CLINICAL RESEARCH ARTICLE

Image-based β-adrenergic sweat rate assay captures minimal cystic fibrosis transmembrane conductance regulator function

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BACKGROUND: There is a need to prognosticate the severity of cystic fibrosis (CF) detected by newborn screening (NBS) by early assessment of CF transmembrane conductance regulator (CFTR) protein function. We introduce novel instrumentation and protocol for evaluating CFTR activity as reflected by β-adrenergically stimulated sweat secretion.
METHODS: A pixilated image sensor detects sweat rates. Compounds necessary for maximum sweat gland stimulation are applied by iontophoresis, replacing ID injections. Results are compared to a validated β-adrenergic assay that measures sweat secretion by evaporation (evaporimetry).
RESULTS: Ten healthy controls (HC), 6 heterozygous (carriers), 5 with CFTR-related metabolic syndrome (CRMS)/CF screen-positive, inconclusive diagnosis (CFSPID), and 12 CF individuals completed testing. All individuals with minimal and residual function *CFTR* mutations had low ratios of β-adrenergically stimulated sweat rate to cholinergically stimulated sweat rate (β/chol) as measured by either assay.
CONCLUSIONS: β-Adrenergic assays quantitate CFTR dysfunction in the secretory pathway of sweat glands in CF and CRMS/ CFSPID populations. This novel image-sensor and iontophoresis protocol detect CFTR function with minimal and residual function and is a feasible test for young children because it is insensible to movement and it decreases the number of injections. It may also assist to distinguish between CF and CRMS/CFSPID diagnosis.

INTRODUCTION

Cystic fibrosis (CF) is a genetically transmitted disease caused by a defective anion channel, CF transmembrane conductance regulator (CFTR). Defective CFTR results in abnormal mucus secretions causing obstruction in the lungs and pancreas, leading to life-threatening lung infections and malnutrition. Thousands of infants in the United States are identified annually through newborn screening (NBS) to be at risk for developing CF and are referred to CF Foundation-accredited centers for confirmation of diagnosis (www.cff.org). The standard sweat chloride test (SCT) uses quantitative pilocarpine iontophoresis to diagnose CF.[1] This test is based on the pathophysiology of defective chloride reabsorption in sweat ducts that causes elevated chloride concentrations in sweat from patients with CF[2] (Supplemental Fig. S1). The SCT is accepted worldwide as the gold standard diagnostic test for CF; however, it was established with patients with severe disease vs. healthy subjects.[3] It assesses CFTR protein function through analysis of the level of chloride content in the sweat collected after 30 min of electrical–chemical stimulation of the skin.[1,4] Sweat chloride ≥60 mmol/L confirms the diagnosis; <30 mmol/L is considered negative or "CF unlikely"; and 30–59 mmol/L is considered intermediate.[5] Intermediate sweat chloride results (and even negative results with two identified *CFTR* mutations detected by NBS) represent a significant challenge to physicians when determining prognosis and the appropriate level of management, because those individuals can potentially develop moderate to severe disease and conversely may not develop CF symptoms at all. Treatment is only initiated upon confirmed diagnosis or presentation of symptoms. Currently, positive NBS children with intermediate range will be followed and standard SCT repeated multiple times along the child's life. Signs and symptoms and confirmed diagnosis have been reported in individuals with negative and intermediate SCT in specific genotypes: D1152H and 3849 + 10kbC>T.[4,6–8]

Since the CF gene was discovered in 1989, >2000 different *CFTR* variants have been identified (http://genet.sickkids.on.ca), and the disease is now better understood as a spectrum expressing mild to severe phenotypes. A broader use of genetic testing in NBS revealed a subset of subjects genetically screen positive, who are asymptomatic, and have negative or intermediate SCT results. These individuals are given a diagnosis of CFTR-related metabolic syndrome (CRMS) or CF screen-positive, inconclusive diagnosis (CFSPID) and they are at risk to develop disease associated with CFTR protein dysfunction in the future.[9] Genotyping is now part of most NBS programs and an important component of confirmation of diagnosis in the United States and in other parts of the world.[5,10] The focus on genotyping is justified by the fact that there are three recently approved drugs capable of partially improving function of the defective protein.[1,11,12] With this shift in paradigm, the standard SCT has become limited in defining diagnosis. Thus, there is a clear need to develop an assay that can better assess CFTR function in vivo, not only to define diagnosis and prognosis in patients with mild to severe disease but also to define potential responses to CFTR-targeted drugs.

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The rate of adrenergically stimulated sweat secretion test provides a unique, essentially non-invasive, physiological approach to determine CFTR function in vivo. Human sweat glands secrete sweat via stimulation of two apparently independent neural pathways: cholinergic and adrenergic. The cholinergic pathway controls thermoregulation and the β-adrenergic pathway is thought to be related to a "fight or flight" response.[13] The cholinergic pathway appears to be independent of CFTR, while the β-adrenergic pathway is highly CFTR-dependent.[14,15] Consequently, β-adrenergic sweat rates can be used as a physiological marker of CTFR activity (Fig. 1). The initial sweat secretion test was developed in adults by measuring the ratio of the rate of evaporation of sweat with an evaporimeter (cyberDERM Inc.) after sequentially stimulating the two pathways independently. Figure 1 shows how the standard SCT differs from the β-adrenergic sweat secretion test. We applied the evaporimeter technology to test 30 children who were screen positive for CF. Although safe and well tolerated, measurements in infants (n = 5) were inaccurate due to movement artifacts, and in the pre-school age group, the sweat rate response to β-adrenergic stimulation (CFTR function) was not statistically different among those with symptoms and deleterious mutations (CF $n = 16$) vs. those who were asymptomatic and with benign variants of the gene (CRMS/CFSPID $n = 10$). Thus, sweat secretion assays as measured by the evaporimeter did not adequately discriminate between verified CF subjects and the CRMS/CFSPID group.[16]

The present work presents new technology and protocol that determine in vivo CFTR function as reflected by β-adrenergically stimulated eccrine sweat secretion. We hypothesize that this new technology and protocol are minimally invasive, suitable to be applied to young children, and sensitive to detect minimal and residual CFTR function. The technology may be used to develop a clinical test to confirm the diagnosis of CF after a positive NBS, to define prognosis of CF screen-positive infants to personalize care, and/or to quantitate responses to CFTR-targeted therapy in vivo.

METHODS

Subjects

The Institutional Review Boards from Children's Hospital Los Angeles (CHLA) and the University of Southern California (USC) approved the study. Written informed consent was obtained from all subjects ≥18 years old, and from the subjects' parents for all minors (<18 years old). Assent was obtained from subjects 7–18 years old. Only non-CF healthy controls (HCs) ≥18 years old were recruited during protocol development. HCs were recruited based on self-reporting of no chronic respiratory symptoms and no family history of CF; they did not have SCT or genotyping. CF and CRMS/CFSPID patients were diagnosed based on accepted CF Foundation (CFF) criteria and recruited from CHLA CF center.[5] The parents of patients who were previously genotyped were also recruited for testing.

Instrumentation

The measurement is based on the change in local electrical capacitance that results from the generation of sweat by eccrine sweat glands stimulated by cholinergic or adrenergic agonists. The capacitive fingerprint module (HF-EM401, HuiFan Technology Co., China) senses the capacitance on each pixel of the detector to produce an image of the fingerprint ridges and valleys, the air gaps in the valleys having a higher capacitance than the ridges. Sweat production decreases the local capacitance and modifies the image. A thin piece of filter paper placed on the sensor masks the texture of the skin surface. The resulting image is generated as
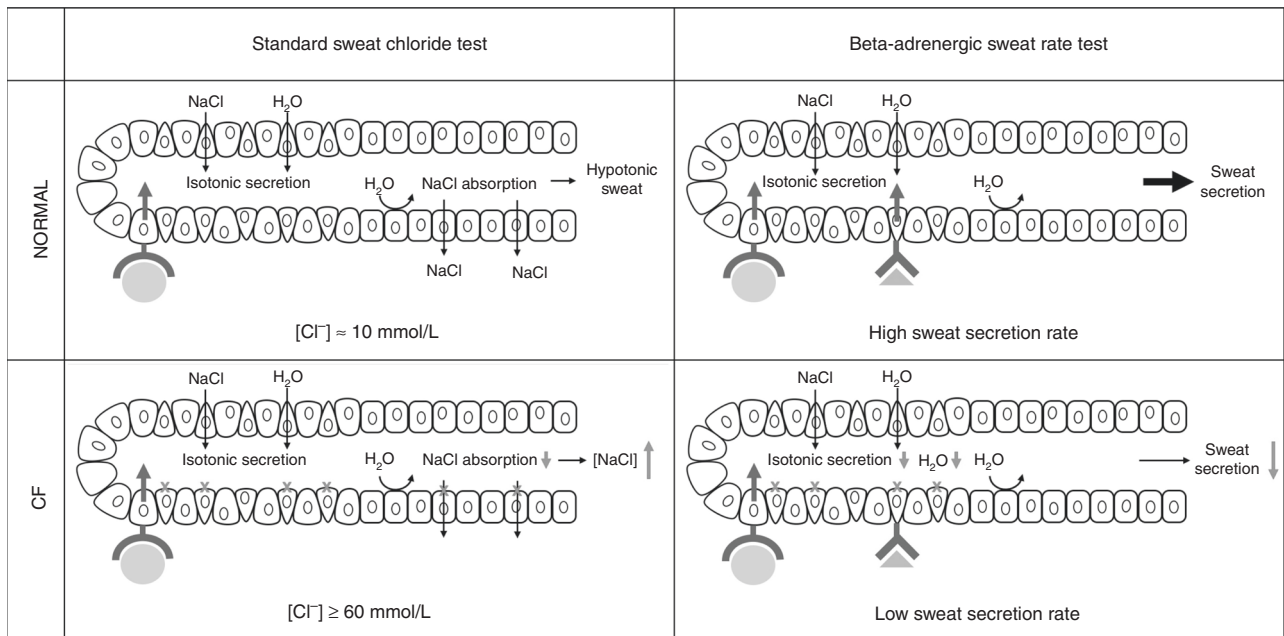

Fig. 1 The two images on the left depicts the traditional sweat chloride test that distinguishes between normal and cystic fibrosis (CF) phenotypes on the basis of the differences in concentration of chloride in the final sweat. Sweating is stimulated by the iontophoresis of a cholinergic stimulus (typically pilocarpine), which triggers both normal and CF glands equally. The normal duct readily reabsorbs NaCl, resulting in the output of hypotonic sweat. In contrast, the CF duct poorly reabsorbs NaCl, yielding sweat with abnormally high NaCl concentrations. The images on the right show the ratiometric sweat rate test; it distinguishes CF from normal based on the defective secretory functions of the CF transmembrane conductance regulator (CFTR)-mediated adrenergic pathway. That is, when an adrenergic stimulus is applied independent of cholinergic stimulus in a normal gland (i.e., cholinergic stimulus is blocked by atropine), sweat is secreted via CFTR-dependent pathway. Because CFTR function is defective in the CF gland, the volume of sweat secreted is significantly decreased or absent. The ratiometric sweat rate test uses the adrenergic sweat rate normalized by the individual's cholinergic sweat rate (hence, "ratiometric") to account for variability in sweat gland density and individual sensitivity to sweat gland stimulation. A higher adrenergic-to-cholinergic ratio indicates better in vivo CFTR functional activity, while a lower ratio indicates impaired CFTR function

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sweat wets the paper non-uniformly as a function of local sweat secretion from individual glands.

To conduct the sweat test, the capacitive sensor module is positioned on the ventral surface of the forearm with a 3D-printed holder that maintains the module in tight contact with the skin with a Velcro strap wrapped around the forearm. Since the image intensity varies with the pressure with which the module is held on the skin, a resistive pressure sensor (FSR 400, Interlink Electronics, Westlake Village, CA) was installed behind the fingerprint module. A cushion of stiff plastic foam was used to normalize the contact pressure between the skin and the sensor module and standardize the contact pressure of the sensor module on all subjects (Fig. 2).

Two types of filter paper (GE Healthcare, Pittsburgh, PA) were used: (1) Whatman AE 99 (120 µm thickness, 8 µm pore size) was used to measure sweat production after cholinergic stimulation. (2) Whatman Cyclopore polycarbonate membrane (7 µm thickness, 0.1 µm pore size) was used after adrenergic stimulation. Two different filter papers were used because the thinner Cyclopore paper allowed detection of small amounts of sweat observed in CF subjects after adrenergic stimulation. However, the thinner paper saturated quickly with the larger amounts of sweat observed in HCs after cholinergic stimulation and therefore required thicker paper.

Image acquisition and analysis

Custom software written in LabVIEW (National Instruments, Austin, TX) was used to transfer the sensor image every 5 s from the fingerprint module controller to the computer using serial communication. The gray-scale images (200×153 pixels) coded on 8 bits were displayed on the computer monitor to visualize the production and accumulation of sweat on the sensor surface. Analysis of the image sequence during a sweat stimulation

Fig. 2 Diagram of the study protocol and equipment. Drugs were administered identically on both arms: pilocarpine 1% by iontophoresis → atropine 5% by iontophoresis → β-adrenergic cocktail (atropine 8.8 µg, aminophylline 940 µg, and isoproterenol 8.0 µg) by intradermal injection. A = drug-soaked felt patches for iontophoresis; B = sweat inducer (A and B were applied to both sides); C–E = image sensor; F = pressure sensor; and G = cyberDERM evaporimeter and evaporimeter probes as applied

protocol and evaluation of the sweat rate was carried out as shown in Fig. 3.

Study protocol
Protocol development
Iontophoresis of pilocarpine 1%: Iontophoresis was performed to decrease the number of intradermal (ID) injections. Initial measurements were performed comparing the standard Pilogel 0.5% (ELITechGroup) and pilocarpine 1% (USC Compounding pharmacy). Maximum sweat rate was measured by a evaporimeter (Model RG1, cyberDERM Inc., Broomall, PA). The subject was seated comfortably with both arms resting on a table. A small area of skin on the ventral surface of the forearms (about $4 \times 4 \text{ cm}^2$) was gently cleaned with distilled water, dried, and covered by a thin layer of mineral oil to minimize transepidermic water loss. Evaporimeter probes were placed on these cleaned areas and secured with Velcro bands. Baseline activity was measured for 5 min. Sweat secretion was then stimulated by iontophoresis of Pilogel® on one arm and subsequently by pilocarpine 1% on the opposite arm. Circular-shaped gels (Pilogel®) or circular-shaped felt patches soaked in pilocarpine 1% solution were mounted on the sweat-inducer iontophoresis discs (Model 3700, Wescor Inc., Logan, UT), which applies 1.5 mA current for 5 min between electrodes to iontophorese drugs transcutaneously into the ID space (Fig. 2).

ID injections compared to iontophoresis: Quinton et al.[14] validated the evaporimeter protocol using subsequent ID injections of carbachol, atropine, and a β-adrenergic cocktail. Serial injections were well tolerated by adults in the validation study, but would be less manageable in a pediatric population. We then compared ID carbachol 0.1 ml = 0.01 µg (Miostat intraocular, Alcon®) and iontophoresed pilocarpine 1% measured by evaporimeter in a similar fashion as described for Pilogel 0.5% and pilocarpine 1% experiments. Similarly, ID atropine 0.2 ml = 8.8 µg (Bausch & Lomb) and atropine 5% (USC Compounding Pharmacy) were compared. Several attempts were made to iontophorese the β-adrenergic cocktail; however, sweat rate results were not reproducible when atropine, aminophylline, and isoproterenol were iontophoresed concurrently or sequentially.

Sweat gland potentiation: We evaluated the use of the ID β-adrenergic cocktail in HCs ($n = 2$) with and without prior "sweat gland potentiation" with ID carbachol followed by ID atropine measured by both methods.[17]

Reproducibility of image sensor: Reproducibility of the sweat response measured by the image sensor was assessed in three HCs who received three ID carbachol at neighboring sites on the ventral surface of the arm. We also map the darkened spots visualized in response to pilocarpine and β-adrenergic cocktail to demonstrate that the same sweat glands were stimulated.

Final protocol. In the final protocol, sweat rate was measured on both arms concurrently (Fig. 2). The subject was seated and skin preparation of the forearms was as described above. Evaporimeter was applied to the right arm and the image sensor to the left arm. Baseline secretion was measured for 5 min. Sweat secretion was then stimulated simultaneously on both arms by iontophoresing pilocarpine 1%, followed by Atropine 5%. The skin was marked with a pen to reposition the probes in the same site. The effect of blocking cholinergic response with atropine was confirmed by the evaporimeter only on the right arm. Once the evaporimeter signal stabilized (usually 2–5 evaporimeter units above baseline), an ID injection of 0.2 ml β-adrenergic cocktail was administered containing: 8.8 µg of atropine, 940 µg of aminophylline (Regent Laboratories Inc.), and 8 µg of isoproterenol (Hospira Inc.). After injecting both sides, the evaporimeter and the image-sensor probes were
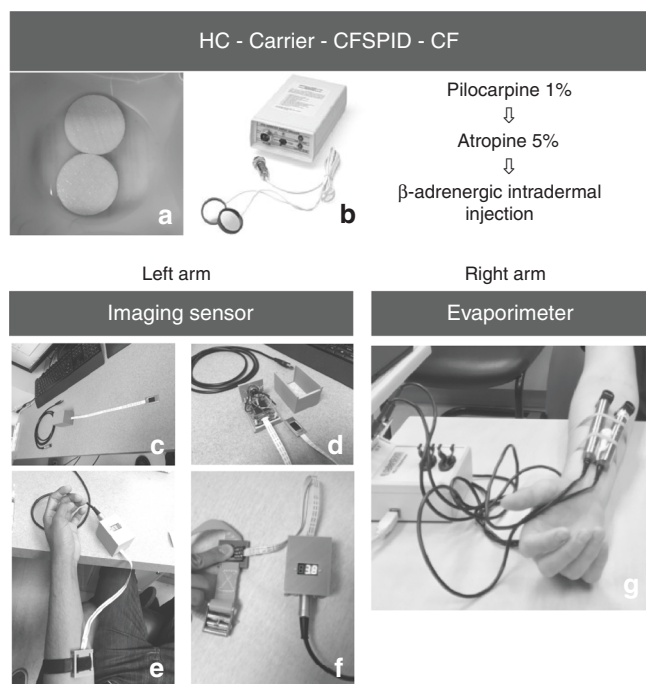
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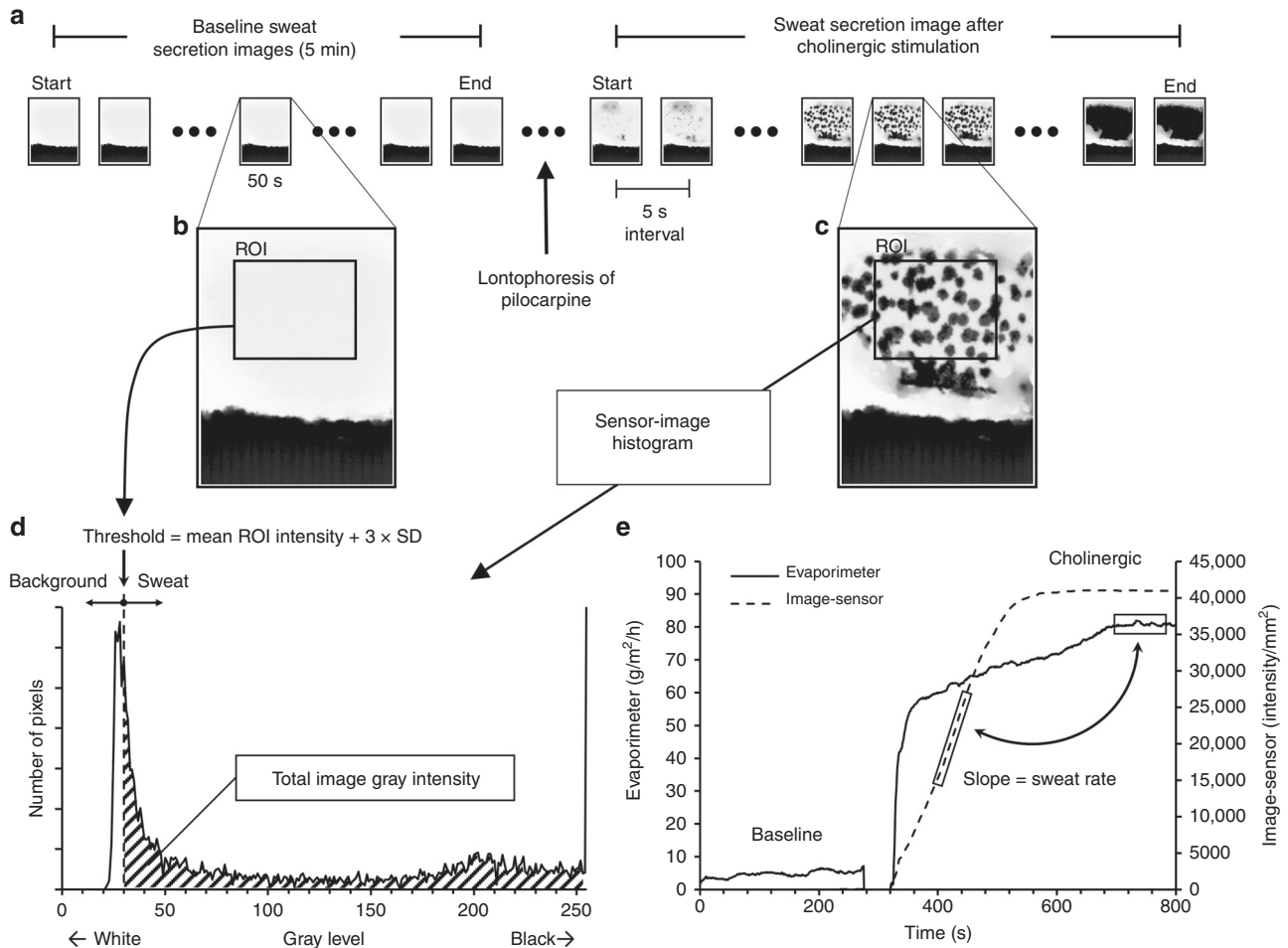DB Salinas et al.

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Fig. 3 Analysis of the image sequence during a sweat stimulation protocol and evaluation of the sweat rate with the image sensor. **a** shows the sequence of images acquired every 5 s during the sweat stimulation protocol. Images collected during baseline conditions are sampled first and are followed by images collected after cholinergic stimulation. The sensor is briefly removed from the measurement site to allow for pilocarpine iontophoresis between the two sequences. The baseline image acquired 50 s after the start of the sequence is examined visually after the data collection is completed to select the largest region of interest (ROI) free of artifacts and edge effects (**b**). The mean and standard deviation of the gray levels of all pixels in the ROI are computed to estimate a gray level threshold, above which a change in the image associated with detection of sweat is measured above the background gray level. In this example, the gray level threshold is 30 on a scale 0–255, where 0 corresponds to white and 255 to black. **c** A typical image collected during cholinergic stimulation displays spotty dark regions in the ROI that correspond to the production of sweat. **d** shows the number of pixels on this image (ordinate) for each gray level between 0 and 255 (abscissa). As more dark spots appear on the image collected during the sweat stimulation sequence, the image shows more and more pixels with elevated gray levels above the threshold. Thus, the right side of the curve gradually rises above 0 and the area under the curve (hashed) increases. The threshold level (30 in the example) is subtracted from each image in the sequence to correct for the background gray level of the image. The sum of all pixel intensities above the threshold (hashed area under the curve) is computed as a measure of the darkness of the image in the ROI. **e** shows a plot of the area under the curve in each image (hashed line) as a function of time. The steepest slope of the curve corresponds to the fastest change in image darkness and is used to represent the maximum rate of sweat production measured with the image sensor. Because different image collection sequences had ROIs of different sizes, the hashed area under the curve in **d**, **e** is scaled by the size of the ROI expressed in mm^2 (right vertical scale). **e** also shows the evaporimeter readings (solid line, left vertical scale) collected on the contralateral site. The maximum rate of sweat evaporation measured by the evaporimeter is observed when the evaporimeter output is maximum and near flat

repositioned over the sites of ID injections on the right and left arms, respectively.

Statistical analysis
Patient characteristics and distribution of maximum sweat rate were described by summary statistics. Mean and standard deviation (SD) were used to describe the variables with a normal distribution, while median and interquartile range was used for the variables with non-normally distributed. Frequency and percentages were used to summarize categorical variables. Maximum sweat rate measurements were natural log-transformed to appropriately adjusting for the non-normal distribution and analysis of variance was used to examine the differences in these measurements for both evaporimeter and image-sensor assays across HCs, carriers, CRMS/CFSPID, and CF groups. Then, multiple comparisons with Tukey's adjustment was used to further assess which of the pairwise comparisons differ in maximum sweat rate measurements. Taking into account that the two methods have different units, z-scores were calculated based on total sample mean for each method in order to assess β/chol ratio difference for all groups. The difference in the z-scores between two methods was assessed by repeated-measure analysis of variance. Statistical significance was set at 5% level with two-tailed test throughout the analyses. All statistical computations were done in Stata/SE 15.1 (StataCorp, College Station, TX).

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RESULTS

Initial results during protocol development

Iontophoresis of pilocarpine 1% and Pilogel 0.5% were equivalent. Iontophoresis of Pilogel 0.5% and pilocarpine 1% on the same HC individuals were equivalent (104 ± 7.4 and 104 ± 13.4 mg $H_2O/m^2/h$, respectively, $n = 5$; $p = 0.986$).

ID injections compared to iontophoresis. ID carbachol 0.1 ml = 0.01 μg and iontophoresed pilocarpine 1% measured by evaporimeter had similar results: 84 ± 11.5 and 82 ± 12.7 mg $H_2O/m^2/h$ ($p = 0.49$, HCs $n = 33$, CF $n = 13$)· atropine-injected vs. iontophoresed blocked 95 and 83%, respectively, of the cholinergic sweat rate ($n = 11$, $p = 0.345$).

Sweat gland potentiation with cholinergic stimulation is necessary for maximum β-adrenergic stimulated rate. When evaluating the use of the ID β-adrenergic cocktail in HC ($n = 2$) with and without prior "sweat gland potentiation," maximum sweat rate in response to adrenergic stimulation without potentiation was about half the rate with potentiation, when measured by both evaporimeter and image sensor: evaporimeter—no potentiation: 40 (36–44) median (95% confidence interval (CI)) and with potentiation: 77 (66–83) ($p = 0.004$). Image sensor—no potentiation: 36 (33–38) and with potentiation: 65 (49–112) ($p = 0.001$), confirming previous findings.[17,18]

Sweat rates measured by the image sensor were reproducible. Sweat rates after injection of carbachol in three different locations on the volar aspect of the arm of three individual subjects showed: subject 1 = 102.57 ± 1.89, subject 2 = 69.66 ± 16.60, and subject 3 = 184.03 ± 19.03 intensity/time/mm². The sequence of sensor images gathered after stimulation of the sweat glands demonstrated the production and gradual accumulation of sweat at the stimulated site. Mapping the darkened spots visualized in response to pilocarpine and β-adrenergic cocktail indicated that the same sweat glands were measured with each repeated stimulation (Fig. 4).

Evaporimeter and image-sensor measurements were comparable. A total of 10 HC, 6 heterozygous (carriers), 5 CRMS/CFSPID, and 12 CF individuals completed testing using the final protocol (Table 1). Group characteristics were as follows: age in years (SD): HC = 32 (13), carrier = 38 (8), CRMS/CFSPID = 8 (1), and CF = 16 (4); race/ethnicity: HC = 100% white/10% Hispanic, carrier = 100% white/67% Hispanic and 33% Caucasian, CRMS/CFSPID = 100% white/60% Hispanic and 40% Caucasian, and CF = 83% white and 17% black/33% Hispanic, 50% Caucasian, and 17% African descent. *CFTR* mutations/variants were identified by NBS and parent DNA testing. Evaporimeter and image-sensor measurements had a linear relationship when quantifying maximum sweat rates in response to β-adrenergic stimulation in HC and CF (Spearman's correlation = 0.8379, Supplemental Fig. S2). There is
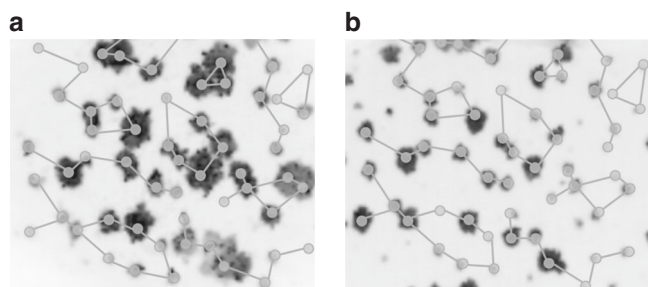
no significant difference in the sweat rate measurements between evaporimetry and image sensor according to z-scores analyses (Table 2).

Minimal and residual function CFTR *mutations yield low β-adrenergic/cholinergic sweat rate ratios.* Table 1 shows individual β/chol ratios measured by both assays. Figure 5 shows individual image-sensor-β/chol ratio plotting and genotype with corresponding *CFTR* I–VI classes and functional classification. All CRMS/CFSPID subjects had one or both *CFTR* mutations classified as residual function as did 2 of the 12 CF subjects (siblings with the same genotype: 3849 + 10kbC>T/935delA). All minimal and residual function *CFTR* mutation combinations had low β/chol ratios when measured by both assays.

β-Adrenergic/cholinergic ratio in natural log from evaporimetry and image-sensor measurements were able to differentiate between CRMS/CFSPID and CF. β/Chol ratio (after natural log transformed) from evaporimetry and image-sensor measurements were equally successful to show significant differences between HC and CRMS/CFSPID, HC and CF, carrier and CRMS/CFSPID, carrier and CF, and CRMS/CFSPID and CF (Fig. 6).

DISCUSSION

The image-sensor and Iontophoresis protocol presented here illustrate initial development of a novel application of technology to detect minimal and residual CFTR function in vivo. The image-sensor assay detects sweat rates in response to β-adrenergic stimulation in individuals diagnosed with CF and CRMS/CFSPID that can be performed on pediatric subjects. As seen previously with the evaporimeter, both groups had low β-adrenergic sweat rate,[16] which suggests CFTR dysfunction in CFTR-dependent sweat secretion in glands of both CF and CRMS/CFSPID children. The advantages of this technology and proposed iontophoresis protocol over other proposed assays are that: (a) it can be completed in <30min, (b) it requires only one ID injection, as iontophoresis of pilocarpine and atropine are applied prior to the β-cocktail injection; (c) the apparatus is insensitive to subject movement, making it more feasible for use in a younger population; and (d) it detects β-induced sweat secretion over the full range of CFTR function, which may assist in differentiating between CF and CRMS/CFSPID diagnosis.

Since the mid-1980s when Sato and Sato[15,18] discovered that CF glands do not secrete sweat in response to β-adrenergic stimuli, but secrete normally after exposure to cholinergic secretagogues, there have been several attempts to develop an assay with detection limits that are low enough to capture differences in β-adrenergic sweat rate responses among CF subjects with different *CFTR* mutations.[17] A ratiometric assay comparing β-adrenergic-to-cholinergic sweat rates using the evaporimeter was validated several years ago for differentiating heterozygotes from CF genotypes.[14] However, evaporimetry was not sufficiently sensitive to differentiate among CF subjects with different levels of disease severity such as pancreatic sufficient vs. insufficient subjects. As there was a significant difference between CFTR-related disorder (which is a single organ disease caused by two *CFTR* mutations) and CF, our group attempted to apply evaporimetry to determine differences between CF and CRMS/CFSPID.[16] To our surprise, the CFTR function in CRMS/CFSPID assayed by this method appeared just as low as in CF individuals. The current image-sensor protocol shows no significant difference with the evaporimeter measurements, but with a slight advantage of a lower detection limit for the CFTR-related response. The image sensor detected β-adrenergic sweat rate responses in two individuals who had *zero* response (consequently zero ratios) when measured by the evaporimeter (genotypes: G330X/G480C and F508del/W1282X). Since these genotypes are known to express very little protein,

Fig. 4 Sweat glands identified by imaging sensor. Mapping shows the same glands were stimulated after cholinergic stimulation (**a**) and after β-adrenergic stimulation (**b**)

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Table 1. Population characteristics and individual data

Diagnosis	Gender/age (years)	*CFTR* mutations	PI (y/n)	SCT (mmol/L)	FEV$_1$	Evap (β/chol)	Sensor (β/chol)
HC	F/22	–	–	–	–	1.214	0.772
HC	M/23	–	–	–	–	0.977	1.313
HC	M/32	–	–	–	–	0.654	0.791
HC	F/43	–	–	–	–	0.925	0.854
HC	M/64	–	–	–	–	1.092	0.784
HC	M/25	–	–	–	–	1.019	0.412
HC	F/26	–	–	–	–	0.510	0.382
HC	M/25	–	–	–	–	1.063	0.595
HC	F/25	–	–	–	–	1–107	0.568
HC	F/26	–	–	–	–	1.028	0.896
Carrier	M/44	F508del	–	–	–	1–139	0.774
Carrier	F/34	3849 + 10kbC>T	–	–	–	0.614	0.827
Carrier	F/46	F508del	–	–	–	0.429	0.204
Carrier	F/25	F508del	–	–	–	0.514	0.300
Carrier	M/37	12(TG)-5T	–	–	–	0.245	1.168
Carrier	F/35	F508del	–	–	–	0.193	0.427
CFSPID	F/6	F508del/R117H/7T	N	46	128%	0.058	0.051
CFSPID	F/8	F508del/11(TG)-5T	N	26	99%	0.054	0.072
CFSPID	F/7	F508del/D1152H	N	51	117%	0.058	0.054
CFSPID	M/6	3272–26A>G/ 12(5T)-5T	N	21	–	0.100	0.048
CFSPID	M/10	F508del/ (TG)11–5T	N	19	–	0.107	0.049
CF	M/21	G330X/G480C	Y	83	71%	0.000	0.045
CF	M/19	F508del/ 3199del6; I148T	Y	123	56%	0.021	0.010
CF	M/20	F508del/R516G	Y	117	47%	0.003	0.005
CF	F/22	F508del/1248 + 1G>A	Y	88	60%	0.058	0.007
CF	M/17	F508del/ 2183delAA>G	Y	64	73%	0.038	0.032
CF	M/15	F508del/F508del	Y	113	76%	0.024	0.014
CF	F/17	F508del/I507del	Y	94	40%	0.066	0.013
CF	F/14	3120 + 1G>A/ c.2516delC	Y	113	84%	0.008	0.011
CF	M/14	W1282X/W1282X	Y	–	85%	0.015	0.014
CF	M/18	F508del/W1282X	Y	–	75%	0.000	0.143[a]
CF	F/11	3849 + 10kbC>T/ 935delA		99	78%	–	0.042
CF	M/7	3849 + 10kbC>T/ 935delA	N	51	115%	0.061	0.003

Groups were defined as follows: non-CF healthy controls (HC), heterozygous for one CFTR mutation (Carriers), cystic fibrosis screen positive, inconclusive diagnosis (CFSPID), and cystic fibrosis (CF). PI was defined based on fecal elastase results (< 200 mcg/g). FEV1 % predicted = most recent measure. Evap β/chol (ratio of maximal sweat rate with β-adrenergic stimulation to maximal rate with cholinergic stimulation measured by evaporimetry). Sensor β/chol (maximal sweat rate ratio in response to β-adrenergic to cholinergic stimulation measured by an image sensor

CFTR cystic fibrosis transmembrane conductance regulator, *HC* healthy control, *CFSPID* cystic fibrosis screen-positive, inconclusive diagnosis, *M* male, *F* female, *N* no, *Y* yes, *SCT* sweat chloride test, *FEV$_1$* forced expiratory volume in 1 s, *β/chol* β-adrenergically stimulated sweat rate to cholinergically stimulated sweat rate, *PI* pancreatic insufficiency, – not available or not assessed

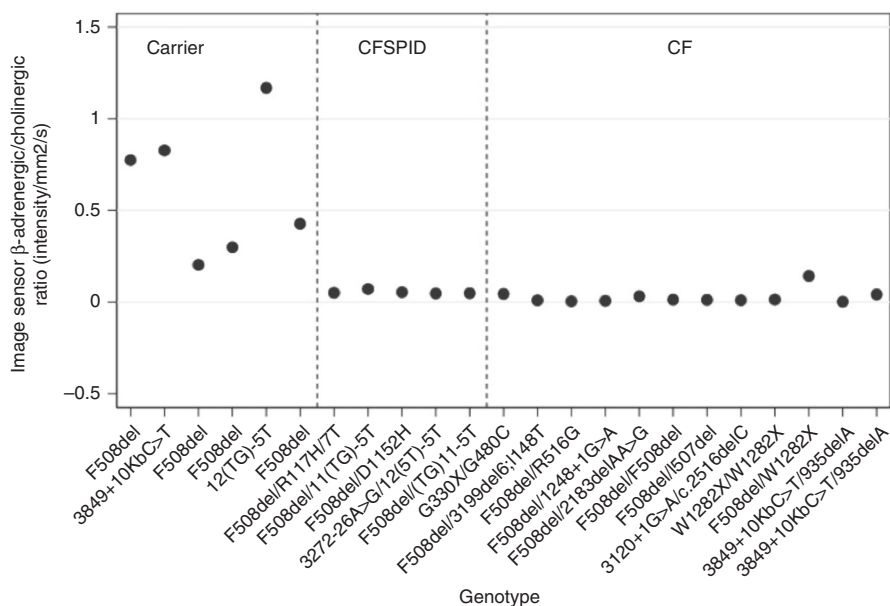[a]Evap and Sensor tests were repeated in this CF subject and results were reproducible

Table 2. Difference between Evaporimetry and Image Sensor (mean (SD))

	Evaporimetry	Image sensor	*p* Value
Baseline *z*-score	0.001 (1.00)	0.03 (1.02)	0.7940
Cholinergic *z*-score	0.0001 (1.00)	0.00002 (1.00)	0.9530
Adrenergic *z*-score	−0.0001 (1.00)	0.00002 (1.00)	0.8762
β/Chol ratio *z*-score	−0.003 (1.00)	0.01 (1.01)	0.7464

β/chol β-adrenergically stimulated sweat rate to cholinergically stimulated sweat rate

these results may be an artifact and not the actual β-adrenergic sweat secretion. However, in view of the cautious phases of protocol development including reproducibility and mapping of the sweat glands, this possibility seems unlikely. The β-adrenergic-induced sweat secretion was low regardless of genotype, CFTR class, or category of residual/minimal function (Fig. 5). Yet, the image sensor detected some level of β-adrenergic sweat rate responses in 100% of CF subjects, while the evaporimeter detected sweat rate in 80% of subjects with residual/minimal CFTR function (Table 1).

An optical ratiometric technology assay also comparing β-adrenergic-to-cholinergic sweat rates has been developed

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Fig. 5 Individual ratios between maximum sweat rate in response to β-adrenergic and cholinergic stimulation, measured by the image sensor in heterozygotes for one cystic fibrosis (CF) transmembrane conductance regulator (*CFTR*) mutation (carrier), cystic fibrosis screen-positive, inconclusive diagnosis (CFSPID), and CF subjects. The abscissa shows subjects' genotype with corresponding *CFTR* functional classification below

	Genotype	CFTR classification	
CFSPID	F508del/R117H-7T	II/IV	Residual function
	3849+10KbC>T/11(TG)-5T	V/V	
	F508del/D1152H	II/IV	
	3272-26A>G/12(5T)-5T	V/V	
	F508del/(TG)11-5T	II/V	
CF	G330X/G480C	I/unassigned	Minimal function
	F508del/3199del6;I148T	II/unassigned/unassigned	
	F508del/R516G	II/unassigned	
	F508del/1248+1G>A	II/I	
	F508del/2183delAA>G	II/I	
	F508del/F508del	II/II	
	F508del/I507del	II/II	
	3120+1G>A/c.2516delC	I/I	
	W1282X/W1282X	I/I	
	F508del/W1282X	II/I	
	3849+10KbC>T/935delA	V/I*	Residual function
	3849+10KbC>T/935delA	V/I*	

*Siblings: the brother was detected by newborn screening and the older sister was diagnosed during sibling screening. please refer to table 1 for phenotype details. These are the only pancreatic sufficient CF patients in this group.

to measure CFTR function in persons with CF-pancreatic sufficiency/insufficiency and with CFTR-related disorder.[17,19] This assay was used to measure the effect of a CFTR modulator (Ivacaftor) on single, individual sweat glands in subjects carrying G551D and R117H-5T.[17,20] β-Adrenergic response was seen in 16–21% of sweat glands of individuals treated with the drug and in no glands in subjects on placebo. This finding suggested that the optical ratiometric method is more sensitive than the evaporimeter to measure sweat rate response to CFTR-targeted therapy.[21] Although highly sensitive, the optical ratiometric assay does not detect β-adrenergic-induced sweat secretion in individuals carrying *CFTR* mutations classified as minimal function and in others with CFTR-related disorder measurements were highly variable (0–0.6).[17,19] Additionally, it requires

maintaining the same position without any movement for a prolonged period of time along with several ID injections, making it less suitable for children.

The image sensor and the evaporimeter were equally successful to differentiate between groups (HC, carrier, CRMS/CFSPID, and CF). Interestingly, the natural log of β/chol ratios showed significant difference between CRMS/CFSPID and CF, suggesting that both methods and this iontophoresis protocol may complement standard SCT in defining the diagnosis of screen-positive children carrying *CFTR* mutations/variants of unknown clinical significance. Another possible application in this NBS population would be to use this assay to monitor CRMS/CFSPID to CF reclassification over time. The current findings are different than our previous work, which showed no difference between CF and
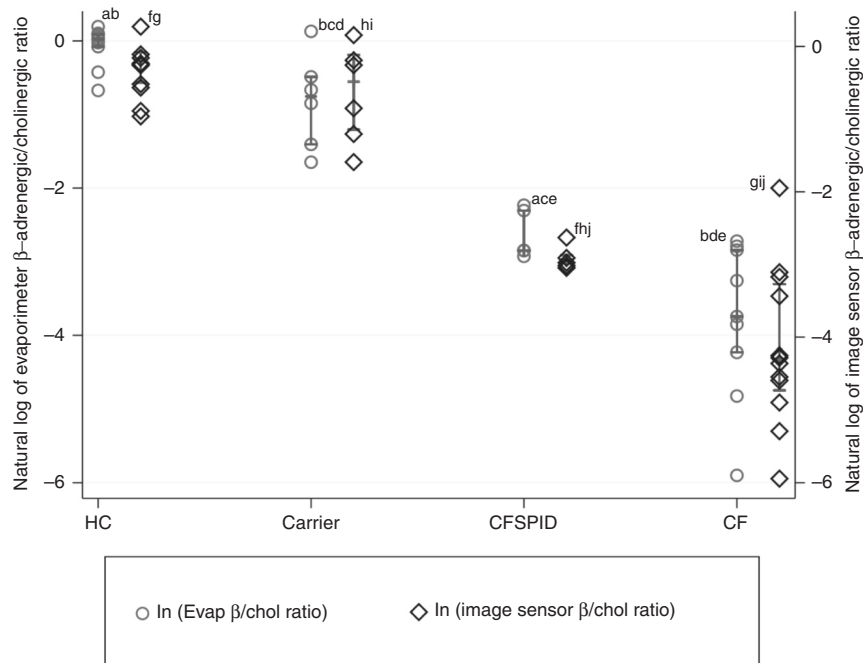
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Fig. 6 β-Adrenergic and cholinergic ratio in natural log from evaporimeter and image-sensor measurements in all four groups non-cystic fibrosis (CF) healthy controls (HC), heterozygotes for one *CFTR* mutation (carrier), CF screen-positive, inconclusive diagnosis (CFSPID), and CF subjects. Letters denote significant comparisons. Evaporimeter: a = HC vs. CFSPID ($p < 0.0001$), b = HC vs. CF ($p < 0.0001$), c = carrier vs. CFSPID ($p = 0.001$), d = carrier vs. CF ($p < 0.0001$), and e = CFSPID vs. CF ($p = 0.026$). Image sensor: f = HC vs. CFSPID ($p < 0.0001$), g = HC vs. CF ($p < 0.0001$), h = carrier vs. CFSPID ($p < 0.0001$), i = carrier vs. CF ($p < 0.0001$), and j = CFSPID vs. CF ($p = 0.022$)

CRMS/CFSPID pre-school age children when sweat rate was measured by evaporimetry after carbachol and β-cocktail injections given on different days. The difference may be explained by several factors: (1) different protocol (sweat glands were not potentiated); (2) different age groups (sweat glands may not be fully developed in 4–6-year-old children); and (3) small samples in both studies. Also, in our experiments the evaporimeter (or the image sensor) did not detect a significant difference between HC and heterozygous (carriers) as previously seen by other groups.[14,17,19] Thus, it is important to plan for a larger study using this new technology and iontophoresis protocol, including all four groups.

The present protocol is better tolerated because it is less invasive, as it uses 1 vs. 3 ID injections. The iontophoresis of pilocarpine and atropine was developed after our group repeated and confirmed that pre-stimulation of the sweat gland with cholinergic drugs potentiates β-adrenergic-induced sweat secretion.[17] Ideally, we would eliminate all injections, but iontophoresis of β cocktail were not reproducible when testing the same individual on the same or different days. A possible explanation is the fact that the three drugs in the cocktail (atropine, aminophylline, and isoproterenol) are not pharmacologically compatible. These compounds have different charges, pH, and aminophylline accelerates degradation of isoproterenol. As in previous protocols β cocktail drugs were mixed immediately before injecting or iontophoresing so that the drug effect would not be compromised. For iontophoresis more promising results were achieved when drugs were iontophoresed sequentially. However, three sequential iontophoresis prolonged the protocol to about one hour and caused skin irritation, which created measurement artifacts and was judged to be more uncomfortable than injections.

Limitations to this work include: (1) Small sample size that completed the final protocol, which can be justified by the fact that this is an introduction to this new technology and protocol; validation studies will require bigger samples (precisely $n = 15$ in

each of the four groups for 80% power). On the other hand, within the CRMS/CFSPID group we recruited a broad representation of genotypes: from individuals carrying benign variants (F508del and (TG)11–5T) to others carrying variants of varying clinical consequence (F508del and D1152H).[22,23] (2) The image sensor does not yield results expressed in physical units. To accommodate the different units, we used z-scores to compare the two methods. (3) There was no direct comparison to the standard SCT. We considered this step irrelevant to the proof of concept of this protocol as these two assays measure different functions of CFTR in the sweat gland (secretory vs. absorptive).

In conclusion, the β-adrenergic assays remain as a valid method to complement the standard SCT, since CFTR secretory rather than absorptive function is assayed. The advantages of the image-sensor and this iontophoresis protocol over previously proposed assays are the detection of CFTR function with minimal and residual function and its practical utility for measurement in young children. The fact that both image-sensor and the evaporimeter assays detect β-adrenergic sweat in CRMS/CFSPID almost as low as in CF suggests CFTR dysfunction in the secretory pathway of sweat glands in both populations. The β/chol ratio (after natural log transform) successfully differentiates between CRMS/CFSPID and CF groups, when measured by both evaporimetry and image sensor. Once these findings are validated in a larger trial and by other groups, the image-sensor assay and iontophoresis protocol can assist in defining CF and CRMS/CFSPID diagnosis after a positive NBS and monitoring for CRMS/CFSPID to CF reclassification over time. Future directions include testing in a larger sample of young children from NBS, and also before and after use of CFTR-targeted drugs.

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AUTHORS CONTRIBUTIONS

D.B.S., J.-M.M., Y.-H.P., B.H., and C.P.W. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: D.B.S, J.-M.M., Y.-H.P., B.H. and E.F.; data acquisition and analysis: D.B.S., Y.-H.P., B.H. and J.-M.M.; interpretation: D.B.S., J.-M.M., Y.-H.P., B.H. and E.F.; drafting of the article: D.B.S., Y.-H.P., B.H., J.-M.M. and C.P.W.; critical revision of the article for important intellectual content: D.B.S., Y.-H.P., B.H., C.P.W. and J.-M.M.; obtained funding: D.B.S., Y.-H.P. C.P.W. and J.-M.M.; statistical analysis: C.P.W.

ADDITIONAL INFORMATION

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