Nicotinamide as potential biomarker for Alzheimer's disease: a translational study based on metabolomics

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Abstract

The metabolic routes altered in Alzheimer's disease (AD) brain are poorly understood. We performed untargeted ^1H -NMR metabolomics in hippocampus of McGill-R-Thy1-APP transgenic (Tg) rats, a model of AD-like cerebral amyloidosis. Three groups of 9 month-old rats were tested: hemizygous Tg+/-, displaying mild amyloid pathology characterized by intraneuronal amyloid β (iA β) accumulation; homozygous Tg+/, showing iA β , senile plaques (extracellular A β deposition) and neuroinflammation, and wild-type (WT). Eighteen metabolites were detected, three of them showed significant differences among genotypes, but only two were specifically assigned to a known molecule: nicotinamide adenine dinucleotide (NAD) and nicotinamide (Nam). Only Tg+/+ rats showed significantly decreased levels of total-NAD, NADH and NAD + as compared to WT, and a significant increase in NAD+/NADH ratio, suggesting an alteration of the redox state, alongside the reduction of all forms of NAD. Transcript levels of NAD-consuming (CD38 and PARP2) and NAD-synthesis (NMNAT2) enzymes were increased in both transgenic genotypes. Next, Nam and NAD were evaluated at the peripheral level by targeted ^1H -NMR analysis in rat plasma, where NAD/H was undetectable, Nam levels were unchanged among genotypes, but Trigonelline (a metabolic product of Nam) was reduced in Tg+/+. Finally, the translational potential of these findings in the rat model was assessed by measuring Nam and Trigonelline levels in plasma of participants in the German longitudinal study on Aging, Cognition and Dementia (AgeCoDe), by targeted GC-El/MS. While trigonelline was undetected, Nam was significantly reduced in AD demented patients respect to cognitively normal participants (controls). This finding in Nam was replicated in a second independent case-control sample drawn from the same AgeCoDe. Next, the predictive value of Nam on disease progression was analyzed in AgeCoDe. Herein, reduction of Nam levels was observed in AgeCoDe participants who progressed to AD dementia ~ 1 year after blood collection, whereas Nam level were not reduced in those who converted afterwards. In summary, this preclinical study suggests that dysregulation of NAD/Nam depends on cerebral amyloid burden, and support the hypothesis that changes observed in the hippocampus may be detected in plasma. Furthermore, the findings in AgeCoDe points toward the potential use of Nam as plasma biomarker for AD.

Findings

Alzheimer's disease (AD) is a progressive neurodegenerative proteinopathy characterized by deposition of amyloid β (A β) and hyperphosphorylated tau protein in the brain of patients. Associated to the cognitive impairments and neurodegeneration, AD brains show metabolic disturbances [2]. However, the metabolic routes altered are poorly understood. As the metabolic pathways are evolutionarily conserved, the metabolic profiles carried out in transgenic (Tg) animal models of AD could be directly translated into human studies [3]. A promising animal model is the McGill-R-Thy1-APP rat [4] expressing the human amyloid precursor protein (APP) with the Swedish and Indiana mutations responsible for familial AD in humans. The hemizygous Tg+/- rats display intraneuronal A β accumulation and mild cognitive and behavioral impairments, representing an excellent model that resembles early stages of AD [5]. The homozygous Tg+/+ rats show the full AD-like-amyloid pathology, accompanied by neuroinflammation

and cognitive impairment, reflecting stages of late AD [4]. While the Tg rat model has been extensively used to explore stages of AD pathology and validation of experimental therapeutic candidates, studies linking the metabolic profile in hippocampus and plasma in association with the degree of amyloid pathology are still lacking. Consequently, this study aimed to characterize metabolic abnormalities in the hippocampus and plasma of homo- and hemizygous McGill-R-Thy1-APP rats by using untargeted metabolomics. Promising findings in the rat were followed up in human plasma samples exploring their potential utility as AD biomarkers.

To determine A β -associated shifts in brain metabolites, we first performed a highly sensitive multiplex ELISA to quantify total A β levels within the hippocampus of a sub-set of Tg rats (n=3-7). The median value of the concentration of A β 40 showed nearly 4-fold increase in Tg+/+ [54.4 [pg/mg] (IQR: 31.8-55.7)] vs. Tg+/- [13.8 [pg/mg] (IQR: 9.8-31.1), $p=0.048$]. For A β 42, the increased was more than 20-fold [48.8 [pg/mg] (IQR: 16.7-153.4)] vs. Tg+/- [2.4 [pg/mg] (IQR: 2.0-7.5), $p<0.035$]. These results confirmed the impact of the two copies of human mutant APP transgene on the accumulation of cerebral amyloid. To identify changes in pathways relevant for the hippocampus of the McGill-R-Thy1-APP rat, we carried out untargeted ¹H-NMR metabolomics on methanol-extracted samples from freshly isolated tissues. From the 18 metabolites detected with this technique (Fig.1A), three showed significant differences between genotypes. However, only two of them were assigned to a specific known metabolite: NAD and nicotinamide (Nam) (Fig.1B). Standard runs analysis confirmed that changes were in NAD and not in its related metabolite NADP (Fig.1C). As previously described for these key molecules in the NAD salvage-pathway, Nam and NAD levels showed an inverse relationship (Fig.1D). Since NMR analysis cannot differentiate between NADH and NAD⁺, we quantified total NAD (NAD_t), NADH and NAD⁺ by a colorimetric kit. One-way ANOVA tests indicated that NAD_t, NADH and NAD⁺ levels differed between genotypes. Post-hoc analyses revealed that Tg+/+ showed significantly lower levels of all NAD forms compared with those observed in WT. While the Tg+/- rat showed an intermediate level for NAD forms, these levels did not reached significance neither with WT nor with Tg+/+ (Fig.2A). These observations suggest that a threshold of A β accumulation is required to affect NAD metabolism. Comparison of NAD⁺/NADH ratios between genotypes were significant in the one-way ANOVA test with NAD⁺/NADH ratio significantly higher in the Tg+/+ compared to WT and Tg+/- (Fig.3B). This observation suggests a clear alteration of the redox state in the brains of the Tg+/+ rats which is probably still incipient in the Tg+/- rat to reach significance. We also evaluated whether changes in gene expression of NAD⁺-consuming and NAD⁺-generating enzymes might explain changes observed in Nam and NAD⁺ [6]. RT-qPCR experiments revealed that in both Tg rats genotypes the mRNA levels of CD38 (member of the cyclic ADP-ribose synthase family) and PARP2 (member of the ADP-ribose transferases family) were increased more than 1.5-fold as compared to WT, whereas transcript levels of PARP1 and SIRT3 (sirtuin) were unaffected (Fig.2C). As to NAD⁺-generating enzymes, gene expression analysis showed that mRNA levels of NMNAT2 were increased 1.5-fold in both Tg rats genotypes when compared to WT (Fig.2C). The NMNAT2 gene is a NAM-monomucleotide adenylyltransferase mainly expressed in brain. Based on these results, both the NAD⁺-consuming and the NAD⁺-generating pathways seem to be activated in AD brain suggesting a potential redox disturbances following the ongoing amyloid pathology. While central

disturbance in NAD⁺ metabolism in Tg rats was observed, its translation to peripheral tissue was unclear. Consequently, ¹H-NMR spectroscopy in plasma from these rats was performed. While NAD/H was undetectable, one-way ANOVA tests did not reveal significant difference in their Nam plasma levels (F(2, 16): 1.77, $p>0.05$). Interestingly, after a database analysis and ¹H-¹H-TOCSY confirmation, we detected levels of Trigonelline (a product of Nam metabolism) [7] in the plasma of the rats that were significantly reduced in Tg+/+ compared to both Tg+/- and WT rats (Fig.3A).

The results in the brain and plasma of the rat prompted us to explore whether these findings can be translated to humans. Herein, we focused our analysis on plasma because they might offer a promising alternative for biomarker in blood. Consequently, levels of Trigonelline and Nam were measured in human plasma samples derived from the longitudinal study AgeCoDe using GC-EL-MS. First, we compared whether plasma levels of 68 participants with AD dementia (AD) showed statistical differences compared to 93 cognitively normal (CN) participants. While Trigonelline was not detected in human plasma, Nam levels were significantly reduced in cases compared to CN (odd ratio (OR)=0.67, $p=0.02$, Fig.3B). In an independent replication sample drawn from AgeCoDe, including 96 CN and 29 AD, Nam showed a protective effect (OR=0.93) which, however, did not reach significance ($p=0.7$, Fig.3B). The meta-analysis of both samples confirmed the protective effect of plasma levels of Nam (OR=0.76, $p=0.04$, Fig.3B). In a second step, we explore whether Nam plasma levels, measured at baseline, are associated with the time to conversion to AD. Consequently, participants were included in the analysis if they have available data on plasma levels of Nam and converted to AD at any of the next three follow-ups for which data was available [Follow up (FU1)=0.94±0.35 years after baseline; FU2=2.43±0.38 years after baseline; FU3=4.13±0.37 years after baseline]. For the analysis, the effect of Nam levels was stratified in tertiles and their effect on time to conversion was visualized by *Kaplan-Meier* survival curves (Fig.3C). This analysis showed that only the high tertile of Nam showed a protective effect progression to AD (HR=0.73, $p=0.04$). However, we also observed that the hazard ratio (HR) is not proportional over time (curves intersect). Thus, while a person with Nam levels in plasma within the high tertile has 27% risk reduction of progressing to AD within the next 2.5 years, this HR is lost afterwards. Supporting this finding, we observed that only participants progressing to AD at FU1 showed significantly lower levels of Nam compared with CN ($p=0.04$, Fig.3D).

NAD⁺ and related metabolites are critical compounds essential to adaptive stress responses and cell survival. Considering the number of enzymes and transcription factors sensitive to the redox potential, NAD/H redox state acquires pathophysiological relevance for aging and neurodegenerative diseases [8-9]. While several studies in mouse models for AD have shown the relevance of the NAD(P)⁺/NAD(P)H homeostasis in the brain, especially in hippocampus and cortex [10-12], few reports have been published on the role of the NAD(P)⁺/NAD(P)H homeostasis in the McGill-R-Thy1-APP rats. Herein, an *in vivo* ¹H-MRS study in McGill-R-Thy1-APP rats identified a complex metabolite differences in hippocampus and frontal cortex of the transgenic rats [13]. In addition, several isolated cortical regions were analyzed in aged animals (15-month-old) using ¹H- and ¹³C NMR spectroscopy and HP-LC showing decreased levels of NAD⁺ in the cingulate cortex of Tg rats [14]. Our study now provides additional supporting evidence

indicating that hippocampal A β burden could determine the degree of NAD⁺ shift in McGill-R-Thy1-APP rat brain. This information is useful because it offers a different perspective on the A β -mediating mechanisms involved in brain energy dysfunction observed in AD.

From a translational point of view, experimental evidence supports that NAD⁺ augmentation can revert cognitive deficits in AD mouse model [15, 16]. Furthermore, research has shown that plasma levels of NAD⁺ decrease significantly with age in humans [17]. These animal and human data have fueled several clinical trials of NAD⁺ precursors which, however, produced inconsistent results [18, 19]. Importantly, our report showed that a metabolic disturbance detected in McGill-R-Thy1-APP rat brain is also found in rat plasma. This finding offers the possibility to use NAD⁺ metabolites as peripheral biomarkers for AD. Our study identified a significant lower level of plasma Nam in AD patients compared to healthy control. This difference is also seen at least 1.5 years before the patients progressed to AD. Thus, Nam levels in plasma may also serve as biomarker for progression to AD. However, it remains to be determined when Nam level increases in plasma before dementia is diagnosed.

Our study has also limitations. For example, rats showed decrease levels of Trigonelline in plasma, whereas human showed reduced levels of Nam. Although little is known about the pathway connecting Nam and Trigonelline, their metabolism seems to be a linked. Trigonelline is the methylation product of nicotinic acid, and this latter metabolite is in constant equilibrium with Nam within the vitamin B3 metabolism [20]. Hence, disequilibrium in this pathway may be reflected in either Trigonelline or Nam levels.

In summary, this is the first report showing a significant decrease of Nam plasma levels in people with AD that is observed couple of years before conversion, thereby suggesting its potential use as biomarker for progression. Further studies in larger cohorts are now needed to confirm our findings and the potential use of Nam as peripheral biomarkers for AD.

Methods

Rat model

Transgenic (Tg) McGill-R-Thy1-APP rats, harboring the human APP751 transgene with the Swedish and Indiana mutation under the control of the murine Thy1.2 promoter, were generated using the HsdBr1: WH Wistar strain [4]. Animals were provided to Fundación Instituto Leloir (FIL) by The Royal Institution for the Advancement of Learning/McGill University, Montreal, Canada, and an in-house colony was established at FIL. Rats' genotypes were determined by real time qPCR as previously described [5]. To avoid the litter effect, groups were made up of pups from 3 to 4 different litters. Homozygous (Tg+/+), hemizygous (Tg+/-), and littermates' wild type (WT) control animals were maintained in polycarbonate cages in a temperature-controlled animal facility with a 12-h dark/light cycle and allowed to consume standard diet and water ad libitum. Only 9-month-old male rats were used for experiments to avoid any potential effects of female estrus cycle. All experimental procedures were performed in accordance with the

guidelines of ARRIVE and OLAW–NIH. The protocol was approved by the local animal care committee (CICUAL # A5168-01).

Hippocampal tissue and plasma collection

Rats were anesthetized with ketamine (50mg/kg) and xylazine (10mg/kg), placed under a guillotine blade, decapitated and brains quickly removed. Hippocampi were dissected on an ice-cold plate, and divided into left and right hemispheres. Each hippocampus was immediately and independently frozen in liquid nitrogen, and stored at -80 °C until used. Blood samples were collected immediately after animals' decapitation in 15 mL heparinized tubes, centrifuged at 3500 xg for 10 min. Plasma was collected, aliquoted and stored at -80 °C until use.

Human plasma samples

Plasma samples were selected from the German study on Aging, Cognition and Dementia (AgeCoDe) biobank [21]. This is a longitudinal study, where participants were recruited in primary care centers in six German cities. Inclusion criteria was to be at least 75 years old and cognitively healthy according to the general practitioner's judgment. Every ~18 months interval participants are followed-up with personal interviews and neuropsychological assessments. To date, nine follow-ups (FUs) were completed, but results from the last one are still in process. Blood samples were obtained at the 3rd visit, processed and store at -80 °C. For this study the 3rd visit is considered the baseline. Controls (n=189) remained cognitively normal until the last FU analyzed, were 83.6 ± 3.1 years old, 64.0% were female and 20.6% were APOE4 carriers. Cases were participants with incident AD (n=68), 86.0 ± 3.6 years old, 64.7% female and 33.8% APOE4 carriers; and prevalent AD (n=29), 84.2 ± 3.1 years old, 75.8% female and 37.9% APOE4 carriers. Participants converting to AD in the next three visits following baseline (FU1, FU2 and FU3) were included in the analysis. At FU1 were 25 participants with age of 84.8 ± 3.5 years old, 80% women, and 28% APOE4 carriers; at FU2 were 37 participants with age of 83.6 ± 2.6 years old, 67.6% women, and 32.4% APOE4 carriers; and at FU3 were 23 participants with age of 82.7 ± 2.6 years old, 60.9% women, and 21.7% APOE4 carriers.

Expression of A β isoforms in rat hippocampus

To quantify human A β 38/40/42 MSD® V-PLEX PLUS A β Peptide Panel 1 kit was used following the manufacturer's instructions. Briefly, hippocampus of Tg rats were processed with illustra triplePrep kit (GE) and Individual protein samples were loaded onto MULTI-SPOT® microplates pre-coated with antibodies specific to the C-termini of A β 38, A β 40 and A β 42 and were detected with SULFO-TAG™-labeled 6E10 antibody. Light emitted upon electrochemical stimulation was read using the MSD QuickPlex SQ120 instrument. Data were analyzed using MSD Workbench 4.0 software. Values of concentration in pg/mg of total protein were expressed as median and interquartil range (IQR). Mann Whitney test was applied to assess significant differences between groups.

NMR Spectroscopy

Frozen hemi-hippocampus were homogenized with a teflon-glass grinder in 2 ml ice-cold 80% methanol and centrifuged at 4 °C for 10 min at 15000xg. Supernatants were collected and dried in a Savant SpeedVac (Thermo Scientific). Plasma proteins were precipitated with 80% methanol (1:2) as described in G. A. Nagana Gowda *et al* [22]. Supernatants were collected and dried in a Savant SpeedVac (Thermo Scientific). Dried hippocampus or plasma samples were solubilized in 0.5 ml sodium phosphate buffer (100 mM dissolved in D₂O, pH = 7.4), supplemented with 3-trimethylsilyl-[2,2,3,3,-2H₄]-propionate (TSP, final concentration 0.33 mM) as chemical shift reference. All NMR experiments were performed at 298 K on a Bruker Avance III spectrometer operating at a proton frequency of 600.3 MHz. ¹H-NMR 1D spectra were acquired using a standard Bruker 1D NOESY pulse program with pre-saturation during relaxation delay and mixing time, and spoil gradients (noesygppr1d). The following experimental parameters were used in all measurements: 256 scans, 1.85 s relaxation delay, 1.36 s acquisition time, 20 ppm spectral width, 10 ms mixing time, and 32 K acquisition points. The NMR data were zero-filled, Fourier transformed, phase corrected using NMRPipe and converted to a Matlab-compatible format for further processing and analysis. All spectra were referenced to TSP (1H δ = 0 ppm) and submitted to water peak elimination, baseline correction, normalization, and scaling. The assignment was achieved using the freely available electronic databases HMDB and BMRB, and subsequently confirmed by 2D spectra including heteronuclear single quantum coherence (HSQC) and total correlation spectroscopy (TOCSY). 2D ¹H-¹H TOCSY spectra were collected with N1=512 and N2=2048 complex data points. The spectral widths for the indirect and the direct dimensions were 9615.4 and 9604.9 Hz, respectively. The number of scans per t1 increment was set to 36. The transmitter frequency offset was 4.7 ppm in both ¹H dimensions. 2D ¹³C-¹H HSQC spectra were collected with N1=512 and N2=2048 complex data points. The spectral widths for the indirect and direct dimensions were 24,906.9 and 12,019.2 Hz, respectively. The number of scans per t1 increment was set to 256. The transmitter frequency offset was 70 ppm in the ¹³C dimension and 4.7 ppm in the ¹H dimension. The ¹H resonances assigned to Trigonelline were based on HMDB database (<https://hmdb.ca/metabolites/HMDB0000875#spectra>) and confirmed by 2D ¹H-¹H TOCSY spectra.

NAD⁺/NADH quantification and NAD salvage pathway enzymes levels determination

NAD levels were measured using NAD/NADH assay kit from Abcam (ab65348). Briefly, hippocampi from WT, Tg(+/-) and Tg(+/+) rats were snap frozen in liquid nitrogen, homogenized in NADH/NAD extraction buffer and filtered through a 10kD spin column (Abcam, ab93349) to remove enzymes. Assay procedure was followed per kit instructions and levels of NADH and NAD⁺ were determined normalized to tissue weight. NAD salvage-pathway enzymes transcript levels were estimated by reverse-transcription quantitative PCR (RT-qPCR). Protein, DNA and RNA were isolated from frozen hemi-hippocampus using Illustra™ TriplePrep Kit (GE Healthcare) following manufacturer's instructions. Briefly, 1-3 ug of total RNA was reverse transcribed using oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen). Sequences of oligonucleotides (F, forward; R, reverse; 5'→3') to assess transcript levels of NAD⁺ consumption enzymes, NAD⁺ synthesis enzymes and a constitutive gene were as follows: CD38 (F 5'-GGTCCCTCAGTGAGCCATTT-3'; R 5'-ATGTCATGAATTACCCAGGC-3'), PARP1 (F 5'-

AGGACCCCATCGATGTCAAC-3'; R 5'-GGTCGCGTGAGTGTTCTTCAC-3'), PARP2 (F 5'-ATGACGTCGTTCAAGCG-3'; R 5'-gtcatctgttgctctgttgcc-3') SIRT3 (F 5'-TGTGGGGTCCGGGAGTATTA-3'; R-5'GTCATCTGTTGCTCTGTTGCC-3'); NMNAT2 (F 5'-TCCCAATATGACCGAGACCAC-3'; R 5'-TTGTGCAGATAATCCCTGGCT-3') and Eukaryotic Translation Elongation Factor 1 Alpha 1 (EEF1A1) (F 5'-AACTGACAAGCCTCTGCGAC-3'; R 5'-GCTTCATGGTGCATTTCCACA-3'). RT-PCR reactions were prepared using PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific) and run following the standard cycling mode (primer T_m > 60°C) instructions in a Light Cycler 480 Instrument II (Roche). Melt curve analysis and agarose gels verified the presence of a single desired PCR product. Absolut quantification was performed using dilutions (1, 1:10, 1:50, 1:100, 1:500 and 1:1000) of a standard sample, which was a pool of 5 ul cDNA of each analyzed sample. The relative amount of transcripts to EEF1A1 was quantified by the $2^{-\Delta\Delta Ct}$ method using MxPro software. The mean $\pm$ SEM relative to WT (=1) were analyzed for each genotype and values above a fold-change of +1.5 were considered different from WT (=1).

Gas chromatography Electron Impact Mass Spectrometry (GC-EI-MS)

Plasma samples were thawed on ice, and 100 ul were extracted with 900 ul of cold extraction buffer containing 40:40:20 methanol:acetonitrile:water [v:v:v]. After 30 min in an orbital mixer at 4°C., samples were sonicated for 10 min in an ice-cooled bath-type sonicator and centrifuged for 10 min at 16000xg at 4°C. Supernatants were collected and dried in a SpeedVac until complete dryness. Standard curves were prepared with concentrations ranging from 0.005 to 50 ug/ml of nicotinamide and trigonelline, processed in the same way as samples. Dried down samples and standards were derivatized using methoxyamine and MSTFA/FAMEs solution (N-methyl-N-trimethylsilyl-trifluoroacetamid/Fatty acid methyl esters) following standard procedures [23, 24]. After that, samples were analyzed in a GC-EI-MS (Q Exactive GC Orbitrap system, ThermoFisher) using a 30-m DB-35MS capillary column. Representative fragments from the GC-EI-MS analysis of Nam were extracted using TraceFinder (Version 4.1, ThermoFischer) and quantified using the linear range of the obtained standard curve. All analysis were performed using peak areas, transformed into Z-scores, for easier comparison among experiments.

Statistical analysis

Differences among groups in the AUC of peaks from ¹H-RMN spectroscopy experiments were analyzed by one-way ANOVA, taking p<0.05 and p<0.0001 as significant according to Dunnett's multiple range test. Data of protein levels of NADt/NADH and NAD⁺ were analyzed by one-way ANOVA tests followed by post-hoc Tukey's multiple comparisons tests. Two-tailed unpaired t-tests were used to compare transcripts expression of those genes that showed more than 1.5-fold change in their expression levels compared to WT (=1). For easier comparison among experiments of rat plasma metabolome a Z-score standardization was applied and subsequently data were analyzed by one-way ANOVA tests followed by post-hoc Tukey's multiple comparisons tests. In all cases, assumption of normality was examined using Kolmogorov-Smirnov or Shapiro-Wilk tests. A probability equal or less than 5 % was considered as significant. All analyses were carried out using GraphPad Prism for Windows (version 7.0). Statistical analysis and plots of data from GC-EI-MS experiments performed with human plasma were done using R-

project v. 4.0.0 (<https://www.R-project.org>) and R-studio v.1.2.5042 (<http://www.rstudio.com/>). Normal distribution was visualized using qqnorm plots, and outliers (defined as mean $\pm$ 3 standard deviation) were eliminated from the analysis (n=2). Linear regression models adjusted for sex, age and apoe4 were used to estimate the association of Nam levels vs CN in the *discovery* and *replication* experiments, as well as in FUs groups. Meta-analysis was performed using the R-package “metafor” [25] and visualized with the general function forest. The cox proportional hazards regression model, which relates time dependent variables, time dependent strata, and multiple events per subject, were performed with the R-package “survival” [26] and “survminer” [27]. Only samples with incident AD at FU1-3 were included, time variable was time to conversion to AD in years, and the event per subject was conversion (no=0, yes=1). Proportional hazard assumption was tested by Schoenfeld’s test, and consequently two cox regressions were performed with a split-time=2.5 years.

Declarations

Ethics approval and consent to participate

The original study protocol with human subjects was approved by the local ethics committees at the following German institutions: University of Bonn; University of Hamburg; University of Duesseldorf; University of Heidelberg/Mannheim; University of Leipzig and the Technical University of Munich. Written informed consent was obtained from all participants.

Consent for publication

Not applicable

Availability of data and material

The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

Conceptualization: MCD, AR, LM; Methodology: MCD, MA, PG, PG, SP, MS, WM, MW, SRH; Statistical Analysis: MCD, MA, PG, PVMA; Investigation: MCD, AR, LM; Resources: MCD, AR, LM; Writing - original draft: MCD, MA, AR, LM; Writing - review & editing: MCD, MA, PG, PVMA, SP, PG, EMC, ACC, MS, WM, MW, SRH, AR, LM; Supervision: AR, L.M.; Project administration: MCD, L.M; Funding acquisition: MCD, ACC, AR, LM All authors read and approved the final manuscript.

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Figures

Figure 1

Untargeted 1H-NMR metabolomics of hippocampus of AD-like amyloid pathology transgenic rats. (A) Typical 600 MHz 1H-NMR spectrum of WT rats (n= 14), representative of all the registered spectra. Assigned resonances of specific metabolites are indicated in red. Expanded views of the spectrum between 3.1-4.4 ppm (a) and 1.7-3.2 ppm (b) are shown. (B) Overlaid of representative 1H-NMR spectra of WT rats (black) (n=14), and Tg+/+ (red) (n=10) in the 8.5-8.0 ppm zone (dashed box in A). The resonances assigned to NAD and Nam protons are indicated. Differences in the AUC among groups (WT; Tg+/- and Tg+/+) were analyzed by one-way ANOVA. Significant differences between WT and Tg+/+ are indicated as significant according to Dunnett's multiple range test. *p<0.05; ****p<0.0001. (C) Overlaid of representative 1H-NMR spectra of WT rats (black, sample), NAD standard (red, upper panel) and NADP standard (red, lower panel). (D) Correlation between NAD and Nicotinamide levels of samples analyzed. The AUC of H6 of NAD and H2 of Nicotinamide were plotted (n=25). The linear regression (dashed line), the Pearson's correlation coefficient and the p-value (two tailed) are shown.

Figure 2

Hippocampal Aβ deposition alters the brain NAD+ metabolism. (A) Bars show mean ± SEM protein levels of total NAD (NADt), NADH and NAD+ in hippocampal homogenates of control (WT; n=3), hemizygous (Tg+/-; n=3) and homozygous (Tg+/+; n=3) transgenic rats. One-way ANOVA tests indicated that NADt,

NADH and NAD⁺ levels differed between genotypes (NADt: F(2, 6): 8.31, p = 0.02; NADH: F(2, 6): 10.76, p = 0.01; NAD⁺: F(2, 6): 4.89, p = 0.05). Post-hoc analyses revealed that Tg^{+/+} showed significantly lower levels of NADt, NADH and NAD⁺ compared with those observed in WT, while Tg^{+/-} did not differ from either WT or Tg^{+/+} in any of the cases. *p<0.05; **p<0.01 (B) Mean NAD⁺/NADH ratio in hippocampal homogenates described in (A). Results are means ± SEM (n=3 rats per group). One-way ANOVA test resulted to be significant (F(2, 6): 9.25; p = 0.01) and post-hoc comparisons revealed that NAD⁺/NADH ratio from Tg^{+/+} was significantly lower than NAD⁺/NADH ratios observed in WT and Tg^{+/-}. *p<0.05. (C) Transcript levels of NAD⁺ consumption enzymes (CD38, PARP1, PARP2 and SIRT3) and NAD⁺ synthesis enzyme (NMNAT2). Each bar represents the mean ± SEM of at least three independent experiments performed by triplicate for each sample normalized by Eukaryotic Translation Elongation Factor 1 Alpha 1 (EEF1A1). The mean ± SEM relative to WT (=1) is shown. Values above the dashed line (+1.5) were considered different from WT (=1). Unpaired Student t-tests showed no significant differences between hemizygous (Tg^{+/-}) and homozygous (Tg^{+/+}) transgenic rats in all genes above 1.5 fold change relative to WT (CD38: t=0.2753, p=n.s.; PARP2: t=0.3588, p=n.s.; NMNAT2: t=1.038, p=n.s.).

Figure 3

Plasma levels of Trigonelline as a potential peripheral biomarker of AD amyloid pathology. (A) Box plots represent the normalized NMR spectral areas of Trigonelline in plasma of WT (n=8), Tg ^{+/-} (n=5), and Tg

+/+ rats (n=6) (one-way ANOVA test: $F(2, 16): 33.5, p < 0.0001$ followed by Tukey post-hoc tests).
**** $p < 0.001$. (B) Meta-analysis forest plot of Nicotinamide plasma levels in human samples. Discovery experiment includes 68 cases and 93 controls. Replication experiment includes 29 cases and 93 controls. Estimates are in Odds Ratios; CI, confidence interval; FE Model, fixed effects meta-analysis results. (C) Kaplan-Meier conversion to AD survival of 85 participants after blood test for Nicotinamide, stratified in high, medium or low levels. High levels of Nicotinamide seem to be a predictor of dementia survival for 2.5 years (HR=0.73, $p=0.04$). Arrows represent the periods of the follow-ups (FU) 1 (0.26-1.68 years), FU2 (1.63-3.08 years) and FU3 (3.39-4.68 years). The dotted lines indicate the time (in years) when the probability of converting to AD is 50%. (D) Box plots represent the normalized GC-ESI-MS spectral areas of Nicotinamide in human plasma of CN (cognitive normal) subjects (n=189); AD (n=85) patients and FU1 (n=25); FU2 (n=37) and FU3 (n=23) participants.* $p < 0.05$