SUPPLEMENTARY MATERIAL

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Supplementary Methods – Extended

Materials and Methods

Commercial reagents were purchased from Sigma-Aldrich (Saint Louis, MO) unless otherwise noted. Adenosine (Ribose-13C5), citric acid (2,2,4,4-D), fumaric acid (1,4-13C2), glucose (U-13C6), glutathione (glycine-13C2,15N), alpha-ketoglutaric acid (13C5), palmitic acid (U-13C16), sodium pyruvate (1-13C), sodium L-lactate (3-13C), succinic acid (13C4), uric acid (1,3-15N2), taurodeoxycholic acid, sodium salt (2,2,4,4,-D), taurochenodeoxycholic acid, sodium salt (2,2,4,4,-D), tauroursodeoxycholic acid, sodium salt (2,2,4,4,-D), taurocholic acid, sodium salt (2,2,4,4,-D), taurolithocholic acid, sodium salt (2,2,4,4,-D), cholic acid (2,2,4,4,-D), deoxycholic acid (2,2,4,4,-D), chenodeoxycholic acid (2,2,4,4,-D), ursodeoxycholic acid (2,2,4,4,-D), glycocholic acid (2,2,4,4,-D), glycochenodeoxycholic acid (2,2,4,4,-D), labeled carnitine standards set (NSK-B), and metabolomics amino acid mix standard (MSK-A2, 2.5 mM) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA) Sphingosine-1-phosphate (D7) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL)

Sample collection

Blood was collected from disomic (n=67; 23 male and 44 female – age range at the time of donation 12 to 76.5 years old) and trisomic (n=30; 13 male and 17 female – age range at the time of donation 0.5 to 53.6 years old) donor volunteers in accordance with the Declaration of Helsinki. Of these 97 individuals, 72 were age matched (specifically 43 D21 and 29 T21 subjects, respectively). RBCs were sorted through centrifugation at 2,000 *g* for 10 min at 4 °C and stored at the Linda Crnic Institute for Down Syndrome biobank (University of Colorado Denver – Anschutz Medical Campus).

UHPLC-MS metabolomics

RBCs (50 µl) were processed as previously described[1] and extensively reported in **Supplementary Methods.** Briefly, sample extracts were analyzed via ultra-high pressure liquid chromatography coupled to mass spectrometry - UHPLC-MS (Vanquish, Q Exactive – Thermo Fisher, San Jose, CA and Bremen, Germany) using C18 reversed phase chromatography with separate runs in positive and negative ion mode, using either electrospray or atmospheric pressure chemical ionization (ESI or APCI)-MS.[2,3] A Kinetex C18 column, 2.1x x150mm, 1.7 µm particle size (Phenomenex, Torrance, CA) was used and equipped with a guard column (SecurityGuard™ Ultracartridge – UHPLC C18 for 2.1 mm ID columns – AJO-8782 – Phenomenex, Torrance, CA) Metabolite assignments and heavy isotopologue detection for absolute quantitation against internal standards were determined against in house standard libraries and KEGG database searches through Maven.[4] Technical reproducibility (CVs) was assessed by monitoring internal heavy labeled standard mixes and the xenometabolite 5-fluorouracil (2.5 µM), as reported.[2]

High-throughput Isocratic method

This method has been extensively described.[2] Briefly, for positive ion mode samples were resolved at 25°C for 3 minutes using an isocratic condition of 5% acetonitrile, 95% water, and 0.1% formic acid flowed at 250 µL/min. The Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated in positive ion mode using electrospray ionization, scanning in Full MS mode (1 µscan) from 60 to 900 m/z at 70,000 resolution, with 4 kV spray voltage, 15 sheath gas, 5 auxiliary gas.

For negative ion mode, samples were resolved at 25°C for 3 minutes using an isocratic condition of 5% acetonitrile and 95% water with 5 mM ammonium acetate flowed at 250 µL/min. The Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated in

negative ion mode using electrospray ionization, scanning in Full MS mode (1 μscan) from 60 to 900 m/z at 70,000 resolution, with 4 kV spray voltage, 15 sheath gas, 0 auxiliary gas.

High-throughput gradient-based methods

The method is a variant of previously published methods.[5] Samples were resolved at 45°C with a gradient elution over 4 minutes, flowing at 450 μL/min. For positive ion mode, Mobile phase A is 0.1% formic acid in water, mobile phase B is 0.1% formic acid in acetonitrile. 5% A and 95% B is held from 0.00 – 0.50 minutes. From 0.50 – 1.10, A increases to 95% A and 5% B. This condition is held from 1.10 – 2.75 minutes. From 2.75 – 3.00 minutes A decreases to the initial condition of 5% A and 95% B and is held from 3.00 – 4.00 minutes. The Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated in positive ion mode using electrospray ionization, scanning in Full MS mode (1 μscan) from 100 to 1500 m/z at 70,000 resolution, with 4 kV spray voltage, 45 sheath gas, 15 auxiliary gas.

For negative ion mode, samples were resolved at 45°C with a gradient elution over 4 minutes, flowing at 450 μL/min. Mobile phase A is 5% acetonitrile and 95% water with 5 mM ammonium acetate, mobile phase B is 95% acetonitrile and 5% water with 1 mM ammonium acetate. 100% A, 0% B is held from 0.00 – 0.50 minutes, then from 0.050 – 1.10 minutes %B is increased to 100% and held from 1.10 – 2.75 minutes. From 2.75 – 3.00 minutes B decreases to the initial condition of 100% A, 0% B and is held from 3.00 – 4.00 minutes. The Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated in negative ion mode using electrospray ionization, scanning in Full MS mode (1 μscan) from 100 to 1500 m/z at 70,000 resolution, with 4 kV spray voltage, 45 sheath gas, 15 auxiliary gas.

Atmospheric Pressure Chemical Ionization (APCI)

Samples were resolved at 45°C with a gradient elution over 4 minutes, flowing at 450 µL/min. Mobile phase A is 0.1% formic acid in water, mobile phase B is 0.1% formic acid in acetonitrile. 5% A and 95% B is held from 0.00 – 0.50 minutes. From 0.50 – 1.10, A increases to 95% A and 5% B. This condition is held from 1.10 – 2.75 minutes. From 2.75 – 3.00 minutes A decreases to the initial condition of 5% A and 95% B and is held from 3.00 – 4.00 minutes. The Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated in positive ion mode using atmospheric pressure chemical ionization, scanning in Full MS mode (1 µscan) from 100 to 1500 m/z at 70,000 resolution, with 5 kV spray voltage, 10 sheath gas, 20 auxiliary gas.

Metabolite assignment and isotopologue quantitation

Calibration was performed prior to analysis using the PierceTM Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific). Acquired data was then converted from .raw to .mzXML file format using Mass Matrix (Cleveland, OH, USA). Metabolite assignments, isotopologue distributions, and correction for expected natural abundances of deuterium, 13C, and 15N isotopes were performed using MAVEN (Princeton, NJ, USA).

Determination of absolute quantities against stable isotope-labeled internal standards

Calculation of absolute quantification for measured metabolites was performed using the following formula: **[light] = (abundance light) / (abundance heavy) * [heavy] [dilution factor]** where [light] = concentration of non-isotopic metabolite, (abundance light) = total area abundance of non-isotopic metabolite, (abundance heavy) = total area abundance of isotopic metabolite, and [heavy] = known concentration isotopic metabolite.

Statistics

Statistical analysis, including T-test (disomic vs trisomic) or Two-way ANOVA (D21 vs T21, either male or female), as well as multivariate analysis (partial least square-discriminant analysis – PLS-DA and hierarchical clustering analysis – HCA) were performed with GraphPad Prism 5.0 and MetaboAnalyst 3.0.[6] Metabolic linkage analyses have been recently described.[7] Briefly, correlative analyses (Pearson or Spearman correlations – r – upon testing for normality distribution of data with Kolmogorov-Smirnov) and calculation of **Δ**|r|>30% deviations were performed with GraphPad Prism 5.0 and Microsoft Excel 2017, while results were plotted with GENE-E (Broad Institute, Boston, MA, USA). Briefly, the underlying assumption of the metabolic linkage analysis[7] is that – even though correlation does not necessarily imply causation - the levels of metabolites from linked pathways are highly correlated owing to biochemical constraints of the enzymatic reactions necessary to consume one metabolite to generate another.

References

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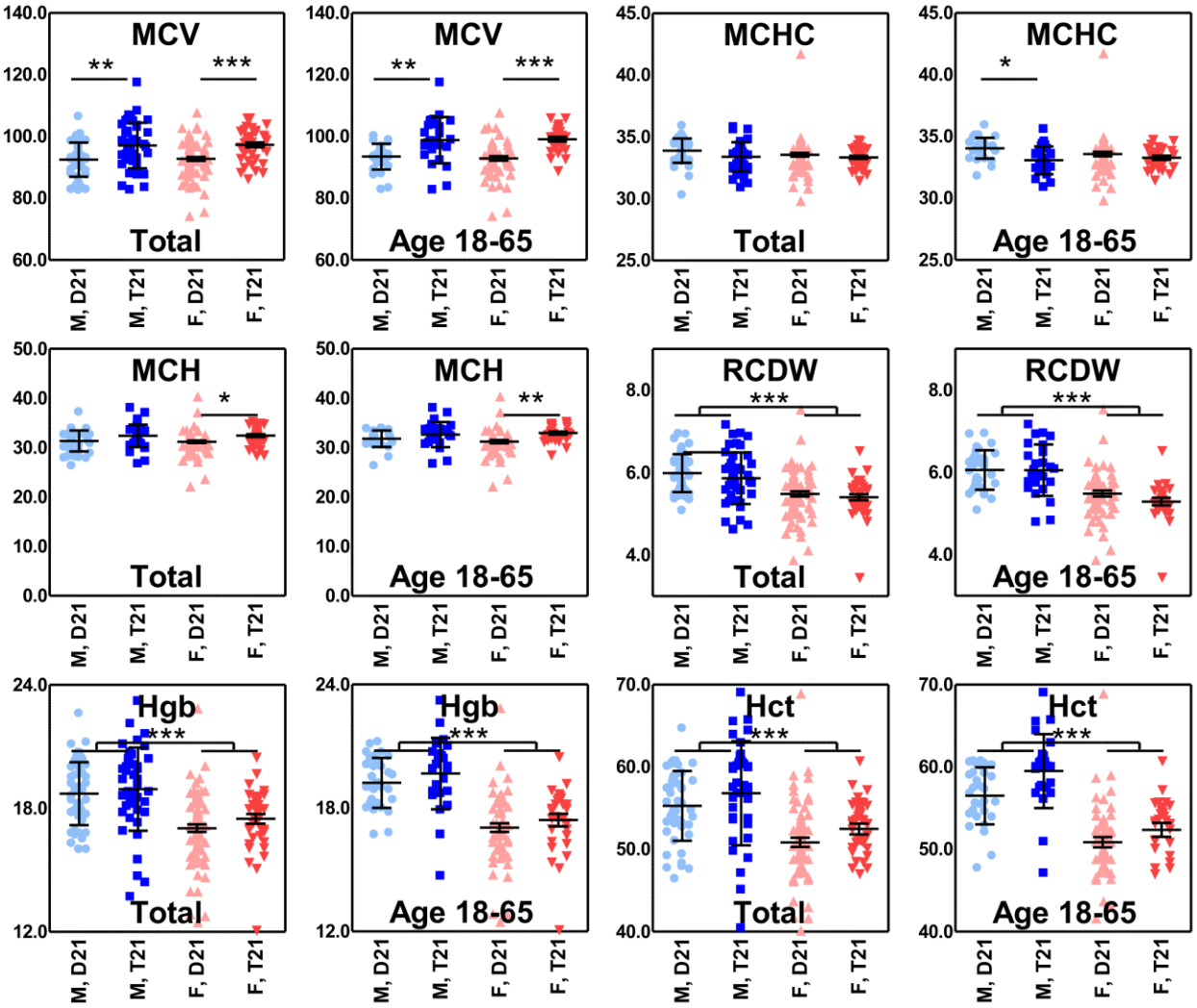
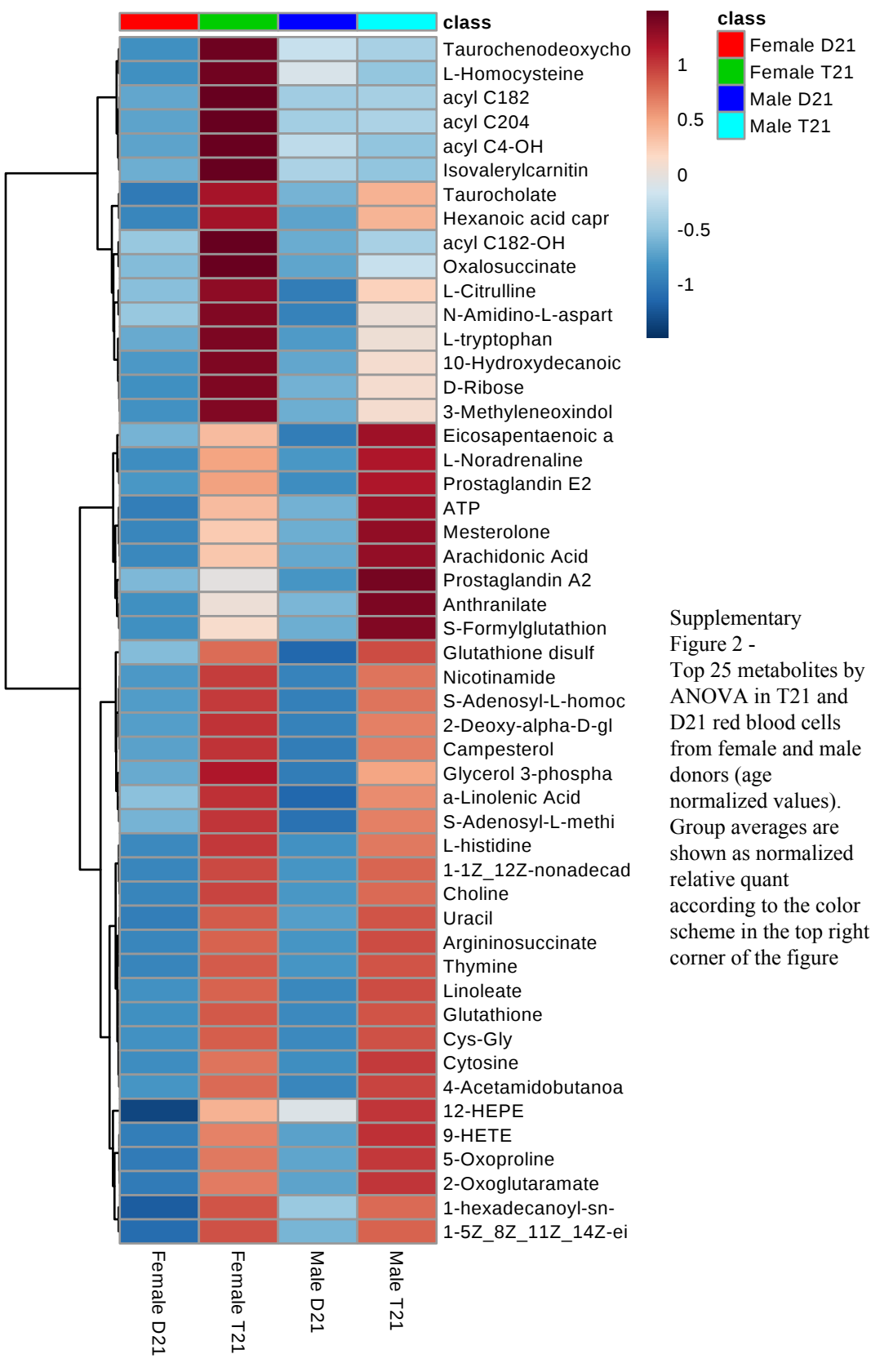
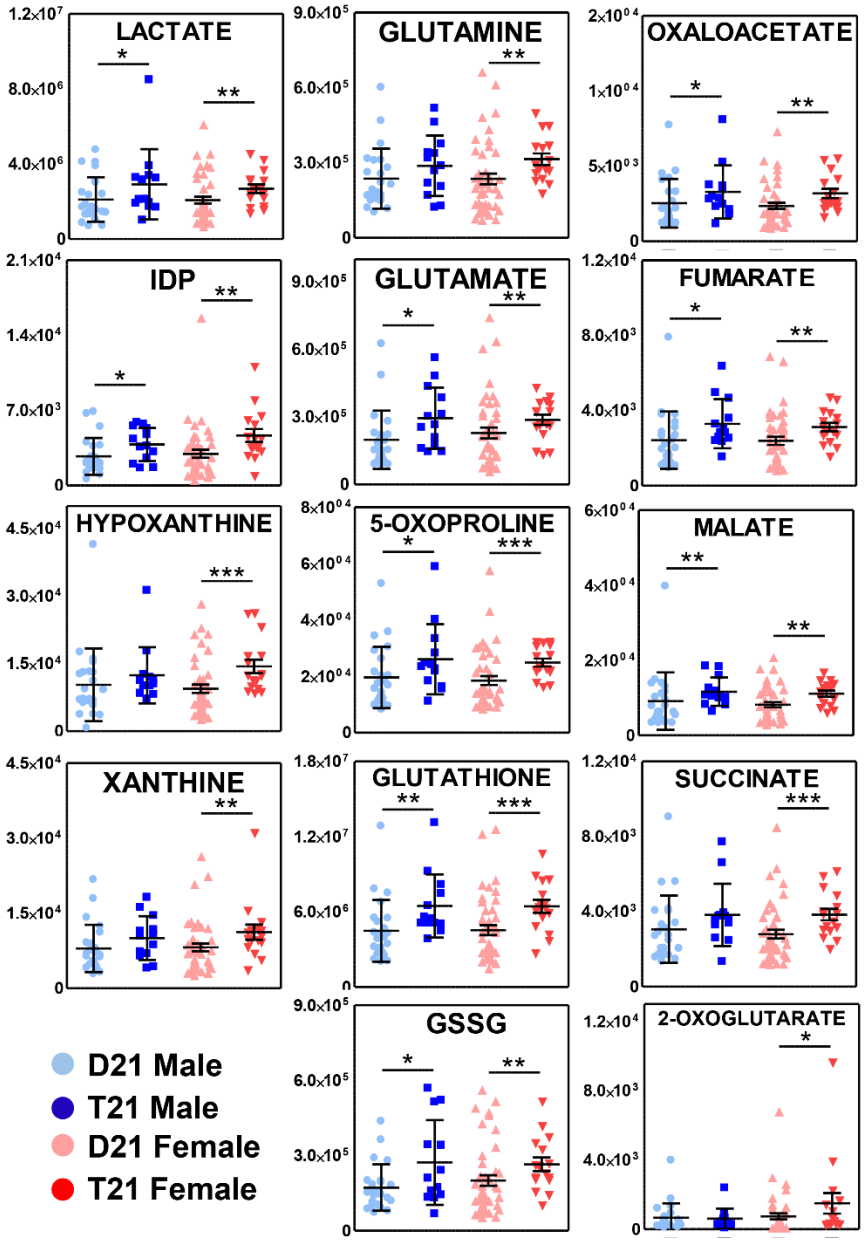
7. D'alessandro A, Nemkov T, Reisz J, et al. Omics markers of the red cell storage lesion and metabolic linkage. *Blood Transfus. Trasfus. Sangue*. 2017;15(2):137–144.



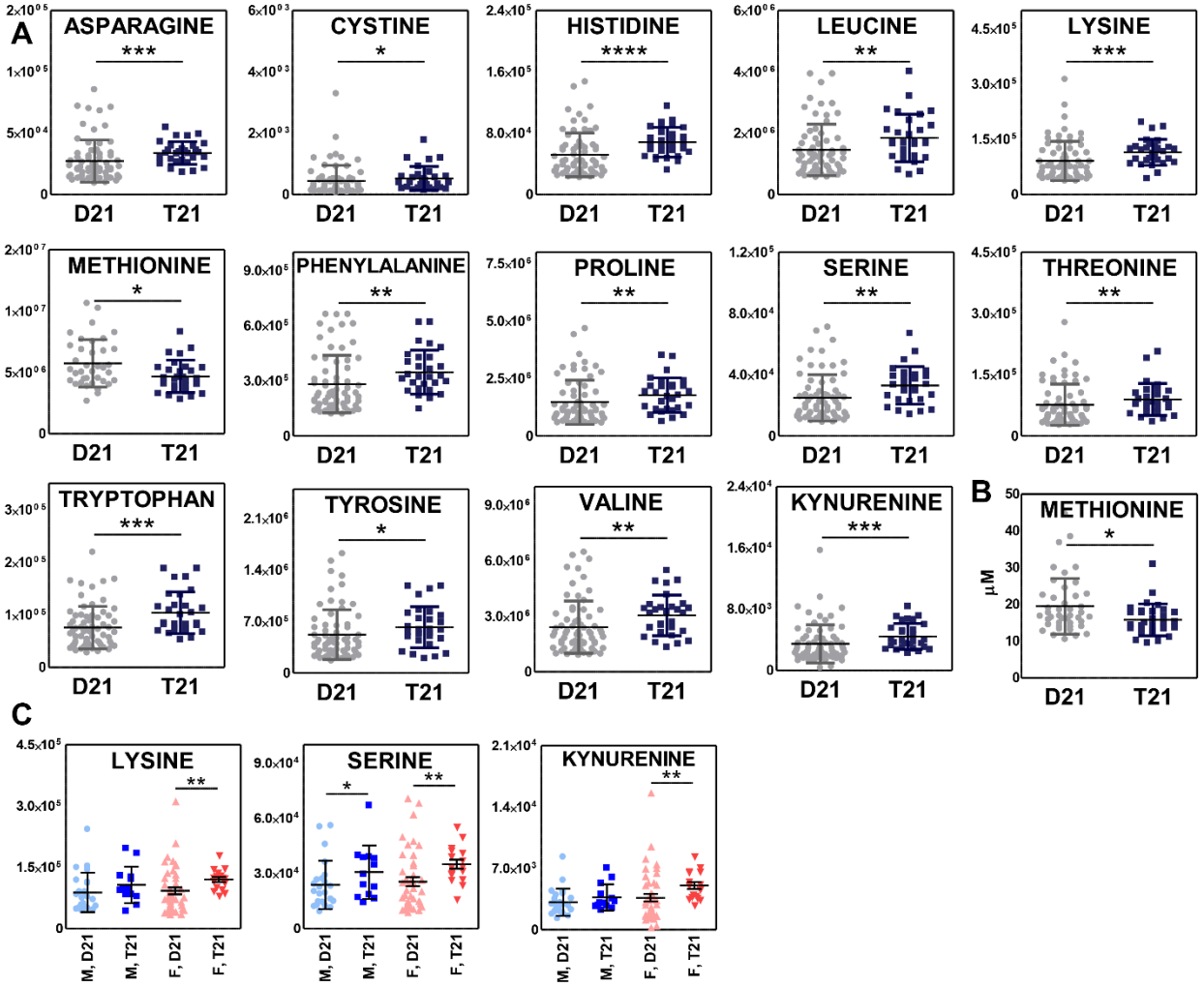
Supplementary Figure 1– Red Blood Cell hematological parameter in the disomic and trisomic population, gender and/or age-matched. Red blood cell mean cell volume (MCV), mean cell hemoglobin concentration (MCH), mean cell corpuscular hemoglobin (MCH), red cell distribution width (RCDW), hemoglobin (Hgb) and hematocrit (Hct) levels in disomic and trisomic individuals (male or female). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ ANOVA.



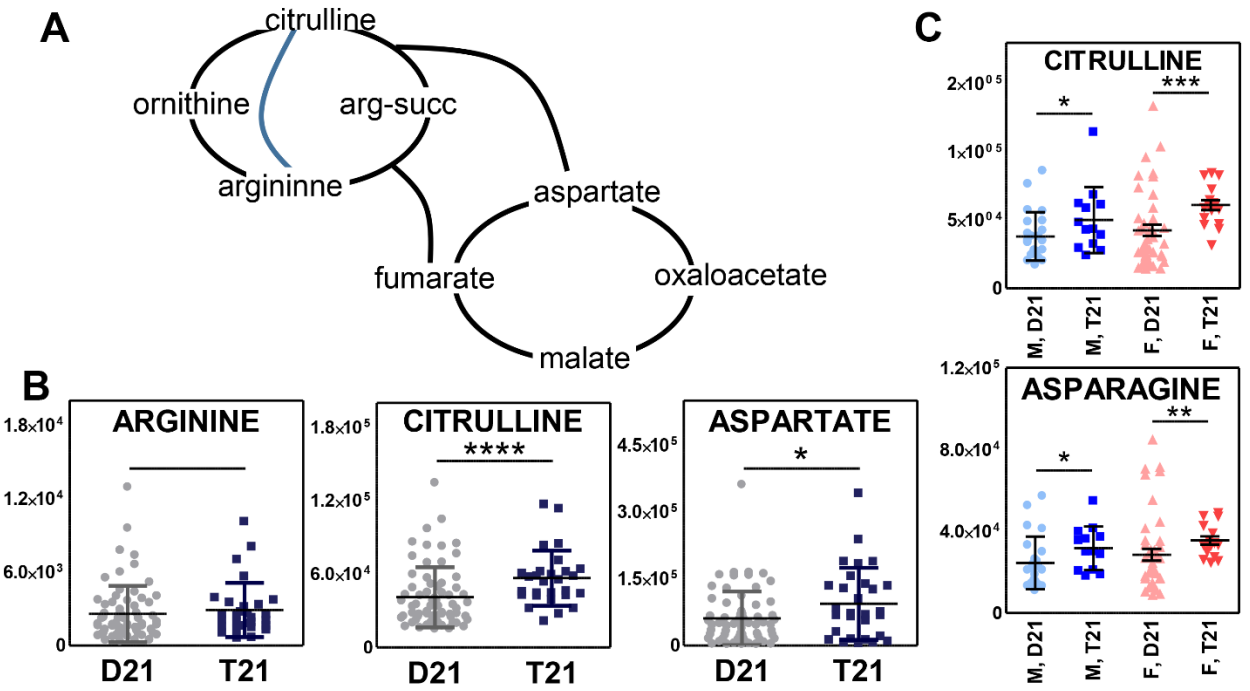
Supplementary Figure 2 - Top 25 metabolites by ANOVA in T21 and D21 red blood cells from female and male donors (age normalized values). Group averages are shown as normalized relative quant according to the color scheme in the top right corner of the figure



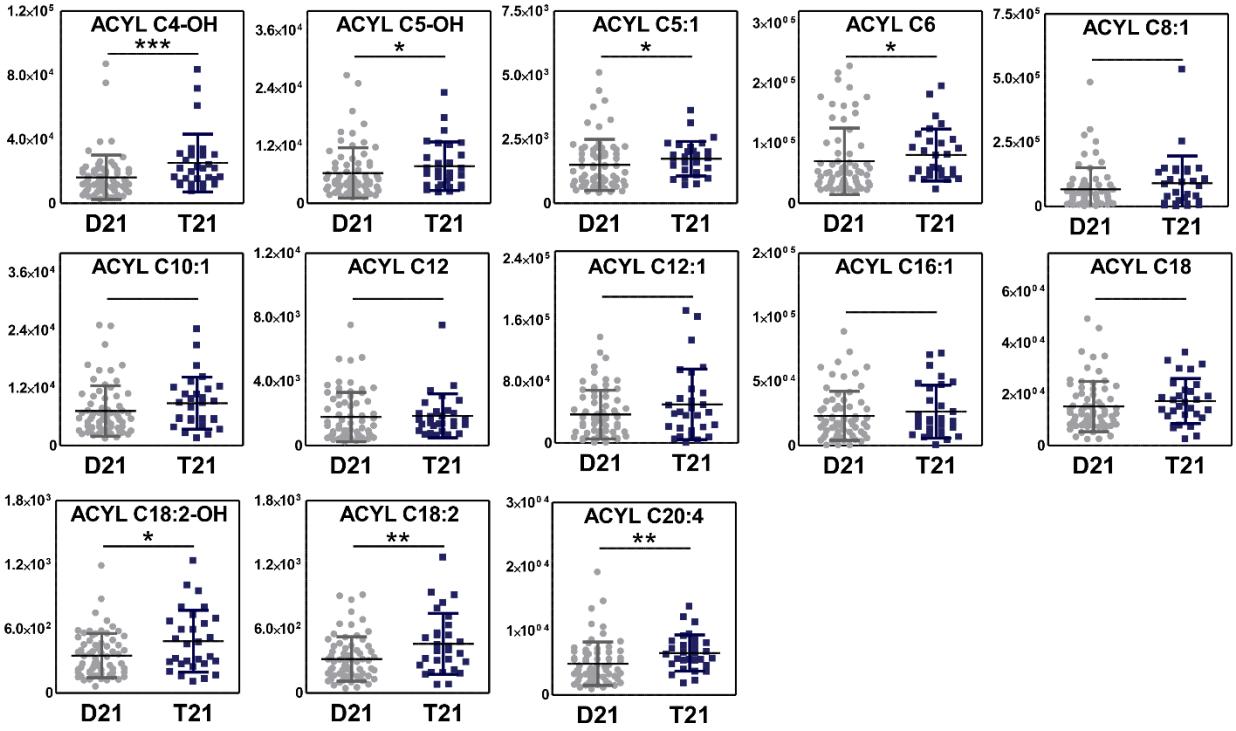
Supplementary Figure 3 – Down Syndrome promotes a significant gender-specific metabolic reprogramming of red blood cells. Levels of metabolites involved in glycolysis, glutathione homeostasis, carboxylic acids and purine metabolism are significantly increased in red blood cells from individuals with trisomy 21 male or female when compared to disomic individuals male or females (y axis = AU normalized by donor age). Color codes for dot plots are indicated in the bottom left corner of the figure. Of note, purine metabolites like IDP, hypoxanthine and xanthine and carboxylic acids like succinate and 2-oxoglutarate underwent significant increases only in RBCs from female trisomic donors. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ ANOVA.



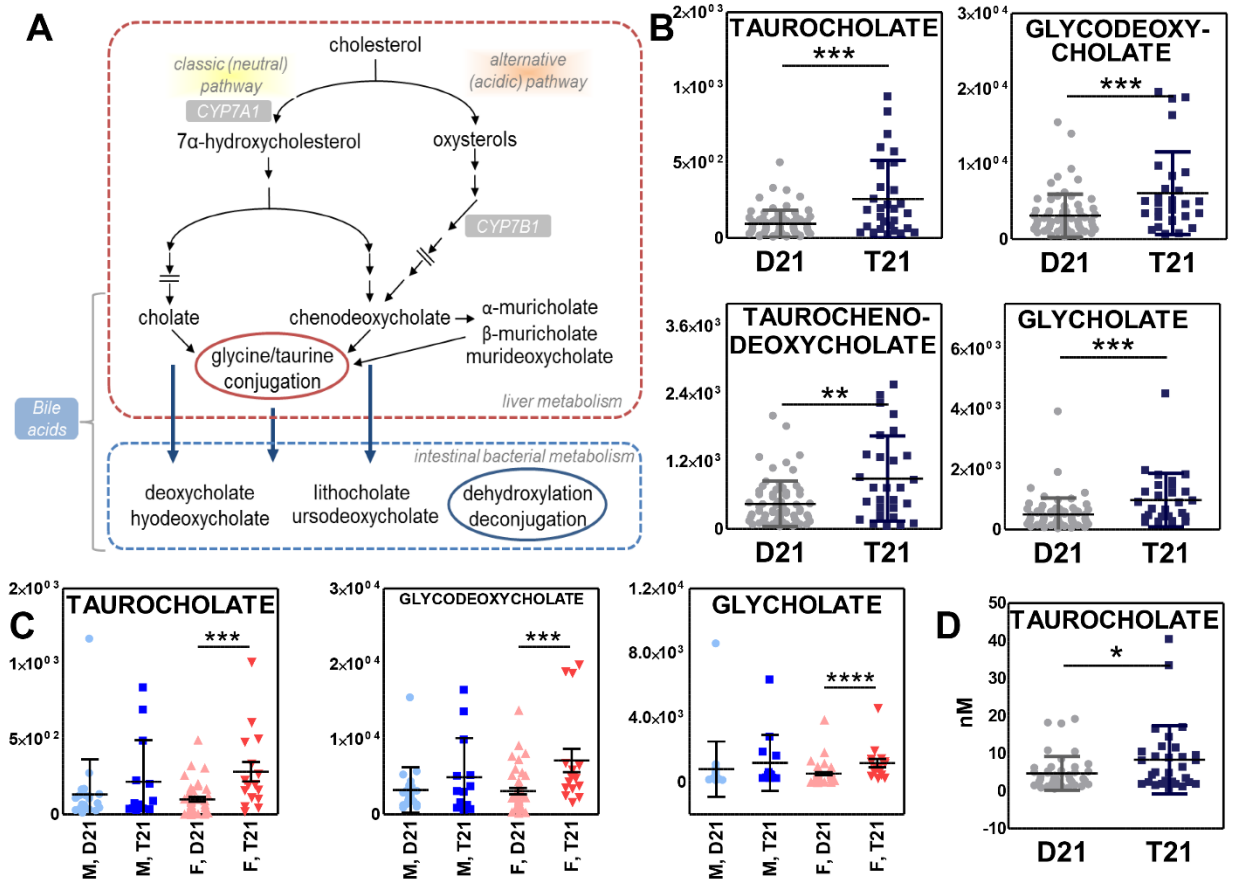
Supplementary Figure 4 - Down Syndrome promotes significant increases in RBC amino acids and tryptophan catabolites. Levels of amino acids are significantly increased in red blood cells from individuals with trisomy 21 (dark blue) when compared to disomic individuals (light grey) (y axis = AU normalized by donor age) (**A**). Notably, methionine levels decreased in T21 RBCs, a finding validated in age matched D21 and T21 RBCs (n=72) with targeted absolute quantification of this amino acids against an internal ^{13}C ^{15}N-methionine standard (**B**). In **C**, some of these changes are gender specific, such as lysine, serine and kynurenine significantly increasing in female trisomic individual but not in male trisomic subjects when compared to gender-matched disomic individuals. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ T-test or ANOVA.



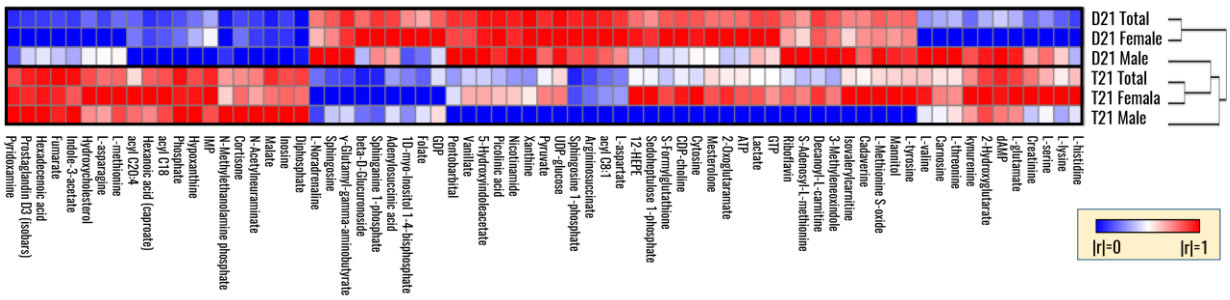
Supplementary Figure 5 - Down Syndrome promotes significant increases in RBC metabolic intermediates of amino acid metabolism (incomplete urea cycle). Levels of amino acids like citrulline, arginine and aspartate are significantly increased in red blood cells from individuals with trisomy 21 (dark blue) when compared to disomic individuals (light grey) (y axis = AU normalized by donor age) (**A**). In **B**, some of these changes are gender specific, such as citrulline and asparagine increasing in female trisomic individual but not in male trisomic subjects when compared to gender-matched disomic individuals. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ **** $p<0.0001$ T-test or ANOVA.



Supplementary Figure 6 – Down Syndrome promotes significant increases in RBC acyl-conjugated carnitines. Several acyl-conjugated carnitines are significantly increased in red blood cells from individuals with trisomy 21 (dark blue) when compared to disomic individuals (light grey). (y axis = AU normalized by donor age) * $p<0.05$; ** $p<0.01$; *** $p<0.001$ T-test or ANOVA.



Supplementary Figure 7 – Down Syndrome promotes significant increases in RBC bile acids. Levels of bile acids (conjugated and de-conjugated – **A**) are significantly increased (**B**) in red blood cells from individuals with trisomy 21 (dark blue) when compared to disomic individuals (light grey). (y axis = AU normalized by donor age). These changes are particularly gender-specific (F = female only – **C**). Results were validated with absolute quantification against internal deuterated standards for bile acids, including taurocholate (**D**). * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001 T-test or ANOVA.



Supplementary Figure 8 – Metabolites correlating with age n male and female disomic or trisomic individuals. The heat map in shows Spearman correlation coefficients of red blood cell metabolites to age in disomic and trisomic individuals (either overall or distinguished by gender – |r| from 0 to >0.75 = blue to red).