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Metabolomic changes in autopsy confirmed Alzheimer's disease

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Abstract

Metabolomics, the global science of biochemistry, provides powerful tools to map perturbations in the metabolic network and enables simultaneous quantification of a large number of metabolites to identify metabolic perturbances that might provide insights into disease. In this pilot study we take a targeted electrochemistry based metabolomics approach where liquid chromatography followed by coulometric array detection enables quantification of over thirty metabolites within key neurotransmitter pathways (dopamine and serotonin) and pathways involved in oxidative stress.

Using samples from post-mortem ventricular cerebrospinal fluid (CSF) (15 AD and 15 non-demented subjects with autopsy confirmed diagnoses) and using regression models, correlations, Wilcoxon rank-sum tests and t-tests we identified alterations in tyrosine, tryptophan, purine, and tocopherol pathways in patients with AD. Reductions in norepinephrine and its related metabolites were also seen, consistent with prior literature. These data support further investigation of metabolomics in larger samples of clinical AD as well as in those with preclinical disease for utility as biomarkers.

Keywords

metabolomics; Alzheimer's; biomarkers; diagnosis; staging; tryptophan; tyrosine; purine

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1. INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia among the elderly, and an estimated 10 million baby boomers may be at risk for AD over the coming decades (1). Despite significant advances over the past two decades, the causes are not yet fully known and treatment remains suboptimal. Although clinical diagnosis based on consensus criteria (2,3) is reasonably accurate, community physicians may fail to diagnose up to 33% of mild dementia cases (4) and some 20% of subjects clinically diagnosed with AD may have other diagnoses at post-mortem. The gold standard is pathologic diagnosis (5) based on severity and topography of beta-amyloid plaques and neurofibrillar tangles. Increasing evidence now suggests that the early pathogenesis of Alzheimer's disease may be protracted with a "prodromal phase" characterized by subthreshold clinical and pathologic changes (6).

Many prior studies using targeted approaches have examined the profound biochemical and pathological alterations in the AD brain (reviewed in 7). Such changes have been speculated to result from a synaptic failure associated with a cascade of cellular events involving abnormal beta-amyloid protein metabolism, tau phosphorylation, oxidative stress, inflammation, mitochondrial dysfunction, neurotransmitter changes, membrane lipid dysregulation, apoptosis, and changes in other proteins/chemicals (7–9). Several ongoing multicenter studies (e.g. ADNI) are also currently examining imaging, genetic, proteomic, and fluid biomarkers in CSF and plasma (9). However, the lack of validated analytical platforms till recently has limited the global biochemical mapping in early AD at a systems level.

Metabolomics, the study of metabolism at the global or "-omics" level, is a new but rapidly growing field that can simultaneously quantify tens to hundreds of small molecules present in a biological sample of interest (10–13). Sophisticated mathematical tools further allow these data to be mined for molecular signals (14). By complementing the more targeted approaches that have looked at one or few biochemical events at a time, it promises to provide a detailed map of the regulation of metabolic pathways as influenced by genome and environment (12,13). We and others have reported initial metabolomic signatures for several disease states, including motor neuron disease, schizophrenia, depression, addictive disorders, Parkinson's disease, Huntington's disease, cardiovascular disorders and diabetes (15–24). However, to date, no study has used a systems biochemistry approach to evaluate multiple metabolomic changes in an autopsy confirmed sample of AD and control subjects. Our primary aims were to examine the feasibility of performing metabolomics in post-mortem ventricular CSF samples, to detect biochemical changes that discriminate definite AD from controls, and to correlate ventricular CSF metabolomics with pathologic staging. As such this was a pilot study and it was not our goal to validate biomarker signatures but merely to probe biochemical pathways in hopes of understanding mechanisms and identifying candidate markers that could be tested more definitively in larger studies of lumbar CSF or plasma.

2. METHODS

2.1 Subjects and CSF samples

Controls and demented subjects were enrolled in the autopsy program of the Joseph and Kathleen Bryan Alzheimer Disease Research Center according to standard protocols approved by the Duke University Medical Center Institutional Review Board (IRB) (25). Autopsies were performed according to institutional guidelines. Postmortem ventricular cerebrospinal fluid (CSF) samples from 30 subjects (15 AD and 15 non-demented subjects) were selected from the brain bank for analyses. All subjects and/or next of kin provided consent for brain autopsy and research prior to death according to published methods.

Samples were selected for analysis so as to prioritize those with the shortest post-mortem interval (PMI) and to match AD and controls as closely as possible on age and gender. All AD patients had been followed in the Bryan ADRC and received diagnostic evaluations by neurologists in the Center. The autopsy non-demented subjects had neuropsychological evaluations on a regular basis and were diagnosed as cognitively normal based on intake evaluations. Controls enrolled in the Brain Autopsy Program were followed annually with detailed neuropsychological testing from the time of enrollment until death. In this sample, the interval between the last clinical assessment and death (in the controls) ranged from 7 days to 425 days (median was 119 days; average 213 days). All were judged clinically normal prior to death.

2.1.1 Neuropathology Staging—Brains were examined neuropathologically and diagnosed as AD or normal according to NIA-Reagan Institute criteria (5). Brains were banked according to approved protocols. Neurofibrillary changes were staged according to Braak stages I-VI (26) and plaque frequency was estimated according to CERAD criteria (27). Within each diagnostic group, we selected a broader range of Braak and CERAD scores to more fully study metabolic correlations with tangle and plaque pathology. Thus, among AD patients, we selected 5 subjects each in Braak stages III, IV, and V. Among the non-demented subjects, there were 7 subjects in stage I, 2 in stage II, and 6 in stage III. The nondemented subjects with Braak stage III were selected since clinically they had no symptoms and pathologically they did not meet AD criteria for plaques.

2.1.2 Clinical Data—All cases had a minimum set of clinical data available, which included: Clinical Diagnosis prior to death, dementia onset, age, certain medications, and other demographic variables. We also collected data on postmortem interval and agonal state. All controls had much more detailed clinical information including detailed neuropsychological information (standard battery of tests) that was repeated annually along with a detailed medical condition update and an update to family history of dementia and neurological disorders

2.1.3 CSF—Ventricular fluid was removed post mortem by entering the lateral ventricle after the bony calvarium is cut but before the skull plate is removed. Collected CSF samples were centrifuged for 10 minutes to remove any particulate debris and then stored in 1 ml aliquots at −80°C. All CSF sample are stored in Nalgene Cryoware polypropylene vials.

2.2 Metabolic profiling

We analyzed postmortem ventricular CSF using liquid chromatography / electrochemical array (LC/ECA) detection as previously described (15). After initial signal processing, 104 metabolites passed our quality control checks for detection. Among these peaks, we were able to determine the corresponding known compounds for 33. In subsequent computational analysis we also considered eight ratios of metabolite levels that, based on prior experience, we believed might reflect differences in metabolomic profiles between controls and Alzheimer patients. These metabolites were in tyrosine, tryptophan, purine and tocopheral pathways.

2.2.1 Sample preparation and metabolomic analysis—Samples were prepared for analysis by extraction in acidified acetonitrile and analyzed by LCECA as previously described (11,15). Briefly, 20 cc of freshly drawn CSF with anticoagulant citrate dextrose (ACD) was centrifuged at 750 × g for 7 minutes to remove red blood cells and stored at −80 ° C. 250 μl sub aliquots of stored samples were mixed with 1 ml of acetonitrile/0.4% acetic acid at −2 0 ° C and vortexed for 45–60sec, then temperature was brought to −15 ° C in a cold block, and vortexed again for 30–45 sec. Samples were centrifuged for 30 min at 21 ,

000 × g at 4 ° C. 1 ml of the resulting supernatant was transferred to a 2 ml screw top vial and evaporated under vacuum. Sufficient vacuum was generated to freeze the sample during this step. The sample was reconstituted in 200 μl of mobile phase A, and 100 μl were loaded into two auto sampler vials, one of which was archived at −80 ° C. Profiles are stable in acetonitrile extract, dried extract and mobile phase diluted extract. During the sample preparation, pools were created from equal volumes of sub aliquots of all samples. All assays were run in sequences that include (a) 10 samples, (b) authentic reference standard mixtures of 80 known compounds, (c) pools of all samples and (d) duplicate preparations of the same sample. Duplicates were spaced at short and long intervals through the run to reflect the performance of the total data base. Run orders of all samples were randomized. The sequences minimized possible analytical artifacts. The pools and duplicates were used to access the precision of the entire data set. Additionally, the pools were used as references for time normalization (stretching).

2.3 Data generation and analyses

All chromatograms in the study were background corrected (BC) to eliminate the base line drift inherent in gradient profiles. By controlling analytical conditions, the location of any particular peak in a 16-channel 110 min chromatogram was held within ±5–30 s through the study. BC files were then sequentially time normalized against a single pool in the middle of the study sequence. A two-step stretching protocol with a multitude of peaks was used. First, proprietary software (ESA, CEAS 512) was used to align 15–20 major peaks in the chromatogram and interpolate the positions between them. Then an additional 20–25 smaller peaks present in most samples were selected from the derivative file and those were realigned, keeping the major peaks in the same position. Selected peaks were aligned within ±0.5 s and non-selected peaks within ±1–1.5 s over the entire 110 min assay.

First, all responses matching the retention and EC signature of compounds in the reference standard were exported in concentration units of ng/ml. Second, all responses matching resolved peaks in the pool of all samples were exported in terms of their relative response to the pool value. The concentrations of these are subsequently estimated by the total coulombs in the peak assuming a molecular weight of 200 and a 2 electron charge transfer.

2.4 Statistical analysis

Although hundreds of metabolites were identified on the LCECA platform we restricted metabolomic analysis to the 33 known metabolites we identified and eight metabolite ratios. We sought to determine (1) if there were significant differences between metabolites and metabolite ratios in controls and Alzheimer's disease patients, (2) if there were significant correlations between metabolites and metabolite ratios and severity/topography of plaques and tangles, (3) if multivariate models could provide additional insight into these differences and correlations. We also sought to determine if the metabolomic profiles were affected by post mortem interval (PMI). T tests and Wilcoxon rank-sum tests were carried out by the R functions t.test and wilcox.test. Spearman rank correlations were computed by the R function cor with method='spearman'. To address the issue of multiple hypothesis testing we calculated Q values using the R qvalue function (package `qvalue') (29). Q values estimate cumulative false discovery rates. For example, in Table 2, the Q value of 0.38 for guanosine (row 7) indicates that an estimated 38% of the compounds to that point are different between Alzheimer's disease patients and non-demented controls simply by chance. These compounds also include acetaminophen, which is significantly different between the two groups but not shown because it is exogenous. False discovery rates provide a helpful measure for determining which results justify further testing. We constructed a multivariate logistic model of disease status (non-demented subject versus Alzheimer patient) as a function of the variables (known compounds, known-compound ratios, and clinical /

demographic measures) shown in Tables 1 and 2. The Results section describes the model selection procedure in detail.

3. RESULTS

Table 1 summarizes the demographic and pathologic characteristics of AD and nondemented subjects. Groups did not differ statistically in mean age, postmortem interval (PMI) or gender ratio. As expected, AD subjects had higher tangle burden (Braak stage) and more extensive/frequent neuritic plaques (CERAD ratings).

3.1 Metabolite differences between controls and Alzheimer subjects

Table 2 shows the mean (sd) metabolite levels and metabolite ratios in AD and nondemented groups. Norepinephrine was significantly decreased in AD (P = .0001, Q=. 005) (Figure 1). Several metabolites showed nominally significant differences between the two groups mostly in the tyrosine, tryptophan, purine and tocopherol pathways.

3.2 Correlations between metabolite levels and Pathologic Staging

Tables 3 and 4 show the nominally significant correlations of metabolites with Braak (tangle) and CERAD (plaque) stages. Depletion of NE along with down regulation of many metabolites within the purine pathway such as hypoxanthie and xanthine and in methionine significantly correlates with tangle formation. These might point to the importance of catecholamines, purines and methylation processes in the process of tangle formation. 5HTP the precursor for serotonin seems to positively correlate with formation of tangles and unfortunately many metabolites within this branch of tryptophan metabolism were not measurable in lumbar CSF samples. Changes in the glutathione system seem to also correlate with tangle formation.

Evaluation of metabolites that correlate with CERAD measurements revealed that depletion of NE, alpha tocopherols and 3 methoxytyramine and increase of serotonin precursor 5HTP and of tyramine seem to correlate with plaque staging. Changes in glutathione system seems to also be related. These observations suggest that catecholamines, oxidative stress and methylation processes might be implicated in process of plaque formation.

3.3 Statistical Discrimination of AD from Non-Demented Subjects

We constructed a multivariate logistic model of disease status (non-demented subject versus Alzheimer patient) as a function of the variables (known compounds, known-compound ratios, and clinical / demographic measures) shown in Tables 1 and 2. Because we had more variables than samples, we first selected an initial set of variables using glmnet (30). This initial set included years of education and gender, but not PMI. We then used this initial set, plus PMI (because of its a-priori possible effects on metabolomic profiles) as the starting model for variable selection using stepAIC (R package MASS) (31). The stepAIC function aims to discover a best model in terms of AIC (Akaike information criterion), by adding or dropping explanatory variables. In this case the initial model used many variables, and stepAIC selected a model with many fewer variables. The selected model uses tryptophan, norepinephrine, and indoleacetic acid, with AIC=8. This model offers complete separation on the training data (Figure 2), and its estimated generalization error (estimated error on a new set of data) is < 10% (the ".632" estimator, Eqn. 7.57, ref. 32).

3.4 Correlations with postmortem interval (PMI)

Seven metabolites (hypoxanthine, tryptophan, tyrosine, xanthine, methionine, glutathione-oxidized, 3-hydroxyanthranilic acid) had nominally significant ($p<0.05$) correlation with PMI with relatively low Q values. Thus, PMI effects may have reduced our ability to detect

group differences or disease correlations in these compounds. For example, hypoxanthine levels were negatively correlated with Braak stage and positively correlated with PMI. But we see no evidence that effects of PMI caused spurious relationships, as PMIs were not significantly different between patients and controls, and the best multivariate model of disease status did not include PMI, even though we provided it as a possible covariate in our maximal, starting model.

4. DISCUSSION

While there have been post-mortem and antemortem neurochemical studies in AD, they have been limited to the study of a few metabolites. The advent of metabolomics now permits analyses of tens to thousands of molecules simultaneously using biological samples and a variety of metabolomics platforms. Since most ADRCs bank CSF samples, it is important to determine whether such samples can yield useful information. We designed this as a pilot study to examine the feasibility of analyzing post-mortem banked CSF for metabolomic information. As such, it was not our goal to identify biomarkers but rather to to detect differences in biochemical pathways and patterns which in turn could yield candidate biomarkers for testing in a future study. To our knowledge, ours is the first metabolomic study of post-mortem CSF in autopsy confirmed AD subjects.

One main finding of our study is that norepinephrine (NE) is significantly depleted in AD patients and that along with its depletion there is down regulation of many metabolites within the dopamine pathway (such as 3 methoxytyramine and DOPAC) leading to end product build up of 3 methoxy-4-hydroxy-phenylethyleneglycol (MHPG) and 3-methoxy-4-hydroxymandelate (VMA) (Figure 3). A second finding is that a model that included tryptophan, NE, and indoleacetic acid, offered complete separation of the groups. MHPG is a major NE metabolite and ratios of MHPG/NE are 4 fold higher in AD patients than controls (data not shown). Tryptophan and indoleacetic acid are both involved in serotonin pathway and serotonergic dysfunction has been implicated in some of the behaviour changes in AD (33). These data are consistent with prior studies (34–40) which have shown that forebrain NE systems are involved in learning and memory (35), and that advancing AD is associated with increased NE turnover and elevated CSF MHPG/NE ratios (39). Dopamine beta-hydroxylase DBH) a marker of noradrenergic neurons is reduced in hippocampus and frontal cortext of AD subjects (37), areas rich in tangles and plaques, respectively. Plasma MHPG has also been noted to be raised in AD (39). Loss of melanin neurons in locus ceuruleus have been shown to be reduced especially in subjects with greatest plaque burden (36,39). Although NE depletion has traditionally been viewed as a downstream effect of plaques and degeneration, some recent data suggests otherwise. Inducing locus ceruleus (LC) degeneration by induction with *N*-(2-chloroethyl)-*N*-ethyl-bromo-benzylamine (dsp4) in APP23 transgenic mice leads to depletion of NE, increase in microglial activation and an increase in amyloid plaques in specific brain regions (40). The loss of NA was also associated with increased cognitive deficits in these animals indicating that NE depletion LC degeneration contributes significantly to AD pathogenesis. Thus our findings not only confirm prior findings, but also suggest that enzymes within the pathway of NE monoamine oxidase, alcohol dehydrogenase, catechol-o-methyl transferasemight be modified in AD. Of interest is the observation that CERAD scales correlate with three metabolites within the same dopamine pathway: tyramine, methoxytyramine and NE. The positive correlation with tyramine and negative correlations with NE and methoxytyramine suggest that enzymes within this pathway are possibly implicated in plaque formation (tyrosine decarboxylase, catechol O-methyltransferase, dopamine beta-monooxygenase among others). The correlation of several purine pathway metabolites (Figure 4) in Braak staging suggests new hypotheses in mechanisms involved in tangle formation.

As with any pilot study, our study is limited by a relatively small sample size, exploratory approach, and our decision to just focus on 33 known metabolites. Our findings need replication in a test set. However, all the reported statistical analyses include steps to account for multiple hypothesis testing, overfitting, and other issues that arise in analysis of a relatively large number of variables compared to the number of observations. In this instance, in each of 30 observations, we consider 46 variables, including metabolites, a-priori-selected metabolite ratios, and the clinical and demographic covariates in Table 1. In Tables 2, 3, 4, we report cumulative false discovery rates in the form of Q values. As discussed above, for a given p value, Q, the cumulative false discovery rate is an estimate of the proportion of p values <= Q that are due to statistical chance. For example, in Table 3 there are 9 metabolites and metabolite ratios that are correlated with Braak stage with Q <= 0.17, indicating that 9 * 0.83 > 7 of the correlations are real, while ~2 are due to chance. Similarly, to address overfitting, we explicitly estimate the generalization error (<10%) of the logistic regression model that relates the response variable (Alzheimer's status) to the independent variables tryptophan, norepinephrine, and indoleacetic acid. This indicates that the model is likely to perform well in a second, replication study – ewhile the model provided complete separation on the training data, we estimate that it would (only) be 90% accurate on new data from the same population. Our observation that several metabolites within the dopamine NE pathway are impaired in AD patients suggests that this pathway might be implicated in disease pathogenesis. The down regulation of NE maybe a part of a cascade of related events. Additional mechanistic studies using proper labels such as stable isotopes can allow for further investigation of the dysregulation of this pathway in AD. The observation that NE is significantly down regulated in animal models of Down's Syndrome and the earlier findings showing central and peripheral changes in NE in AD patients suggest that the NE finding in our study is not an artifact. It is also important to try to determine if alterations in the NE pathway are unique to AD or are common among other CNS diseases. Including other CNS disease controls, such as non-AD dementia would be an important addition to future studies.

Post-mortem ventricular CSF has both advantages and disadvantages compared to ante-mortem lumbar CSF. The main advantage is that the diagnoses are pathologically verified, making it possible to perform biochemical-pathologic correlations. The disadvantage of post-mortem CSF are the confounding effects of cause of death and medical comorbidities at end of life, variable PMI (41), and imperfect ascertainment of clinical cognitive status at time of death. The use of banked materials from autopsy confirmed patients can be highly informative if confounding issues are properly handled and it is for these reasons that all NIH funded Alzheimer Disease Research Centers maintain a brain bank. Since the CSF samples have been stored appropriately most signatures would be highly stable (as supported by prior analyses of banked CSF from the DATATOP study where samples profiled over nine years found stability of metabolites) (28). Lumbar CSF studies of early stage disease could have greater relevance to clinical biomarker development (than ventricular CSF in advanced subjects) but at the time of this study we did not have access to lumbar CSF from MCI subjects. We have since begun studies utilizing lumbar CSF from subjects with MCI, mild AD and controls disease who are being followed to autopsy. Future studies of larger samples must examine these variables more closely.

Lastly, preliminary unpublished data suggests that ventricular CSF has metabolic complexity that parallels the complexity of the brain. Strong correlations between tyrosine and xanthine in the brain and in ventricular CSF but not in lumbar CSF suggests that ventricular CSF might better reflect brain related changes. These early findings need replication in more definitive, metabolomic studies of samples collected from prospective multicenter biomarker studies and clinical trials. It would have also been of interest to examine the locus ceruleus degeneration and its relationship to NE but we were unable to do

it in this study because of the way the brains were sectioned the locus was not examined routinely. Future studies using additional metabolomics platforms will allow us to explore changes in metabolism with report on 500 metabolites in intermediary metabolism allowing generation of additional hypotheses. Of importance is to conduct longitudinal studies to carefully map biochemical changes seen in the normal aging process and compare them to the metabolic changes that happen early in neurodegenerative diseases. Such detailed biochemical studies could provide insights about common and unique pathways modified in CNS disorders and could yield valuable prognostic and diagnostic markers. This study is only a pilot study to demonstrate the feasibility of this concept.

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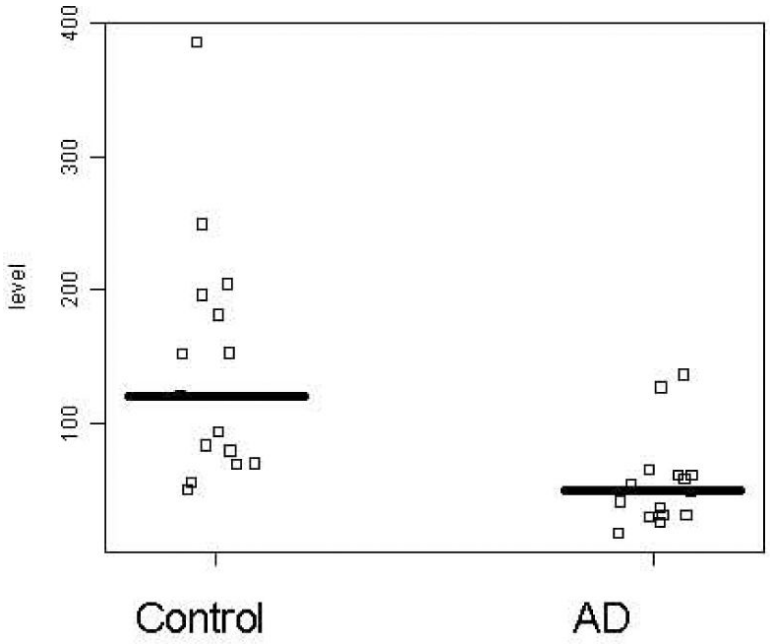

Figure 1.
Norepinephrine levels in non-demented controls and AD patients. Dark horizontal lines indicate medians.

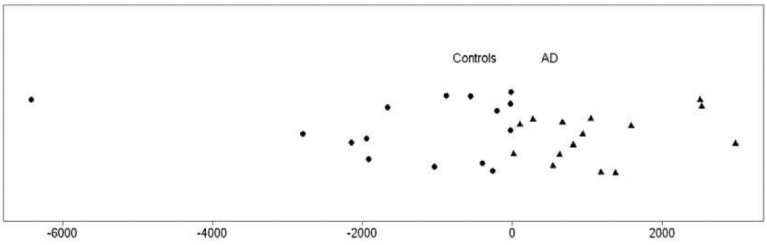

Figure 2.

Linear predictor of AD status from logistic regression on tryptophan, norepinephrine, and indoleacetic acid. Circles=non-demented controls, triangles=AD patients. The *x* axis shows the log odds that a given subject is AD, $log_e(p / (1 - p))$, where p is the model-estimated probability that the sample is from an AD patient. This is the untransformed response variable of the linear regression that underlies the logistic regression.

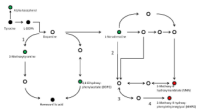

Figure 3.
Differences in tyrosine metabolism patients vs. controls. Green indicates that the mean metabolite level was significantly lower ($P<0.05$) in Alzheimer's patients. Red indicates that the mean metabolite level was significantly higher ($P<0.05$) in Alzheimer's patients.

1. Catechol-o-methyltransferase
2. Monoamine oxidase
3. Alcohol dehydrogenase
4. Catechol-o-methyltransferase

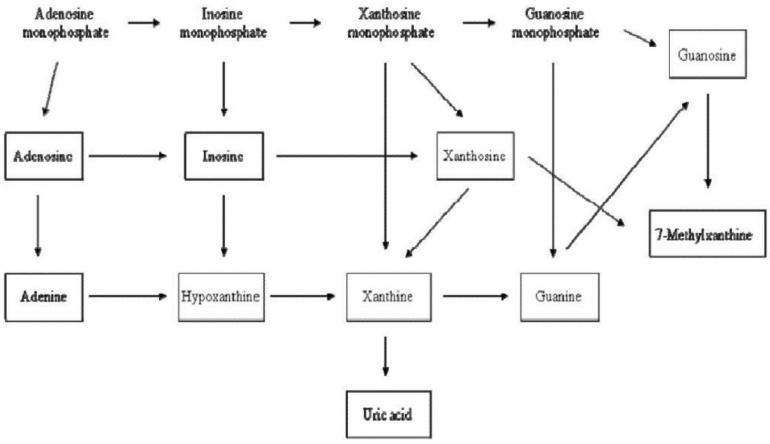

Figure 4.
Metabolites of the Purine Pathway