PURINE-RELATED METABOLITES AND THEIR CONVERTING ENZYMES ARE ALTERED IN FRONTAL, PARIETAL, AND TEMPORAL CORTEX AT EARLY STAGES OF ALZHEIMER'S DISEASE PATHOLOGY

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Abstract

Adenosine, hypoxanthine, xanthine, guanosine, and inosine levels were assessed by HPLC, and the activity of related enzymes 5'-nucleotidase (5'-NT), adenosine deaminase (ADA), and purine nucleoside phosphorylase (PNP) measured in frontal (FC), parietal (PC) and temporal (TC) cortices at different stages of disease progression in Alzheimer's disease (AD) and in age-matched controls.

Significantly decreased levels of adenosine, guanosine, hypoxanthine, and xanthine, and apparently less inosine, are found in FC from the early stages of AD; PC and TC show an opposing pattern, as adenosine, guanosine, and inosine are significantly increased at least at determinate stages of AD whereas hypoxanthine and xanthine levels remain unaltered. 5'-NT is reduced in membranes and cytosol in FC mainly at early stages but not in PC, and only at advanced stages in cytosol in TC. ADA activity is decreased in AD when considered as a whole but increased at early stages in TC. Finally, PNP activity is increased only in TC at early stages.

Purine metabolism alterations occur at early stages of AD independently of neurofibrillary tangles and β-amyloid plaques. Alterations are stage- and region-dependent, the latter showing opposite patterns in FC compared with PC and TC. Adenosine is the most affected of the assessed purines.

Key words: Adenosine, Alzheimer's disease, cerebral cortex, purine metabolism.

Introduction

Alzheimer disease (AD), the most common neurodegenerative disease in adulthood, is characterized neuropathologically by β-amyloid deposition forming plaques and amyloid angiopathy, and hyperphosphorylated tau in neurons with neurofibrillary tangles (NFTs) and pre-tangles, dystrophic neurites of amyloid plaques, and neuropil threads (24, 31). NFTs increase in number and distribution from selected nuclei of the brain stem, and entorhinal and transentorrhinal cortex (stage I-II) to the hippocampus and limbic cortex (stage III-IV), and eventually the neocortex (stages V-VI) (14). Clinical symptoms in AD manifest several years after the appearance of classical neuropathological findings and that AD pathology is not restricted to β-amyloid and tau (13, 26). On the contrary, alteration of multiple metabolic pathways and cell types converge and contribute to the development of AD (26).

Purines and pyrimidines are components of a large number of key molecules. Primary purines adenine and guanosine, and pyrimidines cytosine, thymidine, and uracyl are the core of DNA, RNA, nucleosides, and nucleotides involved in energy transfer (ATP, GTP) and co-enzymes (NADH, $FADH_2$) (6, 32).

Adenosine produced by neurons and astrocytes modulates excitatory and inhibitory neurotransmission, and influences relevant brain functions including sleep and arousal, cognition and memory, and neuronal damage and degeneration, by acting as an extracellular molecular via specific adenosine receptors (8, 29, 53, 55). There are four G-protein coupled receptors for adenosine A_1, A_{2A}, A_{2B} and A_3 (29, 55). Importantly, A_{2A} receptor is necessary for neurodegeneration and memory impairment in animal models of AD and tauopathy (16, 17, 21, 45, 61). Moreover, A_{2A} receptor is necessary and sufficient to trigger memory impairment in adult mice (47, 51).

Adenosine is generated intracellularly by the hydrolysis of AMP and S-adenosyl homocysteine (SAH) by soluble 5'-nucleotidase (5'-NT) and S-adenosyl homocysteine hydrolase, respectively. It can then be phosphorylated to AMP by adenosine kinase

(ADK) or transformed into inosine by adenosine deaminase (ADA). Extracellularly, ATP can be dephosphorylated to AMP by ectonucleoside triphosphate diphospho-hydrolase CD39 and AMP dephosphorylated to adenosine by 5'-nucleotidase CD73. In addition, adenosine can be transported to intracellular and extracellular compartments by specific transporters (28). In the intracellular space, the products of adenosine degradation, such as AMP and inosine, are transformed into inosine or hypoxanthine by AMP deaminase and cytosolic 5'-nucleotidase II (cN-II) or by purine nucleoside phosphorylase (PNP), respectively. Inosine is also able to exit by nucleoside transporter. Hypoxanthine can be oxidized by xanthine oxidase and transformed into xanthine. Inosine, hypoxanthine, and xanthine can also be transported to the extracellular compartment. Guanosine is derived from GMP (guanosine monophosphate) by nucleotidase and can be phosphorylated and deaminated into xanthine (11, 57). Ecto-5'-nucleotidase has a role in the activation of A_1 and A_2 receptors (19, 23, 25, 54). Figure 1 shows some of these metabolic connections. Although not addressed in the present paper, other enzymes are important in adenosine signaling (10, 50, 52). A_1 and A_{2A} receptors are modified in brain in AD (63). A_1 and A_{2A} receptors are increased in frontal cortex with respect to age-matched control (1). A_1 receptors are increased in degenerating neurons and A_{2A} receptors in glial cells of the hippocampus in AD (5). These data contrast with previous studies reporting decreased A_1 receptor in dentate gyrus and CA3 region of the hippocampus in AD patients (34).

Studies on enzymes linked to purine metabolism in AD are scant. AMP deaminase activity is increased in AD with respect to control cases (58). 5'-NT activity is reduced in brain homogenates of AD (37).

Our previous studies have shown altered expression of several genes involved in purine metabolism and abnormal levels of certain purine metabolites in the entorhinal cortex, frontal cortex area 8, and precunneus in AD which are region- and stage-dependent (6). The present study is focused on the assessment of adenosine,

hypoxanthine, xanthine, guanosine, and inosine levels as well as on differences in the activity of related enzymes 5'-NT, ADA, and PNP in three cortical regions -frontal cortex, parietal cortex and temporal cortex- in AD with disease progression and age-matched controls.

Material and Methods

Human samples

Human brain samples were obtained from the Institute of Neuropathology HUB-ICO-IDIBELL Biobank following the Spanish legal regulations (Real Decreto 1716/2011) and the approval of the local ethics committee of the Bellvitge University Hospital. Brains of patients with AD and age-matched controls were obtained at from 3 to 24 h after death and were immediately prepared for morphological and biochemical studies. Special attention was paid to minimize limitations related to molecular studies of the post-mortem brain including combined pathologies, metabolic syndrome, medication that could interfere with biochemical studies, long agonic stress, reduced post-mortem delay and controlled conditions of temperature and tissue processing as detailed elsewhere (27). A slight deterioration of the quality of brain samples is expected to dramatically alter signals related to stress in the brain, such as ATP (56) or adenosine (30). However, brain samples pH value was not an interfering factor in the present study as it has not been related to postmortem delay or time in storage (48). Even considering an optimal scenario, restricted sampling conditions imply not acceptance of certain cases thus reducing the number of suitable cases for study.

During autopsy, half of the brain was fixed in formalin, while the other half was cut into coronal sections 1 cm thick from which selected areas were dissected and immediately frozen on dry ice and stored at -80°C until use. The neuropathological study was carried out on formalin-fixed, de-waxed 4-mm thick paraffin sections of the frontal (area

8), primary motor, primary sensory, parietal and temporal superior, temporal inferior, anterior cingulate, anterior insular, and primary and associative visual cortices; entorhinal cortex and hippocampus; caudate, putamen and pallidum; medial and posterior thalamus; subthalamus; Meynert nucleus; amygdala; midbrain (two levels); pons and medulla oblongata; and cerebellar cortex and dentate nucleus. The sections were stained with hematoxylin and eosin, Luxol fast blue-Klüver Barrera, and for immunohistochemistry to glial fibrillary acidic protein, CD68, and Iba1 for microglia, β-amyloid, phosphorylated tau (clone AT8), αβ-crystallin, α-synuclein, TDP-43, and ubiquitin. AD-related pathology was categorized following the classification of Braak and Braak for neurofibrillary tangle pathology adapted for paraffin sections (simplified as stages I-II, III-IV, and V-V) (12), and the phases of Thal (59) for β-amyloid burden. Special care was taken to use AD cases with no or minimal co-morbidities to avoid bias related to combined pathologies. Cases with added proteinopathies and with vascular pathology other than small blood vessel disease were excluded. Cases with infectious and inflammatory diseases of the nervous system, and systemic metabolic diseases including those linked to chronic ethanol consumption, were not considered suitable in the pathological and control groups. Main characteristics of cases are summarized in Table 1.

Fresh frozen samples of the frontal area 8, parietal area 7, and temporal area 21 were used in this study for the determination of adenosine, guanosine, hypoxanthine, xanthine, and inosine levels, and the enzymatic activity of 5'-nucleotidase, adenosine deaminase, and purine nucleotidase phosphorylase.

Membrane and cytosol fractions purification

Samples were homogenized in 30 volumes of isolation buffer (50 mM Tris HCl, pH 7.4 containing 10 mM $MgCl_2$ and protease inhibitors) in DOUNCE homogenizer (10xA, 10x B). After homogenization, samples were centrifuged for 5 min at 1,000xg in a Beckman JA 20 centrifuge (Coulter, Madrid, Spain). Supernatants were recovered and

centrifuged for 20 min at 27,000xg, and the resulting pellet (membrane fraction) was finally re-suspended in isolation buffer and homogenized again (DOUNCE homogenizer, 10xA, 10xB). Cytosol was concentrated in Vivaspin 20 columns (MW cut off 3 kDa) (GE Healthcare Life Sciences, UK). Columns were cleaned with Milli-Q water and centrifuged for 20 min at 5,000xg. After this, 15 ml of supernatant was added to the columns which were then centrifuged for 110 minutes at 5,000xg at 20°C. The eluted fraction was lyophilized overnight in Vitris Sentry Benchtop 3L Freeze Drying System. This fraction was employed for HPLC analysis.

Protein concentration was determined with the Lowry method using bovine serum albumin as standard.

HPLC procedure

HPLC reagents

Stocks of adenosine, guanosine, hypoxanthine, xanthine, and inosine standards were prepared at 1 mM with water (HPLC-grade) in a final volume of 100 ml. NaOH (100 µl of 1 M) was added to hypoxanthine and xanthine stocks whereas inosine and guanosine were heated for optimal homogenization. All purines were from Sigma (Madrid, Spain), except adenosine which was from Fluka (Madrid, Spain). Purine standards were filtered using a 0.25 µm filter.

Preparation of standard curves and samples

The standard curves were obtained using five concentrations of each purine: 500 µM, 100 µM, 10 µM, 1 µM, and 100 nM. The initial stock was 1 mM and dilutions were prepared with water (HPLC-grade). The samples were reconstituted with water (HPLC-grade) and adjusted to pH between 2 and 3 with 1 M HCl. The final volume of the samples was 400 µl.

HPLC protocol

Chromatographic analysis was performed with Ultimate 3000 U-HPLC (ThermoFisher, Madrid, Spain) and data peaks were processed with Chromaleon 7 (ThermoFisher,

Madrid, Spain). HPLC diode array was used working at a 254 nm wavelength. We employed a C18 column of 4.6 mm x 250 mm, 5 µm particle size. Volumes of 10 µl were injected for the standard and 40 µl for the samples. Two solvents were used for gradient elution: solvent A, 20 mM phosphate buffer solution (pH 5.7), and solvent B 100% methanol. The gradient was initiated at 95% solvent A and 5% solvent B for 11 min, followed by 80% solvent A and 20% solvent B for 9 min, and finally 95% solvent A for 2 min. The total run time was 22 min with a constant flow rate of 0.8 ml/min at 25°C. Retention times for hypoxanthine, xanthine, inosine, guanosine, and adenosine were 3.5, 3.9, 8.4, 9.4, and 15.5 min, respectively. Each purine level was obtained by interpolation from the corresponding purine standard curve. Data were then normalized using the protein concentration of each sample.

Determination of protein concentration in HPLC samples

Protein concentration was measured with Micro BCA Protein Assay (Thermo Scientific, Rockford, USA) using bovine serum albumin as a standard.

Measurement of 5'-nucleotidase activity

5'-NT activity was measured in the membranes and cytosolic fractions. In the cytosol, 5'-NT activity was determined with one-time measurement of V_{max} at a saturated concentration of 500 µM AMP. Membranes and cytosol samples (20 µg protein) were pre-incubated in 180 µl reaction medium containing 50 mM Tris, 5 mM $MgCl_2$, pH 9, at 37°C for 10 min. The reaction was initiated by the addition of 20 µl AMP (final concentrations, 10 µM-1 mM in the case of membranes and 500 µM in the case of cytosol) and stopped after 20 min by adding 200 µl of 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 min and centrifuged at 12,000xg for 4 min at 4°C. The supernatants were used to measure inorganic phosphate released (20) using KH_2PO_4 as P_i standard. Non-enzymatic hydrolysis of AMP was corrected by adding membranes after TCA in order to denaturalize the enzyme. Incubation times and protein concentration were selected in order to ensure the linearity of the reactions. All

samples were run in duplicate. Enzyme activity was expressed as nmol Pi released/min x mg of protein (46).

Measurement of adenosine deaminase activity

ADA activity was measured in the cytosol of AD and control samples with the commercially available ADA activity assay kit (Abcam, London, UK) following the indications of the supplier. This is an assay where inosine formed from the breakdown of adenosine is detected via a multi-step reaction, resulting in the formation of an intermediate that reacts with an ADA probe to generate a fluorescent product that can be quantified at λex / λem of 535/587 nm. The kit measures total activity of Adenosine Deaminase with limit of quantification of 10 μU recombinant Adenosine Deaminase.

ADA activity was calculated as:

$$\Delta RFU_{535/587\ nm} = (RFU_2 - RFU_{2BG}) - (RFU_1 - RFU_{1BG})$$

where $\Delta RFU_{535/587\ nm}$ was used to obtained X pmol of inosine generated by ADA during a reaction time $\Delta T = T_2 - T_1$. RFU_{2BG} and RFU_{1BG} represented background values at reaction times, and RFU_2 and RFU_1 were fluorescence values at the same reaction times.

Adenosine deaminase concentration was calculated as:

$$ADA\ Activity = \frac{X}{\Delta T \times \mu g\ of\ protein} * D = \text{pmol/min/μg=μU/μh}$$

where X was the quantity of inosine extrapolated from the standard curve (pmol), ΔT was the reaction time, μg of protein was the quantity of protein put on each well, and D the dilution factor of each sample.

Measurement of purine nucleotidase phosphorylase activity

PNP activity was measured in the cytosol using Purine Nucleoside Phosphorylase activity assay kit (Abcam, London, UK) following the indications of the supplier. This is an assay where the hypoxanthine formed from the breakdown of inosine is detected via

a multi-step reaction, resulting in the generation of an intermediate that reacts with the PNP Probe. The fluorescent product is measured at λex / λem of 535/587 nm. Limit of quantification is 0.005 µU recombinant PNP. PNP activity was calculated as:

$$\Delta RFU_{535/587\ nm} = (RFU_2 - RFU_{2BG}) - (RFU_1 - RFU_{1BG})$$

where $\Delta RFU_{535/587\ nm}$ was used to obtained Y pmol of hypoxanthine generated by PNP during a reaction time $\Delta T = T_2 - T_1$. RFU_{2BG} and RFU_{1BG} represented background values at reaction times, and RFU_2 and RFU_1 were the fluorescence values at the same reaction times.

PNP concentration was calculated as:

$$PNP\ Activity = \frac{Y}{\Delta T \times \mu g\ of\ protein} * D = \text{pmol/min/µg=µU/µh}$$

where Y was the quantity of hypoxanthine extrapolated from standard curve (pmol), ΔT was the reaction time, µg of protein was the quantity of protein put on each well, and D the dilution factor of each sample.

Statistical analysis

Data were analyzed with GraphPad Prism 6.0 program (GraphPad Software, San Diego, CA, USA). Differences between mean values of two groups (control and AD) were studied with Student's t-test. The difference between mean values of three or more groups (control and AD stages) was evaluated with ANOVA-Fisher's LSD post-test; $p<0.05$ was considered significantly different. Regarding nucleotidase activities, the K_M and V_{max} values were calculated using a nonlinear Michaelis-Menten curve and analyzed using unpaired two-tailed Student's t-test or ANOVA-Fisher's LSD post-test test; $p<0.05$ was considered significantly different. Pearson r correlation coefficients and corresponding P values were obtained by Correlation analysis.

Results

Nucleotide measurement in frontal, parietal, and temporal cortex

Adenosine, guanosine, hypoxanthine, xanthine, and inosine were determined in the frontal, parietal, and temporal cortices in control and total AD cases. To study Alzheimer's progression, AD samples were further divided in three groups, initial (I-II), intermediate (III-IV), and advanced (V-VI) stages of NFT degeneration.

Adenosine was significantly decreased in the frontal cortex in AD (9.22 ± 1.91 pmol/μg prot, $p<0.001$) compared with control cases (24.97 ± 5.21 pmol/μg prot). Lower levels were observed at stages I-II (3.85 ± 3.39 pmol/μg prot, $p<0.01$) when compared to intermediate (9.76 ± 1.03 pmol/μg prot, $p<0.05$) and advanced stages (11.91 ± 4.21 pmol/μg prot, $p<0.05$). In contrast, adenosine levels were increased in the parietal (85.15 ± 7.77 pmol/μg prot, $p<0.05$) and temporal (73.88 ± 7.60 pmol/μg prot, $p<0.05$) cortices in AD compared with corresponding controls (55.45 ± 7.97 and 44.56 ± 5.65 pmol/μg prot, respectively). Comparing the adenosine level in control cases in the three different brain areas we can observe a lower level in frontal cortex (24.97 ± 5.21 pmol/μg prot) than in parietal (55.45 ± 7.97 pmol/μg prot) and temporal (44.56 ± 5.65 pmol/μg prot) cortices (Figure 2).

Guanosine levels were not significantly altered in the frontal cortex in AD cases when compared with controls. Only reduction in the levels of guanosine was found in frontal cortex at early stages (AD I-II: 69.03 ± 18.51 vs C: 166.29 ± 22.40 pmol/μg prot, $p<0.05$). In contrast, guanosine levels in AD were increased in the parietal cortex (AD: 144.20 ± 14.27 vs C: 90.22 ± 13.30 pmol/μg prot, $p<0.05$) and temporal cortex (AD: 146.20 ± 11.40 vs C: 73.80 ± 9.00 pmol/μg prot, $p<0.01$). In the parietal cortex, higher values were observed at advanced stages (AD V-VI: 170.50 ± 40.34 pmol/μg prot, $p<0.05$), whereas in the temporal cortex they were significantly increased at early (AD I-II: 158.30 ± 10.35 pmol/μg prot, $p<0.01$) and advanced (AD V-VI: 154.70 ± 30.15 pmol/μg prot, $p<0.01$) stages of the disease (Figure 2). Contrary to adenosine control values, guanosine level in control frontal cortex (166.29 ± 22.40 pmol/μg prot) was

higher than in the parietal (90.22 ± 13.30 pmol/µg prot) and temporal (73.80 ± 9.00 pmol/µg prot) cortices.

Hypoxanthine levels were decreased only in the frontal cortex at early stages of AD-related pathology (AD I-II: 1901 ± 683 vs C: 3534 ± 408 pmol/µg prot, $p<0.05$). No modifications were identified in the parietal and temporal cortices (Figure 2).

Xanthine levels were decreased in frontal cortex at early stages of AD-related pathology (AD I-II: 1290 ± 439 vs C: 2374 ± 219 pmol/µg prot, $p<0.01$) which resulted in a decrease of xanthine levels in total AD (AD: 1888 ± 181 pmol/µg prot, $p<0.05$) when compared with controls. No differences were observed in the parietal and temporal cortices (Figure 2).

Inosine levels were not significantly altered in the frontal cortex in AD. Increased levels were found in AD parietal cortex (AD: 1083 ± 99 vs C: 735 ± 93 pmol/µg prot, $p<0.05$) and AD temporal cortex (AD: 1030 ± 86 vs C: 610 ± 82 pmol/µg prot, $p<0.01$) when compared with controls. Values were higher at advanced stages in the parietal cortex (AD V-VI: 1260 ± 280 pmol/µg prot, $p<0.05$), and at early (AD I-II: 1133 ± 84 pmol/µg prot, $p<0.01$) and advanced (AD V-VI: 1075 ± 226 pmol/µg prot, $p<0.05$) stages in the temporal cortex (Figure 2).

Enzymatic activities

5'-Nucleotidase (5'-NT), adenosine deaminase (ADA), and purine nucleoside phosphorylase (PNP) activities were measured in the same frontal, temporal, and parietal cortex of AD and control samples used for nucleotide assessment.

5'-NT activity in membrane

Prior to the 5'-NT activity assessment, the possible contribution of alkaline phosphatase to the conversion of AMP into adenosine was evaluated. To this end, the hydrolysis of 500 µM AMP was assayed in the absence or the presence of 100 µM levamisole, a specific inhibitor of alkaline phosphatase, using both control and AD

samples from different membrane and cytosol preparations. No significant differences were found after comparing results obtained in the absence or the presence of the inhibitor which allow to suggest that alkaline phosphatase did not participate in AMP hydrolysis under our conditions (Figure 3).

5'-NT activity was measured at different concentrations of AMP (10 μM to 1 mM) and the results indicated that this activity increased with increasing AMP concentrations until saturation. Data were fitted to Michaelis-Menten model with a non-linear regression and the corresponding V_{max} and K_M values were obtained. Reduced V_{max} and K_M values were found in frontal cortex in AD compared with control cases (V_{max} AD: 0.019 ± 0.002 vs C: 0.034 ± 0.004 nmol Pi/mg prot · min, $p<0.01$; K_M AD: 56.8 ± 7.2 vs C: 152.7 ± 34.7 μM, $p<0.01$). Significant decreased V_{max} and K_M values occurred through all disease stages. No significant differences in parietal or temporal cortices were found in AD (Figure 4).

5'-NT activity in cytosol

In the frontal cortex, cytosolic 5'-NT activity was significantly decreased in the AD group, mainly at early stages of NFT pathology with respect to control cases (AD: 0.009 ± 0.001 vs C: 0.014 ± 0.002 nmol Pi/mg prot · min, $p<0.05$). No other significant modifications were observed in parietal cortex, but decreased 5'-NT activity occurred in the temporal cortex at advanced stages of AD (AD V-VI: 0.007 ± 0.001 vs C: 0.013 ± 0.001 nmol Pi/mg prot · min, $p<0.05$) (Figure 4). 5'-NT activity measured in the present work was not associated to postmortem delay neither in membrane nor cytosolic fractions (Figure 5).

ADA activity

ADA activity was significantly decreased in the frontal cortex in AD cases (AD: 27.25 ± 3.00 vs C: 36.78 ± 3.65 pmol/ μg prot · min, $p<0.05$), but increased in the temporal cortex in AD (AD: 38.21 ± 9.17 vs C: 17.69 ± 2.06 pmol/ μg prot · min, $p<0.05$), mainly

at early stages (AD I-II: 57.92 ± 20.98 pmol/ μg prot · min, $p<0.05$), with no significant differences between AD stages. No modifications in ADA activity were noted in the parietal cortex in AD cases (Figure 6A). ADA activity was not associated to postmortem delay neither in membrane nor cytosolic fractions (Figure 6B).

PNP activity

PNP activity did not show significant differences between control and AD cases in frontal or parietal cortices. PNP activity was significantly increased in the temporal cortex at early stages of AD with respect to control cases (AD I-II: 24.39 ± 9.71 vs C: 9.26 ± 1.43 pmol/ μg prot · min, $p<0.05$) (Figure 7A). ADA activity was not associated to postmortem delay neither in membrane nor cytosolic fractions (Figure 7B).

Discussion

Previous experiments have determined that nucleoside concentrations are unevenly distributed in different human brain areas (15, 40, 41, 43). For instance, similarly to our results, adenosine was found at lower levels in frontal than in parietal (42) or temporal (41) cortex in control human brain samples. In turn, guanosine in frontal cortex has higher levels than in parietal or temporal cortex. Although we have no detailed information to fully explain regional differences in controls in the present study, the different regional activity of enzymes related to purines metabolism could be involved (41). On the other hand, a comparative study reported that levels of hypoxanthine and adenosine are lower in human brain samples (ca. 6 times) as compared to those in rat brain. Levels of xanthine and inosine are slightly higher in rat (ca. 2 times) than in human brain, while the guanosine level is 2 times higher in human brain (44).

Levels of purines have been assessed in the CSF in AD (33, 35, 36). Levels of cGMP but not of cAMP are decreased in AD (60). Altered levels of methionine, tryptophan, and products of tyrosine pathway together with increased xanthine and hypoxanthine co-relate with mild cognitive impairment in AD-related cohorts (36). Other studies show no changes in hypoxanthine but increased levels of xanthine in the CSF in AD (22). Although metabolomics in combination with other measurements can be useful to identify biomarkers of disease progression (49), determinations in the CSF and peripheral blood do not necessarily reflect molecular events in particular brain regions.

Previous studies in AD, showed deregulation with regional variations of adenine phosphoribosyltransferase, deoxyguanosine kinase, RNA polymerase III subunit B, *ENTPD3*, *AK5*, *NME1*, *NME3*, *NME5*, *NME7* and *ENTPD2* messenger RNAs (6). In addition, liquid chromatography mass spectrometry-based metabolomics in the entorhinal cortex identified altered levels of dGMP, glycine, xanthosine, inosine

diphosphate, guanine, and deoxyguanosine (6). Purine metabolism has been reported to be deregulated in patients with major depression, and certain purines exhibit antidepressant properties (2, 7, 38, 39). AD cases in the present series did not have major depressive disease. However, the *locus coeruleus* and the raphe nuclei of the brain stem, major sources of noradrenergic and serotoninergic innervations to the hippocampus, amygdala and neocortex respectively, are altered at early stages of AD-related pathology (4, 13). Accordingly, it has been hypothesized that depression in the elderly and AD can be linked, at least in part, to AD-related pathology in selected nuclei of the brainstem (4).

The present observations reveal three important general aspects. The first of these concerns regional variations: 1. the frontal cortex is the most affected area in AD-related pathology, showing significantly decreased levels of adenosine, guanosine, hypoxanthine, and xanthine, and a tendency of inosine to decrease; and 2. parietal cortex and temporal cortex show an opposing pattern when compared with the frontal cortex, as adenosine, guanosine, and inosine are significantly increased at least at defined stages of AD whereas hypoxanthine and xanthine levels do not show differences in AD-related pathology when compared with controls. The second point concerns adenosine as the purine most commonly affected in all regions: adenosine levels are altered, although in opposing directions, in frontal cortex, and parietal and temporal cortex, followed by guanosine and inosine.

Regarding enzyme activities, 5'-NT V_{max} value is reduced in membranes and cytosol in frontal cortex but not in parietal cortex, and only at advanced stages in cytosol in temporal cortex. ADA activity is decreased in AD when considered as a whole compared with controls but increased at early stages of AD-related pathology in temporal cortex. Finally, PNP activity is increased only in the temporal cortex at early stages of AD-related pathology. Adenosine, guanosine, and inosine can be generated

from AMP, GMP, and IMP, respectively, through the action of 5'-NT. Decreased 5'-NT activity in frontal cortex correlates with decreased levels of these metabolites (although not significant for inosine) in this region in AD cases. Levels of adenosine, guanosine, and inosine in parietal cortex and temporal cortex do not relate to 5'-NT in these regions. Significantly decreased V_{max} and K_M values occurred through all disease stages. No significant differences in parietal or temporal cortices were found in AD (Figure 3). These results have to be interpreted with care, because 5'-NT, a glycosyl phosphatidylinositol-anchored membrane protein, is a dissociation-sensitive enzyme (18, 62, 64). However, it has been previously demonstrated, by means of artificial postmortem delay experiments, that ecto-nucleotidase activity in human brains was stable up to 24 h, indicating the reliability of this tissue for these enzyme determinations (3). In agreement, 5'-NT activity measured in the present work was not associated to postmortem delay neither in membrane nor cytosolic fractions. As the efficiency of the enzyme is defined as $(V_{max}/[E]_{total}) / K_M$, 5'-NT enzyme efficiency could be altered due to the lower V_{max} and the lower K_M found in AD cases versus controls.

It may be speculated that reduced levels of adenosine in frontal cortex in AD underlie increased expression and activity of adenosine A_1 receptors previously described in this region throughout disease progression (1).

Adenosine is transformed into inosine by ADA (11). Therefore, changes in inosine can be linked to alterations of adenosine. Inosine decrease in frontal cortex and decrease in parietal and temporal cortex in AD has the same pattern as that seen for adenosine in these regions. However, ADA activity is not significantly decreased in frontal cortex and significantly increased only in temporal cortex in the present series. Activity of adenosine monophosphate deaminase, another enzyme involved in inositol synthesis, is increased in the temporal cortex in soluble and membrane fractions in AD (58).

PNP catalyzes the conversion of inosine to hypoxanthine and the degradation of guanosine to guanine (9). Therefore, changes in PNP can produce changes in hypoxanthine and guanine levels. PNP activity is increased in temporal cortex at advanced stages of AD-related pathology; however, hypoxanthine level are not altered in temporal cortex and is decreased in frontal cortex at early stages of AD-related pathology. Finally, xanthine is obtained from oxidation of hypoxanthine by xanthine oxidase (11). Levels of hypoxanthine correlate with xanthine in the frontal, parietal, and temporal cortices in the present series.

The third important point identified in this study is the early alteration of purine levels and enzymatic activity in different regions of the three cerebral cortices at stages in which there is no evidence of NFT pathology or β-amyloid deposition in neocortex (Braak stages I-III; Thal phases 0). Reduced levels of adenosine, guanosine, hypoxanthine, and xanthine, and a tendency toward decrease of inosine, occur at stages I-II of NFT pathology in which tau aggregates in the cerebral cortex are limited to the entorhinal and transentorhinal cortices and discrete neurons in the hippocampus. Importantly, adenosine, guanosine, and inosine are increased in the temporal cortex, as is adenosine in the parietal cortex during the same early stages. Moreover, 5'-NT and ADA activities are reduced in frontal cortex at stages I-II.

The present findings are descriptive and do not contemplate possible functional implications. Nor do they pretend to explore the pathogenesis of the disease deeply. However, they show 1: alterations of purine metabolism in the cerebral cortex at early stages of AD-related pathology which are independent of NFTs and β-amyloid plaques; 2: alterations which are stage- and region-dependent, the latter showing opposite patterns in frontal cortex compared with parietal cortex and temporal cortex; 3: adenosine to be the most vulnerable member of the group of assessed purines; and 4:

variable correspondence between metabolite levels and enzymes involved in their synthesis.

These observations point to the likely implications of these particular patterns in the analysis and interpretation of purine modifications in CSF as possible biomarkers in AD and other neurodegenerative diseases.

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Figure legends

Table 1: Summary of the cases; M: male; F: female; P-M delay: postmortem delay between death and tissue processing; FC: Frontal cortex; PC: parietal cortex; TC: temporal cortex; NFT stage: Braak stage of neurofibrillary tangle pathology; Thal: phases of β-amyloid plaques (plaque distribution).

Figure 1: Purine metabolic pathway. E-NTPDases: ecto-nucleoside tri-phosphate diphosphohydrolases; PD, ectophosphodiesterase; ADA: adenosine deaminase; SAHH: S-adenosyl homocysteine hydrolase; SHMT: S-adenosyl methyl transferase; PNP: purine nucleoside phosphorylase; HGPRT: hypoxanthine-guanine phosphoribosyl transferase (modified from (57)).

Figure 2: Adenosine, guanosine, hypoxanthine, xanthine, and inosine levels in frontal cortex (FC), parietal cortex (PC), and temporal cortex (TC) in control (C) and cases with AD-related pathology (AD) at early (AD I-II), intermediate (AD III-IV), and advanced (AD V-VI) stages of NFT pathology. Values are expressed as mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 according to ANOVA - Fisher's LSD test.

Figure 3: Contribution of Alkaline Phosphatase to measured 5'-Nucleotidase (5'-NT) activity. Presence of 100 µM levamisole, a selective alkaline phosphatase inhibitor, during the assay determining 5'-NT activity did not modify the activity of 5'-NT neither in membranes nor cytosolic fraction.

Figure 4: 5'-Nucleotidase (5'-NT) activity in membranes and in cytosol in the frontal cortex (FC), parietal cortex (PC), and temporal cortex (TC) in control (C) and cases

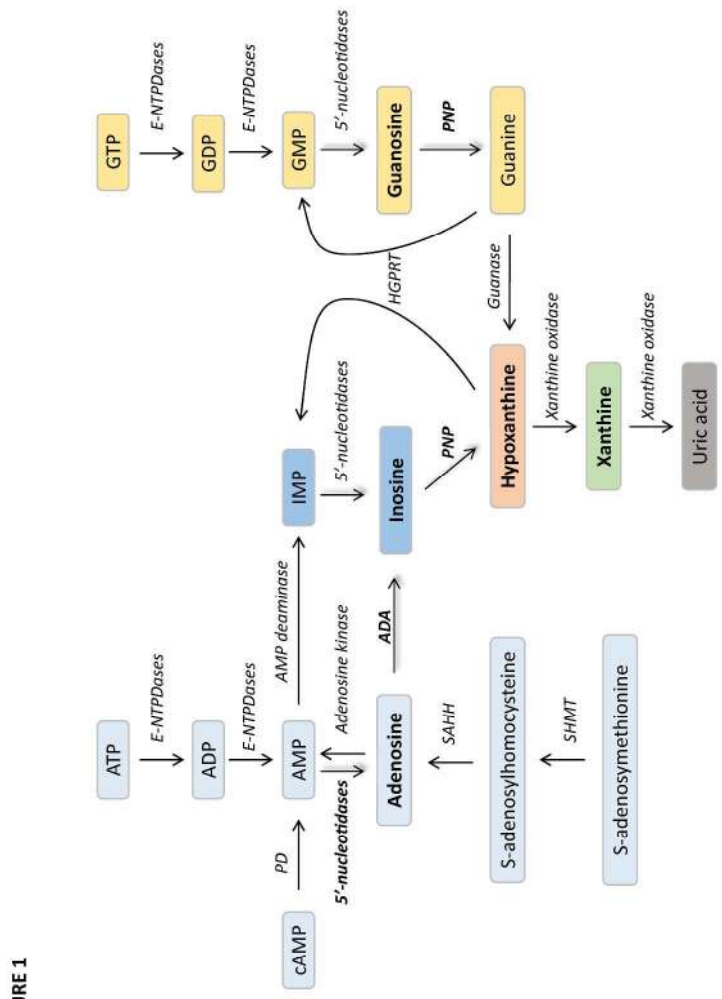
with AD-related pathology (AD) at early (AD I-II), intermediate (AD III-IV), and advanced (AD V-VI) stages of NFT pathology. 5'-NT activity was determined by fitting a Michaelis Menten model using a non-linear regression. Values are expressed as mean ± SEM. *p<0.05 and **p<0.01 significantly different from the control value according to ANOVA - Fisher's LSD test.

Figure 5: Correlation analysis between 5'-Nucleotidase activity and postmortem delay values. 5'-NT activity in membranes and in cytosol from the frontal, parietal and temporal cortices of control and cases with AD-related pathology (Figure 4). r: Pearson's correlation coefficient. P: P value. Straight line: linear regression fit of 5´-NT activity value.

Figure 6: Adenosine deaminase analysis. A. ADA activity in the frontal cortex (FC), parietal cortex (PC), and temporal cortex (TC) in control (C) and cases with AD-related pathology (AD) at early (AD I-II), intermediate (AD III-IV), and advanced (AD V-VI) stages of NFT pathology. Data are mean ± SEM. *p<0.05 significantly different from the control value, according to ANOVA - Fisher's LSD test. B. Correlation analysis between ADA activity and postmortem delay values. r: Pearson's correlation coefficient. P: P value. Straight line: linear regression fit of ADA activity value.

Figure 7: Purine nucleotide phosphorylase analysis. A. PNP activity in the frontal cortex (FC), parietal cortex (PC), and temporal cortex (TC) in control (C) and cases with AD-related pathology (AD) at early (AD I-II), intermediate (AD III-IV), and advanced (AD V-VI) stages of NFT pathology. Data are mean ± SEM. *p<0.05 significantly different from the control value, according to ANOVA - Fisher's LSD test. B. Correlation analysis between PNP activity and postmortem delay values. r: Pearson's correlation coefficient. P: P value. Straight line: linear regression fit of PNP activity value.

Case number	Gender	Age	p-m delay	NFT stage	Thal phase	FC	PC	TC
1	M	70	13h	0	0	✓	✓	✓
2	F	80	21h	0	0	✓	✓	✓
3	M	79	7h	0	0	✓	✓	
4	F	66	8h	0	0	✓	✓	✓
5	M	48	12h	0	0	✓		✓
6	F	65	4h	0	0	✓		
7	F	80	3h 30min	0	0	✓		
8	M	53	3h	0	0	✓		
9	F	46	20h	0	0	✓		
10	M	63	17h	0	0	✓		
11	F	81	4h	0	0		✓	
12	M	71	12h	0	0		✓	✓
13	F	71	8h 30min	0	0		✓	
14	F	69	2h 30min	0	0		✓	✓
15	M	67	20h	0	0		✓	✓
16	M	78	19h	0	0		✓	✓
17	M	80	13h	I	0	✓		
18	M	85	12h	I	0	✓		
19	F	79	3h 30min	I	0		✓	✓
20	F	79	6h 30min	I	0		✓	✓
21	F	79	4h 45min	I	0		✓	✓
22	M	72	10h	II	0	✓		
23	F	97	13h	II	0	✓		
24	M	71	5h 15min	II	0		✓	✓
25	M	76	4h 15min	II	0		✓	✓
26	M	85	3h 45min	II	0		✓	✓
27	M	75	7h 30min	II	0		✓	✓
28	M	74	24h	III	1	✓		
29	F	81	14h	III	1	✓		
30	F	71	6h 45min	III	1		✓	✓
31	M	85	14h	IV	1	✓		
32	F	82	5h	IV	1	✓		
33	F	82	10h	IV	2	✓		
34	F	69	8h	IV	2		✓	✓
35	F	81	5h	IV	2		✓	✓
36	M	64	16h 30min	IV	2			✓
37	M	79	5h	IV	2		✓	
38	F	86	10h	V	3	✓		
39	M	69	6h	V	3	✓		
40	F	78	19h	V	3	✓		
41	M	93	7h 30min	V	3	✓		
42	M	69	20h	V	3	✓		
43	M	93	3h	V	3		✓	✓
44	F	96	17h 30min	V	3		✓	✓
45	F	81	5h 15min	V	3		✓	✓
46	F	56	7h	VI	3		✓	✓
47	F	86	2h 15min	VI	4		✓	✓



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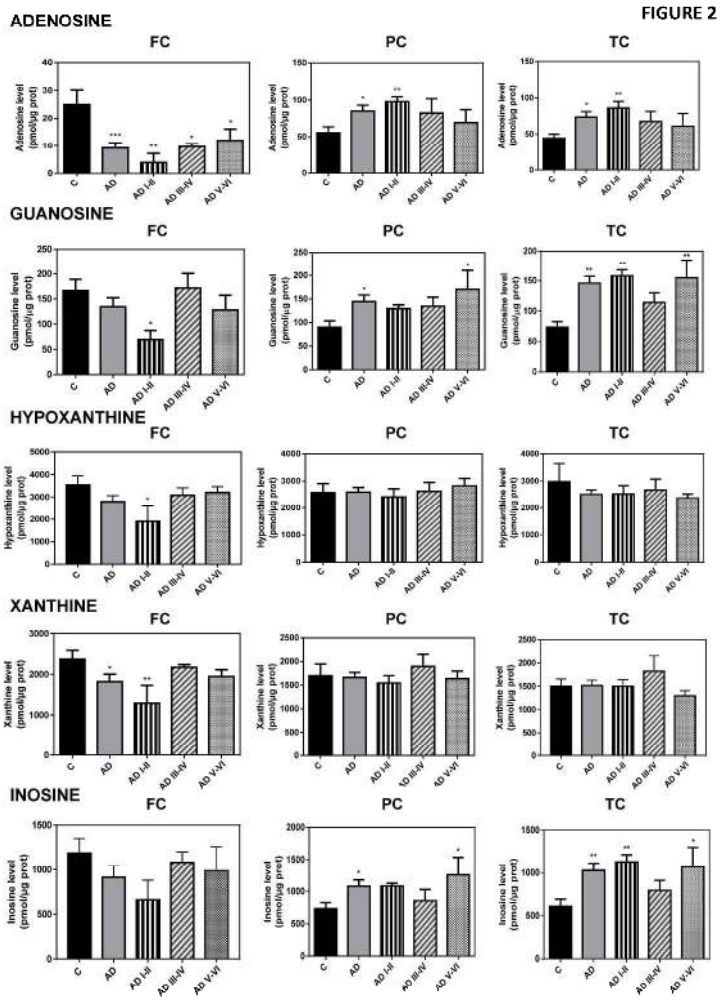

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FIGURE 3

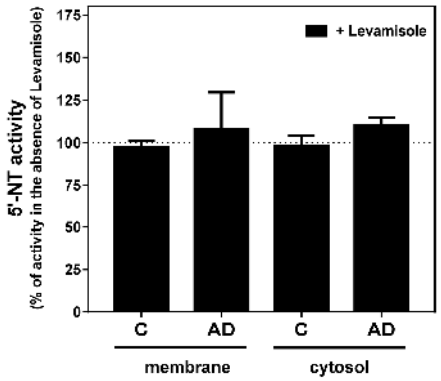

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FIGURE 4

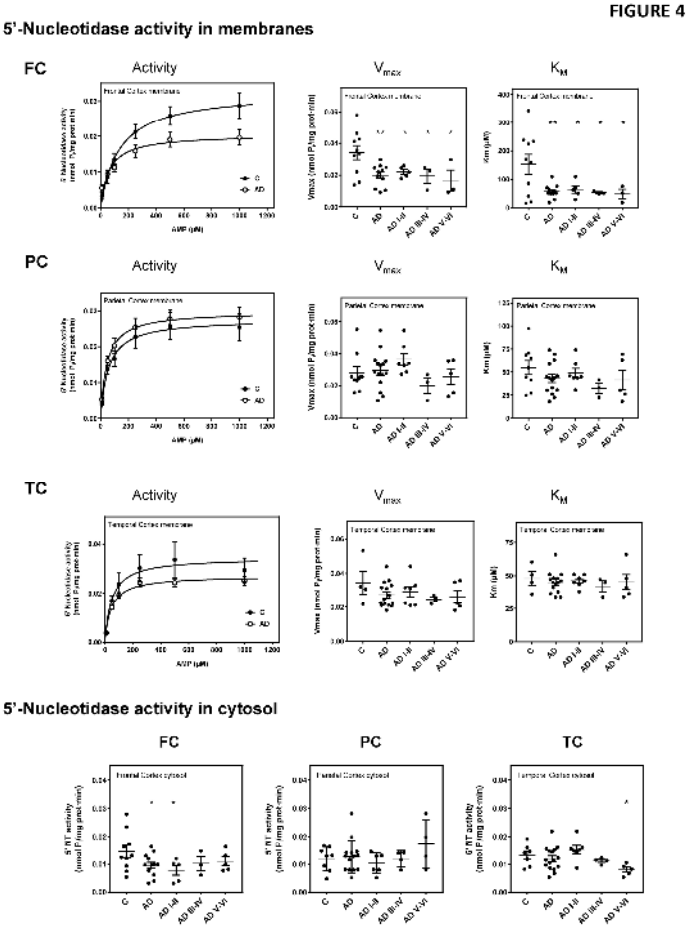

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FIGURE 5

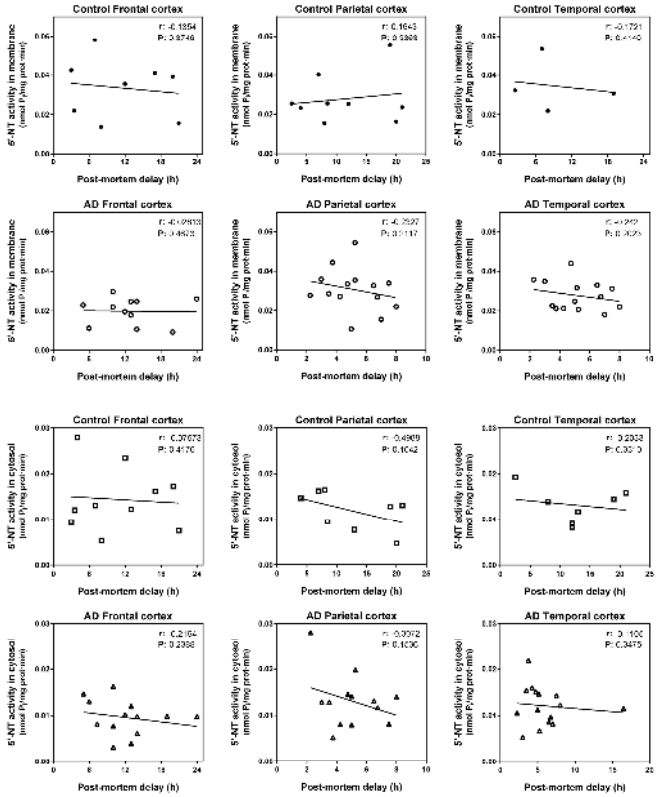

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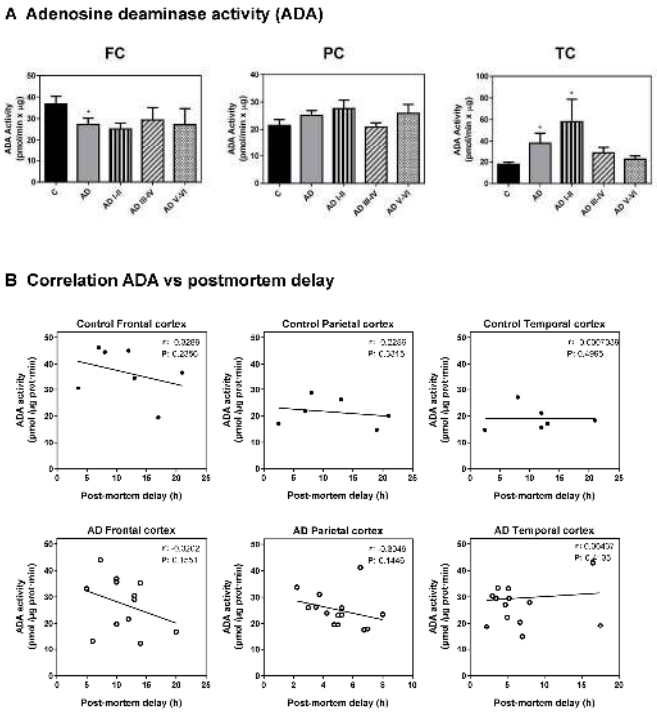
FIGURE 6



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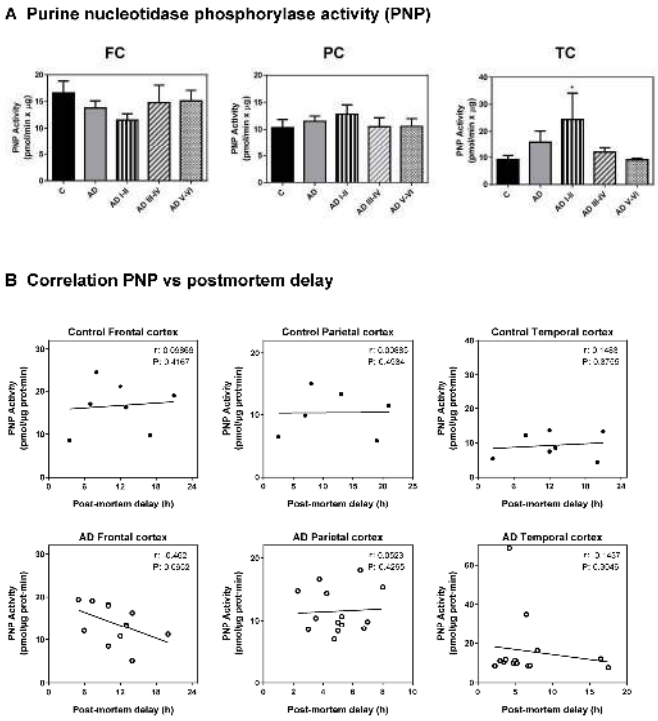

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