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Is NAD$^+$ a key factor in ovarian aging and dysfunction? Insights and uncertainties from current research†

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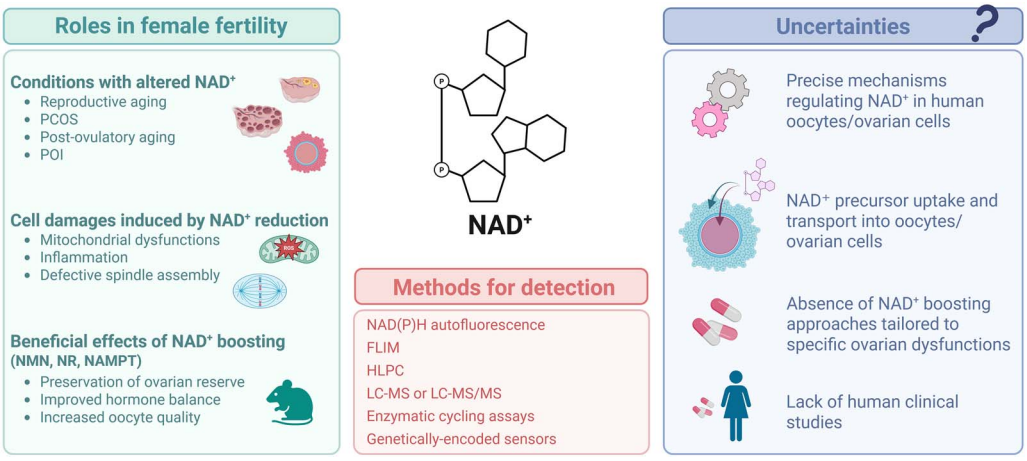
Abstract

Recent findings highlight NAD$^+$ as a central regulator of various cellular processes, including energy metabolism, stress response, and aging. Growing evidence of the benefits associated with dietary NAD$^+$ precursors has elevated NAD$^+$ to a promising therapeutic target for addressing female infertility. This review aims to evaluate existing literature on the mechanisms governing the availability and utilization of NAD$^+$ in the ovaries and its alterations in female reproductive disorders, with a particular focus on ovarian aging and dysfunction including polycystic ovary syndrome and premature ovarian insufficiency. Alongside data from in vivo and in vitro studies on various NAD$^+$ boosters, this review incorporates findings from research on genetic mutations, polymorphisms in human and animal populations, and insights from transgenic animal models. The present work emphasizes that NAD$^+$ deficiency is largely driven by a combination of factors, including heightened consumption, impaired utilization efficiency, and diminished biosynthesis or transport. Based on this analysis, we suggest that the ovary possesses its own unique NAD$^+$ metabolism, but our understanding of its regulatory mechanisms is still in its infancy. Key questions remain unanswered, such as how NAD$^+$ and its precursors are transported into oocytes and ovarian cells, their specific preferences for different NAD$^+$ precursors, as well as the specific changes associated with different ovarian dysfunctions. Finally, we reviewed methods for studying NAD$^+$ metabolism as essential tools for investigating the potential of NAD$^+$ boosting therapies to counteract ovarian aging and dysfunction.

Summary Sentence

This review highlights the critical role of NAD$^+$ metabolism in ovarian function, exploring its regulation, disruption in reproductive disorders, and the therapeutic potential of NAD$^+$-boosting strategies against ovarian aging and dysfunction.

Graphical Abstract



Key words: female fertility, PCOS, oocyte, NAMPT, sirtuins, mitochondria, FLIM, aging.

Introduction

Recent scientific advancements have highlighted NAD (nicotinamide adenine dinucleotide) as a fundamental molecule regulating numerous cellular processes, including energy metabolism, stress response, and aging. As a central player in redox reactions, NAD facilitates the transfer of electrons in metabolic pathways, thereby sustaining cellular energy production. Research over the past decades, and particularly more recent studies, clearly demonstrate that the importance of NAD extends to non-redox functions revealing that NAD metabolism is very complex and dynamic. NAD can be converted into several molecules that play key roles in energy transduction and cell signaling, like nicotinamide adenine dinucleotide phosphate (NADP), nicotinic acid adenine

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dinucleotide phosphate (NAADP), and cyclic ADP-ribose (cADPR). Products of NAD degradation such as nicotinamide (NAM) and n-methyl-nicotinamide have also emerged [1]. NAD pathway metabolites can also serve as substrates for a group of diverse enzymes, including PARPs, sirtuins, CD38, SARM1, and RNA polymerases, which are involved in several aspects of cellular homeostasis [2, 3]. It becomes evident that alterations in NAD levels have a deep influence on cellular bioenergetics, energy metabolism, and cell response to stressing conditions. Dysregulation in NAD metabolism has emerged as a contributing factor in the pathogenesis of several diseases, and the so-called NAD^+ boosting therapy has been proposed as an approach to treat different human diseases [4, 5]. More recently, increasing evidence has underscored the role of NAD^+ in maintaining ovarian function and suggested its potential as a therapeutic target in addressing female infertility [6–8].

The ovaries experience significant oxidative stress due to their dynamic nature, involving cycles of follicular growth, ovulation, and luteal function [9]. These processes necessitate efficient NAD^+-dependent pathways for maintaining mitochondrial health, counteracting oxidative damage, and ensuring proper oocyte quality [10].

Recent findings point to a decline in NAD^+ levels as a hallmark of ovarian aging, correlating with diminished oocyte quality and reduced fertility [8]. NAD^+ depletion is not only associated with aging ovaries but is also implicated in conditions such as polycystic ovary syndrome (PCOS) and premature ovarian insufficiency (POI) [11–14]. These observations have spurred interest in the potential of dietary NAD^+ precursors, such as nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN), to replenish NAD^+ stores and enhance ovarian function [15].

After providing knowledge about the pathways that govern NAD^+ biosynthesis, utilization, and degradation, we will discuss the mechanisms governing the availability and utilization of NAD in the ovaries and its alterations in ovarian aging, PCOS and POI. In addition to data about the effects of NAD^+ boosters, we will focus on findings from research on genetic mutations, polymorphisms and transgenic animal models, as well as on oocytes and granulosa cells. We will also discuss the current techniques for NAD measurement in oocytes and ovarian cells that represent a critical point for addressing unanswered questions in future research about NAD biology in female fertility.

NAD definition and functions

NAD is a crucial molecule in all living cells. It plays a key role as a co-factor in numerous biological pathways, particularly in cellular metabolism, besides serving as a precursor in the pyridine nucleotide family [16, 17]. NAD exists in two forms: NAD^+ (the oxidized form) is able to accept a hydride ion (formally, the anion of hydrogen, H^-) during biochemical reactions, thus forming its reduced form (NADH), that can transport the electrons gained by NAD^+.

Particularly, NAD^+ is the main co-enzyme for proteins implied in hydride transfer-dependent pathways, like metabolic processes including glycolysis, tricarboxylic acid (TCA) cycle, glutaminolysis, and fatty acid oxidation. The enzymes responsible for the catalysis of NAD^+ reduction into NADH are known as oxidoreductases or dehydrogenases. The electrons accepted by NADH in these metabolic reactions are then transferred to the electron transport chain (ETC), participating in ATP generation through mitochondrial oxidative phosphorylation (OXPHOS) [18]. Additionally, NAD^+ can be phosphorylated to generate $NADP^+$ (approximately 10% of the total NAD(H) is phosphorylated at the adenosine riboside site by NAD^+ kinases), which also serves as a hydride acceptor to form NADPH. Despite their structural similarities, NAD(H) and NADP(H) are generally recognized by different enzymes, thus leading to distinct functions. Indeed, NAD(H) is primarily involved in catabolic processes, while NADP(H) represents a key molecule in anabolic reactions requiring reducing power (e.g. reductive biosynthesis of fatty acids, cholesterol and steroids) and in defense mechanisms against both oxidative stress and pathogens infection (e.g. NADP(H) is used by glutathione reductase, NADPH-dependent thioredoxin reductase (NTR)/thioredoxin (Trx), and NADPH oxidase) [19].

NAD was discovered in the first years of twentieth century, when Otto Warburg isolated it from cozymase and demonstrated its involvement in hydrogen transfer and regulation of metabolic rates in yeast extracts [20, 21]. In the early 2000s, researchers showed a growing and renewed interest in studying NAD, especially after the discovery of its novel function as a co-substrate for the enzymes belonging to the sirtuin family, emerging regulators of both metabolic adaptation, response to stress, and longevity [22, 23].

Beyond its role in energy metabolism and sirtuins activity, it has been demonstrated that NAD^+ is the co-substrate of a variety of other enzymes, including NAD^+ glycohydrolases, also indicated as NADases, like cluster of differentiation 38 (CD38), CD157, and sterile alpha and toll/interleukin receptor motif-containing protein 1 (SARM1), and the protein deacylase family of poly(ADP-ribose) polymerases (PARPs) (see section "Enzymatic reactions requiring NAD^+" for more details) [24–26]. NAD^+ also acts as a 5′-terminal cap for bacterial and eukaryotic RNA, by protecting RNA against nucleases in prokaryotes, and marking transcripts for decay in eukaryotes [27–29].

Hence, dynamic changes, due to cellular stress or physiological stimuli, in the levels of NAD^+, which is always in high demand, can impact critical pathways such as epigenetic regulation, DNA repair, circadian rhythm, inflammation, redox balance, and mitochondrial function [30].

NAD^+ production

NAD^+ is primarily localized in the cytosol, mitochondria and nucleus. This is consistent with the compartmentalization of the main enzymes implied in both the biosynthesis and degradation of NAD^+. However, tissues with different metabolic functions have been reported to have variable subcellular NAD^+ distribution, indeed, myocytes show about 75% of the total NAD^+ in mitochondria, while hepatocytes present the majority of NAD^+ in cytoplasm [25, 30, 31]. Besides the subcellular differences in NAD^+ levels, also the expression of NAD^+-producing enzymes and the preference for specific NAD^+ precursors are subjected to organ-specific variations. In particular, the liver and, to a lesser extent, the kidney represent the center of whole-body NAD^+ homeostasis, since all the enzymes responsible for NAD^+ formation are highly expressed in these two organs [32, 33].

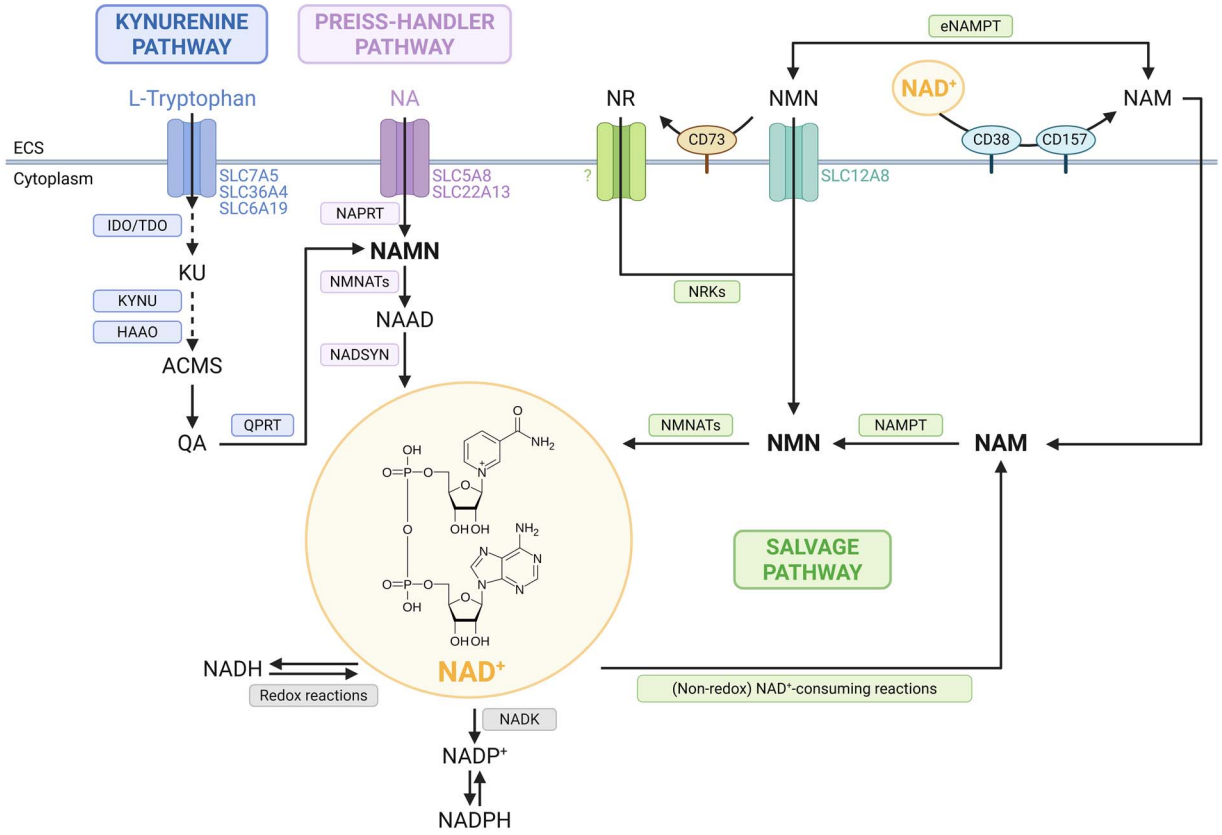

Figure 1. Biosynthetic pathways and molecular fates of NAD^+. NAD^+ levels are sustained by 3 biosynthetic pathways. In the Kynurenine (KU) pathway, NAD^+ is produced from the diet-derived amino acid tryptophan (L-Trp). This latter uses different transporters (SLC7A5, SLC36A4, SLC6A19) to enter the cells, then it is converted to KU by the enzymes indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO). After several biochemical reactions (catalyzed by KYNU and HAAO), KU is transformed in ACMS, which can then spontaneously rearrange into QA. In the final steps, QA is converted into nicotinamide mononucleotide (NAMN), by QPRT; at this point the KU pathway converges with the Preiss–Handler pathway. The Preiss–Handler pathway uses diet-derived NA, entering the cells through SLC5A8 or SLC22A13 transporters, and the NAPRT to generate NAMN. This latter is then converted into NAAD by NMNATs. At the end NAAD is transformed into NAD^+ by NAD^+ synthetase (NADSYN). The third biosynthetic pathway (NAD^+ salvage pathway) recycles the NAM generated as a by-product of the non-redox NAD^+ – consuming reactions. Initially, the intracellular NAMPT converts NAM into NMN, which is then transformed into NAD^+ via the different NMNATs. In the ECS, NAM, generated by the enzymes CD38 and CD157, can be converted into NMN by eNAMPT. At this point, NMN can be directly imported into the cell (for example, via the transporter SLC12A8 in the small intestine) or can be dephosphorylated by CD73 to NR, which can then enter the cell through an un-known nucleoside transporter (indicated by a question mark). Intracellularly, nicotinamide riboside kinases (NRKs) are responsible for the conversion of NR into NMN, which is lastly transformed into NAD^+ by NMNATs, as already described. This figure has been created with Biorender.

As shown in Fig. 1, NAD^+ production occurs via different pathways: the salvage pathway, the Preiss-Handler pathway, and the de novo biosynthesis pathway (also termed as "Kynurenine pathway") [34, 35]. The salvage pathway is the fastest, most energy-efficient, and most adaptable route to produce NAD^+, making it the primary one used by cells, especially under normal physiological conditions [34].

Biosynthesis

Kynurenine pathway

In the de novo/kynurenine (KU) pathway, diet-derived L-tryptophan (L-Trp) is metabolized into KU and further processed to produce quinolinic acid (QA), which is then transformed to nicotinic acid mononucleotide (NAMN). More specifically, once L-Trp enters the enterocytes via the transporters SLC7A5, SLC6A19, and SLC36A4, it is first converted to N-formylkynurenine by tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO). After several biochemical reactions, there is the formation of the intermediate 2-amino-3-carboxymuconate semialdehyde (ACMS), which can spontaneously rearrange into QA [1]. However, ACMS decarboxylase (ACMSD), by converting ACMS to picolinic acid, is able to shift the balance from de novo NAD^+ synthesis pathway toward acetyl-CoA generation, and it has been demonstrated that ACMSD over-expressing mice become dependent on dietary niacin intake for NAD^+ production [36].

A key regulatory step in the KU pathway is the conversion of QA to NAMN, by the enzyme quinolinate phosphoribosyltransferase (QPRT), which commits the process to NAD^+ biosynthesis by converging with the Preiss–Handler pathway [37, 38]. Remarkably, the specific contribution of the de novo synthesis pathway to determine the overall NAD^+ levels is still to be clarified, since outside the liver, most cells do not have all the enzymes necessary to convert L-Trp into NAD^+. Then, the liver releases NAM into circulation, contributing to over 95% of the newly generated NAM found in the bloodstream. This circulating NAM is subsequently taken up and recycled by all cells through the salvage pathway to help maintain NAD levels [33].

Preiss-handler pathway

The Preiss-Handler pathway uses NAMN derived from both the KU pathway and dietary intake of nicotinic acid (NA) (which enters the cells via SLC5A8 or SLC22A13 transporters), to generate NAD^+. In particular, NA is converted by nicotinic acid phosphoribosyltransferase (NAPRT) into NAMN, which is subsequently transformed into nicotinic acid adenine dinucleotide (NAAD) by the enzyme nicotinamide mononucleotide adenylyltransferases (NMNATs). In the final step, NAD synthetase (NADSYN) can amidate NAAD to form NAD^+, by using glutamine as a nitrogen donor [39, 40].

Salvage pathway

The NAD^+ salvage pathway recycles NAM generated as a by-product of the enzymatic activities of non-redox NAD^+-requiring enzymes (see later). Indeed, the intracellular nicotinamide phosphoribosyltransferase (iNAMPT) first converts NAM into NMN. This latter is then transformed into NAD^+ through the activity of different NMNATs. The NAM generated outside the cell by the NAD^+-consuming enzymes CD38 and CD157 can be converted to NMN by the extracellular NAMPT (eNAMPT). NMN can enter the cell via an NMN-specific transporter (e.g. SLC12A8), or can be dephosphorylated to NR by CD73, and then imported into the cells. Finally, as already described for the intracellular NMN pool, it is converted to NAD^+ by NMNATs enzymatic activities [41, 42]. Structurally, iNAMPT and eNAMPT share the same amino acid sequence, but they differ in localization, oligomerization state, and in post-translational modifications. iNAMPT predominantly exists as a functional homodimer in the cytoplasm, while eNAMPT can also be found in monomeric or modified forms in the extracellular space (ECS), where it may undergo modifications, such as glycosylation, that influence its stability and activity [43, 44]. Moreover, eNAMPT was initially considered as a secreted cytokine whose levels increase during infections, known as pre-B-cell colony-enhancing factor (PBEF) [45–47], further supporting the complex network of pathways regulated by NAMPT both at intracellular and extracellular level [48] and its implication in NAD^+ metabolism. NAMPT is also reported under the name of Visfatin (visceral fat-specific adipokine), an adipokine with insulin-mimetic effects [49].

Enzymatic reactions requiring NAD^+

Although the association between NAD^+ levels and health was recognized almost a century ago, only recently low levels of this dinucleotide were linked to numerous disease states, including metabolic and neurodegenerative disorders, besides being related to aging in both mouse models and humans [4, 50–55]. Therefore, understanding the underlying causes and mechanisms of NAD^+ deficiency and how its metabolism can impact the origin of these pathologies are nowadays a matter of debate. In light of this, in addition to the already described pathways of NAD^+ production, a key role in NAD^+ metabolism is played by the enzymatic activities requiring this co-factor, both in redox and non-redox reactions.

Redox reactions

As previously mentioned, the NAD^+/NADH redox couple is critical for numerous electron-exchange-dependent biochemical reactions, especially those involving oxidoreductase-mediated hydride transfer. Although already said, it is worth keeping in mind that NAD^+ is an electron acceptor in these reactions, whereas NADH is an electron donor.

Several redox reactions requiring NAD(H) as a co-substrate are linked to catabolism. Particularly, NAD^+ acts as co-factor for enzymes involved in glycolysis (by glyceraldehyde 3-phosphate dehydrogenase, GAPDH), alcohol metabolism (by alcohol and aldehyde dehydrogenases, ADH and ALDH, respectively), decarboxylation of pyruvate to form acetyl-CoA (through pyruvate dehydrogenase complex, PDC), TCA cycle (via isocitrate, α-ketoglutarate and malate dehydrogenase, IDH, α-KGDH and MDH, respectively), β-oxidation of fatty acids (through 3-hydroxyacyl-CoA dehydrogenase, HADH), and lactate oxidation as part of the Cori cycle in liver (via lactate dehydrogenase, LDH) [56]. LDH usually catalyzes the reverse reaction. Indeed, through the electrons donated by NADH, it can reduce pyruvate to lactate, regenerating NAD^+, to be used in glycolysis. Similarly, during liver gluconeogenesis, GAPDH can catalyze the reverse reaction, with respect to the one described above [30]. In addition, as known from the OXPHOS route, complex I, the first enzyme of the respiratory chain, oxidizes NADH, generated through the TCA cycle in the mitochondrial matrix, leading to reduction of ubiquinone to ubiquinol [57]. At the end, NADH is also the co-enzyme of desaturases, proteins responsible for the synthesis of highly unsaturated fatty acids, so contributing to NAD^+ restoration [58].

NAD^+ consuming reactions

Besides its function as an electron carrier, NAD^+ has also been identified as a co-factor of enzymes essential in various cellular signaling pathways [25, 59]:

- *PARP family*: a group of enzymes composed by 17 members in humans and 16 members in mice, implied in the preservation of genome integrity and DNA repair. The main NAD^+ consumers among the PARP family is PARP1, accounting for about 90% of all the NAD^+ used by PARPs [60]. Although little is known about the mechanisms of substrate recognition and specificity of PARPs, this class of enzymes is responsible for PARylation—a post-translational modification in which mono- or poly-ADP-ribose units are attached to an amino acid acceptor, while simultaneously hydrolyzing NAD^+ into NAM [61]. Most of the scientific papers reported that mono-ADP ribosylation plays a role in cell adhesion, response to virus infection, inflammation, autophagy and the endoplasmic reticulum stress [62, 63]; while, as already mentioned, poly-ADP ribosylation is crucial in various cellular processes, particularly in chromatin regulation, transcription, DNA repair, and the initiation of cell death pathways [64, 65]. However, as recently reviewed by Wu and colleagues, this distinction no longer appears to be so clear-cut [63]. For example, PARP1 and 3 can directly ADP-ribosylate dsDNA and ssDNA breaks by attaching poly-ADPR and mono-ADPR moieties, respectively, to 5′- and 3′-terminal phosphates [65, 66]. The PARP3-dependent mono-ADP ribosylation of DNA may play a role both in non-homologous end joining (NHEJ) DNA damage repair and in shielding breakage sites until recruitment of repair proteins [67].
- *Sirtuin (SIRT) family*: a class of NAD^+-dependent deacylating enzymes, homologous of the yeast silent

information regulator 2 (ySir2), that about 20 years ago was described to enhance yeast lifespan by deacetylating telomere-associated proteins [68]. As evidence of their importance, Sir2 homologues are highly conserved in different organisms, from archaea to humans [69]. Sirtuins catalyze the deacylation of their targets in a two-step process: first NAD^+ is cleaved to NAM and ADPR, and second, the acyl/acetyl group present on the target protein (commonly at a lysine residue) is transferred to ADPR, producing O-acetyl-ADP-ribose (OAADPR) [70]. This latter has been implicated in signaling processes, like redox regulation, gene silencing, and ion channel gating (like transient receptor potential cation channel, subfamily M, member 2, TRPM2) [71]. Of note, NAM has been shown to inhibit both SIRTs and PARPs in a negative feedback loop [72, 73].

The mammalian SIRT family comprises seven protein groups (SIRT1–SIRT7) with different downstream targets, subcellular localizations (i.e. nucleus for SIRT1, and SIRT6; mitochondria for SIRT3, SIRT4 and SIRT5; cytosol for SIRT1, SIRT2 and SIRT5; nucleolus for SIRT7), and enzymatic activities (indeed, some members of the sirtuin family showed demyristoylase, demalonylase, desuccinylase, depalmitoylase, and deglutarylase activities, besides acetyl-lysine deacylating function) [74, 75]. Specifically, SIRT1 and SIRT2 appear to account for approximately one third of the total NAD^+ consumption in epithelial cells at baseline [33]. As extensively reviewed, the action of SIRT1 is mainly associated with oxidative stress and antioxidant cell defense, indeed besides deacetylation of histones, it can modulate transcription factors, like forkhead box (FOXOs), nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), and peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) [76–80]. In apparent contrast with its role in stimulating mitochondrial biogenesis through PGC-1α deacetylation, SIRT1 has also been linked to turnover of dysfunctional mitochondria by mitophagy [81, 82]. Mitochondrial sirtuin SIRT3 can promote the oxidation of amino acids (through glutamate dehydrogenase deacetylation), control the mitochondrial antioxidant environment (through the deacetylation-dependent activation of superoxide dismutase 2—SOD2), help the repair of oxidative mtDNA damage (via the stabilization of 8-oxoguanine-DNA glycosylase 1), and prevent mitochondrial division (through deacetylation of optic atrophy 1 protein (OPA1) [74]. Overall, sirtuin alterations are associated with aging and aging-related disorders, including chronic inflammatory pathologies, and metabolic dysfunctions [11, 74, 83, 84], thus representing key actors in the efforts to investigate how NAD^+ levels can affect cellular homeostasis.

- *CD38*: the most prominent cyclic ADPR (cADPR) synthase, together with its homolog CD157, which converts NAD^+ into NAM and cADPR. CD38 was first designated as a cell-surface protein specific of the immune system cells (i.e. T and B lymphocytes, natural killer (NK) cells, monocytes, macrophages, dendritic cells, and plasma cells), but later it was found to be expressed even in other tissues (e.g. brain, eyes, gut, prostate, muscle, bone, and kidney), with variable expression levels and with both intra- and extra-cellular enzymatic activity [85, 86]. Considering its widespread expression, it is unsurprising that *Cd38*-deficient mice exhibited higher NAD^+ levels in various tissues compared to wild-type animals, even during the aging process [24, 87, 88]. Similar results were obtained upon the administration of apigenin, a CD38 inhibitor, to obese mice, which led to increased NAD^+ levels and an amelioration of several aspects of lipid and glucose homeostasis [89]. cADPR, generated by CD38/CD157 activity, is a potent second messenger able to trigger receptors (e.g. ryanodine receptors) implied in the regulation of Ca^{2+} fluxes [90, 91]. Therefore, cADPR synthases, like CD38 and CD157, can indirectly affect a variety of Ca^{2+}-dependent mechanisms, including muscle contraction, cell proliferation, adaptive and innate immune responses, and glucose-induced secretion of insulin from pancreatic β-cells [85].
- *SARM1*: an enzyme catalyzing NAD^+ hydrolysis and cyclization, together with a base exchange reaction between NA and $NADP^+$ to generate NAADP, a powerful calcium signaling molecule [92]. SARM1 is mainly expressed in neurons, macrophages and T lymphocytes, where it regulates neuronal morphogenesis and inflammation [93, 94]. Moreover, SARM1 plays a key role in the programmed axon degeneration pathway, termed "Wallerian degeneration", in which a metabolic stress or injury induces a microtubule and neurofilament breakdown, leading to a missing transport of the NAD^+ biosynthetic enzyme NMNAT2 towards the distal axon portion [95]. SARM1 is thought to be activated by the reduced levels of NMNAT2, and the accumulation of the NAD^+ precursor NMN, finally leading to a further NAD^+ depletion and axonal degradation [26, 96]. Recently, Miyamoto and co-workers reported that the direct activation of SARM1 by the neurotoxin Vacor is able to trigger also the dendrite degeneration in mouse hippocampal neurons, although by different down-stream pathways as compared to axonal degeneration [97]. Hence, SARM1 is emerging as a putative therapeutic target to prevent or ameliorate traumatic brain injuries and neurodegenerative pathologies.

Taken together, these non-redox reactions of NAD^+ involve the cleavage of the dinucleotide to generate ADPR or second messenger molecules. Since NAD^+ is continuously consumed in these enzymatic activities, a constant NAD^+ biosynthesis must occur. In addition, NAM is an essential NAD^+ precursor together with a product of NAD^+-consuming reactions, representing a common denominator between regulation of NAD^+ biosynthesis and NAD^+-requiring reactions. To further corroborate this interplay, imbalances between these NAD^+-related pathways may lead to an insufficient NAD^+ supply, and eventually to several diseases (as mentioned above). Of note, the supplementation with NAD^+ precursors has been demonstrated to be effective in increasing the overall lifespan of yeast, *C. elegans*, and mice, highlighting the therapeutic potential of enhancing NAD^+ levels in eukaryotes [98–100].

NAD^+ in female fertility

In the last decade, the critical role of NAD^+ metabolism in supporting female fertility has emerged. Mounting evidence suggests that proper NAD supply is essential for a healthy pregnancy and that NAD deficiency is associated

with disruption of embryogenesis, leading to congenital malformation and miscarriage [101–103]. Studies on humans have revealed the occurrence of biallelic mutation of key enzymes involved in NAD biosynthesis, like tryptophan 2,3-dioxygenase (KYNU), 3-hydroxyanthranilic acid dioxygenase (HAAO) or NADSYN1 in aborted fetuses or in newborns with severe malformations, including defects at heart, vertebrae and kidney, collectively referred as to congenital NAD deficiency disorder [101, 104].

Recent studies on mouse models have observed reduced NAD levels in the ovary from reproductively aged mice and have proposed different approaches aimed at enhancing NAD^+ to improve ovarian function and oocyte competence [55, 105, 106]. Nevertheless, knowledge of NAD^+ metabolism in the female reproductive system is still poor when compared with other organs and tissues.

Role of NAMPT in female reproductive system

Early studies in female reproductive system have been conducted on the role of NAMPT as an adipokine with insulin-mimetic effects, as reported in previous paragraph [49]. Indeed, studies on ovarian NAMPT described its ability to modulate steroid secretion and cell proliferation in granulosa cells and explored its role as insulin-mimetic and glucose-lowering agent [107–109], without any reference to NAD^+ metabolism. In addition to granulosa cells, NAMPT has been described in the oocyte and in the follicular fluid [107, 110–112]. Studies on mice have shown that NAMPT is expressed in prepubertal ovaries and has a rise at the postnatal age of 21 days [109]. In addition, NAMPT concentration in follicular fluid has been found to be similar to that in plasma [107, 110, 112], and has been positively correlated with the number of oocytes retrieved in in vitro fertilization (IVF) patients [110]. Studies on livestock/dairy cows have observed that the homozygous haplotype "$Fertil^+$", known to be associated with improved fertility, higher developmental potential and quality of the blastocysts, is correlated to increased NAMPT expression in cumulus cells, in comparison to "$Fertil^-$" haplotype [113]. In accordance with these data, multiomics meta-analysis performed on 100 genomes of goats with different litter rate, a key indicator of production performance in livestock, led to the identification of *NAMPT* among the seven genes with a positive regulation of fertility potential in goats [114]. Moreover, the variations in the intron regions of the *NAMPT* gene seemed to directly influence its expression levels, supporting the hypothesis that reproductive capacities could be related to transcriptional expression levels of the gene [114]. Proteomic analysis revealed that NAMPT expression was positively correlated with ovine prolificacy [115].

Evidence from transgenic mice

The observations above have pointed out the importance of increasing knowledge on absorption, transport and/or processing of precursors or co-factors required for NAD synthesis, by the employment of specific transgenic animal model. In accordance with observations in humans, mice with biallelic mutations in *Kynu*, *Haao* and *NadSyn1* presented NAD deficiency and showed alteration in embryo development, leading to embryo loss or congenital malformations similar to those observed in human patients [101, 104, 116]. By contrast, loss of rate-limiting enzymes in de novo NAD^+ synthesis, *Ido1* or *Qprt* was not associated to any embryo developmental defects but exhibited a reduced reproductive lifespan [25, 40, 117, 118]. Indeed, mice with null ablation for *Ido1* or *Qprt* had a reduced NAD^+ bioavailability at ovarian level, associated with decreased ovarian reserve, irregular estrous cycles, and compromised oocyte quality. These changes were ascribed to increased reactive oxygen species (ROS), altered mitochondrial metabolism and spindle abnormalities, leading to decreased fertilization capacity and impaired embryonic development [118]. Collectively, these changes are suggestive of accelerated aging occurring in the ovaries of knockout mice for *Ido1* or *Qprt* [118, 119].

Interestingly, homozygotic mutation of *Nampt* does not allow embryo/fetal development and survival [120]. To overcome this lethality, conditional transgenic models have been developed to investigate the role of *Nampt* ablation in organs and tissue. *Nampt* silencing in oocytes severely affected NAD^+ level, with deleterious effects on meiosis resumption and completion, spindle assembly, and redox homeostasis [121].

Finally, studies on loss of function of *Slc6a19*, encoding for the neutral amino acid transporter B 0 AT1, which is crucial for L-Trp absorption in intestine and kidneys [122, 123] pointed out that embryo malformations/loss and NAD deficiency due to reduced L-Trp absorption was manifested only if a diet with a restricted content of NAD precursors was administered to pregnant mice [124].

Overall, these studies emphasize that NAD^+ de novo biosynthetic pathways and NAD^+ recycle/salvage pathway have a different impact on embryo survival, suggesting a different influence also on germ cell physiology. This knowledge highlights the importance of a better understanding of specific dysfunctions of NAD^+ production and their contribution to the ovarian altered microenvironment.

Age-related decline of NAD^+ bioavailability and production

The aging process significantly impacts female fertility, which is characterized by a progressive decline in ovarian function in the early 30s, hence much earlier than most somatic tissues, typically showing significant functional decline later in life—often after 50s or beyond [119, 125]. The ovarian aging is crucial in female reproductive aging, leading to negative effects on fertility and overall health. Maternal age is strictly associated with the quality of oocytes, which decreases the likelihood of conception and live births. Oocyte aging results from the prolonged dormancy of oocytes in primordial follicles and exposure to an aging ovarian and follicular environment during maturation. This decline has been described to be primarily linked to mitochondrial dysfunctions, which cause oxidative stress, mitochondrial damage, inadequate energy levels, calcium imbalances, and issues with meiotic spindles. These factors lead to increased rates of aneuploidy, DNA damage, loss of chromosomal cohesion, checkpoint dysfunction, errors in meiotic recombination, and telomere shortening, alongside changes in mitochondrial dynamics and cellular structures [8, 119, 126].

Recent literature has pointed out that NAD^+ availability declines with ovarian aging, as it occurs later in other organs [6, 7, 106, 118].

Indeed, reduced availability of NAD^+ affects both oocyte and somatic ovarian cells, by influencing the activity of NAD^+ consuming enzymes, such as sirtuins and PARPs,

mitochondrial energy production and dynamics, and leading to the generation of oxidative stress [7, 106, 127]. In addition, NAD^+ decline also induces the senescence of ovarian cells, leading to diminished hormone production and reproductive capacity [127], together with increased expression of pro-inflammatory factors [106] and dysregulation of autophagy and proteostasis [128].

Looking for possible dysregulations of NAD^+ production underlying NAD^+ decline with aging, it has been demonstrated that ovarian *Nampt* expression is significantly decreased with advanced maternal age [129]. In accordance with this observation, studies from transgenic mice have revealed that loss of *Ido1* or *Qprt*, both involved in the de novo NAD^+ biosynthesis, resulted in premature ovarian NAD^+ decrease, leading to accelerated ovarian aging, as evaluated by reduced ovarian reserve, irregular oestrus cycles and impaired oocyte quality with reduced development potential [118]. In addition, it has been proposed that the onset of inflammation may be a causative mechanism for NAD^+ depletion, since it leads to overactivation of CD38, an important NAD^+ consuming enzyme [130]. Accordingly, IL-1 or TNF-α knockout mice exhibited a prolonged ovarian lifespan and fertility [131, 132].

In order to clarify molecular changes leading to NAD^+ decline in the aged oocyte, our research group has investigated age-related alterations in NAD^+ biosynthetic and NAD+ consuming pathways. Our study has revealed a down-regulation of NAD^+ biosynthesis, stemming from reduced de novo production and salvage pathways [111]. Specifically, a decline in Qprt, an enzyme that aids in the conversion of quinolinic acid to NAMN for NAD^+ synthesis, was noted, corroborating previous studies linking *Qprt* deletion to accelerated ovarian aging [111, 118]. Additionally, lower levels of *Nadsyn1* in aged oocytes suggested decreased NAD^+ production from NaAD, derived from NAMN. Furthermore, the reduced gene and protein expression of NAMPT, the rate-limiting enzyme in the NAD^+ salvage pathway, highlighted diminished NAD^+ recycling [111]. These alterations in NAD^+ production may disrupt cellular homeostasis by impairing the function of NAD^+-dependent enzymes, e.g. Cd38, members of PARP (Tnks, Tnks2) and SIRTs (Sirt3, Sirt4), thus leading to decreased metabolic processes, including fatty acid and glucose metabolism, alongside compromised DNA repair and oxidative metabolism [111].

Altered NAD^+ bioavailability and production in ovarian dysfunctions

Altered NAD metabolism is increasingly recognized as a factor influencing ovarian function beyond the effects of aging. When NAD metabolism is disrupted, it leads to an imbalance in energy homeostasis and affects the cellular environment of the ovaries [8, 40]. This can result in oxidative stress, inflammation, and impaired mitochondrial function. Such conditions may hinder the growth and maturation of ovarian follicles and reduce the quality of oocytes.

PCOS

Oxidative stress, inflammation, and impaired mitochondrial function in the ovarian microenvironment are particularly relevant in the context of PCOS [133, 134]. PCOS is a complex endocrine disorder affecting 15%–20% of women of reproductive age whose diagnosis is based on the occurrence of at least two out of three signs, namely polycystic ovarian morphology (PCOM), hyperandrogenism and anovulation. Nevertheless, PCOS is a far more complex syndrome being usually associated with insulin resistance, obesity, hirsutism, acne, acanthosis nigricans, and hair loss, with multifactorial causes, involving environmental, genetic, and epigenetic factors, with only symptomatologic therapeutic approaches available today.

Dysfunctional NAD^+ metabolism has been addressed by studies focused on molecular mechanisms underlying ovarian alterations in PCOS. Granulosa cells from patients with PCOS exhibit a marked decrease of NAD^+ content in comparison to control women, with values of respectively 0.10 pmol/10^3 cells and 0.16 pmol/10^3 cells [12]. In accordance, metabolomic analysis of follicular fluids from PCOS patients revealed altered levels of intermediate metabolites of all bioenergetic pathways, including NAD^+-derived metabolites, including NAM [135].

Elevated L-Trp metabolites in the plasma and urine have been observed in PCOS patients, as evidence of abnormal activation of the tryptophan [136, 137]. Moreover, altered L-Trp metabolism has been positively correlated with serum androgen levels, suggesting that it might play an important role [137–139]. In addition, L-Trp metabolism in the ovary relies on the IDO1 enzyme, which can be quickly activated by pro-inflammatory signals, through the p38, NF-κB [140]. The resulting L-Trp breakdown and increased high KU levels have been associated with reduced estradiol and follicle-stimulating hormone (FSH) [140].

Beyond L-Trp catabolism, growing evidence suggests that other NAD^+-related metabolic pathways may also be disrupted in PCOS. Notably, two different bioinformatic analyses confirmed a down-regulation of nicotinate-nicotinamide metabolism pathway and *NAMPT* [141, 142]. These alterations were accompanied by reduced NAD^+ biosynthesis and were mechanistically linked to decreased expression of SIRT2, further highlighting the interplay between impaired redox balance, sirtuin activity, and metabolic dysfunction in the ovarian environment [142]. In this regard alteration of sirtuins expression, in particular SIRT1 have been extensively studied not only in the ovary, but also in other reproductive organs and in the brain of a PCOS mouse model [133, 134, 143–145]. Dysregulation of SIRT1 and SIRT3 in the ovary of PCOS mice indicates the activation of an adaptive response to counteract oxidative and glycative damage associated with mitochondrial dysfunctions, and leads to the activation of the autophagic flux [134]. Collectively, these data suggest that PCOS women have an altered NAD^+ production through the de novo synthesis and salvage pathways, which should be analysed in an integrated approach to the PCOS ovarian microenvironment, in order to identify the triggering event and the compensative strategies implemented by different cells.

Post-ovulatory aging

Another condition affecting oocyte developmental potential is the post-ovulatory aging, which is characterized by the time-dependent deterioration of oocyte quality that occurs if fertilization is not completed within 12 h for rodents and 24 h for monkeys and humans after ovulation [146–148]. In assisted reproductive techniques (ART), post-ovulatory aging significantly affects the quality of retrieved oocytes, with dramatic consequences on ART outcome, in terms of

reduced fertilization rates and embryo quality, and increased risk of aneuploidies [147–150]. It has recently found that oocytes experiencing post-ovulatory aging are characterized by reduced NAD^+ levels [151], supporting previous observations about a reduction of sirtuins, elevated ROS levels, leading to the establishment of oxidative stress that occur during prolonged culture after ovulation [152, 153].

Gonadotoxicity by chemotherapy

Chemotherapy can markedly affect healthy ovaries, particularly influencing ovarian reserve. Indeed, all ovarian follicle populations are off target of most chemotherapeutic agents, leading to a reduced number of primordial follicles. Cytotoxic effects of chemotherapy on ovarian tissue contribute also to decreased hormone production and menstrual irregularities. As a consequence, women may face diminished ovarian function, potentially leading to early menopause and reduced fertility. Molecular changes observed in ovarian cells including oocytes in response to chemotherapeutic agents, i.e. cyclophosphamide, cisplatin, doxorubicin, and γ-irradiation, include changes in sirtuins and PARPs as attempt to repair DNA damage, improve mitochondrial functions and restore oxidative balance and cellular homeostasis [154, 155]. Both Sirtuins and PARPs rely on NAD^+ for their activity, and as expected, a condition of NAD^+ deficiency has been recently described in ovaries from mice exposed to the gonadotoxic agent cyclophosphamide [13, 14], probably due to an intense NAD^+ utilization in order to cope with extensive cellular damage. In addition, in ovaries exposed to chemotherapy, adequate NAD^+ levels are required for activating SIRT2 in order to inhibit NLRP3-mediated cell pyroptosis [14].

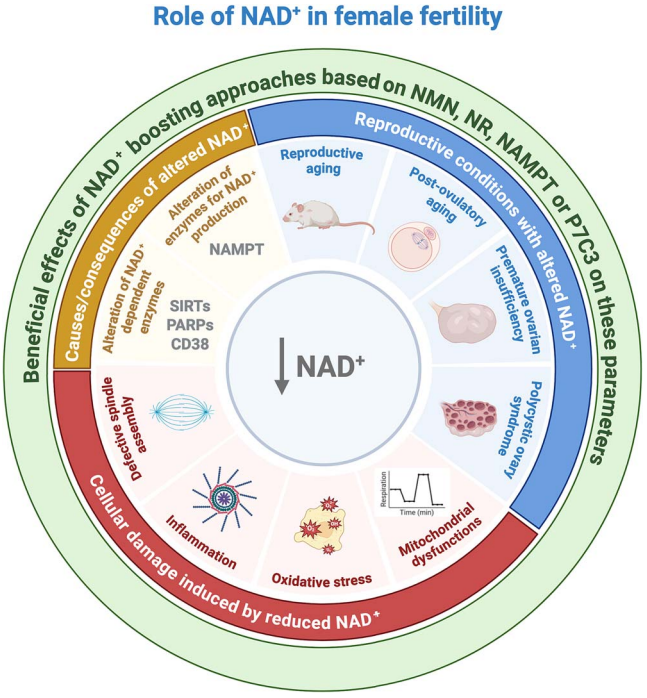

Figure 2. Role of NAD^+ in female fertility. Reproductive aging, post ovulatory aging, premature ovarian insufficiency and polycystic ovary syndrome are characterized by reduced NAD^+ levels. Depletion of NAD^+ may be ascribed to altered activity of NAD^+ producing, e.g. NAMPT, or consuming enzymes, e.g. SIRTs, PARPs, CD38, and leads to mitochondrial dysfunctions, oxidative stress, inflammation and meiotic spindle defects. NAD^+ boosting approaches based on NAD^+ precursors, e.g. NMN and NR, NAMPT administration or stimulation of NAMPT activity by P7C3 restore NAD^+ levels and improve female fertility potential.

NAD^+ boosting approaches in infertility

Mounting evidence from animal studies has highlighted the pivotal role of NAD^+ availability in supporting female reproductive health, particularly during pregnancy and reproductive aging. The essentiality of NAD^+ intake has been underscored by the observation that pregnant mice receiving a diet with restricted content of NAD precursors, e.g. L-Trp and vitamin B3, exhibited NAD deficiency and fetal alterations, including miscarriage and multiple congenital malformations [116]. Studies on transgenic mice for key biosynthetic NAD^+ enzymes revealed that maternal NAD bioavailability, more than embryo genotype, is the primary driver of positive outcome of pregnancy, together with environmental factors, such as NAD precursors acquisition by diet, malnutrition or hypoxic conditions [101, 104, 116, 156].

Numerous preclinical studies have explored exogenous supplementation with NAD^+ precursors as a potential therapeutic strategy to counteract ovarian aging and infertility. Exogenous administration of NAD^+ precursors has been proved to improve ovarian aging, by preserving ovarian reserve, reducing inflammation, and rejuvenating oocyte quality in aged animals, leading to restoration in fertility potential [6, 7, 106, 118, 127, 128, 157, 158]. After ovulation induction, mice receiving NAD^+ precursors produced more mature oocytes, characterized by enhanced meiotic competency and fertilization ability, probably due to improved mitochondrial function, reduced ROS leakage and apoptosis [6, 127, 158] (Fig. 2).

Most studies have relied on oral administration of NMN [6] at 0.5 g/L or 2 g/L, although beneficial effects were also described after intraperitoneal administration of NMN at 500 mg/kg [127]. Interestingly, the low dose of NMN (0.5 g/L) was proved to be more beneficial on oocyte quality and fertility potential rather that the 2 g/L, suggesting that proper dosing of NAD^+ precursors is a relevant issue [6]. Prolonged administration of low NMN concentration for 20 weeks was also able to counteract ovarian senescence, by increasing mitochondria function, autophagy and proteostasis of somatic cells [128]. In addition, NMN administration partially restored FSH and LH levels and relative ratio [127]. Oral administration of high dose of NMN (2 g/L) before and during pregnancy, and throughout lactation did not induce any alterations in the offspring [6].

Similarly to NMN, NR supplementation (200 mg/kg by gavage) restored NAD^+ levels in aging mice, showing an increased number of ovarian follicles and a younger hormonal profile [127]. Moreover, mice receiving NR presented oocytes with physiological levels of ROS, proper spindle assembly and mitochondrial functionality and distribution, which lead to improved reproductive success [7]. As observed with NMN supplementation, NR administration during lactation did not alter the health status of the offspring [159, 160]. Moreover, in vitro NR supplementation attenuated the features of in vitro aged oocytes, e.g. mitochondrial dysfunction, and the subsequent decline in embryonic development potential, likely by NAD^+/SIRT1 signaling, paving the way for its use in clinical practice during ART procedures [151].

Contrasting results on the effects of NMN supplementation on ovarian physiology challenged by chemotherapy have been reported [14, 161]. After NMN administration, increased NAD^+ levels were reported to contribute to the protection of the ovarian reserve challenged by cyclophosphamide (CP), by activating SIRT2 to inhibit NLRP3-mediated pyroptosis [14]. By contrast, another study reported that follicle loss by CP or γ-irradiation was not prevented by NMN administration [161]. Nevertheless, these discrepancies in NMN efficacy may be related to difference in the experimental design in the aforementioned studies, and suggest that a better understanding of changes in NAD^+ metabolism and related pathways induced by different chemotherapeutic agents may support the development of tailored therapeutic approaches with improved fertoprotective activity.

Finally, the administration of exogenous NAMPT has been used as a strategy to enhance ovarian function and fertility potential in 18-month-old aged female mice [105]. This approach resulted in improved expression of proliferative and angiogenic factors at the ovarian level. These changes were also associated with improved embryo development potential in aged mice, whereas no amelioration was reported in embryos from young mice [105].

In vitro stimulation of NAMPT activity by P7C3 may enhance the oocyte quality of aged oocytes by influencing mitochondrial function and by modulating the expression level of genes involved in NAD^+ metabolism [111, 162]. Of note, although P7C3 had beneficial effects on oocyte alterations induced by oxidative stress, it was not able to fully restore mitochondrial bioenergetics altered by prolonged stay in the aged ovarian microenvironment [111]. Additionally, as observed for NAD^+ precursors, high doses of P7C3 may have harmful effects, indicating that establishing a safe dosage for aged oocytes is needed.

Experimental approaches for the study of NAD^+ metabolism in reproductive organs and cells

To identify NAD deficiency and evaluate NAD^+ boosters efficacy, it is essential to accurately quantify NAD^+ and/or NADH. Once the optical properties of NADH have been established, NADH monitoring was made by using the dual-beam spectrophotometer [163, 164]. To provide a method more specific than absorption spectroscopy, fluorescence spectrophotometry in the near-ultraviolet range was applied for NADH measurement. In its reduced form, NADH and NADPH absorb light within the 320–380 nm range and emits fluorescence between 420–480 nm [165]. By contrast NAD^+ is a nonfluorescent molecule due to the presence of a double-bound nitrogen and the meta-directing carbamyl group in its structure, which suppress fluorescence [166, 167]. The corrected excitation peaks of NADH in aqueous solution occur at 260 nm and 340 nm, with fluorescence emission observed at 457 nm [168]. This method based on the autofluorescence emission by reduced form of nicotinamide has been progressively used to quantify NADH, and NADPH as well, in different biological systems, including reproductive tissues and cells. However, this method is limited by a weak signal, reduced sensitivity and specificity, and the potential for cell damage due to the ultraviolet irradiation needed to generate the autofluorescent signal. As a consequence, only in 2007, Dumollard and colleagues successfully measured NADH autofluorescence under confocal or epifluorescence microscope for NAD quantification in oocytes [169]. Since then, many researchers have used confocal microscopy to measure oocyte NAD levels via NADH autofluorescence, as an assessment of oocyte metabolic status [6, 111, 170–178]. Nevertheless, the autofluorescence method cannot discriminate between NADH and NADPH because of their similar spectral properties. Consequently, the acquired autofluorescence signal generally represents the concentration of both cofactors [169].

The introduction of Fluorescence Lifetime Imaging Microscopy (FLIM) marked a significant advancement, since images are generated by measuring the fluorescence decay kinetics at each pixel, allowing spatial mapping of fluorescence lifetimes, independently of fluorophore concentration [179, 180]. Based on their distinct fluorescence lifetimes, FLIM is able to distinguish between free and protein-bound forms of NADH, with the NADH bound form predominantly localized in mitochondria, and its free form primarily found in the cytosol of cells. The quantification of the free/bound NADH ratio has also been correlated with NAD^+/NADH ratio [181–184]. This approach has been successfully employed for the study of NADH consumption and metabolic changes occurring during different stages of follicle development in intact *ex vivo* mouse ovaries [185]. This method preserved the native structural integrity and cellular interactions, allowing compartment-specific measurements in granulosa cells, oocyte nucleus, and cytoplasm, without alteration of cellular metabolism [185–187]. In human cumulus cells (CC), FLIM analysis revealed that $73 \pm 5\%$ of the NAD(P)H signal originated from mitochondria, $16 \pm 1\%$ from the nucleus, and the remaining signal came from the cytoplasm [188, 189].

Given the wide concentration range of NAD^+ and related metabolites in cells (1 μM to 1 mM), chromatographic techniques provide a reliable analytical approach. Since the introduction of ion-exchange chromatography in 1949 [190], these methods have evolved significantly, with HPLC and LC–MS now widely adopted for their high sensitivity and specificity in the analysis of small molecules [191, 192]. LC–MS can simultaneously quantify NAD^+ and its intermediates/precursors in a relatively short time, with each run typically lasting between 10 minutes and an hour [193–195]. In 2017, Bustamante and colleagues developed a method based on liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) that enabled robust and reliable quantification of metabolites in the NAD^+ metabolome, including NAD^+ and NADH, at fmol concentrations per oocyte in pools of 5–10 mouse oocytes [196]. A similar protocol was applied successively to measure NAD^+ and NADH metabolites at pmol concentrations per mg of mouse ovarian tissue [6]. Recently, the ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) allowed the quantification of NAD^+ content in human oocytes [151, 197, 198].

Apart from direct NAD quantification, most studies rely on indirect methods based on enzymes that cycle NAD^+/NADH and amplify the signal, producing a measurable color or fluorescence. Early studies on mouse and fish models measured NADH utilization by glycolytic enzymes for a better understanding of oocyte and early embryo metabolism [199, 200]. Next, bioluminescent assays using reductase enzymes to convert NAD^+/NADH into a luciferase-detectable signal have been introduced. Collectively, fluorometric, colorimetric or bioluminescent methods are cost-effective and highly sensitive, and have been successfully employed in ovaries and in

oocytes, the latter in pools ranging from 12 to 200 oocytes [111, 118, 158, 201, 202]. NAD^+ content was also evaluated in human granulosa cells and in the KGN tumor granulosa cell line by fluorometric assay revealing comparable levels [12].

The study of the enzymes involved in the biosynthesis of NAD^+ represent also an indirect way to measure NAD^+ content [111]. Building on these findings, the advancement of single-cell transcriptome analysis via RNA-seq now enables the use of atlas-derived data in predictive bioinformatics approaches to explore the expression of genes involved in NAD^+ biosynthesis at single-cell resolution [203], e.g. in ovarian cells from women with different reproductive disfunctions.

In the end, the genetically encoded NAD^+/NADH sensors, e.g. Peredox, SoNar [204, 205] represent promising methods for NAD detection due to their ability to enable dynamic, real-time monitoring of redox changes in living cells. Unlike conventional approaches, these sensors can be targeted to specific subcellular compartments, providing precise insights into NAD^+/NADH levels in different organelles, while their ratiometric properties allow for quantitative and non-invasive measurements [206]. To our knowledge, no studies have explored the use of these sensors to analyse reproductive cells.

Future prospectives

From the above observations, NAD^+ metabolism should be considered essential for female reproductive health, impacting embryogenesis, oocyte quality, and overall fertility. Indeed, a condition of NAD^+ deficiency in the ovarian microenvironment is linked to age-related reproductive decline, PCOS and anticancer drug-related POI, underscoring the importance of metabolic health in female fertility. From genetic insights and analysis of transcripts in aged oocytes, it emerged that reduction in NAD^+ biosynthetic enzymes may play a relevant role in ovarian dysfunctions as a main cause of compromised cellular homeostasis and diminished activity of NAD^+-dependent enzymes like sirtuins and PARPs. These changes might lead to impaired energy metabolism, DNA repair, and resistance to oxidative stress responsible for reduced oocyte competence. In this context, the multifaceted role of NAMPT is relevant. This enzyme is expressed in granulosa cells, oocytes, and follicular fluid and correlates positively with oocyte yield and fertility in both humans and livestock. Beyond its role as a critical enzyme in the NAD^+ salvage pathway, NAMPT has been involved in steroid secretion and glucose metabolism. In PCOS, altered NAD^+ metabolism is associated with disrupted KU pathway and deficiencies in granulosa cells and correlates with impaired oocyte development, hormonal imbalances, and dysregulated sirtuins. The finding of NAD^+ depletion as an effect of anti-cancer drugs on ovarian reserve further emphasizes the key role of this metabolite in response to cellular damage. Although the outcomes are inconsistent across different chemotherapeutic agents, boosting NAD^+ mitigates some effects. Moreover, NAD^+ levels are significantly reduced in oocytes as a consequence of post-ovulatory aging thereby exacerbating oxidative stress, mitochondrial dysfunction, and sirtuin reduction.

In addition, NAD^+-boosting strategies based on dietary precursors such as NMN and NR have demonstrated beneficial effects in animal models. These include improvements in mitochondrial activity, hormonal balance, oocyte competence, and preservation of ovarian reserve, thus offering a promising therapeutic avenue.

Our analysis suggests that the ovary has a distinct NAD^+ metabolism, but despite advances, several uncertainties remain. Notably, the regulation of NAD^+ metabolism in human ovarian cells is still poorly understood. Although preclinical data indicate strong links between NAD^+ availability and fertility, as well as between NAMPT levels and oocyte quality, it is unclear whether these findings fully translate to humans. NAMPT's dual role, as NAD^+ salvage pathway enzyme and an adipokine, adds another layer of complexity, especially in disorders like PCOS where endocrine and metabolic pathways intersect.

A major limitation in the current literature is the lack of well-designed human clinical studies directly evaluating the effects of NAD^+ modulation on fertility. Most of the current understanding derives from animal models, in vitro experiments, and omics-based associations. While NAD^+ levels have been quantified in human granulosa cells and oocytes using advanced techniques such as UPLC-MS/MS, interventional trials assessing NAD^+ precursors or NAMPT-targeting molecules in women facing reproductive aging or other ovarian dysfunctions are lacking.

Additionally, the precise mechanisms of NAD^+ precursor uptake, transport, and compartmentalization within oocytes and ovarian somatic cells remain elusive. This includes the identification of cell-type specific NAD^+ requirements and potential differential responses to NMN, NR, or other metabolic modulators. Moreover, potential risks or adverse effects associated with chronic NAD^+ supplementation, including metabolic imbalances, excessive activation of NAD^+-dependent enzymes, or unintended modulation of redox signaling, are not yet fully understood. Methodologically, while recent advances in FLIM and genetically encoded sensors offer exciting potential for real-time subcellular NAD^+/NADH monitoring, these tools have not yet been systematically applied to reproductive tissues. Integrating these high-resolution approaches with single-cell transcriptomics and metabolomics could greatly enhance our understanding of redox dynamics across various stages of oocyte maturation and folliculogenesis.

In conclusion, while the current body of research strongly implicates NAD^+ metabolism as a critical factor in ovarian function and aging, translating these findings into clinical benefits will require targeted, multidisciplinary efforts. Clinical trials in women are urgently needed to validate the efficacy and safety of NAD^+-targeting strategies in improving fertility outcomes. Furthermore, research must continue to clarify the nuanced interactions between NAD^+ pathways, endocrine signals, inflammatory mediators, and reproductive aging, to enable the development of personalized and effective reproductive interventions.

Conflicts of interest: The authors declare no competing interests.

Data availability

No new data were generated or analysed in support of this research.

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