PHYSIOLOGICAL GENOMICS

RESEARCH ARTICLE

Metabolic and genetic imbalance of the homocysteine-methionine cycle in trisomy 21

Beatrice Vione,[1]* **Bassam Lajin,**[3]* **Francesca Antonaros,**[1] **Michela Cicilloni,**[1] **Francesca Catapano,**[2] **Chiara Locatelli,**[4] **Maria Chiara Pelleri,**[1] **Allison Piovesan,**[1] **Lorenza Vitale,**[1] **Gian Luca Pirazzoli,**[5] **Pierluigi Strippoli,**[1] **Luigi Tommaso Corvaglia,**[2,4] **Giuseppe Ramacieri,**[6] and **Maria Caracausi**[1]

[1]*Unit of Cellular and Applied Biology, Department of Biomedical and Neuromotor Sciences (DIBINEM), University of Bologna, Bologna, Italy;* [2]*Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna, Italy;* [3]*Institute of Chemistry, ChromICP, University of Graz, Graz, Austria;* [4]*Neonatal Intensive Care Unit, IRCCS Azienda Ospedaliera-Universitaria di Bologna (A.O.U.BO), Bologna, Italy;* [5]*IRCCS Institute of Neurological Sciences of Bologna - AUSL Bologna, Bologna, Italy; and* [6]*Speciality School of Child Neuropsychiatry-Alma Mater Studiorum, University of Bologna, Bologna, Italy*

Abstract

The homocysteine-methionine cycle is involved in the critical human cellular functions, such as proliferation and epigenetic regulation. S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) metabolites are synthesized in this metabolic cycle, and their levels are finely regulated to ensure proper functioning of key enzymes controlling the cellular growth and differentiation. SAM and SAH levels were found altered in the plasma of subjects with trisomy 21 (T21), but how this metabolic dysregulation influences the clinical manifestation of T21 phenotype has not been previously described. SAM and SAH quantifications were performed in urine samples of 58 subjects with T21 and 48 controls (N) through liquid chromatography with tandem mass spectrometry. SAH resulted slightly more excreted in urine of subjects with T21 (T21/N mean ratio = 1.16, P value = 0.021), although no difference was found in SAM levels. Metabolite urine levels were compared with those previously observed in plasma, in which higher amounts of SAM and SAH were found. In addition, we examined if an association between the levels of SAM and SAH in T21 and the expression levels of genes involved in their production/utilization exists using the transcriptome map of blood samples of T21 and N subjects. The analysis showed overexpression of 44 methyltransferase genes responsible for the conversion of SAM to SAH, of two genes involved in SAH utilization, adenosylhomocysteinase-like 1, adenosylhomocysteinase-like 2, and of one gene involved in SAM utilization, adenosylmethionine decarboxylase 1. These data support the hypothesis that T21 genetic imbalance is responsible for SAM and SAH excess, which may be involved in the T21 phenotypic features.

NEW & NOTEWORTHY S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) are critical metabolites for the fundamental cellular functions, such as proliferation and epigenetic regulation. For the first time, their levels were quantified in the urine of subjects with trisomy 21 (T21) and compared with euploid controls (N). These dosages were compared with their plasma levels, and the expression of genes involved in SAM and SAH production/utilization was further investigated in the differential blood transcriptome map of T21 versus N samples.

Down syndrome; gene expression; homocysteine-methionine cycle; metabolism; trisomy 21

INTRODUCTION

Trisomy 21 (T21) or Down syndrome (DS) (OMIM 190685) is a chromosomal anomaly caused by the presence of an extra copy of human chromosome 21 (Hsa21) (1) and occurs with a frequency of 1 in every 1,000–1,100 newborn children around the world (2). Individuals with T21 may manifest many comorbidities affecting numerous systems, delineating a highly complex and variable phenotype (3, 4). The two phenotypic features always present in individuals with DS are *1*) distinctive facial features and *2*) intellectual disability (ID), which is present with high intersubjective variability.

Although DS is the most frequent genetic cause of ID (5), the pathogenetic mechanism underlying cognitive delay is unclear.

It is known that T21 contributes to metabolic alterations that can be crucial for the onset of pathological phenotypes associated with DS (6–8). In 2018 and later in 2020, a clearly different metabolic profile between trisomic and euploid subjects (N) was also identified, highlighting that several significantly altered metabolites are produced at the beginning or during the Krebs cycle (9, 10).

The first to suggest that DS could be considered a metabolic disease was Jérôme Lejeune, who observed altered one-

carbon metabolism in subjects with DS and in trisomic cell lines (11, 12). Over the years, the alteration of the one-carbon cycle in DS at different levels was confirmed by several authors (13–20).

One-carbon metabolism consists of several interconnected biochemical pathways driven by folate and homocysteine-methionine cycles, in which one-carbon groups at different oxidation states are used. It is involved in many processes, including DNA synthesis through purine and thymidylate generation, amino acid homeostasis, antioxidant generation, and epigenetic regulation (21), supporting critical cellular functions, such as cell proliferation and mitochondrial respiration (22). It is known that one-carbon metabolism plays a role in the proper neurodevelopment of the individual. When it is impaired, for example, in cerebral folate deficiencies (CFD), neuropsychiatric pathologies may occur (23).

S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) are metabolites produced in the homocysteine-methionine cycle of the one-carbon pathway (Fig. 1) (24). SAM is produced by transferring the adenosyl moiety of ATP to methionine, then, after removing a methyl group that is added to other substrates, it is converted to SAH (25), and for this reason, it is considered the universal methyl donor (24, 26). The removal of the methyl group is mediated by methyltransferases, which may add it to nucleic acid molecules (DNA or RNA methyltransferases), phospholipids, and proteins (protein methyltransferases) differentiating between protein lysine methyltransferases (PKMTs) if the amino acid methyl-acceptor is lysine or protein arginine methyltransferases (PRMTs) if the methyl-acceptor is arginine (25, 27). In addition, there are methyltransferases acting on the small molecules, i.e., the catechol *O*-methyltransferase (COMT), which catalyzes the

methyl transfer to the catecholamines for neurotransmitter degradation (24, 28).

Finally, SAH is hydrolyzed by AHCY (adenosylhomocysteinase) to adenosine (Ado) and L-homocysteine (Hcy). There is a fine enzymatic regulation of these reactions in which SAH is a feedback inhibitor of SAM-dependent methyltransferases, even if it has been shown that some methyltransferases are more susceptible than others to its inhibitory function (27). The SAM/SAH ratio is a well-known indicator of cellular methylation potential, and when it is decreased may correlate with reduced methylation capacity (24, 27, 30). Moreover, an accumulation of SAH in body fluids is associated with vascular disease, tissue damage, and neurological symptoms (25, 31–34).

The role of homocysteine-methionine cycle was already discussed as necessary for the synthesis of cholinergic and adrenergic mediators (35), for the construction of neurons, and for isolation of substances. These mechanisms make a high consumption of monocarbons, so if their supply is not sufficient, brain functions might be impaired (36, 37).

An excess of SAM and SAH with a decreased SAM/SAH ratio in the plasma of subjects with DS compared with N was recently identified (38). It has also been reported that normal fractional excretion compared with that of creatinine is 93% for SAM and 39% for SAH, with a high ratio of SAM to SAH in N urine (39).

In this work, SAM and SAH levels in urine samples of 58 DS and 48 N were determined and compared with plasma analyses of the same subjects carried out by an immunoenzymatic approach enzyme-linked immunosorbent assays (ELISA) (38). In addition, we examined if an association between the higher plasma levels of SAM and SAH in DS versus N and the expression levels of genes involved in their production/utilization exists using the transcriptome map generated by RNA-sequencing (RNA-seq) on blood samples of DS and N subjects (29).

Figure 1. Schematic representation of the methionine-homocysteine cycle. The metabolites studied in our analysis (SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine) are reported in bold. The genes encoding for enzymes that produce or use two metabolites of interest in blood tissue are also indicated: *1*) the genes showing an expression ratio (DS/N) >0.76 and <1.30 in the RNA-sequencing analysis (*MAT2A*, methionine adenosyltransferase 2 A; *MAT2B*, methionine adenosyltransferase 2 noncatalytic beta subunit; *AHCY*, adenosylhomocysteinase) are reported in bold; *2*) the genes showing an expression ratio ≥1.3 (*AMD1*, adenosylmethionine decarboxylase 1; *AHCYL1*, adenosylhomocysteinase like 1; *AHCYL2*, adenosylhomocysteinase like 2) are considered overexpressed and are reported in bold followed by the symbol ↑; and *3*) SAM-dependent methyltransferase gene over- or underexpression details are provided in the Supplementary Table S2A. Gene expression data are derived from Antonaros et al. (29).

MATERIALS AND METHODS

Ethics Approval and Consent to Participate

The Independent Ethics Committee of the Hospital—University of Bologna Policlinico S. Orsola-Malpighi, Italy has granted the ethical approval for this study (No.: 39/2013/U/Tess). We obtained informed written consent from all participants to collect urine and blood samples and clinical data. Concerning minors, the consent was collected from their parents. All procedures were carried out in accordance with the Ethical Principles for Medical Research involving Human Subjects of the Helsinki Declaration.

Case Selection

The study has been proposed to all subjects consecutively admitted to the Neonatology Unit of the "Istituto di Ricovero e Cura a Carattere Scientifico, Azienda Ospedaliero-Universitaria di Bologna (IRCCS AOUBO)", Italy in the context of routine follow-up provided for DS.

For the assay of SAM and SAH in urine, we have selected a group of subjects with a diagnosis of DS with homogeneous or mosaic T21, and a control group (N) selected among siblings of subjects with DS and without evidence of abnormal karyotype or pathologies. For both groups, we have selected
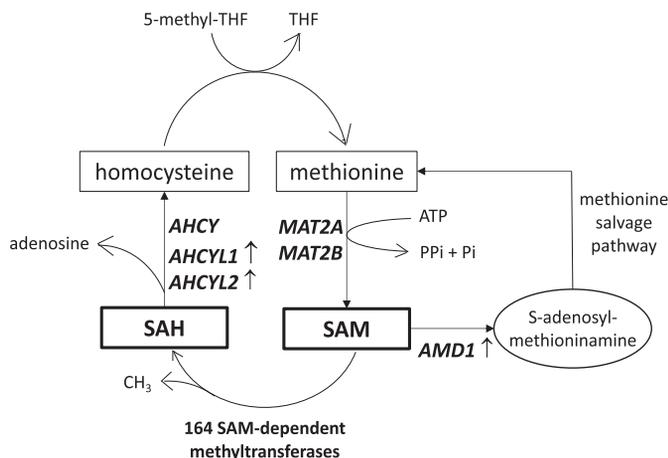
subjects who have an adequate amount of urine to determine SAM and SAH metabolites, and who were similar in age and sex. The urine samples selected were treated within 2 h of collection and with an apparently good physical state (clear urine after centrifugation).

A total of 106 subjects were selected. DS group consists of 58 subjects, 35 males (M) and 23 females (F) with a mean age of 12.7-yr old [standard deviation (SD) = 5.9] and age range from 3 yr to 28 yr; the N group consists of 48 subjects, 32 M and 16 F with a mean age of 14.4 yr (SD = 7.1), and an age range from 2 yr to 31 yr. Concerning the N group, 20 subjects were siblings of 17 subjects in the DS group.

For every collected sample, parents filled out a form with information about the current fasting state, last meal, concomitant diseases, and consumed medications (Supplemental Dataset S1).

SAM and SAH quantification values in plasma were retrieved from a previous work (38). These plasma samples were collected on the same day as the urine samples, for some of the subjects (see Supplemental Dataset S1). Both plasma and urine SAH levels were available for 30 DS subjects and 15 N subjects, whereas plasma and urine SAM levels were available for 8 DS subjects and 11 N subjects. Plasma values were converted from ng/mL to nM (Supplemental Dataset S1), according to the formula [(ng/mL)·1,000]/MW, where MW is the molecular weight (g/mol). The MW of SAM is 398.44 g/mol, MW of SAH is 384.412 g/mol.

Urine Sample Preparation

Preanalytical treatment of urine samples followed standard operating procedures (40). All procedures were conducted carefully in a sterile environment to avoid contaminations.

Urine samples were collected in a sterile plastic cup with a lid and kept refrigerated at $+4°C$ if immediate processing was not possible. They were treated within 2 h of collection. The sample was transferred to a new tube and centrifuged at 2,500 g for 5 min at $+4°C$. After centrifugation, filtration by a 0.20 μm cutoff filter was performed to avoid contamination of the metabolome with soluble molecules derived from cellular components. The filtered urine was transferred to sterile cryovials, making 1.0 mL aliquots. All urine samples were rapidly stored in liquid nitrogen and were ready for subsequent analysis.

LC-MS/MS Analysis

We used an Agilent 1260 Infinity II LC system (Agilent Technologies, Waldbronn, Germany) for chromatographic separation, consisting of a Multisampler (G7167A), quaternary 1260 Infinity II Flexible Pump (G7104C), and Multicolumn Thermostat (G7116A). The separation was carried out using a Zorbax Eclipse Plus C18 RRHD column (50 mm × 2.1 mm, 1.8 μm particle size, Agilent Technologies, Waldbronn, Germany). For mass spectrometric detection, the ultra high performance liquid chromatography (UHPLC) column was connected with PEEK tubing (0.005″ ID × ca. 60 cm long) to a triple quadrupole Ultivo LC/TQ system (G6465B). Chromatographic separation was performed isocratically with a mobile phase containing 0.1% vol./vol. heptafluorobutyric acid and 10% acetonitrile (Sigma-Aldrich, Steinheim, Germany). The column was temperature-controlled at 30°C, the mobile phase flow rate was

set at 0.4 mL·min^{-1}, and the injection volume was 1.0 μL. Instrumental parameters for the electrospray ionization tandem mass spectrometric detector (ESIMS/MS) were as follows: nebulizer gas temperature: 350°C; nebulizer gas flow: 10 L·min^{-1}; nebulizer pressure: 35 psi; sheath gas: 400°C; sheath gas: 12 min^{-1}; fragmentor voltage: 50 V; collision energy: 20 eV; capillary voltage: 3,000 V. SAM and SAH were quantified using the mass transitions 399 → 250 and 385 → 136, respectively.

Urine was thawed, mixed by vortexing, and centrifuged for 15 min at 10,000 g. To minimize matrix effects, 100 μL of the urine supernatant was diluted with 900 μL of water. The combination of this dilution with the used small injection volume (1.0 μL) was sufficient to eliminate the matrix effects, as indicated by recovery experiments where recoveries were found within the range of 85%–115%. The limit of detection was 0.01 μM (calculated based on the S/N = 3 method).

Statistical Analyses

To choose the correct test to perform the statistical analysis, we first checked whether our data followed a normal distribution using the Kolmogorov–Smirnov test of the online Social Science Statistics software (https://www.socscistatistics.com/tests/kolmogorov/).

To know if there was any difference between DS and N group for age or sex, we performed an unpaired t test and Fisher's test, respectively.

Later, we analyzed the statistical influence of age, sex, and fasting status on metabolite levels; specifically, to test if a link between age and metabolite levels exists, we performed a linear correlation, to test if sex and fasting/nonfasting state affects the urinary and plasma levels we performed the unpaired t test for parametric variables, and Kruskal–Wallis test for nonparametric variables.

Once the previous analyses were performed, we investigated whether there were different distributions of SAH and SAM levels in the different groups (DS and N groups) using unpaired t test.

Finally, we performed a bivariate correlation or partial bivariate correlation, checked for the effect of chronological age between SAM and SAH levels in the urine [SAM in urine (uSAM) and SAH in urine (uSAH)] and plasma [SAM in plasma (pSAM) and SAH in plasma (pSAH)] samples of the DS and N groups.

Statistical analyses were carried out with SPSS Statistics software (IBM, Version 28 for Mac OS X) and were performed using the data available in Supplemental Dataset S1. For all results, a value $P < 0.05$ was considered statistically significant. An $R < 0.4$ was considered as weakly correlated, $0.4 < R < 0.7$ as moderately correlated, and $R > 0.7$ as strongly correlated.

Blood Transcriptome Map and Selection of Genes Related to SAH and SAM

By using a recently published differential blood transcriptome map obtained by analyzing RNA-sequencing data of 4 DS and 4 N samples (29), we identified and analyzed the expression pattern of genes involved in the homocysteine-methionine cycle for the production/utilization of SAH and SAM metabolites.

To select the human SAM-dependent methyltransferase genes, whose enzymes use SAM to donate the methyl group,

obtaining SAH, we performed the search "GENES" for "Methyltransferase" and "hsa" for "Methyltransferase" on Kyoto Encyclopedia of Genes and Genomes database (KEGG Pathway); we found 189 methyltransferase genes. Then we searched for the record "methyltransferase [MH] AND metabolism [MH] AND "methyl donor" with the filters "Humans," "Review," and "English" on National Center for Biotechnology Information (NCBI); we found 15 articles from three of which we retrieved 15 new methyltransferase genes to our list (41–43). Each gene was checked as a SAM-dependent methyltransferase by searching the gene name on the NCBI PubChem database and eventually on NCBI Gene and NCBI PubMed following the links on the Gene page. We deleted 13 genes that were methyltransferase not SAM-dependent, and then 27 genes that were not expressed in blood tissue (29).

Finally, to select the other genes that create and use SAM and SAH, we performed the search "S-adenosylmethionine" and "hsa" on KEGG Pathway and then "S-adenosylhomocysteine" and "hsa," and we found seven genes, one of which was not expressed in blood tissue, so we selected a total of six genes.

RESULTS

SAM and SAH Quantifications and Statistical Analyses

The 58 subjects with DS and 48 N subjects selected for the urine sample analysis resulted in comparable age (Cohen's $D = -0.263$, $P = 0.189$) and sex (Fisher's test $P = 0.548$).

Considering that children's hydration status can affect the metabolites analyzed in urine samples, uSAM and uSAH concentrations (μM) were adjusted for specific gravity (SG).

In the DS group, uSAM mean concentration value was 8.87 μM (SD = 2.21), and uSAH mean concentration value was 0.59 μM (SD = 0.18) (Table 1, Supplemental Dataset S1). DS uSAM/uSAH ratio was 15.03. In the N group, uSAM concentration mean value was 8.81 μM (SD = 2.39), and uSAH concentration mean value was 0.51 μM (SD = 0.19) (Table 1, Supplemental Dataset S1). The N uSAM/uSAH ratio was 17.27.

The levels of the urine metabolites analyzed follow a normal distribution and are not affected by fasting state, sex, or age with the exception of uSAH levels, which have a moderate statistically significant correlation with age in both DS ($P = 0.002$ and $R = 0.405$) and N groups ($P = 0.004$ and $R = 0.413$). Plasma metabolite levels follow a normal distribution in DS group, whereas pSAH and pSAM in N group do not; moreover, they are not affected by fasting state, sex, or age in plasma (Supplemental Table S1, A–D). The descriptive analyses performed with SPSS Statistics on plasma and urine metabolites did not highlight the presence of strong outliers among the analyzed values.

Unpaired t test showed that mean DS/N ratio values are statistically different for uSAH, pSAH, and pSAM (Supplemental Table S1E).

Correlation analyses showed that uSAH levels are moderately correlated with uSAM values both in DS ($R = 0.59$, $P < 0.001$) and N ($R = 0.63$, $P < 0.001$) groups (Supplemental Table S1F).

Blood Transcriptome Map

Using a recently published differential blood transcriptome map between DS and N samples (29), we have analyzed the expression of genes involved in SAM and SAH production/utilization (Supplemental Table S2) with the aim of finding a relationship between metabolite levels observed in plasma, urine, and gene behavior.

The Supplemental Table S2, A and B show the lists of 170 genes found and the mean expression values in the DS (Expression A) and N (Expression B) samples together with the DS/N ratio (Expression A/B). In total, 164 out of 170 genes are SAM-dependent methyltransferase genes, and 35 of which were also present in the list of SAM-dependent methyltransferases made by Clarke and Banfield (27) (Supplemental Table S2A). Seven of 164 methyltransferases were excluded from the analyses because their expression levels were calculated from only one sample group (DS or N), leading to a final number of 157 SAM-dependent methyltransferases analyzed. Among the six genes selected that are not for methyltransferases, two genes are SAM-producers (MAT2A and MAT2B), one uses

Table 1. *SAM and SAH concentration values and ratios*

	DS				N			
	SAH Concentration (μM) in Urine Adjusted for Specific Gravity	SAM Concentration (μM) in Urine Adjusted for Specific Gravity	SAH Concentration (μM) in Plasma Vione et al. (38)	SAM Concentration (μM) in Plasma Vione et al. (38)	SAH Concentration (μM) in Urine Adjusted for Specific Gravity	SAM Concentration (μM) in Urine Adjusted for Specific Gravity	SAH Concentration (μM) in Plasma Vione et al. (38)	SAM Concentration (μM) in Plasma Vione et al. (38)
Mean	0.59	8.87	16.96	21.64	0.51	8.81	5.40	14.95
SD	0.18	2.21	8.08	4.55	0.19	2.39	4.83	7.28
Median	0.58	8.33	16.56	21.53	0.48	8.41	4.19	13.01

	Urine		Plasma	
	SAH	SAM	SAH	SAM
Mean ratio, DS/N	1.16	1.01	3.14	1.45
Median ratio, DS/N	1.21	0.99	3.95	1.66
P value	0.021	0.913	<0.001	0.019

Mean, median, and standard deviation (SD) are shown for each metabolite in plasma and urine of Down syndrome (DS) and control (N) groups. In addition, the ratios between DS and N mean and median values of SAM and SAH are shown. The results of unpaired t test for SAH and SAM concentration values in plasma and urine samples, both in DS and N groups, are also shown. SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

SAM (*AMD1*), and the last three use SAH (*AHCY, AHCYL1,* and *AHCYL2*) (Supplemental Table S2*B*).

Analyzing the differential gene expression levels between the DS and N group, we found that 34 of 157 SAM-dependent methyltransferase genes and 3 of 6 other genes have a DS/N ratio ≥1.30, which we can consider overexpressed in the DS group, whereas 10 of 157 SAM-dependent methyltransferase genes have a ratio ≤0.76, which we can consider underexpressed in the DS group (44). None of the other six genes is underexpressed. The remaining genes (113 of 157 and 3 of 6) have DS/N ratios >0.76 and <1.30, so we can consider them normally expressed (44).

DISCUSSION

The homocysteine-methionine cycle has been found to be altered at different levels in DS (24), and given the lack of literature on their level in DS biological samples, in this work, SAM and SAH imbalances in urine samples of 58 DS and 48 N subjects were checked by LC/MS-MS method. SAM and SAH levels in urine were already analyzed in subjects with renal insufficiency versus controls (31, 45, 46), but it is the first time that the levels of these metabolites are analyzed in urine samples of subjects with DS. The mean value of uSAM and uSAH in our cohort of N is close to those already published by Kruglova et al. (45) (20 healthy subjects, mean age = 58.5 yr; SAM = 10.2 μM; SAH = 0.89 μM), although a small variation is probably due to the different mean age of the enrolled subjects. This variation is coherent with our analyses that showed uSAH levels moderately correlated with age.

We also compared these results with those obtained in our previous work conducted on plasma samples of DS and N subjects (38) (Supplemental Dataset S1) to observe if the excess of these metabolites observed in plasma is also detectable in urine. The uSAH levels are 16% higher in DS subjects (Table 1) compared with N subjects, whereas the uSAM levels are equal between the two groups. In plasma, SAH and SAM levels are much higher in DS subjects (Table 1) compared with N subjects. Indeed, these data show that pSAH DS/N mean ratio is 2.71 times higher than uSAH DS/N mean ratio, so there is a greater discrepancy of the pSAH levels between DS and N groups than the uSAH levels. A similar pattern exists for SAM, indeed pSAM DS/N mean ratio versus uSAM DS/N mean ratio is 1.44 times higher. The comparison between these two detection studies allows us to compare the amount of SAM and SAH that remains in the circulation and that is excreted. This gives us a better knowledge of the metabolism of SAM and SAH in DS, highlighting that SAH levels are higher in both urine and plasma DS samples.

In addition, even if it is known that 60% of SAH is reabsorbed in renal tubules (45), a decrease of SAM/SAH ratio (0.87) was detected in our DS urine samples. The decrease of SAM/SAH ratio is probably due to the slightly but statistically significant higher level of SAH in DS compared with N urine samples. Surely, the reason for higher SAH level in DS urine needs to be investigated further, indeed the decrease of SAM/SAH ratio was also previously associated with chronic kidney disease (45, 46), and DS subjects have a four- to fivefold higher risks than healthy controls of developing it due to congenital urologic or renal function and structure abnormalities, such as renal hypoplasia (47, 48).

The decrease of the SAM/SAH ratio was also associated with depressive tendencies in the elderly population (49), accumulation of phosphorylated tau protein in the central nervous system (50), melatonin deficiency, and hypomethylation of DNA, RNA, and histones (51) emphasizing the importance of SAM and SAH homeostasis.

It would have been interesting to correlate SAM and SAH with methionine and Hcy levels in urine, but the metabolome analysis through untargeted nuclear magnetic resonance on a cohort of 67 DS versus 26 N urine samples did not detect methionine (9), and recent works already discussed that Hcy, like many other amino acids, is reabsorbed almost completely (>97%) in primary urine, whereas >90% of SAM and ~40% of SAH are normally excreted (45, 46). Another interesting information is the lack of a relationship between SAH or SAM and Hcy levels in plasma samples, already searched for by Vione et al. (38). These data indicate that the relationship between the increase of both SAH and Hcy in plasma of N subjects reported by Clarke and Banfield (27) does not occur in DS.

SAM is the principal methyl donor for many cellular methyltransferase reactions, and SAH is the demethylation product of SAM. The excess of SAH itself acts with negative feedback on methyltransferases that are inhibited by high levels of SAH (28). Indeed, SAH analogs were used as antiproliferative molecules through their action as inhibitors of methyltransferase enzymes (28). The congruence between the antiproliferative effects given by the administration of the analogs of this molecule and the hypocellularity typical of DS, also characterized by a very low incidence of cancer (52) might be related to the high level of SAH circulating molecules in DS subjects (53, 54).

To investigate the role of the genomic imbalance, due to the third copy of Hsa21, on the altered metabolic profile observed in DS, we analyzed the expression of genes involved in SAH and SAM production or utilization (Fig. 1). The analysis of the transcriptome conducted on blood samples of DS and N subjects (29) showed 164 genes involved in the SAM/SAH production and utilization (Supplemental Table S2, *A* and *B*). Specifically, 157 of the 164 genes encode for methyltransferases (Supplemental Table S2*A*) that carry out the basic mechanism for the conversion of SAM to SAH and consequently for the control of their concentration levels. Observing the expression values is interesting to note that 44 of 157 methyltransferases are differentially expressed genes (DEGs) (28%), and 34 of 44 methyltransferases are overexpressed. Overexpression of methyltransferases, which convert SAM to SAH, could be consistent with higher SAH levels.

Moreover, we found that the *AMD1* gene, whose enzyme uses SAM to start the methionine salvage pathway, and *AHCYL1* and *AHCYL2*, whose enzymes seem to be involved in the reaction that metabolizes SAH to create adenosine and Hcy (55), are overexpressed (Fig. 1, Supplemental Table S2*B*).

Focusing on Hsa21 genes, we have found only two differentially expressed genes encoding for methyltransferases: *PRMT2* (DS/N = 1.60), encoding for protein arginine methyltransferase 2, and *SETD4* (DS/N = 1.42), encoding for SET domain containing 4 (SETD4), a histone methyltransferase. PRMT2 regulates various biological processes, including transcription, posttranscriptional events, cellular apoptosis, cell

morphogenesis, and immune responses, through mechanisms such as methylation, coactivation, and specific structural interactions. PRMT2 predominantly exhibits oncogenic tendencies in malignant tumors, but under specific circumstances, it can also demonstrate tumor-suppressive characteristics, displaying a bidirectional regulatory nature (56). SETD4 functions as a key regulator in multiple tumor types by catalyzing histone lysine methylation. It regulates cell proliferation indeed, it was shown to be downregulated in prostate cancer (57).

Successively, we focused on the expression levels of the SAM-dependent methyltransferases that are more susceptible to SAH levels, as declared by Clarke and Banfield (27) in 2001 (Supplemental Table S2). We have found the overexpression of the genes *CAMKMT, GNMT, HNMT, TPMT, ICMT, PCMT1,* and the underexpression of the genes *TRMT61A* and *GAMT*. We might expect that the underexpressed methyltransferases undergo a greater reduction in their activity due to high levels of SAH, whereas the overproduced methyltransferase activities might be compensated by high SAH levels. Among the underexpressed methyltransferase gene, *TRMT61A* encodes for an enzyme that enables mRNA (adenine-N1-)-methyltransferase activity. It is involved in mRNA methylation and results to be elevated levels in hepatocellular carcinoma (HCC) (58). GAMT is a methyltransferase that, using SAM as the methyl donor, converts guanidinoacetate to creatine phosphate necessary for the synthesis of ATP, the main source of cellular energy. Defects in this gene have been implicated in neurological syndromes and muscular hypotonia, probably due to creatine deficiency and accumulation of guanidinoacetate in the brain of affected individuals (59).

Among the genes involved in the production/utilization of SAM and SAH, *AMD1* gene encodes a key enzyme which, using SAM as substrate, is involved in the biosynthesis of polyamines, essential molecules for cellular proliferation and survival (60, 61). It was reported that when overexpressed, a negative feedback mechanism is activated, in particular, the protein inhibits the translation of itself (62). An alteration of this mechanism of control seems to be the cause of high translational levels of the protein, which were associated with cell proliferation and tumorigenesis (63).

The overexpression of *AHCYL1* and *AHCYL2* genes was also observed in the blood transcriptome map of subjects with DS. They are both paralogs of the *AHCY* gene, whose expression in turn is not altered in DS versus N. AHCYL1 is a SAH hydrolase-like protein that also interacts with inositol 1,4,5-trisphosphate receptor, type 1 (IP$_3$R), suppressing IP$_3$R activation through competition with IP$_3$ for the common binding site on IP$_3$R (64). In humans, AHCYL1 functions as a tumor suppressor (64) and also interacts with microtubule-associated protein tau (MAPT), studied to be involved in Alzheimer's disease, and seems to control AHCYL1 function (65). AHCYL2 is the SAH hydrolase-like 2 protein, also called adenosylhomocysteinase 3, and has a different function than AHCYL1; in particular, it regulates the electrogenic sodium/bicarbonate cotransporter SLC4A4 activity (66).

Conclusions

In this study, we measured for the first time SAM and SAH levels in urine samples from DS and N subjects and compared them with their previous detection in plasma samples (38). In addition, we further investigated the behavior of genes involved in SAM and SAH production/utilization using a blood transcriptome map of DS and N subjects (29). This study demonstrated that SAH is significantly higher in both urine and plasma samples of DS subjects, and this alteration might be considered a metabolic imbalance typical of DS. The excess circulating SAH might be due to the genetic imbalance of genes involved in its production/utilization found in the blood samples of DS subjects. Gene expression data are difficult to interpret, due to the high amount of genes involved in the reactions and the fine regulation mechanisms involved, such as the retroactive inhibition of SAH on the SAM-dependent methyltransferase enzymes (27, 67), and the allosteric activation of cystathionine-β-synthase (CBS) enzyme and the inhibition of methylenetetrahydrofolate reductase (MTHFR) enzyme carried out by SAM (68, 69), two enzymes that have a role in the homocysteine-methionine cycle as well as particularly studied in DS (70).

Because of the possible involvement of some methyltransferases on the hypocellularity and also on neurological development, testing their enzymatic activities might be a future perspective of this study. In this way, an additive inhibitory effect of multiple enzyme activities induced by SAH excess in DS might be revealed.

DATA AVAILABILITY

Source data for this study are openly available at https://doi.org/10.17605/OSF.IO/BXHCG.

SUPPLEMENTAL MATERIAL

Supplemental Dataset S1, and Supplemental Tables S1 and S2: https://doi.org/10.17605/OSF.IO/BXHCG.

ACKNOWLEDGMENTS

REFERENCES

1. **Lejeune J**, **Gautier M**, **Turpin R**. [Study of somatic chromosomes from 9 mongoloid children]. *C R Hebd Seances Acad Sci* 248: 1721–1722, 1959.

2. **Lukowski AF**, **Milojevich HM**, **Eales L**. Cognitive functioning in children with down syndrome: current knowledge and future directions. *Adv Child Dev Behav* 56: 257–289, 2019. doi:10.1016/bs.acdb.2019.01.002.

3. **Roper RJ**, **Reeves RH**. Understanding the basis for Down syndrome phenotypes. *PLoS Genet* 2: e50, 2006. doi:10.1371/journal.pgen.0020050.

4. **Bull MJ**. Down syndrome. *N Engl J Med* 382: 2344–2352, 2020. doi:10.1056/NEJMra1706537.

5. **Romano C**. Genetics and clinical neuroscience in intellectual disability. *Brain Sci* 12: 338, 2022. doi:10.3390/brainsci12030338.

6. **Chango A**, **Mircher C**, **James SJ**, **Réthoré MO**, **Nicolas J-P**. [One carbon metabolism and trisomy 21: analysis of the genetic polymorphism]. *Ann Biol Clin (Paris)* 60: 647–653, 2002.

7. **Orozco JS**, **Hertz-Picciotto I**, **Abbeduto L**, **Slupsky CM**. Metabolomics analysis of children with autism, idiopathic-developmental delays, and Down syndrome. *Transl Psychiatry* 9: 243, 2019. doi:10.1038/s41398-019-0578-3.

8. **Dierssen M**, **Fructuoso M**, **Martínez de Lagrán M**, **Perluigi M**, **Barone E**. Down syndrome is a metabolic disease: altered insulin signaling mediates peripheral and brain dysfunctions. *Front Neurosci* 14: 670, 2020. doi:10.3389/fnins.2020.00670.

9. **Caracausi M**, **Ghini V**, **Locatelli C**, **Mericio M**, **Piovesan A**, **Antonaros F**, **Pelleri MC**, **Vitale L**, **Vacca RA**, **Bedetti F**, **Mimmi MC**, **Luchinat C**, **Turano P**, **Strippoli P**, **Cocchi G**. Plasma and urinary metabolomic profiles of Down syndrome correlate with alteration of mitochondrial metabolism. *Sci Rep* 8: 2977, 2018. doi:10.1038/s41598-018-20834-y.

10. **Antonaros F**, **Ghini V**, **Pulina F**, **Ramacieri G**, **Cicchini E**, **Mannini E**, **Martelli A**, **Feliciello A**, **Lanfranchi S**, **Onnivello S**, **Vianello R**, **Locatelli C**, **Cocchi G**, **Pelleri MC**, **Vitale L**, **Strippoli P**, **Luchinat C**, **Turano P**, **Piovesan A**, **Caracausi M**. Plasma metabolome and cognitive skills in Down syndrome. *Sci Rep* 10: 10491, 2020. doi:10.1038/s41598-020-67195-z.

11. **Lejeune J**. [Biochemical investigations and trisomy 21 (author's transl)]. *Ann Genet* 22: 67–75, 1979.

12. **Lejeune J**. Pathogenesis of mental deficiency in trisomy 21. *Am J Med Genet Suppl* 7: 20–30, 1990. doi:10.1002/ajmg.1320370705.

13. **Rosenblatt DS**, **Whitehead VM**, **Matiaszuk NV**, **Pottier A**, **Vuchich MJ**, **Beaulieu D**. Differential effects of folinic acid and glycine, adenosine, and thymidine as rescue agents in methotrexate-treated human cells in relation to the accumulation of methotrexate polyglutamates. *Mol. Pharmacol* 21: 718–722, 1982. doi:10.1016/S0026-895X(25)14917-1.

14. **Ueland PM**, **Refsum H**, **Christensen B**. Methotrexate sensitivity in Down's syndrome: a hypothesis. *Cancer Chemother Pharmacol* 25: 384–386, 1990. doi:10.1007/BF00686245.

15. **Peeters MA**, **Rethore MO**, **Lejeune J**. In vivo folic acid supplementation partially corrects in vitro methotrexate toxicity in patients with Down syndrome. *Br J Haematol* 89: 678–680, 1995. doi:10.1111/j.1365-2141.1995.tb08390.x.

16. **Obeid R**, **Hartmuth K**, **Herrmann W**, **Gortner L**, **Rohrer TR**, **Geisel J**, **Reed MC**, **Nijhout HF**. Blood biomarkers of methylation in Down syndrome and metabolic simulations using a mathematical model. *Mol Nutr Food Res* 56: 1582–1589, 2012. doi:10.1002/mnfr.201200162.

17. **Song C**, **He J**, **Chen J**, **Liu Y**, **Xiong F**, **Wang Y**, **Li T**. Effect of the one-carbon unit cycle on overall DNA methylation in children with Down's syndrome. *Mol Med Rep* 12: 8209–8214, 2015. doi:10.3892/mmr.2015.4439.

18. **Vitale L**, **Serpieri V**, **Lauriola M**, **Piovesan A**, **Antonaros F**, **Cicchini E**, **Locatelli C**, **Cocchi G**, **Strippoli P**, **Caracausi M**. Human trisomy 21 fibroblasts rescue methotrexate toxic effect after treatment with 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate. *J Cell Physiol* 234: 15010–15024, 2019. doi:10.1002/jcp.28140.

19. **Funk RS**, **Talib NJ**, **Zimmerman KO**, **van Haandel L**, **Becker ML**. Altered folate homeostasis in children with down syndrome: a potential basis for enhanced methotrexate toxicity. *J Pediatr* 221: 235–239, 2020. doi:10.1016/j.jpeds.2020.01.032.

20. **Antonaros F**, **Lanfranchi S**, **Locatelli C**, **Martelli A**, **Olivucci G**, **Cicchini E**, **Carosi Diatricch L**, **Mannini E**, **Vione B**, **Feliciello A**, **Ramacieri G**, **Onnivello S**, **Vianello R**, **Vitale L**, **Pelleri MC**, **Strippoli P**, **Cocchi G**, **Pulina F**, **Piovesan A**, **Caracausi M**. One-carbon pathway and cognitive skills in children with Down syndrome. *Sci Rep* 11: 4225, 2021. doi:10.1038/s41598-021-83379-7.

21. **Lyon P**, **Strippoli V**, **Fang B**, **Cimmino L**. B vitamins and one-carbon metabolism: implications in human health and disease. *Nutrients*. 12: 2867, 2020. doi:10.3390/nu12092867.

22. **Mattson MP**, **Shea TB**. Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders. *Trends Neurosci* 26: 137–146, 2003. doi:10.1016/s0166-2236(03)00032-8.

23. **Pope S**, **Artuch R**, **Heales S**, **Rahman S**. Cerebral folate deficiency: analytical tests and differential diagnosis. *J Inherit Metab Dis* 42: 655–672, 2019. doi:10.1002/jimd.12092.

24. **Caracausi M**, **Ramacieri G**, **Catapano F**, **Cicilloni M**, **Lajin B**, **Pelleri MC**, **Piovesan A**, **Vitale L**, **Locatelli C**, **Pirazzoli GL**, **Strippoli P**, **Antonaros F**, **Vione B**. The functional roles of S-adenosyl-methionine and S-adenosyl-homocysteine and their involvement in trisomy 21. *Biofactors* 50: 709–724, 2024. doi:10.1002/biof.2044.

25. **Barić I**, **Erdol S**, **Saglam H**, **Lovrić M**, **Belužić R**, **Vugrek O**, **Blom HJ**, **Fumić K**. Glycine N-methyltransferase deficiency: a member of dysmethylating liver disorders? *JIMD Rep* 31: 101–106, 2017. doi:10.1007/8904_2016_543.

26. **Mentch SJ**, **Locasale JW**. One-carbon metabolism and epigenetics: understanding the specificity. *Ann N Y Acad Sci* 1363: 91–98, 2016. doi:10.1111/nyas.12956.

27. **Clarke S**, **Banfield K**. S-Adenosylmethionine-dependent Methyltransferases. In: *Homocysteine in Health and Disease*, edited by Carmel R, Jackobsen DW. Cambridge University Press, 2001. p. 63–78.

28. **Zhang J**, **Zheng YG**. SAM/SAH analogs as versatile tools for SAM-dependent methyltransferases. *ACS Chem Biol* 11: 583–597, 2016. doi:10.1021/acschembio.5b00812.

29. **Antonaros F**, **Zenatelli R**, **Guerri G**, **Bertelli M**, **Locatelli C**, **Vione B**, **Catapano F**, **Gori A**, **Vitale L**, **Pelleri MC**, **Ramacieri G**, **Cocchi G**, **Strippoli P**, **Caracausi M**, **Piovesan A.** The transcriptome profile of human trisomy 21 blood cells. *Hum Genomics* 15: 25, 2021. doi:10.1186/s40246-021-00325-4.

30. **Petrossian TC**, **Clarke SG.** Uncovering the human methyltransferasome. *Mol Cell Proteomics* 10: M110.000976, 2011. doi:10.1074/mcp.M110.000976.

31. **Loehrer FM**, **Angst CP**, **Brunner FP**, **Haefeli WE**, **Fowler B.** Evidence for disturbed S-adenosylmethionine: S-adenosylhomocysteine ratio in patients with end-stage renal failure: a cause for disturbed methylation reactions? *Nephrol Dial Transplant* 13: 656–661, 1998. doi:10.1093/ndt/13.3.656.

32. **Kerins DM**, **Koury MJ**, **Capdevila A**, **Rana S**, **Wagner C.** Plasma S-adenosylhomocysteine is a more sensitive indicator of cardiovascular disease than plasma homocysteine. *Am J Clin Nutr* 74: 723–729, 2001. doi:10.1093/ajcn/74.6.723.

33. **Valli A**, **Carrero JJ**, **Qureshi AR**, **Garibotto G**, **Bárány P**, **Axelsson J**, **Lindholm B**, **Stenvinkel P**, **Anderstam B**, **Suliman ME.** Elevated serum levels of S-adenosylhomocysteine, but not homocysteine, are associated with cardiovascular disease in stage 5 chronic kidney disease patients. *Clin Chim Acta* 395: 106–110, 2008. doi:10.1016/j.cca.2008.05.018.

34. **Liu S**, **Liao H**, **Dai X**, **Guo H**, **Wang D**, **Xia M**, **Ling W**, **Xiao Y.** Association between plasma S-adenosylmethionine and risk of mortality in patients with coronary artery disease: A cohort study. *Am J Clin Nutr* 114: 1360–1370, 2021. doi:10.1093/ajcn/nqab210.

35. **Lejeune J.** On the mechanism of mental deficiency in chromosomal diseases. *Hereditas* 86: 9–14, 1977. doi:10.1111/j.1601-5223.1977.tb01207.x.

36. **Lejeune J.** Les tables de la Loi de la vie. *Acad Natl dei Lincei (Rome)* 11: 287–293, 1985.

37. **Lejeune J**, **Rethoré MO**, **de Blois MC**, **Maunoury-Burolla C**, **Mir M**, **Nicolle L**, **Borowy F**, **Borghi E**, **Recan D.** [Metabolism of monocarbons and trisomy 21: sensitivity to methotrexate]. *Ann Genet* 29: 16–19, 1986.

38. **Vione B**, **Ramacieri G**, **Zavaroni G**, **Piano A**, **La Rocca G**, **Caracausi M**, **Vitale L**, **Piovesan A**, **Gori C**, **Pirazzoli GL**, **Strippoli P**, **Cocchi G**, **Corvaglia L**, **Locatelli C**, **Pelleri MC**, **Antonaros F.** One-carbon pathway metabolites are altered in the plasma of subjects with Down syndrome: relation to chromosomal dosage. *Front Med (Lausanne)* 9: 1006891, 2022 [Erratum in *Front Med (Lausanne)* 11: 1432559, 2024]. doi:10.3389/fmed.2022.1006891.

39. **Stabler SP**, **Allen RH.** Quantification of serum and urinary S-adenosylmethionine and S-adenosylhomocysteine by stable-isotope-dilution liquid chromatography-mass spectrometry. *Clin Chem* 50: 365–372, 2004. doi:10.1373/clinchem.2003.026252.

40. **Bernini P**, **Bertini I**, **Luchinat C**, **Nincheri P**, **Staderini S**, **Turano P.** Standard operating procedures for pre-analytical handling of blood and urine for metabolomic studies and biobanks. *J Biomol NMR* 49: 231–243, 2011. doi:10.1007/s10858-011-9489-1.

41. **Williams AC**, **Cartwright LS**, **Ramsden DB.** Parkinson's disease: the first common neurological disease due to auto-intoxication? *QJM* 98: 215–226, 2005. doi:10.1093/qjmed/hci027.

42. **Obeid R.** The metabolic burden of methyl donor deficiency with focus on the betaine homocysteine methyltransferase pathway. *Nutrients* 5: 3481–3495, 2013. doi:10.3390/nu5093481.

43. **Fenwick MK**, **Ealick SE.** Towards the structural characterization of the human methyltransferome. *Curr Opin Struct Biol* 53: 12–21, 2018. doi:10.1016/j.sbi.2018.03.007.

44. **Pelleri MC**, **Cattani C**, **Vitale L**, **Antonaros F**, **Strippoli P**, **Locatelli C**, **Cocchi G**, **Piovesan A**, **Caracausi M.** Integrated quantitative transcriptome maps of human trisomy 21 tissues and cells. *Front Genet* 9: 125, 2018. doi:10.3389/fgene.2018.00125.

45. **Kruglova MP**, **Grachev SV**, **Bulgakova PO**, **Ivanov AV**, **Virus ED**, **Nikiforova KA**, **Fedoseev AN**, **Savina GD**, **Kubatiev AA.** Low S-adenosylmethionine/S-adenosylhomocysteine ratio in urine is associated with chronic kidney disease. *Lab Med* 51: 80–85, 2020. doi:10.1093/labmed/lmz035.

46. **Kruglova MP**, **Ivanov AV**, **Virus ED**, **Bulgakova PO**, **Samokhin AS**, **Fedoseev AN**, **Grachev SV**, **Kubatiev AA.** Urine S-adenosylmethionine are related to degree of renal insufficiency in patients with chronic kidney disease. *Lab Med* 52: 47–56, 2021. doi:10.1093/labmed/lmaa034.

47. **Padoan F**, **Stifano R**, **Brugnara M**, **Guarnaroli M**, **Piazza M**, **Lauriola S**, **Piacentini G**, **Pietrobelli A**, **Pecoraro L.** Kidney function and size in children with down syndrome: a cross-sectional study. *Nephrology (Carlton)* 30: e70007, 2025. doi:10.1111/nep.70007.

48. **Ranchin B**, **Bidault V**, **Zekre F**, **DeMul A**, **Sanlaville D**, **Bacchetta J.** Kidney and urological involvement in Down syndrome: frequent, underestimated, but associated with impaired quality of life and risk of kidney failure. *Pediatr Nephrol* 39: 347–355, 2024. doi:10.1007/s00467-023-05986-y.

49. **Xiong Y**, **Zhou G**, **Zhou L**, **Guo M**, **Lu H.** Serum molecules of the folate-driven one-carbon cycle as indicators of depressive tendencies in the elderly. *Heliyon* 10: e36980, 2024. doi:10.1016/j.heliyon.2024.e36980.

50. **Popp J**, **Lewczuk P**, **Linnebank M**, **Cvetanovska G**, **Smulders Y**, **Kölsch H**, **Frommann I**, **Kornhuber J**, **Maier W**, **Jessen F.** Homocysteine metabolism and cerebrospinal fluid markers for Alzheimer's disease. *J Alzheimers Dis* 18: 819–828, 2009. doi:10.3233/jad-2009-1187.

51. **Indika N-LR**, **Deutz NEP**, **Engelen MPKJ**, **Peiris H**, **Wijetunge S**, **Perera R.** Sulfur amino acid metabolism and related metabotypes of autism spectrum disorder: a review of biochemical evidence for a hypothesis. *Biochimie* 184: 143–157, 2021. doi:10.1016/j.biochi.2021.02.018.

52. **Osuna-Marco MP**, **López-Barahona M**, **López-Ibor B**, **Tejera ÁM.** Ten reasons why people with Down syndrome are protected from the development of most solid tumors -a review. *Front Genet* 12: 749480, 2021. doi:10.3389/fgene.2021.749480.

53. **Borchardt RT.** S-Adenosyl-L-methionine-dependent macromolecule methyltransferases: potential targets for the design of chemotherapeutic agents. *J Med Chem* 23: 347–357, 1980. doi:10.1021/jm00178a001.

54. **Kennedy BP**, **Bottiglieri T**, **Arning E**, **Ziegler MG**, **Hansen LA**, **Masliah E.** Elevated S-adenosylhomocysteine in Alzheimer brain: influence on methyltransferases and cognitive function. *J Neural Transm (Vienna)* 111: 547–567, 2004. doi:10.1007/s00702-003-0096-5.

55. **Huang W**, **Li N**, **Zhang Y**, **Wang X**, **Yin M**, **Lei QY.** AHCYL1 senses SAH to inhibit autophagy through interaction with PIK3C3 in an MTORC1-independent manner. *Autophagy* 18: 309–319, 2022. doi:10.1080/15548627.2021.1924038.

56. **Min Z-Q**, **Jiang M-J**, **Liu X-L**, **Yuan S-P**, **Chen P-A**, **Wang C-H**, **Chen Y-J**, **Dai X-P.** Advances in research on protein arginine methyltransferase 2: functions and diseases. *Protein Pept Lett* 31: 25–42, 2024. doi:10.2174/0109298665281395231211060535.

57. **Wang C**, **Wang T**, **Li K-J**, **Hu L-H**, **Li Y**, **Yu Y-Z**, **Xie T**, **Zhu S**, **Fu D-J**, **Wang Y**, **Zeng X-Z**, **Liu F-P**, **Chen H**, **Chen Z-S**, **Feng N-H**, **Liu J**, **Jiang Y**, **Zhao S-C.** SETD4 inhibits prostate cancer development by promoting H3K27me3-mediated NUPR1 transcriptional repression and cell cycle arrest. *Cancer Lett* 579: 216464, 2023. doi:10.1016/j.canlet.2023.216464.

58. **Wang Y**, **Cao K.** KDM1A promotes immunosuppression in hepatocellular carcinoma by regulating PD-L1 through demethylating MEF2D. *J Immunol Res* 2021: 9965099, 2021. doi:10.1155/2021/9965099.

59. **Mercimek-Mahmutoglu S**, **Pop A**, **Kanhai W**, **Fernandez Ojeda M**, **Holwerda U**, **Smith D**, **Loeber JG**, **Schielen PCJI**, **Salomons GS.** A pilot study to estimate incidence of guanidinoacetate methyltransferase deficiency in newborns by direct sequencing of the GAMT gene. *Gene* 575: 127–131, 2016. doi:10.1016/j.gene.2015.08.045.

60. **Brooks WH.** Autoimmune diseases and polyamines. *Clin Rev Allergy Immunol* 42: 58–70, 2012. doi:10.1007/s12016-011-8290-y.

61. **Morrison LD**, **Becker L**, **Kish SJ.** S-adenosylmethionine decarboxylase in human brain. Regional distribution and influence of aging. *Brain Res Dev Brain Res* 73: 237–241, 1993. doi:10.1016/0165-3806(93)90143-x.

62. **Yordanova MM**, **Loughran G**, **Zhdanov AV**, **Mariotti M**, **Kiniry SJ**, **O'Connor PBF**, **Andreev DE**, **Tzani I**, **Saffert P**, **Michel AM**, **Gladyshev VN**, **Papkovsky DB**, **Atkins JF**, **Baranov PV.** AMD1 mRNA employs ribosome stalling as a mechanism for molecular memory formation. *Nature* 553: 356–360, 2018. doi:10.1038/nature25174.

63. **Xu L**, **You X**, **Cao Q**, **Huang M**, **Hong L-L**, **Chen X-L**, **Lei L**, **Ling Z-Q**, **Chen Y.** Polyamine synthesis enzyme AMD1 is closely associated with tumorigenesis and prognosis of human gastric cancers. *Carcinogenesis* 41: 214–222, 2020. doi:10.1093/carcin/bgz098.

64. **Ando H**, **Kawaai K**, **Mikoshiba K.** IRBIT: a regulator of ion channels and ion transporters. *Biochim Biophys Acta* 1843: 2195–2204, 2014. doi:10.1016/j.bbamcr.2014.01.031.

65. **Wischhof L**, **Adhikari A**, **Mondal M**, **Marsal-Cots A**, **Biernat J**, **Mandelkow EM**, **Mandelkow E**, **Ehninger D**, **Nicotera P**, **Bano D.** Unbiased proteomic profiling reveals the IP3R modulator AHCYL1/IRBIT as a novel interactor of microtubule-associated protein tau. *J Biol Chem* 298: 101774, 2022. doi:10.1016/j.jbc.2022.101774.

66. **Yamaguchi S**, **Ishikawa T.** AHCYL2 (long-IRBIT) as a potential regulator of the electrogenic Na($+$)-HCO3(-) cotransporter NBCe1-B. *FEBS Lett* 588: 672–677, 2014. doi:10.1016/j.febslet.2013.12.036.

67. **Perna AF**, **Ingrosso D**, **Zappia V**, **Galletti P**, **Capasso G**, **De Santo NG.** Enzymatic methyl esterification of erythrocyte membrane proteins is impaired in chronic renal failure. Evidence for high levels of the natural inhibitor S-adenosylhomocysteine. *J Clin Invest* 91: 2497–2503, 1993. doi:10.1172/jci116485.

68. **Bhatia M**, **Thakur J**, **Suyal S**, **Oniel R**, **Chakraborty R**, **Pradhan S**, **Sharma M**, **Sengupta S**, **Laxman S**, **Masakapalli SK**, **Bachhawat AK.** Allosteric inhibition of MTHFR prevents futile SAM cycling and maintains nucleotide pools in one-carbon metabolism. *J Biol Chem* 295: 16037–16057, 2020. doi:10.1074/jbc.RA120.015129.

69. **Zuhra K**, **Augsburger F**, **Majtan T**, **Szabo C.** Cystathionine-β-synthase: molecular regulation and pharmacological inhibition. *Biomolecules* 10: 697, 2020. doi:10.3390/biom10050697.

70. **Antonaros F**, **Olivucci G**, **Cicchini E**, **Ramacieri G**, **Pelleri MC**, **Vitale L**, **Strippoli P**, **Locatelli C**, **Cocchi G**, **Piovesan A**, **Caracausi M.** MTHFR C677T polymorphism analysis: a simple, effective restriction enzyme-based method improving previous protocols. *Mol Genet Genomic Med* 7: e628, 2019. doi:10.1002/mgg3.628.