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The Sweat Metabolome of Screen-Positive Cystic Fibrosis Infants: Revealing Mechanisms beyond Impaired Chloride Transport

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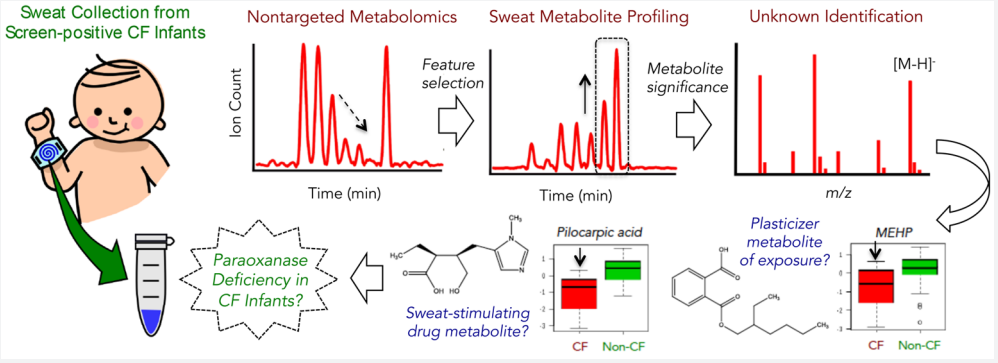
Supporting Information



ABSTRACT: The sweat chloride test remains the gold standard for confirmatory diagnosis of cystic fibrosis (CF) in support of universal newborn screening programs. However, it provides ambiguous results for intermediate sweat chloride cases while not reflecting disease progression when classifying the complex CF disease spectrum given the pleiotropic effects of gene modifiers and environment. Herein we report the first characterization of the sweat metabolome from screen-positive CF infants and identify metabolites associated with disease status that complement sweat chloride testing. Pilocarpine-stimulated sweat specimens were collected independently from two CF clinics, including 50 unaffected infants (e.g., carriers) and 18 confirmed CF cases. Nontargeted metabolite profiling was performed using multisegment injection−capillary electrophoresis−mass spectrometry as a high throughput platform for analysis of polar/ionic metabolites in volume-restricted sweat samples. Amino acids, organic acids, amino acid derivatives, dipeptides, purine derivatives, and unknown exogenous compounds were identified in sweat when using high resolution tandem mass spectrometry, including metabolites associated with affected yet asymptomatic CF infants, such as asparagine and glutamine. Unexpectedly, a metabolite of pilocarpine, used to stimulate sweat secretion, pilocarpic acid, and a plasticizer metabolite from environmental exposure, mono(2-ethylhexyl)phthalic acid, were secreted in the sweat of CF infants at significantly lower concentrations relative to unaffected CF screen-positive controls. These results indicated a deficiency in human paraoxonase, an enzyme unrelated to mutations to the cystic fibrosis transmembrane conductance regulator (CFTR) and impaired chloride transport, which is a nonspecific arylesterase/lactonase known to mediate inflammation, bacterial biofilm formation, and recurrent lung infections in affected CF children later in life. This work sheds new light into the underlying mechanisms of CF pathophysiology as required for new advances in precision medicine of orphan diseases that benefit from early detection and intervention, including new molecular targets for therapeutic intervention.

INTRODUCTION

Cystic fibrosis (CF) is a life-shortening and multiorgan autosomal recessive disease characterized by pancreatic insufficiency and recurrent lung infections that contribute to growth failure and progressive respiratory dysfunction.[1] However, life expectancy and quality of life are improved considerably with early diagnosis[2] as it allows for therapeutic interventions to be initiated before the onset of the first debilitating symptoms.[3] Early detection is achieved by inclusion of CF within expanded newborn screening (NBS) programs across many western countries given its prevalence in the Caucasian population.[4] Moreover, growing evidence has demonstrated the cost-effectiveness and efficacy of nutritional

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supplementation on later growth, lung function, and survival for individuals diagnosed through NBS as compared to symptomatically.[5,6] In most cases, NBS for CF adopts a two-tier screening algorithm based on an immunoreactive trypsinogen (IRT) test on a dried blood spot collected shortly after birth followed by DNA analysis for a panel of common disease-causing mutations of the CF transmembrane conductance regulator (*CFTR*) gene in the population.[3,7] However, the low specificity of IRT results in a high rate of false positives, whereas genetic testing leads to identification of unaffected carriers who comprise a majority (>70%) of screen-positive CF infants.[8] As a result, sweat chloride testing is required for confirmatory diagnosis of all screen-positive CF cases, including neonates having highly elevated IRT concentrations without identifiable CFTR mutations.[9]

Sweat chloride remains the gold standard for CF diagnosis with an accepted cutoff limit of ≥60 mmol/L for affected infants since its introduction almost 60 years ago.[10] Quantitative pilocarpine-stimulated iontophoresis via gel disks placed on the forearm of screen-positive CF infants is a noninvasive approach for localized sweat collection using microbore tubing as required for eccrine secretion analysis of major electrolytes from skin. However, ambiguous results complicate clinical decision-making especially when chloride levels are intermediate (30−59 mmol/L), which is defined as a CF-screen positive inconclusive diagnosis or CF-SPID.[11] The latter indeterminate result includes carriers, individuals with mild manifestations of the disease, and even patients who will later be diagnosed with CF.[12] Additionally, the highly variable phenotypes of CF are not clearly explained by sweat chloride levels or CFTR genotype alone.[13,14] Therefore, new biomarkers are needed to complement sweat chloride testing as a way to improve the prognosis and/or treatment monitoring of CF patients. As downstream biochemical products of gene expression that also reflect dietary and lifelong environmental exposures, metabolites are closely associated with clinical outcomes, which highlights their great potential as biomarkers for presymptomatic diagnosis of human diseases, including insights into disease mechanisms.[15,16] For instance, metabolites from dried blood spot extracts serve as biomarkers for early detection of inborn errors of metabolism using tandem mass spectrometry (MS/MS) technology.[17] In the case of CF, the sweat gland allows for direct assessment of CFTR function as compared to other less accessible organs, such as the lungs or pancreas.[18] However, the human sweat metabolome remains largely uncharacterized to date,[19] being composed primarily of water, electrolytes, urea, and lactate, as well as some amino acids and organic acids.[20] Only a few studies have reported nontargeted metabolite profiling of human sweat,[21−26] but none have been focused on infants using standardized sweat collection methods within a clinical setting.

We present a comprehensive characterization of sweat from screen-positive CF infants (<3 months), including several unknown compounds reported in sweat for the first time. Residual pilocarpine-stimulated sweat samples collected from CF affected (sweat chloride ≥60 mmol/L; 2 *CFTR* mutations) and unaffected infants (sweat chloride <30 mmol/L; 1 or no *CFTR* mutation) were analyzed using multisegment injection−capillary electrophoresis−mass spectrometry (MSI-CE-MS), which offers a high throughput platform for analysis of polar/ionic metabolites with quality assurance that is ideal for volume-restricted biospecimens.[27] Unknown metabolites were identified by high resolution MS/MS and confirmed with authentic standards.[28] This study demonstrates that sweat metabolites beyond chloride are associated with CF disease status in affected yet asymptomatic infants.

RESULTS

The Sweat Metabolome of Screen-Positive CF Infants. Nontargeted characterization of the sweat metabolome from screen-positive CF infants was performed by MSI-CE-MS when using a dilution trend filter on a pooled sweat sample that also served as a quality control (QC). As demonstrated in Figure S1, authentic metabolites from sweat were readily annotated based on their characteristic temporal signal pattern when using a serial injection format in MSI-CE-MS provided that they satisfied three major criteria, namely, their relative ion responses were measured with adequate precision and linearity with no signal detected in blank sample.[27] This rigorous filtering process resulted in a total of 64 unique compounds (i.e., 35 cations and 29 anions) after rejection of spurious, background, irreproducible, and redundant ion signals (e.g., in-source fragments, isotopes, and adducts derived from same metabolite) that constitute a majority of signals generated in ESI-MS. Sweat metabolites detected consistently in screen-positive CF infants comprised a diverse range of compounds, including amino acids, dipeptides, organic acids, fatty acids and several exogenous chemicals, such as paraben-based preservatives (e.g., methylparaben) from the gel pad, and a synthetic blue dye (e.g., FD&C blue no. 1) used for visualization of sweat collection following pilocarpine-stimulated iontophoresis. All molecular features in sweat were annotated based on their unique mass-to-charge ratio and relative migration time (m/z:RMT), including accurate mass and isotopic distribution to determine their most likely molecular formula with low mass error (<5 ppm). Confidence levels for metabolite identification are presented in Table S1 according to recommendations from the Metabolomics Standards Initiative.[29] Unambiguous identification was achieved based on comigration after spiking, as well as MS/MS spectral matching using authentic chemical standards, which was the case for more than 70% of annotated sweat metabolites. For instance, two unknown compounds of significance in this study were subsequently identified as pilocarpic acid (PA) and mono(2-ethylhexyl)phthalic acid (MEHP) as they were confidently assigned (level 1) with standards that displayed consistent m/z and RMT, in addition to MS/MS spectra with matching scores over 90% (Figure S2). In cases when commercial standards were not available, unknown compounds were tentatively identified (e.g., glycylglycine, level 2) based on comparative matches with MS/MS spectral databases. When no MS/MS matches were found in public databases or literature search, in silico MS/MS fragmentation prediction using CFM-ID[28] was performed for putative candidates in conjunction with identification of a likely metabolite class based on assignment of characteristic product ions and/or neutral losses from their MS/MS spectra (e.g., m/z 168.0770, RMT 0.733, ESI+, level 3, assigned as an amino acid derivative). Additionally, a total of eight compounds remained unknown with no defined chemical structure or metabolite class assignment, which were annotated only in terms of their most probable molecular formula (e.g., m/z 194.1380, RMT 0.802, ESI+, level 4).

Batch-Correction Adjustment and Probabilistic Quotient Normalization. Individual sweat samples were distributed into two analytical batches due to time required in collecting an adequate number of sweat samples from two

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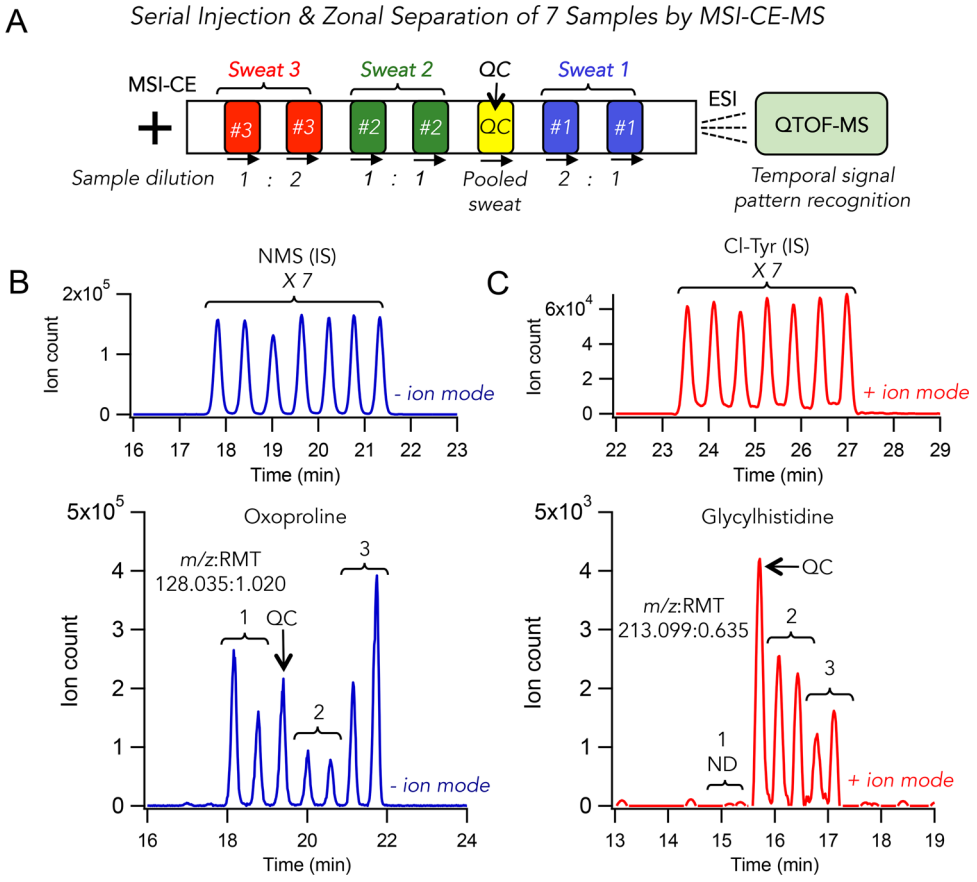

Figure 1. Temporal signal pattern recognition for high throughput metabolite profiling with quality assurance when using MSI-CE-MS. (A) Serial injection configuration and zonal separation used for analysis of seven sweat specimens from screen-positive CF infants, which was performed under negative and positive ion mode detection for acidic and cationic metabolites, respectively. Three pairs of sweat specimens were analyzed in duplicate and diluted with a unique pattern to encode mass spectral information temporally within a separation. A single pooled sweat sample serving as QC was also injected randomly at a different position (position 3 in this case) for each run. (B) Representative EIE for oxoproline (m/z 128.0352, ESI−) showing the peak pattern expected for each sample with differences in ion responses reflecting biological variance in sweat metabolite concentrations. (C) EIE for glycylhistidine (m/z 213.0990, ESI+), where one of the sample pairs (sample #1) was not detected (ND) reflecting high between-subject variance. Ion responses and migration times for all sweat metabolites were normalized to an internal standard (IS, 20 μM) used in negative (NMS) and positive (Cl-Tyr) ion mode in order to correct for differences in sample injection volume on-column, where each sweat metabolite was annotated based on its characteristic m/z:RMT.

hospital sites for CF affected infants with a low incidence rate in the population (≈1:3600). When combining results from both batches, a stepwise change in normalized ion signal to internal standard (i.e., relative peak areas, RPA) was observed for some compounds, due to a batch effect caused by long-term system drift of the instrument. Batch effects constitute a common problem in metabolomic studies when using electrospray ionization (ESI)-MS[30] even when implementing standard operating procedures, including daily preventative maintenance and mass calibration. In order to improve signal comparability across batches, an adjustment algorithm based on empirical Bayesian frameworks[31] was used to obtain batch-corrected RPA including the pooled QC sweat samples that were analyzed in every serial injection run by MSI-CE-MS together with three pairs of randomly assigned infant sweat samples that are measured in duplicate (Figure 1). Batch correction improved the precision of QC signals measured for some metabolites while others were somewhat negatively affected (Figure S3A,B). This resulted in a modest improvement in the median relative standard deviation (RSD) for all sweat metabolites from 25% (9−121%) to 24% (11−94%) when comparing RPA and batch-corrected RPA for all QCs (n = 24). Nevertheless, overall signal comparability across batches was improved as reflected by a greater overlap between QC samples in a 2D scores plot by principal component analysis (PCA) when comparing data before and after batch correction (Figure S3C,D). Therefore, this approach was adopted to reduce nonbiological experimental variation within the data set. Another aspect that contributes to unwanted variability is between-subject differences in hydration status and/or sweat rate that impact the effective concentration of sweat metabolites.[19] In this case, probabilistic quotient normalization (PQN)[32] was explored as a way to correct for underlying sweat dilution variability, using the QC within each MSI-CE-MS run as a reference sample to calculate the most probable relative dilution for individual sweat specimens. Overall, the CF affected (n = 18) and unaffected (n = 50) groups had a median RSD of 60−80% by batch correction or PQN normalization reflecting large between-subject biological variability that is considerably greater than technical variance for QCs (median RSD = 20−24%) as shown in Table S2. Overall, within-group biological variance was metabolite-dependent, ranging from 42 to 258% for CF and 33 to 228% for unaffected infants when considering batch-

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Table 1. Summary of Study Cohort Characteristics for Screen-Positive CF Infants Identified by NBS Using a Two-Tiered Screening Algorithm[a]

variable	non-CF (n = 50)	CF (n = 18)
sex		
female: no.	25	9
male: no.	25	9
age (days): range (median ± IQR)	11–60 (22 ± 7)	9–95 (18.5 ± 16)
birth wt (g): range (mean ± SD)	2232–5310 (3594 ± 540)	2650–4290 (3385 ± 430)
IRT (ng/mL): range (median ± IQR)	48.3–350.0 (63.2 ± 33.1)	95.0–376.0 (137.8 ± 54.9)
gestational age (weeks): range (median ± IQR)	37.0–41.3 (40.0 ± 1.6)	37.3–41.3 (40.0 ± 3.0)
chloride (mmol/L): range (median ± IQR)	6–28 (13 ± 7)	60–103 (92 ± 17)
CFTR genotype		
0 mutations	8	
1 mutation: DF508/null	29	
1 mutation: non-DF508/null	13	
2 mutations: DF508/DF508		6
2 mutations: DF508/non-DF508		8
2 mutations: non-DF508/non-DF508		4
collection site		
McMaster[b]	31	13
Sick Kids[c]	19	5
pancreatic status (fecal elastase)		
pancreatic sufficient	50	3
moderate pancreatic disorder		2
pancreatic insufficient		13

[a]Most continuous variables, with the exception of birth weight, were non-normally distributed (Shapiro–Wilk, $p < 0.05$), and described in terms of their median and interquartile ranges (IQR). IRT ($p = 1.03 \times 10^{-7}$) and chloride ($p = 7.85 \times 10^{-17}$) concentrations were the only continuous variables significantly different between the CF and non-CF infants (Mann–Whitney U test, $p < 0.05$). [b]McMaster Children's Hospital. [c]The Hospital for Sick Children.

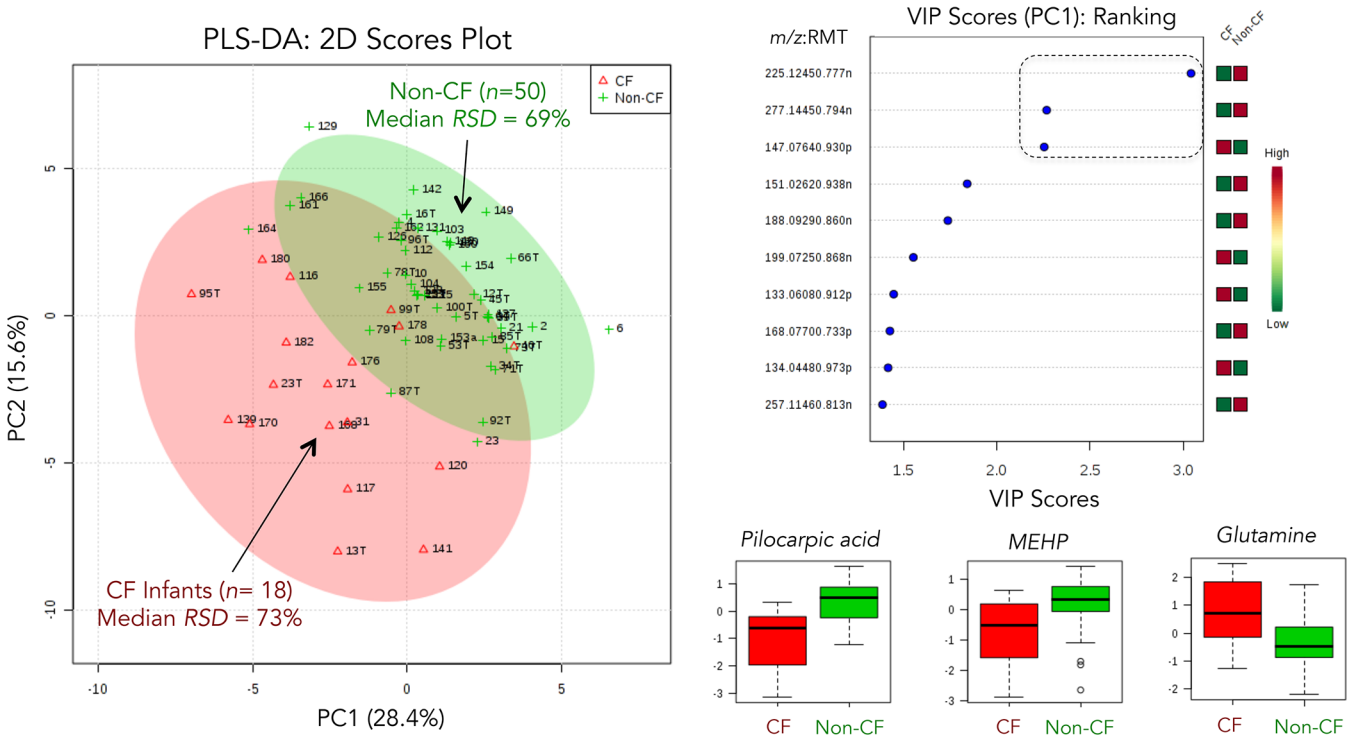

Figure 2. Overall biological variance when discriminating between affected (n = 18) and nonaffected (n = 50) screen-positive CF infants as depicted in a 2D scores plot when using PLS-DA with cross-validation (R^2 = 0.685; Q^2 = 0.472) and permutation testing (p = 0.006). Multivariate analysis of batch-corrected RPAs was used for selection of top-ranked sweat metabolites associated with CF in affected infants based on variable importance in projection (VIP scores >2.0), such as pilocarpic acid, MEHP, and glutamine. Sweat metabolites that were detected in at least 75% of individual sweat samples with adequate precision in QC samples were retained in the final data matrix, resulting in 54 polar/ionic metabolites from 64 sweat metabolites originally identified. All data was log-transformed and autoscaled after exclusion of an extreme outlier from the non-CF infant group.

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Table 2. Top-Ranked Sweat Metabolites Comparing Batch-Corrected Data for Affected and Unaffected Screen-Positive CF Infants[a]

m/z:RMT:mode	compound ID	p-value[b]	effect size	fold-change[c]	q-value
225.1245:0.777:n	pilocarpic acid	1.12×10^{-6}*[d]	0.55	0.37	6.06×10^{-5}**[e]
133.0608:0.912:p	asparagine	3.88×10^{-5}*	0.48	7.18	1.05×10^{-3}**
277.1445:0.794:n	MEHP	2.67×10^{-4}*	0.43	0.50	4.81×10^{-3}**
147.0764:0.930:p	glutamine	5.44×10^{-4}*	0.41	2.16	7.34×10^{-3}**
168.0770:0.733:p	amino acid derivative[f]	1.92×10^{-3}	0.37	0.54	2.07×10^{-2}**
151.0402:0.755:n	methylparaben	6.14×10^{-3}	0.33	0.57	5.52×10^{-2}
188.0929:0.860:n	unknown	7.19×10^{-3}	0.32	0.51	5.55×10^{-2}
134.0448:0.973:p	aspartic acid	1.03×10^{-2}	0.31	1.66	6.95×10^{-2}
213.0990:0.635:p	glycylhistidine	1.63×10^{-2}	0.29	2.05	9.61×10^{-2}
199.0725:0.868:n	unknown	1.78×10^{-2}	0.29	2.04	9.61×10^{-2}
163.0719:0.827:p	glycylserine[f]	3.09×10^{-2}	0.26	1.77	1.51×10^{-1}
215.0673:0.866:n	unknown	3.42×10^{-2}	0.26	1.72	1.54×10^{-1}

[a]Correction for multiple hypothesis testing is done by FDR ($q < 0.05$) or Bonferroni adjustment ($p < 9.26 \times 10^{-4}$) using Mann−Whitney U test. [b]Two-tailed exact p-values. [c]Fold-change based on median batch-corrected RPAs for CF/non-CF. [d](*) Compounds significantly different after Bonferroni correction. [e](**) Compounds significantly different based on FDR. [f]Compound or chemical class tentatively identified.

corrected RPA, which was selected as the optimum approach to adjust for long-term system drift.

Differential Metabolite Levels in CF Affected and Nonaffected Infants. This study comprised a sex-balanced cohort of infants with normal birth weight and gestational age, who were all presumptive (i.e., screen-positive) CF cases from NBS prior to confirmatory sweat chloride tests at two regional pediatric hospitals in the province of Ontario. A summary of the study cohort characteristics is presented in Table 1 for confirmed CF cases with elevated sweat chloride (≥60 mmol/L), high IRT, and two *CFTR* mutations, and screen-positive yet unaffected CF infants with low sweat chloride (<30 mmol/L), who are largely carriers with a single identified disease-causing *CFTR* mutation. Figure S4 highlights that affected screen-positive CF infants were distinguished primarily by their elevated sweat chloride as compared to unaffected infants, whereas birth weight and age at sweat testing between two groups were the same ($p > 0.05$). A highly heterogeneous genotype was notable among confirmed CF cases, mainly homozygotes and compound heterozygotes for *DF508*, as well as a few cases with other less common mutations. A variable disease phenotype among CF infants is also reflected by three cases of pancreatic sufficiency and two of borderline pancreatic disorder (100−200 μg/g fecal elastase), although the majority of CF infants were pancreatic insufficient. For unaffected screen-positive infants, the majority were carriers of a single *DF508* allele or other mutations, while eight infants had no mutation identified from the provincial NBS panel consisting of 39 disease-causing mutations and three variants. In order to effectively visualize overall trends in the sweat metabolomic data, Figure 2 depicts a 2D scores plot from a partial least squares discriminant analysis (PLS-DA) for differentiation of the metabolic phenotype in affected ($n = 18$) from unaffected ($n = 50$) screen-positive CF infants based on log-transformed and autoscaled batched-corrected data. Overall, 54 sweat metabolites were consistently detected in ≥75% of individual sweat samples (i.e., 10 sweat metabolites were excluded). The PLS-DA model was validated by permutation testing ($p = 0.006$; $n = 1000$) with good accuracy and robustness following cross validation ($R^2 = 0.685$; $Q^2 = 0.472$) despite considerable biological variance (RSD ≈ 70%), with three top-ranked metabolites (VIP scores >2.0) largely responsible for group class discrimination, namely, PA, MEHP, and L-glutamine (Gln).

Additionally, a comparison of metabolites in sweat samples from affected and nonaffected screen-positive CF infants was performed using nonparametric univariate statistical analysis since a large fraction of sweat metabolites (≈80%) deviate from a normal distribution based on a Shapiro−Wilk test ($p < 0.05$), including PA, MEHP, and Gln. Table 2 summarizes the most significant metabolites based on batch-corrected RPAs, including p-values (Mann−Whitney U test), effect sizes (estimated from z-scores), average fold-change (FC), and false discovery rate (FDR, q-values). Metabolites were considered significant following a Bonferroni correction ($p < 9.26 \times 10^{-4}$) or FDR ($q < 0.05$) to correct for multiple hypothesis testing. The top-ranked metabolites obtained for batch-corrected data were remarkably consistent with those for noncorrected RPAs (Table S3) and PQN (Table S4), although overall significance was dependent on the type of data treatment. For comparison, sweat chloride levels in the CF and non-CF groups were significantly different as expected ($p = 7.85 \times 10^{-7}$, effect size = 0.76, average FC = 7.1), performing extremely well in this case since samples with intermediate chloride levels were not included in this study. Overall, four sweat-derived metabolites were found to be significantly associated with CF in infants, namely, PA, L-asparagine (Asn), MEHP, and Gln as shown in boxplots and receiver operating characteristic (ROC) curves in Figure 3, which were also confirmed to be independent of sex, gestational age, birth weight, age at sweating testing, or hospital collection site (Mann−Whitney U test, $p > 0.05$) as summarized in Table S5.

Responsivity to Drug Exposure and Xenobiotic Elimination Reflect CF Disease Status. Two unexpected results from this study were the discovery that PA, a hydrolysis product from the sweat-stimulating drug pilocarpine, and MEHP, a metabolite from the ubiquitous plasticizer bis(2-ethylhexyl) phthalate (DEHP), differentiate CF disease status among screen-positive infants. In order to evaluate if these exogenous compounds were metabolized in vivo or represent hydrolysis artifacts from sampling or background contamination, pilocarpine gel disk extracts and blanks for the collection device were analyzed by MSI-CE-MS (Figure S5). The median fraction of pilocarpine hydrolyzed to PA in gel disks was 0.3% (0.1−1.2%), indicating that enzyme-mediated

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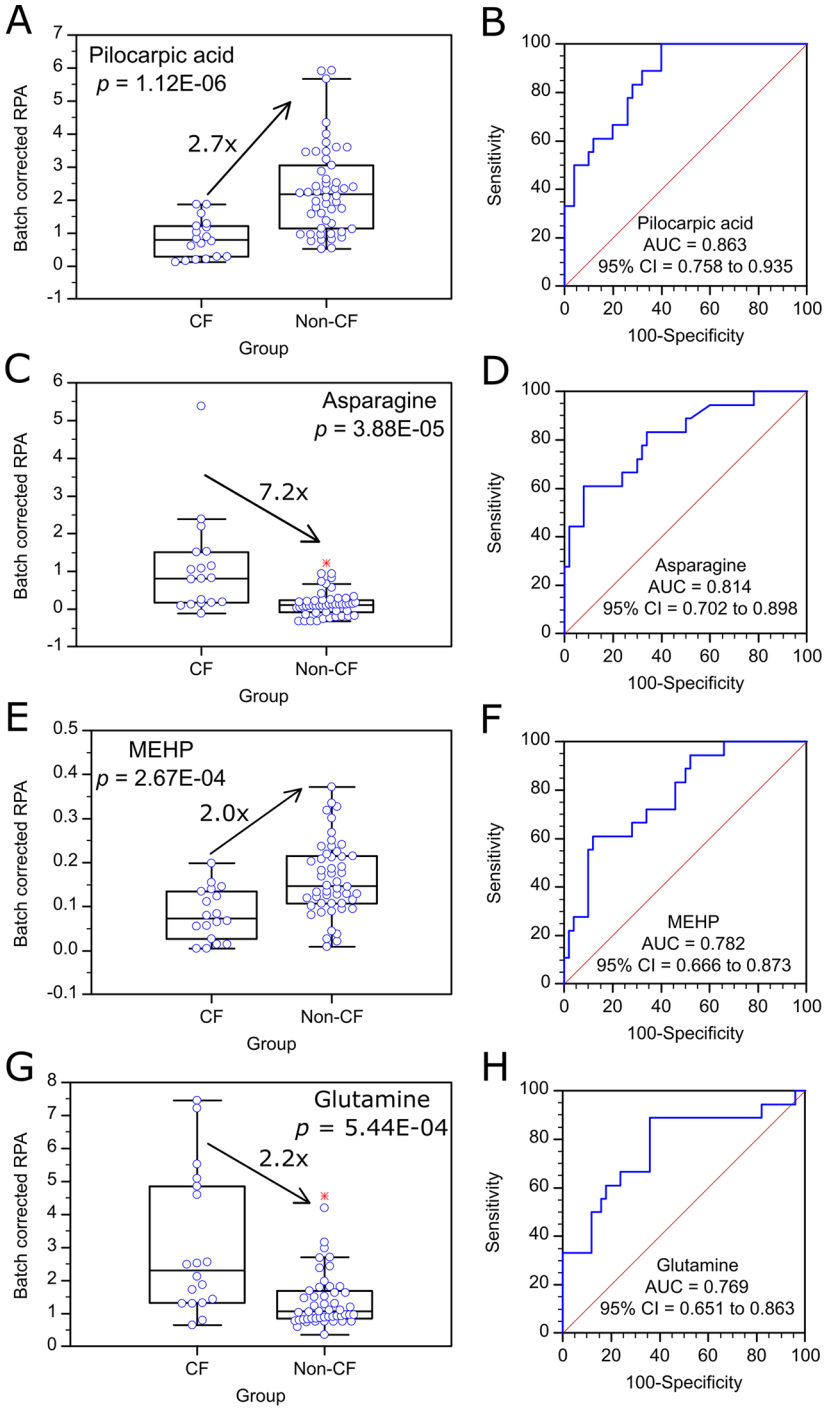

Figure 3. Boxplots with scatter plot overlays and receiver operating characteristic (ROC) curves for the four top-ranked sweat metabolites in screen-positive CF infants. Plots compare differentiating metabolites ($q < 0.05$) in affected ($n = 18$) and unaffected ($n = 50$) screen-positive CF infants based on batch-corrected relative peak areas (RPA), including PA (A, B), Asn (C, D), MEHP (E, F), and Gln (G, H). ROC curves indicate the area under the curve (AUC) and their 95% confidence interval (95% CI). For comparison, the ROC curve for sweat chloride had an AUC = 1.0 with a median fold-change of 7.1 and $p = 7.85 \times 10^{-7}$ in affected CF cases relative to unaffected screen-positive infants (non-CF).

hydrolysis was likely responsible for PA measured in sweat from CF unaffected infants (median = 1.1%, 0.2−11.5%, $p = 4.47 \times 10^{-5}$), although the extent of hydrolysis was similar to background for CF infants (median = 0.3%, 0.1−4.5%, $p = 6.62 \times 10^{-1}$). Other compounds detected in gel disk extracts included the preservatives methylparaben and propylparaben. The blanks for the collection device also contained the synthetic blue dye used for sweat visualization, whereas the fatty acids lauric acid and capric acid were likely derived from the plastic collection tube. However, the concentration of MEHP in the blank (median = 1.60 μmol/L in 55 μL, 1.35−1.71 μmol/L) was significantly lower than levels measured in unaffected CF infants (median = 4.9 μmol/L, 0.8−14.5 μmol/L, $p = 4.91 \times 10^{-5}$), although no difference was again observed for CF infants (median = 3.0 μmol/L, 0.7−6.6 μmol/L, $p = 8.72 \times 10^{-2}$). Overall, about 88% of all sweat samples had MEHP above blank-limited concentrations supporting the premise that MEHP was predominately derived from infant

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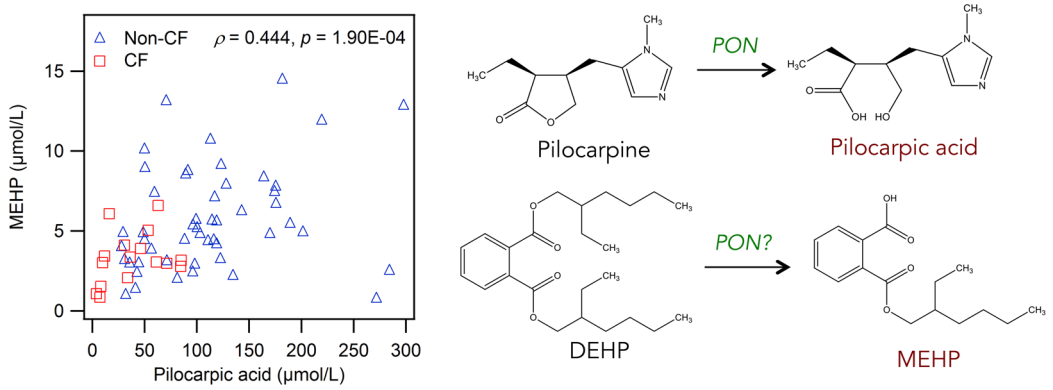

Figure 4. Scatter plot highlighting the correlation between PA and MEHP in sweat from screen-positive CF infants. A Spearman rank correlation analysis confirmed a significant association ($p < 0.05$) between these two exogenous metabolites. These hydrolysis byproducts of pilocarpine and DHEP are largely generated in vivo by the arylesterase/lactonase enzyme, human paraoxanase (PON), that is likely deficient in CF affected infants ($n = 18$) relative to unaffected screen-positive CF controls ($n = 50$).

sweat, notably among unaffected CF controls. Figure S6 confirms that MEHP was consistently measured with good precision (RSD < 6%) without background contributions during spray formation in both QC and dilution trend filter runs that include blanks. Furthermore, Figure 4 depicts a positive correlation between these two exogenous sweat metabolites based on a Spearman's rank correlation analysis ($\rho = 0.444$; $p = 1.90 \times 10^{-4}$) when comparing concentrations of MEHP and PA in sweat that is reflected in trends from boxplots using transformed data with supervised multivariate analysis (Figure 2) and original data using nonparametric statistical methods (Figure 3), whereas Gln and Asn had a weaker positive correlation among infant sweat samples analyzed ($\rho = 0.277$; $p = 3.69 \times 10^{-2}$). However, MEHP ($\rho = -0.241$; $p = 5.09 \times 10^{-2}$) and PA ($\rho = -0.327$; $p = 6.55 \times 10^{-3}$) had only modest negative correlations with sweat chloride concentrations measured independently by a chloridometer, in contrast to positive correlations of Asn ($\rho = 0.366$; $p = 4.35 \times 10^{-3}$) and Gln ($\rho = 0.194$; $p = 1.24 \times 10^{-1}$) with sweat chloride (Table S6). Additionally, a comparison was performed between sweat samples included in the first (9 CF and 31 non-CF infants) and second batches (9 CF and 19 non-CF infants) separately. For the first data batch, PA, MEHP, and Asn were found to be statistically significant ($q < 0.05$), whereas in the second batch, only PA and Gln were differentially expressed among screen-positive CF infants, although the same trends observed in the first batch were consistent for all four metabolites (Table S7). Nevertheless, the consistent trends in metabolite rankings for highly variable sweat specimens from screen-positive CF infants with diverse *CFTR* genotypes and phenotypes, collected from two different hospitals and analyzed across different batches over time, supports that these compounds are robust biomarkers reflecting CF disease status, rather than spurious findings or products of other underlying differences between affected CF and unaffected infant groups.

DISCUSSION

Sweat offers a promising biofluid for chemical analysis as it enables noninvasive sampling and continuous biomonitoring for assessment of disease biomarkers, chemical exposures, and drug metabolism.[19] Although various passive sweat collection devices/materials have been developed to date,[26] quantitative pilocarpine-stimulated iontophoresis remains the gold standard for confirmatory diagnosis of CF within a clinical setting based on elevated sweat chloride (≥60 mmol/L).[18] Herein, we present the first nontargeted characterization of the sweat metabolome among screen-positive CF infants demonstrating that several metabolites are associated with CF disease status in addition to sweat chloride. Similar to other metabolomics studies performed in sweat samples from healthy adults,[21−26] most of the metabolites identified from infants are composed of polar/ionic metabolites, including amino acids, organic acids, amino acids derivatives, dipeptides, and purine derivatives, including a number of exogenous compounds derived from sweat collection, diet, cosmetics, or environmental exposure. A majority of the 64 sweat metabolites, rigorously filtered from background ions and spurious signals after applying a dilution trend filter, were conclusively (level 1) or tentatively (level 2) identified, as summarized in Table S1. Only a small number of molecular features were associated with a probable metabolite class (level 3) or having no known chemical structure apart from a most likely molecular formula (level 4). Despite high biological variability of pilocarpine-stimulated sweat specimens measured within both groups of screen-positive infants (median RSD of about 70%) and occurrence of batch effects during data acquisition, a panel of four discriminating metabolites were found to be significantly associated with CF status ($q < 0.05$), which were largely consistent when comparing original results from measured relative ion responses prior to and after batch correction or following PQN normalization.

Interestingly, two exogenous metabolites in sweat, PA and MEHP, were both found to be present at higher concentration levels in unaffected screen-positive CF infants relative to confirmed CF cases. Indeed, these compounds were detected in all sweat samples analyzed while applying standardized cleaning procedures on forearms prior to sweat chloride testing. PA is a hydrolysis byproduct from the sweat stimulating and muscarinic cholinergic agent pilocarpine, which is metabolized by the enzyme human paraoxonase 1 (PON 1).[33] Although a small residual fraction of pilocarpine was found to be hydrolyzed to PA in gel disks, a far larger fraction was detected only in sweat samples from the majority of unaffected CF infants supporting the hypothesis that enzyme-mediated hydrolysis of the lactone moiety of pilocarpine is likely occurring in vivo within the sweat gland, which is impaired in CF infants. Similarly, MEHP is a hydrolyzed monoester derived from the plasticizer DEHP, which is used in the production of polyvinyl chloride (PVC) plastics, and is ubiquitously present in food packages, toys,

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personal care products, and medical devices.[34] MEHP has been found in human blood, urine, and sweat,[35] as well as in amniotic fluid, suggestive of prenatal exposures to the developing fetus.[36] In fact, infants have been shown to have the highest total intake of DHEP relative to other age groups.[37] Previous studies in older populations have reported urinary MEHP levels of up to 2.6 μmol/L,[38] including evidence that sweat may be a preferred route of excretion in comparison to urine with a ratio of MEHP concentrations in sweat to urine of about 4.6.[35] Similar to PA, only a small residual amount of MEHP originated from the plastic sweat collection tube when assessing the collection device blank. Similar to PA, unaffected CF infants were found to have significantly higher MEHP concentrations than the blank, which is likely attributed to in vivo metabolism of circulating DEHP and secretion of MEHP in sweat. The enzyme PON 1 is a nonspecific arylesterase/lactonase associated with xenobiotic detoxification and lipid metabolism[39] that has been implicated in modifying phthalate exposure on fetal development.[40] Indeed, maternal exposure to phthalates has also been associated with increased risk for childhood overweight/obesity.[41] As a result, we hypothesize that PON 1 expression and/or activity may be impaired in CF given the observations that both PA and MEHP are depleted in sweat specimens from CF affected infants (Figure 3) while having a significant degree of correlation (Figure 4) among sweat samples suggesting that they are likely metabolized via a common arylesterase/lactonase action. Indeed, recent studies have found that other PON isoforms present in serum and airway epithelial cells inhibit *Pseudomonas aeruginosa* infection by hydrolyzing their quorum-sensing molecules (e.g., *N*-acylhomoserine lactones), which control virulence factors and biofilm formation.[42] Griffin et al.[43] reported lower expression of genes for PON 2 in bronchoalveolar lavage fluid in CF patients with *Pseudomonas aeruginosa* infection, which indicates an association between PON and early lung infection in CF. Historically, PON was the first reported gene linkage associated with CF along with other polymorphic biomarkers prior to the discovery of the *CFTR* gene in 1989.[44,45] Indeed, CF is an inherited disease whose phenotypic variability is affected by not only specific mutations of the *CFTR* gene but also epigenetic and pleiotropic modifier genes.[46] Herein, we suggest that PON deficiency is prevalent among affected CF infants early in life prior to the occurrence of lung infections, a hypothesis that will be tested in future studies. Indeed, a putative link between these two xenobiotic metabolites in sweat and PON deficiency in this study also suggests a greater susceptibility to intoxication, oxidative stress, and inflammation among CF infants that could be mediated via specific therapeutic agents that activate the enzyme.

Asn and Gln were two endogenous sweat metabolites that differentiated confirmed CF cases from unaffected infants who were mainly identified as carriers having a single disease-causing *CFTR* mutation. To the best of our knowledge, no previous report has described a direct association between these conditionally essential and physiologically important amino acids and CF. Although lower levels of Gln have been found in circulating neutrophils in CF children compared to non-CF, no alterations were identified in plasma concentrations,[47] whereas Gln supplementation in CF patients produced no clear effect on markers of pulmonary inflammation.[48] Metabolite concentrations in sweat are dependent on solute partitioning during sweat production, including metabolites that are dependent on or independent of sweat rate, actively or passively transported from blood to sweat, or even generated within the sweat gland as part of its own metabolism.[49] Both Gln and Asn are cotransported by cationic/neutral amino acid transport systems that have been shown to be sodium and chloride dependent in human tissue[50] in order to maintain amino acid homeostasis given their myriad roles in regulating cell metabolism and function.[51] An earlier study in healthy men has indicated that amino acid excretion and/or duct reabsorption is compound-dependent, including Gln levels consistently lower in sweat compared to plasma with a mean fold-change of 33, whereas Asn concentrations were highly variable yet generally higher in sweat with a mean fold-change of 2.5.[52] More insight into the clinical significance of these metabolites in CF requires future studies involving collection of paired sweat and plasma samples from screen-positive infants. Our work suggests that disease-causing *CFTR* mutations that disrupt sodium and chloride reuptake in the sweat gland may also impair transport of these neutral amino acids, which were positively correlated with sweat chloride concentrations.

In summary, nontargeted metabolite profiling of sweat samples from CF affected and nonaffected infants identified by newborn screening revealed the presence of several discriminating metabolites (AUC > 0.75) associated with CF infants that are complementary to sweat chloride testing. Impaired chloride conduction in CF may impact transport of other nutrients that are also regulated by these same electrolytes in the sweat gland, such as Asn and Gln. Importantly, we demonstrate a potential association between CF disease status and sweat excretion of PA and MEHP that are largely generated in vivo following sweat stimulation by pilocarpine and environmental exposure to DHEP, respectively. These two exogenous compounds were strongly correlated in sweat and likely associated with a deficiency in paraoxanase activity in affected CF infants that is an arylesterase/lactonase mediating lipid metabolism, inflammation, and bacterial biofilm formation relevant to persistent lung infections later in life. Study limitations included a small sample size in the case of CF affected infants without an independent hold-out test cohort for further validation. However, we adopted a rigorous data filtering approach using a validated methodology[53] while correcting for multiple hypothesis testing, batch correction, and sampling bias/background contamination to reduce false discoveries when comparing results to a screen-positive yet unaffected CF infant group as control with sweat samples collected independently from two different hospital sites. Future work is planned to evaluate sweat metabolites within a larger multicenter cohort, including determination of reference levels for sweat metabolites and cutoff limits for biomarker candidates. Sweat metabolites that can predict disease progression among screen-positive infants having an ambiguous/intermediate sweat chloride test result will also be examined within a prospective study. This study demonstrates the rich information content derived from human sweat, which can reveal mechanisms in disease pathophysiology in CF beyond defective chloride transport, as well as differential responsivity to drug administration and lifelong chemical exposures. Sweat metabolomic studies can also identify new molecular targets for therapeutic intervention in precision medicine in order to elicit positive clinical outcomes for responsive CF patients especially when introduced early in life.

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■ ASSOCIATED CONTENT

Supporting Information

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Methods, Tables S1−S7, and Figures S1−S6 (PDF)

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