Rectal organoid morphology analysis (ROMA) as a promising diagnostic tool in cystic fibrosis

Senne Cuyx [ORCID] ,[1,2] Anabela Santo Ramalho [ORCID] ,[1] Nikky Corthout,[3,4] Steffen Fieuws,[5,6] Eva Fürstová,[7] Kaline Arnauts,[8,9] Marc Ferrante,[8,10] Catherine Verfaillie,[9] Sebastian Munck,[3,4] Mieke Boon,[1,2] Marijke Proesmans,[1,2] Lieven Dupont,[11,12] Kris De Boeck,[1,2] François Vermeulen [ORCID] ,[1,2] On behalf of the Belgian Organoid Project

For numbered affiliations see end of article.

Correspondence to
Dr François Vermeulen, Department of Development and Regeneration, Woman and Child Unit, CF research lab, KU Leuven, Leuven, Flanders, Belgium; francois.vermeulen@uzleuven.be

ABSTRACT

Diagnosing cystic fibrosis (CF) when sweat chloride is not in the CF range and less than 2 disease-causing *CFTR* mutations are found requires physiological CFTR assays, which are not always feasible or available. We developed a new physiological CFTR assay based on the morphological differences between rectal organoids from subjects with and without CF. In organoids from 167 subjects with and 22 without CF, two parameters derived from a semi-automated image analysis protocol (rectal organoid morphology analysis, ROMA) fully discriminated CF subjects with two disease-causing mutations from non-CF subjects (p<0.001). ROMA, feasible at all ages, can be centralised to improve standardisation.

INTRODUCTION

The diagnosis of cystic fibrosis (CF), an autosomal recessive disease caused by CF transmembrane conductance regulator (*CFTR*) gene mutations, relies on abnormal (≥60 mmol/L) sweat chloride concentration (SCC) and/or two disease-causing *CFTR* mutations, as defined by the CFTR2 database.[1 2] In some subjects either with CF compatible symptoms or after neonatal screening, the diagnosis cannot be confirmed nor excluded. For patients with only intermediately elevated (30–60 mmol/L) SCC and *CFTR* mutation(s) of varying or unknown clinical consequence, second-line diagnostic tests (nasal potential difference (NPD) and intestinal current measurements (ICM)) are advocated to further explore CFTR function and assist the diagnosis. These tests are not readily available nor feasible at all ages.[1]

Rectal organoids are 3D structures grown from intestinal stem cells in a mucosal sample obtained through rectal biopsy. CFTR protein expression is maintained in and determines the morphology of these organoids, inducing swelling of non-CF organoids through salt and water accumulation in the lumen surrounded by a cellular layer, while CF organoids have no lumen as described by the Beekman group.[3 4]

We quantified morphological differences to explore the ability of rectal organoid morphology analysis (ROMA) to differentiate organoids from subjects with a clear-cut diagnosis of CF from those of subjects without CF, giving proof of concept for ROMA as a diagnostic test for CF.

METHODS

Organoids from a convenience cohort of 212 subjects were collected and imaged by one researcher blinded to subject characteristics. Twenty-three subjects were excluded due to low-quality images. Organoids of 167 subjects with CF and two disease-causing *CFTR* mutations[2] and 22 non-CF subjects were analysed (online supplemental file 1 and 2.1).

Rectal biopsies and organoid cultures were performed as previously described (online supplemental file 2.2).[4] No adverse events were reported. In total, 32 wells per subject were plated under basal conditions (no CFTR modulators nor forskolin added) and images of each well were acquired after overnight growth and calcein staining.

Two indices were calculated using imaging software (NIS-Elements AR Analysis 5.02.00) to quantify organoid morphology: the intensity ratio (IR) measures the presence or absence of a central lumen, and the circularity index (CI) quantifies the roundness of the organoids (figure 1 and online supplemental file 2.3).

Discriminant analysis was applied to differentiate between CF and non-CF subjects using IR and CI. Mann-Whitney and Fisher exact tests were used for between-group comparisons.

RESULTS

The IR and CI differed (p<0.001) between CF and non-CF (table 1). Perfect discrimination (AUC=1) was obtained with a linear discriminant analysis (figure 2). Analysis of only eight wells chosen randomly out of the 32 showed almost identical results (AUC=1) (online supplemental file 2.4).

No correlation between ROMA indexes and age was found, nor differences in IR and CI between subjects with CF in the lowest versus three highest SCC quartiles (<87 and ≥87 mmol/L, respectively). The IR (p=0.007), but not CI (p=0.419), was different between PI (pancreatic insufficient) and PS (pancreatic sufficient) subjects with CF. For additional statistical analyses, see online supplemental files 2.4 and 3.

$$IR = \frac{\text{intensity}_{\text{central part of organoid}}}{\text{intensity}_{\text{whole organoid}}}$$

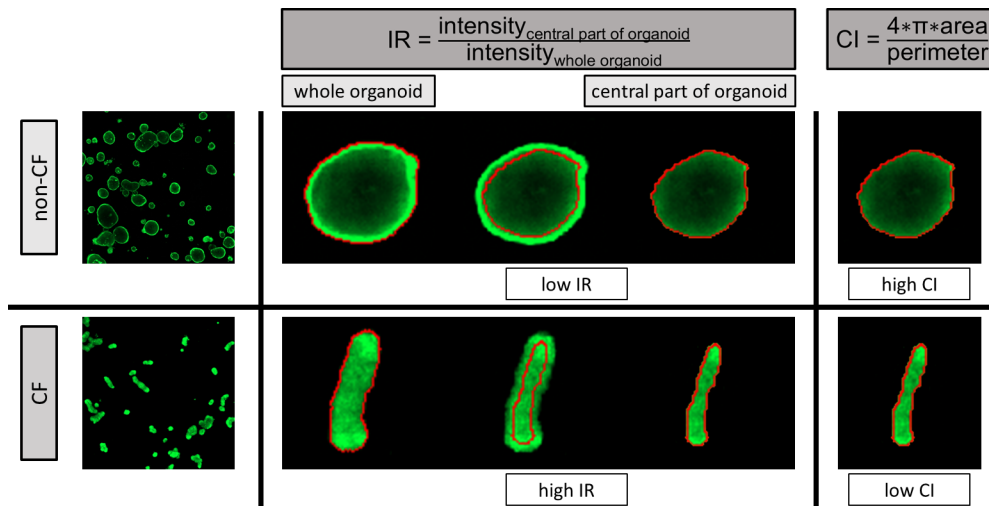$$CI = \frac{4 * \pi * \text{area}}{\text{perimeter}}$$

Figure 1 Images of rectal organoids from subjects without (upper panels) and with CF (lower panels). Illustration of the methods to calculate the two indexes, IR (intensity ratio; central panel) and CI (circularity index; right panel), used to quantify morphological differences between rectal organoids of subjects with and without CF. IR measures the presence or absence of a central lumen, calculated in three steps: (1) calculate the global fluorescence intensity of the organoids: erode 1 pixel (2.5 µm) to remove the surrounding 'halo' around each structure and measure the mean fluorescence intensity of the remaining whole organoid; (2) calculate the lumen fluorescence intensity of the organoids: erode 10 pixels from each structure to remove the cellular border from the organoids and measure the mean fluorescence intensity of the remaining structure; (3) IR is equal to (intensity central part of organoid)/(intensity whole organoid) and is higher in CF than in non-CF organoids. CI quantifies the roundness of the organoids, defined as (4×π×area)/perimeter, which is lower in CF than in non-CF organoids. CF, cystic fibrosis; IR, intensity ratio; CI, circularity index.

DISCUSSION

Both IR and CI, calculated with ROMA, discriminated subjects with clinical CF and two disease-causing *CFTR* mutations from non-CF subjects. This makes ROMA appealing as an additional physiological CFTR assay to assist in the diagnosis of CF, with a discriminative ability comparable with that reported for SCC and other physiological CFTR assays such as NPD or ICM.[5 6]

Similarly to SCC,[5] mean IR is different between PS and PI subjects with CF, although not fully discriminative. Residual CFTR function, translating in pancreatic sufficiency, leads to the presence of a small lumen in the organoids and thus lower IR, without altering the organoids' irregular shape quantified by CI.

The current study is monocentric and needs replication, but samples have already been successfully received from other Belgian and international centres. Before ROMA can be proposed as diagnostic test for CF, more data are needed, especially from patients with a clinical CF diagnosis but without two disease-causing *CFTR* mutations, and from prospective analysis in subjects with unresolved CF diagnosis, including infants with CF screening positive, inconclusive diagnosis (CFSPID). The absence of a gold standard will need to be taken into account, as with other CFTR physiological assays.

Rectal suction biopsy for ROMA can easily be performed in general hospitals with low complication rates,[7] even in infants (with CFSPID). Quality requirements for biopsies are lower for ROMA[8] than for ICM,[9] as only the presence of viable crypts is needed to grow an organoid culture, rather than an intact epithelium required for electrophysiology. Biopsies can thus be shipped to a central laboratory for analysis,[8] which ensures standardisation together with the semi-automated nature of the analysis.

Possible issues with ROMA include the complexity and cost of organoid culture. However, standardised protocols are available for organoid culture and technique portability has been demonstrated.[8] Reducing the number of wells from 32 to 8 resulted in virtually identical results and would make ROMA more cost-efficient. Using rectal instead of airway tissue is both an advantage (CFTR specific, no influence of disease state)[1] and a disadvantage (possible differences in CFTR function between organs). The delay of 1–2 months between taking the biopsy and availability of results is rarely an issue as these cases are often not urgent. This waiting time is similar to that of extensive genetic analysis.

Further work will have to assess ROMA as a diagnostic test

Table 1 Baseline characteristics of the subjects and indexes calculated using rectal organoid morphology analysis (ROMA)

	CF	Non-CF	p value
n	167	22*	
IR	1.11 (0.93–1.34)	0.76 (0.61–0.88)	<0.001
CI	0.59 (0.49–0.70)	0.79 (0.73–0.84)	<0.001
Age (years)	18 (0–60)	44 (0–77)	<0.001
Gender	85 male (51%)	11 male (50%)	>0.999
	82 female (49%)	11 female (50%)	
SCC (mmol/L) (n=164)	97.61 (36–160)		
SCC low (<87 mmol/L) or high (≥87 mmol/L)	41 low (25%)		
	123 high (75%)		
Pancreatic status (n=165)	28 PS (17%)		
	137 PI (83%)		

n or mean and range.

*7 carriers, 3 non-carriers, 2 autosomal dominant polycystic kidney disease, 6 ulcerative colitis, 1 polyp screening, 3 healthy controls included in a study about inflammatory bowel disease.

CF, cystic fibrosis; IR, intensity ratio; CI, circularity index; SCC, sweat chloride concentration; PI, pancreatic insufficient; PS, pancreatic sufficient
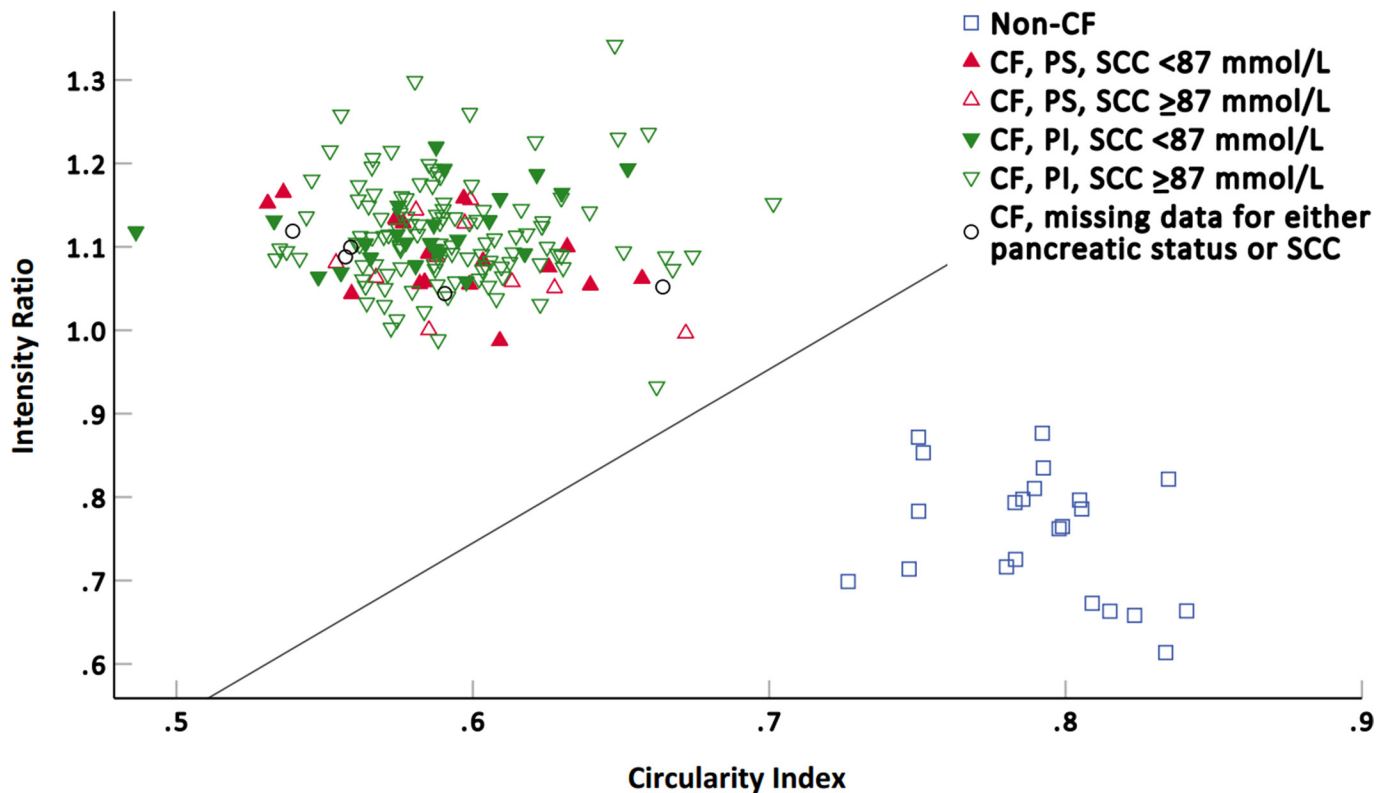
Figure 2 Intensity ratio (IR) and circularity index (CI) values of each subject according to disease status, pancreatic status and sweat chloride concentration. The line represents the optimal discrimination line obtained by linear discriminant analysis. CF, cystic fibrosis; PI, pancreatic insufficient; PS, pancreatic sufficient; SCC, sweat chloride concentration.

in patients with equivocal diagnosis. Beyond diagnosis, organoids used for ROMA can be biobanked for later personalised medicine research for the newly diagnosed patients with CF.[10] ROMA could also contribute to measuring modulator efficiency, as restoring CFTR function to high levels causes the appearance, before any stimulation, of a central lumen and swelling to a more round shape,[3] [4] measured by IR and CI, respectively.

Author affiliations
[1]Department of Development and Regeneration, Woman and Child Unit, CF Research Lab, KU Leuven, Leuven, Flanders, Belgium
[2]Department of Pediatrics, Pediatric Pulmonology, University Hospital of Leuven, Leuven, Flanders, Belgium
[3]VIB Bio Imaging Core, VIB KU Leuven Center for Brain & Disease Research, Leuven, Belgium
[4]Department for Neuroscience, KU Leuven, Leuven, Flanders, Belgium
[5]Interuniversity Center for Biostatistics and Statistical Bioinformatics, KU Leuven, Leuven, Flanders, Belgium
[6]Interuniversity Center for Biostatistics and Statistical Bioinformatics, Hasselt University, Hasselt, Limburg, Belgium
[7]Department of Pediatrics, 2nd Faculty of Medicine, Motol University Hospital, Praha, Czech Republic
[8]Department of Chronic Diseases and Metabolism (CHROMETA), Translational Research Center for Gastrointestinal Disorders (TARGID), KU Leuven, Leuven, Flanders, Belgium
[9]Department of Development and Regeneration, Stem Cell Institute Leuven (SCIL), KU Leuven, Leuven, Flanders, Belgium
[10]Department of Gastroenterology and Hepatology, University Hospital of Leuven, Leuven, Flanders, Belgium
[11]Department of Chronic Diseases, Metabolism and Ageing; Pneumology, University Hospital of Leuven, Leuven, Flanders, Belgium
[12]Department of Respiratory Diseases, University Hospital of Leuven, Leuven, Flanders, Belgium

ORCID iDs
Senne Cuyx http://orcid.org/0000-0002-6473-8966
Anabela Santo Ramalho http://orcid.org/0000-0003-3143-3661
François Vermeulen http://orcid.org/0000-0003-2303-2470

REFERENCES

1 De Boeck K, Vermeulen F, Dupont L. The diagnosis of cystic fibrosis. *La Presse Médicale* 2017;46:e97–108.
2 CFTR2 [Internet]. Available: https://www.cftr2.org/ [Accessed cited 2019 May 30].
3 Dekkers JF, Wiegerinck CL, de Jonge HR, *et al*. A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nat Med* 2013;19:939–45 https://pubmed.ncbi.nlm.nih.gov/23727931/
4 Dekkers JF, Berkers G, Kruisselbrink E, *et al*. Characterizing responses to CFTR-modulating drugs using rectal organoids derived from subjects with cystic fibrosis. *Sci Transl Med* 2016;8:344ra84 https://pubmed.ncbi.nlm.nih.gov/27334259/
5 Wilschanski M, Dupuis A, Ellis L, *et al*. Mutations in the cystic fibrosis transmembrane regulator gene and in vivo transepithelial potentials. *Am J Respir Crit Care Med* 2006;174:787–94 https://pubmed.ncbi.nlm.nih.gov/16840743/
6 Derichs N, Sanz J, Von Kanel T, *et al*. Intestinal current measurement for diagnostic classification of patients with questionable cystic fibrosis: validation and reference data. *Thorax* 2010;65:594–9 http://www.ncbi.nlm.nih.gov/pubmed/20627915
7 Friedmacher F, Puri P. Rectal suction biopsy for the diagnosis of Hirschsprung's disease: a systematic review of diagnostic accuracy and complications. *Pediatr Surg Int* 2015;31:821–30 https://link.springer.com/article/
8 Vonk AM, van Mourik P, Ramalho AS, *et al*. Protocol for application, standardization and validation of the forskolin-induced swelling assay in cystic fibrosis human colon organoids. *STAR Protoc* 2020;1:100019.
9 Servidoni MF, Sousa M, Vinagre AM, *et al*. Rectal forceps biopsy procedure in cystic fibrosis: technical aspects and patients perspective for clinical trials feasibility. *BMC Gastroenterol* 2013;13:91.
10 Ramalho AS, Fürstová E, Vonk AM. Correction of CFTR function in intestinal organoids to guide treatment of cystic fibrosis. *Eur Respir J [Internet]* 2020.