Ion concentrations in nasal airway surface liquid: a prediction model for the identification of cystic fibrosis carriers*

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Abstract

Background: Cystic fibrosis (CF) carriers seem to have a higher risk to develop chronic rhino-sinusitis (CRS), although the full underlying mechanisms are unknown.
Ion concentrations in nasal airway surface liquid (ASL) may be influenced by the heterozygosity for CF gene mutation, with possible impacts on the development of CRS.

Methods: A cheap and feasible standardized technique was designed to measure the ion levels in nasal ASL. With this purpose we collected, under basal conditions, samples from the nasal cavity of 165 adults: 14 homozygous for CF, 83 carriers and 68 healthy controls. Sodium (Na) and Chlorine (Cl) concentrations were then evaluated among different groups.

Results: Statistical analysis revealed a significant difference of Na and Cl values between controls and carriers and between controls and homozygotes. Receiver operating characteristic (ROC) curves and derived indicators (Youden's index and Area Under the Curve, AUC) were used to further evaluate the diagnostic capability of Na and Cl concentrations to differentiate heterozygotes from controls. ROC curves demonstrated that the optimal diagnostic cut-off value of Na is at 124, and the optimal cut-off value of Cl is at 103,2.

Conclusion: ASL sampling can be considered a new diagnostic tool for providing quantitative information on nasal ion composition. According to our findings, Na and Cl concentrations of nasal ASL could represent a useful tool to assess heterozygotes and healthy controls.

Key words: Cystic fibrosis, chronic rhinosinusitis, airway surface liquid

Introduction

Cystic fibrosis (CF) is the most common inherited autosomal recessive disease in Europe and North America [(1)]. This genetic disorder is caused by the mutation in the CF transmembrane conductance regulator (CFTR) gene on chromosome 7, which encodes for CFTR protein, a transmembrane cAMP-activated chloride channel [(2,3)]. Alterations of CFTR protein result in a defective secretion of chloride and bicarbonate ions and subsequently altered functions of epithelial sodium channels (ENaC), which manifest with thicker mucus and more viscous secretions [(4)].

In Italy, the estimated prevalence of CF in 2016 was 8.8/100,000 residents, with territorial heterogeneity [(5)]. Bizzoco et al. hypothesized a CF carrier frequency between 1:26 and 1:30 (about 3% of the Italian general population) [(6)]. According to literature, Caucasian populations showed a higher prevalence of CF com-

pared to other ethnic groups [7-10].
While in the past the heterozygous status was not recognized to be associated with morbidity, recent studies suggested possible haploinsufficiency due to low levels of CF gene product and related adverse health effects [11].
Among ENT manifestations, CRS seems to be more frequent in CF carriers than the general population [1], possibly due to the reduced activity of CFTR and the resultant impact on electrolytes transport, but detailed data on ions of nasal airway surface liquid (ASL), the thin layer of fluid that covers the airways, in patients with CFTR mutation are scant.
On that basis, we designed an innovative method to collect and analyze electrolytes concentration in nasal ASL, with the aim to detect electrolytes ion levels in different conditions (homozygosis, heterozygosis, and health condition) and to determine optimal cut-off values for discrimination of CF carriers.

Materials and methods

Ethical approval

All procedures performed were in accordance with the ethical standards of the institutional research committee (Ethics Committee at the University Hospital of Ferrara, ref. no. 150295) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Participants

The nature of this study and its procedures, performed in the Rhinology Outpatient Clinic of Ear, Nose and Throat Department at the Ferrara University Hospital, Italy, were explained to all participants, who provided written consent.
We recruited 15 homozygotes followed in the Regional Center of diagnosis and therapy of CF, Bufalini Hospital, in Cesena.
Then, we selected 92 carriers among the parents of CF patients. The diagnosis of both carriers and homozygotes conditions were confirmed by blood CFTR genetic analyses.
The control population was composed of 78 healthy volunteers (without a family history of CF).
All participants, including CF homozygotes, did not report medical history for acute nasal symptoms in the 15 days before the analysis or previous sinus surgery; furthermore, their endoscopic evaluation did not reveal evidence of nasal polyps.

Collection of clinical samples

During the rhinologic evaluation, nasal secretions were collected from the nasal cavity of all patients using neuro-patties (Codman® neurosurgical patties, 1/2" x 3", Johnson & Johnson medical ltd). To avoid contamination from the lacrimal secretions a neuro-patty was placed in the middle meatus, between inferior and middle turbinate, and it was left in situ for 30 minutes, to absorb the ASL. The patty was then removed and placed into a specific collection plastic tube with an insert and a cap (Salivette SARSTEDT), commonly used to obtain high-quality saliva diagnostic samples.
The devices were stored in a refrigerator at 2-4 ° C for max 24 hours and centrifuged at 2000 rpm for 15 minutes. Once the liquid portion was extracted, it was analyzed by common laboratory instruments.

Quantitative analysis

The ISE module of the Roche/Hitachi Cobas 8000 analyser, a fully automated system for description clinical chemistry analysis of body fluids, based on ion-selective electrodes was used for the quantitative determination of sodium, potassium, and chloride ions.
The system is a part of the technology platform of Clinical Biochemistry (Core Lab), accredited Laboratory of Clinical Chemistry and Microbiology, at the Ferrara University Hospital, in Ferrara, Italy.
Exiguous samples (<100 mcL), with excess mucus or organized material, were discarded.
Each 100 mcL of the fluid sample was diluted to 200 mcL of double-distilled water (1:3), in order to obtain the minimum volume required by the system (300 mcL) and to guarantee the availability of multiple samples for every single subject.
The investigation aimed to determine the correct measures of Chlorine (Cl), Sodium (Na) and potassium (K). Cl and Na values were easily detectable, while K levels were very negligible and therefore not taken into account.

Statistical analysis

The Shapiro-Wilk test was used to test the normality of the distribution of the continuous variables. In the case of symmetric distribution, the variables will be represented with the mean and standard deviation (SD) or, in the case of non-normal distributions, with the median value and interquartile range. Categorical data were expressed as total numbers and percentages.
Mann Whitney test was used to analyze the difference in Cl and Na between heterozygotes and controls. The Receiver Operating Characteristic (ROC) curve, the area under the curve (AUC) was used to measure the potential ability of the test to discriminate the heterozygous status for CFTR gene by identification of Cl and Na levels. Thresholds for Cl and Na were defined as the optimal cut-off that maximized the distance to the identity (diagonal) line in the ROC curve according to Youden's J statistic. The sensitivities and specificities of different Na and Cl levels were analyzed to determine the point with the maximum Youden's Index (J= sensitivity + specificity - 1), namely the point of the curve for which the predictive value is maximized. (12)
AUC values can range from 0.5 to 1, where 0.5 denotes a poor diagnostic test and 1 denotes an excellent diagnostic test. All

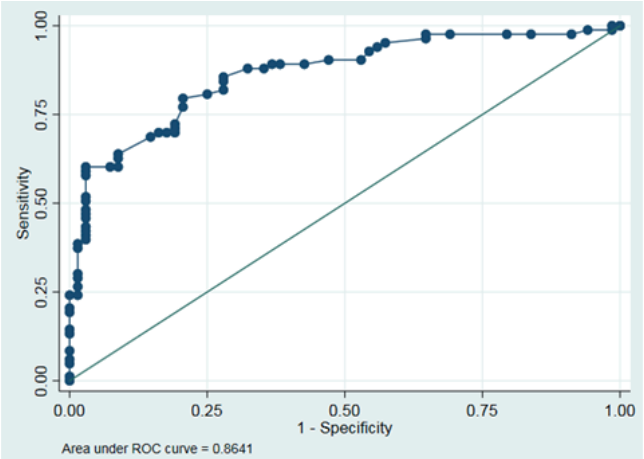

Figure 1. ROC curve for Na levels: Youden's J statistic: 124. Sensitivity: 0.79 confidence interval:0.69-0.87. Specificity: 0.45 confidence interval 0.67-0.88.

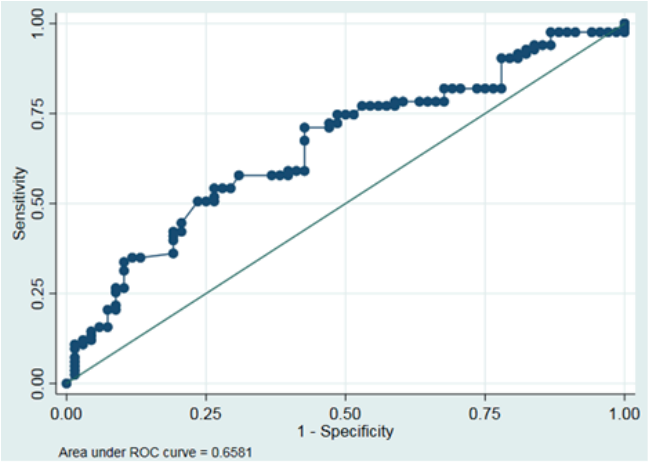

Figure 2. ROC curve for Cl levels: Youden's J statistic: 103,2. Sensitivity: 0.77 confidence interval:0.66-0.86. Specificity: 0.45 confidence interval 0.33-0.58.

analyses were performed using Stata 15.1 SE (Stata Corporation, College Station, Texas, USA). P value <0.05 was defined as statistically significant.

Results

All subject involved in the study considered the collection technique tolerable and none of them reported side effects (including epistaxis or mucosal trauma). 165 over 185 total samples were considered valuable for examination. Twenty samples were discarded since exiguous, or due to the presence of mucus excess or organized material. Therefore, after samples' selection, the final study population included 68 volunteers recruited among medical students, representing the control cohort, 83 carriers, parents of CF patients, and 14 homozygotes followed in the Regional Center of diagnosis and therapy of CF, Bufalini Hospital, in Cesena.

Male to female ratio was 1:1,17. Median age was 32.8 (range ±11.2) years old in woman and 34.2 (range ±11.3) years old in men.

Values of Na and Cl of ASL for each group of subjects are shown in Table 1.

Statistical analysis revealed a significant difference (p< 0.0001) of Sodium and Chlorine values between the control group (Na 114 mmol/l, Cl 105 mmol/l) and the CF carriers (Na 143 mmol/l, 112 mmol/l) and between control group and CF homozygotes (Na 134 mmol/l, Cl 119 mmol/l), with increased concentration of these ions in both CF patients and carriers.

Sodium values increased significantly (p=0.04) in CF carriers in comparison to Homozygotes, while Chlorine values between these groups showed a lower variability (p=0.09).

For Na levels, the AUC was 0.86 (Figure 1) with a Youden's Index of 124 (Sensitivity: 0.79, confidence interval:0.69-0.87; Specificity 0.45, confidence interval 0.67-0.88). The AUC of Cl was 0.66 (Figure 2) with a Youden's Index of 103,2 (Sensitivity: 0.77 confidence interval:0.66-0.86, Specificity: 0.45 confidence interval 0.33-0.58).

The combination of Na and Cl levels revealed an AUC of 0.82, with a Youden index of 242 (sensitivity: 0.746; specificity: 0.763).

Discussion

To our knowledge, this research presents the largest cohort of subjects ever studied, so far, for ionic ASL composition, and the only one that collects ASL with a standardized protocol from the middle meatus, in order to save samples uncontaminated by tears.

The designed method showed to be feasible, minimally invasive, well-tolerated and easily reproducible, as it is based on the use of common and cheap clinical tools. The measurements on nasal ASL electrolytes showed a significant difference between the three cohorts of subjects: both Cl and Na increased significantly in carriers and homozygous patients in comparison to the gene-

Table 1. Sodium and Chlorine levels (mmol/l) of ASL of CF homozygotes, control group and carriers.

	CF homozygotes (14)	Control Group (68)	Carriers (83)	Pvalue
Cl levels Median [1Q- 3Q]	119 [105.25- 127]	100.45 [93.05 -111.55]	112 [100 -122.5]	<0.001
Na levels Median [1Q -3Q]	133 [118 -157]	114 [102 -123.5]	143 [126 -164.5]	<0.001

ral population.
The ROC curve applied on the large population of controls and heterozygotes suggested that Na concentration superior to the Youden's index may represent a useful cut off to detect potential CF carriers or at least patients that could be addressed for further investigations.
A full ENT evaluation comprehensive of ASL analysis could help to identify patients suitable for genetic assessment, alone or in association to other red flag signs such as peculiar radiological phenotypes. As observed in previous studies, a maxillofacial CT scan of CF heterozygous appears to be characterized by diffuse thickening of sinus mucosa without inflammatory polyps [13] and the evidence of significantly smaller frontal and maxillary sinus size compared to healthy subjects [14].
Despite the differences with healthy subjects, ionic levels in CF homozygotes ASL were not extremely diverse from those of CF heterozygotes. This finding can likely be influenced by the exiguous number of homozygotes in comparison to the number of carriers; in our opinion, additional researches with a larger cohort of homozygotes, are needed to further investigate ion concentration in this group.
In recent decades, CRS has been reported to have s significant impact on life quality in North America and Europe; any effort to detect CRS predisposing factors is desirable, particularly in order to understand its pathogenesis and to improve and customize treatments.
The latest studies focused on the application of biomarkers for the identification of difficult to treat CRS, with promising results [15].
In this context, the analysis of ASL may provide further support for the recognition of specific patient subgroups affected by CRS, such as CF carriers [16].
Since the identification of the CFTR gene in 1989, more than 2000 different mutations have been detected [17]. The CFTR expression at the level of the pseudostratified epithelium of upper airways contributes to the production of the ASL which covers the surface of mucosal cells and that represents an important layer of defence by exogenous microorganisms [18-20].
The first reports on quantitative analysis of the nasal mucus dated back to the seventies but, despite the potential clinical implication, studies on ASL electrolytes are limited and frequently discordant [21-23]. The absence of specific buffers, the challenging analysis of ASL with common laboratory instruments and the lack of standardized procedures probably contributed to the paucity of investigations in this field.
In 1996 Smith et al. [24] analyzed the concentration of Cl in ASL collected from the nasal cavity. Instructions on the fluid collection were not provided. The study, according to previous data published by Joris et al. (1993) and Gilljamet al. (1989) regarding Cl levels on trachea and bronchi fluids, showed that the Cl concentration in CF patients (182 2 ± 10 mM) was higher than in control group (132 ± 3 mM) [25,26].
Hull et al. in 1998 performed the evaluation of Na and Cl in 10 young patients with CF and in 10 healthy subjects. ASL was sampled, after wearing a nasal clip for 5 minutes, by touching the inferior turbinate with nitrocellulose strips. There were no significant differences in the nasal ASL sodium or chlorine concentration in the CF subjects compared to those found in the controls, but it was found a trend of an increased chlorine concentration in the CF group [27].
In contrast with these reports, Knowles et al. in 1998 evaluated the ASL ionic components demonstrating no differences in ionic composition between CF and healthy subjects [28]. However, these results may be influenced by the collection protocol, based on the placement of filter paper pledgets under the inferior turbinate, with a possible tears contamination, and by the small sample size.
Our technique is based on a simple and feasible method, that can guide the clinician to select patients for a genetic test, thus reducing healthcare costs.

Conclusion

CFTR gene mutations in heterozygotes appear to influence the ion composition of nasal ASL; in particular, according to our findings, Na and Cl concentrations of nasal ASL could represent a useful tool to assess between heterozygotes and healthy controls. Future studies are required to investigate the mechanism underlying these findings and their clinical implication.

Authorship contribution

NM: original idea and procedures planning; VF: manuscript preparation; AC: ion technical analysis; FB, AR: patients' selection; CF, ADL, VI, MB, AC: clinical examination and collection a of ASL samples. GV, CM: data analysis and statistical evaluation; FS, SP: supervised the project. All authors contributed to the clinical project, provided critical feedback, discussed the results and contributed to the elaboration of the manuscript.

Conflict of interest

There are no conflicts of interest to declare.

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