

## Predisposition to atherosclerosis in children and adults with trisomy 21: biochemical and metabolomic studies

Predyspozycje do miażdżycy u dzieci i dorosłych z trisomią 21: badania biochemiczne i metabolomiczne

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### Abstract

**Introduction:** Atherosclerosis, a precursor to cardiovascular disease (CVD), is deeply intertwined with lipid metabolism. The metabolic process in the Down syndrome (DS) population remain less explored.

**Aim of the study:** This study examines the lipid profiles of DS in comparison to their siblings (CG), aiming to uncover potential atherosclerotic and CVD risks.

**Material and methods:** The study included 42 people with DS (mean age 14.17 years) and the CG – 20 individuals (mean age 15.92 years). Anthropometric measurements: BMI, BMI SDS, and TMI were calculated. Lipid profile (LP) and metabolomics were determined.

**Results:** LP: DS display significantly reduced HDL (DS vs. CG:  $47 \pm 10$  vs.  $59 \pm 12$  mg/dl;  $p = 0.0001$ ) and elevated LDL ( $104 \pm 25$  vs.  $90 \pm 22$  mg/dl;  $p = 0.0331$ ). Triglycerides, APO A1, and APO B/APO A1 ratio corroborate with the elevated risk of CVD in DS. Despite no marked differences in: TCH and APO B, the DS group demonstrated a concerning BMI trend. Of 31 identified metabolites, 12 showed statistical significance (acetate, choline, creatinine, formate, glutamine, histidine, lysine, proline, pyroglutamate, threonine, tyrosine, and xanthine). However, only 8 metabolites passed the FDR validation (acetate, creatinine, formate, glutamine, lysine, proline, pyroglutamate, xanthine).

**Conclusions:** Down syndrome individuals show distinct cardiovascular risks, with decreased HDL and increased LDL levels. Combined with metabolomic disparities and higher BMI and TMI, this suggests an increased atherosclerosis risk compared to controls.

**Key words:** atherosclerosis, cardiovascular disease, lipid profile, Down syndrome, metabolomics.

### Introduction

Atherosclerosis is a chronic inflammatory disease, marked by the accumulation of lipids and fibrous materials within arterial walls, leading to cardiovascular disease (CVD) – one of the major health concerns worldwide. The development of this disease is influenced by a combination of genetic factors, environmental exposures, and various metabolic processes. Notably, lipid metabolism plays a significant role. Excessive levels of low-density lipoprotein cholesterol (LDL), known as “bad cholesterol”, accumulate in arterial walls, forming the basis of atherosclerotic plaque [1]. Conversely, high-density lipoprotein (HDL), termed “good cholesterol”, helps in removing chole-

sterol from arterial plaques, directing it towards elimination from the body [2]. The relationship between apolipoprotein A1 (APO A1) and apolipoprotein B (APO B) is crucial in understanding atherosclerotic risk [2]. Besides, emerging studies have highlighted the roles of homocysteine (HCY) and lipoprotein(a) (LP(a)) in vascular health, given their correlation with arterial damage and atherosclerosis [3, 4].

Metabolomics, which focuses on identifying and quantifying small molecules in biological samples, has become instrumental in cardiovascular research [5–12]. Through metabolomics, researchers can gain a clearer picture of the numerous metabolic processes taking place at a cellular level. In the context of atherosclerosis, this approach has identified new path-

ways and molecules of interest [13–16]. Specific metabolites, including acetate, choline, and xanthine, might offer insights into the relationship between Down syndrome (DS) metabolism and atherosclerosis [17–19].

There's a notable gap in our understanding when considering unique populations like those with trisomy 21 (DS). Historically, individuals with DS display a reduced incidence of atherosclerosis [20, 21], but the metabolic reasons behind this remain elusive.

This study seeks to fill the knowledge gap by providing a comprehensive evaluation of the lipid profiles and related metabolites in individuals with DS. We aimed to discern whether the DS population (study group; DS) is at a heightened risk for atherosclerosis and, by extension, CVD, by comparing their lipid and metabolomic profiles to a control group (CG) – their siblings.

## Material and methods

### *Study design and participants*

The participants comprised a study group (DS) of 42 children and adolescents with DS (17 females; mean age of 14.17 ± 7 years old) and the control group (CG) of 20 siblings of the individuals with DS (10 females; mean age of 15.92 ± 9 years old). The study protocol adhered to the principles of the Declaration of Helsinki, and the Bioethics Committee of Wrocław Medical University approved it (KB 674/2020). The inclusion criteria for the DS were patients diagnosed with DS (due to genetic test results) while for the CG, it was being a sibling of an individual DS. There were no specific exclusion criteria. Written informed consent was obtained from the parents or legal guardians of all the participants before the data collection and anthropometric measurements. Administrative approvals were also secured from each institution involved to access the necessary participants' data.

### *Data collection*

Participants were actively recruited from various regions across Poland. Upon receiving written informed consent from the parents or guardians, these recruited children and adolescents were invited for an anthropometric examination and blood collection in a pediatric clinic located in Wrocław, Poland.

### *Anthropometric measurements*

The methodology and procedures for anthropometric measurements have been detailed in our prior research [22].

### *BMI Standard Deviation Score (SDS) and Tri-Ponderal Mass Index (TMI) Calculation*

BMI was converted into SDS in line with our previously outlined methodologies [20]. The TMI was calculated according to the methodology described by Peterson *et al.* [23]. For this study, the threshold to determine elevated TMI was set at the 95<sup>th</sup> percentile – specifically, values surpassing 18.8 kg/m<sup>3</sup> were considered elevated for boys, while for girls, the 95<sup>th</sup> percentile value was set at 19.7 kg/m<sup>3</sup> [23]. Before proceeding

with the TMI analysis, participants aged below 7 years were excluded from the dataset. This led to the exclusion of nine individuals: 6 from the DS and 3 from the CG. Upon extending the analysis of the TMI to a larger cohort, we incorporated participants older than 18 years by categorizing them within the 17-year-old bracket. This methodological choice was made to expand our sample size and assess broader patterns without the age restrictions strictly imposed by the traditional TMI guidelines [23].

### *Biochemical tests*

All laboratory parameters were determined under fasting conditions. For lipid profile routine laboratory methods were used. APO A1 and APO B were measured by immunoturbidimetric method with goat anti-human apolipoprotein A1 antibody and anti-human apolipoprotein B antibody, respectively, whereas for Lp(a) determination particle enhanced immunoturbidimetric test was applied. Homocystein was measured with Diazyme Laboratories, Poway, USA reagent kit on the Konelab 20i analyser (ThermoScientific, Vantta, Finland). The research results were analyzed according to the established reference ranges presented in Table I.

### *Metabolomic tests*

#### *Samples*

Metabolomic studies were conducted at the Department of Biochemistry, Molecular Biology, and Biotechnology, Faculty of Chemistry, Wrocław University of Science and Technology. Serum samples were received from the Medical University of Wrocław and were stored at –80°C before analysis.

#### *Samples preparation*

The volume of 300 µl serum was transferred into the new Eppendorf tube. To each sample, 600 µl of methanol was added. After 1 min mixing, the samples were stored at –20°C for 20 min and centrifugated (15 min, 11000 rpm, 4°C). Next, 750 µl of supernatant was transferred into the new Eppendorf tube, and samples were evaporated (1100 rpm, 4.5 h, 40°C). After that, 600 µl of PBS buffer (0.5 M, 50% D<sub>2</sub>O, pH = 7.4, TSP = 0.03 mM) were added to each sample and mixed for 3 min. After centrifugation (5 min, 11000 rpm, 4°C), 550 µl of solution were transferred into 5-mm NMR tubes (Norell®) for measurements. Before analysis, the samples were stored at 4°C.

#### *<sup>1</sup>H NMR spectroscopy analysis of the bacterial metabolites*

Standard <sup>1</sup>H NMR experiments were performed on a Bruker AVANCE II 600.58 MHz spectrometer equipped with a 5 mm NMR probe at 298 K. All one-dimensional <sup>1</sup>H NMR spectra were carried out using the noesy1dpr (in Bruker notation) pulse sequence by suppression of water resonance by presaturation. Acquisition parameters were as follows: spectral width, 19.82 ppm; the number of scans, 128; acquisition time, 2.75 s per scan; relaxation delay, 3.5 s; and time-domain points, 65.5 K. The spectra were referenced to the TSP resonance at 0.0 ppm and manually corrected for phase and baseline (Mes- tReNova v. 14.0.2).

**Table I.** Reference ranges used for result interpretation

Parameter	Age/Sex	Below norm	Norm	Above norm
TCH [mg/dl]	Children	×	< 170	≥ 170
	Adults	×	< 200	≥ 200
HDL [mg/dl]	Children	≤ 35	> 35	×
	Adults (women)	< 50	≥ 50	×
	Adults (men)	< 40	≥ 40	×
LDL [mg/dl]	Children	×	< 110	≥ 110
	Adults		< 100	≥ 100
TG [mg/dl]	Children	×	< 100	≥ 100
	Adults	×	< 150	≥ 150
HCY [umol/L]	x	×	< 15	≥ 15
APO A1 [mg/dl]	Women	< 108	108-125	> 125
	Men	< 104	104-202	> 202
APO B [mg/dl]	x	< 50	50-150	> 150
Lp(a)	x		< 30	≥ 30
APO B/APO A1	Women	×	< 0.8	≥ 0.8
	Men	×	< 0.9	≥ 0.9

TCH – total cholesterol; HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides; APO A1 – apolipoprotein A1; APO B – apolipoprotein B; HCY – homocysteine; Lp(a) – lipoprotein(a); APO B/APO A1 – ratio.

*Data processing and multivariate statistical data analysis*

Data analysis was performed utilizing Statistica version 13 software. The normality of the data distribution was assessed using the Shapiro-Wilk test. Subsequent analyses were guided by the results of this normality test. In instances where the data followed a normal distribution, the Student's t-test was employed. Alternatively, when data exhibited a non-normal distribution, the Mann-Whitney U test was utilized. To identify relationships between variables, correlation matrices were created. Pearson's correlation was employed for data with a normal distribution, whereas Spearman's correlation was used for data with a non-normal distribution. The level of statistical significance was set at  $p < 0.05$ . All spectra were exported to Matlab (Matlab v. 8.3.0.532) for preprocessing. Regions affected by solvent suppression were excluded (4.7–5.1 ppm) and alignment procedures involving the correlation of optimized warping (COW) and interval correlation shifting (icoshift) algorithms were applied [24, 25]. The spectra consisted of 8910 data points and were normalized using the probabilistic quotient method to overcome the issue of dilution [26]. The multivariate and statistical data analysis were performed on a set of the 30 as-

signed metabolites (methanol wasn't included in the analysis, because it was used for the extraction). The concentration of metabolite measured by NMR was obtained as the sum of the intensities of the no-overlapping resonances (or a part of partly overlapping resonances). The input for SIMCA-P software was a transformed data matrix (v 17.0.2, Umetrics, Umeå, Sweden). The data sets were unit variance scaled before the chemometric analysis. For T21 samples, principal component analysis (PCA), and partial least square analysis (OPLS) were carried out. The OPLS-DA model reliability was tested with CV-ANOVA at the level of significance of  $\alpha < 0.05$ . Univariate analysis was performed by the use of MATLAB software (v R2014a, Mathworks Inc.) by use of Student's t-test (equal/unequal variance) for data originated from a normal distribution, and Mann-Whitney-Wilcoxon test was performed for data that does not meet these requirements. The normality of distribution was assessed by the Shapiro-Wilk test. The Student's t-test was performed for metabolites recurring in both samples. The correction for multiple comparisons was preceded by the Benjamini-Hochberg procedure (FDR). All univariate statistics were carried out at the level of significance of  $\alpha < 0.05$ .

## Results

### Anthropometric data

**BMI:** The DS demonstrated a higher mean BMI and BMI SDS than the CG. Despite the absence of significant statistical disparities, it's imperative to highlight the contrast in overweight and obesity prevalence: about half of the DS group compared to just 20% in the CG. This accentuates the prominent weight concerns in the DS group. For a comprehensive view, refer to Tables II and IV.

**TMI:** The mean TMI for the DS was significantly higher than the CG ( $16.22 \pm 4.04 \text{ kg/m}^3$  vs.  $13.67 \pm 1.59 \text{ kg/m}^3$ ;  $p = 0.02$ ) emphasizing a potential concern regarding body fat distribution within the DS compared to the CG.

### Biochemical tests

Basic descriptive statistics are presented in Table II. Percentage data regarding specific parameters in both groups are presented in Table III.

**HDL:** The DS displayed a significantly lower concentration compared to the CG ( $46.88 \pm 10.37 \text{ mg/dl}$  vs.  $59.19 \pm 12.28 \text{ mg/dl}$ ;  $p = 0.0001$ ). **Triglycerides (TG):** The DS had a noticeably elevated median TG level than the CG ( $87.02 \text{ mg/dl}$  vs.  $63.34 \text{ mg/dl}$ ;  $p = 0.0588$ ). **LDL:** A heightened concentration was observed in the DS when juxtaposed with the CG ( $104.30 \pm 25.41 \text{ mg/dl}$  vs.  $89.78 \pm 22.43 \text{ mg/dl}$ ;  $p = 0.0331$ ). **APO A1:** The DS exhibited a markedly lower concentration than the CG ( $119.64 \pm 20.17 \text{ mg/dl}$  vs.  $158.21 \pm 40.61 \text{ mg/dl}$ ;  $p = 0.000005$ ).

**Other parameters:** Statistical examination revealed no significant contrasts between DS and CG for **TCH** ( $p = 0.3747$ ); **APO B** ( $p = 0.1612$ ); **HCY** ( $p = 0.5320$ ); **Lp(a)** ( $p = 0.3466$ ), **APO B/APO A1 Ratio** ( $p = 0.3466$ ).

### Differential correlations between DS and CG

The correlation patterns among the various parameters can be found in Table V.

**Homocysteine:** Present in DS with correlations to body metrics, absent in CG; **HDL:** In DS, mainly correlated with APO

**Table II.** Comparative parameters analyzed for the DS and CG

Parameter	DS (n = 42)			CG (n = 20)			p-value
	Mean	Median	SD	Mean	Median	SD	
Age [years]	14.17	14.50	6.7	15.92	15.25	8.58	0.3804
Body weight [kg]	44.57	48.75	19.3	49.52	51.35	23.80	0.3852
Body height [m]	1.37	1.42	0.2	1.50	1.59	0.26	<b>0.0377</b>
BMI [kg <sup>2</sup> /m]	22.20	21.70	5.9	20.20	20.79	5.36	0.2006
BMI SDS	0.79	1.01	1.6	-1.19	0.23	7.91	0.1264
BMI PC	70.56	84.34	31.4	60.97	58.78	29.67	0.1283
TCH [mg/dl]	182.08	180.47	34.5	173.64	174.27	35.15	0.3747
HDL [mg/dl]	46.99	47.08	10.4	59.19	61.62	12.28	<b>0.0001</b>
LDL [mg/dl]	104.30	101.41	25.4	89.78	90.05	22.43	<b>0.0331</b>
TG [mg/dl]	97.57	87.02	53.4	68.42	63.34	27.58	<b>0.0588</b>
APOA1 [mg/dl]	119.64	119.14	20.2	158.21	149.69	40.61	<b>0.0000</b>
APOB [mg/dl]	67.40	64.18	16.8	61.08	63.60	15.52	0.1612
HCY [umol/l]	9.60	9.40	2.6	10.17	9.64	3.07	0.5320
Lp(a) [mg/dl]	22.63	7.66	33.8	21.24	4.27	38.39	0.3466
APOB/APOA1	0.58	0.54	0.2	0.39	0.40	0.10	0.3466
TMI [kg/m <sup>3</sup> ]	16.22	15.22	4.0	13.67	13.30	1.59	<b>0.0200</b>

DS – study group; CG – control group; N – amount of participants; SD – standard deviation; BMI – body mass index; SDS – standard deviation scores; PC – percentiles; TCH – total cholesterol; HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides; PO A1 – apolipoprotein A1; APO B – apolipoprotein B; HCY – homocysteine; Lp(a) – lipoprotein(a); APO B/APO A1 – ratio; TMI – tri-ponderal mass index

**Table III.** Group demographic characteristics

Group	TCH (%)	HDL (%)	LDL (%)	TG (%)	APOA1 (%)	APOB (%)	HCY (%)	Lp(a) (%)	APOB/APOA1 (%)
DS									
Below norm	×	12.5	×	10	21.95	12.20	×	×	×
Norm	51.22	87.5	46.34	90	78.05	87.80	97.56	80.49	90.244
Above norm	48.78	×	53.66	×	×	×	2.44	19.51	9.756
CG									
Below norm	×	10	×	×	5	10	×	×	×
Norm	80	90	90	95	90	90	95	80	100
Above norm	20	×	10	5	5	×	5	20	×

DS – study group; CG – control group; PC – percentile; TCH – total cholesterol; HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides; APO A1 – apolipoprotein A1; APO B – apolipoprotein B; HCY – homocysteine; Lp(a) – lipoprotein(a); APO B/APO A1 – ratio

**Table IV.** Percentage distribution of participants in the study and control groups across different BMI categories

Group	N	< 3 <sup>rd</sup> PC		3–90 <sup>th</sup> PC		90–97 <sup>th</sup> PC		> 97 <sup>th</sup> PC	
		Underweight		Normal weight		Overweight		Obese	
		n	%	n	%	n	%	n	%
Study	42	2	4,76	21	50	11	26,19	8	19,05
Control	20	1	5	15	75	3	15	1	5

PC – percentile

A1; in CG, correlated with age and BMI; **TCH:** DS had multiple correlations (e.g., LDL, APO B, Lp(a)); CG's were fewer and included age; **APO B:** In DS, primarily linked to lipids; in CG, showed broader links including age and body metrics; **APO A1:** DS correlations centered around lipids; in CG, they were more diverse, involving HDL, TCH, TG, LDL, APO B; **LDL:** DS showed correlations including APO B and Lp(a); CG had fewer, like age; **APO B/APO A1 Ratio:** In DS, both positive and negative correlations were seen, including TCH and BMI metrics. In CG, it was mainly linked to Lp(a).

#### Metabolomics

In total, 31 metabolites were identified (3-hydroxybutyrate, acetate, acetone, alanine, choline, creatinine, ethanol, formate, glucose, glutamine, glycine, histidine, isobutyrate, isoleucine, lactate, leucine, lysine, methionine, methylamine, oxypurinol, phenylalanine, proline, pyroglutamate, pyruvate, succinate, threonine, tryptophan, tyrosine, valine, xanthine, methanol).

All assignments were verified using the following databases (HMDB). Information about a chemical shift for each metabolite is available in Table VI. The representative <sup>1</sup>H NMR spectrum is presented below (Fig. 1).

#### Metabolites relative concentration and statistical analysis

For each metabolite, relative concentration and standard deviation were obtained. Statistical analysis was performed to obtain statistically important metabolites. Two groups were compared – Study Group (DS; 39 samples) and the Control Group (CG; 20 samples) (Table VI). The results showed that 12 metabolites were statistically important according to *p*-value (acetate, choline, creatinine, formate, glutamine, histidine, lysine, proline, pyroglutamate, threonine, tyrosine, and xanthine), while only 8 metabolites were statistically important according to FDR results, where choline, tyrosine, threonine, and histidine didn't pass the Benjamini-Hochberg procedure

**Table V.** Correlation matrices in DS and CG

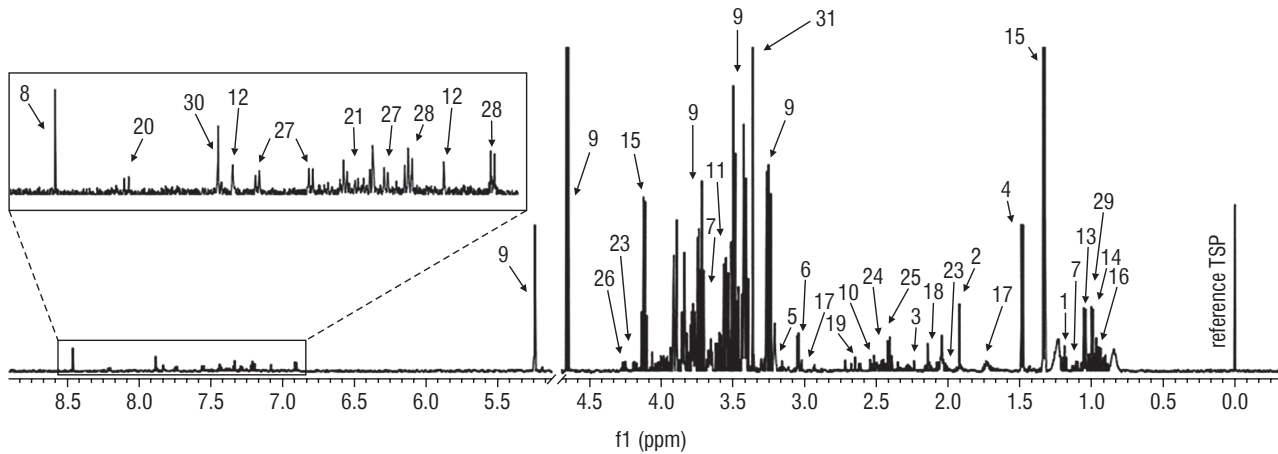
Age [years]	Body weight [kg]	Body height [m]	BMI [kg/m <sup>2</sup> ]	BMI SDS	BMI PC	HDL [mg/dl]	TCH [mg/dl]	TG [mg/dl]	LDL [mg/dl]	APO A1 [mg/dl]	APO B [mg/dl]	HCY [μmol/L]	Lp(a) [mg/dL]	APO
SG	<b>0.8136</b>	<b>0.7702</b>	<b>0.6346</b>	0.3048	0.3042	0.0164	0.0797	-0.2599	0.1028	-0.0533	0.0776	B/APO	0.0789	0.1083
Body weight [kg]	<b>0.8136</b>	<b>0.8649</b>	<b>0.8246</b>	<b>0.5697</b>	<b>0.6195</b>	-0.0973	0.0726	-0.0211	0.0805	-0.1658	0.0437	<b>0.3422</b>	0.0599	0.1470
Body height [m]	<b>0.7702</b>	<b>0.8649</b>	<b>0.4637</b>	0.2493	<b>0.3850</b>	-0.0899	0.1431	0.0064	0.1477	-0.0871	0.0381	0.2624	0.0252	0.0843
BMI [kg/m <sup>2</sup> ]	<b>0.6346</b>	<b>0.8246</b>	<b>0.4637</b>	<b>0.7808</b>	<b>0.7187</b>	-0.0816	-0.0769	-0.1017	-0.0570	-0.1777	-0.0369	<b>0.3103</b>	0.0221	0.0941
BMI SDS	0.3048	<b>0.5697</b>	0.2493	<b>0.7808</b>	<b>0.9044</b>	-0.1521	-0.1938	0.0694	-0.1401	<b>-0.3901</b>	-0.1233	<b>0.3929</b>	0.0186	0.1269
BMI PC	0.3042	<b>0.6195</b>	<b>0.3850</b>	<b>0.7187</b>	<b>0.9044</b>	-0.2572	-0.0361	0.0662	0.0172	<b>-0.3552</b>	-0.0078	<b>0.3916</b>	0.0127	0.2014
HDL [mg/dl]	0.0164	-0.0973	-0.0899	-0.0816	-0.1521	-0.2572	0.0694	-0.2596	-0.1167	<b>0.7767</b>	-0.1838	-0.0406	-0.1681	<b>-0.6457</b>
TCH [mg/dl]	0.0797	0.0726	0.1431	-0.0769	-0.1938	-0.0361	0.0694	<b>0.4511</b>	<b>0.9695</b>	<b>0.3575</b>	<b>0.8622</b>	0.0605	<b>0.4036</b>	<b>0.5318</b>
TG [mg/dl]	-0.2599	-0.0211	0.0064	-0.1017	0.0694	0.0662	-0.2596	<b>0.4511</b>	<b>0.4158</b>	-0.0446	<b>0.4307</b>	0.0798	0.2477	<b>0.4676</b>
LDL [mg/dl]	0.1028	0.0805	0.1477	-0.0570	-0.1401	0.0172	-0.1167	<b>0.9695</b>	<b>0.4158</b>	0.1857	<b>0.9139</b>	0.1007	<b>0.4480</b>	<b>0.6756</b>
APO A1 [mg/dl]	-0.0533	-0.1658	-0.0871	-0.1777	<b>-0.3901</b>	<b>-0.3552</b>	<b>0.7767</b>	-0.0446	0.1857	0.1252	0.0126	-0.0066	-0.0066	<b>-0.5088</b>
APO B [mg/dl]	0.0776	0.0437	0.0381	-0.0369	-0.1233	-0.0078	-0.1838	<b>0.4307</b>	<b>0.9139</b>	0.1252	0.1354	0.1354	<b>0.4840</b>	<b>0.7737</b>
HCY [μmol/l]	0.1516	<b>0.3422</b>	0.2624	<b>0.3103</b>	<b>0.3929</b>	<b>0.3916</b>	-0.0406	0.0798	0.1007	0.0126	0.1354	0.2352	0.2352	0.1252
Lp(a) [mg/l]	0.0789	0.0599	0.0252	0.0221	0.0186	0.0127	-0.1681	<b>0.4036</b>	<b>0.4480</b>	-0.0066	<b>0.4840</b>	0.2352	0.2352	0.1672
APO B/APO A1	0.1083	0.1470	0.0843	0.0941	0.1269	0.2014	<b>-0.6457</b>	<b>0.5318</b>	<b>0.4676</b>	<b>-0.5088</b>	<b>0.7737</b>	0.1252	0.1252	0.1672
CG														
Age [years]	<b>0.6812</b>	<b>0.7840</b>	<b>0.5701</b>	-0.0526	-0.0526	-0.0526	<b>0.4963</b>	<b>0.5197</b>	-0.0658	0.3489	<b>0.6922</b>	0.2117	0.0677	0.3698
Body weight [kg]	<b>0.6812</b>	<b>0.7840</b>	<b>0.5701</b>	<b>0.9025</b>	0.4415	0.4415	0.3679	0.3467	0.2745	0.2546	<b>0.5246</b>	0.3556	-0.0903	0.3225
Body height [m]	<b>0.7840</b>	<b>0.9440</b>	<b>0.8206</b>	0.2385	0.2385	0.2385	0.4189	0.3961	0.2377	0.2568	<b>0.5701</b>	0.4066	-0.0143	0.3849
BMI [kg/m <sup>2</sup> ]	<b>0.5701</b>	<b>0.9025</b>	<b>0.8206</b>	<b>0.6767</b>	<b>0.6767</b>	<b>0.6767</b>	<b>0.4776</b>	0.2446	0.0961	0.2140	0.3563	0.2843	-0.0797	0.1798
BMI SDS	-0.0526	0.4415	0.2385	<b>0.6767</b>	1.0000	1.0000	0.1759	0.2060	-0.0902	-0.0331	0.1624	0.0015	-0.2586	-0.0638
BMI PC	-0.0526	0.4415	0.2385	<b>0.6767</b>	1.0000	1.0000	0.1759	0.2060	-0.0902	-0.0331	0.1624	0.0015	-0.2586	0.0822
HDL [mg/dl]	<b>0.4963</b>	0.3679	0.4189	<b>0.4776</b>	0.1759	0.1759	<b>0.6143</b>	0.1160	0.3681	<b>0.7091</b>	0.4012	-0.0960	0.0677	-0.2483
TCH [mg/dl]	<b>0.5197</b>	0.3467	0.3961	0.2446	0.2060	0.2060	<b>0.6143</b>	<b>0.4445</b>	<b>0.9469</b>	<b>0.7603</b>	<b>0.8361</b>	-0.1313	0.2120	0.2289
TG [mg/dl]	-0.0658	0.2745	0.2377	0.0961	-0.0902	-0.0902	0.1160	<b>0.4445</b>	0.3561	<b>0.4978</b>	0.2937	-0.0576	0.0346	-0.1000
LDL [mg/dl]	<b>0.4845</b>	0.2376	0.3059	0.0697	-0.0331	-0.0331	0.3681	<b>0.9469</b>	0.3561	<b>0.5977</b>	<b>0.8589</b>	-0.1178	0.3534	0.4216
APO A1 [mg/dl]	0.3489	0.2546	0.2568	0.2140	-0.0316	-0.0316	<b>0.7091</b>	<b>0.7603</b>	<b>0.4978</b>	<b>0.5977</b>	<b>0.5508</b>	-0.3302	0.2947	-0.3450
APO B [mg/dl]	<b>0.6922</b>	<b>0.5246</b>	<b>0.5701</b>	0.3563	0.1624	0.1624	<b>0.8361</b>	0.2937	<b>0.8589</b>	<b>0.5508</b>	<b>0.5508</b>	-0.0628	0.4376	<b>0.5651</b>
HCY [μmol/l]	0.2117	0.3556	0.4066	0.2843	0.0015	0.0015	-0.0960	-0.1313	-0.0576	-0.1178	-0.3302	-0.0628	-0.2121	0.2965
Lp(a) [mg/l]	0.0677	-0.0903	-0.0143	-0.0797	-0.2586	-0.2586	0.0677	0.2120	0.0346	0.3534	0.2947	0.4376	-0.2121	<b>0.5837</b>
APO B/APO A1	0.3698	0.3225	0.3849	0.1798	-0.0638	0.0822	-0.2483	0.2289	-0.1000	0.4216	-0.3450	0.2965	<b>0.5837</b>	

DS – study group; CG – control group; BMI – body mass index; SDS – standard deviation scores; PC – percentiles; TCH – total cholesterol; HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides; APO A1 – apolipoprotein A1; APO B – apolipoprotein B; HCY – homocysteine; Lp(a) – lipoprotein(a); APO B/APO A1 – ratio

**Table VI.** Statistical analysis of metabolite levels: distinguishing between Study Group (DS) and Control Group (CG) using  $p$ -value and FDR criteria

Metabolite	$p$ -value	FDR	Mean/ Median DS	Mean/ Median DS	RSD DS [%]	SD DS	RSD CG [%]	SD CG
Creatinine	1.67E-07	5.01E-06	0.111	0.203	24.69	0.027	26.30	0.053
Pyroglutamate	6.10E-07	9.15E-06	0.231	<b>0.677</b>	14.19	0.033	30.58	0.207
Glutamine	1.75E-06	1.75E-05	1.075	<b>0.120</b>	12.39	0.133	28.54	0.034
Formate	8.21E-06	5.31E-05	<b>2.246</b>	0.153	15.38	0.345	14.18	0.022
Proline	8.85E-06	5.31E-05	<b>1.630</b>	<b>2.033</b>	22.65	0.369	45.64	0.928
Acetate	1.28E-05	6.40E-05	<b>0.115</b>	<b>0.208</b>	25.57	0.029	38.83	0.081
Xanthine	3.30E-05	1.41E-04	0.117	0.143	15.61	0.018	17.57	0.025
Lysine	1.97E-04	7.38E-04	<b>0.125</b>	0.276	19.68	0.025	18.80	0.052
Choline	1.82E-02	6.06E-02	<b>5.280</b>	1.997	26.47	1.398	22.63	0.452
Tyrosine	2.07E-02	6.20E-02	0.224	<b>0.789</b>	15.28	0.034	15.66	0.124
Threonine	2.55E-02	6.95E-02	<b>1.041</b>	0.308	22.51	0.234	21.59	0.067
Histidine	4.21E-02	1.05E-01	0.152	0.134	19.66	0.030	24.15	0.032
Acetone	7.95E-02	1.84E-01	<b>0.692</b>	<b>0.687</b>	60.33	0.418	155.21	1.066
Succinate	1.30E-01	2.79E-01	0.249	<b>0.292</b>	43.86	0.109	34.91	0.102
Ethanol	1.48E-01	2.96E-01	0.528	0.503	10.83	0.057	14.48	0.073
Valine	2.27E-01	4.25E-01	1.140	<b>0.904</b>	15.30	0.174	20.03	0.181
Pyruvate	2.80E-01	4.94E-01	<b>0.326</b>	<b>0.271</b>	31.84	0.104	22.55	0.061
Isoleucine	3.11E-01	5.18E-01	0.328	0.308	22.31	0.073	20.37	0.063
3-hydroxybutyrate	3.46E-01	5.19E-01	0.148	0.142	16.56	0.025	17.25	0.024
Methionine	3.33E-01	5.19E-01	0.132	<b>0.100</b>	16.18	0.021	24.70	0.025
Glucose	3.66E-01	5.22E-01	<b>0.267</b>	5.208	15.88	0.042	14.30	0.745
Oxypurinol	3.83E-01	5.22E-01	<b>0.101</b>	<b>0.149</b>	33.28	0.033	28.64	0.043
Phenylalanine	4.04E-01	5.26E-01	0.264	0.274	14.78	0.039	18.52	0.051
Lactate	5.59E-01	6.99E-01	<b>0.129</b>	16.235	36.54	0.047	25.42	4.127
Isobutyrate	7.79E-01	8.71E-01	0.708	<b>0.186</b>	18.04	0.128	22.79	0.042
Methylamine	7.84E-01	8.71E-01	0.053	0.054	23.50	0.012	29.45	0.016
Tryptophan	7.78E-01	8.71E-01	0.150	0.152	18.82	0.028	17.31	0.026
Alanine	9.46E-01	9.46E-01	2.282	2.289	17.22	0.393	19.48	0.446
Glycine	9.04E-01	9.46E-01	<b>1.148</b>	<b>1.083</b>	22.13	0.254	21.98	0.238
Leucine	9.30E-01	9.46E-01	0.937	<b>0.138</b>	17.90	0.168	17.82	0.025

DS – study group; CG – control group



**Figure 1.** The representative 1D  $^1\text{H}$  NMR spectra of T21 sample (1 – 3-hydroxybutyrate, 2 – acetate, 3 – acetone, 4 – alanine, 5 – choline, 6 – creatinine, 7 – ethanol, 8 – formate, 9 – glucose, 10 – glutamine, 11 – glycine, 12 – histidine, 13 – isobutyrate, 14 – isoleucine, 15 – lactate, 16 – leucine, 17 – lysine, 18 – methionine, 19 – methylamine, 20 – oxypurinol, 21 – phenylalanine, 22 – proline, 23 – pyroglutamate, 24 – pyruvate, 25 – succinate, 26 – threonine, 27 – tryptophan, 28 – tyrosine, 29 – valine, 30 – xanthine, 31 – methanol)

## Discussion

The findings of this study provide an elucidative insight into the biochemical and metabolic parameters that might predispose children and young adults with DS to atherosclerosis and subsequent CVD.

### *BMI implications on health profiles*

The DS group displayed a higher tendency towards overweight and obesity compared to the CG group, a trend linked to considerable health risks including cardiovascular and metabolic issues [27, 28]. The heightened prevalence of increased weight in the DS population points to their potential vulnerability to related health complications [29, 30]. In contrast, the CG group showed a more balanced BMI, possibly due to a mix of metabolic, genetic, and lifestyle factors [31]. It is critical to recognize that being underweight, although less common in both groups, can also lead to health issues [32]. To analyze adolescent body fat distribution more accurately, the study introduced the TMI metric [23]. This method suggests the DS group might be prone to dangerous fat distribution patterns, indicative of greater health risks including visceral fat accumulation. The findings emphasize the need for targeted interventions and monitoring strategies to counter potential health threats.

### *HDL-cholesterol and its cardiovascular implications*

The DS group exhibited notably lower HDL cholesterol levels compared to their CG siblings, potentially increasing their risk of cardiac issues due to the associated higher likelihood of developing atherosclerosis, a precursor to CVD [33]. However, the HDL distribution was similar between the two groups, suggesting that both groups might still benefit from the cardiovascular protective features generally associated with optimal HDL properties. It's important to consider not just the HDL

concentration but also its functionality, which includes aspects like particle size and antioxidant enzymes, in assessing its cardioprotective potential [34, 35].

### *LDL-cholesterol: atherogenic risk*

The DS group showed a significant increase in LDL levels compared to the CG group, which is worrisome given that LDL-cholesterol is a well-established indicator for cardiovascular risk and even slight elevations can substantially increase this risk over time [36, 37]. These elevated levels could mean a higher susceptibility to arterial plaque build-up and consequently, a greater risk of CVD [38]. The observed discrepancy prompts further exploration into the metabolic or genetic factors in individuals with DS that might be causing these higher LDL levels, indicating a potential predisposition to atherosclerotic developments [39].

### *Triglycerides: a borderline concern*

While our study reveals an approaching significance in TG levels between the DS and CG. Elevated TG levels have been universally recognized as a marker for cardiovascular risk [40]. In the DS population, understanding the particular significance of these levels is crucial due to their unique metabolic and genetic profiles. A seminal paper by Capone *et al.* [39] highlighted the increased frequency of congenital heart disease among individuals with DS. With such a pre-existing predisposition, even borderline variations in TG levels can compound potential risks.

### *Balancing atherogenic and anti-atherogenic particles*

The DS group exhibited significantly lower APO A1 levels compared to the CG group, which corroborates with the reduced HDL cholesterol findings and indicates a diminished defensive mechanism against atherosclerosis [41, 42]. Further, the DS group had a higher APO B/APO A1 ratio, revealing an



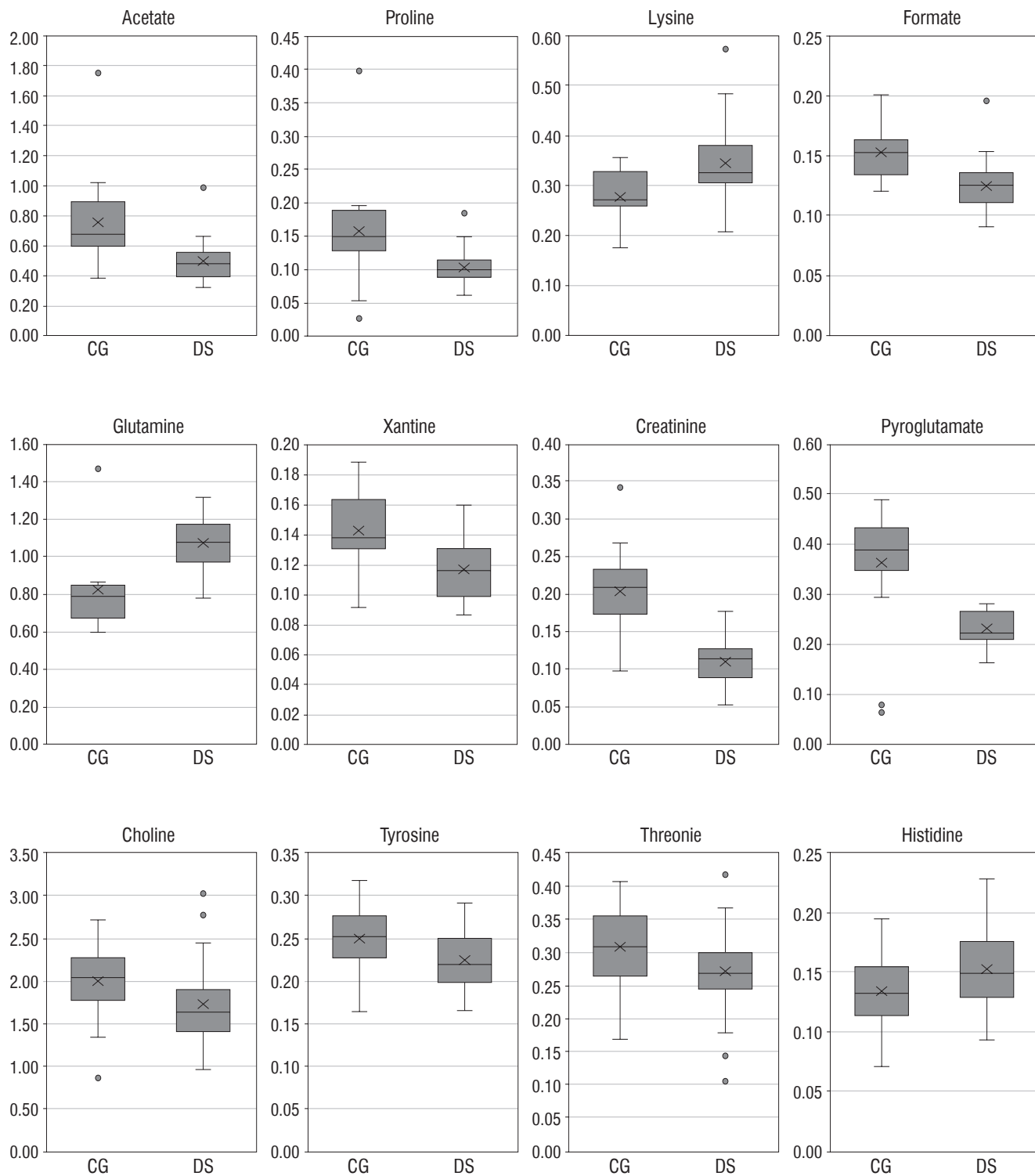
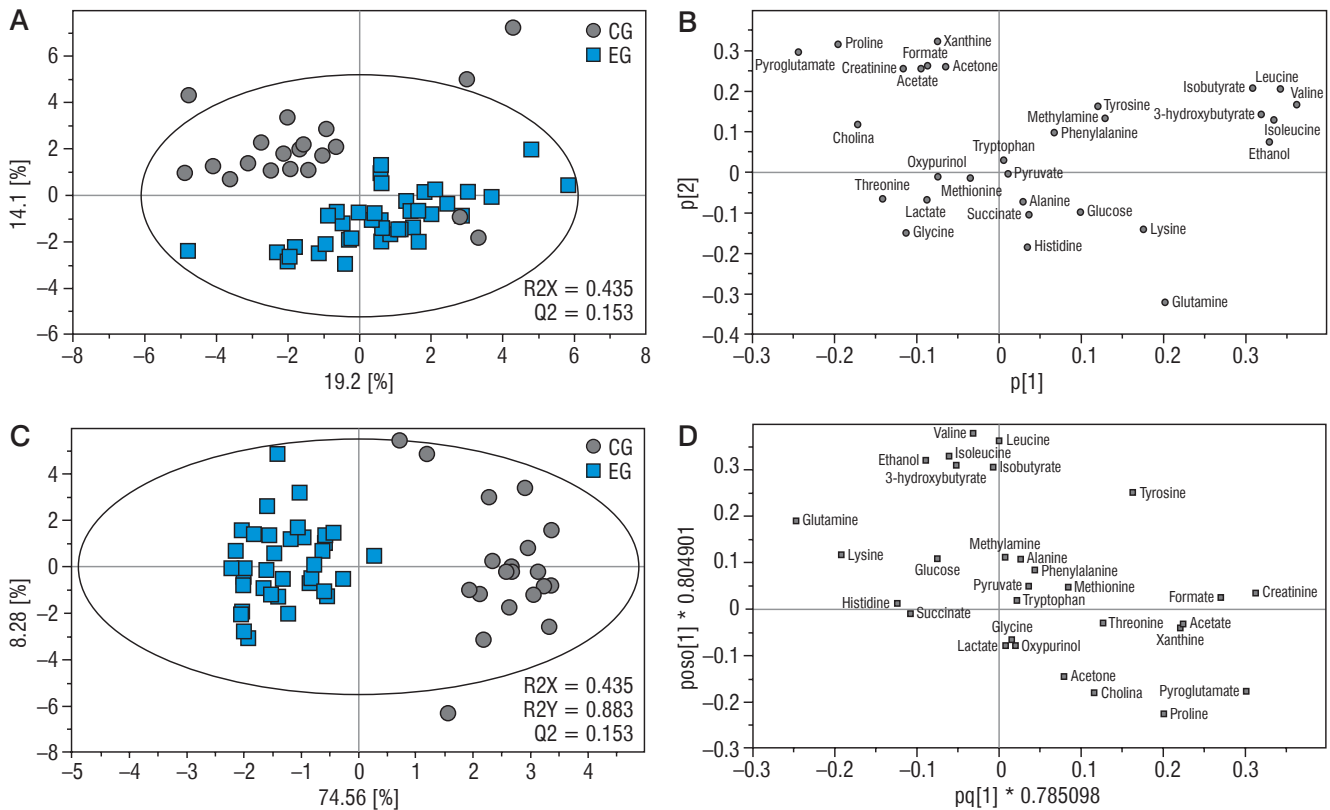


Figure 2. The differences in relative concentration for statistically important metabolites

DS – study group, CG – control group



**Figure 3.** PCA model plot (A) with corresponding loading plot (B) and OPLS-DA model (C) of 1H NMR data of T21 samples

increased predisposition to cardiovascular risks as this ratio serves as a recognized predictor of such risks [43]. Despite a distinct difference in the mean ratios between the groups, it was observed that a larger percentage of the DS group had ratios above the norm, highlighting a noticeable shift towards higher values and emphasizing the augmented risk of CVD in the DS group.

*Other parameters*

While TCH, APO B, HCY, and Lp(a) did not display pronounced differences between the two groups, a deeper analysis into their distributions can reveal nuanced disparities [44]. Even if average values appear similar, the proportion of individuals within or outside the recommended ranges can vary significantly between groups [45]. This underscores the importance of evaluating both overarching trends and individual data distributions to comprehensively understand cardiovascular health in the DS [46]. Such patterns hint at the potential for hidden cardiovascular risks, even when overall averages seem reassuring, emphasizing the need for a holistic assessment approach [47].

*Interconnected metabolic relationships: insights from correlation patterns in study and control groups*

In the DS group, homocysteine’s connection to body metrics hints at a relationship between body composition and met-

abolic implications for cardiovascular health. There is a notable complex relationship between the APO B/APO A1 ratio and various health markers, emphasizing the necessity for a comprehensive view of metabolic health. In the CG, age appears to significantly influence lipid metabolism, impacting various lipid and apolipoprotein markers and consequently cardiovascular risk assessment. The observed relationships between different lipid markers, such as the interplay between APO A1 and other markers, suggest a balanced lipid regulatory environment and reiterate LDL’s central role in cholesterol transport. Moreover, associations between HDL and factors like age and BMI necessitate further investigation into the effects of lifestyle or dietary habits on beneficial cholesterol. The connection between the APO B/APO A1 ratio and Lp(a) also indicates underlying lipid metabolic pathways deserving of further study.

*Discussion on metabolomic analysis in the context of atherosclerosis and cardiovascular disease risk*

Twelve out of 31 metabolites showed significant differences between the DS and CG groups, but applying the Benjamini-Hochberg procedure for FDR correction highlighted eight metabolites with physiological relevance, offering a deeper understanding of potential health implications. Acetate variations point to altered lipid metabolism, which is associated with the formation of atherosclerotic plaques and increased CVD risks

[48, 49]. Creatinine fluctuations indicate that apart from kidney function, muscle metabolism and its impact on cardiac performance should be considered [50]. Format is linked with amino acid metabolism and hints at changes in methylation pathways that, when disrupted, can lead to vascular inflammation and early atherosclerosis. Glutamine, crucial for protein synthesis and cellular energy, suggests broader metabolic shifts potentially affecting vascular health and stress responses [51, 52]. Lysine is vital for collagen synthesis essential for arterial wall structure, and its deficiency could result in weaker arterial walls prone to plaque accumulation [53, 54]. Proline, also involved in collagen production, can affect vessel flexibility, with inconsistent levels potentially leading to hypertension and early atherosclerosis stages [55]. Pyroglutamate plays a role in glutamate metabolism, possibly influencing vascular tone and responsiveness. Xanthine oscillations reflect purine metabolism state, where increased uric acid levels are associated with higher risks of hypertension and vascular inflammation, raising CVD risks [4]. These insights suggest a complex interplay of various metabolic pathways influencing cardiovascular health in the DS group.

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### *Oxidative stress and its implications in DS*

Oxidative stress is a known contributor to the pathogenesis of atherosclerosis and CVD. Relevant to this discussion is our previous work where we demonstrated that individuals with DS exhibit higher levels of oxidative stress compared to their siblings without DS [22]. This increased oxidative stress in the DS population might be a significant factor contributing to their distinctive cardiovascular risk profile.

## Conclusions

Individuals with DS demonstrate distinct cardiovascular risk profiles, marked by reduced HDL cholesterol and heightened LDL cholesterol levels, pointing to an increased susceptibility to atherosclerosis and CVD. Their metabolomic analysis further emphasizes potential metabolic imbalances that could predispose them to vascular complications. Moreover, a pronounced tendency towards elevated BMI and TMI in DS group signals additional health challenges. It can be concluded that individuals with DS might be at a greater risk of developing atherosclerosis compared to the CG.

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