

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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Germline heterozygous *PTEN* mutations cause subsets of Cowden syndrome (CS) and Bannayan-Riley-Ruvalcaba syndrome (BRRS); these subsets are characterized by high risks of breast, thyroid, and other cancers and, in one subset, autism spectrum disorder (ASD). Up to 10% of individuals with *PTEN*^{MUT} CS, CS-like syndrome, or BRRS have germline *SDHx* (succinate dehydrogenase, mitochondrial complex II) variants, which modify cancer risk. *PTEN* contributes to metabolic reprogramming; this is a well-established role in a cancer context. Relatedly, *SDH* sits at the crossroad of the electron transport chain and tricarboxylic acid (TCA) cycle, two central bioenergetic pathways. Intriguingly, *PTEN*^{MUT} and *SDH*^{MUT} individuals have reduced *SDH* catalytic activity, resulting in succinate accumulation; this indicates a common genotype-independent biochemical alteration. Here, we conducted a TCA targeted metabolomics study on 511 individuals with CS, CS-like syndrome, or BRRS with various genotypes (*PTEN* or *SDHx*, mutant or wild type [WT]) and phenotypes (cancer or ASD) and a series of 187 population controls. We found consistent TCA cycle metabolite alterations in cases with various genotypes and phenotypes compared to controls, and we found unique correlations of individual metabolites with particular genotype-phenotype combinations. Notably, increased isocitrate ($p = 1.2 \times 10^{-3}$), but reduced citrate ($p = 5.0 \times 10^{-4}$), were found to be associated with breast cancer in individuals with *PTEN*^{MUT}/*SDHx*^{WT}. Conversely, increased lactate was associated with neurodevelopmental disorders regardless of genotype ($p = 9.7 \times 10^{-3}$); this finding was replicated in an independent validation series ($n = 171$) enriched for idiopathic ASD (*PTEN*^{WT}, $p = 5.6 \times 10^{-4}$). Importantly, we identified fumarate ($p = 1.9 \times 10^{-2}$) as a pertinent metabolite, distinguishing individuals who develop ASD from those who develop cancer. Our observations suggest that TCA cycle metabolite alterations are germane to the pathobiology of *PTEN*-related CS and BRRS, as well as genotype-independent ASD, with implications for potential biomarker and/or therapeutic value.

Cowden syndrome (CS [MIM: 158350]) and Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM: 153480]) represent two well-defined *PTEN*-related overgrowth disorders. CS is a multisystem autosomal dominant disorder characterized by elevated lifetime risks for both benign and malignant tumors of the breast and thyroid, as well as neurodevelopmental disorders such as autism spectrum disorder (ASD).^{1–3} CS is difficult to clinically recognize due to the extensive degree of phenotypic variability and the existence of individual features which mimic normal variation or other disorders.⁴ BRRS is a rare autosomal dominant congenital disorder classically characterized by macrocephaly in combination with intestinal hamartomatous polyposis, vascular malformations, lipomas, hemangiomas, and genital freckling.⁵ Similar to CS, individuals with BRRS can present with a spectrum of mild to severe phenotypic manifestations, including neurodevelopmental delays.⁶

The tumor suppressor gene phosphatase and tensin homolog (*PTEN* [MIM: 601728]), which encodes a dual-specificity phosphatase which classically counteracts the PI3K/AKT/mTOR growth-promoting cascade, is the first CS and

BRRS susceptibility gene.⁷ Germline *PTEN* mutations occur in ~25% of CS/CS-like individuals who meet International Cowden Consortium (ICC) operational diagnostic criteria (Table S1)⁸ and in up to ~60% of BRRS cases.⁹ More recent studies have shown that up to 20% of individuals with ASD and macrocephaly have germline *PTEN* mutations.^{3,10–12} In the *PTEN* wild type (WT) subset of CS/CS-like individuals (who meet full diagnostic criteria minus one), ~10% harbor germline heterozygous variants in genes encoding three of the four subunits of succinate dehydrogenase or mitochondrial complex II (*SDHB* [MIM: 185470], *SDHC* [MIM: 602413], and *SDHD* [MIM: 602690]; collectively referred to as *SDHx*).¹³ Mitochondrial complex II lies at the crossroads between the electron transport chain and the tricarboxylic acid (TCA or Krebs) cycle, catalyzing the oxidation of succinate to fumarate. Individuals harboring *SDHx* variants show an increased risk of breast cancer and papillary thyroid cancer that surpasses the risk mediated by mutant *PTEN* alone.¹⁴ Functional studies show that *SDHx* variants result in ROS-mediated stabilization of HIF-1 α , destabilization and decreased protein levels of p53 due to defective interaction with NQO1, and

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Table 1. Demographic and Clinical Characteristics of 511 Cowden/Cowden-Like Syndrome and Bannayan-Riley-Ruvalcaba Syndrome Cases

median age at consent (range)	45 (1–89)
median CC score (range)	13 (0–69)
Gender	
female	350 (68.5%)
male	161 (31.5%)
Genotypes	
<i>PTEN</i>	309
<i>SDHx</i>	79
<i>PTEN/SDHx</i>	11
<i>PTEN/SDHx</i> wildtype	112
Macrocephaly	
yes	337 (65.9%)
no	116 (22.7%)
unknown	58 (11.4%)
Neurodevelopmental Features	
macrocephaly	108
autism spectrum disorder	45
global developmental delay	64
variable delay	27
mental retardation	12
learning disabilities	10
Cowden Component Malignancies	
breast cancer	142 (27.8%)
thyroid cancer	99 (19.4%)
renal cell cancer	50 (9.8%)
endometrial cancer	48 (9.4%)
colon cancer	16 (3.1%)
melanoma	13 (2.5%)
Other Malignancies	
ovarian cancer	24 (4.7%)
testicular cancer	3 (0.6%)
bladder cancer	1 (0.2%)
other gastrointestinal cancer	2 (0.4%)

resistance to apoptosis.¹⁴ Indeed, mitochondrial dysfunction is not an unusual suspect at the crux of metabolic perturbations which lead to tumorigenesis.^{15–17}

Biochemical screening of organic acids identified elevated plasma succinate levels in a pilot series of 55 CS/CS-like individuals with *PTEN*, *SDHB*, and *SDHD* mutations compared to controls.¹⁶ Mutations in *SDHB* and *SDHD* make sense in terms of succinate accumulation due to mitochondrial complex II dysfunction. However, the finding of succinate accumulation in *PTEN* mutation

positive (*SDHx* WT) individuals intriguingly suggests a common biochemical perturbation in CS/CS-like individuals independent of genotype. A subsequent biochemical analysis of urine and plasma organic acids and amino acids in another pilot series of 69 individuals with ASD, with or without macrocephaly and in the presence or absence of germline *PTEN* mutations, did not reveal genotype- or phenotype-driven associations with metabolite levels.¹⁸ However, the lack of elevated succinate levels in the ASD cases compared to the original series of CS/CS-like individuals without ASD reflects potential differences in metabolite signatures in a phenotype-dependent manner, and that may have been missed due to the limited sample size in each of the two pilot series. Thus, our current study sought to identify common and distinct TCA cycle metabolite alterations in an expanded prospective series of CS, CS-like, and BRRS individuals to ascertain genotype- and/or phenotype-dependent metabolic signatures.

Five hundred eleven eligible CS, CS-like and BRRS individuals were identified based on genotypic and phenotypic characteristics (Table 1) and in accordance with our research protocol 8458-PTEN, approved by the Cleveland Clinic Institutional Review Board. Both females (68.5%) and males (31.5%) were represented, and the median age at consent was 45 years (range 1–89). The Cleveland Clinic (CC) score, roughly an estimate of age-related disease burden,⁸ ranged from 0 to 69 (median 13), corroborating the broad spectrum of phenotypic burden and *PTEN* germline mutation status. Of 511 research participants, 309 (60.5%) harbored germline pathogenic *PTEN* mutations, 79 (15.5%) harbored germline *SDHx* mutations or variants, and 112 (21.9%) met clinical diagnostic criteria but were WT for both *PTEN* and *SDHx*. Neurodevelopmental disorders and solid tumors were the two predominant clinical phenotypic features. Among all cases, 111 (21.7%) had neurodevelopmental features, chief of which were ASD and developmental delays (DD). Of these 111, the median age at consent was 7 years (range 1–62). A total of 285 (55.8%) individuals had a cancer diagnosis, with 145 (50.9%) of those having second malignant primary neoplasms (SMNs). In the remaining 124 cases without cancer and without ASD or DD, the median age at consent was 39 years (range 1–74). We also identified 98 population controls (median age 37; range 2–76) who were WT for *PTEN* and *SDHx* (Table S2).

We first sought to investigate whether TCA metabolite levels (Figure S1) are altered in CS/CS-like and BRRS cases compared to population controls, while taking *PTEN/SDHx* mutation status, age, and gender into consideration. Immortalized lymphoblastoid cell lines (LBLs) from case and control peripheral blood samples ($n = 780$) were generated following standard procedures and then cultured *in vitro* for metabolic profiling (see Supplemental Material and Methods in Supplemental Data). Extracted metabolites were measured through the use of liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis. Sample measurements were obtained in five

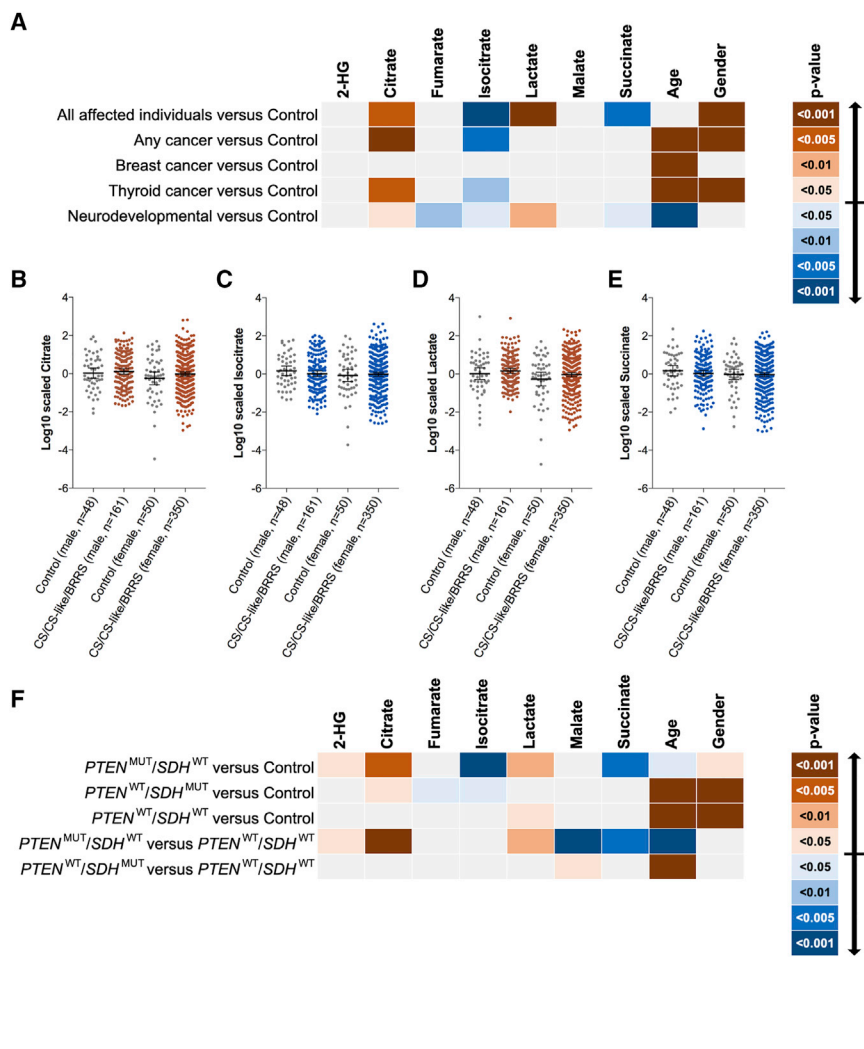


Figure 1. Alteration of TCA Cycle Metabolites in CS, CS-like syndrome, or BRRS Cases Compared to Population Controls

A general logistic regression model was applied to evaluate the association between metabolite levels and genotype and/or phenotype, with age and gender as covariates. Heatmaps (A and F) depict TCA cycle metabolites (top label) and significance of associations relative to various phenotypic or genotypic group comparisons (left label). Blue color corresponds to an association with decreased metabolite levels, whereas orange color corresponds to an association with increased metabolite levels. Color intensity corresponds to different thresholds of p value significance (scale on the right).

(A) Heatmap depicting the association between TCA cycle metabolite levels and phenotypes, irrespective of genotype status, compared to population controls.

(B–E) Stratified gender depiction of increased citrate and lactate, but decreased isocitrate and succinate in all CS, CS-like syndrome, or BRRS cases ($n = 511$) compared to population controls ($n = 98$). Metabolite levels were log-transformed and center-scaled to have a mean of 0 and a standard deviation of 1. The line in the middle of each scatter plot is plotted at the mean. Error bars represent the 95% confidence intervals.

(F) Heatmap depicting the association between TCA cycle metabolite levels and genotypes, irrespective of phenotype status. The first three rows represent comparisons between individuals of different genotypes and population controls. The last two rows represent comparisons between individuals of various genotypes.

batches and normalization was applied before we performed global comparisons. Quality control measures resulted in seven out of nine TCA metabolites (citrate, isocitrate, succinate, fumarate, malate, lactate, and 2-hydroxyglutarate) with reliable signal-to-noise estimates (Figure S1). To look for correlations between genotypic and/or phenotypic features and metabolite levels, we applied linear regression models controlling for age and gender (see Supplemental Material and Methods in Supplemental Data).

First, we looked for metabolite associations with phenotype, irrespective of genotype status. As such, we found that increased citrate ($p = 2.9 \times 10^{-3}$) and lactate ($p = 6.1 \times 10^{-4}$) but decreased isocitrate ($p = 3.0 \times 10^{-5}$) and succinate ($p = 2.8 \times 10^{-3}$) levels were associated with CS/CS-like or BRRS compared to controls (Figure 1A–E). Moreover, increased citrate ($p = 6.8 \times 10^{-4}$) but decreased isocitrate ($p = 1.9 \times 10^{-3}$) levels were correlated with having a cancer diagnosis (any malignancy) relative to controls. These observations, compared to controls, remained consistent for individuals diagnosed with thyroid cancer (increased citrate, $p = 1.9 \times 10^{-3}$; decreased isocitrate, $p = 7.2 \times 10^{-3}$), but not for those diagnosed with breast

cancer. We focus our analyses on breast and thyroid cancers because they are the most prevalent component malignancies reported in *PTEN* mutation positive CS.¹⁹ Considering the subset of individuals with ASD/DD (Table 1), we observed similar correlation patterns, i.e., increased citrate ($p = 4.6 \times 10^{-2}$) and lactate ($p = 9.7 \times 10^{-3}$), along with decreased isocitrate ($p = 3.2 \times 10^{-2}$) and succinate ($p = 1.3 \times 10^{-2}$); but we additionally noted the distinct association of those with ASD/DD with decreased fumarate ($p = 7.2 \times 10^{-3}$). These data indicate that TCA cycle metabolite alterations show a clear and consistent pattern in CS/CS-like and BRRS cases with various phenotypic features compared to controls.

Second, we sought to determine whether TCA cycle metabolite levels may be influenced by underlying genotype status. We first compared metabolites from individuals with germline *PTEN* mutations (*WT* for *SDHx*, *PTEN*^{MUT}/*SDH*^{WT}) to metabolites from controls (*WT* for both *PTEN* and *SDHx*). Our analysis showed germline *PTEN* mutation as a genetic feature associated with increased 2-hydroxyglutarate ($p = 2.4 \times 10^{-2}$), citrate ($p = 1.8 \times 10^{-3}$), and lactate ($p = 7.2 \times 10^{-3}$), but decreased isocitrate ($p = 5.0 \times 10^{-4}$) and succinate ($p = 4.3 \times 10^{-3}$)

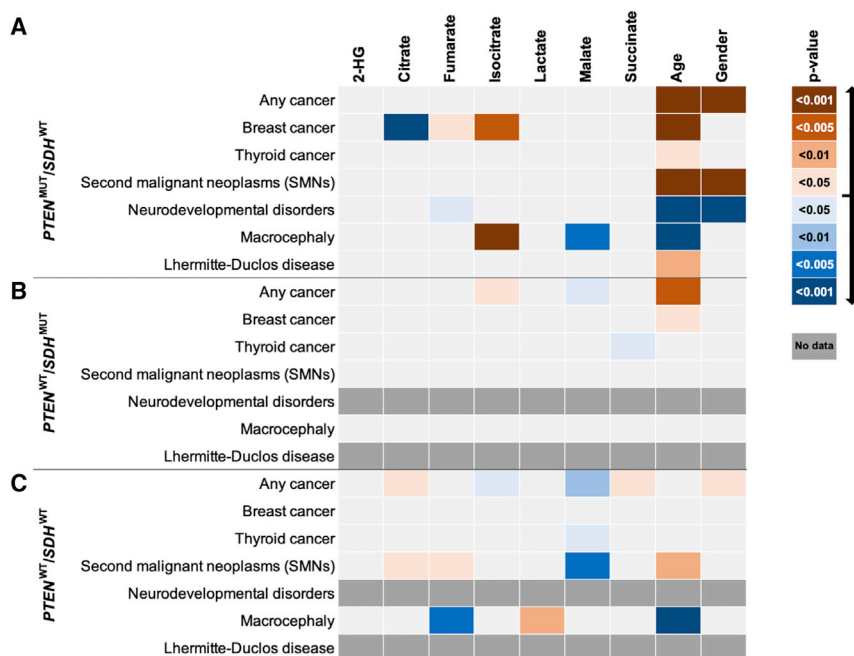


Figure 2. Altered TCA Cycle Metabolites Correlate with Distinct Genotype-Phenotype Combinations

Heatmaps depicting stratified analyses to determine associations among distinct metabolites and specific phenotypic features within the $PTEN^{MUT}/SDH^{WT}$ (A), $PTEN^{WT}/SDH^{MUT}$ (B), and $PTEN^{WT}/SDH^{WT}$ (C) groups, compared to cases who have identical genotypes, respectively, but who do not have the phenotype of interest. A general logistic regression model was applied with age and gender as covariates. Heatmaps depict TCA cycle metabolites (top label) and significance of associations relative to various phenotypic or genotypic group comparisons (left label). Blue color corresponds to an association with decreased metabolite levels, whereas orange color corresponds to an association with increased metabolite levels. Color intensity corresponds to different thresholds of p value significance (scale on the right). Dark gray (no data) fields indicate the absence of cases fulfilling the corresponding genotype/phenotype combinations.

(Figure 1F). Relatedly, increased citrate ($p = 1.9 \times 10^{-2}$) but reduced fumarate ($p = 4.4 \times 10^{-2}$) and isocitrate ($p = 1.9 \times 10^{-2}$) were associated with germline $SDHx$ variants (WT $PTEN$, $PTEN^{WT}/SDH^{MUT}$) compared to controls. Interestingly, CS/CS-like and BRRS individuals who are WT for both $PTEN$ and $SDHx$ ($PTEN^{WT}/SDH^{WT}$) only showed weak association with increased lactate levels ($p = 1.3 \times 10^{-2}$). To elucidate whether these differences are indeed genotype-dependent (versus linked to the presence of a disease state in cases relative to controls), we compared TCA metabolite levels between mutant and WT individuals. Relative to WT CS, CS-like syndrome, or BRRS cases ($PTEN^{WT}/SDH^{WT}$), increased 2-hydroxyglutarate ($p = 1.4 \times 10^{-2}$), citrate ($p = 2.5 \times 10^{-4}$), and lactate ($p = 1.1 \times 10^{-2}$) but reduced malate ($p = 6.0 \times 10^{-4}$) and succinate ($p = 4.3 \times 10^{-3}$) were associated with the $PTEN^{MUT}/SDH^{WT}$ genotypic feature. However, only increased malate ($p = 3.8 \times 10^{-2}$) was associated with the $PTEN^{WT}/SDH^{MUT}$ genotype compared to $PTEN^{WT}/SDH^{WT}$ cases. Interestingly, increased 2-hydroxyglutarate and decreased malate were distinctive metabolites in $PTEN^{MUT}/SDH^{WT}$ compared to WT individuals. So far, these genotype-level analyses suggest that $PTEN$ and $SDHx$ are strong predictive genetic features for TCA metabolite levels in cases versus controls (Figure 1F).

Because genotype is the dominant risk factor contributing to the metabolite differences of cases versus controls, we performed stratified analyses to tease out whether specific metabolites are associated with specific phenotypic features within defined genotypic subgroups. Within the $PTEN^{MUT}/SDH^{WT}$ subset of individuals, we found increased levels of fumarate ($p = 3.0 \times 10^{-2}$) and isocitrate ($p = 1.2 \times 10^{-3}$) but reduced citrate ($p = 5.0 \times 10^{-4}$) to be associated with breast cancer compared to $PTEN^{MUT}/SDH^{WT}$ individuals

without breast cancer (Figure 2A). Additionally, decreased fumarate ($p = 1.8 \times 10^{-2}$) was correlated with the presence of ASD/DD. Finally, increased isocitrate ($p = 1.0 \times 10^{-3}$) but decreased malate ($p = 1.4 \times 10^{-3}$) were both correlated with the presence of macrocephaly. Within the subset of individuals with $SDHx$ variants ($PTEN^{WT}/SDH^{MUT}$), we observed increased isocitrate ($p = 1.9 \times 10^{-2}$) but decreased malate ($p = 1.6 \times 10^{-2}$) to be associated with a cancer diagnosis, compared to individuals with the same genotype but without cancer. Additionally, reduced levels of succinate ($p = 3.2 \times 10^{-2}$) were specifically associated with the presence of a thyroid cancer diagnosis compared to $PTEN^{WT}/SDH^{MUT}$ cases without thyroid cancer (Figure 2B).

The third subset of analyses focuses only on $PTEN$ and $SDHx$ WT CS/CS-like and BRRS cases ($PTEN^{WT}/SDH^{WT}$). Intriguingly, although we did not observe notable associations, except for increased lactate, in this WT subset of individuals compared to controls (Figure 1F), multiple associations were observed when specific phenotypic features were taken into consideration (Figure 2C). These metabolite differences appear to be purely associated with phenotypic features in $PTEN^{WT}/SDH^{WT}$ CS, CS-like syndrome, or BRRS individuals. Increased citrate ($p = 1.9 \times 10^{-2}$) and succinate ($p = 3.6 \times 10^{-2}$) but decreased isocitrate ($p = 3.9 \times 10^{-2}$) and malate ($p = 9.4 \times 10^{-3}$) were associated with the presence of cancer (any malignancy) in WT CS, CS-like syndrome, or BRRS cases compared to WT cases without cancer. Second, decreased malate ($p = 1.2 \times 10^{-2}$) was specifically associated with the presence of a thyroid cancer diagnosis in $PTEN^{WT}/SDH^{WT}$ CS, CS-like syndrome, or BRRS individuals. Interestingly, increased citrate ($p = 3.9 \times 10^{-2}$) and fumarate ($p = 3.5 \times 10^{-2}$) but reduced malate ($p = 3.6 \times 10^{-3}$) correlated with the presence of SMNs in

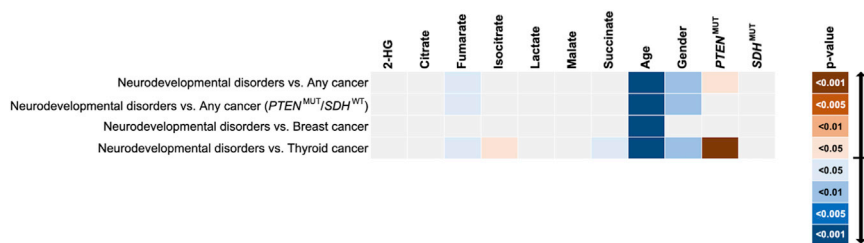


Figure 3. TCA Metabolite Signatures in Individuals with Neurodevelopmental Disorders Compared to Those with Cancer

A general logistic regression model was applied to evaluate the association of metabolite levels in neurodevelopmental disorders (ASD/DD) versus cancer, with age, gender, and genotype as covariates. Heatmap depicts TCA cycle metabolites (top label) and significance of associations (top label) and significance of associations (scale on the right).

relative to various phenotypic and/or genotypic group comparisons (left label). Blue color corresponds to an association with decreased metabolite levels, whereas orange color corresponds to an association with increased metabolite levels. Color intensity corresponds to different thresholds of p value significance (scale on the right).

these $PTEN^{WT}/SDH^{WT}$ CS, CS-like syndrome, or BRRS cases compared to CS, CS-like syndrome, or BRRS cases with the same genotype but without SMNs. Finally, increased lactate ($p = 1.1 \times 10^{-2}$) but decreased fumarate ($p = 4.5 \times 10^{-3}$) were associated with the presence of macrocephaly in $PTEN^{WT}/SDH^{WT}$ CS, CS-like syndrome, or BRRS individuals. Overall, these stratified analyses corroborate the impact of genotype-phenotype interactions on the identity and levels of altered TCA metabolites in CS/CS-like and BRRS individuals.

We next sought to explore whether metabolic differences exist among individuals belonging to the two predominant phenotypic features, neurodevelopmental disorders and cancer (Table 1). Indeed, decreased fumarate ($p = 1.9 \times 10^{-2}$) was associated with the presence of ASD/DD compared to those with any cancer (Figure 3). This observation remains consistent (decreased fumarate, $p = 3.2 \times 10^{-2}$) if we consider the subset of individuals with germline $PTEN$ mutations ($PTEN^{MUT}/SDH^{WT}$) for both phenotypes. Interestingly, in addition to an association with decreased fumarate ($p = 4.3 \times 10^{-2}$), we also observed decreased succinate ($p = 3.9 \times 10^{-2}$) but increased isocitrate ($p = 4.0 \times 10^{-2}$) to be associated with the presence of ASD/DD compared to the presence of a thyroid cancer diagnosis. These observations indicate that particular TCA metabolites, consistently fumarate, may be pertinent to distinguish individuals with neurodevelopmental disorders compared to those with cancer.

Since the majority of our accrued cases with syndromic ASD/DD (96.4%) harbored germline $PTEN$ mutations, it became prudent to evaluate whether TCA metabolite levels are also altered in individuals with idiopathic neurodevelopmental disease. We hence evaluated TCA metabolite levels in an independent series of cases enriched for idiopathic ASD, with or without macrocephaly (Table 2). This series also included 89 healthy individuals consisting of unaffected members of the probands' families. To investigate whether metabolic changes are $PTEN$ -genotype-dependent, we also included an independent series of individuals with ASD who harbor germline $PTEN$ mutations. If TCA cycle metabolite alterations are dependent on the $PTEN$ genotype, then we would expect distinct metabolite profiles between individuals with ASD who have germline $PTEN$ mutations and those with ASD who are WT for $PTEN$. Regardless

of genotype or head circumference, increased lactate ($p = 5.6 \times 10^{-4}$) but decreased isocitrate ($p = 3.6 \times 10^{-2}$) and succinate ($p = 6.6 \times 10^{-3}$) were associated with the presence of ASD compared to non-ASD controls (Figure 4A). These observations remained consistent among various subsets of individuals (with or without macrocephaly and with or without germline $PTEN$ mutations); these results suggest that the metabolic alterations within the TCA cycle are ASD state dependent but $PTEN$ status independent. Interestingly, increased isocitrate ($p = 4.2 \times 10^{-2}$) but decreased citrate ($p = 4.9 \times 10^{-2}$) and succinate ($p = 2.1 \times 10^{-2}$) were associated with the presence of macrocephaly (without ASD), and isocitrate showed a correlation in the opposite direction to what we had observed for individuals with ASD (decreased levels), including those with macrocephaly (macro-ASD). Notably, elevated lactate levels were consistently associated with ASD ($p = 2.0 \times 10^{-5}$) compared to non-ASD controls (Figure 4B). Additionally, family-based analyses corroborated the correlation of elevated lactate with ASD relative to non-ASD members within the same family (Figure S2). Overall, this association was consistent in 23 out of 39 (59%) families with ASD (8/18 [44.4%] with idiopathic normo-ASD, 8/11 [72.7%] with idiopathic macro-ASD, and 9/13 [69.2%] with $PTEN^{MUT}$ macro-ASD).

Overall, we found multiple associations between TCA metabolite profiles and genotypic and phenotypic features within CS/CS-like and BRRS individuals. TCA metabolite measurements were obtained from blood-derived lymphoblastoid cell lines (LBLs). While LBL interrogation to represent the germline is well accepted in the heredity field, it is tempting to speculate whether such metabolite alterations are also consistent in an affected tissue-specific manner. However, presuming identical effects within affected tissues and/or global effects on the whole organism may be a simplistic view for a complex process that warrants further investigation. Indeed, systematic studies investigating concordance of metabolite levels between affected tissues (e.g., breast cancer) and a surrogate source (e.g., blood, urine) are lacking. Additionally, we note that the opposite risk direction of age being younger age for neurodevelopmental features ($p = 9.4 \times 10^{-13}$) versus older age for cancer ($p = 1.4 \times 10^{-11}$) is due to the inherent nature of our cohort, which includes individuals presenting with

Table 2. Demographic and Clinical Characteristics of the ASD Validation Series (n = 171)

Control (non-ASD)	89 (52%)
female	54 (60.7%)
male	35 (39.3%)
median age at consent (range)	41 (2–67)
Pan-ASD	70 (41%)
Idiopathic Normo-ASD	41 (59%)
Female	6 (14.6%)
male	35 (85.4%)
median age at consent (range)	8 (2–45)
Idiopathic Macro-ASD	15 (21%)
female	0 (0.0%)
male	15 (100%)
median age at consent (range)	12 (5–38)
<i>PTEN</i> Macro-ASD	14 (20%)
female	5 (35.7%)
male	9 (64.3%)
median age at consent (range)	7 (2–24)
Macrocephaly or <i>PTEN</i> (no-ASD)	12 (7%)
macrocephaly	7 (58%)
female	4 (57.1%)
male	3 (42.9%)
median age at consent (range)	42 (35–61)
Macro- <i>PTEN</i> (no ASD)	5 (42%)
female	4 (80.0%)
male	1 (20.0%)
median age at consent (range)	34 (3–47)

ASD/DD or BRRS at a younger age (median age at consent 7 years, range 1–62) than that of individuals presenting with a cancer phenotype (median age at consent 52 years, range 3–89). Interestingly, we have shown particular individual TCA metabolites to be implicated in tumorigenic phenotypes. For example, within the *PTEN*^{MUT}/*SDH*^{WT} subset of individuals, we found increased levels of isocitrate ($p = 1.2 \times 10^{-3}$) but reduced citrate ($p = 5.0 \times 10^{-4}$) to be associated with breast cancer, compared to *PTEN*^{MUT}/*SDH*^{WT} individuals without breast cancer. In the TCA cycle, citrate is converted to isocitrate via cis-aconitate through the Fe-S cluster enzyme aconitase or aconitate hydratase.²⁰ While aconitase activity is inhibited, leading to citrate accumulation in normal prostate epithelium,²¹ catalytic activity is restored in prostate cancer.^{22,23} Importantly, this metabolic shift toward decreased citrate is a useful marker for distinguishing normal from transformed prostate epithelia. Additionally, aconitase expression was found to be an independent prognostic factor in gastric cancer.²⁴ Interestingly, aconitase inhibition has been observed in

fumarate hydratase (FH) deficient cancer cells due to elevated fumarate,²⁵ where FH is another TCA enzyme catalyzing fumarate to malate conversion. Whether perturbations in individual TCA metabolites will have a global impact on the TCA cycle and ATP production is a hypothesis warranting further investigation.

Intriguingly, TCA metabolite alterations were also observed in individuals with idiopathic ASD, suggesting that the TCA cycle may be a common pathway impacted in syndromic and sporadic neurodevelopmental disorders. We found elevated lactate to be consistently associated with ASD/DD compared to population controls and/or related family members who do not have ASD. Indeed, dysregulation of mitochondrial metabolism has been previously reported in ASD. Several studies have shown elevated levels of lactate or increased lactate/pyruvate ratio in children with autism.^{26–30} Additionally, *in vivo* magnetic resonance spectroscopic imaging demonstrated elevated lactate in the brains of individuals with ASD,³¹ as was observed in lymphoblastoid cell lines derived from individuals with ASD/DD in this study. Some studies speculate that ASD with mitochondrial dysfunction represents a distinct subgroup of individuals with frank mitochondrial disease.³² Certainly, longitudinal studies will be important to evaluate dynamic levels of lactate in individuals with ASD and correlations with the natural history of the disease. Importantly, the contribution of *PTEN* dysfunction to metabolic reprogramming and increased lactate production (i.e., Warburg effect) have been extensively studied in the context of carcinogenesis.³³ Although we observed elevated lactate levels to be associated with ASD regardless of the *PTEN* genotype, we speculate that individuals with germline *PTEN* mutations may have elevated levels of lactate at baseline, potentially resulting in intrinsic “adaptation” to these levels. However, another possibility is variable tissue-specific levels of lactate that could influence organ-specific manifestations in individuals with germline *PTEN* mutations.

Finally, an outstanding clinical challenge is the ability to predict among germline *PTEN* mutation carriers, who will develop neurodevelopmental disorders chief of which is ASD versus cancer.³⁴ While studies have shown distinct *PTEN* protein structural and functional characteristics of germline *PTEN* mutations in those with ASD versus those with cancer,^{35–40} absolute *PTEN* genotype-phenotype correlations are lacking and downstream clinical phenotypes difficult to predict in humans.³⁴ Here, we show that decreased fumarate is associated with ASD/DD versus cancer. In the TCA cycle, fumarate is formed from succinate oxidation by succinate dehydrogenase. Fumarate is then converted to malate by FH. Intriguingly, germline heterozygous mutations in *FH* (MIM: 136850) cause the neoplasia predisposition syndrome hereditary leiomyomatosis and renal cell cancer (HLRCC [MIM: 150800]), resulting in pro-tumorigenic fumarate accumulation.⁴¹ Indeed, TCA cycle intermediates, traditionally linked to oxidative phosphorylation, are now clearly shown to act in signal

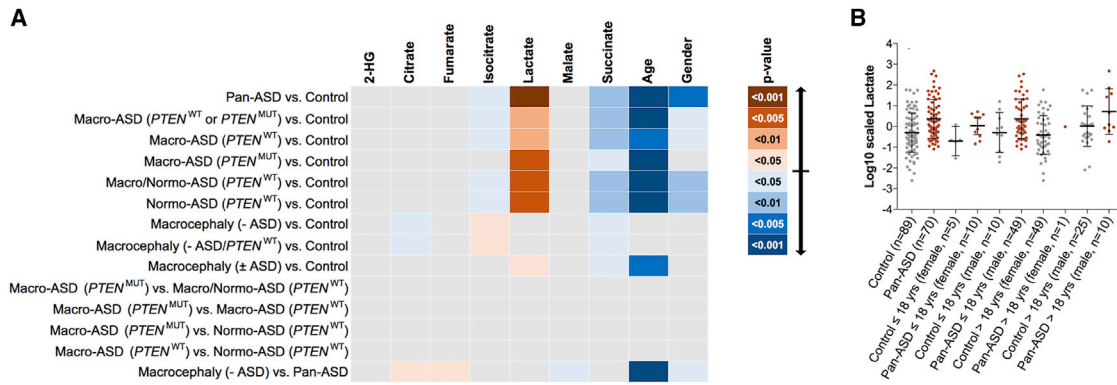


Figure 4. ASD Validation Series and Relevance of TCA Cycle Metabolites to Idiopathic Disease

(A) Evaluation of TCA metabolite levels in an independent series of cases ($n = 82$) enriched for idiopathic ASD, with or without macrocephaly, as well as $n = 89$ healthy individuals consisting of unaffected (non-ASD, control) members of the probands' families. Heatmap depicts associations among distinct metabolites and specific genotype/phenotype combinations compared to non-ASD healthy controls or among ASD cases (within-cases comparisons). A general logistic regression model was applied, and age and gender were covariates. Heatmap depicts TCA cycle metabolites (top label) and significance of associations relative to various phenotypic or genotypic group comparisons (left label). Blue color corresponds to an association with decreased metabolite levels, whereas orange color corresponds to an association with increased metabolite levels. Color intensity corresponds to different thresholds of p value significance (scale on the right).

(B) Stratified age and gender depiction of the association of increased lactate in ASD, irrespective of genotype or macrocephaly, compared to non-ASD controls. Metabolite levels were log-transformed and center-scaled to have a mean of 0 and a standard deviation of 1. The line in the middle of each scatter plot is plotted at the mean. Error bars represent the standard deviations.

transduction as well. Excessive accumulation of succinate or fumarate have been shown to suppress the homologous recombination (HR) DNA-repair pathway required for the resolution of DNA double-strand breaks and for maintenance of genomic integrity.¹⁷ The role of fumarate in neurodevelopmental disorders is less understood. However, fumarate is involved in the metabolism of glutamate, an excitatory neurotransmitter. Abnormalities in excitatory and inhibitory synaptic signaling have been implicated in the pathobiology of ASD.⁴² Recently, alterations in gut glutamate metabolism have been shown to be associated with gut microbiome composition in children with ASD.⁴³ In addition to reduced fumarate, the latter study found notably decreased 2-keto-glutaramic acid and L-aspartic acid in the guts of children with ASD compared to children with typical development. It is therefore tantalizing to speculate whether fumarate may be a pertinent predictive biomarker to distinguish individuals who will develop ASD/DD versus those who will develop cancer. Overall, our data suggest that TCA cycle metabolite alterations are germane to the pathobiology of *PTEN*-related CS, CS-like syndrome, or BRRS and to that of genotype-independent ASD, with potential implications for risk prediction for the individual and for therapeutic metabolic correction.

Supplemental Data

Supplemental Data can be found online at <https://doi.org/10.1016/j.ajhg.2019.09.004>.

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Declaration of Interests

The authors declare no competing interests.

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Web Resources

OMIM, <https://www.omim.org/>

RStudio version 1.1.463, <https://www.rstudio.com>

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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-like¹ (**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% CO₂ 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.

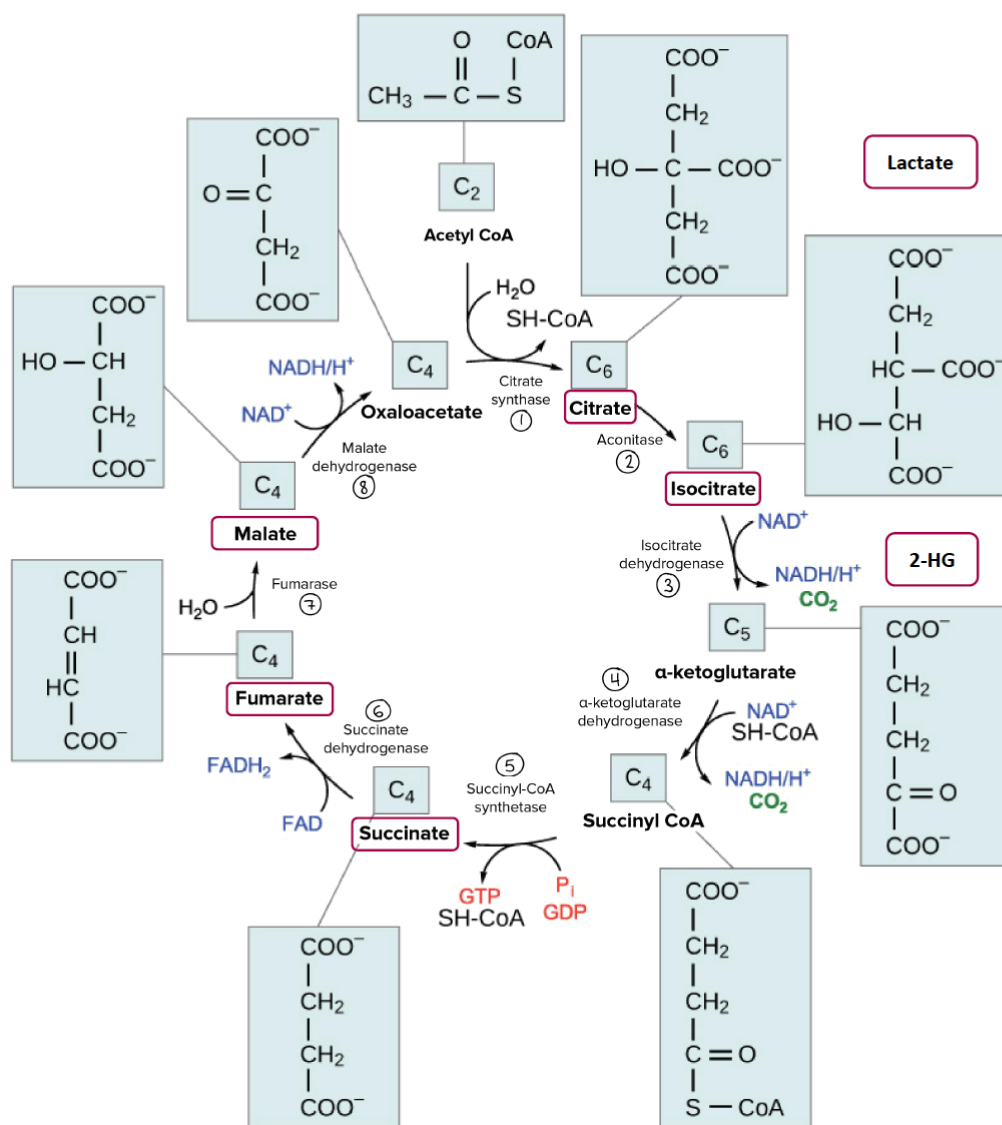


Figure S1. High confidence Tricarboxylic Acid Cycle (TCA) metabolites measured from case- and control-derived lymphoblastoid cell lines

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](#)).

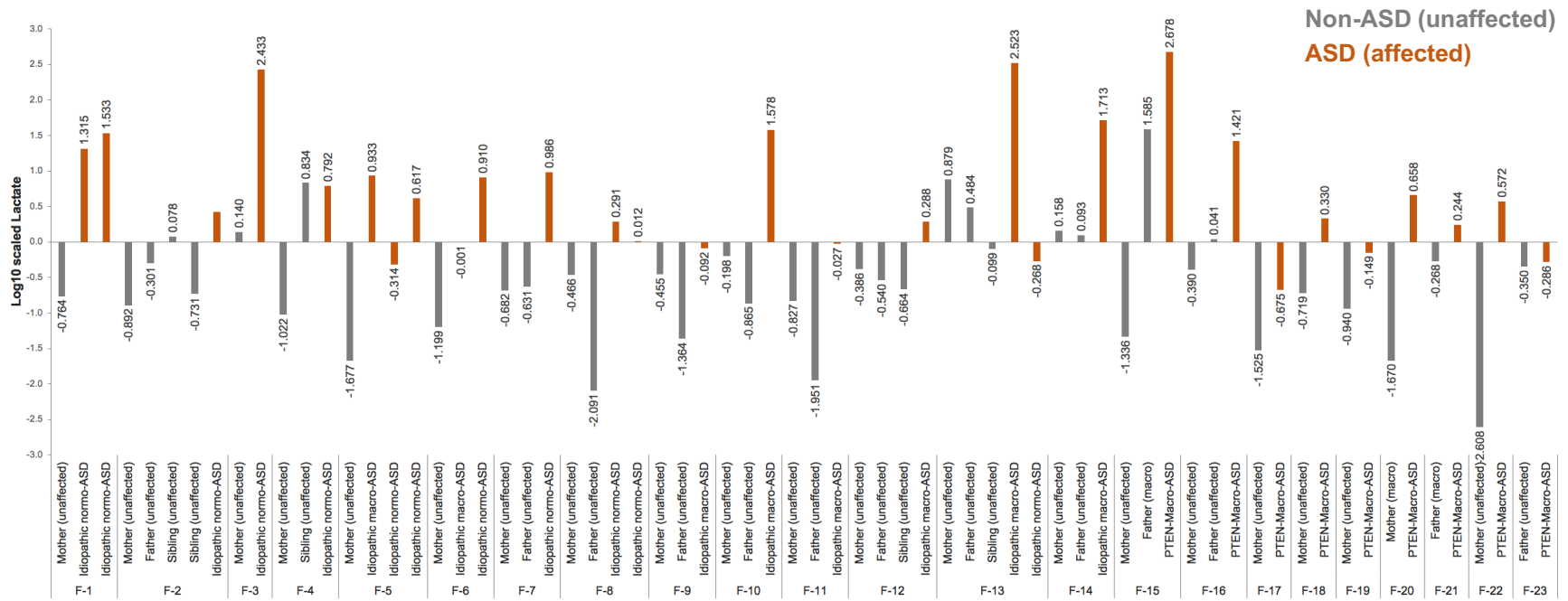


Figure S2. Family-based analysis of normalized lactate levels

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.

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

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

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