Folate pathway metabolites are altered in the plasma of subjects with Down syndrome: relation to chromosomal dosage

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Abstract

Down syndrome (DS) is the most common chromosomal disorder, and it is caused by trisomy of chromosome 21 (Hsa21). Subjects with DS can show a large heterogeneity of phenotypes and congenital defects and the most constant clinical features present are typical facies and intellectual disability (ID). Jérôme Lejeune was the first who hypothesized that DS could be a metabolic disease and he noted an alteration of the folate pathway (part of the one-carbon cycle) in trisomic cell lines and subjects with DS. Comparing DS with other metabolic diseases characterized by ID and altered folate pathway he hypothesized a possible correlation among them. Recently, a nuclear magnetic resonance (NMR) analysis of the detectable metabolic part in plasma and urine samples was performed, comparing a group of subjects with DS and a group of control subjects. The data showed a clear difference in the concentration of some metabolites (all involved in central metabolic processes) for the DS group, which was sometimes in agreement with gene dosage expected proportions (3:2). The aim of this work is to underline metabolic differences between subjects with DS and control subjects in order to better understand the dysregulation of the folate pathway in DS. For the first time, we performed enzyme-linked immunosorbent assays (ELISAs) to identify the concentration of 4 different intermediates of the one-carbon cycle, namely tetrahydrofolate (THF), 5-methyl-THF, 5-formyl-THF and S-adenosyl-homocysteine (SAH) in plasma samples obtained from 153 subjects with DS and 54 euploid subjects. Results highlight specific alterations of some folate pathway related metabolites. The relevance of these results for the biology of intelligence and its impairment in trisomy 21 is discussed leading to the proposal of 5-methyl-THF as the best candidate for a clinical trial aimed at restoring the dysregulation of folate pathway in trisomy 21 and improving cognitive skills of subjects with DS.

Introduction

Down syndrome (DS) or trisomy 21 (T21) is the most frequent aneuploidy occurring in human live births (OMIM 190685) and the most common genetic cause of intellectual disability (ID) (Antonarakis, 1991). Its association with the presence of an extra copy of a chromosome 21 (Hsa21) was discovered by Jerôme Lejeune and coll., who was the first to establish a link between ID and a chromosomal abnormality (Lejeune et al., 1959).

Subjects with T21 can present a variety of congenital defects and comorbidities. Only a few features occur in every individual with DS, and they are facial dysmorphology, small and hypocellular brain and cognitive impairment with highly variable severity (Roper and Reeves, 2006).

Lejeune was the first who hypothesized that DS could be considered a metabolic disease and that the folate pathway part of one-carbon cycle could be involved in the pathogenesis of ID. Caracausi and coll. (Caracausi et al., 2018) conducted a nuclear magnetic resonance (NMR) analysis of the metabolome in plasma and urine samples, comparing trisomic and euploid subjects (siblings of subjects with DS) to study metabolic processes possibly changed in DS as a result of the genetic imbalance. The multivariate analysis of the NMR metabolomic profiles showed a clear difference between the two groups, confirmed in a more recent study in plasma samples of a greater cohort (Antonaros et al., 2020). The univariate analysis showed a significant alteration of some metabolites. Most of the altered concentrations reflect the 3:2 gene dosage model, suggesting effects caused by the presence of three copies of Hsa21. It is interesting to note that several metabolites found increased in this study are involved in central metabolic processes such as fumarate and succinate in the Krebs cycle, pyruvate and lactate in glycolysis, and formate that is an end product of one-carbon metabolism in the mitochondrion.

In 1979 Lejeune published a pivotal study (Lejeune, 1979) in which he reasoned that several metabolic diseases cause ID and, comparing DS with them, he hypothesised that a disturbance of the one-carbon cycle could occur in DS. He thought that trisomic cells were intoxicated by an excess of gene products, which was caused by the presence of the additional Hsa21 (Lejeune, 1990). He investigated the genes located on Hsa21, and he found that several enzymes involved in the one-carbon metabolism were encoded by genes located on this chromosome, in particular: cystathionine β-synthase (*CBS*) and phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase (the three subunits of GART enzyme). Moreover,

the gene for the main transporter of folate, solute carrier family 19 member 1 gene (*SLC19A1*), is also located on Hsa21.

Further studies performed by Lejeune and coll. (Lejeune et al., 1986) demonstrated that methotrexate (MTX) was twice as toxic in T21 lymphocytes as compared to control cells, in agreement with the observation of Peeters and coll. (Peeters et al., 1986) of an increased toxicity of MTX in the leukaemia therapy in children with DS compared to euploid subjects. MTX is a cytotoxic drug used as a chemotherapy agent and immune system suppressant (Chabner et al., 1985;Morgan et al., 1987). MTX is a folate analogue which inhibits the activity of the dihydrofolate reductase (DHFR) enzyme involved in the one-carbon metabolic cycle (Lejeune et al., 1986).

In 2019 Vitale and coll. (Vitale et al., 2019) evaluated the rescue effect on MTX toxicity mediated by folate and some of its derivatives in primary human fibroblast cell lines obtained from euploid and T21 donors. This study shows that 5-methyl-tetrahydrofolate (5-methyl-THF) and 5-formyl-tetrahydrofolate (5-formyl-THF or folinic acid) treatments have significant protective effects on both euploid and T21 cells during MTX treatment.

In a recent survey, erythrocyte folate concentrations in children with DS were compared with a cohort of subjects with juvenile arthritis as euploid control, in order to understand if the MTX treatment toxicity in children with DS is related to altered erythrocyte folate concentrations. They reported a lower concentration level of total folate, 5-methyl-THF and 5,10-methenyl-THF in subjects with DS. These reductions in erythrocyte folates were also associated with a decrease in short-chain folate polyglutamation 5-methyl-THFGlu3-6 and a corresponding increase in longer chain 5-methyl-THFGlu7-10 (Funk et al., 2020). These data suggest that in trisomic cells there is a biological adaptation to folate deficiency by an increased level of long-chain folate polyglutamation; indeed, increased expression of folylpolyglutamate synthetase (*FPGS*) gene was already reported in *vitro* and in animal models (Ifergan et al., 2004;Pelleri et al., 2018;Funk et al., 2020).

In subjects with DS, folate metabolism was considered to be imbalanced by several authors (Lejeune, 1979;Rosenblatt et al., 1982;Peeters et al., 1995;Song et al., 2015;Funk et al., 2020). In particular, subjects with DS commonly present lower blood levels of vitamin B12, folic acid and erythrocyte folates and higher blood level of homocysteine compared to healthy controls (Ueland et al., 1990;Song et al., 2015).

Folate metabolism plays at least two separate roles. It is critical for one-carbon metabolism: the process by which one-carbon groups at different oxidation states are used for purine and thymidylate synthesis and for the remethylation of homocysteine to methionine,

allowing several transmethylation reactions (see Figure 1). Moreover, reactions of folate metabolism play a role in the catabolism of choline and at least three amino acids: histidine, serine and glycine (Brosnan et al., 2015). These biochemical processes, in turn, support critical cellular functions such as cell proliferation, mitochondrial respiration and epigenetic regulation (Mattson and Shea, 2003).

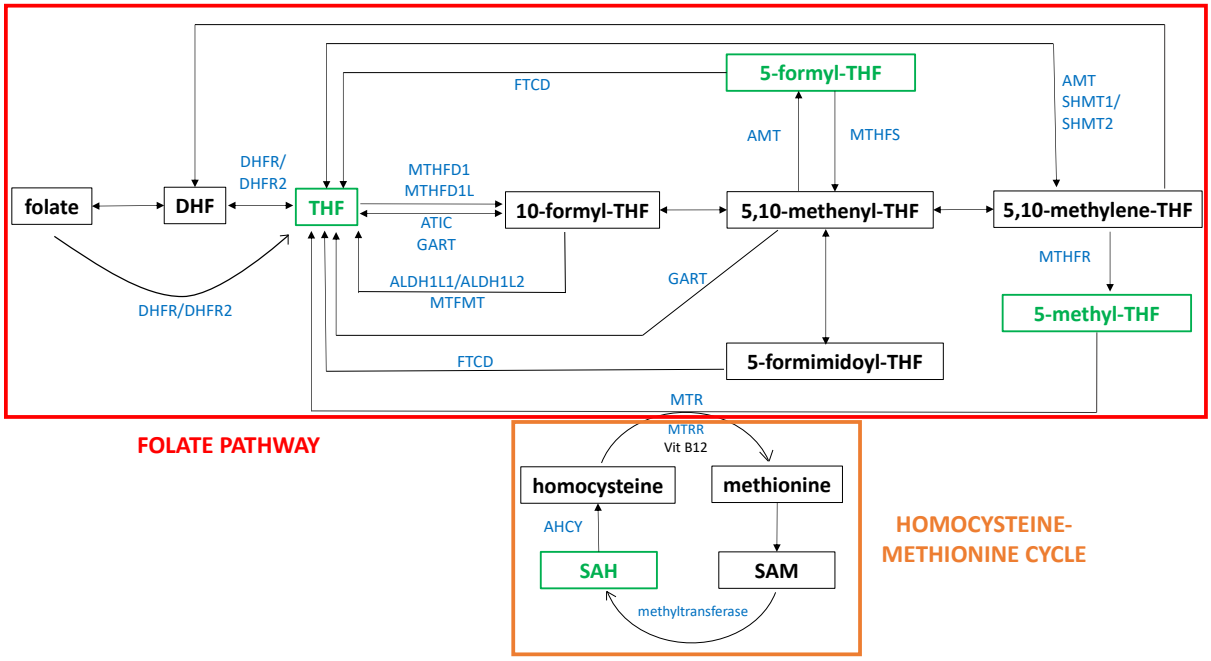

Figure 1. One-carbon pathway. The figure shows a schematic representation of folate pathway (in red) and homocysteine-methionine cycle (in orange), both part of one-carbon pathway. The metabolites analysed in our study are reported in green and the enzymes directly involved in their production or transformation are indicated with the official symbol reported in Gene NCBI (https://www.ncbi.nlm.nih.gov/gene). Alternative forms of enzyme are shown together separated by "/". The schematic representation was realised starting from "One carbon pool by folate - Homo sapiens" of KEGG pathway database (https://www.genome.jp/kegg/pathway.html).

In the present study, we conducted enzyme-linked immunosorbent assays (ELISAs) to collect for the first time the concentration of 4 different intermediates of one-carbon metabolism which are tetrahydrofolate (THF), 5-methyl-THF, 5-formyl-THF and S-adenosyl-homocysteine (SAH) in plasma samples obtained from 153 subjects with DS and 54 euploid subjects as control.

Materials and methods

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 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.

Case selection

A total of 207 subjects were selected for this study, including 153 subjects with DS and 54 subjects as control, selected among siblings of subjects with DS and without evidence of abnormal karyotype. The study has been proposed to all subjects consecutively admitted to the Day Hospital of the Neonatology Unit, IRCCS Sant'Orsola-Malpighi Polyclinic, Bologna, Italy, in the context of routine follow up provided for DS.

Inclusion criteria for DS group were diagnosis of DS with homogeneous or mosaic T21, availability of an adequate amount of plasma to perform at least one ELISA assay and a similar mean age as close as possible to control group. Concerning control group, the inclusion criteria were availability of an adequate amount of plasma to perform at least one ELISA assay and a similar mean age as close as possible to DS group. The blood samples selected were treated within two hours of collection and plasma samples contaminated by residual erythrocytes after the treatment were excluded.

DS group consists of 90 males (M) and 63 females (F) with a mean age of 11.58 years old (standard deviation, SD=6.76) and an age range from 3.0 to 37.9; control group consists of 30 M and 24 F with a mean age of 14.53 years old (standard deviation, SD=6.64) and an age range from 3.3 to 31.5. Concerning the control group, 28 subjects were siblings of 23 subjects in the DS group.

For every collected sample, parents filled out a form with information about the current fasting state, last meal, consumed medications (see Supplementary Dataset 1).

Blood sample preparation

Blood samples were collected in EDTA-coated blood collection tubes, kept at room temperature and treated within two hours from blood draw. Sample was transferred to a new

tube and centrifuged at 1,250 g for 10 min to separate corpuscular fraction from plasma. The plasma was isolated and centrifuged for a second time at 800 g for 30 min to eliminate residual debris. The supernatant was collected and transferred into new tubes avoiding contact with pellets or the bottom of the tube and divided in aliquots. All plasma samples were rapidly stored in a -80°C freezer.

Every delayed treatment of the sample following its transfer to the university laboratory (>2 hours) was recorded, as one of the exclusion criteria from the analysis was sample treatment after two hours from the draw. The other one was evident contamination of plasma by hemoglobin due to hemolysis after the treatment. All procedures were conducted carefully to avoid contamination during the different steps and any anomalies like different plasma colour or precipitates after centrifugation were noted and considered in further analysis.

Enzyme-linked immunosorbent assay (ELISA)

We evaluated the quantitative measurement of THF, 5-methly-THF, 5-formyl-THF and SAH plasma concentrations using specific ELISA kits manufactured by MyBioSource (San Diego, California, USA). We performed 5 ELISA assays to collect concentration values of THF, 5-methly-THF and 5-formyl-THF and 4 ELISA assays to collect concentration values of SAH.

"General Tetrahydrofolic Acid (THFA) ELISA assay", "Human 5-methyltetrahydrofolate ELISA assay" and "Enzyme-linked Immunosorbent assay for 5-formyl-THF (folinic acid)" kits are based on a competitive inhibition enzyme technique, while "Human S-Adenosyl-Homocysteine (SAH) ELISA assay" kit employs a double antibody sandwich technique.

Ninety-six well plates were used for all the assays and all standard and plasma samples were tested in duplicate. Due to preliminary studies, plasma samples used for the measurement of 5-methyl-THF were diluted 1:10 using Sample Dilution Buffer supplied by the kit and plasma samples used for the measurement of SAH were diluted 1:5 using Sample Diluent supplied by the kit. The standard samples were provided by the kits and were reconstituted and serially diluted as suggested.

In order to perform the assays, the manufacturer instructions of each kit were followed, and the final spectrophotometric reading was carried out by microplate reader (Perkin Elmer Wallac 1420 Victor 2 Multi-Label) settled at a wavelength of 450 nm.

In order to create the standard curve for each ELISA assay, standard absorbance (O.D.) mean values were plotted on the x-axis and the known standard concentration values

were plotted on the y-axis using Microsoft Excel and following manufacturer instructions. The transformation of standard concentration values in their logarithm (Log_{10}) is required to build the standard curves in THF and 5-formyl-THF ELISA assays. The subtraction of the background O.D. mean value ("standard 8") from the O.D. mean values of the other standards and plasma samples is required in SAH ELISA assay.

The polynomial trend lines of each plot were created using Excel, and the resulting polynomial equations ($y = a + bx + cx^2$) were used to determine metabolite concentrations of plasma samples using interpolation.

Statistical analyses

Statistical analyses were carried out with SPSS Statistics (IBM, Version 25 for Mac OS X) and were performed using the data available in Supplementary Dataset S1. For all results, a $p<0.05$ 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.

A first descriptive analysis was made using SPSS Statistics software in order to obtain a general view of the data included in Supplementary Dataset S1. It was performed with SPSS Statistics software as follows: from the leading software Menu, we selected "Analyze" and then "Descriptive analysis", we then chose "Frequencies", we included our metabolites data in the "variables" box and finally we chose "Quartiles", "Percentiles", "Mean", "Median", "Modal value", "Standard deviation", "Variance", "Range", "Minimum", "Maximum" in the "Statistics" box.

The presence of strong outliers in metabolite level distribution was performed with SPSS Statistics software as follows: from the leading software Menu we selected "Analyze" and then "Descriptive statistics", we then chose "Explore", and included metabolite levels in "Dependent List", finally in "Statistics section" we selected the "Outliers" and "Percentiles" options. SPSS Statistics software indicates strong outliers with an asterisk in the graph.

For each metabolite we performed an unpaired student t-test between DS and control group using the "Graph Pad" t-test calculator online (https://www.graphpad.com/quickcalcs/ttest1.cfm). In order to perform the analysis, we chose "Enter or paste up to 2000 rows", then we chose "Unpaired t-test", entered for each DS metabolite data in "Group one" column and control data in "Group two" column and in the end, we chose "Calculate now".

A linear correlation was used to determine if the correlation between age and metabolite levels existed. It was performed with SPSS Statistics software as follows: from the

leading software Menu, we selected “Analyze” and then “Correlate”, we then chose “Bivariate correlation”, and finally we included our data in the “variables” box.

The correlations between sex and metabolite levels and between fasting/non-fasting state and metabolite levels were investigated with an unpaired t-test. Unpaired t-test was performed with SPSS Statistics software as follows: from the leading software Menu we selected “Analyze” and then “Compare means”, we then chose “independent-samples T-test” and finally we included our data in the “test variables” box and inserted “Sex” or “Fasting/no fasting” in the “grouping variable” box. Correlations between sex and SAH concentration and fasting and SAH concentration in control group were investigated with Mann-Witney U test using SPSS Statistics software as follows: “Analyze” and then “Non parametric tests”, we then chose “independent-samples”, “Customize analysis” and finally we included “Folic Acid data” in the “test variables” box and inserted “Sex” or “Fasting” in the “grouping variable” box.

SPSS Statistics software was used to perform a linear correlation between the level of each metabolite and the levels of all the other metabolites. Briefly, from the main Menu of the “SPSS Statistics” software, we selected “Analyze” and then “Correlation”; we chose “Bivariate” and finally we inserted our data in the main box. Partial correlation analyses checked for the effect of chronological age and sex were used to investigate associations between the level of the involved metabolites and other metabolites. These analyses were performed as follows: from the main Menu of the “SPSS Statistics” software, we selected “Analyze” and then “Correlation”; we chose “Partial correlation” and finally we inserted our data in the main box and inserted “Age” in the “Check by” box. The Heat Map figures representing metabolite correlations were generated using JMP Pro software (Version 14 of the SAS System for Mac OS X, SAS Institute Inc., Cary, NC, USA).

Results

Enzyme-linked Immunosorbent Assay (ELISA)

We selected 153 subjects with DS and 54 euploid subjects. Concerning subjects with DS, only one has T21 mosaicism at 94%. In the DS group we obtained the concentration values of THF in 108 subjects, of 5-methyl-THF in 132 subjects, of 5-formyl-THF in 79 subjects and of SAH in 89 subjects. In the control group we obtained the concentration values of THF in 38 subjects, of 5-methyl-THF in 34 subjects, of 5-formyl-THF in 25 subjects and of SAH in 10 subjects (see Supplementary Dataset 1). It was not possible to obtain the metabolite dosage in each selected subject for several reasons: the amount of plasma was not sufficient, the O.D. mean values of plasma samples were out of the range, or there was a technical problem during the assay.

Concerning the O.D. mean values, those that were higher or lower than the O.D. mean values of standard sample ranges were not taken into consideration and they are indicated in red in Supplementary Tables 1, 2, 3 and 4. In total we did not consider the concentration values of 9 plasma samples in THF ELISA assays, 59 plasma samples in 5-formyl-THF assays and 31 plasma samples in SAH ELISA assays.

In order to assess the intra-wells variability of the assays in duplicate, the Coefficient of Variation of the O.D. values expressed as percentage (%CV) was reported for each sample in Supplementary Tables 1, 2, 3 and 4. The %CV was calculated dividing the standard deviation of the two measurements for the mean of the two measurements and multiplying for 100. The mean of the %CV values also named Intra-Assay CV is 9.57% for THF assays, 6.71% for 5-methyl-THF assays, 7.24% for 5-formyl-THF assays and 11.50% for SAH assays. Out of a total of 191 samples assayed, there are 40 samples that ranged between 15.49% and 53.23% of %CV in THF assays; out of a total of 207 samples assayed, there are 16 samples that ranged between 15.02% and 29.79% in 5-methyl-THF assays; out of a total of 141 samples assayed, there are 15 samples that ranged between 15.44% and 36.29% in 5-formyl-THF assays; out of a total of 135 samples assayed, there are 43 samples that ranged between 15.10% and 46.36% in SAH assays.

The coefficient of determination (R^2) of the standard curves ranged between 0.9697 and 0.9881 for THF (see Supplementary Table 1), between 0.9423 and 0.9707 for 5-methyl-THF (see Supplementary Table 2), between 0.9048 and 0.9433 for 5-formyl-THF (see Supplementary Table 3) and between 0.9987 and 0.9997 for SAH (see Supplementary Table 4).

Statistical analyses

Five strong outliers were identified among 5-formyl-THF concentration values that are reported in red in Supplementary Dataset 1.

The main results of descriptive analyses without strong outliers are shown in Table 1. The detailed results of descriptive analysis with and without strong outliers are shown in Supplementary Table 5.

		DS				control			
		THF (ng/mL)	5-methyl-THF (ng/mL)	5-formyl-THF (pg/mL)	SAH (ng/mL)	THF (ng/mL)	5-methyl-THF (ng/mL)	5-formyl-THF (pg/mL)	SAH (ng/mL)
n	Valid	108	132	78	89	38	34	21	10
	Missing	45	21	75	64	16	20	33	44
Mean		41.120	50.578	151.070	6.788	60.326	45.369	127.414	3.146
Median		32.769	49.940	138.091	6.490	56.272	42.744	137.984	2.330
SD		29.565	9.267	31.187	2.855	32.577	12.301	45.622	1.899

Table 1. Main results of descriptive analysis of DS and control group excluding strong outliers. The values of subjects with Down syndrome (DS) are reported on the left of the table and the values of normal control subjects (control) are reported on the right of the table. For each metabolite the number (n) of plasma samples valid or missing are reported. Also the mean, median and standard deviation (SD) values are shown. The detailed results of descriptive analysis with and without strong outliers are shown in Supplementary Table 5.

The results of unpaired student t-test between DS and control concentration values of each metabolite showed that the difference between DS and control concentrations is statistically significant for all metabolites when the strong outliers are considered (see Supplementary Table 6), while the significance is lost for 5-formyl-THF when the strong outliers are not considered (see Table 2).

	THF	5-methyl-THF	5-formyl-THF	SAH
p-value	0.001	0.0072	0.4686	0.0002
t-value	3.3532	2.7215	0.7275	3.927
df	144	164	97	97
SD	5.728	1.914	7.047	0.927

Table 2. Unpaired student t-test between DS and control group concentration values of each metabolite excluding strong outliers. Significant p-values (<0.05) are reported in red in the first line. T-value, degrees of freedom (df) and the standard deviation (SD) are reported in the other lines.

Figure 2 shows the variation of metabolite concentrations in subjects with DS and normal control subjects excluding strong outliers. In Supplementary Figure 1 the variation of 5-formyl-THF concentration in subjects with DS and normal control subjects is reported including strong outliers.

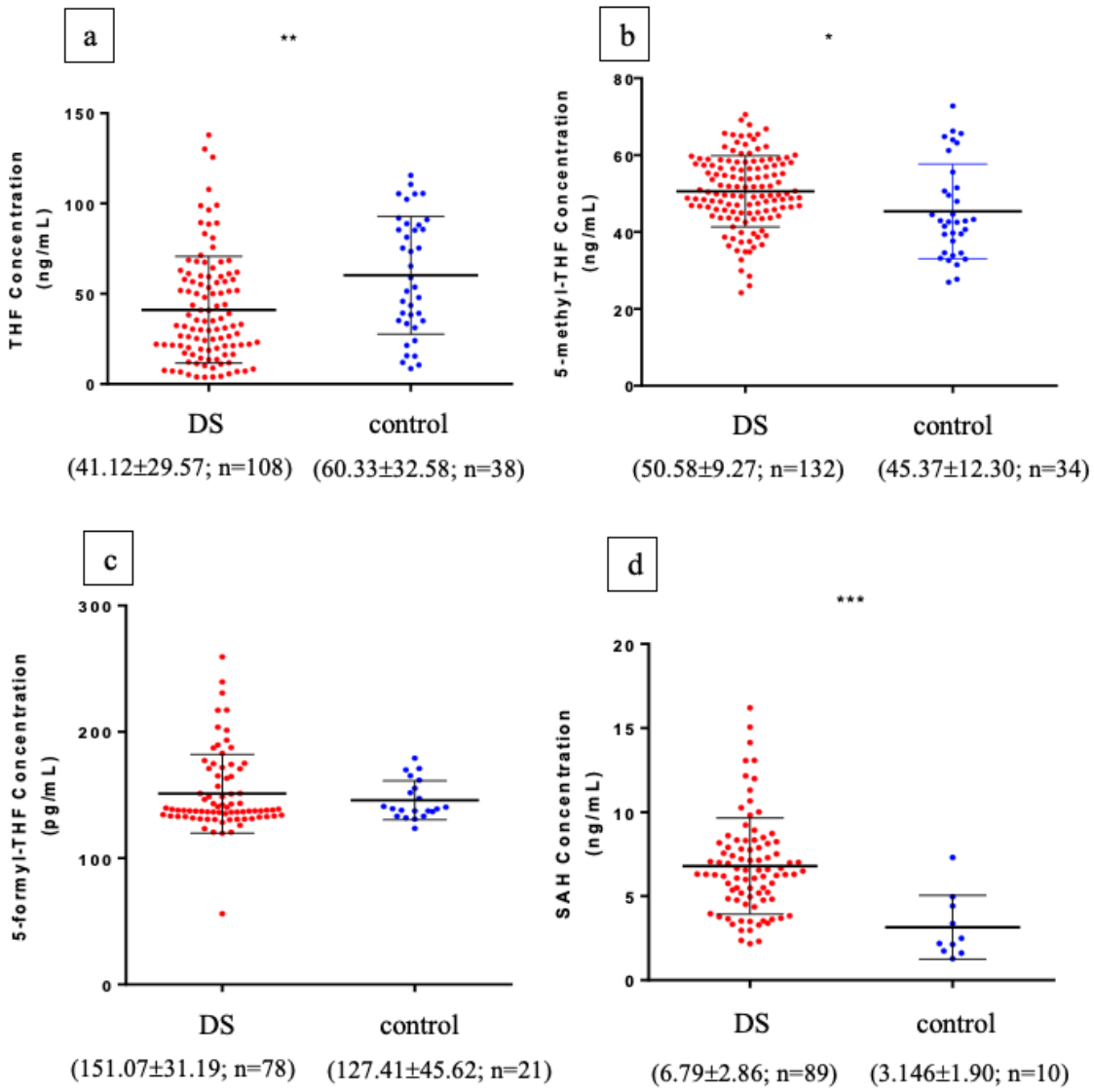

Figure 2. Metabolite concentrations in subjects with DS and normal control subjects. The graphs report metabolite plasma levels of each subject in the study. On the x-axis there is the subdivision of subjects in DS and control groups. Subjects with DS are represented like red dots and normal control subjects are represented like blue dots. On the y-axis the concentration of the metabolite in ng/mL or pg/mL is reported. The asterisks above the graph indicate the level of statistical significance (*=p<0.05; **=p<0.005; ***=p<0.0005). The middle black lines indicate the mean concentration values for each group and the external black lines indicate standard deviation (SD) values. The mean concentration, SD values and the number of subjects (n) are reported below each graph for DS and control groups. Figure 2a shows THF concentrations; Figure 2b shows 5-methyl-THF concentrations; Figure 2c shows 5-formyl-THF concentrations excluding strong outliers; Figure 2d shows SAH concentrations. The graphs were created with GraphPad Prism software v.6.0 (San Diego, CA).

In Table 3 the ratio of mean concentration values between DS and control groups is reported.

	THF	5-methyl-THF	5-formyl-THF		SAH
			Including outliers	Excluding outliers	
Mean ratio DS/control	0.68	1.11	1.23	1.04	2.16

Table 3. Ratio of mean concentration values between DS and control groups. For each metabolite in the study the ratios of mean concentration levels between DS and control groups (Mean ratio DS/control) is reported. For 5-formyl-THF the ratio including and excluding strong outliers are both showed.

A statistically significant moderate correlation was found between age and THF concentration levels in control group (r=0.403 and p-value=0.012). The significance was lost in DS group. Whether or not the strong outliers were considered did not change the results (see respectively Supplementary Table 7 and Table 4).

	DS				control			
	THF	5-methyl-THF	5-formyl-THF	SAH	THF	5-methyl-THF	5-formyl-THF	SAH
r	0.104	-0.088	0.166	0.171	0.403	0.271	0.239	0.318
p-value	0.286	0.313	0.145	0.108	0.012	0.121	0.296	0.371

Table 4. Bivariate correlation between age and each concentration levels in DS and control groups excluding strong outliers. The table reports for each metabolite the results of the statistical analyses in DS and control groups indicated by Pearson correlation coefficient (r) and two-tailed significance (p-value). Significant r (>0.4) and p-values (<0.05) are reported in red.

The unpaired t-tests between sex and metabolite did not identify correlation with metabolite concentrations when the strong outliers are included in the analyses (see Supplementary Table 8a). The results evidenced a statistically significant difference (p-value of assumed equal variances=0.028) of 5-formyl-THF concentration in DS group when the strong outliers are not considered, but in control group the significance is lost (see Supplementary Table 8b). Mann-Witney U test between sex and SAH concentration in control group also did not report a statistically significant correlation (see Supplementary Table 8b).

The unpaired t-tests between fasting/non-fasting state and metabolite levels did not report some statistically significant correlations (see Supplementary Table 9a and 9b). Mann-Witney U test between fasting/non-fasting state and SAH concentration in control group also did not report a statistically significant correlation (see Supplementary Table 9b).

A statistically significant moderate positive correlation (r=0.556 and p-value=0.017) between 5-methyl-THF concentration and 5-formyl-THF concentration levels was found in the control group when the strong outliers were included in the analysis (see Supplementary Table 10a and Supplementary Figure 2). The correlation was not maintained in DS group (see Supplementary Table 10a and Supplementary Figure 2) and also when the same statistical analysis was performed excluding strong outliers (see Supplementary Table 10b and 10c and Figure 3). A statistically significant negative correlation was found between 5-formyl-THF plasma levels and THF (r=-0.530 and p-value=0.011) and SAH (r=-0.514 and p-value=0.024) plasma levels in female DS group excluding strong outliers, while the correlation was lost in the male DS group (see Figure 3a and Supplementary Table 10b) and the control group (see Figure 3b Supplementary Table 10c).

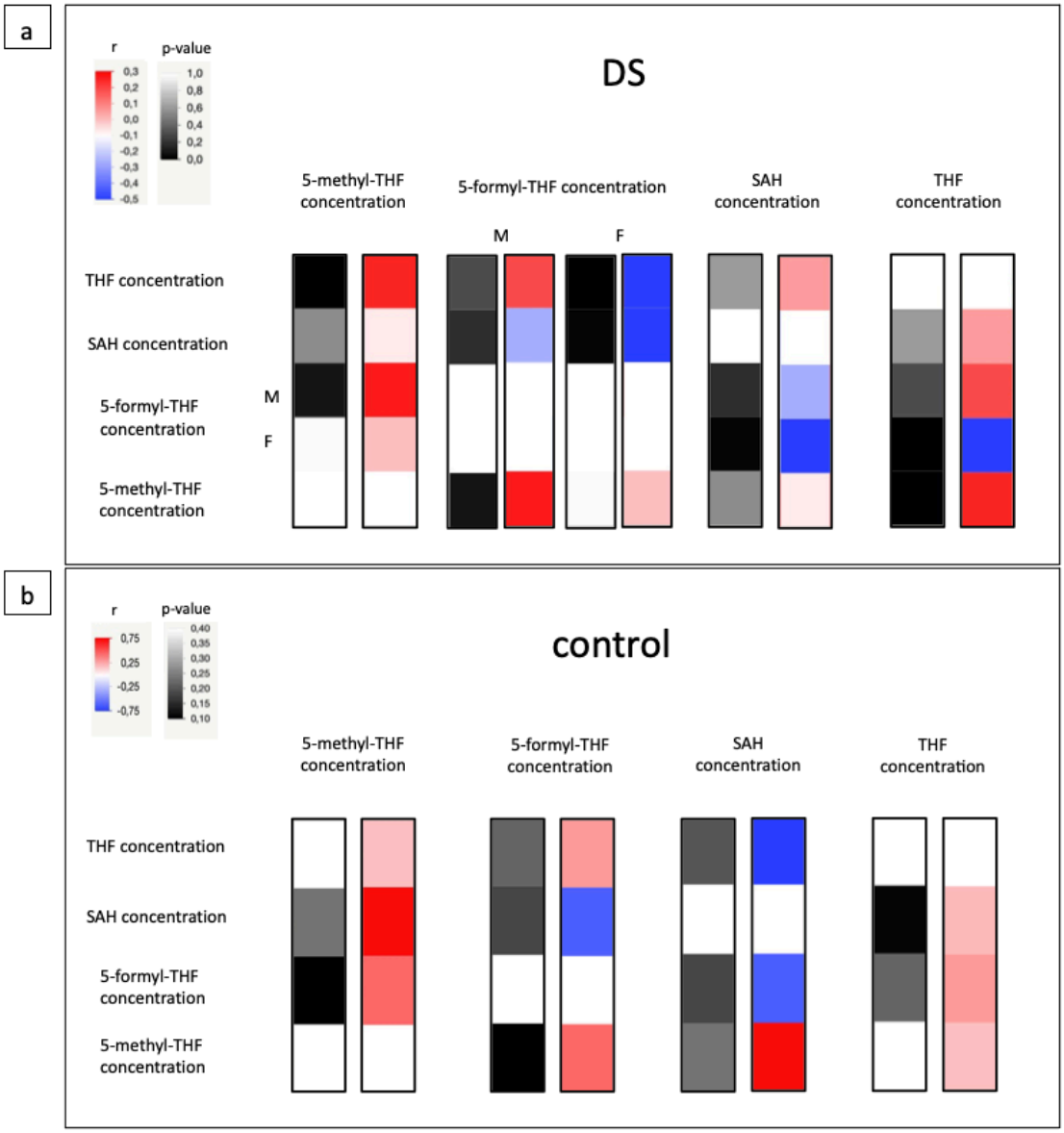

Figure 3. Heat Map figure of bivariate correlation between levels of each metabolite and levels of all the other metabolites excluding strong outliers. Figure 3a presents bivariate correlations in DS group (for complete data see Supplementary Table 10b and 10c) and Figure 3b presents bivariate correlation in the control group (for complete data see Supplementary Table 10c). At the top left of the figures the color codes for Pearson correlation coefficient (r) and two tailed-significance (p-value) are reported. For each metabolite indicated above, two bars are shown: the first represents p-values and the second r values. Every bar is divided in four segments corresponding to the four correlations with other metabolites. Concerning 5-formyl-THF in DS group, the partial correlation checked for sex is reported thus four bars are shown and every bar is divided in five segments.

Discussion

Our results highlight plasma level alteration of some intermediates of one-carbon metabolism in a group of subjects with DS compared to a group of euploid subjects as control. We reported a statistically significant difference of THF, 5-methyl-THF and SAH plasma levels between DS and control groups (see Table 2). Concerning 5-formyl-THF, the difference is not statistically significant when the strong outliers are excluded (see Table 2), while it is statistically significant when the strong outliers are included (see Supplementary Table 6).

THF is the metabolic active form of folates in cells and it serves as one-carbon carrier in most folate-mediated reactions (Zheng and Cantley, 2019). THF is the folate form most interconnected in the folate pathway and it is the product of ten enzymatic reactions as shown in Figure 1. Moreover, Lejeune analyzed the genetic diseases causing biochemical alterations that affect the nervous system and he found out that many biochemical dysregulations had an effect on THF metabolism and its chemical connections (Lejeune, 1979). Our results suggest that THF plasma concentration is lower than normal in subjects with DS and the mean concentration ratio between DS and control groups is 0.68 (see Table 3), that is a 2:3 ratio, suggesting a possible correlation with the imbalanced original event or the presence of a third Hsa21. The starting event leading to the 3:2 or 2:3 metabolite imbalances observed in DS could be the activity of critical gene determinants included in the Hsa21 region associated with diagnosis of DS (highly restricted Down syndrome critical region, HR-DSCR) (Pelleri et al., 2016;Pelleri et al., 2019) and still unknown.

5-methyl-THF is the most important folate form in blood circulation and the only form able to cross the blood-brain barrier (Ducker and Rabinowitz, 2017). Our results suggest that 5-methyl-THF plasma concentration is slightly higher than normal in subjects with DS and the mean concentration ratio between DS and control groups is 1.11 (see Table 3), that is a 1:1 ratio.

5-formyl-THF is a reserve of one-carbon units in cells, and it does not play a direct biosynthetic role (Ducker and Rabinowitz, 2017). Our results suggest that 5-formyl-THF plasma concentration is equal in subjects with DS compared to normal control subjects and the mean concentration ratio between DS and control groups is 1.04 excluding strong outliers (see Table 3), that is a 1:1 ratio.

SAH is part of homocysteine-methionine cycle in which the universal methyl donor named S-adenosyl-methionine (SAM) is transformed in SAH by methyltransferases (Mentch and Locasale, 2016) (see Figure 1). Our results suggest that SAH plasma concentration is much higher than normal in subjects with DS and the mean concentration ratio between DS

and control groups is 2.16 (see Table 3), that is a 2:1 ratio. The dosage of SAM would have been interesting to perform, but it was not possible due to technical problems with the ELISA kit purchased.

Abnormal folate metabolism has been causally linked with many diseases. Cerebral folate deficiency (CFD), caused by folate receptor autoantibodies or germline mutations such as in *FOLR1* (folate receptor alpha), *SLC46A1* (solute carrier family 46 member 1) or *MTHFR* genes, is manifested in neurological impairments and shows deficiency of 5-methyl-THF in the cerebrospinal fluid (CSF) in the presence of low or undefined peripheral blood folate levels (Watkins and Rosenblatt, 2012;Pope et al., 2019). Thus, folate metabolism plays a crucial role in the brain, but is still poorly defined.

Recently, Antonaros and coll. explored the role of five metabolite levels involved in one-carbon pathway (homocysteine, folate, vitamin B12, uric acid, creatinine) in the intellectual impairment of children with DS. The findings highlighted that specific vitamin B12 and homocysteine thresholds corresponded respectively with better and worse cognitive scores in children with DS suggesting a role in their cognitive development (Antonaros et al., 2021).

Today the research about DS is focusing on the improvement of cognitive status (Antonarakis, 2017). Several groups have tried to improve cognitive impairment of subjects with DS using different compounds in clinical trials (Bianchi et al., 2010;Moon et al., 2010;Braudeau et al., 2011;Das et al., 2013;de la Torre et al., 2016). Even if some of them showed interesting results, as of today none of these compounds are used as effective treatments of cognitive impairment in DS.

It was demonstrated that folate deficiencies contribute to several neuropsychiatric disorders and that can be corrected by folate supplementation (Kronenberg et al., 2009;Nazki et al., 2014;Ramaekers et al., 2016). Two clinical trials tried to use 5-formyl-THF supplementation (alone and together with other compounds) to improve psychomotor and language development in young subjects with DS, but they did not evidence any significant clinical differences compared to placebo group (Ellis et al., 2008;Blehaut et al., 2010;Mircher et al., 2020). The search results for "Down syndrome" [MeSH Terms] AND "folic acid" [MeSH Terms] in Pubmed.gov (https://pubmed.ncbi.nlm.nih.gov/) showed no other clinical trials about folates and DS have been found.

Our results confirmed that there is a dysregulation of the folate pathway that could be related to cognitive impairment. We speculate that a clinical trial with the supplementation of folates different from 5-formyl-THF in subjects with DS can be very interesting to realize.

We demonstrated that 5-formyl-THF plasma level is not imbalanced in DS group, while THF, 5-methyl-THF and SAH are imbalanced.

Folinic acid (5-formyl-THF) has been in use for the longest time for the treatment of CFDs (Irons et al., 1987) and is currently the most used folate form for the treatment of CFDs (Pope et al., 2019). The use of 5-methyl-THF was more recently proposed (Li et al., 2008;Knowles et al., 2016) and is considered to be the most efficient way to normalize CSF 5-methyl-THF concentrations (Pope et al., 2019). From our results, 5-methyl-THF plasma concentration is not decreased in subjects with DS, but it does not seem to be a mark of CFD because subjects with CFD can show normal peripheral blood folate levels (Pope et al., 2019).

5-methyl-THF is the only folate form able to cross the blood-brain barrier (Ducker and Rabinowitz, 2017) and it is the most reduced folate form (Nelson, 2017). It is well absorbed in the intestinal tract and its bioavailability is not influenced by additional enzymatic steps (Pope et al., 2019). Moreover, 5-methyl-THF is necessary for the formation of SAM by the regeneration of methionine from homocysteine (see Figure 1). SAM is a powerful methylating agent in several biosynthetic reactions, and its methyl group is the most reactive in the one-carbon cycle (Nelson, 2017).

For all these reasons, we propose 5-methyl-THF as the best candidate for a clinical trial that aims to improve cognitive status of subjects with DS. Nevertheless, we advise against administrating 5-methyl-THF until the effective dosage in correcting the altered values is identified.

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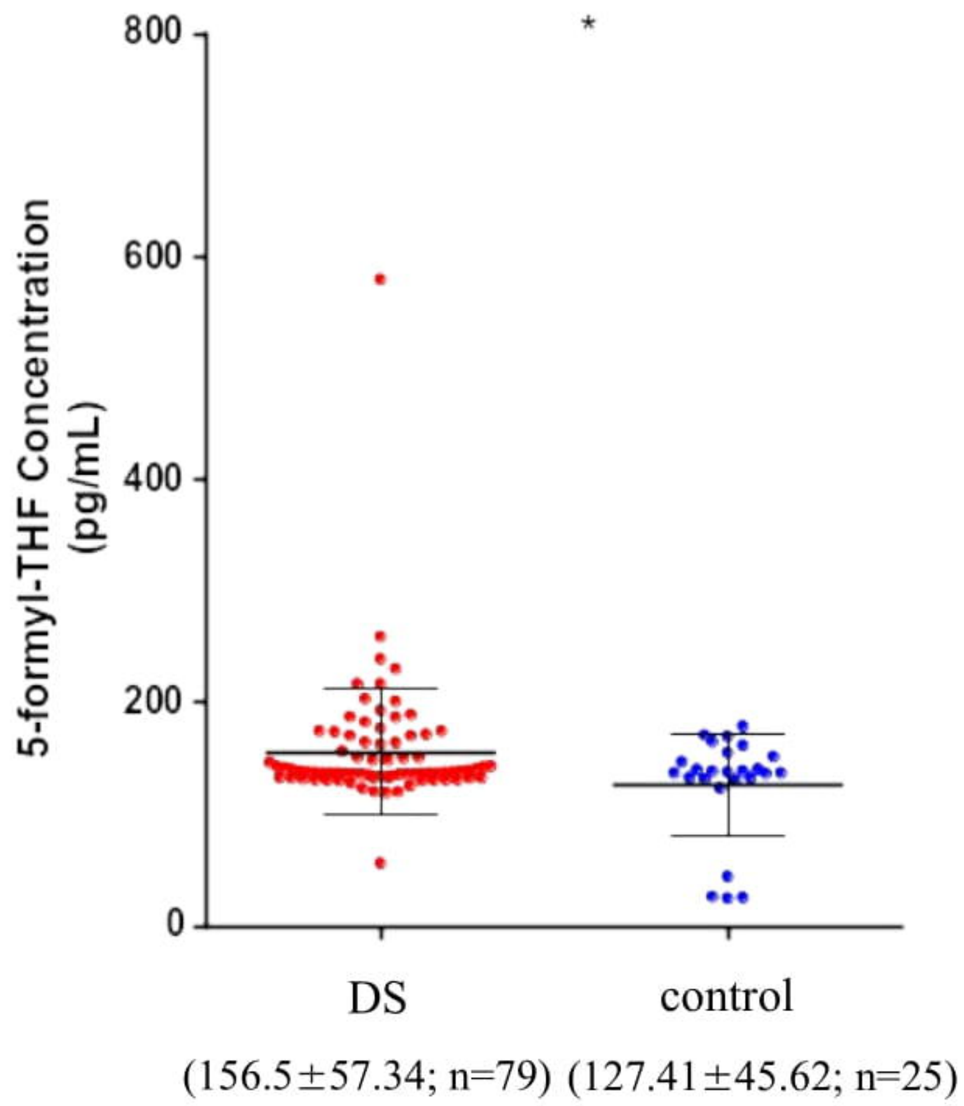
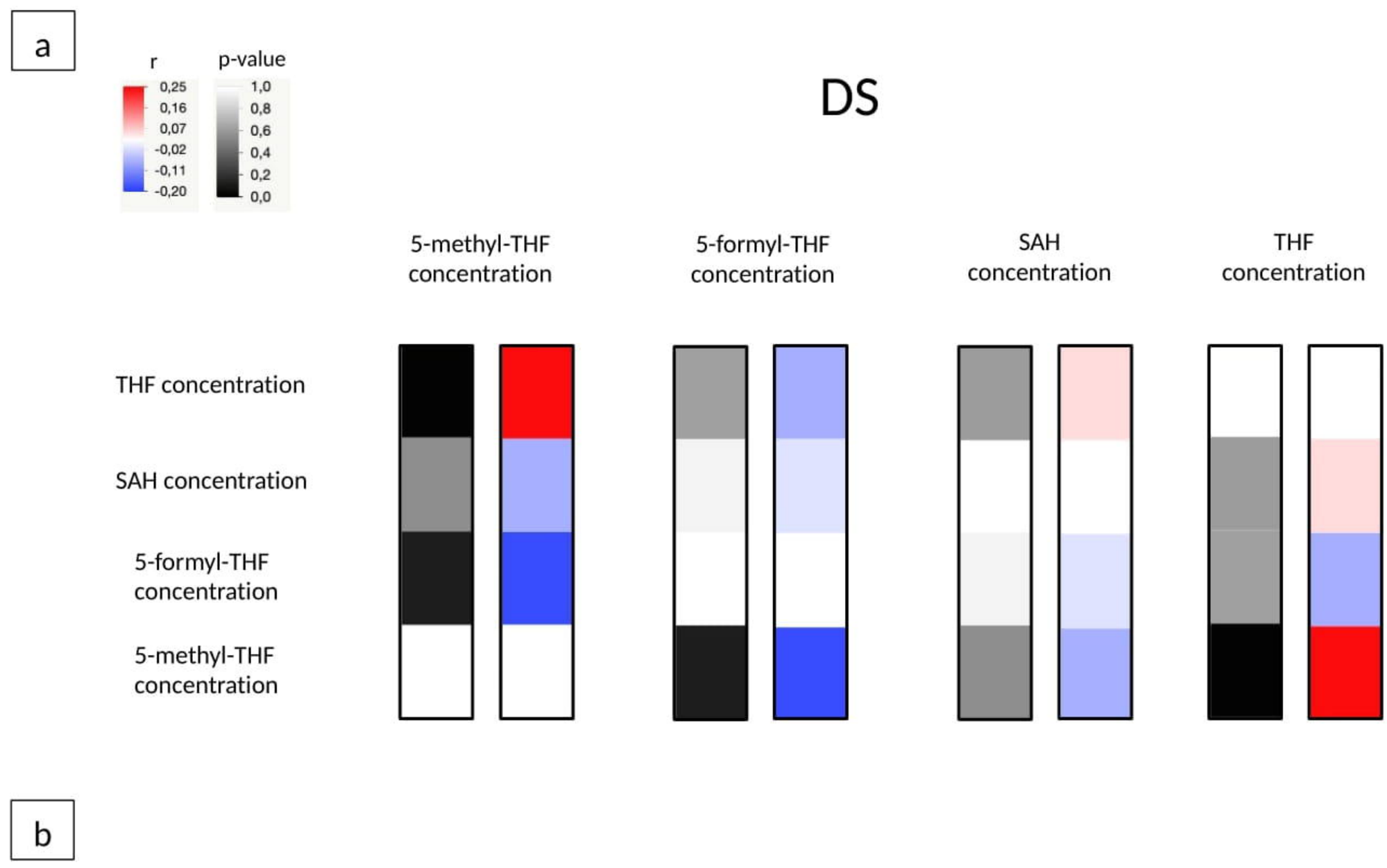
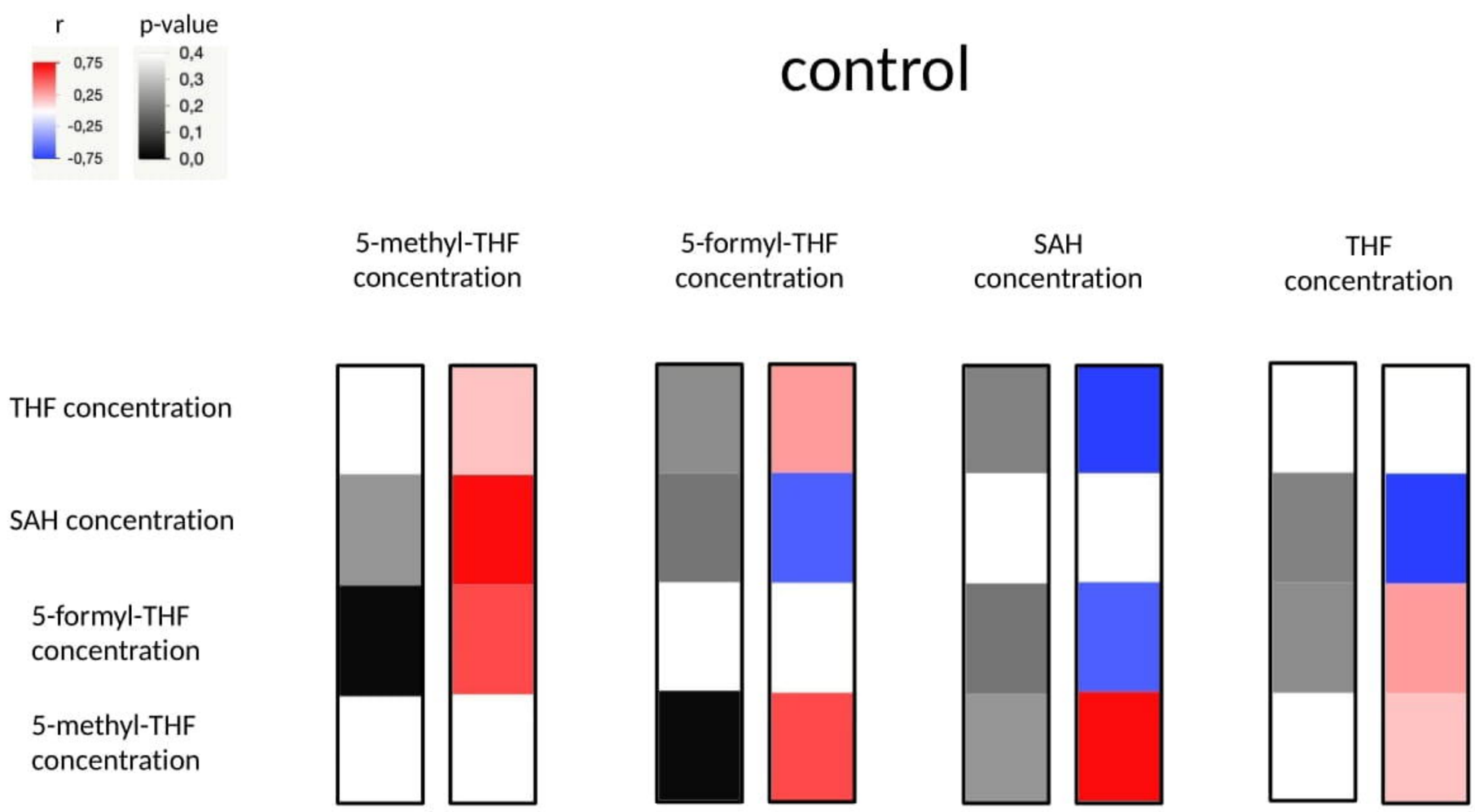


Supplementary Figure 1. 5-formyl-THF concentration in subjects with DS and normal control subjects including strong outliers. The graph reported 5-formyl-THF plasma concentrations of each subject in the study. On the x-axis there is the subdivision of subjects in DS and control groups. Subjects with DS are represented like red dots and normal control subjects are represented like blue dots. On the y-axis the concentration of the metabolite in ng/mL is reported. The middle black lines indicate the mean concentration values for each group and the external black lines indicate standard deviation (SD) values. The mean concentration, SD values and the number of subjects (n) are reported below the graph for DS and control groups. The difference in concentration of this metabolite is statistically significant (*=$p<0.05$). The graph was created with GraphPad Prism software v.6.0 (San Diego, CA).





Supplementary Figure 2. Heat Map figure of bivariate correlation between levels of each metabolite and levels of all the other metabolites including strong outliers. Supplementary Figure 2a presents bivariate correlation in DS group and Supplementary Figure 2b presents bivariate correlation in control group (for complete data see Supplementary Table 10a). At the top left of the figures the color codes for Pearson correlation coefficient (r) and two tailed-significance (p-value) are reported. For each metabolite indicated above, two bars are shown: the first represents p-values and the second r values. Every bar is divided in four segments corresponding to the four correlations with other metabolites.