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ORIGINAL INVESTIGATION

Secretory IgA-mediated immune response in saliva and early detection of *Pseudomonas aeruginosa* in the lower airways of pediatric cystic fibrosis patients

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Abstract

Pseudomonas aeruginosa (*Pa*) detection in the paranasal sinuses may help to prevent or postpone bacterial aspiration to the lower airways (LAW) and chronic lung infection in cystic fibrosis (CF). We assessed the ability of an ELISA test for measurement of specific *Pa* secretory IgA (sIgA) in saliva (a potential marker of sinus colonization) to early detect changes in the *Pa* LAW status (indicated by microbiological sputum or cough swab culture and specific serum IgG levels) of 65 patients for three years, in different investigation scenarios. Increased sIgA levels were detected in saliva up to 22 months before changes in culture/serology. Patients who remained *Pa*-positive had significantly increased sIgA levels than patients who remained *Pa*-negative, both at the baseline (39.6 U/mL vs. 19.2 U/mL; $p = 0.02$) and at the end of the follow-up (119.4 U/mL vs. 25.2 U/mL; $p < 0.001$). No association was found between sIgA levels in saliva and emergence or recurrence of *Pa* in the LAW. A positive median sIgA result in the first year of follow-up implied up to 12.5-fold increased risk of subsequent *Pa* exposure in the LAW. Our test detected early changes in the *P. aeruginosa* LAW status and risk of exposure to *P. aeruginosa* in the LAW with two years in advance. Comparison with sinus culture is needed to assess the test's ability to identify CF patients in need of a sinus approach for *Pa* investigation, which could provide opportunities of *Pa* eradication before its aspiration to the lungs.

Keywords Cystic fibrosis · *Pseudomonas aeruginosa* · Respiratory tract infections · Diagnosis · Secretory IgA · Saliva

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Introduction

The early diagnosis of *Pseudomonas aeruginosa* chronic lung infection remains a major challenge in the clinical routine of cystic fibrosis (CF). Microbiological respiratory culture is the reference diagnostic method, but limitations exist as only 35–40% of the patients are able to spontaneously expectorate a representative sputum sample [1, 2], and the utility of cough swabs as an alternative for surveillance is controversial, since they can underestimate the bacterial presence in the lungs [3].

Pseudomonas aeruginosa IgG serology as a surrogate has been long discussed and proved useful for screening patients with chronic lung infection and at risk of developing thereof [4, 5]. However, the potential of serology for the early *P. aeruginosa* detection is a matter of debate [6], and the humoral response may rise when a mucoid biofilm-producing *P. aeruginosa* strain is already well established in the CF lungs [7].

The recent "unified airways" hypothesis suggests the upper airways (UAW), namely the paranasal sinuses, as the first niche and main reservoir of *P. aeruginosa* strains causing intermittent colonization, even after a certain period of antibiotic eradication therapy, and bacterial detection and treatment in the UAW might prevent or postpone its aspiration to the lower airways (LAW), where it ultimately leads to chronic lung infection [8–10]. Nevertheless, sampling the paranasal sinuses with sinus aspiration (the reference method) can be invasive, time-consuming, painful, and expensive [9], therefore, unfeasible in the clinical routine of most CF centers. Antibody-based enzyme-linked immunosorbent assay (ELISA) techniques for measurement of the secretory IgA (sIgA)-mediated immune response in nasal lavage and saliva have been tested for diagnostic purposes, as they can reflect the local immune response in the sinus mucosa, and proved potentially useful for differentiation among *P. aeruginosa* chronic infection, intermittent colonization, and absence of infection in the lungs [11], as well as for ruling out chronic lung infection [12].

In the present study, we assessed the usefulness of a previously standardized ELISA test for measurement of specific *P. aeruginosa* sIgA in saliva [12] for early detection of changes in the *P. aeruginosa* colonization/infection status in the lungs, indicated by changes in microbiological culture and serum IgG results, in a three-year longitudinal follow-up.

Methods

Patients' classification

The study took place at the CF Reference Centre of the University of Campinas Teaching Hospital (Hospital de Clínicas—HC Unicamp). A total of 70 patients with confirmed CF diagnosis [13] and without chronic *P. aeruginosa* lung infection were initially enrolled, out of whom two patients moved to other CF centres, two patients asked to be excluded from the study, and one patient lost follow-up. The final sample included 65 patients.

Pseudomonas aeruginosa lung colonization/infection definition relied on modified Leeds criteria [12, 14], as follows: *never colonized* (no history of *P. aeruginosa* in microbiological culture with negative screening serum IgG result), *free of infection* (history of *P. aeruginosa* in microbiological culture, but not in the last 12 months prior the study, with negative serum IgG), *intermittent colonization* (*P. aeruginosa* isolated in < 50% of cultures in the last 12 months with negative serum IgG, or no isolation of *P. aeruginosa* with positive serum IgG), and *chronic infection* (*P. aeruginosa* isolated in < 50% of cultures in the last 12 months with positive serum IgG, or *P. aeruginosa* isolated in ≥ 50% of cultures in the last 12 months regardless of the screening IgG result).

LAW microbiological assessment

A minimum of four microbiological LAW cultures were performed per patient/year, and sputum and cough swabs were the samples of reference. Samples were sent to the HC Unicamp Microbiology Lab and cultured in MacConkey agar for 24 h at 37 °C. Identification was based on colonial morphology (no lactose fermentation, metallic sheen, and green diffusible pigment), grape-like odor, positive oxidase reaction, and growth at 42 °C [15]. The biochemical identification was confirmed by automated methods using the BD Phoenix™ System (Becton–Dickinson, Franklin Lakes, NJ, United States), according to the manufacturer's instructions.

Measurement of *P. aeruginosa*-specific serum IgG and salivary sIgA

Levels of *P. aeruginosa*-specific serum IgG (used as a complementary test for *P. aeruginosa* detection in the LAW) and sIgA in saliva were measured using previously described ELISA tests [12, 16]. Blood samples were collected in 4.0 mL blood tubes without anticoagulant additives and centrifuged (2500 rpm/15 min) for obtaining the serum samples. Saliva samples were collected using Salivette® cotton swabs (Sarstedt, Nümbrecth, Germany). The swab is placed in the patient's mouth for 3 min and transferred to an appropriate tube for centrifugation (3000 rpm/10 min) and obtainment of clear saliva.

A 96-well ELISA plate was coated overnight with a commercially available pooled *P. aeruginosa* antigen (St-Ag; Statens Seruminstitut, Copenhagen, Denmark) diluted 1:2000 in carbonate–bicarbonate buffer (Na_2CO_3 10.0 nmol/L + $NaHCO_3$ 28.0 nmol/L; pH 9.5). The St-Ag consists of 64 water-soluble antigens from 17 different O-groups of *P. aeruginosa*, obtained by sonication [5]. The plate was rinsed for three times with a rinsing buffer composed of phosphate-buffered saline (NaCl 137.0 mmol/L + Na_2HPO_4 10.0 mmol/L + KH_2PO_4 1.8 mol/L; pH 7.4) added with 0.1% Tween 20 (PBS-T). After rinsing, nonspecific antibody-binding sites on the plate were blocked with PBS-T added with 0.1% bovine serum albumin for 1 h and the plate was rinsed twice. Samples diluted 1:800 (serum) and 1:8 (saliva) were added for 1 h, followed by a three-time rinsing and addition of horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-human IgG (P0214, diluted 1:4000) and IgA (P0216, diluted 1:2000) (Dako Denmark A/S, Glostrup, Denmark), for antibody measurement in serum and saliva, respectively. Tetramethylbenzidine (TMB; Sigma-Aldrich, St. Louis, MO, United States) was added as the substrate,

for 10 and 20 min, for plate-containing serum and saliva samples, respectively, and reactions were stopped after addition of sulfuric acid (H_2SO_4) 1.0 mol/L. The absorbances were read at 450 nm on an ELISA reader (Labsystems, Helsinki, Finland), and the absorbance values were converted into U/mL. Cut-off values of 28.2 U/mL and 47.2 U/mL determined a positive result for serum IgG and sIgA in saliva, respectively [12].

After screening, patients were seen on a quarterly basis for three years and monitored for changes in the *P. aeruginosa* LAW status and in the sIgA levels in saliva, which were measured at least once a year. A change in the *P. aeruginosa* status was considered upon conversion in either microbiology or serology results. Changes in the sIgA levels were analyzed in different scenarios, according to patients' *P. aeruginosa* LAW status at the end of the follow-up. First, patients were divided into three groups: Patients who remained *P. aeruginosa*-negative, i.e., never colonized or free of *P. aeruginosa* LAW colonization/infection ($Pa_{(neg)}$); patients whose *P. aeruginosa* LAW status were positive (intermittent colonization) at the baseline but became negative at the end of the follow-up ($Pa_{(Pos-neg)}$), and patients who became or kept *P. aeruginosa*-positive ($Pa_{(Pos)}$), i.e., intermittently colonized or chronically infected. In a second scenario, patients who kept/became *P. aeruginosa*-negative were evaluated for changes in the sIgA levels according to isolated episodes of *P. aeruginosa* LAW colonization during the study, being divided into two groups: those who had at least one positive result for *P. aeruginosa* culture or serum IgG during the follow-up and those who did not have any positive result indicating novel or recurrent colonization. In a third scenario, patients were grouped according to their history of exposure to *P. aeruginosa* in the LAW. In this case, patients who were free of infection and intermittently colonized were grouped together as having history of *P. aeruginosa* exposure, and patients with no microbiological or serological history of *P. aeruginosa* were classified as not having history of *P. aeruginosa* exposure. Status was changed from not exposed to exposed upon at least one conversion in microbiological culture or serology during the follow-up.

Statistical analysis

Baseline sIgA levels were compared according to the baseline *P. aeruginosa* status (never colonized, free of infection, and intermittent colonization), and according to the *P. aeruginosa*-related outcome in the different scenarios, using the non-parametric Mann–Whitney *U* test (for two independent samples) and the one-way Kruskal–Wallis *H* test (for more than two independent samples). Variations in the sIgA levels within each group were assessed using the non-parametric Wilcoxon test (for two different periods) and the Friedman test (for more than two different periods). Differences between percentages were assessed using the Chi-square test. Risk analyses were performed by calculating the odds-ratios (ORs) and their 95% confidence intervals (CI). For all tests, a p value lower than 0.05 was considered statistically significant.

Ethical aspects

The study was approved by the ethics committee of the School of Medical Sciences, University of Campinas (technical opinion number: 931.170). All patients who agreed to participate provided written informed consent. When the patient was under 18 years old, written informed consent was provided by his/her legal guardian, and the patient (when literate) provided written informed assent.

Results

Out of 65 enrolled patients, 31 were male and 34 were female; the median age was 4.5 years old (0.4–18.7). Sputum was collected from 12 patients, and, in 53 patients, the respiratory sample was collected with cough swab. Twenty-eight patients were intermittently colonized, 20 were free of infection, and 17 were never colonized with *P. aeruginosa*

Table 1 Patients enrolled in the study

P. aeruginosa status	Intermittent colonization	Free of infection	Never colonized	p value[b]
N	28	20	17	–
Gender (M/F)	12/16	10/10	9/8	–
Median age (range)	3.2 (0.4–14.1)	6.6 (0.7–18.7)	4.3 (0.4–16.3)	–
Median (serum IgG)[a]	11.0 (1.7–145.0)	7.2 (2.5–22.9)	6.2 (3.3–17.1)	0.284
Median (sIgA)[a]	27.1 (3.2–424.5)	37.1 (3.1–223.1)	15.0 (2.8–44.2)	0.037
% sIgA positivity	46.0	35.0	0.0	0.004

Gender, age, *P. aeruginosa* status, median baseline sIgA levels in saliva and percentage of sIgA positivity

[a]Given in units per mL (U/mL)

[b]One-way Kruskal–Wallis *H* test

(Table 1). Both median baseline sIgA levels in saliva and rate of sIgA positivity were significantly higher in patients who were free of infection (37.1 U/mL; $p=0.014$ and 35%; $p=0.007$) and intermittently colonized (27.1 U/mL; $p=0.03$ and 46%; $p=0.001$) when compared to the never colonized group, which did not show sIgA positivity (Table 1).

During the study, five patients who were free of infection at the baseline changed the *P. aeruginosa* LAW status to intermittent colonization, two changed to chronic infection, and 13 remained free of infection. Episodes of recurrent colonization were seen in 54% of the latter. The rate of sIgA positivity in this group at the end of the follow-up was 60%, and all patients who were sIgA-positive at the baseline kept positive sIgA levels until the end of the study. Eight patients who were never colonized developed *P. aeruginosa* colonization during the follow-up, and three of them were intermittently colonized at the end of the follow-up, with a rate of sIgA positivity of 41.2%. Seven patients from the intermittently colonized group maintained this status, two developed chronic lung infection, and 19 became free of infection. The rate of sIgA positivity at the end of the follow-up was 64.3% (Fig. 1).

Out of 12 patients who were able to expectorate sputum, seven (58%) were intermittently colonized at the baseline. Two of them became free of infection, two became chronically infected, and three kept intermittently colonized at the end of the follow-up. Two patients were never colonized at the baseline and remained so during the study, and three patients were free of infection, one of whom became intermittently colonized at the end of the follow-up. Overall, patients who were sputum producers had significantly higher median sIgA levels in saliva at the baseline (46.7 vs. 19.2 U/mL; $p=0.014$), as well as in the first (69.4 vs. 39.5 U/mL; $p=0.042$) and second (131.3 vs. 45.4 U/mL; $p=0.031$) years, but not in the last year of follow-up (108.9 vs. 51.8 U/mL; $p=0.068$), compared to patients whose samples were collected with cough swab.

First scenario: sIgA levels in saliva according to changes in the *P. aeruginosa* LAW status

After three years of follow-up, microbiological respiratory culture and serum IgG results indicated 27 patients who remained *P. aeruginosa*-negative ($Pa_{(neg)}$), 19 who were *P. aeruginosa*-positive and became *P. aeruginosa*-negative ($Pa_{(pos-neg)}$), and 19 who remained or became *P. aeruginosa*-positive ($Pa_{(pos)}$). Median baseline sIgA levels in saliva were 19.2 U/mL (2.8–155.1), 25.4 U/mL (3.2–424.5) and 39.6 U/mL (3.7–223.1), respectively. Statistical significance was found between the $Pa_{(pos)}$ and $Pa_{(neg)}$ groups ($p=0.02$). The median sIgA levels significantly increased within the $Pa_{(pos)}$ and $Pa_{(neg)}$ groups in the last year of follow-up, compared to the baseline. The median values (range; p value) were 25.2 U/mL (10.1–420.8; $p=0.003$), 51.3 U/mL (16.7–285.1;

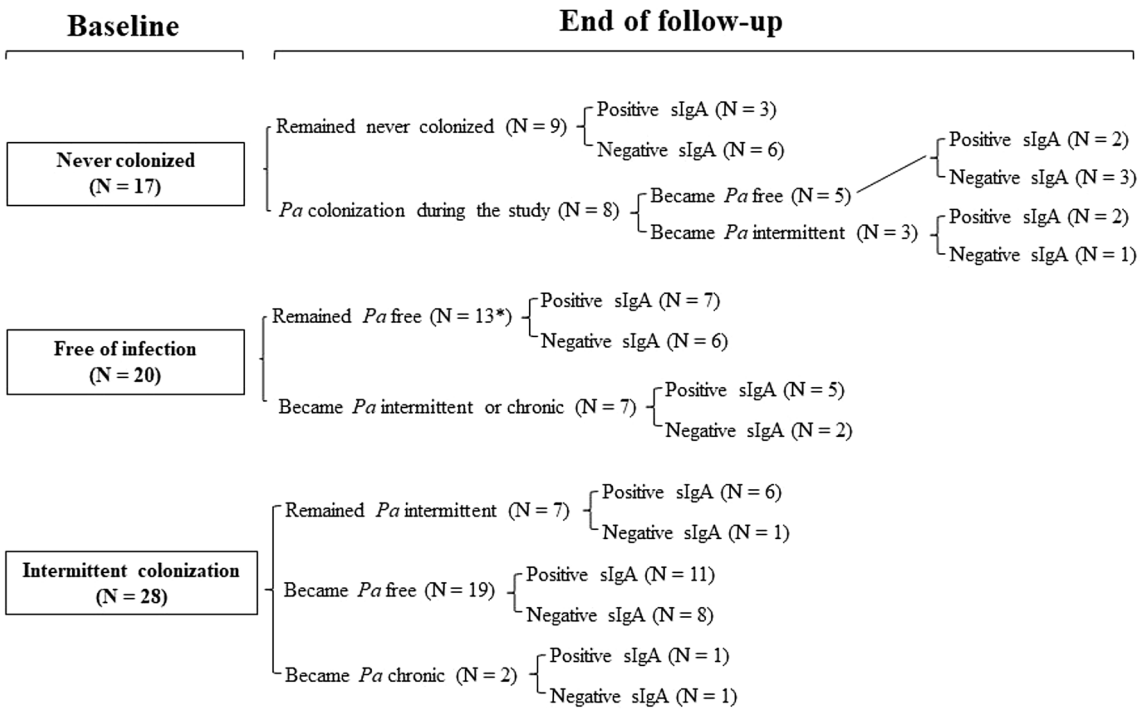

Fig. 1 CF patients enrolled in the study classified according to their baseline *P. aeruginosa* LAW status. Outcomes and sIgA positivity at the end of the follow-up (*54% of the patients in this group had *P. aeruginosa* recurrence during the follow-up)

$p = 0.07$) and 119.4 U/mL (15.6–655.8; $p = 0.01$) in the $Pa_{(neg)}$, $Pa_{(pos-neg)}$ and $Pa_{(pos)}$ groups, respectively. Significantly higher sIgA levels were observed in the $Pa_{(pos)}$ group ($p = 0.005$) at the end of the follow-up (Fig. 2).

Second scenario: change in the sIgA levels according to novel or recurrent LAW colonization with *P. aeruginosa*

Among patients from the $Pa_{(neg)}$ and $Pa_{(pos-neg)}$ groups ($n = 46$), 22 had at least one novel or recurrent episode of LAW colonization during the follow-up. Median sIgA levels in these patients were elevated in all study periods when compared to patients without novel or recurrent colonization; however, statistical significance was reached only in the first year of follow-up. Within both groups, the median sIgA levels were significantly higher in the last year than at the baseline (Table 2). Within the $Pa_{(pos-neg)}$ group, 9/19 (47%) patients did not have recurrent *P. aeruginosa* LAW colonization during the follow-up, but their median sIgA levels in saliva were significantly higher in the last year of follow-up than at the baseline.

Among 24 patients who did not have novel or recurrent *P. aeruginosa* lung colonization, one patient in the $Pa_{(neg)}$ group had chronic *Stenotrophomonas maltophilia* lung colonization and other four patients (three in the $Pa_{(neg)}$ group and one in the $Pa_{(pos-neg)}$ group) had at least three isolations of other Gram-negative bacteria (*S. maltophilia, Haemophilus influenzae* and *Burkholderia cepacia* complex) in microbiological culture during the follow-up. When these five patients were excluded, the median sIgA levels in the second year of follow-up became significantly higher in the $Pa_{(pos-neg)}$ group; however, no statistically significant differences other than that were observed (Table 2).

Out of the patients who were *P. aeruginosa*-negative at the baseline, 20 converted their sIgA levels and

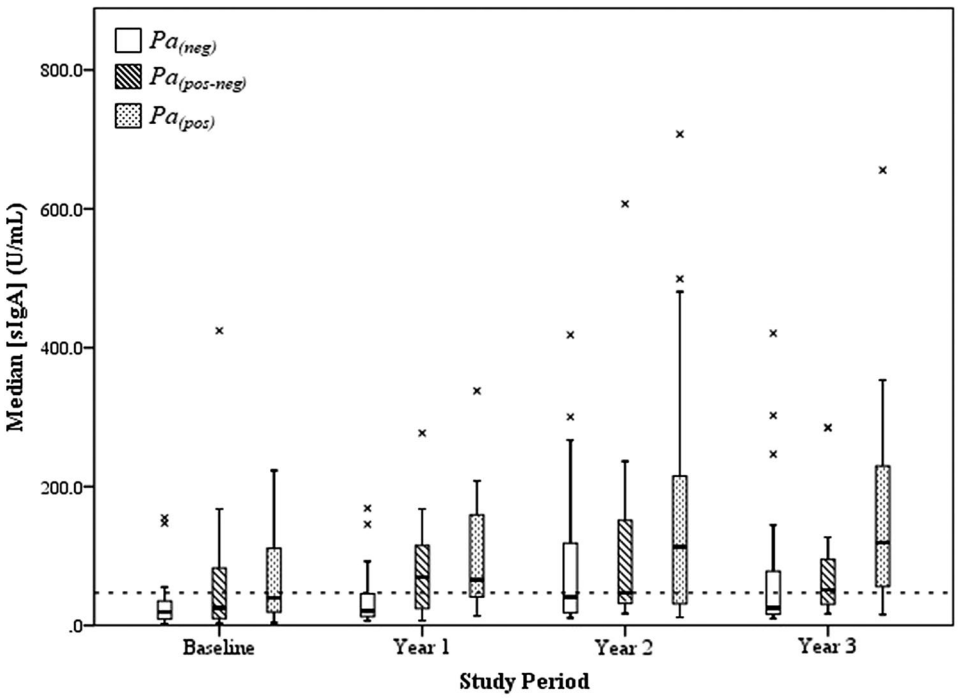

Fig. 2 Distribution of the median sIgA levels in saliva at the baseline and in the three years of follow-up, according to changes in the *P. aeruginosa* LAW status (indicated by changes in microbiological culture and serum IgG results). $Pa_{(neg)}$: patients who were *P. aeruginosa*-negative at the baseline and kept this status until the end of the follow-up; $Pa_{(pos-neg)}$: patients who were *P. aeruginosa*-positive at the baseline and became negative at the end of the follow-up; $Pa_{(pos)}$: patients who were *P. aeruginosa*-positive at the baseline and remained positive until the end of the follow-up. The dotted line corresponds to the cut-off value of the sIgA ELISA test (47.2 U/mL)

Table 2 Changes in the sIgA levels in saliva according to development of novel or recurrent *P. aeruginosa* colonization in the LAW during the follow-up (indicated by changes in microbiological respiratory culture and/or serum IgG results)

P. aeruginosa recurrence	Yes	No	*p* value[b]	*p* value[b,c]
Median [sIgA] baseline	24.2 (2.8–223.1)	21.3 (3.1–424.5)	0.260	0.164
Median [sIgA] Year 1	55.0 (6.9–337.8)	21.9 (6.6–277)	0.011	0.020
Median [sIgA] Year 2	96.7 (11.0–708.0)	40.8 (14.0–607.0)	0.269	0.043
Median [sIgA] Year 3	73.0 (10.1–655.8)	28.1 (11.0–285.0)	0.116	0.138
p value[a]	0.002	< 0.001		

[a]Friedman test

[b]Mann–Whitney *U* test

[c]After excluding patients with chronic or recurrent colonization with other Gram-negative bacteria

maintained them positive until the end of the follow-up, 15 of whom had novel/recurrent colonization or ended the study as *P. aeruginosa*-positive. In 10 of these patients, the sIgA test was able to detect *P. aeruginosa* before ($n=8$) or simultaneously ($n=2$) to changes in microbiological respiratory culture or serum IgG results, with a mean interval of 9.5 months (0–22.1). In five patients, the sIgA response in saliva rose after detection by culture/IgG, with a mean interval of 6.4 months (3–14). Other five patients maintained positive sIgA levels in saliva without converting culture or IgG results until the end of the follow-up.

Third scenario: changes in the sIgA levels in saliva according to the history of *P. aeruginosa* exposure in the LAW

In this scenario, 48 and 17 patients were classified as having and not having history of *P. aeruginosa* LAW exposure at the baseline, respectively. During the follow-up, eight patients were exposed to *P. aeruginosa*, irrespective of their *P. aeruginosa*-related outcome at the end of the follow-up. Both sIgA levels and rate of sIgA positivity were significantly higher in patients who had *P. aeruginosa* exposure in all the study periods, except in the second year (Table 3; Fig. 3). A positive median sIgA result in the first year of follow-up implied significantly increased risks of *P. aeruginosa* exposure both in the second year (OR 12.5; CI: 1.5–104.6) and in the third year of follow-up (OR 9.2; CI: 1.1–78.8).

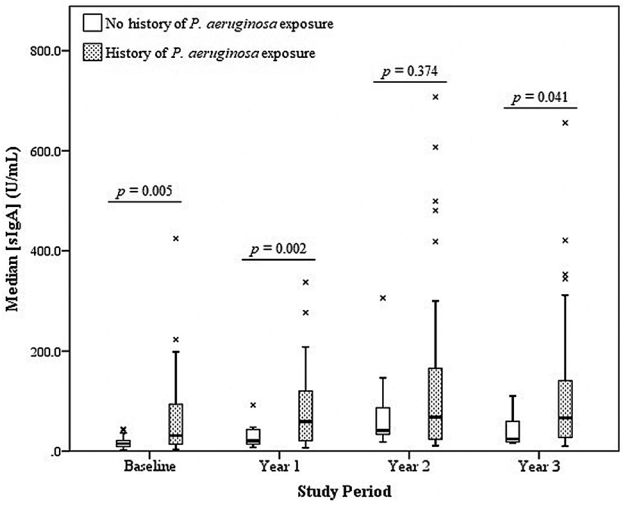

Fig. 3 Distribution of the median sIgA levels in saliva at the baseline and in the three years of follow-up according to the patients' history of *P. aeruginosa* exposure during the study. The dotted line corresponds to the cut-off value of the sIgA ELISA test (47.2 U/mL)

Discussion

Chronic bacterial sinusitis affects most CF patients and *P. aeruginosa* is commonly isolated in cultures of sinus secretion [17–19]. However, the impact in life quality seems to be low and sinus symptoms tend to be underreported by CF patients [20–22]. The high sIgA/IgG ratio in the paranasal sinuses is likely to be the main reason for this, given the low inflammatory response triggered by sIgA upon the encounter with the pathogen in the sinus

Table 3 Changes in the sIgA levels in saliva and in the percentage of sIgA positivity according to the patients' history of *P. aeruginosa* exposure during the follow-up

History of *P. aeruginosa* exposure	Exposed	Not exposed	*p* value[a]
Baseline			
N	48	17	–
% sIgA positivity	41.7	0	< 0.001
Median [sIgA] (U/mL)	31.1 (3.1–424.5)	15.0 (2.8–44.2)	0.005
Year 1			
N	49	16	–
% sIgA positivity	59.2	12.5	0.001
Median [sIgA] (U/mL)	59.1 (6.6–337.8)	20.7 (7.5–92.2)	0.002
Year 2			
N	54	11	–
% sIgA positivity	55.6	45.5	0.54
Median [sIgA] (U/mL)	68.0 (10.8–707.6)	41.4 (18.3–306.2)	0.374
Year 3			
N	56	9	–
% sIgA positivity	60.7	33.3	0.124
Median [sIgA] (U/mL)	66.7 (10.1–655.8)	24.4 (16.0–110.4)	0.041

[a]Mann–Whitney *U* test (1-tailed)

mucosa [9, 23]. *P. aeruginosa*-specific sIgA is detectable in saliva [11, 12] and ELISA-based tests may be useful to identify *P. aeruginosa* foci in the CF paranasal sinuses and the risk of downward lung colonization, as sinus secretions are aspirated to the lungs [24]. Thus, we aimed to assess the ability of our sIgA ELISA test to early detect *P. aeruginosa* emergence in the LAW. To better separate the patients according to their *P. aeruginosa* LAW status, given the limited number of microbiological cultures annually performed at our center (mean number of four per patient), our classification criteria relied on both microbiological culture and specific serum IgG to *P. aeruginosa*, where one test complements the other.

Elevated *P. aeruginosa*-specific sIgA levels in saliva samples from 36% of patients free of infection (Table 1) are not unexpected, since these patients had at least one previous exposure to *P. aeruginosa*, and, even after bacterial eradication in the LAW, the sIgA response in saliva may indicate memory production [25, 26] or, most likely, persistent focus of sinus colonization. This is most evident with the maintenance of positive sIgA levels in this specific group alongside to their considerable rate of recurrent *P. aeruginosa* LAW colonization during the study. Initially, the test also proved able to rule out any current or past exposure to *P. aeruginosa* in the LAW, as no sIgA positivity was seen in the never colonized group, corroborating our first assessment of the test, which found a negative predictive value of 92% for ruling out chronic lung infection [12]. However, at the end of the follow-up, 33% of patients who maintained this status had positive sIgA levels (Fig. 1; Table 3).

Concerning clinical outcomes, the use of different investigation scenarios provided us with a clearer assessment of the test's usefulness. We firstly hypothesized that patients from the $Pa_{(pos)}$ group would have significantly higher sIgA levels in saliva than patients from the $Pa_{(neg)}$ group both at the baseline and in the last year of follow-up, which was confirmed (Fig. 2). The absence of statistical difference between the $Pa_{(pos)}$ and $Pa_{(pos-neg)}$ was not considered a surprise and is in accordance with recent microbiological findings of CF patients who underwent lung transplant, which found a significant correlation between pretransplant sinus cultures and posttransplant BAL cultures, with 19/21 patients with *P. aeruginosa*-positive sinus culture having positive BAL culture [27]. Other study [28] demonstrated that 42% of patients had the same colonizing pathogens of pretransplant LAW cultures recovered in nasal lavage cultures after transplant. Clinical benefits of the sinus approach for CF patients have been reported by the Copenhagen CF study group. Patients who underwent sinus surgery combined with systemic and postoperative topical antibiotic treatment showed lung function improvement and significantly decreased frequency of lung colonization with *P. aeruginosa* and other Gram-negative bacteria 1–3 years after surgery [10, 29]. These results support the potential role of the paranasal sinuses as a niche for downward colonization or recolonization of the LAW.

Interestingly, there was no significant difference in the sIgA levels in saliva between the $Pa_{(neg)}$ and $Pa_{(pos-neg)}$ groups at the end of the follow-up. We hypothesized that this could be explained by isolated episodes of novel or recurrent LAW colonization during the study in both groups, which led us to the second investigation scenario. Nevertheless, despite the significantly elevated median sIgA levels in the first year of follow-up in patients who had *P. aeruginosa* recurrence, no significant associations were found between sIgA levels and recurrent colonization, given the considerable percentage of patients from the $Pa_{(neg)}$ group with positive sIgA at the end of the follow-up (Table 2). Corroborating what was speculated in our cross-sectional analysis [12], these results strongly suggest that patients from the $Pa_{(neg)}$ compose a group of risk for development of *P. aeruginosa* LAW colonization. This hypothesis was confirmed in our third investigation scenario, where a positive median sIgA result in the first year of follow-up entailed 12.5- and ninefold increased risks of exposure to *P. aeruginosa* in the LAW one and two years later, respectively. It is worth mentioning that, despite the absence of positive results of culture and/or serum IgG, patients who are free of LAW infection are not free of *P. aeruginosa* exposure as they probably have established immune memory to *P. aeruginosa* due to the previous episodes of colonization or infection, although no studies have assessed memory formation against CF lung infections so far. Therefore, in this specific scenario, this group of patients was placed together with intermittently colonized patients, who are also exposed to the bacterium. Moreover, the significant rise of sIgA levels in $Pa_{(neg)}$ patients, who did not have recurrent *P. aeruginosa* LAW colonization (theoretically meaning successful antibiotic therapy), prevents the test from being considered a marker of *P. aeruginosa* eradication in the LAW.

Although the St-Ag contains antigens that are cross-reactive with other Gram-negative bacteria and cross-reactions have been previously reported [30–32], this did not appear to be a major problem in the present study, as no statistically significant difference in the sIgA levels between patients who had and who did not have recurrent *P. aeruginosa* lung colonization was observed at the end of the follow-up, even after excluding patients who were chronically or recurrently colonized with other Gram-negative bacteria. In most patients who became *P. aeruginosa*-positive or had novel or recurrent colonization during the study, the sIgA response was detected in saliva before or simultaneously to culture/serum IgG. However, there were cases in which the pathogen was first detected in the LAW and more than 25% of patients ended the follow-up as *P. aeruginosa*-positive without positive sIgA levels in saliva. In these patients, the recolonization source may be the conducting airways of the

lungs, where *P. aeruginosa* is not eradicated by antibiotic therapy, grows slowly in biofilm aggregates or as microcolonies due to the lower oxygen concentration, followed by an inflammatory response activated in a lesser degree [33–35]. Such discrepancies were also found in microbiology-based studies, and the sampling method seems to play a role, as less invasive methods, such as nasal lavage, may not well represent all the paranasal sinuses [36]. In fact, the agreement between *P. aeruginosa* presence in the UAW and LAW seems to vary from 19 to 55%, depending on the sampling method [18, 37, 38]. Yet, a single specific sinus may not capture bacteria located in other sinuses [39]. Likewise, it is not possible to categorically state that the *P. aeruginosa* presence in a given sinus can elicit a detectable sIgA-mediated response in saliva. On the other hand, the rates of genotypic compatibility of samples recovered from the upper and lower airways are high [18, 19, 40]. In brief, this suggests that the paranasal sinuses do act like bacterial reservoirs, but they are not the only ones.

This is the first study in the American continent to assess the usefulness of an ELISA test for detection of *P. aeruginosa*-specific sIgA in saliva. Naturally, there are limitations. First, this was a single-center study with a low number of patients and samples, which hampers more robust statistical analyses. Second, the study lacks the comparison of the sIgA response in saliva with a microbiological assessment of the paranasal sinuses, an approach that is not routinely performed in our CF center and is needed to confirm the true potential of sIgA in saliva as a marker of persistent sinus colonization. Third, the low number of patients who developed chronic infection prevented the assessment of the test's ability to early detect the onset of *P. aeruginosa* chronic LAW infection, which we aim to evaluate in a larger follow-up. Overall, the results show an easy, feasible, and rapid test, which, in a short period, detected changes in the *P. aeruginosa* LAW status and risk of *P. aeruginosa* exposure in the LAW with two years in advance, therefore corroborating the unified airways hypothesis. Although its diagnostic usefulness must be better evaluated, our ELISA test emerges as a potential alternative to identify patients in need of investigation of *P. aeruginosa* sinus colonization, which, in turn, could open a window of opportunity to eradicate the bacteria in this compartment before their aspiration to the lungs.

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Compliance with ethical standards

Conflict of interest None to declare.

References

1. Ramsey BW, Wentz KR, Smith AL, Richardson M, Williams-Warren J, Hedges DL et al (1991) Predictive value of oropharyngeal cultures for identifying lower airway bacteria in cystic fibrosis patients. Am Rev Respir Dis 144:331–337. https://doi.org/10.1164/ajrccm/144.2.331
2. Sagel SD, Kaspner R, Osberg I, Sontag MK, Accurso FJ (2001) Airway inflammation in children with cystic fibrosis and healthy children assessed by sputum induction. Am J Respir Crit Care Med 164:1425–1431. https://doi.org/10.1164/ajrccm.164.8.2104075
3. Seidler D, Griffin M, Nymon A, Koeppen K, Ashare A (2016) Throat swabs and sputum culture as predictors of *P. aeruginosa* or *S. aureus* lung colonization in adult cystic fibrosis patients. PLoS One 11:e0164232. https://doi.org/10.1371/journal.pone.0164232
4. Pressler T, Frederiksen B, Skov M, Garred P, Koch C, Høiby N (2006) Early rise of anti-pseudomonas antibodies and a mucoid phenotype of *Pseudomonas aeruginosa* are risk factors for development of chronic lung infection—a case control study. J Cyst Fibros 5:9–15. https://doi.org/10.1016/j.jcf.2005.11.002
5. Pressler T, Karpati F, Granström M, Knudsen PK, Lindblad A, Hjelte L et al (2009) Diagnostic significance of measurements of specific IgG antibodies to *Pseudomonas aeruginosa* by three different serological methods. J Cyst Fibros 8:37–42. https://doi.org/10.1016/j.jcf.2008.08.002
6. Mauch RM, Levy CE (2014) Serum antibodies to *Pseudomonas aeruginosa* in cystic fibrosis as a diagnostic tool: a systematic review. J Cyst Fibros 13:499–507. https://doi.org/10.1016/j.jcf.2014.01.005
7. Mauch RM, Jensen P, Moser C, Levy CE, Høiby N (2018) Mechanisms of humoral immune response against *Pseudomonas aeruginosa* biofilm infection in cystic fibrosis. J Cyst Fibros 17:143–152. https://doi.org/10.1016/j.jcf.2017.08.012
8. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N et al (2012) Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol 10:841–851. https://doi.org/10.1038/nrmicro2907
9. Aanaes K (2013) Bacterial sinusitis can be a focus for initial lung colonisation and chronic lung infection in patients with cystic fibrosis. J Cyst Fibros 12:S1–S20. https://doi.org/10.1016/S1569-1993(13)00150-1
10. Alanin MC, Aanaes K, Høiby N, Pressler T, Skov M, Nielsen KG et al (2016) Sinus surgery postpones chronic gram-negative lung infection: cohort study of 106 patients with cystic fibrosis. Rhinology 54:206–213. https://doi.org/10.4193/Rhino15.347
11. Aanaes K, Johansen HK, Poulsen SS, Pressler T, Buchwald C, Høiby N (2013) Secretory IgA as a diagnostic tool for *Pseudomonas aeruginosa* respiratory colonization. J Cyst Fibros 12:81–87. https://doi.org/10.1016/j.jcf.2012.07.001
12. Mauch RM, Rossi CL, Aiello TB, Ribeiro JD, Ribeiro AF, Høiby N et al. Secretory IgA response against *Pseudomonas aeruginosa* in the upper airways and the link with chronic lung infection in cystic fibrosis. Pathog Dis 2017. https://doi.org/10.1093/femspd/ftx069
13. Athanazio RA, da Silva Filho LVRF, Vergara AA, Ribeiro AF, Riedi CA, Procianoy EDFA et al. Brazilian guidelines for the diagnosis and treatment of cystic fibrosis. J Bras Pneumol

2017;43(3):219–245. https://doi.org/10.1590/S1806-37562017000000065
14. Lee TWR, Brownlee KG, Conway SP, Denton M, Littlewood JM (2003) Evaluation of a new definition for chronic *Pseudomonas aeruginosa* infection in cystic fibrosis patients. J Cyst Fibros 2:29–34. https://doi.org/10.1016/S1569-1993(02)00141-8
15. Gilligan PH, Kiska DL, Appleman MD (2006) Cumitech 43, Cystic fibrosis microbiology. Coordinating editor, Appleman MD. ASM Press, Washington, D.C.
16. Mauch RM, Rossi CL, Ribeiro JD, Ribeiro AF, Nolasco da Silva MT, Levy CE (2014) Assessment of IgG antibodies to *Pseudomonas aeruginosa* in patients with cystic fibrosis by an enzyme-linked immunosorbent assay (ELISA). Diagn Pathol 9:158–165. https://doi.org/10.1186/s13000-014-0158-z
17. Bonestroo HJC, de Winter-de Groot KM, van der Ent CK, Arets HGM (2010) Upper and lower airway cultures in children with cystic fibrosis: do not neglect the upper airways. J Cyst Fibros 9:130–134. https://doi.org/10.1016/j.jcf.2010.01.001
18. Muhlebach MS, Miller MB, Moore C, Wedd JP, Drake AF, Leigh MW (2006) Are lower airway or throat cultures predictive of sinus bacteriology in cystic fibrosis? Pediatr Pulmonol 41:445–451. https://doi.org/10.1002/ppul.20396
19. Mainz JG, Naehrlich L, Schien M, Ka M, Schiller I, Mayr S et al (2009) Concordant genotype of upper and lower airways *P. aeruginosa* and *S. aureus* isolates in cystic fibrosis. Thorax 64:535–540. https://doi.org/10.1136/thx.2008.104711
20. Illing EA, Woodworth BA (2014) Management of the upper airway in cystic fibrosis. Curr Opin Pulm Med 20:623–631. https://doi.org/10.1097/MCP.0000000000000107
21. Chan DK, McNamara S, Park JS, Vajda J, Gibson RL, Parikh SR (2016) Sinonasal quality of life in children with cystic fibrosis. JAMA Otolaryngol Neck Surg 142:743. https://doi.org/10.1001/jamaoto.2016.0979
22. Le C, McCrary HC, Chang E (2016) Cystic fibrosis sinusitis. Adv Otorhinolaryngol 79:29–37. https://doi.org/10.1159/000444959
23. Johansen HK, Aanaes K, Pressler T, Nielsen KG, Fisker J, Skov M et al (2012) Colonisation and infection of the paranasal sinuses in cystic fibrosis patients is accompanied by a reduced PMN response. J Cyst Fibros 11:525–531. https://doi.org/10.1016/j.jcf.2012.04.011
24. Nelson J, Karempelis P, Dunitz J, Hunter R, Boyer H (2018) Pulmonary aspiration of sinus secretions in patients with cystic fibrosis. Int Forum Allergy Rhinol 8:385–388. https://doi.org/10.1002/alr.22043
25. Cerutti A (2010) IgA changes the rules of memory. Science 328:1646–1647. https://doi.org/10.1126/science.1192488
26. Brandtzaeg P (2013) Secretory IgA: designed for anti-microbial defense. Front Immunol 4:222. https://doi.org/10.3389/fimmu.2013.00222
27. Choi KJ, Cheng TZ, Honeybrook AL, Gray AL, Snyder LD, Palmer SM et al (2018) Correlation between sinus and lung cultures in lung transplant patients with cystic fibrosis. Int Forum Allergy Rhinol 8:389–393. https://doi.org/10.1002/alr.22067
28. Morlacchi LC, Greer M, Tudorache I, Blasi F, Welte T, Haverich A et al. The burden of sinus disease in cystic fibrosis lung transplant recipients. Transpl Infect Dis 2018:e12924. https://doi.org/10.1111/tid.12924
29. Aanaes K, Johansen HK, Skov M, Buchvald FF, Hjuler T, Pressler T et al (2013) Clinical effects of sinus surgery and adjuvant therapy in cystic fibrosis patients—can chronic lung infections be postponed? Rhinology 51:222–230. https://doi.org/10.4193/Rhino12.207
30. Høiby N (1975) Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand J Immunol 4:187–196. https://doi.org/10.1111/j.1365-3083.1975.tb03825.x
31. Høiby N, Collins MT, Espersen F, Hertz JB, Hoff GE, Schiotz PO (1987) Taxonomic application of crossed immunoelectrophoresis. Int J Syst Bacteriol 37:229–240. https://doi.org/10.1099/00207713-37-3-229
32. Johansen HK, Kovesi TA, Koch C, Corey M, Høiby N, Levison H. *Pseudomonas aeruginosa* and *Burkholderia cepacia* infection in cystic fibrosis patients treated in Toronto and Copenhagen. Pediatr Pulmonol 1998;26:89–96. https://doi.org/10.1002/(SICI)1099-0496(199808)26:2%3C89::AID-PPUL3%3E3.0.CO;2-C.
33. Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC et al (2002) Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J Clin Invest 109:317–325. https://doi.org/10.1172/JCI13870
34. Bjarnsholt T, Jensen P, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB et al (2009) *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol 44:547–558. https://doi.org/10.1002/ppul.21011
35. Kragh KN, Alhede M, Jensen PO, Moser C, Scheike T, Jacobsen CS et al (2014) Polymorphonuclear leukocytes restrict growth of *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients. Infect Immun 82:4477–4486. https://doi.org/10.1128/IAI.01969-14
36. Møller ME, Alanin MC, Grønhøj C, Aanæs K, Høiby N, von Buchwald C (2017) Sinus bacteriology in patients with cystic fibrosis or primary ciliary dyskinesia: a systematic review. Am J Rhinol Allergy 31:293–298. https://doi.org/10.2500/ajra.2017.31.4461
37. Dosanjh A, Lakhani S, Elashoff D, Chin C, Hsu V, Hilman B (2000) A comparison of microbiologic flora of the sinuses and airway among cystic fibrosis patients with maxillary antrostomies. Pediatr Transpl 4:182–185. https://doi.org/10.1034/j.1399-3046.2000.00114.x
38. Lavin J, Bhushan B, Schroeder JW (2013) Correlation between respiratory cultures and sinus cultures in children with cystic fibrosis. IJPO 77:686–689. https://doi.org/10.1016/j.ijporl.2013.01.018
39. Lucas SK, Yang R, Dunitz JM, Boyer HC, Hunter RC (2018) 16S rRNA gene sequencing reveals site-specific signatures of the upper and lower airways of cystic fibrosis patients. J Cyst Fibros 17:204–212. https://doi.org/10.1016/j.jcf.2017.08.007
40. Ciofu O, Johansen HK, Aanaes K, Wassermann T, Alhede M, von Buchwald C et al (2013) *P. aeruginosa* in the paranasal sinuses and transplanted lungs have similar adaptive mutations as isolates from chronically infected CF lungs. J Cyst Fibros 12:729–736. https://doi.org/10.1016/j.jcf.2013.02.004

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