Journal of Cystic Fibrosis xxx (xxxx) xxx

Contents lists available at ScienceDirect

Journal of Cystic Fibrosis

journal homepage: www.elsevier.com/locate/jcf

Short Communication

Optical Measurements of Sweat for in Vivo Quantification of CFTR Function in Individual Sweat Glands

Davide Treggiari[a], Karina Kleinfelder[b], Marina Bertini[a], Gloria Tridello[a], Arianna Fedrigo[b], Emily Pintani[a], Patrizia Iansa[a], Antonella Casiraghi[c], Paola Minghetti[c], Marco Cipolli[a], Claudio Sorio[b], Paola Melotti[a,*]

[a] Cystic Fibrosis Centre, Azienda Ospedaliera Universitaria Integrata Verona, piazzale Stefani, 1, 37126 Verona, Italy
[b] Department of Medicine, University of Verona, Division of General Pathology, Strada Le Grazie 8, 37134 Verona, Italy
[c] Department of Pharmaceutical Sciences, Università degli Studi di Milano, via G. Colombo 71, Milan, 20133, Italy

ARTICLE INFO

Article history:
Received 8 October 2020
Revised 1 February 2021
Accepted 6 March 2021
Available online xxx

Keywords:
Sweat glands
CFTR-targeted therapy
image analysis
cystic fibrosis

ABSTRACT

Optical measurement of CFTR-dependent sweat secretion stimulated by a beta-adrenergic cocktail (C-phase) vs. CFTR-independent sweat secretion induced by methacholine (M-phase) can discriminate cystic fibrosis (CF) patientts from controls and healthy carriers by the ratio of sweat rate in the C-phase vs. the M-phase (C/M ratio). However, image analysis is experimentally demanding and time-consuming. Here, sweat droplet number (SDN) in the C-phase, corresponding to the number of sweat-secreting glands, was a statistically significant predictor for detecting the effects of CFTR-targeted therapy. We show that in 44 non-CF subjects and 110 CF patients, SDN in the C-phase provides a linear readout of CFTR function that is more sensitive than that using the C/M ratio. In CF patients, increased SDN in the C-phase during treatment with (LUMA/IVA) was associated with a trend toward improved lung function (FEV1). Our method is suitable for multicenter monitoring of the effects of CFTR modulators.

Abbreviations

CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
SDN	sweat droplet number
C-phase	CFTR-dependent sweat secretion stimulated by a beta-adrenergic Cocktail
M-phase	CFTR-independent sweat secretion induced by Methacholine
C/M ratio	ratio of sweat rate in C-phase vs. sweat rate in M-phase
HC	healthy carriers
F	F508del mutation
M	mutations with Minimal CFTR function
R	mutations with Residual CFTR function
G	Gating mutations
LUMA/IVA	Lumacaftor/Ivacaftor
FEV1	Forced Expiratory Volume in 1 Second

1. Introduction

Sweat Cl^- is the gold standard for the diagnosis of CF; its limitations for diagnosis and monitoring the effects of CFTR-targeted drugs include “borderline” results and intra-subject variability [1]. CFTR-dependent sweating is induced by beta-adrenergic stimulation upon increased intracellular cAMP, whereas CFTR-independent sweating is mediated by cholinergic stimulation that induces Ca^{2+}-dependent Cl^- secretion [2]. Defective CFTR affects sweat production in response to beta-adrenergic stimulation. In CF patients impaired Cl^- diffusion and NaCl reabsorption increase sweat Cl^- and Na^+ concentrations [3]. An optical ratiometric evaluation of CFTR-dependent vs. CFTR-independent sweating has been developed to support controversial diagnosis and monitoring CFTR function during CFTR-targeted treatments [4–9]. Following implementation, we discriminated CF patients from non-CF subjects with 100% sensibility and healthy carriers (HC) from controls with 82% sensitivity and 86% specificity [4]. However, the evaluation of sweat droplets and calculation of the sweat rate based on the camera monitoring of stained sweat droplets formation are experimentally demanding, need skilled operators and dedicated software for image analysis,

* Corresponding author.
E-mail addresses: davide.treggiari@univr.it (D. Treggiari), karina.kleinfelderfonatanesi@univr.it (K. Kleinfelder), marina.bertini@alice.it (M. Bertini), gloria.tridello@aovr.veneto.it (G. Tridello), arianna.fedrigo@univr.it (A. Fedrigo), emily.pintani@aovr.veneto.it (E. Pintani), patrizia.iansa@aovr.veneto.it (P. Iansa), antonella.casiraghi@unimi.it (A. Casiraghi), paola.minghetti@unimi.it (P. Minghetti), marco.cipolli@aovr.veneto.it (M. Cipolli), claudio.sorio@univr.it (C. Sorio), paola.melotti@aovr.veneto.it (P. Melotti).

https://doi.org/10.1016/j.jcf.2021.03.003
1569-1993/

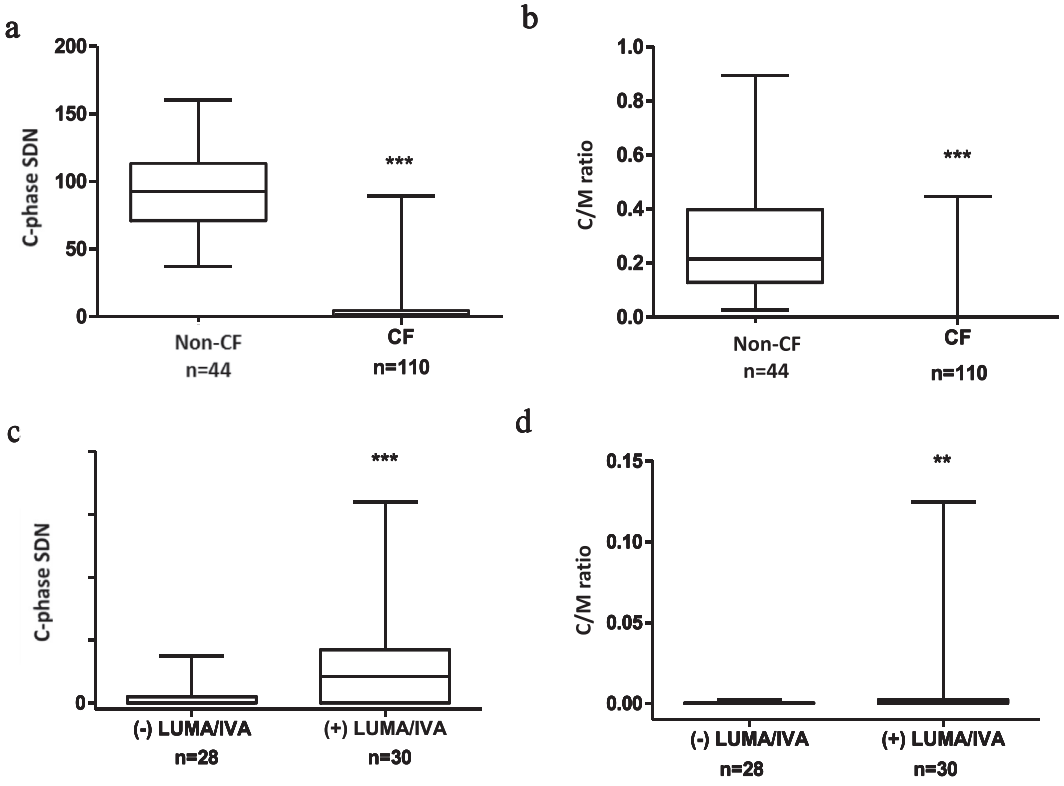

Fig. 1. Comparison of SDN with C/M ratio. a) Box and whisker plot (min-max) of SDN measured in the C-phase in CF patients compared to that in non-CF subjects ($p<0.0001$). b) C/M ratio in CF patients compared to that in non-CF subjects ($p<0.0001$). A comparison of SDN and C/M ratio in patients treated with LUMA/IVA. c) Changes in SDN in subjects without and with this treatment (n=28 and 30, respectively, $p=0.0004$). d) Changes in C/M ratio within the same group ($p=0.0021$). Differences were tested with the Mann-Whitney non-parametric test.

which are still under development. Recently, using a multilinear regression model, we showed that SDN in the C-phase is a statistically significant predictor (p-value <0.01) for the mean C/M ratio [10,11].

2. Materials and Methods

2.1. Study population

At the CF Center of Verona, we tested 44 non-CF (controls and HC) subjects and 110 CF patients following written informed consent according to the local Ethical Committee's rules (project#CFTR028). In a subgroup of 45 CF patients (F508del+/+), 17 were tested only during treatment with Lumacaftor/Ivacaftor (LUMA/IVA) (Vertex Pharmaceuticals, Boston, MA, USA) according to the clinical use (mean: 47.66 months; 25th-75th percentile: 29.10-68.85), 15 were tested without treatment, and 13 were tested with/without treatment (mean: 19.07 months; 25th-75th percentile: 7.54-30.06).

2.2. Optical ratiometric measurement of CFTR-dependent sweat

We followed a previously reported method [4] described in brief as follows: intradermal micro-injection (100 μL) of methacholine stimulated CFTR-independent sweating in the M-phase, and beta-adrenergic agonists induced CFTR-dependent sweat secretion in the C-phase following an intradermal microinjection of aminophylline and isoprenaline with atropine as a cholinergic inhibitor (Supplementary Material). Individual sweat glands were totaled by visually counting single spherical sweat droplets ("bubbles") in a water-saturated oil layer including dispersed, water-soluble blue dye particles (350 μL in a 1 cm diameter well). Single glands were mapped (ImageJ software) on pictures acquired 10 or 30 min after microinjections of methacholine or beta-adrenergic cocktail, respectively, and the sweat volume secreted per minute and the C/M ratio were calculated.

2.3. Statistical analysis

Graphpad (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. Inter-group comparisons between non-CF and CF subjects focused on differences in SDN and C/M ratios. Statistical analysis was performed using the non-parametric Mann-Whitney test applied to individual comparisons; $p<0.05$ was considered statistically significant.

3. Results

SDN was acquired by counting single sweat droplets secreted from individual glands in the C-phase. Performances of SDN and C/M ratio were compared between non-CF (n=44) and CF (n=110) subjects. The descriptive statistics are summarized in Table 1. SDN was able to discriminate between non-CF and CF subjects ($p<0.0001$) (Fig. 1a), as well as the C/M rate, ($p<0.0001$) (Fig. 1b). SDN means, but not C/M ratios, were significantly different in F508del+/+ homozygotes (1.31; 25^{th}–75^{th} percentile: 0.24–2.38; n=31) versus all the other genotypes (6.11; 25^{th}–75^{th} percentile: 2.40–9.81; n=60) (Fig. 2).

The high sensitivity and easy detection of SDN, together with our previous identification by multilinear regression model of SDN as a good predictor of C/M ratio, suggest the use of SDN and its comparison with C/M ratio for detecting CFTR function improvement during CFTR-targeted therapy. In F508del+/+ patients tested before treatment with LUMA/IVA the mean±SD of

Table 1
Descriptive statistics of SDN and C/M ratio. AUC: area under the curve; CI: confidence interval; SDN, sweat droplet number; C/M ratio, ratio of sweat rate in C-phase vs. sweat rate in M-phase

	SDN		C/M ratio	
	Non-CF (n=44)	CF (n=110)	Non-CF (n=44)	CF (n=110)
mean±sd	93.91±28.00	4.53±11.44	0.2795±0.2016	0.0090±0.0471
95% CI of mean	85.29 to 102.5	2.37 to 6.69	0.2182 to 0.3408	0.000103 to 0.01791
AUC	0.9950	0.9862		
p-value	<0.0001	<0.0001		
sensitivity%	97.27	96.36		
specificity%	100	100		

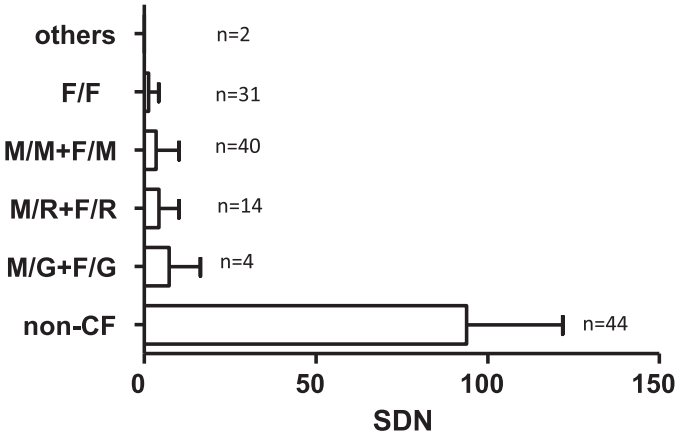

Fig. 2. SDN in different CFTR genotypes. SDN mean values ± SD (horizontal bars) in the C-phase of CF patients in the absence of CFTR-modulator therapy are different according to the CFTR genotypes. Abbreviations: F, F508del; M, mutations with minimal CFTR function [13]; R, mutations with residual CFTR function [14]; and G, gating mutations. SDN mean values in F/F homozygotes vs. all other CFTR genotypes are significantly different (p=0.0093), according to Mann-Whitney non-parametric test. C/M ratio mean values did not significantly differ in F/F homozygotes vs. all other genotypes (p= 0.20); data not shown.

SDN was 0.94±1.90, whereas the mean C/M ratio ranged from 0.0000 to 0.0022 (n=28). During treatment with LUMA/IVA, the mean SDN increased to 6.41±8.16 and the mean C/M ratio ranged from 0.0000 to 0.1248 (n=30). Although both analyses showed significant changes, SDN appeared to be more sensitive (n=28–30, mean diff.=5.46, 95% CI=3.15–7.77, p=0.0004; Fig. 1c) than C/M ratio (n=28–30, mean diff.=0.0057, 95% CI=-0.00251–0.013, p=0.0021; Fig. 1d). Additionally, in the subgroup of 13 CF patients with/without LUMA/IVA therapy, SDN but not C/M ratio discriminated the responsive group (p=0.0103 and 0.1993, respectively, paired test). SDN showed remarkably lower variability (mean CV% diff.=-72.8, p=0.0148) than C/M ratio.

As SDN seemed capable of identifying the response to the treatment, we sub-grouped our patients into “responders” (SDN increase >3) and “non-responders” (SDN increase ≤3) with the aim of investigating the possible association of increased SDN with improved clinical outcomes. The therapy had similar durations in responders and non-responders (mean±SD 34.6±20.58 and 35.09±25.19 months, respectively). Responders showed a trend toward improved lung function (absolute increase of % FEV1) (mean=2.01; median=1.95; n=15), which was higher than that in non-responders (mean=0.68; median=-0.74; n=13), although they had a lower decrease in sweat Cl^- than that in non-responders (17.61% vs. 20.59%, respectively; not statistically significant).

4. Conclusions

Our results demonstrate that SDN in the C-phase provides a linear readout of CFTR function, overlapping with C/M ratio, for the ability to discriminate between CF and non-CF subjects.

Of note, SDN revealed the effects of LUMA/IVA therapy on CFTR function more efficiently than the C/M ratio. The evaporimetry-based analysis of beta-adrenergic sweat did not detect significant effects of Ivacaftor in CF patients [8,12], whereas in the original optical ratiometric beta-adrenergic assay additional evaluations were required [5]. Our study proposes SDN as a rapid and sensitive outcome for detecting the response to CFTR-targeted therapies, suitable for multicenter studies. Considering the levels of lung function improvement and sweat Cl^- decrease, we expect higher changes in SDN in patients treated with more effective drugs than LUMA/IVA, such as Ivacaftor+Tezacaftor±Elexacaftor. Such changes *in vivo* could complement *in vitro* theratyping for rare genotypes that still lack approved targeted drugs. SDN represents an innovative outcome of a rapid, reproducible, robust, and relatively inexpensive optical measurement of CFTR-dependent sweating, consistent with the impact on CFTR function in different genotypes [13,14] and with clinical outcomes during CFTR-targeted treatments. It facilitates repeatability across different operators and across different sites (Supplementary Material). This might allow further improvement of this bioassay by developing a dedicated image-analysis software, by reducing time and (although minimal) invasiveness following eventual reduction of microinjections during follow-up. Normalization with the M-phase seems redundant in individual patients since we always evaluate the same glands. Cholinergic potentiation of the C-phase was reported at intermediate times, but seemed undetectable at 30 min (end of C-phase in our assay); cholinergic treatment in our assay was shorter than that in the previous study [5]. Therefore, we hypothesized that potentiation did not affect our results. Certainly, further studies are required to verify whether eventual skipping of the M-phase during follow-up might significantly affect sweat rate and/or SDN in our setting. Testing larger skin areas and both forearms every time might improve the test and compensate for the eventual lower efficiency of beta-adrenergic induction in the absence of cholinergic pretreatment.

Our study could support and facilitate the multicenter clinical application of this test by simplifying image analysis while preserving or improving test sensitivity and specificity.

Declaration of potential conflict of interest

Paola Melotti was paid for her expert testimony, and Marco Cipolli received a research grant from Vertex Pharmaceuticals.

Author contributions

The authors contributed as follows: Davide Treggiari: methodology, investigation, resources, and writing of the original draft; Karina Kleinfelder: methodology, investigation, and resources; Marina Bertini: investigation and validation; Gloria Tridello: formal analysis; Arianna Fedrigo: investigation and resources; Paola Minghetti: methodology, investigation, and writing; Antonella Casiraghi: methodology, investigation, and writing; Marco Cipolli: supervision and funding acquisition; Emily Pintani: data curation;

Patrizia Iansa: data curation; Claudio Sorio: writing, review, and editing and supervision; Paola Melotti: conceptualization and writing, review and editing, supervision, and funding acquisition.

Acknowledgements

This work was supported by: Lega Italiana Fibrosi Cistica-Associazione Veneta Onlus; Cystic Fibrosis Foundation (award:ASSAEL08A0); Italian Cystic Fibrosis Research Foundation (grant FFC#5/2016, Delegazione FFC Imola-Romagna).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2021.03.003.

References

[1] Vermeulen F, Lebecque P, De Boeck K. T. Leal Biological variability of the sweat chloride in diagnostic sweat tests: A retrospective analysis. J Cyst Fibros 2017;16(1):30–5.
[2] Reddy MM, Bell CL, Quinton PM. Evidence of two distinct epithelial cell types in primary cultures from human sweat gland secretory coil. Am J Physiol 1992;262(4):891–8.
[3] Sato K, Sato F. Defective beta-adrenergic response of cystic fibrosis sweat glands in vivo and in vitro. J. Clin. Invest. 1984;73:1763–71.
[4] Bergamini G, GTridello ECalcaterra, SCeri MTagliasacchi, FBianchi FMonti, Masciadri A, Laudanna E, Peserico D, Sorio E, VEsposito TLeal, Assael BM, Sorio C, Melotti P. Ratiometric sweat secretion optical test in cystic fibrosis, carriers and healthy subjects. J Cyst Fibros 2018;17(2):186–9.
[5] Char JE, Wolfe MH, Cho HJ, Park IH, Jeong JH, Frisbee E, Dunn C, Davies Z, Milla C, Moss RB, Thomas EAC, Wine JJ. A Little CFTR Goes a Long Way: CFTR-Dependent Sweat Secretion From G551D and R117H-5T Cystic Fibrosis Subjects Taking Ivacaftor. Plos ONE 2014;9(2).
[6] Wine JJ, Char JE, Chen J, Cho HJ, Dunn C, Frisbee E, Joo Nam Soo, Milla Carlos, Modlin Sara E, Park Il-Ho, Thomas Ewart AC, Tran Kim V, Verma Rohan, Wolfe Marlene H. In Vivo Readout of CFTR Function: Ratiometric Measurement of CFTR-Dependent Secretion by Individual, Identifiable Human Sweat Glands. Plos ONE 2013;8(10).
[7] Behm JK, Hagiwara G, Lewiston NJ, Quinton PM, Wine JJ. Hyposecretion of Beta-adrenergically Induced Sweating in Cystic Fibrosis Heterozygotes. Pediatric research 1987;22(3):271–6.
[8] Rowe SM, Heltshe SL, Gonska T, Donaldson SH, Borowitz D, Gelfond D, Sagel SD, Khan U, Mayer-Hamblett N, Van Dalfsen JM, Joseloff E, Ramsey BW. GOAL Investigators of the Cystic Fibrosis Foundation Therapeutics Development Network.Clinical Mechanism of Cystic Fibrosis Transmembrane Conductance Regulator Potentiator Ivacaftor in G551-D mediated Cystic Fibrosis. American Journal of Respiratory and Critical Care Medicine 2014;190(2):175–84.
[9] Salinas DB, Peng Y-H, Horwich B, Wee CP, Frisbee E, Maarek J-M. Image-based β-adrenergic sweat rate assay captures minimal cystic fibrosis conductance regulator (CFTR) function. Pediatr Res 2020;87(1):137–45.
[10] P. Lecca, M. Bertini, R. Vicentini, E. Pintani, C. D'Orazio, V. Esposito, K. Kleinfelder, C. Sorio and P. Melotti Multilinear Regression Analysis of Sweat Secretion Volume in Cystic Fibrosis Patients. Proceeding of the 23rd Conference of FRUCT Association, Helsinki: 497-504; 2018.
[11] Melotti P, Lecca P, Esposito V, Bertini M, Vicentini R, Pintani E, D'Orazio C, Sorio C. Measurements of beta adrenergic vs cholinergic sweat rates in single human sweat glands. NACFC 2018. Colorado Convention Center (CCC) in Denver, CO. Pediatric Pulmonology 2018;53 suppl.2: 158.
[12] Kim J, MFarahmand CDunn, Davies Z, Frisbee E, CMilla JJ. Wine Evaporimeter and Bubble-Imaging Measures of Sweat Gland Secretion Rates. PLoS One 2016;11(10).
[13] Middleton PG, Mall MA, Dřevínek P, Lands LC, McKone EF, Polineni D, Ramsey BW, Taylor-Cousar JL, Tullis E, Vermeulen F, Marigowda G, McKee CM, Moskowitz SM, Nair N, Savage J, Simard C, Tian S, Waltz D, Xuan F, Rowe SM, Jain R. VX17-445-102 Study Group Elexacaftor–Tezacaftor–Ivacaftor for Cystic Fibrosis with a Single Phe508del Allele. N Engl J Med 2019;381(19):1809–19.
[14] Han ST, Rab A, Pellicore MJ, Davis EF, McCague AF, Evans TA, Joynt AT, Lu Z, Cai Z, Raraigh KS, Hong JS, Sheppard DN, Sorscher EJG.R.. Cutting Residual function of cystic fibrosis mutants predicts response to small molecule CFTR modulators. JCI Insight 2018.