Clinica Chimica Acta 581 (2026) 120747

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

One-carbon pathway gene expression analyses in blood samples of subjects with trisomy 21

Beatrice Vione [a], Alessandro Maria Gaudesi [a], Francesca Antonaros [a,*], Michela Cicilloni [a], Lorenza Vitale [a], Allison Piovesan [a], Maria Chiara Pelleri [a], Pierluigi Strippoli [a], Giacomo Sperti [b], Giuseppe Ramacieri [c,d], Francesca Catapano [c], Pietro Paradisi [e], Gian Luca Pirazzoli [f], Luigi Tommaso Corvaglia [g], Chiara Locatelli [b,1], Maria Caracausi [a,1]

[a] Unit of Cellular and Experimental Biology, Department of Biomedical and Neuromotor Sciences (DIBINEM), University of Bologna, 40126 Bologna, Italy
[b] Neonatal Intensive Care Unit, IRCCS Azienda Ospedaliero Universitaria di Bologna, 40138 Bologna, Italy
[c] Department of Medical and Surgical Sciences (DIMEC), University of Bologna, 40138 Bologna, Italy
[d] Speciality School of Child Neuropsychiatry - Alma Mater Studiorum, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy
[e] Speciality School of Paediatrics - Alma Mater Studiorum, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy
[f] Medical Department, Maggiore Hospital, Largo Nigrisoli 2, 40133 Bologna, Italy
[g] Neonatal Intensive Care Unit, Department of Medical and Surgical Sciences (DIMEC), IRCCS Azienda Ospedaliero Universitaria di Bologna, University of Bologna, 40138 Bologna, Italy

ARTICLE INFO

Keywords:
Blood
Gene expression
Metabolism
One-carbon pathway
Trisomy 21

ABSTRACT

Trisomy 21 (T21) is the genetic cause of Down syndrome (DS), and the presence of extra genetic material causes altered expression of genes located on chromosome 21 (Hsa21) and others, with effects as altered levels of metabolic reaction products. The one-carbon pathway plays a central role in correct human neurodevelopment and was found to be altered in DS and neurological impairments of different entities. In this work, the expression of 42 genes involved in the one-carbon cycle was analyzed in blood samples from 10 subjects with T21 and 10 euploid (N) subjects. Additionally, plasmatic concentration of methylcobalamin (MeCbl) was evaluated in 10 subjects with T21 and 7 N subjects.

The results showed that 13 genes out of 42 were differentially expressed: 11 were over-expressed (*ABCC3*, *ABCC4*, *ARMT1*, *CTH*, *FOLR2*, *GART*, *ICMT*, *PRMT2*, *SETD4*, *SLC19A1,* and *NSD2*) and 2 were under-expressed (*NSUN3* and *TRMT112*). Among these, 4 over-expressed genes are located on Hsa21 (*GART*, *PRMT2*, *SETD4*, and *SLC19A1*). Statistical analyses revealed significant correlations between gene expression data, highlighting interconnections among genes. Finally, MeCbl shows a slight statistically significant reduction in the T21 group.

In conclusion, the presence of three copies of Hsa21 leads to the dysregulation of gene expression associated with the one-carbon cycle. This dysregulation affects genes located on both Hsa21 and other chromosomes resulting in metabolic alterations. Additionally, new gene interconnections were discovered that have not been previously reported in the literature.

1. Introduction

Trisomy 21 (T21) is the genetic cause of Down syndrome (DS) and represents the most frequent aneuploidy occurring in live human births (OMIM190685) and the most common genetic cause of intellectual disability (ID) [1–3]. The association of DS with the presence of an extra

* Corresponding author at: Unit of Cellular and Experimental Biology, Department of Biomedical and Neuromotor Sciences (DIBINEM), University of Bologna, via Belmeloro 8, 40126 Bologna, Italy.
E-mail addresses: beatrice.vione2@unibo.it (B. Vione), alessandro.gaudesi@studio.unibo.it (A.M. Gaudesi), francesca.antonaros2@unibo.it (F. Antonaros), michela.cicilloni2@unibo.it (M. Cicilloni), lorenza.vitale@unibo.it (L. Vitale), allison.piovesan2@unibo.it (A. Piovesan), mariachiara.pelleri2@unibo.it (M.C. Pelleri), pierluigi.strippoli@unibo.it (P. Strippoli), giacomo.sperti@studio.unibo.it (G. Sperti), giuseppe.ramacieri2@unibo.it (G. Ramacieri), francesca.catapano3@unibo.it (F. Catapano), pietro.paradisi2@unibo.it (P. Paradisi), gianluca.pirazzoli@ausl.bologna.it (G.L. Pirazzoli), luigi.corvaglia@unibo.it (L.T. Corvaglia), chiara.locatelli@aosp.bo.it (C. Locatelli), maria.caracausi2@unibo.it (M. Caracausi).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.cca.2025.120747
Received 17 July 2025; Received in revised form 14 November 2025; Accepted 26 November 2025
Available online 27 November 2025

copy of chromosome 21 (Hsa21) was discovered in 1959 by Jérôme Lejeune and coll. [4].

T21 causes the presence in three copies of more than 400 genes located on Hsa21, of which 228 genes are known protein-coding genes [5]. Based on the most straightforward model of gene expression in T21, genes on Hsa21 should be over-expressed with a 3:2 ratio (T21/euploid condition) but there is evidence of under-expressed Hsa21 genes and alterations of genes located on chromosomes other than Hsa21 [6]. It is well known that this global state of dysregulation leads to the DS phenotype, but the association between specific genetic determinants and clinical characteristics that can be manifested is still poorly understood [6–9]. Differential gene expression analyses have been extensively used to determine the expression levels of Hsa21 genes and of the whole genome in T21 tissues and cell cultures compared with euploid condition and to investigate their involvement in the molecular mechanisms that may be related to the pathogenesis of DS [6,10–12]. These studies often show contrasting results, probably due to different tissue specificities, developmental stages, experimental conditions, platforms and statistical techniques [6,13,14].

Therefore, the presence of extra genetic material in T21, causing altered expression of genes located on Hsa21 and other chromosomes, leads to downstream effects such as altered levels of proteins and metabolites [15–18].

In individuals with DS, a discriminant metabolic profile detected by untargeted NMR was demonstrated and mitochondrial metabolism resulted particularly altered [15,16]. Among the altered molecules, formate was revealed one of the one-carbon donors which metabolically connects mitochondrial and cytoplasmatic one-carbon metabolism. Indeed, one-carbon pathway reactions are interconnected and compartmentalized in the cytoplasm, in the mitochondria, and in the nucleus [19]. Bringing these results together with the scientific thoughts of Lejeune [20] about the alteration of one-carbon pathway in DS, successive metabolism analyses were conducted by enzyme-linked immunosorbent assays (ELISA) and showed the significant alteration of several one-carbon pathway molecules not detectable by untargeted NMR method [18]. This pathway is crucial as it consists of interlinked biochemical processes driven by the cycles of folate and homocysteine-methionine. Its central role is the transfer of one-carbon groups from carbon donors as serine to carbon acceptors as nucleotides or methyl groups through the passage for folate intermediates. One-carbon cycle results involved in several processes, including DNA replication and repair through purine and thymidylate generation, amino acid homeostasis, redox balance, and epigenetic regulation through homocysteine-methionine cycle [21–23]. It supports cell growth and critical cellular functions such as mitochondrial respiration and is fundamental for rapidly proliferating cells [24].

For these reasons, it plays a central role in correct human neurodevelopment and if impaired like in cerebral folate deficiencies (CFD), various neurological impairments may occur, depending on the specific metabolic paths involved [25]. One-carbon pathway is finely regulated by gene expression and particularly relevant downstream mechanisms, such as substrate/product availability. At the same time, this metabolism can itself impact cellular transcription, due to the synthesis of the universal methyl donor S-Adenosyl-methionine (SAM) that through methyltransferase (MT) enzymes, transfers its methyl group to many different substrates, including DNA molecules or histone proteins that are fundamental for epigenetic regulation of gene expression [24,26].

The dysregulation of the one-carbon pathway in T21 was revealed by several authors [27–32]. Recently, we analyzed plasma samples of DS subjects compared with euploid controls (N) and we identified a decreased level of tetrahydrofolate (THF) and an increased level of SAM and S-Adenosyl-homocysteine (SAH) with a decreased SAM/SAH ratio in DS [18,33].

Regarding gene expression dysregulation of the one-carbon pathway in DS, it is known that several genes involved in this metabolism are located on Hsa21, as cystathionine β-synthase (*CBS*), phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase (*GART*) and solute carrier family 19 member 1 (*SLC19A1*), of which an over-expression in specific tissues has been demonstrated [12,34–36].

This work aimed to investigate the imbalance of one-carbon pathway genes in T21 and the possible relationship between gene expression data and metabolite concentrations in T21.

2. Experimental procedures

2.1. Ethics statement

The Independent Ethics Committee of the Hospital - University of Bologna Policlinico S. Orsola-Malpighi Italy has granted the ethical approval for this study (number: 39/2013/U/Tess). We obtained an informed written consent from all participants to collect blood samples and clinical data in the context of the yearly routine follow-up provided for subjects with T21. Concerning minors, the consent was collected from his/her parents. All procedures were carried out in accordance with the Ethical Principles for Medical Research Involving Human Subjects of the Helsinki Declaration.

2.2. Blood processing and RNA extraction

Blood samples from T21 and N subjects were collected in ethylenediaminetetraacetic acid (EDTA)-coated blood collection tubes, kept at room temperature, and treated within two hours from blood draw. A volume of 1–1.5 mL of blood was centrifuged at 1200*g* for 10 min, plasma fraction was removed, 5 mL of denaturing solution [37] was added to the remaining blood fraction (buffy coat and red blood cells), and then stored at −20 °C until RNA extraction. The plasma samples were again centrifuged at 800*g* for 30 min and the supernatants were isolated in aliquots and stored at −80 °C.

Total RNA extraction from blood deprived of plasma was performed with the method of Chomczynski and Sacchi [37] and the RNA quantity and integrity were verified through RNA ScreenTape Assay for TapeStation Systems (Agilent 4150/4200 Santa Clara, CA, USA).

2.3. Selection of RNA and plasma samples

We selected RNA samples extracted from blood deprived of plasma from 20 Caucasian subjects (10 subjects with DS and 10 N subjects) with similar age range between the T21 and N groups, with sufficient RNA concentration values to perform all the reactions and an RNA integrity equivalent (RINe) > 6.5 (Supplementary Dataset 1 A).

Regarding plasma samples, we selected 17 Caucasian samples (10 from subjects with DS and 7 from N subjects) treated within 2 h from blood draw, not contaminated from erythrocytes and a volume sufficient to perform the analyses (Supplementary Dataset 1B). The subjects from which plasma samples were isolated are different from the subjects from which RNA blood samples were extracted, except for one DS and one N subject (Supplementary Dataset 1).

For both RNA and plasma samples, we preferentially selected biological samples from the same subjects studied in our previous work [18].

Control samples were selected among siblings of subjects with DS and without evidence of abnormal karyotype.

2.4. Selection of one-carbon genes

To obtain the list of all genes involved in one-carbon pathway, we performed searches in two databases: KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway (https://www.genome.jp/kegg/), and NCBI (National Center for Biotechnology Information) PubMed (https://pubmed.ncbi.nlm.nih.gov/). From KEGG pathway database we

extracted the genes encoding for proteins of the following gene categories: "one-carbon pool by folate" genes (hsa00670); "cysteine and methionine metabolism" genes (hsa00270), of which we selected "Methionine degradation" (M00035) and "Cysteine biosynthesis" (M00338); "antifolate resistance" genes (hsa01523). We also searched the term "Methyltransferase" on "KEGG GENES" in order to have a list of all the known MTs; the term "carrier" selecting for carrier of one-carbon pathway metabolites; the term "folate receptor". We also searched all the pathways in which the following metabolites are involved on "KEGG COMPOUND" database: folic acid (FA); dihydrofolic acid; THF; 10-formyl-THF; 5,10-methenyl-THF; 5-formyl-THF (5-f-THF); 5-methyl-THF (5-m-THF); methionine; cysteine; homocysteine (Hcy); SAM; cystathionine; SAH; 5,10-formimidoyl-THF. To be sure to have found all the one-carbon pathway genes, we conducted the following search on NCBI PubMed: methyltransferase [MH] AND metabolism [MH] AND "methyl donor", filtering for Humans, Review and English language; metabolism [MH] AND carrier AND one-carbon, filtering for Humans, Review and English language; metabolism [MH] AND receptor AND one-carbon, filtering for Humans, Review and English language. The function of all MT enzymes thus obtained was analyzed using NCBI PubChem (https://pubchem.ncbi.nlm.nih.gov/) in order to eliminate MTs not dependent on SAM but involved in other metabolic pathways. In addition, we eliminated genes of uncertain function (gene symbol beginning with "LOC" prefix).

Then, using the T21 *vs* N human blood cell transcriptome map obtained by RNA-SEq. [12], we selected one-carbon pathway genes to be analyzed by real time RT-PCR using the following criteria: number of samples from which the expression datum derives $\geq$50 % of total; standard deviation (SD) $<$ 100 % of gene expression values in T21 or N group, this means that the intersample variability of the expression value of each gene is less than 100 % of the mean value; reads per kilobase of transcript per million mapped reads (RPKM) $>$ 1 of gene expression values in both T21 and N group, to handle read mapping uncertainty [38]; gene expression ratio (ER) $\leq$ 0.76 and $\geq$ 1.30 refer to a previous careful analysis of differentially expressed genes (DEGs) between different types of trisomy 21 and normal tissues in which was considered the biological significance of the gene expression ratios near to 3:2 (1.5) or 2:3 (0.67) due to the stimulatory or inhibitory effects, respectively, of the extra copy of Hsa21. Moreover, in order to account for natural variation in gene expression, threshold values have been extended to 1.3 or 0.76, respectively [11].

2.5. DNase treatment and real time RT-PCR

After RNA extraction, we used a DNase treatment (Invitrogen by Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove possible contaminating genomic DNA (gDNA) and we quantified RNA again using Nanodrop spectrophotometer (NanoDrop™ OneC Microvolume UV–Vis Spectrophotometer, Thermo Fisher Scientific). Then we proceeded to reverse transcription (RT) (1–2 μg of RNA) using SuperScript™ III (Thermo Fisher Scientific).

We performed real time RT-PCR to ensure that RT was properly done using exon-exon primers and that complementary DNA (cDNA) was not contaminated by gDNA using intron-spanning primers (Supplementary Table 1). Sybr Select Master Mix 2× for CFX (Thermo Fisher Scientific) in a total volume of 20 μL was used according to the manufacturer's instructions. Real time RT-PCR was performed in duplicate for each sample using 10 ng per sample, by CFX96 instrument (Bio-Rad Laboratories, Hercules, CA, USA). A duplicate, negative reaction was included for each target.

For the gene expression study, we used 96 PCR custom well plates having primer pairs for the selected genes lyophilized in the wells (one plate for each cDNA sample) with PrimePCR SYBR Green Assay (Bio-Rad Laboratories) and we followed the manufacturer's instructions. Real time RT-PCR was performed in technical duplicate for each sample using 10 ng per sample in a total volume of 20 μL, by CFX96 instrument (Bio-Rad Laboratories). A duplicate, negative reaction was included for each target.

To identify the best possible reference housekeeping genes, we performed a search on NCBI Pubmed using "HKG AND RT-qPCR" in the search bar and a list of possible housekeeping genes was compiled [39–41]. Then we selected the best housekeeping genes based on gene ER of T21 *vs* N in the range of 0.9–1.2, SD as low as possible and RPKM $>$ 1 [12].

For the gene expression analysis, we used the Livak method of $2^{-\Delta\Delta Ct}$ [42].

2.6. OMIM database analysis

For each gene included in the analysis, we performed a search using the gene symbol in OMIM (Online Mendelian Inheritance in Man) database. Then, we clicked on "Phenotype MIM number" and reported the genetic diseases and phenotypes associated for each gene paying attention to neurological and hematological phenotypic features.

2.7. ELISA assay

We evaluated the quantitative measurement of methylcobalamin (MeCbl) plasma concentrations using a specific ELISA kit (BlueGene, Shanghai, China) based on a competitive enzyme technique. The specifications were: sensitivity, 0.1 ng/mL; intra-assay precision, Coefficient of Variation (CV) $<$ 10 %; inter-assay precision, CV $<$ 12 %; no significant cross-reactivity or interference between MeCbl and analogues was observed. Other metabolite concentrations were retrieved from the work of Vione and coll. [18]. The %CV was calculated dividing the standard deviation of the two measurements for the mean of the two measurements and multiplying by 100.

2.8. Statistical analyses

All statistical analyses were conducted using IBM SPSS Statistics software (Version 25 for Mac OS X). A two-tailed p-value $<$ 0.05 was considered statistically significant. Pearson's correlation coefficient (r) was used to assess the linear relationship between the two continuous variables. Correlations were interpreted as weak ($r < 0.4$), moderate ($0.4 \leq r < 0.7$), or strong ($r \geq 0.7$), in accordance with standard conventions.

To determine if there was any difference between the DS and N groups for age or sex, we performed a Mann-Whitney U test and Fisher's test, respectively.

Outlier detection was performed on ΔCt values (prior to expression ratio calculations) and on molecule concentrations using the interquartile range (IQR) method. Specifically, values below Q1 – 1.5 × IQR or above Q3 + 1.5 × IQR were flagged as outliers. Only strong outliers were removed from subsequent analyses to minimize data loss and preserve statistical power.

For gene expression data, we assessed the agreement between real time RT-PCR and RNA-Seq expression ratios using bivariate correlation and a Bland-Altman plot. To create the Bland-Altman plot, the data obtained by the two measurements were imported into SPSS in column format (one variable for each method). For each pair of values, the mean (X-axis) and the difference between the two values (Y-axis) were calculated. Subsequently, a scatter plot was created to represent the differences as a function of the means. The bias line (mean of the differences) and the limits of agreement (± 1.96 * SD of the mean of the differences) were added to the graph.

The normality of variable distributions was assessed using the Kolmogorov-Smirnov test. For variables normally distributed, bivariate Pearson correlation was used to examine associations with age, and independent sample t-tests were used to test differences between sex or fasting groups. Equality of variances for the t-test was verified using Levene's test. For non-normally distributed variables, non-parametric

tests (Mann-Whitney *U* test or Kruskal-Wallis test) were applied as appropriate.

To assess the relationships among all study variables, we performed pairwise linear correlation analyses. For variables significantly associated with chronological age, partial correlation analyses were used to control for the effects of age. Similarly, for variables influenced by sex or fasting state, we performed correlation analyses while controlling for these covariates in order to reduce potential confounding.

To control for multiple hypothesis testing, *p*-values obtained from the association analyses were adjusted using the Benjamini-Hochberg false discovery rate (FDR) procedure. All raw p-values were first arranged in ascending order and assigned a rank i, where i ranges from 1 to N, and N is the total number of tests. For each ranked p-value, an intermediate value was calculated as (pi × N) / i. To ensure a non-decreasing sequence of adjusted values, the minimum of each intermediate value and all larger-ranked values was taken to obtain the final FDR-adjusted q-values. Calculations were performed in Microsoft Excel by sorting p-values in ascending order, assigning ranks, computing the Benjamini-Hochberg term, and applying a cumulative minimum function from the largest to the smallest rank. Genes with q-values < 0.05 were considered statistically significant after FDR correction.

Finally, MeCbl concentration levels were compared between the T21 and N groups using an independent samples *t*-test.

3. Results

3.1. Selection of RNA and plasma samples

Regarding RNA samples used to perform the gene expression study, a total of 20 Caucasian subjects were selected: 10 subjects with DS with a mean age of 13.3 years old, including 7 males and 3 females and 10 N subjects with a mean age of 11.3 years old, including 9 males and 1 female, who were selected among the siblings of subjects with DS enrolled in the study (Supplementary Dataset 1 A). Eighteen out of 20 blood samples had corresponding metabolic data related to one-carbon pathway. These data have been integrated into the present study.

Regarding plasma samples used to assay MeCbl concentration, a total of 17 Caucasian subjects were selected: 10 subjects with DS with a mean age of 10.7 years old, including 5 males and 5 females and 7 N subjects with a mean age of 20.7 years old, including 2 males and 5 females, who were selected among the siblings of subjects with DS enrolled in the study (Supplementary Dataset 1B). Twelve out of 17 blood samples had corresponding metabolic data related to one-carbon pathway [18]. These data have been integrated into the present study.

We obtained both RNA and plasma samples from only 2 subjects.

3.2. Selection of one-carbon genes

The analysis on “KEGG PATHWAY” database integrated with NCBI PubMed research results allowed us to extrapolate a list of 277 genes involved in one-carbon pathway. Using NCBI PubChem, 7 genes referring to MTs not dependent on SAM but involved in other metabolic pathways were eliminated. In addition, we eliminated 10 genes of uncertain function (with “LOC” prefix). Applying the following selection criteria, we obtained a list of 260 genes (Supplementary Dataset 2 A).

The genes of our interest were further selected based on the T21 *vs* N human blood cell transcriptome map [12]. In particular, we excluded 28 genes because their gene expression was not detectable in blood cells; 18 genes because their expression values derived from a number of samples < 50 % of the total in either T21 or N group; 3 genes because their expression values had SD > 100 %; 24 genes because their expression values had RPKM < 1; 135 genes with T21/N ERs > 0.76 and < 1.30; 10 genes because they had only predicted functions, or custom primers were not available, or their function was biochemically far from the KEGG pathway in which the metabolites investigated are involved [18].

In total, we have selected 42 genes (Supplementary Table 2). Among these, 6 genes were under-expressed in T21 samples (ER ≤ 0.76) and 36 were over-expressed (ER ≥ 1.3) [12].

3.3. Real time RT-PCR

No abnormal amplifications were showed in real time RT-PCR reactions using intron spanning primers to exclude contamination by gDNA and normal detectable amplifications were obtained using exon-exon primers to confirm RT (Supplementary Table 1).

For our analyses, 3 genes (*HDAC1*, *PPIA*, *YWHAZ*) were selected as reference genes among the 7 genes resulting from the NCBI PubMed search (Supplementary Dataset 2B).

Strong outliers in ΔCq values were searched for using SPSS software and 7 of them (4 among ΔCq in the T21 group and 3 among ΔCq in the N group) were found and excluded from further analyses (reported in red in Supplementary Table 3).

Using real time RT-PCR, among the 42 selected genes involved in one-carbon pathway, we identified 13 differentially expressed genes (DEGs). In particular, 11 genes showed an ER ≥ 1.3 (*ABCC3*, *ABCC4*, *ARMT1*, *CTH*, *FOLR2*, *GART*, *ICMT*, *PRMT2*, *SETD4*, *SLC19A1*, *NSD2*), 2 genes showed an ER ≤ 0.76 (*NSUN3* and *TRMT112*) and the remaining showed an ER < 1.3 and > 0.76.

Comparing the T21 *vs* N human blood cell transcriptome map data obtained by RNA-Seq (Supplementary Table 2) [12] with the data obtained here by real time RT-PCR, we found that 11 out of 42 genes maintain the same ER pattern in both analyses, of which 10 resulted over-expressed (*ABCC3*, *ABCC4*, *ARMT1*, *CTH*, *FOLR2*, *GART*, *ICMT*, *PRMT2*, *SETD4*, *SLC19A1*) and 1 under-expressed (*NSUN3*). Among them, there are 4 genes which encode for transporter proteins (*ABCC3*, *ABCC4*, *FOLR2*, *SLC19A1*), 2 genes which encode for enzymes involved in the production/utilization of one-carbon pathway metabolites (*CTH*, *GART*) and 5 genes which encode for methyltransferase enzymes (*ARMT1*, *ICMT*, *NSUN3*, *PRMT2*) or aid methyltransferase activity (*SETD4*) (Supplementary Tables 2 and 3, Fig. 1). The genes located on Hsa21 (*GART*, *PRMT2*, *SETD4* and *SLC19A1*) maintain an ER ≥ 1.3 in both analyses (real time RT-PCR and RNA-Seq).

Twenty-nine genes resulting with an altered ER in the RNA-Seq transcriptome map showed a normal ER (>0.76 and < 1.30) in real time RT-PCR. Finally, 1 gene over-expressed in the transcriptome map resulted under-expressed in real time RT-PCR (*TRMT112*) and, on the contrary, one gene under-expressed in the RNA-Seq transcriptome map resulted over-expressed in the real time RT-PCR (*NSD2*); both of them encode for methyltransferase enzymes (Supplementary Tables 2 and 3, Fig. 1).

3.4. OMIM database analysis

Among the 42 genes analyzed in this study, 9 genes resulted associated with genetic mendelian disorders (Supplementary Table 4), 4 of which resulted altered from real time RT-PCR (*CTH*, *NSD2*, *NSUN3*, *SLC19A1*) (Supplementary Table 3, Fig. 1). *CTH*, *NSUN3* and *SLC19A1* are associated to an autosomal recessive inheritance, *NSD2* to an autosomal dominant inheritance.

The 9 genes found in OMIM resulted associated with neurological symptoms and in particular, for 8 of them the central nervous system is involved, while for the last, the peripheral nervous system is involved. Developmental delay, motor impairment and mental retardation of varying degrees are very common phenotypic characteristics. In addition, 5 out of 9 genes also manifest hematological phenotypes, 2 of which resulted altered by real time RT-PCR (*NSUN3*, *SLC19A1*). Different kinds of anemia are a common phenotypic characteristic.

For all of them, the mutation causes a loss of function of the encoded proteins.

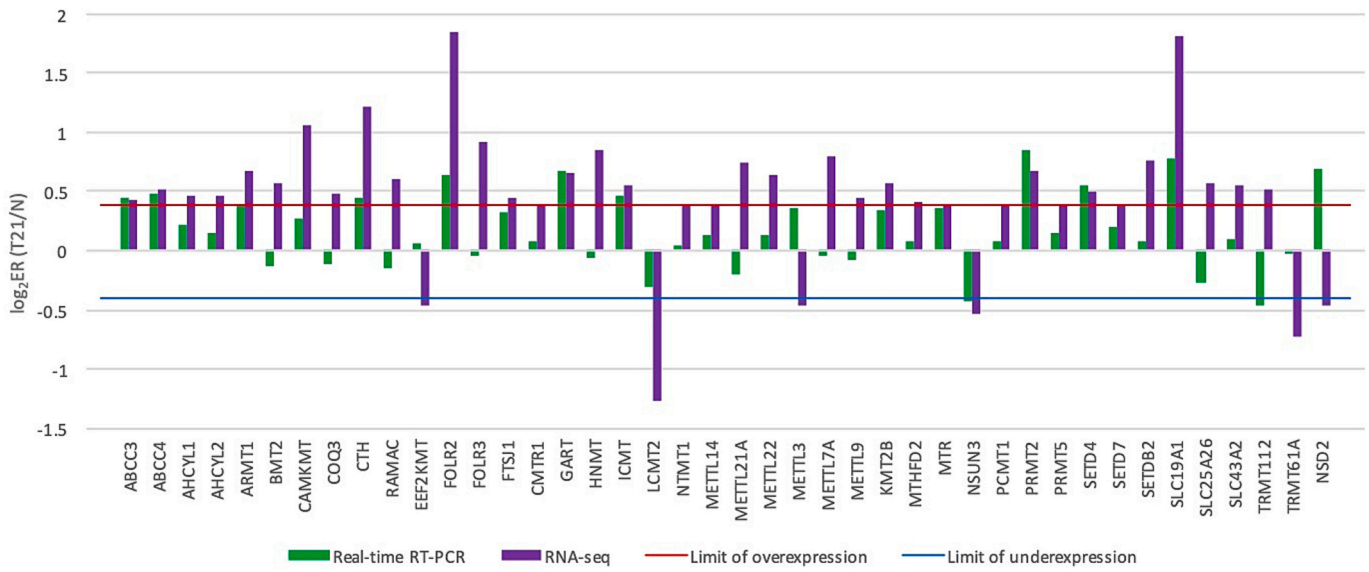

Fig. 1. Comparison between $\log_2$ T21/N expression ratio (ER) of real time RT-PCR results (in green) and RNA sequencing (RNA-Seq) results (in violet). The red line represents the ER threshold above which the genes are considered over-expressed. The blue line represents the ER threshold below which the genes are considered under-expressed. The real-time RT-PCR was conducted on cDNA from 10 trisomic and 10 euploid samples, and technical replicates were performed for each biological sample (see Supplementary Table 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. ELISA assay

We obtained the MeCbl plasma concentration level in 10 subjects with T21 and in 7 N subjects.

To assess the intra-replicate variability of the assays in duplicate, the coefficient of variation of the optical density (O.D.) values expressed as percentage (%CV) was reported for each sample in Supplementary Tables 5. The mean of the %CV values also named intra-assay CV was 8.67 %. To obtain a standard curve in order to quantify the MeCbl plasma levels, the coefficient R^2 value was determined for polynomial of grade 2 (quadratic) curve corresponding to 0.90 (Supplementary Table 5).

3.6. Statistical analyses

The 10 subjects with DS and 10 N subjects selected for the expression analysis resulted comparable in age (Mann-Whitney *U* test: $p = 0.405$, U = 61.5) and sex (Fisher's test: $p = 0.582$). The 10 subjects with DS and 7 N subjects selected for the MeCbl analysis resulted to not be comparable in age (Mann-Whitney U test: $p = 0.0136$, U = 10.0) but comparable in sex (Fisher's test: $p = 0.622$).

Concordance analysis between real-time RT-PCR and RNA-Seq data revealed an average bias of −0.3321, with concordance limits ranging from −1.40 to +0.74, indicating a systematic tendency for RNA-Seq to overestimate gene expression levels compared to real time RT-PCR. Spearman's correlation ($r = 0.410$, $p = 0.007$) suggests moderate concordance in the relative classification of expression levels between the two methods. However, the width of the concordance limits and the presence of 3 outliers (*FOLR2*, *SLC19A1*, and *NSD2*) suggest non-negligible variability, making the methods not fully interchangeable in absolute quantitative terms (Supplementary Fig. 1). We found *FOLR2* and *SLC19A1* both presenting an over-expression (ER ≥ 1.3) in both types of data, but with higher levels in RNA-Seq one (*FOLR1*: real time RT-PCR ER = 1.56 and RNA-Seq ER = 3.60; *SLC19A1* real time RT-PCR ER = 1.73 and RNA-Seq ER = 3.50), while *NSD2* resulted over-expressed in the real time RT-PCR, but under-expressed in the RNA-Seq data (real time RT-PCR ER = 1.62 and RNA-Seq ER = 0.72).

Starting from 42 gene expression data (Supplementary Table 3) and 6 associated metabolic data (THF, 5-mTHF, 5-f-THF, SAH, FA, vitamin B12; Supplementary Dataset 1 A [18]), 2 strong outliers were found among metabolic data: one for FA (ng/mL) and one for vitamin B12 (or cobalamin, Cbl) (pg/mL) (in red in Supplementary Dataset 1 A). They were excluded from the following analyses. The results of descriptive analyses without strong outliers are shown in Table 1.

The Kolmogorov-Smirnov test highlighted the presence of 4 variables whose distribution was significantly different from normal: *FOLR3* and *MTHFD2* ERs ($p = 0.002$ and $p = 0.02$ respectively) among gene expression data and SAH and FA concentration levels ($p = 0.018$ and $p = 0.013$) among metabolite levels data thus for the following two sample *t*-test we used non-parametric tests (Mann-Whitney or Kruskal-Wallis).

From the bivariate correlation between all variables and age, ERs of *ABCC4* ($p = 0.034$ and $r = -0.669$), *COQ3* ($p = 0.049$ and $r = 0.635$), *EEF2KMT* ($p = 0.015$ and $r = 0.737$), *FTSJ* ($p < 0.001$ and $r = 0.875$) and *SLC25A26* ($p = 0.015$ and $r = 0.738$) resulted significantly correlated (Supplementary Table 6A). From the two sample *t*-test, ER of *METTL7A* ($p = 0.017$) and *NSD2* ($p = 0.004$) resulted different for fasting/non-fasting state groups (Supplementary Table 6B e 6C), while ERs of *ARMT1* ($p = 0.009$), *FOLR2* ($p = 0.043$), *METTL22* ($p = 0.013$), *METTL7A* ($p = 0.0024$) and *PCMT1* ($p = 0.005$) resulted different for male/female groups (Supplementary Table 6D e 6E).

Finally, we performed the bivariate correlation between all 48 variables (Supplementary Table 7). For age-related variables we performed a partial correlation (Supplementary Table 7B), for fasting and sex-related variables we performed bivariate correlations separating the two groups (fasting/non-fasting and male/female; Supplementary Table 7C and 7D respectively).

Application of the Benjamini–Hochberg FDR procedure to the unified dataset of *p*-values identified a small subset of gene ERs that remained significant after correction for multiple testing (Supplementary Table 8). Using a q-value threshold of 0.05, seven genes (*AHCYL2*, *LCMT2*, *METTL3*, *MTR*, *SETD4*, *SETDB2*, and *TRMT61A*) showed at least one comparison with an FDR-adjusted q-value below the significance cutoff (Table 2).

Regarding MeCbl concentration data (Supplementary Table 5) and associated metabolic data (THF, 5-m-THF, 5-f-THF, SAH, SAM, FA, Cbl, Hcy; Supplementary Dataset 1B [18]), a preliminary analysis detected 2 outliers in the set of Cbl values (T21 group) that were excluded from further elaborations. The Kolmogorov-Smirnov test highlighted the presence of 2 variables whose distribution was significantly different

Table 1
Descriptive analyses of all variables using SPSS Statistics. Variables related to subjects with trisomy 21 whose blood samples were used to investigate the expression ratios of the selected one-carbon genes. n = number of samples; min = minimum value; max = maximum value; SD = standard deviation. THF = tetrahydrofolate, 5-m-THF = 5- methyl-tetrahydrofolate, 5-f-THF = 5-formyl-tetrahydrofolate, SAH=S-Adenosyl-homocysteine, FA = folic acid, Cbl = cobalamin or vitamin B12. Data on THF, 5-m-THF, 5-f-THF, SAH, FA, Cbl come from [18].

Variables	n	min	max	mean	SD
ABCC3	10	0.63	2.64	1.35	0.64
ABCC4	10	0.65	1.97	1.39	0.47
AHCYL1	10	0.83	1.47	1.17	0.21
AHCYL2	10	0.68	1.45	1.10	0.21
ARMT1	9	1.06	1.78	1.31	0.25
BMT2	10	0.63	1.42	0.91	0.23
CAMKMT	9	0.76	1.67	1.21	0.27
COQ3	10	0.47	1.39	0.92	0.29
CTH	10	0.70	2.56	1.36	0.51
RAMAC	10	0.69	1.06	0.90	0.12
EEF2KMT	10	0.21	1.69	1.04	0.45
FOLR2	10	0.86	1.91	1.56	0.41
FOLR3	10	0.26	3.53	0.97	1.10
FTSJ1	10	0.66	1.85	1.25	0.38
CMTR1	10	0.55	1.42	1.06	0.24
GART	10	1.02	1.97	1.60	0.29
HNMT	10	0.50	1.89	0.96	0.38
ICMT	10	0.69	1.89	1.38	0.36
LCMT2	10	0.34	1.17	0.80	0.25
NTMT1	10	0.66	1.45	1.03	0.23
METTL14	10	0.63	1.57	1.09	0.27
METTL21A	10	0.77	1.10	0.87	0.10
METTL22	10	0.88	1.38	1.09	0.17
METTL3	10	0.82	1.88	1.28	0.41
METTL7A	10	0.66	1.61	0.97	0.29
METTL9	9	0.72	1.14	0.95	0.16
KMT2D	10	0.76	1.82	1.26	0.30
MTHFD2	10	0.86	1.90	1.06	0.31
MTR	10	0.51	1.87	1.28	0.37
NSUN3	10	0.37	1.57	0.74	0.35
PCMT1	9	0.94	1.27	1.06	0.11
PRMT2	10	1.32	2.36	1.81	0.33
PRMT5	10	0.50	1.93	1.10	0.39
SETD4	10	0.74	2.41	1.47	0.47
SETD7	10	0.74	1.38	1.15	0.20
SETDB2	10	0.57	1.45	1.06	0.28
SLC19A1	10	1.00	2.80	1.73	0.60
SLC25A26	10	0.39	1.37	0.83	0.32
SLC43A2	10	0.66	1.74	1.07	0.29
TRMT112	10	0.45	0.96	0.73	0.15
TRMT61A	10	0.43	1.53	0.98	0.34
NSD2	10	0.96	2.52	1.62	0.52
THF (ng/mL)	10	3.82	61.94	34.51	17.43
5-m-THF (ng/mL)	10	41.35	65.37	52.36	7.91
5-f-THF (pg/mL)	9	128.34	259.46	169.50	47.92
SAH (ng/mL)	10	3.69	16.19	8.88	4.71
FA (ng/mL)	7	4.50	7.70	6.43	1.14
Cbl (pg/mL)	9	143.00	407.00	277.44	89.22

than normal: Cbl in T21 group and THF in N group, while due to paucity of measures the normality test failed for several molecules (THF, 5-f-THF, SAH, SAM, Hcy in the T21 group and 5-m-THF, 5-f-THF, SAH, SAM, FA, Cbl, Hcy for N group) and they were thus excluded from the analysis. The retained data was first studied by descriptive statistical analysis (Table 3).

Two sample *t*-test showed significant difference between MeCbl concentrations in T21 and N groups ($p = 0.044$) with a mean and median ratio T21/N = 0.957. All the variables have also been tested for any relationship with sex and fasting, through two sample *t*-test or Mann-Whitney test, and also age through bivariate correlation: the only significant relationships found are between FA and fasting state in the T21 group ($p = 0.011$) and between MeCbl and sex in the N group ($p = 0.047$), even though any other further analysis to normalize the results by sex or fasting state was not possible due to insufficiency of data.

Table 2
Significant p-values corrected by Benjamini-Hochberg (BH) false discovery rate (FDR) method. The *p*-values resulting from the bivariate correlations between all variables studied were corrected using the BH FDR procedure, and the q-values of those still significant (q-value <0.05) are reported in this table. Genes located on Hsa21 are reported in bold.

Variable 1	Variable 2	p-value	q-value	r
MTR	*AHCYL2*	0.000002	0.003	0.973
SETDB2	*LCMT2*	0.000045	0.020	0.942
TRMT61A	*LCMT2*	0.000057	0.020	0.939
TRMT61A	*METTL3*	0.000075	0.020	0.934
TRMT61A	***SETD4***	0.000067	0.020	0.936

Table 3
Descriptive analyses of all variables related to subjects whose blood samples were used to investigate the methylcobalamin (MeCbl) concentration levels. In 2a the variables related to T21 group are showed, in 2b the variables related to control (N) group are showed. n = number of samples, min = minimum value, max = maximum value, SD = standard deviation, 5-m-THF = 5- methyl-tetrahydrofolate, 5-f-THF = 5-formyl-tetrahydrofolate; FA = folic acid, Cbl = cobalamin or vitamin B12. Data on 5-m-THF, FA, Cbl and THF come from [18].

a

T21	n	min	max	mean	median	SD
MeCbl (ng/mL)	10	7.175	8.407	7.802	7.780	0.371
5-m-THF (ng/mL)	6	39.588	58.934	51.323	51.570	7.119
FA (ng/mL)	6	5.000	13.800	7.800	7.350	3.216
Cbl (pg/mL)	5	314.000	354.000	342.800	349.000	16.453

b

N	n	min	max	mean	median	SD
MeCbl (ng/mL)	7	7.934	8.536	8.149	8.124	0.225
THF (ng/mL)	5	35.138	105.413	77.314	102.354	37.075

MeCbl was not influenced by age in both the DS and N groups, even though there was a statistically significant difference in age between the two groups. The hypothesis that the metabolites could be correlated to MeCbl has been tested through bivariate correlation, but with no significant results.

4. Discussion

In this work, the expression of genes strictly involved in the one-carbon pathway was studied in T21 blood samples compared with euploid controls. We identified 260 genes involved in the one-carbon cycle, and we further selected 42 genes differentially expressed in a previous blood transcriptome map created with data from RNA-SEq. [12]. The gene expression study was performed on 42 genes, in 10 T21 *vs* 10 N blood samples. The number of RNA samples analyzed was determined through a rigorous selection process based on the homogeneity of the two groups in terms of age and gender, the possibility to integrate gene expression data with metabolic data derived from previous analysis of the same individual blood sample [18], and RINe and concentration values (details in the "Experimental procedures" section).

Comparing the data obtained by real time RT-PCR with the differential blood transcriptome map data [12], we found that 11 genes maintain the same ER pattern in both analyses (over-expressed or under-expressed in both analyses). The concordance analysis (Supplementary Fig. 1) indicated a systematic tendency for the RNA-Seq method to overestimate gene expression levels compared to real time RT-PCR. Spearman's correlation ($r = 0.41$, $p = 0.007$) suggests moderate concordance in the relative classification of expression levels between the two methods. However, the width of the concordance limits suggests non-negligible variability, making the methods not fully

interchangeable in absolute quantitative terms.

Focusing on DEGs located on Hsa21 (*GART*, *SLC19A1*, *PRMT2*, *SETD4*) (Table 4), there is insufficient literature regarding their involvement in the alteration of one-carbon pathway in trisomy 21. The protein encoded by the *GART* gene is a trifunctional polypeptide which is required for *de novo* purine biosynthesis, involved in the conversion of 10-formyl-THF into THF and it is widely known to be increased at expression and protein levels in T21 [34]. It was previously identified that THF plasma level is decreased in subjects with DS (T21/N median ratio = 0.66) [18], in contrast to *GART* gene over-expression data (real time RT-PCR ER = 1.60; RNA-Seq ER = 1.58). The correlation between gene expression and metabolite levels is not always directly identifiable, and a recent work did in fact demonstrate that *GART* mRNAs are regulated by ncRNA in T21 hippocampus samples [43]. This might explain the lack of correlation between *GART* expression and THF metabolite in blood. Furthermore, THF is the most interconnected folate form in the one-carbon pathway [18], thus its final concentration could not be influenced by the increased expression of *GART* gene.

The second over-expressed gene mapping on Hsa21 is *SETD4* (real time RT-PCR ER = 1.47; RNA-Seq ER = 1.42), a SAM-dependent lysine MT gene which can methylate proteins and histone proteins, for example enabling histone H4K20 MT activity. *SETD4* is predicted to be involved in several processes, including positive regulation of inflammatory response interacting with interferon signalling [44] and it is known that interferon response is activated in DS [14]. Moreover, *SETD4* seems to have a still poorly understood role in many other processes such as cell proliferation and migration and the regulation of stem cell quiescence by promoting heterochromatin formation [45] [33].

The third over-expressed Hsa21 gene is *PRMT2* (real time RT-PCR ER = 1.81; RNA-Seq ER = 1.60), a SAM-dependent MT gene encoding for an enzyme that converts SAM into SAH. PRMT2 enzyme is important in post-translational methylation of arginine residues that is involved in different cellular processes, including transcriptional regulation, RNA metabolism, DNA repair and signal transduction [33,46,47]. It is known that PRMT2 interacts with RB1 to regulate *E2F* transcriptional activity. PRMT2 repressed *E2F1* transcriptional activity in an RB1-dependent manner, delaying cell cycle progression from G1 to the S phase [47]. In the differential transcriptional map, *RB1* is over-expressed (ER = 1.40) and *E2F1* tends to be under-expressed (ER = 0.77) [12].

Finally, *SLC19A1* (or *RFC*) gene encodes the transmembrane protein which transports intracellularly reduced folates including 5-m-THF and methotrexate (MTX); thus it was hypothesized that increased *SLC19A1* expression in various body tissues of individuals with DS may result in increased intracellular MTX transport contributing to the MTX toxicity of DS subjects [48,49]. In our analyses, it results over-expressed (real time RT-PCR ER = 1.73; RNA-Seq ER = 3.50). *SLC19A1* is associated, with AR inheritance, to folate-responsive megaloblastic anemia with demyelination and immunodeficiency with occasional global developmental delay. Megaloblastic anemia displays with a decreased capacity of MTX transport activity, suggesting an impaired folate transport into hematopoietic cells. T-cells from immunodeficient patients, instead, show reduced production of various cytokines but, most interestingly, they hardly proliferate *in vitro* in conditions of FA depletion (OMIM, Table of Contents: 600424) (Supplementary Table 4).

It is interesting to note that among the differentially expressed genes identified in this study, *CTH*, *NSD2*, *NSUN3*, and *SLC19A1* are genes described in OMIM as responsible for developmental delay when carrying loss-of-function mutations, even though only *NSUN3* resulted in under-expressed. Loss-of-function mutations in both alleles of *NSUN3* are accountable for the combined oxidative phosphorylation deficiency 48 (AR disorder, https://omim.org/entry/619012), whose phenotype is also characterized by increased plasma lactate levels. High lactate levels have been documented in DS plasma samples [15], and this phenomenon demonstrates that the connection between altered mitochondrial metabolism in DS [15] and the one-carbon cycle metabolic and genetic imbalance is worth exploring.

The correlation analyses suggest the presence of an altered functional biological network in trisomy 21. A total of five correlations were identified involving seven genes, all of which are part of the homocysteine-methionine cycle (Table 2). Among these, *SETD4* is the only gene located on Hsa21. It presents an ER of 1.47, indicating overexpression compatible with a gene dosage effect (a ratio close to 1.5), and aligning with what is expected for genes on Hsa21 in the absence of transcriptional compensation [8]. The *SETD4* gene plays a role in the methylation of lysine on histone proteins, which can influence chromatin accessibility and transcriptional stability [50]. The heightened expression of *SETD4* in T21 may trigger a cascade effect on gene regulation. Indeed, we found that the ER of *SETD4* correlates with that of *TRMT61A* (ER = 0.98; q = 0.02; $r = 0.936$; Table 4), a tRNA methyltransferase, thereby influencing translation stability and efficiency [51]. In turn, *TRMT61A* correlates with the ER of *METTL3* (ER = 1.28; q = 0.02; $r = 0.934$), a mRNA N6-adenosine-methyltransferase that plays a role in controlling mRNA stability, splicing, and translation, and it was reported to influence metabolism-related diseases [52]. *TRMT61A* also correlates with the ER of *LCMT2* (ER = 0.80; q = 0.02; $r = 0.939$), a methyltransferase that catalyzes the final stage of the synthesis pathway

Table 4

Real-time RT-PCR results with statistically significant correlations. In this table only the differentially expressed genes (DEG) in real time RT-PCR are reported together with gene name, expression ratio (ER) resulting from real-time RT-PCR, ER resulting from RNA-SEq. [12] and results of bivariate analyses, taking in consideration only correlation with $q < 0.05$. MT = methyltransferase enzyme. Genes located on Hsa21 are reported in bold. Complete results are shown in Supplementary Tables 3, 7 and 8.

Gene symbol	Gene name	Function	ER real time RT-PCR	ER RNA-Seq	Correlations $q < 0.05$
ABCC3	ATP binding cassette subfamily C member 3	transporter	1.35	1.35	
ABCC4	ATP binding cassette subfamily C member 4	transporter	1.39	1.43	
ARMT1	acidic residue methyltransferase 1	MT	1.31	1.59	
CTH	cystathionine gamma-lyase	production/utilization of 1-C metabolites	1.36	2.32	
FOLR2	folate receptor beta	transporter	1.56	3.60	
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	production/ utilization of 1-C metabolites	1.60	1.58	
ICMT	isoprenylcysteine carboxyl methyltransferase	MT	1.38	1.47	
NSUN3	NOP2/Sun RNA methyltransferase 3	MT	0.74	0.69	
PRMT2	protein arginine methyltransferase 2	MT	1.81	1.60	
SETD4	SET domain containing 4	MT	1.47	1.42	***TRMT61A***: r = 0.936, q = 0.02
SLC19A1	solute carrier family 19 member 1	transporter	1.73	3.50	
TRMT112	tRNA methyltransferase activator subunit 11–2	MT	0.73	1.42	
NSD2	tRNA methyltransferase 61 A	MT	1.62	0.72	

of the hypermodified nucleoside wybutosine, which is present at position 37 of the tRNA for phenylalanine [53]. This modification is reported to influence the stability of codon-anticodon pairing indirectly and, consequently, the accuracy of protein translation [54]. In turn, *LCMT2* correlates with the ER of *SETDB2* (ER = 1.06; $q = 0.02$; $r = 0.942$), a histone methyltransferase involved in H3K9 methylation and implicated in transcriptional repression [55].

SETD4 (along with *TRMT61A*, *METTL3*, *LCMT2*, and *SETDB2*) catalyzes the conversion of SAM to SAH. It is possible that its hyperactivity could lead to increased demand for SAM and an accumulation of SAH. We previously detected that the concentration of SAH in the plasma of individuals with DS is approximately four times higher than in controls [18]. SAH is reported to be a cytotoxic molecule and an inhibitor of methyltransferase enzymatic activity [56].

The last correlation was found between the ER of *AHCYL2* and *MTR* (ER *AHCYL2* = 1.10; ER *MTR* = 1.28; $q = 0.003$; $r = 0.973$). AHCYL2 converts SAH to homocysteine [57], while MTR converts 5-methyl-THF and homocysteine to methionine [58]. These two enzymes, therefore, help regulate the SAM/SAH ratio. Although the ER of both *MTR* and *AHCYL2* resulted not correlated with *SETD4*, the increased expression of *MTR* detected may suggest an attempt to boost SAM production to maintain cellular methylation balance. Even though *MTR* has not been classified as DEG in our expression analyses, its ER is 1.28 (Supplementary Table 3), very close to the established over-expression threshold of 1.30.

No correlations were identified between gene expression and metabolite data. In fact, according to some literature evidence, it is challenging to establish a direct correlation between gene expression and metabolites in the one-carbon pathway, due to its complex regulation [24].

For the first time, the plasmatic MeCbl concentration was studied in T21 samples. Another set, different from that used for gene expression analysis, consisting of 10 T21 plasma samples and 7 euploid controls, was selected from the plasma samples already analyzed in our previous research to integrate the metabolic data previously obtained related to the one-carbon pathway [18]. MeCbl indeed plays a central role in one-carbon pathway as the active form of Cbl and co-factor of methionine synthase (MTR), which represents the coupling enzyme between the folate cycle and methionine cycle [58]. The obtained mean and median ratio of MeCbl concentration between DS and N group is 0.96 ($p = 0.044$), thus showing a statistically significant slight reduction in T21 group. This result should be confirmed by a wider study involving a larger number of subjects. The slight decrease of MeCbl concentration in the T21 group, together with a slight increase in 5-m-THF (a substrate of the reaction), a decrease in THF (a product of the reaction) [18], and the ER of *MTR* of 1.28 (encoding for the enzyme of the reaction) indicate the context of an imbalanced reaction. It was not possible to test statistical analyses between MeCbl plasma levels and gene ERs due to the absence of samples in which both analyses were performed.

5. Conclusions

This work aimed to investigate the imbalance of one-carbon pathway genes in trisomy 21. We started from a group of one-carbon cycle genes differentially expressed in the blood transcriptome map and performed further real-time RT-PCR analysis using a different and larger group of samples in order to exclude the possible biological differences between groups [59] and further narrow down the set of one-carbon genes to focus on and perform further experimental studies in the future.

The results showed that the three-copy presence of Hsa21 drives dysregulation of the expression of genes involved in the one-carbon cycle, located on both the same and other chromosomes, potentially leading to the alterations found at the metabolic level in DS. The data appear to reveal an expression imbalance of many methyltransferase genes, two of which are located on Hsa21 (*SETD4* and *PRMT2*) and could lead to epigenetic alteration and abnormal regional methylation found in trisomy 21 tissues [60,61].

The sample size for the gene expression and MeCbl metabolism analyses remains a limitation of the study, although the statistical results have been corrected for multiple testing and the SD% of the MeCbl data is extremely low.

Finally, given the known role of the one-carbon pathway in neurodevelopment, these explorative findings could be further investigated in other T21 tissue models. A T21 neuronal cell model will be developed from T21 induced pluripotent stem cells, and cerebrospinal fluid samples will be used in the future to investigate the metabolite levels of the one-carbon pathway in biological models more suitable to study the molecular basis of intellectual disability. However, blood can be considered a general collector of one-carbon metabolites from the whole organism, and fundamental alterations of this ubiquitous pathway are expected to be reflected in most tissues.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2025.120747.

Author contribution

B.V. processed the biological samples, performed data analysis, and contributed to writing the original draft. A.G. processed the biological samples and performed statistical analyses. F.A. edited the manuscript and revised the original draft. Mi.C., L.V., A.P., and G.L.P. validated the data and revised the original draft. M.C.P. and P.S. contributed to the funding acquisition and revised the original draft. G.S., G.R., F.C., P.P., and L.T.C. enrolled the subjects in the study and provided the samples. C.L. enrolled the subjects and co-supervised the work. Ma.C. ideated the work, wrote the original draft, performed data analysis, and supervised the work. All the authors critically contributed to the research and reviewed and edited the final draft.

Funding and additional information

Vione B., Ramacieri G., Catapano F., and Paradisi P. fellowships were funded by donations as acknowledged in the section below.

This study has been realized with the co-financing, in particular regarding the fellowships for M.Ci. and F.A., of the Ministry of University and Research in the framework of PNC “DARE - Digital lifelong prevention project” (PNC0000002 – CUP B53C22006450001), regarding the fellowships for G.S, has been realized with the co-financing of the Ministry of University and Research in the framework of PNC “DARE - Digital lifelong prevention project” (PNC0000002 – CUP B53C22006490001). The views and opinions expressed are solely those of the authors and do not necessarily reflect those of the European Union, nor can the European Union be held responsible for them.

Patient consent for publication

Case details, personal information or images are not included in this manuscript.

CRediT authorship contribution statement

Beatrice Vione: Writing – original draft, Methodology, Investigation, Data curation. **Alessandro Maria Gaudesi:** Methodology. **Francesca Antonaros:** Writing – review & editing, Methodology, Formal analysis. **Michela Cicilloni:** Writing – review & editing, Validation. **Lorenza Vitale:** Writing – review & editing, Validation. **Allison Piovesan:** Writing – review & editing, Formal analysis. **Maria Chiara Pelleri:** Writing – review & editing, Funding acquisition. **Pierluigi Strippoli:** Writing – review & editing, Validation, Funding acquisition. **Giacomo Sperti:** Writing – review & editing, Methodology, Investigation. **Giuseppe Ramacieri:** Writing – review & editing, Methodology, Investigation. **Francesca Catapano:** Writing – review & editing, Data curation. **Pietro Paradisi:** Writing – review & editing, Data curation.

Gian Luca Pirazzoli: Writing – review & editing, Validation. **Luigi Tommaso Corvaglia:** Writing – review & editing, Validation. **Chiara Locatelli:** Writing – review & editing, Validation, Supervision, Investigation. **Maria Caracausi:** Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

Ethics approval and consent to participate

The Independent Ethics Committee of the Hospital - University of Bologna Policlinico di S. Orsola Italy has granted the ethical approval for this study (number: 39/2013/U/Tess). We obtained an informed written consent from all participants to collect urine and blood samples and clinical data. Concerning minors, the consent was collected from his/her parents. All procedures were carried out in accordance with the Ethical Principles for Medical Research Involving Human Subjects of the Helsinki Declaration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors heartily thank all the children and their families who participated in the study.

We are very grateful to Donatella Pascai and Giuseppina Dibenedetto for their expert organization and performance of blood sampling, as well as to Cristiana Forni for her expert collaboration.

This study has been realized with the co-financing of the Ministry of University and Research in the framework of PNC "DARE - Digital lifelong prevention project" (PNC0000002 – CUP B53C22006450001). The views and opinions expressed are solely those of the authors and do not necessarily reflect those of the European Union, nor can the European Union be held responsible for them.

We are especially grateful to the "Fondazione Umano Progresso", Milano, for their valuable support to our research on trisomy 21, as well as to: the Mele Family; "Gruppo Arzdore di Dozza" and the Costa Family; "Associazione Vola con Martin oltre il 21 ODV", Mandello del Lario (LC); Gabriele's dad and mum; "Associazione Sette per te ventuno - Non abbiate paura" onlus; "Fondazione Rosa Prístina", Pisa; Paolo Ciaroni, main organizer of the car meetings "Top Down 2024", VII Edition, devoted to spider cars; "Associazione Amicorum", Cassano Magnago; the parents of Eleonora De Vita: Maurizio De Vita and Daniela Passerini, and the family of Giancarlo and Gabriella Bandera; "Unità Pastorale di Reda", "Parrocchie di S. Stefano in Corleto e S. Maria in Basiago", "Gruppo Dcuore"; "Monastero delle Clarisse di San Biagio, Forlì"; "Il popolo di Elisa Schira"; the Authors and the Editors of the book "Come fiori in un prato"; Jonatan Benvenuti and "Uniamoci per la Ricerca" initiative; Rotary Club Livorno Sud e Colline Pisano Livornesi; Rotary Club Cascina e Monte Pisano; Fondazione Mario Marianelli di Santa Croce sull'Arno (PI); "Associazione Capirsi Down ODV", Monza; Filippo Bono; Sichim Alfa Srl; Marco Bonafaccia, President of AMRP Onlus; A. MA.DOWN-Associazione Marchigiana Persone Down, Porto Potenza Picena (MC); "3x21 I sogni di Saveria Aps" and "Piramis Onlus"; "Amici di Simone ODV", Chiavari; Maria Aluigi; Maria Ilde Lomoro; "Associazione Cromosoma della Felicità APS", Chieti; "Gruppo Giovani Oratorio Narzole"; Ezio Meroni; Simone Bombardi and "Associazione Culturale Compagnia dell'Anello", Forlì; Maurizio Funazzi; Maria Grazia Verand-Bassani; Mimmo and Friends, dinner on 27/01/2024 at Toscanella di Dozza; Maurizio Poloni and Young Doctors in Rimini; Mario Gargantini, "Centro Culturale Marcello Candia", Melzo (MI); Giorgio Carpenito's Family.

We are deeply grateful for their generous bequest in favour of our research to: Mrs. M.T.; to Mrs. Franca Troffei, in memory of Massimo Marchini.

We are very grateful to all the other people that have very kindly contributed through individual donations to support this research, in particular: Giuseppe Angelillo; Gaia Berlanda; Ornella Carciani; Carlo; Andrea Conti; Mariadele Conti; the family of Desirèe Donato; Simone Ferriani; the Grandparents of Carlotta and Maddalena; Anastasiya Koval; Domenico Lascala; Nicoletta Lastella; "Liceo scientifico A. Carrel" - "Fondazione V. Grossman"; Gilberto Lisi; the Mattia's Uncles; Nicolette; the Parents of Desiree; the Parents of Margherita; Domenico Ricci; Ernesto Salzano; Claudio Sandonà; Simona Serattini; Vincenzina; Alessandro Volta.

We are grateful to Kirsten Welter for her kind and expert revision of the manuscript.

Data availability

The data of the participants, who provided informed consent and were approved by the ethics committee, are available in anonymized form in Supplementary Dataset 1. Further information can be obtained upon request from the corresponding author.

References

[1] S.E. Antonarakis, Parental origin of the extra chromosome in trisomy 21 as indicated by analysis of DNA polymorphisms. Down syndrome collaborative group, N. Engl. J. Med. 324 (13) (1991) 872–876.
[2] J.L. Down, Observations on an ethnic classification of idiots. 1866, Ment. Retard. 33 (1) (1995) 54–56.
[3] C. Romano, Genetics and clinical neuroscience in intellectual disability, Brain Sci. 12 (3) (2022).
[4] J. Lejeune, M. Gauthier, R. Turpin, Human chromosomes in tissue cultures, C. R. Hebd. Seances Acad. Sci. 248 (4) (1959) 602–603.
[5] A. Piovesan, et al., Human protein-coding genes and gene feature statistics in 2019, BMC. Res. Notes 12 (1) (2019) 315.
[6] A. Letourneau, et al., Domains of genome-wide gene expression dysregulation in Down's syndrome, Nature 508 (7496) (2014) 345–350.
[7] B. Conrad, S.E. Antonarakis, Gene duplication: a drive for phenotypic diversity and cause of human disease, Annu. Rev. Genomics Hum. Genet. 8 (2007) 17–35.
[8] S.E. Antonarakis, Down syndrome and the complexity of genome dosage imbalance, Nat. Rev. Genet. 18 (3) (2017) 147–163.
[9] E.M. Torres, Consequences of gaining an extra chromosome, Chromosome Res. 31 (3) (2023) 24.
[10] F. Guedj, et al., An integrated human/murine transcriptome and pathway approach to identify prenatal treatments for Down syndrome, Sci. Rep. 6 (2016) 32353.
[11] M.C. Pelleri, et al., Integrated quantitative transcriptome maps of human trisomy 21 tissues and cells, Front. Genet. 9 (2018) 125.
[12] F. Antonaros, et al., The transcriptome profile of human trisomy 21 blood cells, Hum. Genomics 15 (1) (2021) 25.
[13] L.H. Do, W.C. Mobley, N. Singhal, Questioned validity of gene expression dysregulated domains in Down's syndrome, F1000Res 4 (2015) 269.
[14] K.D. Sullivan, et al., Trisomy 21 consistently activates the interferon response, Elife (2016) 5.
[15] M. Caracausi, et al., Plasma and urinary metabolomic profiles of Down syndrome correlate with alteration of mitochondrial metabolism, Sci. Rep. 8 (1) (2018) 2977.
[16] F. Antonaros, et al., Plasma metabolome and cognitive skills in Down syndrome, Sci. Rep. 10 (1) (2020) 10491.
[17] F. Antonaros, et al., One-carbon pathway and cognitive skills in children with Down syndrome, Sci. Rep. 11 (1) (2021) 4225.
[18] B. Vione, et al., One-carbon pathway metabolites are altered in the plasma of subjects with Down syndrome: relation to chromosomal dosage, Front Med (Lausanne) 9 (2022) 1006891.
[19] A.S. Tibbetts, D.R. Appling, Compartmentalization of mammalian folate-mediated one-carbon metabolism, Annu. Rev. Nutr. 30 (2010) 57–81.
[20] J. Lejeune, et al., Metabolism of monocarbons and trisomy 21: sensitivity to methotrexate, Ann. Genet. 29 (1) (1986) 16–19.
[21] M.P. Mattson, T.B. Shea, Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders, Trends Neurosci. 26 (3) (2003) 137–146.
[22] C.E. Clare, et al., One-carbon metabolism: linking nutritional biochemistry to epigenetic programming of long-term development, Annu. Rev. Anim. Biosci. 7 (2019) 263–287.
[23] P. Lyon, et al., B vitamins and one-carbon metabolism: implications in human health and disease, Nutrients 12 (9) (2020).
[24] B. Petrova, et al., Regulatory mechanisms of one-carbon metabolism enzymes, J. Biol. Chem. 299 (12) (2023) 105457.
[25] S. Pope, et al., Cerebral folate deficiency: analytical tests and differential diagnosis, J. Inherit. Metab. Dis. 42 (4) (2019) 655–672.
[26] M. Caracausi, et al., The functional roles of S-adenosyl-methionine and S-adenosyl-homocysteine and their involvement in trisomy 21, Biofactors 50 (4) (2024) 709–724.

[27] J. Lejeune, Biochemical investigations and trisomy 21 (author's transl), Ann. Genet. 22 (2) (1979) 67–75.
[28] D.S. Rosenblatt, et al., 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 (3) (1982) 718–722.
[29] M.A. Peeters, M.O. Rethore, J. Lejeune, In vivo folic acid supplementation partially corrects in vitro methotrexate toxicity in patients with Down syndrome, Br. J. Haematol. 89 (3) (1995) 678–680.
[30] P.M. Ueland, H. Refsum, B. Christensen, Methotrexate sensitivity in Down's syndrome: a hypothesis, Cancer Chemother. Pharmacol. 25 (5) (1990) 384–386.
[31] R. Obeid, et al., Blood biomarkers of methylation in Down syndrome and metabolic simulations using a mathematical model, Mol. Nutr. Food Res. 56 (10) (2012) 1582–1589.
[32] R.S. Funk, et al., Altered folate homeostasis in children with Down syndrome: a potential basis for enhanced methotrexate toxicity, J. Pediatr. 221 (2020) 235–239.
[33] B. Vione, et al., Metabolic and genetic imbalance of the homocysteine-methionine cycle in trisomy 21, Physiol. Genomics 57 (9) (2025) 566–574.
[34] G. Brodsky, et al., The human GARS-AIRS-GART gene encodes two proteins which are differentially expressed during human brain development and temporally overexpressed in cerebellum of individuals with Down syndrome, Hum. Mol. Genet. 6 (12) (1997) 2043–2050.
[35] T. Panagaki, et al., Overproduction of H(2)S, generated by CBS, inhibits mitochondrial complex IV and suppresses oxidative phosphorylation in Down syndrome, Proc. Natl. Acad. Sci. U. S. A. 116 (38) (2019) 18769–18771.
[36] G. Lubec, et al., Increased expression of human reduced folate carrier in fetal Down syndrome brain, J. Neural Transm. Suppl. 67 (2003) 95–103.
[37] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1) (1987) 156–159.
[38] B. Li, et al., RNA-Seq gene expression estimation with read mapping uncertainty, Bioinformatics 26 (4) (2010) 493–500.
[39] Y. Panahi, et al., Selection of suitable reference genes for analysis of salivary transcriptome in non-syndromic autistic male children, Int. J. Mol. Sci. 17 (10) (2016).
[40] A. Cherubini, F. Rusconi, L. Lazzari, Identification of the best housekeeping gene for RT-qPCR analysis of human pancreatic organoids, PloS One 16 (12) (2021) e0260902.
[41] A.K. Badekila, P. Rai, S. Kini, Identification and evaluation of an appropriate housekeeping gene for real time gene profiling of hepatocellular carcinoma cells cultured in three dimensional scaffold, Mol. Biol. Rep. 49 (1) (2022) 797–804.
[42] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (4) (2001) 402–408.
[43] H. Zhao, et al., Competing endogenous RNAs crosstalk in Hippocampus: a potential mechanism for neuronal developing defects in Down syndrome, J. Mol. Neurosci. 74 (2) (2024) 32.
[44] Y. Liu, et al., TBK1-stabilized ZNF268a recruits SETD4 to methylate TBK1 for efficient interferon signaling, J. Biol. Chem. 299 (12) (2023) 105428.
[45] S. Ye, et al., SET domain-containing protein 4 epigenetically controls breast Cancer stem cell quiescence, Cancer Res. 79 (18) (2019) 4729–4743.
[46] N. Katsanis, M.L. Yaspo, E.M. Fisher, Identification and mapping of a novel human gene, HRMT1L1, homologous to the rat protein arginine N-methyltransferase 1 (PRMT1) gene, Mamm. Genome 8 (7) (1997) 526–529.
[47] V. Cura, J. Cavarelli, Structure, Activity and function of the PRMT2 protein arginine methyltransferase, Life (Basel) 11 (11) (2021).
[48] J.W. Taub, Y. Ge, Down syndrome, drug metabolism and chromosome 21, Pediatr. Blood Cancer 44 (1) (2005) 33–39.
[49] L. Vitale, et al., Human trisomy 21 fibroblasts rescue methotrexate toxic effect after treatment with 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate, J. Cell. Physiol. 234 (9) (2019) 15010–15024.
[50] Y. Wang, Z. Shen, Unmasking the Mammalian SET Domain-Containing Protein 4, NAR Cancer, 2022 vol. 4(3): p. zcac021.
[51] M. Alriquet, et al., Deficiency of N1-adenine methyltransferase aggravates RNA and protein aggregation, Cells 14 (17) (2025).
[52] L. Cui, et al., The role of m6A methyltransferase METTL3 in metabolism-related diseases: mechanism and clinical implications, Pharmacol. Res. 221 (2025) 107962.
[53] A. Noma, et al., Biosynthesis of wybutosine, a hyper-modified nucleoside in eukaryotic phenylalanine tRNA, EMBO J. 25 (10) (2006) 2142–2154.
[54] P. Perche-Letuvée, et al., Wybutosine biosynthesis: structural and mechanistic overview, RNA Biol. 11 (12) (2014) 1508–1518.
[55] J. Padeken, S.P. Methot, S.M. Gasser, Establishment of H3K9-methylated heterochromatin and its functions in tissue differentiation and maintenance, Nat. Rev. Mol. Cell Biol. 23 (9) (2022) 623–640.
[56] J. Janisiak, et al., Protein arginine methyltransferase (PRMT) inhibitors-AMI-1 and SAH are effective in attenuating rhabdomyosarcoma growth and proliferation in cell cultures, Int. J. Mol. Sci. 22 (15) (2021).
[57] M.A. Caudill, et al., Intracellular S-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine beta-synthase heterozygous mice, J. Nutr. 131 (11) (2001) 2811–2818.
[58] T.J. McCorvie, et al., The complex machinery of human cobalamin metabolism, J. Inherit. Metab. Dis. 46 (3) (2023) 406–420.
[59] Y.L. Lee, et al., Gene expression profiles of the one-carbon metabolism pathway, J. Genet. Genomics 36 (5) (2009) 277–282.
[60] I.S. Muskens, et al., The genome-wide impact of trisomy 21 on DNA methylation and its implications for hematopoiesis, Nat. Commun. 12 (1) (2021) 821.
[61] B.I. Laufer, et al., Whole genome bisulfite sequencing of Down syndrome brain reveals regional DNA hypermethylation and novel disorder insights, Epigenetics 14 (7) (2019) 672–684.