The American Journal of Human Genetics, Volume *105*

Supplemental Data

Distinct Alterations in Tricarboxylic Acid Cycle Metabolites Associate with Cancer and Autism Phenotypes in Cowden Syndrome and Bannayan-Riley-Ruvalcaba Syndrome

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Supplemental Material and Methods

Research participants and clinical data

A total of 511 individuals clinically diagnosed with Cowden syndrome (CS), Cowden-like syndrome (CS-like), and Bannayan-Riley-Ruvalcaba syndrome (BRRS) were prospectively accrued in accordance with our research protocol 8458-*PTEN*, approved by the Cleveland Clinic Institutional Review Board (IRB). To be eligible for enrolment in protocol 8458-*PTEN*, inclusion criteria include meeting at least the relaxed International Cowden Consortium (ICC) operational diagnostic criteria, meaning full diagnostic criteria minus one feature and these individuals are referred to as CS-like[1] (**Table S1**); having macrocephaly plus autism spectrum disorder/developmental delay/mental retardation and/or penile freckling; or presence of a known pathogenic germline *PTEN* mutation.[2] For each consented participant, we reviewed medical records, including pedigrees, clinical genetic testing reports, and clinical notes associated with cancer genetics and/or genetic-counseling visits, where applicable. We included 98 population controls anonymously accrued through protocol 8458-*PTEN* with demographic data annotations (**Table S2**). Written informed consents were obtained from all research participants.

PTEN and *SDHx* mutation and deletion analysis

All research participants underwent *PTEN* and *SDHx* (*SDHB*, *SDHC*, *SDHD*) mutation and deletion analysis. Genomic DNA was extracted from peripheral blood leukocytes of all eligible research participants using standard protocols (https://www.lerner.ccf.org/gmi/gmb/). Mutation and deletion analysis were performed as previously reported.[3] For mutation positive individuals, only cases with pathogenic germline mutations were included in this study. To be conservative, individuals with *PTEN* promoter variants were considered as mutation positive only if the underlying variants have been shown to be associated with CS or known to affect PTEN function.[4-7]

Cell lines and culture conditions

Immortalized lymphoblastoid cell lines (LBLs) from cases or control peripheral blood samples were generated by the Genomic Medicine Biorepository (GMB) of the Cleveland Clinic Genomic Medicine Institute (GMI, Cleveland, OH) following standard procedures (https://www.lerner.ccf.org/gmi/gmb/). Cells were subsequently grown in RPMI-1640 supplemented with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin and maintained at 37°C and 5% CO_2 culture conditions. All cell lines remained anonymized and devoid of any participant identifiers (only number-coded) during the duration of the experiments.

Measurement of TCA cycle metabolites

Each non-adherent lymphoblastoid cell line (LBL) was seeded at a density of 10 million cells per T75 flask. Cells were allowed to grow overnight and then collected into 50 ml conical tubes. We spun down the cell suspension at 1000 RPM for 5 minutes in a cooled centrifuge (4°C), discarded the supernatant, and added 1 ml of ice-cold PBS to wash the pellet. The cell suspension was then transferred to 1.5 ml tubes, spun down at 1000 RPM for 5 minutes at 4°C to remove the supernatant. Each cell pellet was lysed with 1 ml of an ice-cold mixture of 80% methanol in water, followed by three freeze-thaw cycles on dry ice. Insoluble material was pelleted after two consecutive centrifugation cycles at 13000 RPM for 15 minutes in a cooled centrifuge. Samples were then dried by vacuum centrifugation overnight and stored at -80°C until all batches were processed. Dried samples were resuspended in 100 μl sterile water containing 10 μg/ml internal standards (deuterated citrate and deuterated succinate). Samples were vortexed and then centrifuged at 13000 RPM for 10 minutes in a cooled centrifuge. We transferred 40 μl of the supernatant into high-performance liquid chromatography (HPLC) vials (Thermo Scientific SUN-SRi, Pittsburgh, PA) for injection and subsequent liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis. Targeted metabolomics

measurements were conducted at the Lerner Research Institute Proteomics and Metabolomics Core (Cleveland Clinic Lerner Research Institute, Cleveland, OH).

Data normalization and statistical analyses

All metabolite measurements were log-transformed and center-scaled to have a mean of 0 and a standard deviation of 1 before further analysis. A general logistic regression model was applied to evaluate the association between metabolite levels and genotype/phenotype. Metabolites together with age and gender were used as predictors, and genotype or phenotype as the dichotomous outcome variable. Genotype was placed in the regression model for cancer versus neurodevelopmental disorder comparison. Full model was set up with backward stepwise elimination of non-significant variables, as relevant to metabolites and/or genotypes. Analyses were conducted using RStudio version 1.1.463 (https://www.rstudio.com) and R glm library from Stats v3.6.0 package. Adjusted $p<0.05$ was considered as significant.

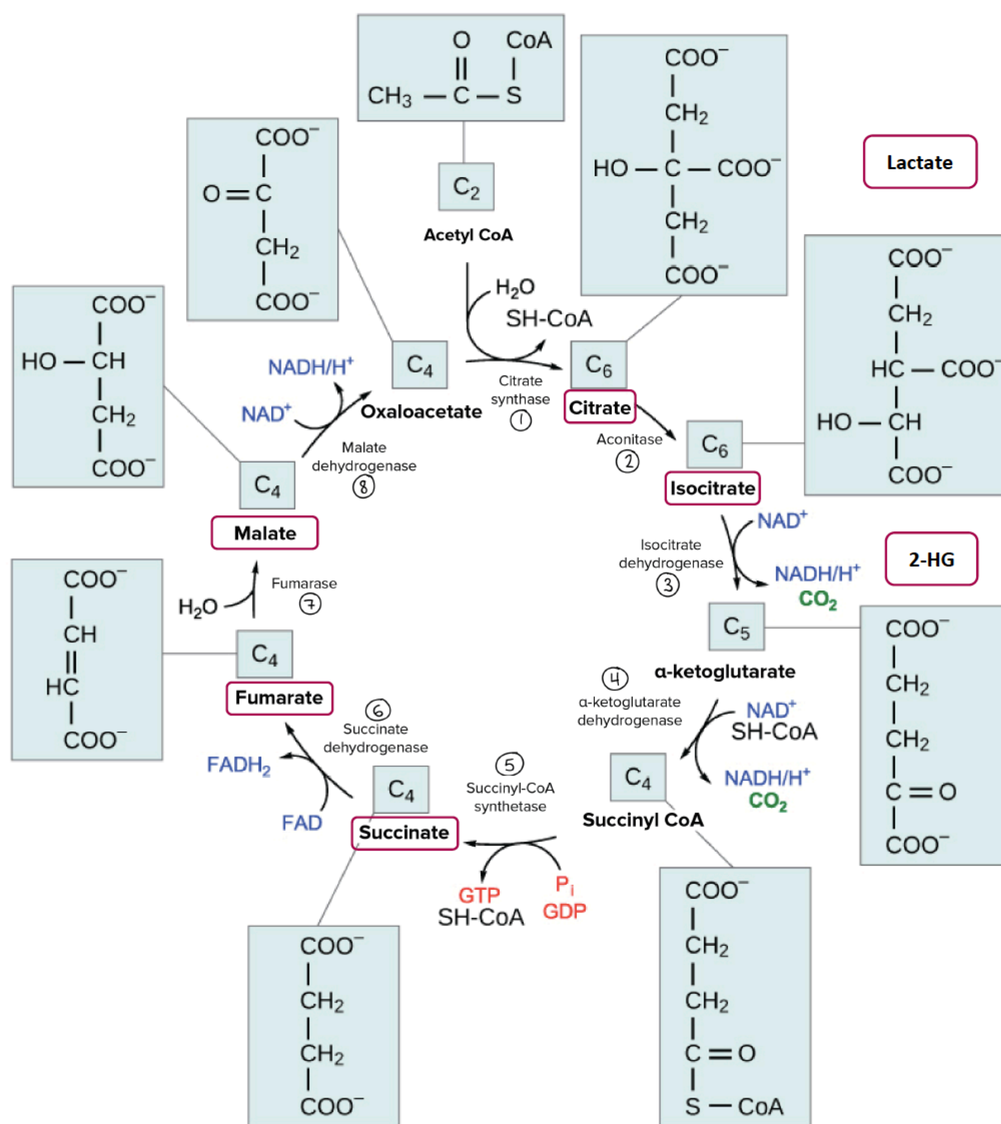

Figure S1. High confidence Tricarboxylic Acid Cycle (TCA) metabolites measured from case- and control-derived lymphoblastoid cell lines

TCA cycle metabolites measured from lymphoblastoid cell lines (LBLs) include citrate, isocitrate, succinate, fumarate, malate, lactate, and 2-hydroxyglutarate (2-HG). The other metabolites could not be reliably measured from LBLs. Image modified from OpenStax College, Biology (Oxidation of pyruvate and citric acid cycle, Figure 2, CC BY 3.0).

Figure S2. Family-based analysis of normalized lactate levels

Family-based analyses of normalized lactate levels, including ASD-affected individuals (orange bars) and unaffected non-ASD members (gray bars) within each family. Affected individuals include those with idiopathic normo-ASD, idiopathic macro-ASD, and *PTEN*^{MUT} macro-ASD. F-1 through F-23 correspond to anonymized family IDs.

<table>
<tr><th>Pathognomonic</th><th>Major</th><th>Minor</th></tr>
<tr><td>Adult Lhermitte-Duclos disease (LDD)
Mucocutaneous lesions
Trichilemmomas, facial
Acral keratoses
Papillomatous papules
Mucosal lesions</td><td>Breast carcinoma
Thyroid carcinoma (non-medullary), especially follicular thyroid carcinoma
Macrocephaly (occipital frontal circumference ≥ 97th percentile)
Endometrial carcinoma</td><td>Other thyroid lesions (e.g., adenoma, multinodular goiter)
Mental retardation (i.e., IQ ≤ 75)
Gastrointestinal hamartomas
Fibrocystic breast disease
Lipomas
Fibromas
Genitourinary tumours (especially renal cell carcinoma)
Genitourinary malformations
Uterine fibroids</td></tr>
<tr><th colspan="3">Operational diagnosis in an individual</th></tr>
<tr><td colspan="3">Any of following:
Mucocutaneous lesions alone, if ≥ six facial papules (three of which must be trichilemmomas)
Cutaneous facial papules and oral mucosal papillomatosis
Oral mucosal papillomatosis and acral keratoses
≥ Six palmoplantar keratoses
≥ Two major criteria (one of which must be macrocephaly or LDD)
One major and ≥ three minor criteria
≥ Four minor criteria</td></tr>
<tr><th colspan="3">Operational diagnosis in a family where one individual is diagnostic for CS</th></tr>
<tr><td colspan="3">Any one pathognomonic criterion
Any one major criterion ± minor criteria
Two minor criteria
History of Bannayan-Riley-Ruvalcaba syndrome (BRRS)</td></tr>
</table>

Table S1. International Cowden Consortium (ICC) operational diagnostic criteria

Gender	
Female	50 (51%)
Male	48 (49%)
Median age at consent (range)	37 (2-76)
Race	
American Indian or Alaska Native	1 (1.0%)
Asian	6 (6.1%)
Black or African American	13 (13.3%)
White	67 (68.4%)
Mixed	7 (7.1%)
Unknown/Other	4 (4.1%)

Table S2. Demographic characteristics of 98 population controls anonymously accrued through IRB protocol 8458-*PTEN*

Supplemental References

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4. Teresi, R.E., Zbuk, K.M., Pezzolesi, M.G., Waite, K.A., and Eng, C. (2007). Cowden syndrome-affected patients with PTEN promoter mutations demonstrate abnormal protein translation. Am J Hum Genet 81, 756-767.
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