in high DAG levels[10,82]. High DAG activates protein kinase C and D1, which phosphorylates AMPK alpha1 S487[83] and alpha2 S491[84], respectively. The phosphorylation of these sites inhibits AMPK activity.

## NICOTINAMIDE IS A SUBSTRATE FOR NAD$^+$ SYNTHESIS, AND A PRODUCT AND INHIBITOR OF SIRTUINS

The metabolic cascade of NAD$^+$ synthesis from nicotinamide is shown in Figures 4 and 7. Nicotinamide is incorporated into the synthetic salvage cascade with phosphoribosyl diphosphate (PRPP), which is a product of the pentose phosphate pathway[66,70]. Production of PRPP requires ATPs[70]. The nicotinamide incorporation reaction by NAMPT itself does not require ATP, but this reaction is strongly activated when coupled with ATP hydrolysis[85]. The NMNAT reaction that converts NMN to NAD$^+$ also uses ATP.

Endogenous nicotinamide is mainly produced by sirtuin family enzymes and PARP (poly ADP-D-ribosyl polymerase or NAD$^+$ ADP-ribosyltransferase)[1,4]. Both enzymes mediate gene repair, DNA stability[73] and increase under stress conditions, such as fasting and aging. They use NAD$^+$ as the substrate and produce nicotinamide as a product. The Km for NAD$^+$ of sirtuin family members is 100–200 μmol/L, whereas that for PARP1 is 20–60 μmol/L[4]. The estimated nuclear (cytosol)
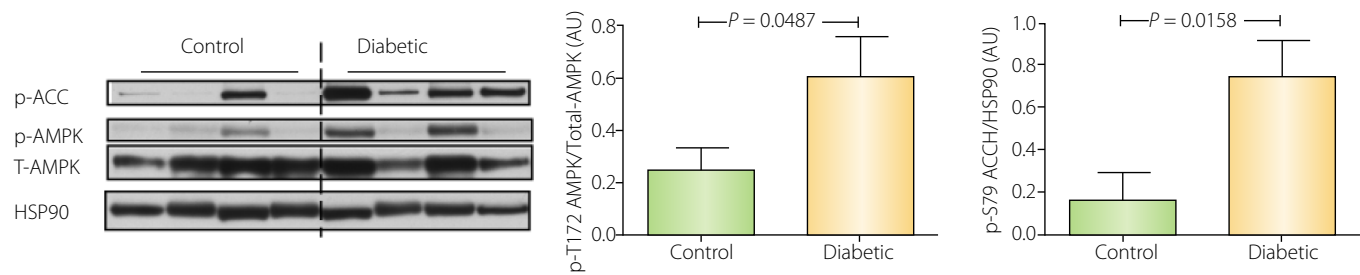
Retain from 6 months STZ diabetic rats

**Figure 5** | Activation of adenosine monophosphate-activated protein kinase (AMPK) in the retina from 6 months streptozotocin (STZ) diabetic rats. Male Wistar rats with bodyweight of 150 g were obtained from Charles River (Boston, MA, USA). Diabetes was induced by a single injection of 50 mg/kg STZ. At 6 months' duration of diabetes, rats were killed with a lethal dose of pentobarbital and their eyes were quickly removed. The retina was sampled and frozen in liquid nitrogen. The whole retina was ground with Western blot sample buffer, then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blotting. Type 1 insulinopenic diabetes showed an increased phospho-Thr172 AMPK and phospho-Ser79 ACC (AMPK substrate) in the retina, which reflects decreasing energy levels.

NAD$^+$ levels are approximately 70 µmol/L[4]. Therefore, increasing NAD$^+$ levels would increase sirtuin family enzyme activity, but not PARP1. Both enzymes can be inhibited by nicotinamide. The IC50 for sirtuins is reported to be 30–70 µmol/L[86], whereas PARP1 can be inhibited 90% by 1 mmol/L nicotinamide[87]. In the case of DNA damage, PARP is activated. Because of the higher IC50 for nicotinamide inhibition and low Km for NAD$^+$, the PARP enzyme can consume cellular NAD$^+$ to the levels low enough to cause ATP depletion and cell death.

## NICOTINAMIDE CATABOLITES AND THE EFFECTS OF DIABETES

Unused nicotinamide for the salvage reaction is mainly methylated and oxidized, and excreted in urine (Figure 7). The major nicotinamide catabolites found in human urine are N-methyl-2 or 4-pyridone-5-carboxamide (2 or 4-YP) and N-methylnicotinamide[88]. In both rodent models of type 2 diabetes (*db/db* mouse; obese Zucker rat) and human type 2 diabetes, urinary section of N-methylnicotinamide and 2-YP are increased[89]. This suggests downregulation of nicotinamide salvage activity. The conversion of N-methylnicotinamide to 2- or 4-PY is mediated by aldehyde oxidase[88], which produces H$_2$O$_2$ as a by-product. In humans, this enzyme is widely expressed in many tissues and cells[90]. In *Caenorhabditis elegans*, this enzyme is responsible for a transient increase of H$_2$O$_2$ production, and is required for the lifespan extension effects of Sir2.1[91]. Such hormesis action (short and mild stress, which is beneficial for health) could occur in higher organisms too. In humans, physical exercise promotes health benefits because of increasing reactive oxygen species (hormesis). Skeletal muscle produces and secretes interleukin-6, which is known to activate AMPK[92,93]. Consistent with this scenario, anti-oxidant treatment was shown to inhibit exercise's beneficial effects[94]. However, sustained oxidative stress could be harmful. Thus, production of high

nicotinamide and H$_2$O$_2$ could damage the cells (Figure 7). Indeed, some suggest that nicotinamide overload causes oxidative stress and insulin resistance[95,96]. There is a distinct possibility that oxidative stress produced by this reaction contributes to DNA damage and PARP activation, which could form a vicious cycle.

The enzyme that converts nicotinamide to N-methylnicotinamide, nicotinamide n-methyltransferase (NNM) utilizes the universal methyl donor S-adenosyl-L-methionine as a co-substrate, and because of this, it might control local S-adenosyl-L-methionine concentration and methylation reactions, including phosphatase and epigenetics[97].

## CONTROL OF NAD$^+$ SYNTHESIS BY SUBSTRATE PRPP AND DIABETES

Nicotinamide is not the only molecule determining NAD$^+$ synthesis by NAMPT. The reaction also requires PRPP, which is another substrate, and is considered as rate limiting. PRPP comes from ribose-5-phosphate, which is the product of the pentose phosphate pathway (Figures 4,7)[70]. The production of ribose-5-phosphate, PRPP and NAD$^+$ synthesis was shown to be regulated by the transketolase reaction. Phosphorylation of trasketolase at T382 by protein kinase B greatly activates the enzyme activity and NAD$^+$ production[98]. Thus, insufficient protein kinase B activity in diabetes likely contributes to the reduction of transketolase activity. Transketolase activity requires thiamine as the co-factor[70]. In both type 1 and 2 diabetes, plasma thiamine concentration is reported to be decreased by 75% as a result of high renal clearance[99,100].

## NICOTINAMIDE, NAD$^+$ SYNTHESIS AND SIRT1-AMPK CASCADE

AMPK activators have been shown to increase NAD$^+$ levels, which results in SIRT1 activation (Figures 3,7)[65]. There are a few points we would like to highlight here based on our data.
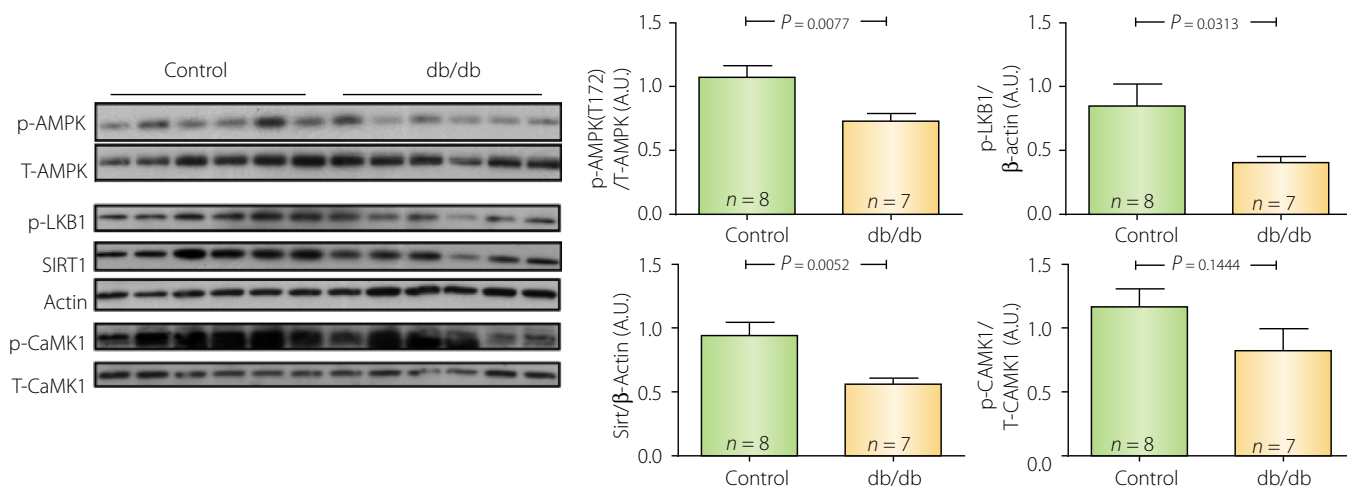
Retina from 1 month diabetes in db/db mice

**Figure 6** | Suppression of sirtuin-1 (SIRT1)–liver kinase B1 (LKB1)–adenosine monophosphate-activated protein kinase (AMPK) cascade in *db/db* type 2 diabetic mice. In contrast to streptozotocin rats (Figure 5), retinas from 3 month-old *db/db* mice with 1 month of confirmed diabetes (vs non-diabetic *db/w*+ littermates) showed decreased pAMPK, pLKB1 and Sirt1 protein levels.
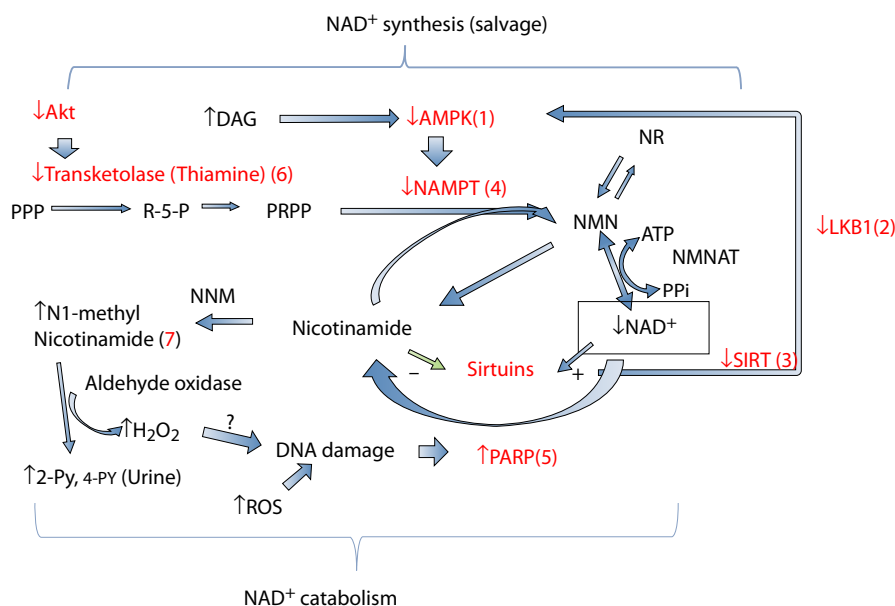


**Figure 7** | Effects of diabetes on nicotinamide adenine dinucleotide (NAD$^+$) metabolisms. In type 2 diabetes, reduced sirtuin-1 (SIRT1)–liver kinase B1 (LKB1)–adenosine monophosphate-activated protein kinase (AMPK) cascade (1–3) decreases nicotinamide phosphoribosyltransferase (NAMPT) expression (4), which decreases NAD$^+$ synthesis. Increased reactive oxygen species (ROS) activates poly-adenosine diphosphate ribosyl-polymerase (PARP) (5) and increases NAD$^+$ consumption. This reaction occurs strongly in insulinopenic type 1 diabetes. In both diabetes, decreased plasma thiamine and reduced transketolase activity might cause impaired phosphoribosyl diphosphate (PRPP) production (6). Excessive nicotinamide increases nicotinamide catabolites N1-nicotinamide, N-methyl-2-pyridone-5-carboxamide (2-PY), 4-PY production (7) and produces more H$_2$O$_2$ by aldehyde oxidase.

We found that AMPK activation in HepG2 cells incubated with 1 mmol/L metformin or 15 µmol/L resveratrol for 16 h increased NAD$^+$ levels by approximately 30% (Figure 8a). Additionally, the messenger ribonucleic acid expression of the rate-limiting enzyme, NAMPT, was increased in 2–5 h by incubation with 1 mmol/L 5-aminoimidazole-4-carboxamide ribonucleotide, an AMPK activator, although the effect was transient (Figure 8b). Increased NAD$^+$ levels with 1 mmol/L

metformin were also observed in wild-type mouse embryonic fibroblasts, whereas no increase was observed in AMPK α1&2 double knockout mouse embryonic fibroblasts (Figure 9a). These results clearly show that AMPK activators increase NAD+ in an AMPK-dependent fashion. We also found that addition of 10 mmol/L nicotinamide in the culture media (5 mmol/L glucose minimal essential medium) was more effective at increasing NAD+ (Figure 8a). We assessed the Km values of NAMPT for nicotinamide using wild-type mouse embryonic fibroblasts and human embryonic kidney 293 (HEK293) cells that were transfected with a human NAMPT1 plasmid. We found a 10-fold higher Km for nicotinamide in human NAMPT (10 µmol/L) than in mouse NAMPT (0.9 µmol/L; Figure 9b). These values are in good agreement with some of the values previously reported[101,102]. This means that human cells do not efficiently utilize nicotinamide for synthesis of NAD+ if the concentration of nicotinamide is low.

Therefore, would incubation with high nicotinamide benefit human cells exposed to high glucose? We assessed this effect on cellular senescence in cultured human retinal pericytes. When human retinal pericytes were incubated with 25 mmol/L glucose for 2 weeks, a percentage of senescent cells (senescence-associated galactosidase-positive cells) was increased by fourfold (Figure 10). This increase was further augmented by an addition of 1 mmol/L nicotinamide. In contrast, the addition of 20 µmol/L resveratrol in 25 mmol/L glucose medium completely prevented the increase. The results are consistent with that NAD+ dysregulation might occur under high glucose, and under this condition, nicotinamide worsens the *in vitro* aging.

## SUMMARY OF THE PLAUSIBLE MECHANISMS OF DYSREGULATION OF NAD+ METABOLISM IN DIABETES

As mentioned in the beginning, dysregulation of NAD+ metabolism occurs in various tissues in diabetes, which might be the

etiology of diabetic complications and type 2 diabetes. Increased NAD+ catabolism for DNA repair appears to be a primary cause for the decrease in NAD levels, especially in insulinopenic type 1 diabetes models, such as the streptozotocin-induced diabetic rat (Figure 11). Continuous low levels of tissue NAD+ and ATP in this model attest that NAMPT-mediated compensatory mechanism somehow does not work properly, albeit even with activation of AMPK. The reason is still not clear. ATP levels might be too low to spare for NAD+ synthesis. Imai *et al.*[103] and others found that the conversion ratio of tryptophan to nicotinamide was lower in streptozotocin- and alloxan-induced diabetes, suggesting that de novo synthesis of NAD+ from tryptophan appears to be impaired. This phenomenon was not observed in a type 2 diabetes model; Goto-Kakizaki rats[104].

In type 2 diabetes animal models, in addition to an increase in NAD consumption by PARP, inactivation of AMPK is another factor (Figure 11). Impaired AMPK activity was reported in type 2 diabetes in skeletal muscle[105], and fat tissue in obese individuals[106]. As aforementioned, activation of protein kinase C and protein kinase D by increased DAG is likely a part of the mechanism.

## NAD+ TARGETING TREATMENTS FOR PATIENTS WITH DIABETES

Ideally, what we need are methods to increase NAD+ levels without increasing nicotinamide or its catabolites (Figure 12). Increasing expression of intracellular NAMPT is the primary goal to do so. Thus, AMPK activation is the key target for type 2 diabetes. It was shown in humans that aerobic, as well as resistance, exercise, which both activate skeletal muscle AMPK, increased the expression of NAMPT in skeletal muscle by 25–30%[107]. In contrast, it failed to do so in adipose tissue, suggesting that the modality of AMPK activation must be specific for the target tissue in which AMPK needs to be activated. Thus, starvation should activate AMPK and increase
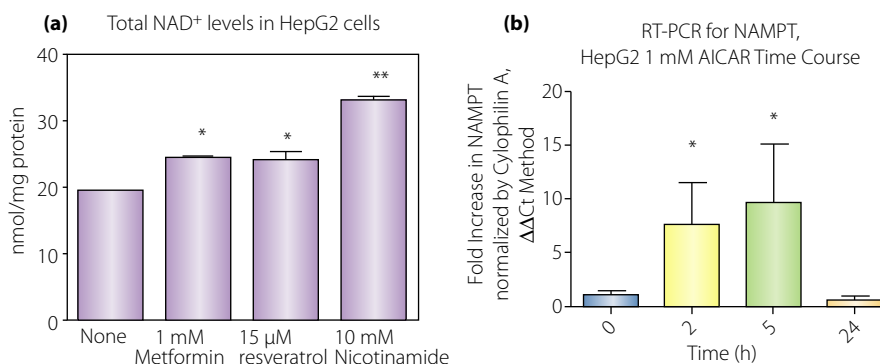


**Figure 8** | Adenosine monophosphate-activated protein kinase (AMPK) activation, nicotinamide adenine dinucleotide (NAD+) levels and nicotinamide phosphoribosyltransferase (NAMPT) expression. (a) HepG2 cells cultured in minimal essential medium were incubated with the indicated concentration of reagents overnight. Total NAD+ was then assessed in these cells. AMPK activators, metformin and resveratrol increased NAD levels (*n* = 3, *P < 0.05 vs none). 10 mmol/L nicotinamide without AMPK activators could also increase NAD+ levels (*n* = 3, **P < 0.01 vs none). (b) NAMPT expression was stimulated by1 mmol/L 5-aminoimidazole-4-carboxamide ribonucleotide (an AMPK activator). NAMPT messenger ribonucleic acid expression was increased seven- to tenfold by activation of AMPK (*n* = 3, *P < 0.05) in 2–5 h, but returned to baseline at 24 h.
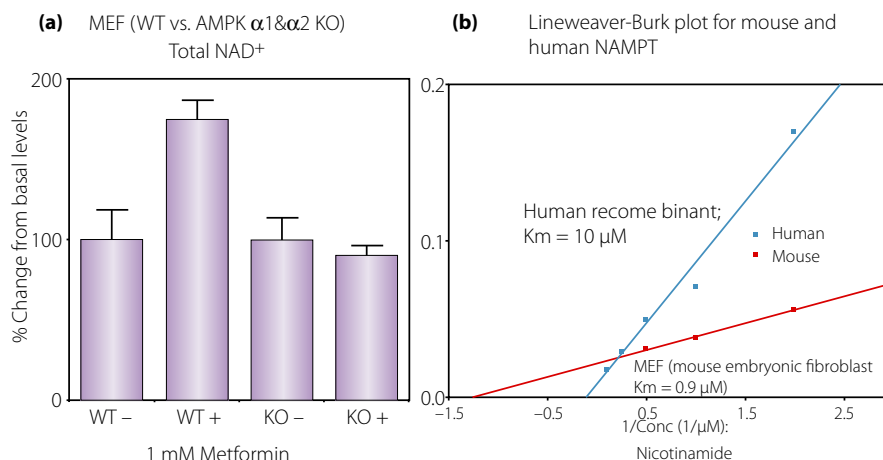
Figure 9 | (a) Requirement of adenosine monophosphate-activated protein kinase (AMPK) for increasing nicotinamide adenine dinucleotide (NAD+) levels by metformin. Mouse embryonic fibroblasts (MEF) were incubated with(+)/without(−) 1 mmol/L metformin overnight and NAD+ was assessed. Metformin was effective to increase NAD+ in wild-type MEFs, but ineffective in AMPK α1&2 double knockout MEFs. (b) Km for nicotinamide of mouse versus human nicotinamide phosphoribosyltransferase (NAMPT). Mouse NAMPT Km was determined from cell protein homogenates of wild-type MEFs. Human NAMPT1 was overexpressed in HEK293 cells and protein homogenates were used to determine the Km for nicotinamide. An average of two measurements were used to draw the plots. Estimated Km for nicotinamide was 0.9 μmol/L for mouse NAMPT and 10 μmol/L for human NAMPT.

NAMPT activity in the liver. In diabetes patients, in addition to exercise and caloric restriction, the effect can be mimicked by taking metformin. Metformin activates AMPK in many tissues[78]. This activation was attributed to decreased fat accumulation and glucose production in the liver of obese and diabetic mice, although the latter appears to be AMPK independent, as shown in AMPK-null mice[108]. In addition to metformin, we previously showed that glucagon-like peptide-1 can activate AMPK in endothelial cells[109]. Sodium–glucose cotransporter 2 inhibitor, canagliflozin, is also found to activate AMPK through inhibiting mitochondria[110]. As insulin potentially suppresses SIRT1-AMPK activity, increasing insulin levels in type 2 diabetes might work negatively.

Resveratrol activates both SIRT1 and AMPK[14,18]. SIRT1 activation occurs by: (i) direct binding at the N-terminus, which activates it in a substrate-specific manner[111]; (ii) resveratrol's phosphodiesterase inhibitor activity[112,113], which leads to increasing cyclic AMP-dependent protein kinase activity, then phosphorylating SIRT1 at Ser434[114]; and (iii) binding lamin A, which stabilizes SIRT1 nuclear localization[115]. AMPK activation is induced by: (i) activation of the SIRT1–LKB1–AMPK cascade[15]; (ii) ATP synthesis inhibitor activity[116]; and (iii) phosphodiesterase inhibitor action, which increases cyclic AMP, leading to increased intracellular $Ca^{2+}$ levels through exchange protein directly activated by cyclic AMP-$Ca^{2+}$/calmodulin-dependent protein kinase kinase-β, which activates AMPK[112]. In humans, bioavailability of resveratrol is a major problem. Previously, now a defunct company, Sirtris developed SRT501 a proprietary formulation of resveratrol to increase bioavailability. In a phase Ib study, patients with type 2 diabetes treated for 28 days with SRT501 showed significantly lower fasting glucose levels (https://www.gsk-studyregister.com/study/SRT-501-006?legacy=true). Similar studies have been carried out and recently systematically reviewed[117], suggesting that resveratrol consistently lowered fasting blood glucose, insulin levels, homeostatic model assessment index and blood pressure. However, the effects on glycated hemoglobin were negligible. As compared to SRT501, the bioavailability of other currently available resveratrols is currently unknown. Quercetin might have similar properties[18], but the efficacy has not been well studied.

For fatty acid toxicity, the acyl group in acetyl coenzyme A can be sequestered by free carnitine. It was shown that both carnitine or acetyl-L-carnitine are effective in improving insulin-mediated glucose disposal in humans[118]. Acetyl-L-carnitine and propyonyl-L-carnitine were tested on various complications in diabetic animal models, showing their efficacy. We previously reported that the levels of DAG can be decreased by acetyl-L-carnitine[23,24], which also normalized suppression of energy utilization by $Na^+$-$K^+$-ATPase and redox change. Currently, acetyl-L-carnitine is prescribed for diabetic neuropathy in some countries. There is borderline efficacy for this condition[119–121].

In case glycemic levels are not well controlled, polyol pathway activation is the main source of NAD+/NADH change. Activation of polyol pathway was postulated to be the main cause for PARP activation in diabetic rats[122,123]. This observation was not examined in human diabetes. However, aldose reductase inhibitor could be prescribed for the treatment.

It is not clear whether a flux from ribose-5-P of pentose phosphate pathway to PRPP is decreased in human diabetes. Based on decreased plasma thiamine levels observed, this might be occurring. At least supplementation with thiamine, or the more active compound, benfotimine, is not harmful.
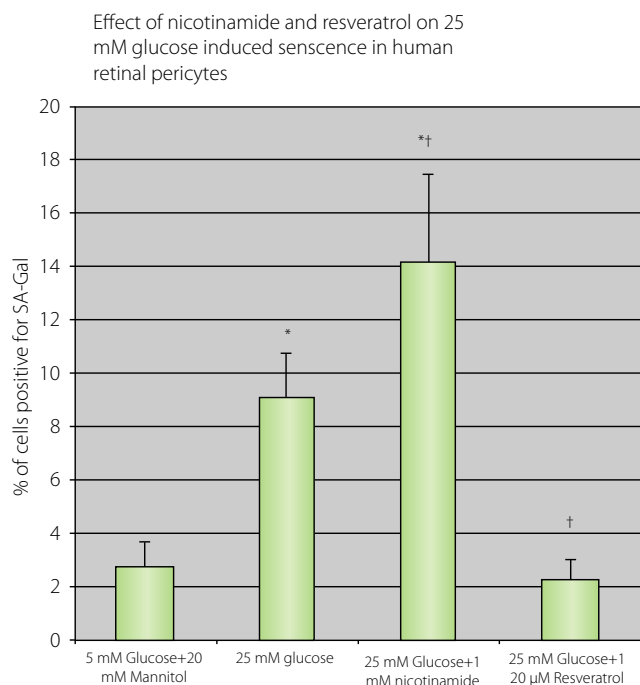
Effect of nicotinamide and resveratrol on 25 mM glucose induced senscence in human retinal pericytes



**Figure 10** | Effects of 25 mmol/L glucose and addition of 1 mmol/L nicotinamide or 25 μmol/L resveratrol on premature senescence in culture human retinal pericytes. Human retinal pericytes were cultured in 5% fetal bovine serum with EGM2 media for 10 days with the addition of indicated concentration of reagents. The cells were fixed and senescence associated galactosidase staining was carried out. Then, 25 mmol/L glucose increased a number of senescence cells by threefold (*$P < 0.05$ vs 5 mmol/L glucose, $n = 4$). The addition of 1 mmol/L nicotinamide in the medium further increased (*$P < 0.05$ vs 5 mmol/L glucose and †$P < 0.05$ vs 25 mmol/L glucose, $n = 4$); 20 μmol/L resveratrol treatment eliminated 25 mmol/L glucose effects (†$P < 0.05$ vs 25 mmol/L glucose).

Benfotiamine was shown to prevent diabetic retinal and kidney complications in diabetic animals[124,125]. In human diabetes, benfotiamine was tested on diabetic neuropathy and found to have borderline efficacy[126,127].

## NICOTINAMIDE AND ITS RELATED COMPOUNDS FOR HUMAN DIABETES

Enzymatic activities involving nicotinamide metabolism varies depending on species and even on strains in rodents. Thus, the effects of nicotinamide and its related compounds cannot be extrapolated from rodents to humans. Despite of effectiveness in rodent models, nicotinamide did not produce a positive result in human diabetes. The European Nicotinamide Diabetes Intervention Trial failed to show any preventative effect of nicotinamide for the onset of type 1 diabetes[75]. Rather, there is a report suggesting that nicotinamide administration causes insulin resistance in human[128]. Zhou *et al.*[95] reported that N-methylnicotinamide by itself causes insulin resistance, and suggested that nicotinamide overload might cause type 2 diabetes. In agreement with this observation, human adipose tissue
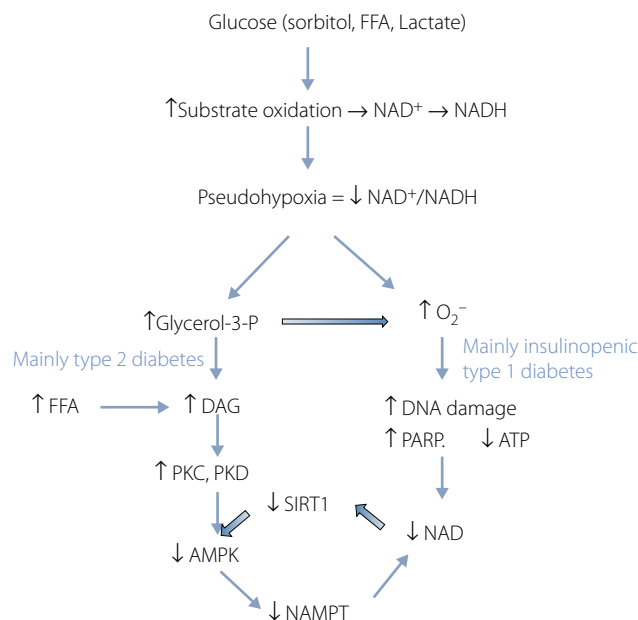


**Figure 11** | Summary of the cascade leading to lower nicotinamide adenine dinucleotide (NAD⁺) levels in diabetes. High glucose increases sorbitol levels by aldose reductase. Oxidation of sorbitol, free fatty acid (FFA) and lactate cause production of excess NADH. This leads to a low NAD⁺/NADH ratio (pseudohypoxia), which mainly causes activation of two pathways: diacylglycerol (DAG)–protein kinase C (PKC) and (DAG)–protein kinase D (PKD) cascade, which occur preferentially in type 2 diabetes and high levels of oxidative stress that occur in insulinopenic type 1 diabetes. Both cascades decrease NAD⁺ levels. ATP, adenosine triphosphate; DNA, deoxyribonucleic acid; PARP, poly adenosine diphosphate-D-ribosyl polymerase; SIRT1, sirtuin 1.

NNMT, the enzyme that produces N-methylnicotinamide, activity correlates positively with adiposity and insulin resistance[129], and serum N-methylnicotinamide levels are associated with obesity and diabetes[130].

Nicotinamide riboside, which is contained in cow milk[131], has recently been tested in humans[132,133], and showed increased blood NAD⁺ levels. It produces NMN by the action of nicotinamide ribose kinase[131] (Figure 4) or nicotinamide by purine nucleotide phosphorylase. If NR goes through the nicotinamide ribose kinase reaction, then PRPP is not required nor is it controlled by the NAMPT reaction.

In rodents, NR increases skeletal muscle NAD⁺ levels. However, this is not the case for humans. This might be related to the different Km for nicotinamide, as aforementioned. NR 1 g per day given to older people for 21 days [70–80] increased NAD⁺ levels in the blood, but not in skeletal muscle[134]. Blood and skeletal muscle nicotinamide levels were not increased, whereas methylnicotinamide and N-methyl-2-pyridone-5-carboxamide (2-PY) levels were increased in muscle, blood and urine. This study suggests that NNM activity might be high enough not to accumulate nicotinamide. Another study in which NR 2 g/day was given to obese and insulin-resistant men for 12 weeks also
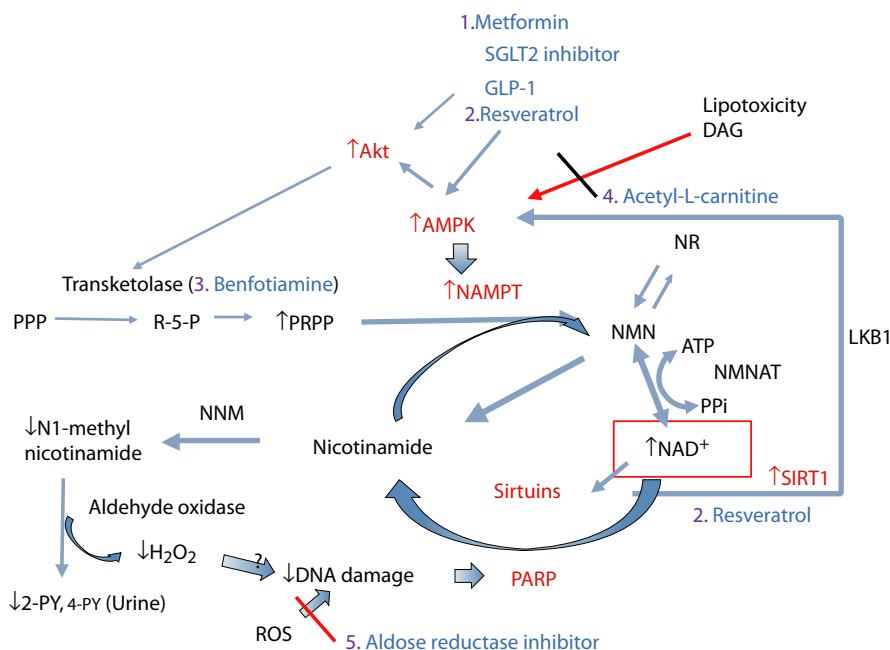
**Figure 12** | Proposed treatments to correct nicotinamide adenine dinucleotide (NAD$^+$) levels in diabetes. Figure 7 showed abnormalities of NAD$^+$ metabolism occurring in diabetes. To correct these abnormalities, proposed regimens are: (1) metformin, sodium–glucose cotransporter 2 (SGLT2) inhibitor, glucagon-like peptide-1 (GLP-1); (ii) resveratrol, other polyphenols or sirtuin-activating compoundssirtuin-activating compounds (STACs) to activate sirtuin 1 (SIRT1)–adenosine monophosphate-activated protein kinase (AMPK) for type 2 diabetes; (iii) benfotiamine to boost substrate (phosphoribosyl diphosphate [PRPP]) production; (iv) acetyl-L-carnitine to block diacylglycerol (DAG) production and lipotoxicity; and (v) aldose reductase inhibitor if glucose control is inadequate. 2-PY, N-methyl-2-pyridone-5-carboxamide; Akt, protein kinase B; AMPK, AMP-activated protein kianse; ATP, adenosine triphosphate; DNA, deoxyribonucleic acid; LKB1, liver kinase B1; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; NNM, nicotinamide n-methyltransferase; PPi, pyrophosphate; PPP, pentose phosphate pathway; R-5-P, ribose-5-P; ROS, reactive oxygen species.

showed no increase in NAD$^+$ levels in skeletal muscle, nor in mitochondrial content and function[135]. Instead, it reduces NAMPT protein expression by 15%. This study also found skeletal muscle NAMPT protein levels were negatively correlated with plasma C-peptide during oral glucose tolerance test and fasting insulin levels. Collectively, the study did not prove the efficacy of NR, rather it appears to suppress SIRT1-AMPK. Neither study reported insulin levels nor glucose tolerance test.

More recently, NMN was tested in rodents[39,136]. Compared with NR, the metabolism of orally administered NMN is not fully understood. It is believed that unlike NR, NMN does not penetrate cell membranes because of phosphate group[1,4]. *In vivo*, CD73 (NADase) removes the phosphate group producing NR, which can be incorporated in cells (Figure 4)[1,4,5]. The compound has not been extensively tested in humans, but similar results of NR might be expected.

Collectively, in light of potential harmful effects of nicotinamide catabolites, nicotinamide-related compound is not recommended for diabetes treatment until more studies are carried out.

## POTENTIAL DRUGS IN DEVELOPMENT
NNMT inhibitor has been developed and tested in mice[137,138]. Inhibition of the enzyme might divert nicotinamide from catabolism toward more NAD$^+$ synthesis. NNMT overexpression decreases NAD$^+$ levels and causes fatty-liver in mice[139]. This enzyme inhibition should reduce a consumption of the universal methyl donor, S-adenosyl-L-methionine, used for nicotinamide methylation, and reduces the production of S-adenosyl-L-homocysteine. Potentially, it reduces atherogenic homocysteine levels and facilitates an induction of epigenetic change by methylation. A potential side-effect is to increase nicotinamide levels that inhibit sirtuins.

A direct AMPK activator has been developed, and is in clinical trials for efficacy and safety[140]. A direct NAMPT activator was identified recently[141], but is still in a very early stage of development. Imeglimin is a new antidiabetic drug that recently finished phase III clinical testing in Japan. In rodents, the drug increases mitochondrial numbers, and reduces oxidative stress and DAG production, and maintains high carnitine levels in high-fat high sucrose mice[142]. If the similar effects are observed in humans, it should benefit NAD$^+$ metabolism.

## MONITORING THE 'HEALTHINESS' OF NAD$^+$ METABOLISM
How can we assess NAD$^+$ metabolism and the effectiveness of interventions in humans? In healthy conditions, our hypothesis

is that nicotinamide production by PARP or sirtuins is low, and produced nicotinamide should be preferentially used for $NAD^+$ salvage processes and not for catabolic processes. Therefore, 'healthiness' might be monitored by measuring levels of tissue $NAD^+$ and levels of urinary nicotinamide catabolites. In humans, measuring tissue $NAD^+$ is very challenging in the clinical setting. Blood $NAD^+$ could be used as a surrogate maker, which reflects red blood cell $NAD^+$ content. Previously we found the RBC $NAD^+$ content varies from 100 to 200 nmol/g Hb, and NADH levels are approximately 1/10th of $NAD^+$. It can be assessed by various methods, including enzyme cycling assay[143]. Urinary nicotinamide catabolites (N-methylnicotinamide, 2-PY and 4-PY) can be assessed by high-performance liquid chromatography[144] and nuclear magnetic resonance[134]. Consistent with our hypothesis, nicotinamide catabolites are known to be increased by type 2 diabetes in both rodent models and humans[89]. In addition, a recent report showed that C57BL/6 mice fed with a high-fat diet had a two- to threefold increase in 2-PY and 4-PY, as compared with mice fed a normal chow diet, and those levels were significantly reduced by treatment with metformin + vildagliptin (dipeptidyl peptidase inhibitor)[145], also attesting our hypothesis. It is known that both $NAD^+$ salvage activity[146] and nicotinamide catabolites[147] show diurnal variations as a result of circadian rhythm. Thus, timing for measuring these metabolites will be an issue. Previous studies did not take this into an account. After standardization of the timing, NAD metabolism 'health index' can be assessed as (blood $NAD^+$) / (urinary nicotinamide catabolites), which are expected to be low with diabetes and become higher in healthy individuals. A previous study giving NR to humans[134] showed that blood $NAD^+$ levels increased two- to threefold, but urine methylnicotinamide and 2-Py levels increased eight- to tenfold. This suggests NR supplementation did not improve 'healthiness.' This interpretation agrees with another NR study that showed decreased NAMPT expression.

## CONCLUDING REMARKS

In this review article, we pointed out that the etiology of $NAD^+$ dysregulation remarkably coincides with the cascades postulated for the etiology of diabetic complications and other abnormalities induced by redox change. We also presented the new possibility that the nicotinamide catabolic cascade might play an important role, especially in oxidative stress production and epigenetic change in diabetes. For the treatment of type 2 diabetes and its complications, we recommend first to take AMPK-activating medicines, such as metformin, glucagon-like peptide-1 agonist and sodium–glucose cotransporter 2 inhibitor. In particular, clinical data showing the ketogenic effect of sodium–glucose cotransporter 2 inhibitor seems to alleviate fatty acid toxicity and to reverse selective insulin resistance in the liver by mobilizing peripheral fat to the liver. In many countries, benfotiamine and acetyl-L-carnitine are available, which might help. If glucose control is not sufficient, aldose reductase inhibitors could be taken, although they are only

available in certain countries. Taking a nicotinamide supplement, whether NR or NMN, at a high dose is not currently recommended until safety is established. However, with other regimens to improve 'healthiness' of $NAD^+$ metabolism, it might be useful, as shown in a recent case report that acetyl-L-carnitine and nicotinamide treatment could prevent the development of type 1 diabetes[148].

## DISCLOSURE

The authors declare no conflict interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Data S1** | Supplemental materials.